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TRAITEMENT DES TUMEURS OSSEUSES PRIMITIVES PAR L'ACIDE ZOLEDRONIQUE

THESE DE DOCTORAT

Ecole Doctorale de CHIMIE BIOLOGIE
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Liste des abréviations

RGD : peptide arginine, glycine, acide aspartique
TGFβ : Tumor Growth Factor β
IGF : Insulin like Growth Factor
TNF: Tumor Necrosis Factor
BMP: Bone Morphogenetic Protein
TRAP: Tartrate Resistant Acid Phosphatase
PTH: ParaTHormone
PTHrp: PTH related protein
IL-6: Interleukin 6
M-CSF: Macrophage-Colony Stimulating Factor
RANK: Receptor Activator of Nuclear factor κB
RANKL: RANK Ligand
OPG: Osteoprotegerin
FGF: Fibroblast Growth Factor
PDGF: Platelet Derived Growth Factor
Rb: Retinoblastoma
CDK: Cyclin Dependent Kinase
BRCA1: Breast Cancer 1
HES: Hémalun, Eosine, Safran
MDR: Multi Drug Resistant
PPi: PyroPhosphate endogène
N-BPs: Nitrogen containing BisphosPhonates
FPPs: Farnesyl diphosphate synthase
GGPP: GeranylGeranyldiphosphate
AIF: Apoptosis Inducing Factor
Endo G: Endonuclease G
FAK: Focal Adhesion Kinase
Zol: Zoledronic Acid
UFT: Uracile+Tégafure
i.v: intraveineux
ET-1: Endotheline-1
ABC: ATP-Binding Cassette
MRP: Multidrug Resistant associated Protein
GSH: Gluthation
MXR: Mitoxantrone Resistance associated transporter
LRP: Lung Resistant Protein
BCRP: Breast Cancer Resistant Protein
PXR: Pregnate Xenobiotic Receptor
P-gp: glycoprotein-P
FAK: Focal Adhesion Kinase
mTOR: mammalian Target Of Rapamycin

Introduction

I. Le Tissu Osseux

Le tissu osseux est un tissu conjonctif spécialisé composé d'une fraction organique et d'une fraction minérale. Son organisation et sa dynamique assurent sa fonction mécanique de soutien et son rôle dans l'homéostasie minérale. Il revêt une importance capitale pour l'organisme tant sur le plan biomécanique que sur le plan métabolique. En effet il est le support mécanique essentiel du squelette, permet la locomotion, transmet les forces issues de la contraction musculaire d'une partie du corps à une autre pendant le mouvement et assure la protection des organes internes. Enfin, il joue un rôle extrêmement important dans le maintien de l'homéostasie phosphocalcique, minérale et cellulaire car il est un réservoir métabolique de sels minéraux, (en particulier de calcium et de phosphore) et contribue ainsi à la régulation de la composition des fluides extracellulaires. Par ailleurs, le tissu osseux renferme la moelle osseuse, siège de l'hématopoïèse. Le tissu osseux est hautement spécialisé et est caractérisé par sa dureté et son apparente rigidité mais il n'est pas pour autant figé. En effet tout au long de la vie, l'os est constamment détruit puis reconstruit au cours des différentes phases du remodelage osseux : sa fraction organique est produite par les ostéoblastes et détruit par les ostéoclastes. Ce tissu est capable de réparation, d'adapter sa masse, sa forme, et ses propriétés intrinsèques à des modifications d'ordre biomécanique. Un bon équilibre entre les phases de résorption et de formation permet à l'os de conserver ses propriétés mécaniques.

A/ Organisation du tissus osseux

L'os sec et dégraissé est formé de 70% de substances minérales et de 30% de substances organiques. Ces deux fractions ne sont pas simplement juxtaposées mais au contraire liées l'une à l'autre.

La fraction organique est constituée essentiellement de fibres de collagène de type I, représentant environ 90 % de la phase organique de l'os. Ces fibres sont entourées d'une substance fondamentale interfibrillaire (Gokhale JA 2001). Dans le tissu osseux lamellaire les fibres de collagène sont arrangées de façon parallèle au sein d'une même lamelle osseuse, mais leur orientation varie d'une lamelle à l'autre. La substance fondamentale interfibrillaire est constituée de composants variés tels que des glycoprotéines (ostéonectine, ostéopontine, sialoprotéine osseuse), des protéines contenant des résidus d'acide glutamique carboxylé (ostéocalcine), des phosphoprotéines, des phospholipides, des protéoglycanes, des cytokines

et des facteurs de croissance. La plus abondante des protéines est l'ostéocalcine qui représente 10 à 20 % des protéines non collagéniques. Elle jouerait un rôle dans l'attraction des ostéoclastes dans les foyers de résorption et dans le processus de minéralisation. Plusieurs protéines non collagéniques telles que l'ostéopontine, la sialoprotéine et la fibronectine renferment une séquence arginine-glycine-acide aspartique (RGD). Cette séquence RGD caractérise les protéines d'adhérence cellulaire et est reconnue par certaines protéines membranaires appelées intégrines (Ruoslahti 1991). Ainsi, les intégrines présentes à la surface des ostéoblastes permettraient leur attachement à la matrice extracellulaire. Des facteurs de croissance et des cytokines (transforming growth factor β ou TGFβ, insulin-like growth factor ou IGF, tumor necrosis factor ou TNF, les interleukines et les bone morphogenic proteins ou BMP) sont également présents en petites quantités dans la matrice osseuse. Ces facteurs protéiques jouent un rôle important dans l'activation et la différenciation cellulaire et interviennent dans le couplage entre la formation et la résorption osseuse.

La fraction minérale de la matrice osseuse confère à l'os sa rigidité et sa résistance mécanique et représente une importante réserve minérale. En effet, environ 99 % du calcium de l'organisme, 85 % du phosphore et entre 40 et 60 % du sodium et du magnésium sont incorporés dans les cristaux qui constituent la substance minérale osseuse (Glimcher 1992). Elle est essentiellement composée de phosphate de calcium cristallisé sous forme d'hydroxyapatite. Les cristaux d'hydroxyapatite ont une forme hexagonale, aplatie et sont disposés dans les espaces interfibrillaires. Leur nombre et leur taille s'accroissent lentement au cours du processus de minéralisation secondaire succédant à la minéralisation primaire qui elle-même suit immédiatement la synthèse de matrice par les ostéoblastes.

Organisation macroscopique

L'os long chez l'adulte est constitué d'une partie centrale cylindrique appelée diaphyse, et de deux extrémités élargies et arrondies appelées épiphyses, couvertes de cartilage articulaire. Des régions coniques, appelées métaphyses connectent la diaphyse à chaque épiphyse. On distingue l'os cortical ou compact et l'os trabéculaire ou spongieux, l'ensemble étant entouré d'une enveloppe externe richement vascularisée, le périoste, absent uniquement au niveau des articulations. Il est présent partout sauf au niveau des articulations. Cette enveloppe joue un rôle fondamental dans la croissance en longueur et surtout circonférentielle des os. Chez l'enfant elle est constituée de deux couches. Une couche

superficielle fibreuse vascularisée et d'une couche profonde contenant des cellules souches et des préostéoblastes. (*Figure 1*).

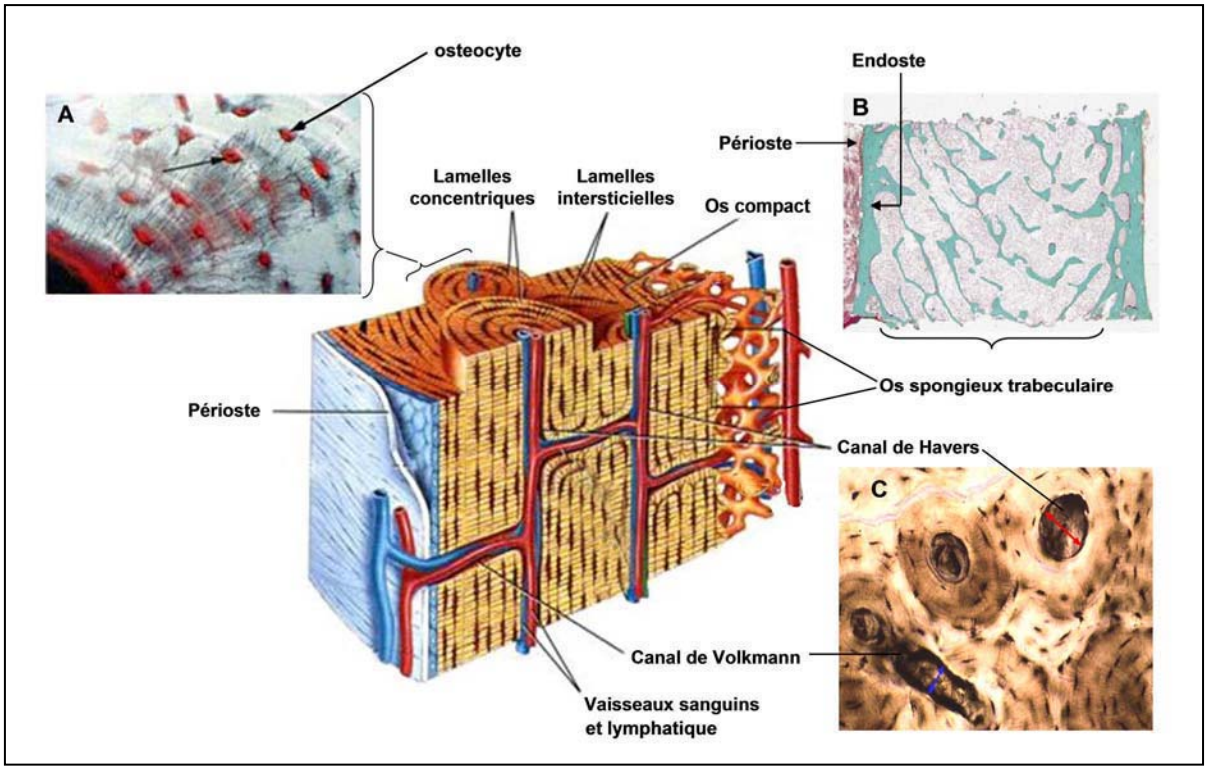

Figure 1 : **Organisation structurale de l'os**

A : coloration d'ostéocytes figés dans les lamelles concentriques de l'unité structural de l'os compacte : l'ostéon. B : réseau trabéculaire limité à l'endoste par les deux corticales (composées d'os compact) recouvertes de périoste. Coloration au trichrome de Goldner. C : canal de Havers et canal de Volkmann vue en coupe.

Os compact

L'os compact est également appelé os cortical, il constitue la diaphyse des os longs et l'enveloppe des os plats et courts. Il représente environ 80-85% du tissu osseux et constitue une enveloppe résistante composée par la juxtaposition d'ostéons cylindriques de 200 à 300 µm de diamètre alignés parallèlement à la diaphyse. Chaque ostéon (unité structurale de l'os compact) est composé de lamelles concentriques (*Figure 1*). Les fibres de collagène sont orientées parallèlement les unes aux autres de façon à conférer au tissu cortical une résistance mécanique optimale. Chaque ostéon est centré par un canal dit " de Havers " de 50 µm de diamètre. Dans les ostéons primaires le canal de Havers est délimité par les cellules ostéoprogénitrices et les ostéoblastes. Ces cellules, assurant le comblement du canal durant la maturation des ostéons, se trouvent emmurées dans la matrice extracellulaire dans une logette appelée ostéoplaste et prendront alors le nom d'ostéocytes. Les canaux de Havers sont reliés entre eux par des canaux transversaux dit " de Volkmann " ; chaque canal est composé d'un vaisseau innervé qui communique à la fois avec la vascularisation périostée et les vaisseaux de la moelle osseuse (*Figure 1-2*).

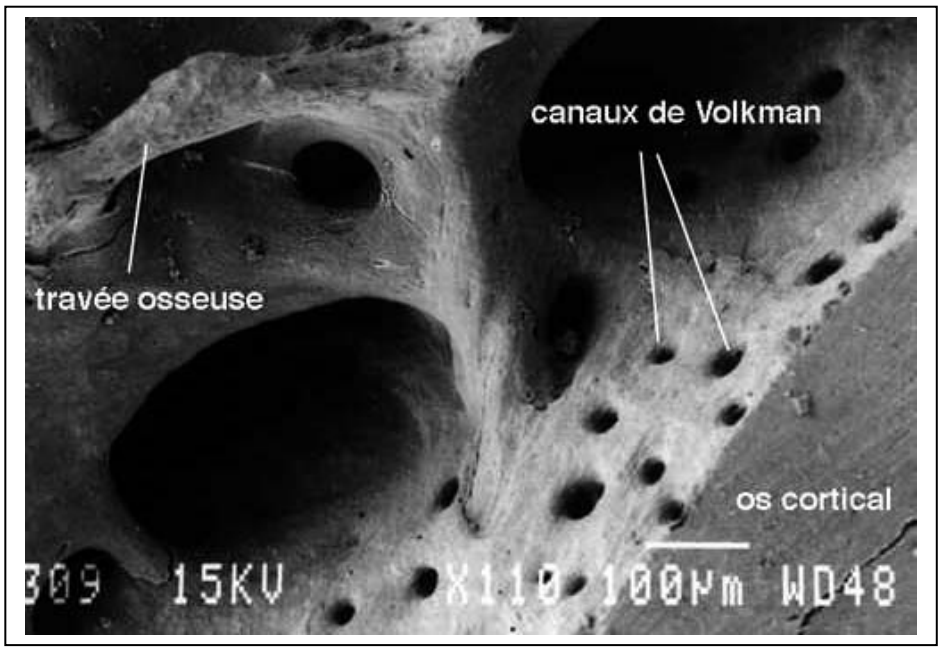

Figure 2 : **Jonction entre os tabéculaire et os cortical observée par microscopie électronique à balayage.** (D'après le laboratoire de biologie du tissu osseux l'université Jean monnet, st Etienne)

Os trabeculaire

L'os trabéculaire est également appelé os spongieux. Il est constitué d'un réseau de travées osseuses composées de plaques de tissu minéralisé reliées entre elles par des arches. Entre les travées osseuses se positionne la moelle hématopoïetique représentant environ 75% du volume total de l'os spongieux. De par sa structure, l'os trabéculaire dispose d'une large surface d'échange ($7m^2$) avec la moelle osseuse, lui permettant ainsi d'effectuer au mieux ses fonctions hématopoïetiques (*Figure 1-2*). A l'interface de la moelle osseuse et du tissu minéralisé se trouve les cellules ostéoblastiques jouant un rôle essentiel dans les échanges entre ces deux entités.

B/ L'Ostéoblaste

L'ostéoblaste est la principale cellule sécrétrice des constituants de la matrice organique. Son origine est médullaire et il dérive d'une cellule souche mésenchymateuse pluripotente commune aux chondrocytes, fibroblastes, myocytes et adipocytes (Owen 1988) essentiellement présentes chez l'adulte dans le périoste et le stroma de la moelle osseuse. De forme allongée ou cuboïdale, les ostéoblastes tapissent la surface osseuse en cours de formation. La différenciation vers l'une ou l'autre voie implique l'expression de facteurs de transcription spécifiques. Ainsi, l'expression de Sox-9 induit la différentiation des cellules dans la voie chondroblastique, celle de Myo-D dans la voie myoblastique et celle de PPARγ2 dans la voie adipocytaire (Pittenger et al. 1999). Une série complexe d'étapes de prolifération et de différenciation conduisent aux ostéoblastes matures depuis les cellules souches mésenchymateuses (*Figure* 3). Leur cytoplasme renferme un abondant réticulum endoplasmique granuleux, un appareil de Golgi très développé et de nombreuses mitochondries témoins d'une synthèse protéique importante. Leur fonction principale est la synthèse de la trame protéique de l'os. L'engagement de cellules précurseurs vers la voie ostéoblastique implique de nombreux facteurs de transcription. Ainsi, l'importance du complexe AP-1 (constitué d'homo ou hétérodimères de facteurs de transcription de la famille Jun/Fos) dans le développement du tissu osseux a été mise en évidence dans des souris présentant une surexpression de c-Fos, caractérisées par la transformation des ostéoblastes et l'apparition d'ostéosarcomes (Grigoriadis et al. 1993; David et al. 2005). Au contraire, des

souris fos -/- présentent un retard de croissance, une ostéopétrose, des défauts dans le remodelage osseux et une hématopoïèse altérée (Wang 2005). L'activité de ce complexe AP-1, très intense dans les ostéoblastes proliférant, diminue fortement lors de la différenciation terminale en ostéoblastes. Les ostéoblastes reposent sur une couche de tissu constitué de collagène, de protéines non collagéniques (ostéocalcine, décorine) et de glycosaminoglycanes, non encore minéralisé appelé tissu ostéoïde. Ce tissu sera minéralisé dans un second temps (chez l'homme en moyenne 10-20 jours après la déposition de la matrice). A l'issue de la période de formation osseuse, la majorité des ostéoblastes (65%) meurent par apoptose, dans le cas contraire les ostéoblastes peuvent devenir des cellules bordantes ou subir une différentiation terminale en ostéocytes.

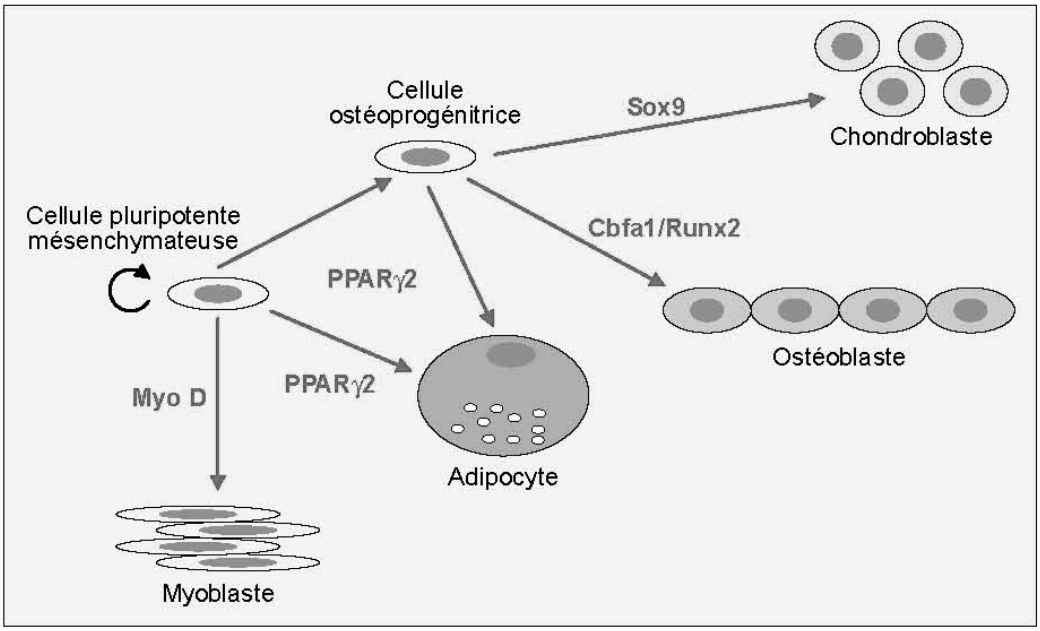

Figure 3 : **Les ostéoblastes proviennent de la différenciation de cellules souches mésenchymateuses** (D'après Marie P.J, Médecine/Sciences 2001)

Les cellules bordantes ont pour origine des ostéoblastes mis au repos susceptibles, s'ils sont sollicités, de redevenir des ostéoblastes actifs. Ils recouvrent les surfaces osseuses en phase quiescente. Ce sont des cellules aplaties, avec un cytoplasme pauvre en organites, en particulier en mitochondrie et REG, dont la principale fonction serait d'assurer la communication entre la surface osseuse, l'environnement cellulaire et les ostéocytes emmurés dans la matrice osseuse. Elles jouent également un rôle durant la phase initiale du remodelage

osseux. Sous l'effet de certains stimuli, elles libèrent la surface osseuse permettant ainsi l'attraction des ostéoclastes (Chambers et al. 1985).

Les ostéocytes proviennent de la différenciation terminale de certains ostéoblastes emmurés dans le tissu osseux, à l'intérieur d'une lacune périostéocytaire appelé ostéoplaste. 10 à 20% des ostéoblastes deviennent des ostéocytes soit 25000/mm^3 de tissu osseux (*Figure 4*). Au cours de cette différenciation, les ostéoblastes perdent une grande quantité de leurs organites. Les ostéocytes possèdent de nombreux et fins prolongements cytoplasmiques qui leur permettent d'établir des contacts avec les autres ostéocytes et les cellules bordantes qui recouvrent la surface osseuse. Les substances nutritives qui proviennent du sang atteignent les ostéocytes en diffusant soit dans les canalicules autour des prolongements cellulaires, soit dans les cellules elles-mêmes, passant de l'une à l'autre par des jonctions communicantes (GAP). Ce mode de diffusion peu efficace impose aux ostéocytes de se trouver à moins de 0,2 mm d'un vaisseau sanguin. Cette limitation explique la taille des travées de l'os spongieux et la structure de base de l'os compact : l'os haversien. Les ostéocytes interviennent essentiellement dans la transmission des signaux mécano-sensoriels et dans les échanges entre les cellules et le microenvironnement (Aarden et al. 1994). Ils régulent la formation osseuse *via* la sécrétion de sclérostine qui constitue un marqueur spécifique des ostéocytes (Poole et al. 2005; van Bezooijen et al. 2005).

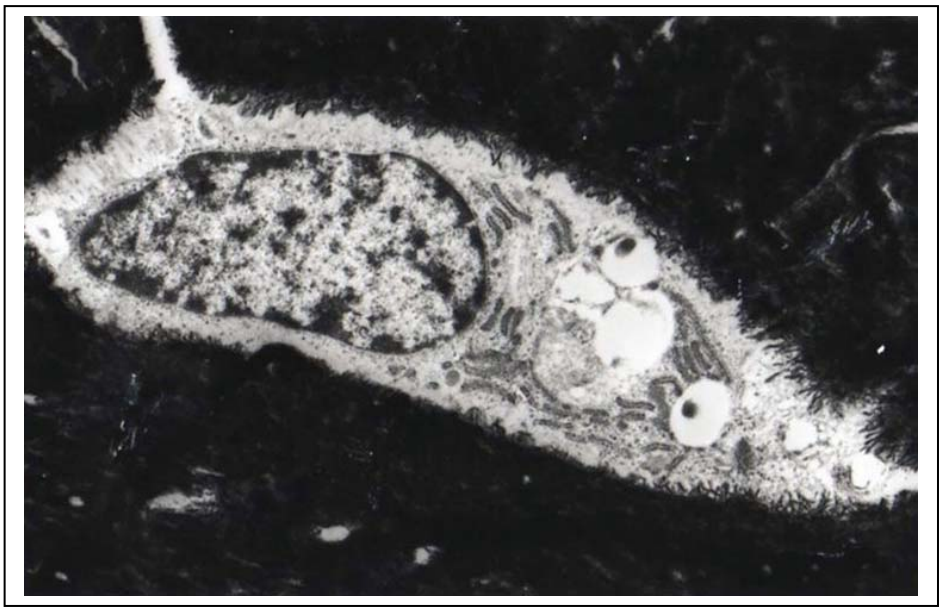

Figure 4 : **Ostéocyte observé par microscopie électronique à transmission**.

C/ L'Ostéoclaste

Les ostéoclastes sont les cellules responsables de la résorption osseuse. L'ostéoclaste est une grosse cellule multinucléée pouvant renfermer de 2 à 30 noyaux et riche en enzymes lysosomales telles la phosphatase acide tartrate résistante (TRAP) ou la cathepsine k et des collagénases. D'origine hématopoïétique, ils appartiennent à la famille des monocytes/macrophages. Ces cellules sont polarisées, le noyau se trouvant à l'opposé de la surface osseuse tandis que la zone au contact de la matrice développe des replis membranaires, appelés bordure en brosse. L'ostéoclaste est observable au fond des lacunes de résorption appelées lacunes de Howships, au contact de la matrice osseuse calcifiée *(figure 5)*. La zone de contact est caractérisée par la présence de nombreuses vacuoles intracytoplasmiques et une bordure plissée. À ce niveau, l'ostéoclaste est capable d'acidifier le milieu extracellulaire par la sécrétion de protons grâce à des pompes à protons, qui solubilisent la fraction minérale. La digestion de la trame organique s'effectue ensuite sous l'action des enzymes protéolytiques contenues dans les lysosomes (Blair et al. 1986).

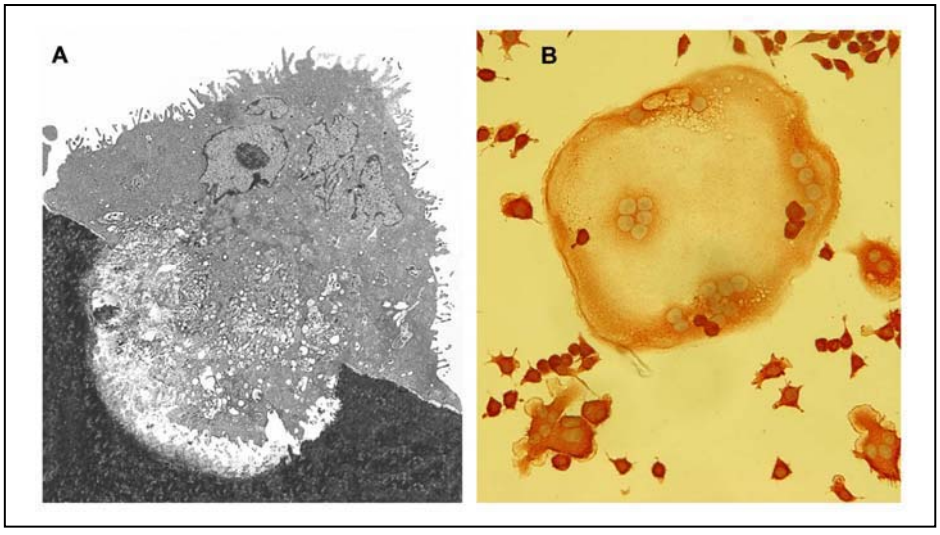

Figure 5 : **Ostéoclaste résorbant issu d'une tumeur à cellules géantes et cultivé sur une pastille de dentine observé au microscope électronique à transmission (A) et au microscope optique (B).**

D/ Le remodelage osseux

Tout au long de la vie, l'os est le siège d'un remaniement permanent. Ce processus permet au tissu osseux de s'adapter aux différentes contraintes mécaniques, d'assurer l'homéostasie minérale et assure la cicatrisation des fractures. Cette activité de remodelage donne naissance aux éléments de base de l'os cortical appelés ostéons mais également à l'os spongieux (Frost 1969). Le remodelage osseux se déroule selon une séquence d'évènements bien précis en un même site résultant de l'activité de plusieurs types cellulaires. Le remodelage débute par une phase d'activation des ostéoclastes qui conduit à la résorption osseuse suivie d'une phase de transition qui aboutit au recrutement des cellules ostéoprogénitrices, à l'activation des ostéoblastes puis une phase d'invertion et enfin une phase de formation d'une nouvelle matrice osseuse (Hill 1998). A chaque instant, environ 5% des surfaces intracorticales et 20% des surfaces trabéculaires sont le siège d'un remodelage. Ce processus implique un couplage étroit entre résorption et formation. La durée moyenne d'une séquence de remodelage est de 4 à 6 mois (Parfitt 1992).

Phase d'activation : La séquence du remodelage débute en un point d'une surface osseuse quiescente recouverte par des cellules bordantes *(figure 6)*. Ces cellules seraient capables de percevoir un signal d'initiation dont la nature exacte est inconnue mais qui pourrait être de nature hormonale ou cytokinique, comme c'est le cas dans l'ostéoporose où une diminution du taux d'estrogène entraîne une augmentation du nombre et de l'activité des ostéoclastes (Pacifici 1998). Enfin elle pourrait relever d'un stimulus mécanique (altération de l'architecture locale de l'os : fracture, prise de poids…) (Turner et al. 1998). La captation de ce signal conduirait à la dégradation de la fine couche de matrice non minéralisée située sous les cellules bordantes, exposant ainsi la matrice minéralisée à l'action des ostéoclastes. En effet les cellules bordantes ont une activité métabolique très réduite, mais peuvent se multiplier ou se différencier à nouveau en ostéoblastes fonctionnels sous l'influence de stimuli mécaniques ou moléculaires. Lors de la phase d'activation, sous l'action des facteurs ostéorésorbants [PTH, vitamine D3 et prostaglandine E2,…], les ostéoblastes se rétractent et laissent la place aux précurseurs mononucléés des ostéoclastes, ou pré-ostéoclastes, qui peuvent adhérer à la matrice.

Phase de résorption : Elle débute par l'activation des précurseurs ostéoclastiques présents dans la moelle osseuse au site de remodelage et conduit à leur différenciation en ostéoclastes matures et à leur attachement à la surface osseuse *(figure 6)*. Des études récentes ont montré que la différenciation et l'activité ostéoclastiques sont modulées par des facteurs libérés par les cellules stromales de la lignée ostéoblastique. Parmi les facteurs synthétisés, l'interleukine 6 (IL-6) et le macrophage-colony stimulating factor (M-CSF) sont des stimulateurs de la résorption. Les cellules stromales ostéoblastiques expriment également RANK ligand (RANKL) qui stimule l'ostéoclastogenèse en agissant sur RANK (Receptor Activator of Nuclear Factor κB) situé à la surface des précurseurs ostéoclastiques mononucléés. A l'inverse, l'ostéoprotégérine (OPG), facteur soluble également produit par les cellules ostéoblastiques agit comme un antagoniste de RANK ligand (Simonet et al. 1997; Lacey et al. 1998). Une fois attaché à la matrice osseuse, l'ostéoclaste crée un microenvironnement acide qui permet la dissolution de la phase inorganique qui précède la dégradation de la fraction protéique de la matrice osseuse grâce à l'équipement enzymatique de l'ostéoclaste. La lacune de résorption ainsi créée est appelée lacune de Howship *(figure 6)*. La phase de résorption dure environ 30 jours (Parfitt 1992).

Phase de réversion : Le couplage entre résorption et formation intervient durant la phase de transition. La dégradation de la matrice entraîne une augmentation du taux de calcium intracellulaire des ostéoclastes qui va provoquer une désorganisation des podosomes et par conséquent le détachement de l'ostéoclaste. Les ostéoclastes qui se détachent de la surface osseuse meurent par apoptose vraisemblablement médiée par le système Fas/Fas ligand (Kavanagh et al. 2006). Ces ostéoclastes sont alors remplacés par des cellules mononucléées de type macrophagique qui vont éliminer les derniers résidus de matrice présents dans le fond de la lacune. Il a été suggéré que les facteurs favorisant la formation osseuse soient intégrés dans la matrice osseuse et relargués durant la phase de résorption. Cela concernerait en particulier les insulin-like growth factors (IGF), les fibroblast growth factors (FGF), le transforming growth factor-β (TGF β), les bone morphogenic proteins (BMP) et le platelet derived growth factor (PDGF). La surface osseuse ainsi libérée par les ostéoclastes et correspondant au fond de la lacune de Howship est riche en éléments tels que l'ostéopontine qui activeraient les ostéoblastes.

Phase de formation : Elle débute par la prolifération des cellules ostéoprogénitrices qui vont ensuite tapisser le fond de la lacune de Howship *(figure 6)*. Les ostéoblastes vont alors

synthétiser les constituants de la matrice protéique osseuse. Puis après un délai de 10 à 15 jours, ce tissu se minéralise par le dépôt de cristaux d'hydroxyapatite dans les espaces interfibrillaires du collagène. Cette étape de minéralisation comporte une phase rapide appelée minéralisation primaire puis se poursuit plus lentement pendant une durée variable appelée minéralisation secondaire. La durée de la minéralisation secondaire est liée à la fréquence du remodelage. La durée de la phase d'ostéoformation est de 4 à 5 mois.

Phase quiescente : Une fois la phase de formation achevée, les ostéoblastes qui en partie meurent par apoptose ou sont emmurés dans la matrice osseuse sous forme d'ostéocytes, laissent la place aux cellules bordantes qui vont recouvrir la surface osseuse *(figure 6)* et demeurer quiescentes jusqu'à une prochaine activation des ostéoclastes.

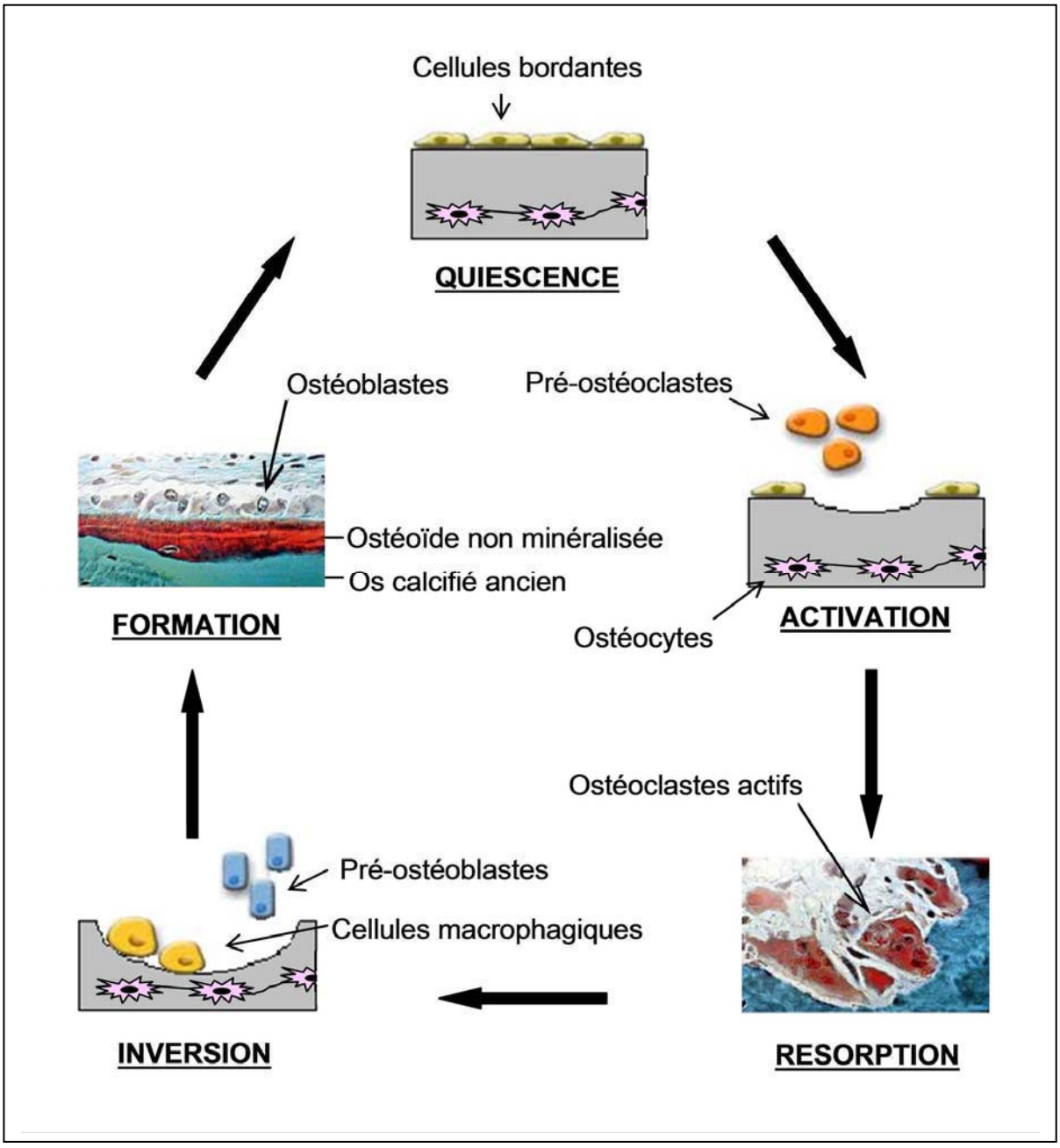

Figure 6 : **Etapes du remodelage osseux**

II. Cancer et Ostéosarcome

A/ Généralités sur le cancer

L'Agence Internationale de Recherche sur le Cancer (IARC), dépendant de l'Organisation Mondiale de la Santé (OMS), recense chaque année dans le monde plus de 10 millions de nouveaux cas de cancer et 6 millions de décès, représentant 12% des décès dans le monde. L'incidence des cancers pourrait augmenter de 50 % d'ici à 2020 pour atteindre 15 millions de nouveaux cas chaque année, notamment en raison du vieillissement de la population. Dans les pays industrialisés, ces pathologies affectent toutes les classes d'âge : le cancer est désormais la première cause de mortalité avant 65 ans. En France, les différents types de cancer sont responsables de 26 % des décès, 31 % chez l'homme, 21 % chez la femme. Ils sont la 1ère cause de décès chez l'homme (maladies cardiovasculaires 29 %) et la 2ème chez la femme après les maladies cardiovasculaires (38 %). Ils sont la 1ère cause de décès chez la femme entre 20 et 60 ans. Les maladies regroupées sous le terme "cancer" sont extrêmement fréquentes et posent un problème de santé publique, dans la mesure où une personne sur deux atteintes de cancer décède de la pathologie.

Le cancer est dû à des déficiences dans les mécanismes qui contrôlent habituellement la croissance et la prolifération des cellules. Au cours du développement normal et tout au long de la vie, des systèmes de contrôle génétique régulent l'équilibre entre naissance et mort des cellules en réponse à des signaux qui soit induisent la prolifération, soit l'inhibent, soit conduisent à la mort des cellules. Ce système de régulation détermine la taille du corps adulte et la vitesse de croissance aboutissant à cette taille. La perte de régulation cellulaire qui conduit au cancer est due à une lésion génétique. Des mutations dans deux grandes classes de gènes sont impliquées dans la transformation d'une cellule en cellule tumorale : les proto-oncogènes d'une part et les anti-oncogènes ou gènes suppresseurs de tumeurs d'autre part.

Oncogènes

Tout gène cellulaire, appelé proto-oncogène est susceptible de devenir, par suite d'une modification qualitative ou quantitative, *un gène transformant*, c'est-à-dire un gène capable de conférer expérimentalement un phénotype cancéreux (transformation) à une cellule normale eucaryote (La notion de phénotype cancéreux sera détaillée plus tard). Les oncogènes sont répartis en 6 grandes classes en fonction des oncoprotéines pour lesquelles ils codent : les facteurs de croissance, les récepteurs transmembranaires de facteurs de croissance, les protéines G ou protéines membranaires liant le GTP, les tyrosines protéines-kinases membranaires, les protéines-kinases cytosoliques et les protéines à activité nucléaire qui contrôlent la transcription de gènes cibles en interagissant avec l'ADN.

Anti-Oncogènes

Les gènes suppresseurs de tumeurs sont aptes à inhiber la croissance cellulaire lorsqu'ils sont introduits par transfection dans les cellules tumorales. Cette propriété s'explique par la capacité de ces gènes à réguler négativement le cycle cellulaire et/ou à induire l'apoptose ou mort cellulaire programmée. Les anti-oncogènes agissent principalement en phase G1/S. Cette transition G1/S est sous la dépendance des facteurs de transcription de la famille E2F qui contrôlent l'expression de gènes indispensables à la phase S de synthèse de l'ADN (Bartek et al. 2004). Les protéines de la famille E2F existent sous forme libre, ou sous forme inactive complexée à la protéine RB. L'aptitude de la protéine RB à fixer les facteurs de transcription E2F dépend de son état de phosphorylation. En effet, lorsque la protéine RB est non phosphorylée, elle est active et peut fixer les facteurs E2F, il en résulte un blocage de la transition G1/S. Lorsque la protéine RB est phosphorylée, elle devient inactive et est incapable de fixer la protéine E2F qui, libérée, permet la transition G1/S. La phosphorylation de RB est elle-même sous la dépendance de complexes protéiques jouant le rôle de verrous moléculaires au niveau de la transition entre les différentes phases du cycle (Harbour et al. 2000). Ces complexes sont composés d'unités régulatrices, les cyclines, et d'unités catalytiques, les kinases dépendantes de cyclines ou CDK (Cyclin Dependant Kinase). L'association de ces deux unités constitue le complexe actif. Les complexes cyclines/CDK sont eux-mêmes régulés par des protéines inhibitrices (p15, p16, p18, p19 et p21, p57 et p27), qui agissent en se fixant sur les CDKs, et donc en empêchant la constitution du complexe actif (Pestell et al. 1999). Le gène p21, inhibiteur universel de CDK, est régulé au niveau

transcriptionnel par la protéine p53. Les gènes RB, p16 et p53 interviennent sur la même voie biologique, qui régule la transition G1/S (Harper et al. 1993). La protéine p53 régule la transcription de nombreux gènes dont certains (*bax*) régulent l'apoptose (Lane 1992). Les altérations moléculaires à l'origine de la perte de fonction des gènes suppresseurs dans les tumeurs sont variées. Il peut s'agir de mutations ponctuelles, de délétions, d'insertions, d'anomalies de méthylation des promoteurs inhibant la transcription. La voie biologique contrôlant le cycle cellulaire au niveau de la transition G1/S et passant par les gènes suppresseurs p53, p16 et RB, est la voie la plus fréquemment altérée dans les cancers (Harper et al. 1993). Par exemple, l'inactivation constitutionnelle du gène suppresseur RB est à l'origine des formes héréditaires de rétinoblastome et représente également un facteur de risque génétique pour le développement d'ostéosarcomes (Feugeas et al. 1996). Chez l'adulte, les mutations somatiques de RB sont observées dans les cancers du sein ou du poumon à petites cellules. Le mélanome malin familial peut résulter de mutations constitutionnelles de p16 ou de CDK et les mutations somatiques de p16 sont très fréquemment retrouvées dans les tumeurs solides. Les mutations somatiques de p53 représentent l'altération moléculaire la plus fréquemment observée dans les tumeurs solides et les mutations constitutionnelles de ce gène constituent la base moléculaire du syndrome de Li-Fraumeni, syndrome prédisposant à un très large spectre de tumeurs incluant en particulier des sarcomes des tissus mous, des ostéosarcomes (Miller et al. 1996), des tumeurs du système nerveux central et des cancers du sein. Les altérations constitutionnelles de BRCA1 (breast cancer 1) sont à l'origine des formes héréditaires de cancers du sein et de l'ovaire (Yarden et al. 2002). Les mutations somatiques de bax ont été identifiées dans des tumeurs du colon.

Développement des tumeurs solides

Le cancer se développe fréquemment à la suite de mutations dues à une exposition tout au long de la vie à des agents cancérigènes, ces mutations surviennent dans des cellules somatiques, qui par conséquent ne sont pas transmises à la génération suivante. Par contre, certaines mutations héritées, transmises par la lignée germinale, augmentent la probabilité d'apparition d'une cellule cancéreuse. Les formes héréditaires résultent essentiellement de l'inactivation constitutionnelle de gènes suppresseurs mais elles sont parfois secondaires à une activation constitutionnelle de proto-oncogènes ou à une inactivation constitutionnelle des gènes de réparation de l'ADN. La plupart des cancers surviennent après l'altération par des carcinogènes ou par des erreurs dans la copie ou la réparation de l'ADN, quand ces mutations

somatiques s'ajoutent aux mutations héritées, le cancer résultant est particulièrement agressif (Nyberg et al. 2002; Frank et al. 2004). Cependant, une mutation seule aboutit rarement à un cancer. Dans la majorité des cas, une série de mutations est nécessaire pour créer un type cellulaire proliférant de plus en plus rapidement, étant de ce fait de plus en plus sujet à des erreurs de synthèse ou de réparation de l'ADN conduisant à des mutations supplémentaires (Frank et al. 2004). Ces mutations conduisent à l'apparition du phénotype cancéreux correspondant à l'acquisition de tout ou partie des 6 propriétés cellulaires fondamentales suivantes : perte de l'inhibition de contact, inhibition de l'apoptose, angiogenèse continue, insensibilité aux inhibiteurs de croissance, potentiel illimité de réplication, indépendance vis-à-vis des facteurs de croissance et des signaux de survie liés à l'adhérence (insensibilité à l'anoikis).

Dissémination métastatique des cellules tumorales

Le processus de cancérogenèse peut être divisé en trois étapes : une première étape dite d'initiation qui consiste comme nous l'avons vue précédemment en une dérégulation génomique de multiples évènements mineurs aboutissant à une dérégulation majeure. Il en résulte une transformation cellulaire. Une deuxième étape de promotion qui est le résultat d'interactions entre cytokines (facteurs de croissance) et leurs récepteurs, aboutissant à une perte de l'homéostasie tissulaire et l'émergence de clones cellulaires transformés et enfin la troisième étape d'invasion locale qui est à l'origine du phénomène de dissémination métastatique : elle résulte d'interactions entre le stroma et l'épithélium.

Le processus métastatique est complexe et l'invasion de nouveaux tissus n'est pas aléatoire mais dépendant de la cellule qui métastase et du tissu colonisé. Cette phase se caractérise par la diffusion de cellules tumorales dans l'organisme, avec atteinte, à distance, d'autres organes et est spécifique des tumeurs malignes. D'une façon générale, une métastase se définit comme l'apparition, en un autre point de l'organisme, d'une lésion identique au processus pathologique préexistant. Une métastase cancéreuse ou tumeur secondaire est un foyer cellulaire situé à distance de la tumeur initiale, de même nature qu'elle et sans relation de contiguïté avec elle. Les métastases marquent l'état avancé du processus cancéreux, contre-indiquant en général toute exérèse. Les voies de dissémination métastatique sont multiples : tout d'abord la transplantation, qui est un mode de « dissémination artificiel », est un transport mécanique d'un fragment tumoral par un instrument, par exemple, lors de la biopsie d'une tumeur ; en réalité c'est un mode de dissémination exceptionnel chez l'homme.

Ensuite on trouve la dissémination lymphatique qui est la voie la plus fréquente de dissémination des carcinomes (par exemple de la prostate, de l'ovaire et du sein), mais peut se rencontrer également au cours des sarcomes (par exemple osseux, musculaire ou du tissu conjonctif). Les cellules tumorales envahissent les vaisseaux lymphatiques, migrent dans la lumière par un phénomène voisin de la diapédèse et gagnent le sinus périphérique du ganglion lymphatique le plus proche dans lequel elles se multiplient. Après avoir atteint le premier relais ganglionnaire, les cellules tumorales gagnent le canal thoracique ou la grande veine lymphatique et enfin la circulation sanguine (*Figure 7*). Enfin la dernière voie de dissémination est l'extension hématogène. Cette voie est consécutive à l'effraction de la paroi vasculaire sanguine par les cellules tumorales. Cette effraction est d'autant plus facile que les vaisseaux du stroma ont une paroi mince et qu'il existe, dans certaines tumeurs (sarcomes), des lacunes vasculaires bordées de cellules tumorales. La diffusion par voie sanguine est commune aux sarcomes et aux carcinomes. Cette extension hématogène est le mode d'extension métastatique aux organes relais, en particulier le poumon, le foie et les os.

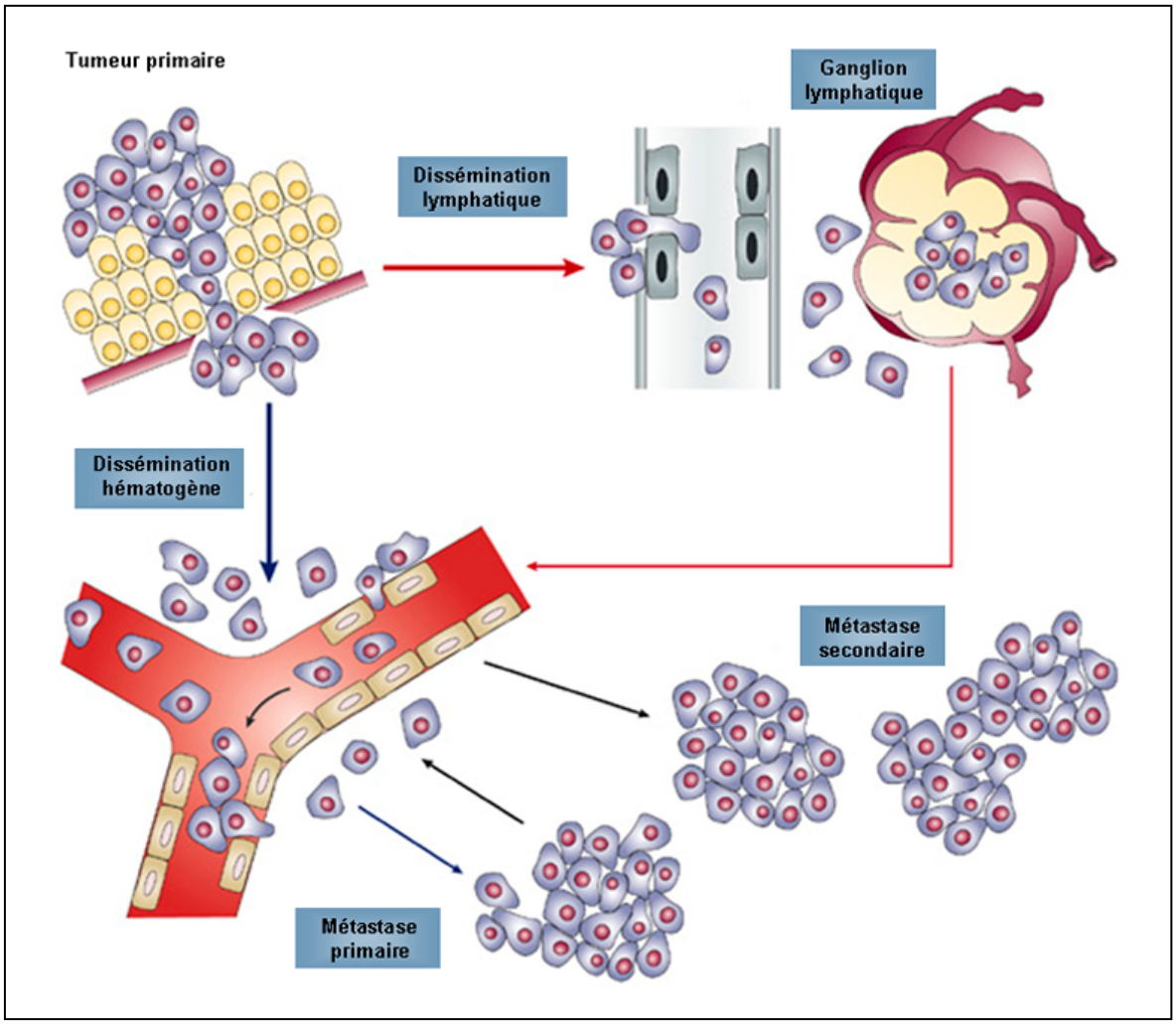

Figure 7 : **Modèle de dissémination métastatique**

Les cellules tumorales peuvent se disséminer à partir du site de la tumeur primaire *via* deux voies : d'une part la dissémination lymphatique (flèches rouges) dans laquelle un premier foyer métastatique se constitue dans les ganglions lymphatiques avant de se disséminer à nouveau pour former des métastases secondaires. D'autre part, la dissémination peut être hématogène (flèches bleues). Une dissémination hématogène secondaire est possible à partir de métastases primaires en direction de sites distants de métastases secondaires (flèches noires). (Pantel et al. 2004)

B/ L'Ostéosarcome

Comme nous l'avons vu, le tissu osseux est un tissu vivant en continuel remaniement grâce à un processus de remodelage assuré par deux types cellulaires : les ostéoclastes qui résorbent la matrice extracellulaire et dissolvent le minéral et les ostéoblastes qui synthétisent une nouvelle matrice et induisent sa minéralisation. L'équilibre entre l'activité ostéoblastique et l'activité ostéoclastique permet le maintien de l'homéostasie phosphocalcique et de la masse osseuse au cours de la vie (Manolagas 2000). Tout dérèglement de l'équilibre entre apposition et résorption osseuse est lié à l'apparition de pathologies ostéocondensantes (ostéopétrose, ostéosarcome) ou ostéolytiques, pouvant être métaboliques (par exemple l'ostéoporose) ou tumorales (par exemple le cancer du sein).

Des cas d'ostéosarcomes ont été décrits sur des momies de l'Égypte ancienne datant de plus de 5000 ans (Capasso 2005) avec la possibilité d'une intervention chirurgicale sous forme d'amputation. Un cas également bien documenté d'ostéosarcome, ayant le profil radiologique typique en « feu d'herbes », a été observé sur le fémur d'un jeune péruvien datant de 1100 après JC (*Figure 8*).

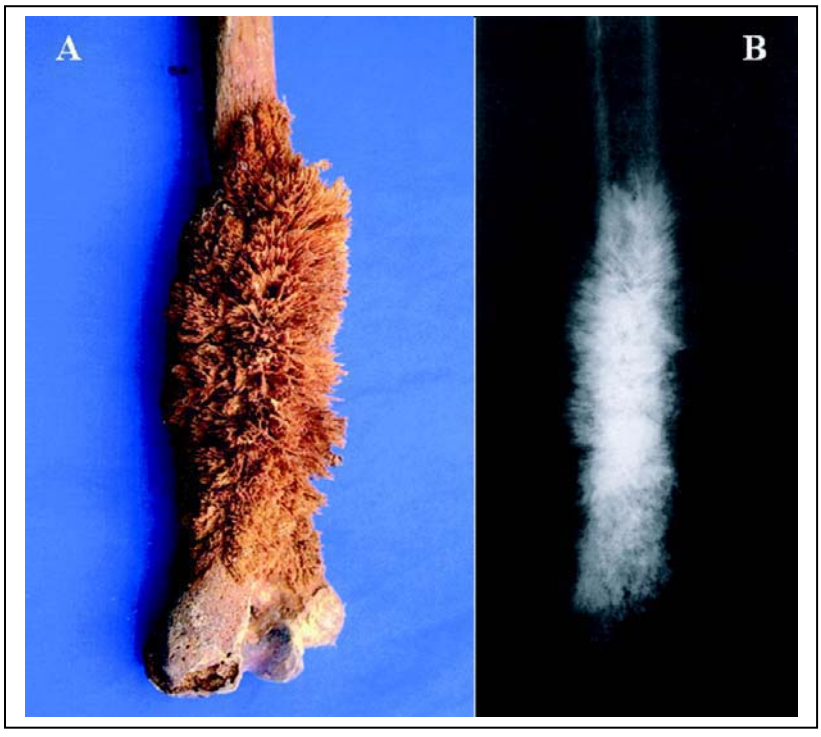

Figure 8 : **Osteosarcome du fémur chez un jeune péruvien datant de 1100 après JC (*a*), profil radiologique typique en « feu d'herbes » (*b*). (Capasso 2005)**

L'Organisation Mondiale de la Santé (OMS) définit l'ostéosarcome comme une tumeur maligne caractérisée par la formation directe d'os ou de substance ostéoïde par les cellules tumorales (Schajowicz 1993) (*figure 9*). Trois groupes sont identifiés selon leur localisation, les ostéosarcomes de surface, ceux de siège intracortical et les ostéosarcomes intramédullaires ou centraux qui sont les plus fréquents. Les ostéosarcomes intracorticaux sont extrêmement rares et seules des observations isolées sont rapportées. Les ostéosarcomes développés à la surface de l'os sont généralement de bon pronostic, et les ostéosarcomes centraux regroupent différentes variantes histologiques qui, à l'exception de la forme dite « bien différenciée intramédullaire », sont toutes de haute malignité.

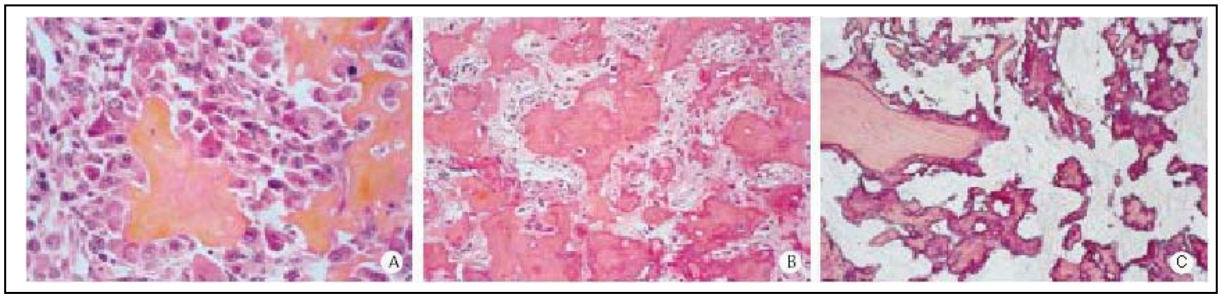

Figure 9 : **Préparations histologiques d'un ostéosarcome**

A : Aspect typique d'un ostéosarcome, constitué d'ostéoblastes tumoraux aux anomalies cytonucléaires marquées, aux mitoses nombreuses, élaborant une substance ostéoïde formant deux épaisses travées qu'elles entourent. Coloration hémalun-éosine-safran (HES), grossissement x 400. B : Dans d'autres secteurs de la même tumeur, l'aspect diffère, associant de nombreuses travées osseuses de taille et de forme variables. Coloration HES, grossissement x 200. C **:** Résection après chimiothérapie d'induction. Pour ce bon répondeur, les cellules tumorales ont disparu, remplacées par un tissu fibreux hyalin et des travées osseuses de disposition irrégulière. Une travée osseuse normale résiduelle, en partie résorbée, persiste (en haut et à gauche). Coloration hémalun-éosine-safran (HES), grossissement x200. (JM Guinebretière 2001)

L'ostéosarcome est la plus fréquente des tumeurs malignes primitives de l'os avec une incidence annuelle d'environ 4,8 nouveaux cas par million d'enfants de moins de 20 ans aux USA (Linet et al. 1999) et 3.6 nouveaux cas par million d'enfant de moins de 15 ans an France (Desandes et al. 2004). Soixante pour cent des ostéosarcomes surviennent dans la seconde décade de la vie, ils sont exceptionnels avant 6 ans mais peuvent survenir à tout âge après cet âge. Il y a une relative prédominance masculine avec un sex-ratio moyen de 1,4 : 1. L'âge de survenue au moment de la poussée de croissance pubertaire, la localisation souvent

métaphysaire de ces tumeurs et l'incidence élevée des ostéosarcomes chez les chiens de grande taille suggèrent un lien entre la croissance osseuse et la survenue des ostéosarcomes. L'étiologie est encore inconnue à ce jour, même si de nombreuses théories ont été émises à partir d'observations expérimentales. On suspecte par exemple des origines virales, traumatiques, chimiques, ou encore des erreurs de réplication de l'ADN, mais aucune n'a été confirmée chez l'homme. On constate également une plus grande fréquence de développement chez les sujets de grande taille et à l'inverse une plus faible incidence dans les populations asiatiques et latino-américaines. L'ostéosarcome secondaire, se rencontre chez l'adulte ayant préalablement développé une maladie de Paget, une dysplasie fibreuse, une tumeur à cellules géantes ou suite à une radiothérapie (Guinebretière JM 2001). L'ostéosarcome, peut dans de très rares cas être familial et correspond à la présence d'un gène de prédisposition aux tumeurs. On peut citer le syndrome de Li-Fraumeni qui correspond à l'anomalie innée de l'un des allèles du gène p53, l'enfant développant de multiples tumeurs aussi bien sarcomateuses (os et tissus mous) que carcinomateuses ; également le rétinoblastome bilatéral, maladie caractérisée par des tumeurs oculaires bilatérales et où les ostéosarcomes s'observent 100 fois plus fréquemment que dans la population « normale ».

Les anomalies cytogénétiques observées dans les ostéosarcomes sont caractérisées par leur grande complexité (*Figure 10*). Il n'existe pas de lésion génétique spécifique reconnue, mais une fréquente atteinte des locus des gènes p53 (environ 50% des patients) (Sandberg et al. 2003) et du rétinoblastome (environ 70% des patients) a été observée (Wadayama et al. 1994; Fuchs et al. 2002). Une étude réalisée sur une cohorte de 29 patients atteints d'ostéosarcome a ainsi montré que 38% d'entre eux présentent une ou plusieurs altérations génétiques touchant des molécules impliquées dans le contrôle de la transition G1-S, et donc dans l'entrée dans le cycle cellulaire (Lopez-Guerrero et al. 2004). Il s'agit principalement de mutations du gène RB, p53, et/ou de l'amplification des gènes MDM2 (inhibiteur de p53) et CDK4 (Cycline D4, nécessaire à la transition G1-S). Une activation du proto-oncogène c-*myc* (control de la prolifération/différenciation, adhérence et apoptose) (Sandberg et al. 2003) ainsi que du facteur de transcription Ap-1 (homo ou hétérodimère de c-Jun et c-Fos régulant des gènes essentielles pour la prolifération et la différenciation des ostéoblastes) (Papachristou et al. 2003) a également été observée dans des ostéosarcomes.

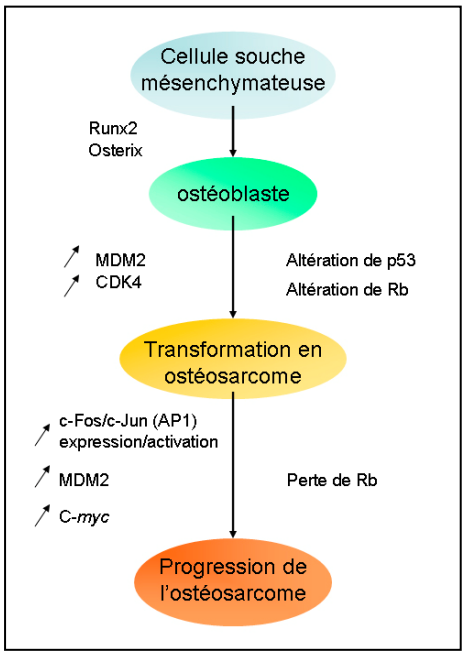

Figure 10 : **Mécanismes moléculaires et génétiques impliqués dans le développement et la progression des ostéosarcomes.**

Cette tumeur est majoritairement localisée au genou et à l'épaule, avec une atteinte presque toujours métaphysaire des os longs ; en effet moins de 10% des ostéosarcomes surviennent à la diaphyse et les localisations épiphysaires sont rares (*figure 11*). Il existe aussi des ostéosarcomes du tronc et du crâne mais pratiquement jamais des pieds ou des mains. D'un point de vue clinique, la principale manifestation est la douleur au site tumoral, irradiant vers les articulations de voisinage. Une masse palpable apparaît plus tardivement, sensible à la palpation, pouvant gêner la mobilité de l'articulation selon sa taille. A un stade plus tardif, des signes inflammatoires cutanés et une stase veineuse sont visibles.

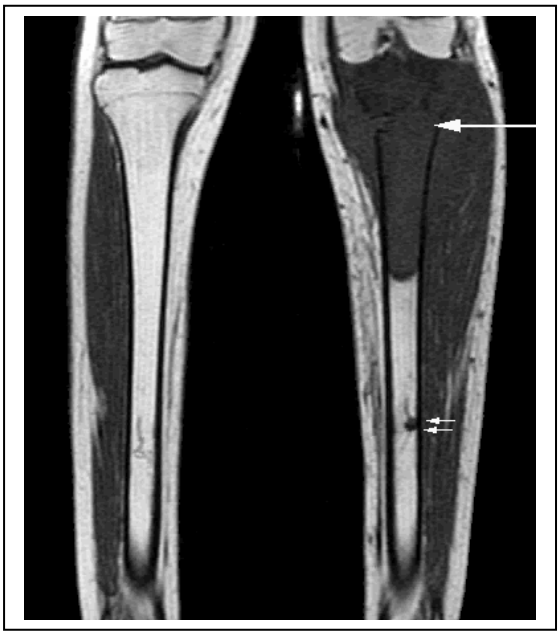

Figure 11 **: Ostéosarcome d'un patient âgé de 15 ans, IRM:** atteinte épiphyso-métaphysaire du tibia (grande flèche) avec skip métastase diaphysaire (petite flèche). (IGR Villejuif)

Le développement d'un ostéosarcome entraîne, comme nous l'avons vu précédemment, la formation directe d'os ou de substance ostéoïde mais également, dans un certain nombre de cas, une ostéolyse maligne (*figure 12*) qui résulte d'une hyperactivité ostéoclastique, consécutive à la sécrétion de cytokines d'origine tumorale, telles que l'IL-1, IL-6, les Tumor Necrosis Factor α et β (TNFα et β), un peptide associé à la parathormone ou Parathormone related peptide (PTH-rP) et des facteurs de croissance comme le Transforming Growth Factor β (TGFβ).

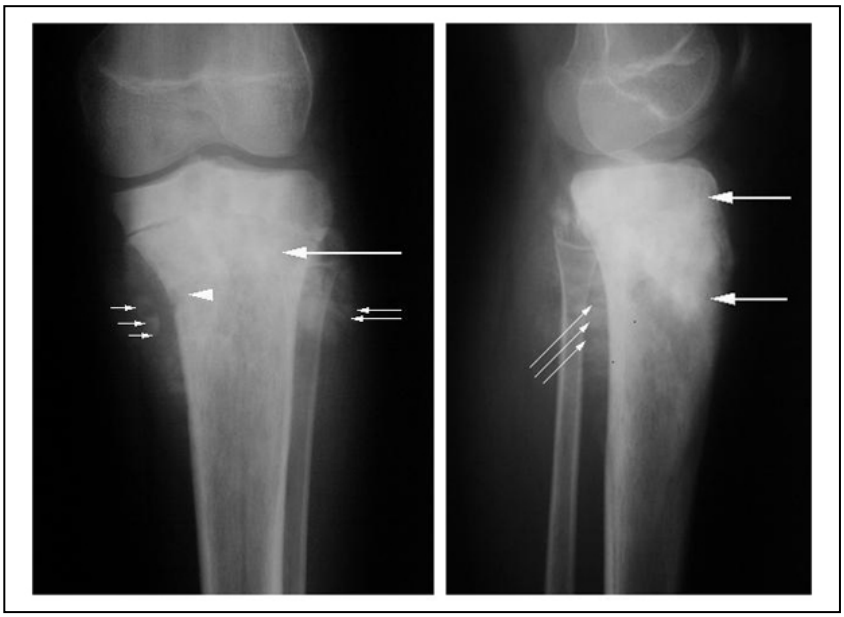

Figure 12 : **Ostéosarcome d'un patient âgé de 15 ans, Radiographies sans préparation du tibia.** Ostéocondensations hétérogènes de la métaphyse et de l'épiphyse tibiale supérieure (flèches), ostéolyse corticale (tête de flèche), Ostéogénèse dans les parties molles péri osseuses (petites flèches). (IGR Villejuif)

L'action de ces différents facteurs est soit directe soit indirecte, et dans ce cas, elle passe par l'activation des ostéoblastes puis par l'interaction RANKL/RANK. La résorption osseuse libère à son tour des facteurs de croissance [TGFβ et Insulin-like Growth Factor (IGF-1)] piégés dans la matrice extracellulaire. Les produits de dégradation de la matrice stimulent alors la prolifération maligne et un cercle vicieux s'installe entre la prolifération tumorale et l'ostéolyse associée (*figure 13*).

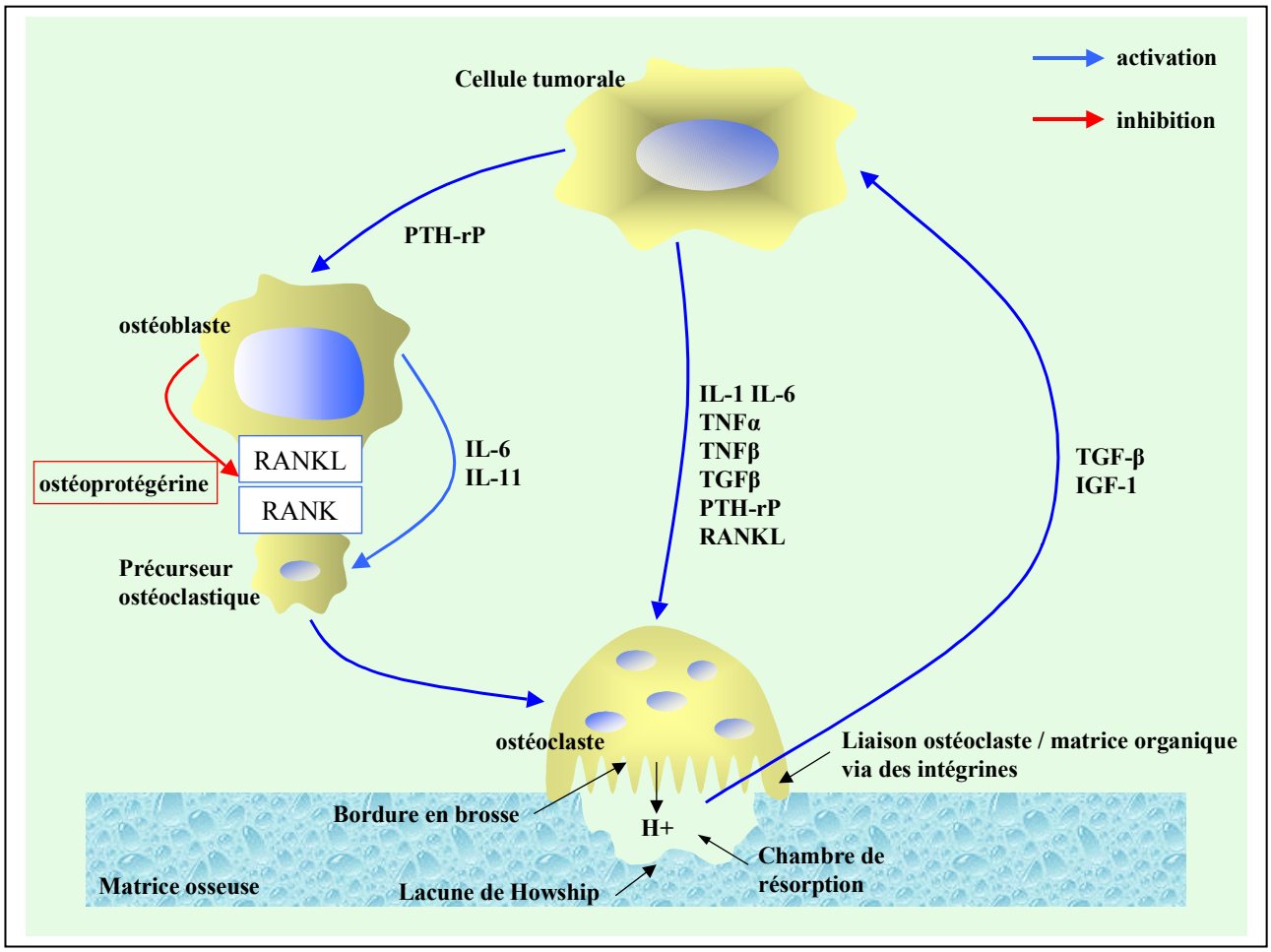

Figure 13 **: Cercle vicieux entre prolifération tumorale et hyper-résorption osseuse d'origine maligne.**

Entre résorption et prolifération tumorale s'instaure un cercle vicieux dans lequel le TGFβ et l'IGF-1 libérés de la matrice lors de l'hyper-résorption osseuse stimulent l'activité et la prolifération des cellules tumorales, qui à leur tour produisent des facteurs, qui vont activer la résorption osseuse, directement ou *via* les ostéoblastes.

Traitement des ostéosarcomes

Actuellement, la survie des patients atteints d'ostéosarcome a été considérablement augmentée grâce à la polychimiothérapie, qui repose sur des cures alternées combinant différents agents pharmacologiques. Les protocoles thérapeutiques actuels correspondent à la succession d'une chimiothérapie d'induction, chirurgie et chimiothérapie adjuvante. La réduction tumorale qu'elle induit rend possible la préservation du membre au moyen de techniques de chirurgie conservatrice, tout en assurant une résection carcinologiquement satisfaisante (Philip T et al. 2000). La chimiothérapie postopératoire est adaptée en fonction de la qualité de la réponse histologique à la chimiothérapie préopératoire. Ce paramètre pronostique essentiel est exprimé généralement en pourcentage de cellules vivantes résiduelles au sein de la tumeur primitive, par exemple chez un bon répondeur moins de 10% de cellules tumorales résiduelles sont retrouvées *(figure 14)*. Cette prise en charge permet aujourd'hui d'obtenir des taux de survie à 5 ans variant entre 50 et 70% selon les séries (Alapetite et al. 2001; Guinebretière JM 2001). La radiothérapie, quant à elle, n'est que rarement utilisée du fait de la radiorésistance de ce type de tumeur.

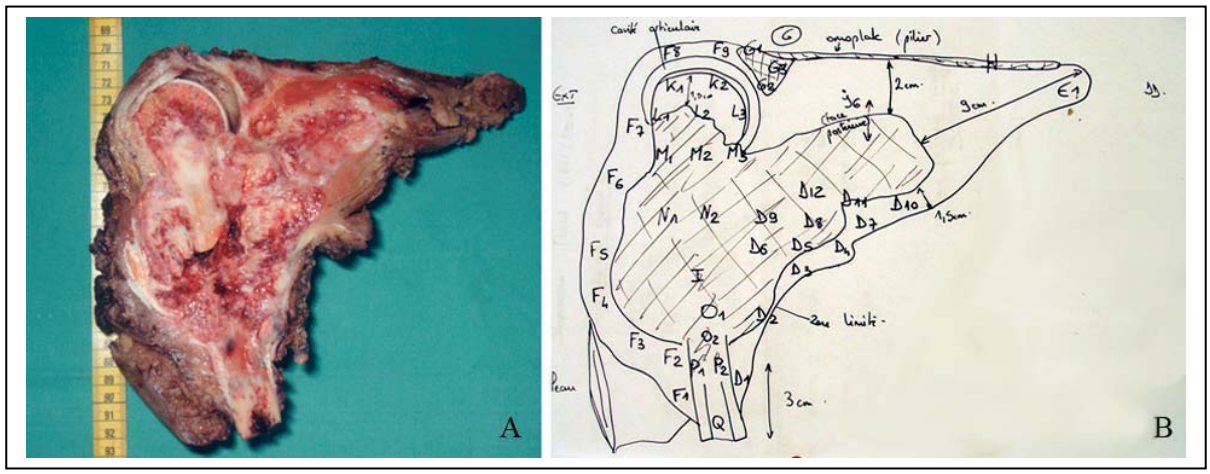

Figure 14 : **Résection tumorale de hanche après chimiothérapie et analyse anatomopathologique associée.**

A : Résection monobloc de la hanche

B : Lors de l'examen microscopique, le pathologiste précise, pour chacun des prélèvements, la présence ou non de la tumeur et le pourcentage de cellules résiduelles pour les différentes zones est retranscrites sur le schéma. Peuvent ainsi être calculées la somme de tous les taux et surtout la moyenne qui, en plus de l'âge et d'autres facteurs, sert au choix du type de chimiothérapie adjuvante.

L'objectif de la chimiothérapie cytotoxique administrée par voie générale est de détruire la tumeur en place mais également d'inhiber le phénomène métastatique. La majorité des tumeurs osseuses malignes sont diagnostiquées en phase localisée. La chimiothérapie en phase localisée de la maladie permet un traitement précoce de la maladie micrométastatique, qui peut être ultérieurement responsable de la rechute et du décès du patient ; une réduction de la taille de la tumeur facilitant le geste du chirurgien et par conséquent une chirurgie de conservation du membre (Alapetite et al. 2001). Lorsque des métastases pulmonaires surviennent d'emblée, leur traitement est identique à celui des formes localisées en matière de chimiothérapie et de chirurgie de la tumeur. Après celle-ci, une exérèse chirurgicale des lésions pulmonaires est proposée dans la mesure où elle est techniquement réalisable, c'est la seule façon d'obtenir des rémissions à long terme dans ces formes à très mauvais pronostic.

Les quatre molécules les plus utilisés (plus de 20% de réponse en monothérapie) dans le traitement des ostéosarcomes sont le méthotrexate à haute dose, la doxorubicine, le cisplatine et l'ifosfamide (van Oosterom et al. 1992).

-Le méthotrexate (utilisé chez les jeunes patients) est un agent cytotoxique anti-métabolite qui agit en inhibant une enzyme-clé de la synthèse de la thymidine, la dihydrofolate réductase. Après l'injection de méthotrexate à doses élevées (supérieures à 100 mg/m^2), il est nécessaire d'administrer l'antidote de ce médicament qui est l'acide folinique, afin de prévenir les principaux effets secondaire qui sont la toxicité rénale et neurologique centrale d'insuffisance rénale (Blackwell 1995).

-La doxorubicine est un agent cytotoxique considéré initialement comme un agent intercalant, qui agit également en inhibant des enzymes impliquées dans le désenroulement de l'acide désoxyribonucléique (ADN), les topo-isomérases de type II. Les principaux effets secondaires immédiats de ce médicament sont la toxicité hématologique et la toxicité muqueuse, cette toxicité dépend de la dose et du schéma d'administration (Link et al. 1986).

-Le cisplatine est un agent cytotoxique alkylant qui agit en induisant la formation de « ponts inter- et intrabrins » dans les chaînes d'ADN. Ce médicament est généralement administré sur un mode séquentiel avec une dose par cure de 80 à 120 mg/m^2. Les principaux effets secondaires de ce médicament sont la toxicité hématologique, la toxicité néphrologique, une

toxicité neurologique périphérique et auditive. Cette toxicité impose l'administration d'une hyperhydratation saline pendant l'administration du cisplatine (Cvitkovic E 1993).
L'ifosfamide est un agent cytotoxique alkylant qui agit en induisant la formation de ponts interbrins en se fixant sur le N7 des guanines. Ce médicament est généralement administré sur un mode séquentiel. Les principaux effets secondaires de ce médicament sont la toxicité hématologique, l'alopécie, la toxicité neurologique, la toxicité néphrologique et la toxicité vésicale (Cvitkovic E 1993). Cette dernière toxicité impose l'administration conjointe de mesna et d'une hyperhydratation alcaline. Le cyclophosphamide a également été utilisé dans les programmes de chimiothérapie adjuvante, et de phase II pour les ostéosarcomes. Le taux de réponse en monothérapie est cependant faible et l'efficacité du protocole est discutée (Mosende et al. 1977).

Malheureusement, une absence de réponses aux drogues anti-tumorales est fréquemment observée entraînant le développement de métastases pulmonaires puis le décès du patient. Il est donc nécessaire de trouver une approche alternative aux traitements classiques de l'ostéosarcome.

C/ Chondrosarcome

Le chondrosarcome est défini pour la première fois par Lichtenstein et Jaffe en 1943 (Lichtenstein L 1943). Il s'agit d'un sarcome dont les cellules tumorales sont associées à une matrice cartilagineuse. Cette composante cartilagineuse peut être associée à des cellules fibroblastiques ; en revanche, il ne doit pas y avoir de formation d'os tumoral. La localisation dans la pièce osseuse peut être centrale (endomédullaire) ou périphérique (à la surface de la corticale).

Le chondrosarcome est la troisième tumeur osseuse maligne primitive la plus fréquente après l'ostéosarcome et le sarcome d'Ewing. Il représente de 11 à 22 % des tumeurs osseuses malignes primitives et présenterait une légère prédominance masculine (Dahlin 1978). L'âge de survenue de ces tumeurs se situe principalement entre 40 et 70 ans. La croissance très lente de ces tumeurs leur permet une installation insidieuse ce qui conduit à une apparition tardive des symptômes. Si tout le squelette peut être atteint, les zones les plus fréquemment concernées sont le bassin (30%) et le fémur (20%) (Lee et al. 1999).

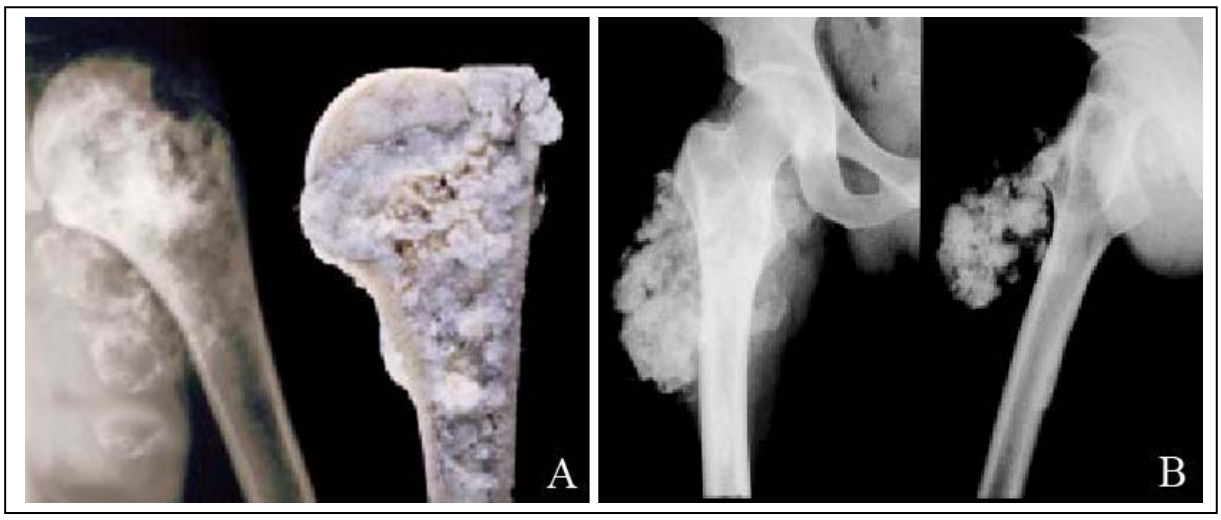

Figure 15 : **Aspect morphologique et radiographique des chondrosarcomes**

A : Chondrosarcome sur humérus, lobules tumoraux blanc nacré, gris-bleu

B : Chondrosarcome périosté du fémur. Calcification floconneuse

(Anract et al. 2001)

L'aspect radiologique est celui d'une tumeur détruisant partiellement l'os, mal délimitée, associée à une tumeur des parties molles. Dans plus de deux tiers des cas des calcifications sont visibles dans la matrice tumorale. Les calcifications floconneuses, dites en « pop-corn » sont les plus typiques (*Figure 15*). Macroscopiquement les chondrosarcomes sont habituellement des tumeurs de grande taille, lobulées de couleur blanc nacré ou gris-bleu, aux contours souvent mal définis (*figure 15*). Histologiquement, des lobules tumoraux de taille et de formes irrégulières sont observés. Des fragments d'os spongieux résiduel, englobés par la prolifération, persistent parfois au sein de ces lobules. Les chondroblastes tumoraux ont un noyau volumineux, à chromatine densifiée. Les aspects de binucléation sont fréquents. Une activité mitotique peut parfois être individualisée, essentiellement dans les tumeurs de haut grade (*figure 16*). Ces caractéristiques histologiques et cytologiques sont utilisées pour établir le grade des chondrosarcomes comme l'ont proposé O'Neal et Ackerman (O'Neal et al. 1952). Les chondrosarcomes de grade I, de faible malignité, sont les plus difficiles à différencier des chondromes. Leur cellularité est plus importante que celle d'un chondrome. Les cellules cartilagineuses sont peu atypiques avec un noyau densifié. L'importance de la cellularité tumorale et des atypies cytonucléaires augmente dans les grades II (malignité intermédiaire) et III (haute malignité).

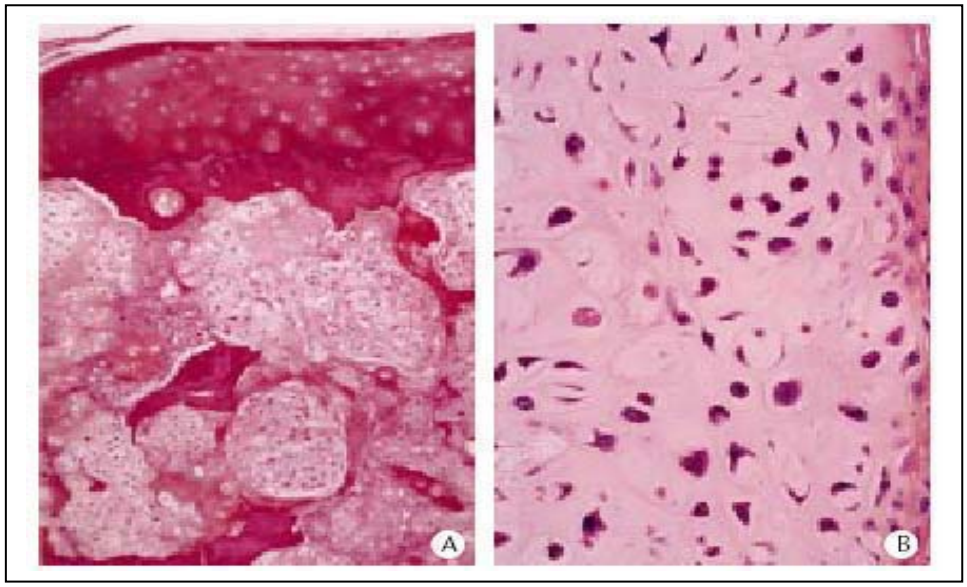

Figure 16 : **préparation histologique d'un chondrosarcome**

A : Chondrosarcome constitué de lobules cartilagineux infiltrant les espaces médullaires et englobant de l'os spongieux résiduel (faible grossissement). B : Chondrocytes tumoraux avec noyau volumineux et chromatine densifiée et aspects de binucléation (fort grossissement). (Anract et al. 2001)

Traitement des chondrosarcomes

Le traitement chirurgical représente le traitement exclusif des chondrosarcomes. En effet, la chimiothérapie n'a aucune efficacité sur les chondrosarcomes. Cela est probablement dû à la diffusion médiocre de ces produits dans la matrice cartilagineuse qui est peu ou pas vascularisée. Des travaux expérimentaux de Wyman et al (Wyman et al. 1999) suggèrent également que les cellules de chondrosarcome expriment le gène multidrug-resistance-1 (Mdr-1). De plus la radiothérapie est quant à elle inefficace, en effet, le chondrosarcome est considéré comme une tumeur radiorésistante (Springfield et al. 1996), en partie à cause de l'absence de l'anti-oncogène $p16^{ink4}$ (Moussavi-Harami et al. 2006).

Le taux de survie des patients varie entre 50 et 60% à 10 ans et des métastases, principalement pulmonaires surviennent chez 10 à 25% des patients (le volume tumoral étant un facteur pronostique important pour l'apparition des métastases). De plus le fort taux de récidive associé à la morbidité de la résection chirurgicale en zone saine font qu'il est nécessaire, comme pour l'ostéosarcome, de mettre en place une approche alternative aux traitements classiques du chondrosarcome.

III. Objectif de la thèse

Le tissu osseux est un tissu conjonctif complexe en perpétuel remaniement, reposant sur l'équilibre de deux mécanismes: la formation et la résorption osseuse. Tout déséquilibre entre ces processus est à l'origine de pathologies qui peuvent être ostéocondensantes ou ostéolytiques, bénignes ou malignes. L'ostéosarcome est la plus fréquente des tumeurs osseuses primitives et est très souvent associée à une ostéolyse. Les traitements actuels, qui consistent en une résection chirurgicale de la tumeur et en une polychimiothérapie (associant principalement le méthotrexate, le cisplatine, la doxorubicine et l'ifosfamide), ont largement contribué à la progression du taux de survie des patients. Mais la forte toxicité des molécules de chimiothérapie utilisées, souvent mal tolérées chez les jeunes patients, dont la médiane d'âge est de 18 ans, ainsi que le mauvais pronostic associé à la détection de métastases pulmonaires, font qu'il est nécessaire de développer de nouvelles approches thérapeutiques des ostéosarcomes.

Le développement d'un ostéosarcome entraîne comme nous l'avons vu précédemment la formation directe d'os ou de substance ostéoide mais également, dans un certain nombre de cas, une ostéolyse maligne qui résulte d'une hyperactivité ostéoclastique, consécutive à la sécrétion de cytokines d'origine tumorale. L'action de ces différents facteurs est soit directe soit indirecte, et dans ce cas, elle passe par l'activation des ostéoblastes puis par l'interaction RANKL/RANK. La résorption osseuse libère à son tour des facteurs de croissance piégés dans la matrice extracellulaire. Les produits de dégradation de la matrice stimulent alors la prolifération maligne et un cercle vicieux s'installe entre la prolifération tumorale et l'ostéolyse associée.

Dans ce contexte, notre hypothèse est le développement de protocoles thérapeutiques utilisant une molécule anti-tumorale et une molécule anti-résorption osseuse afin de briser le cercle vicieux en ses deux points les plus sensibles. Les bisphosphonates et en particulier l'acide zolédronique représente un candidat de choix du fait de sa très forte activité anti-résorption osseuse et de son affinité pour les sites d'ostéolyse. De plus un certain nombre d'études commencent à évoquer un potentiel effet anti-tumoral de l'acide zolédronique. Ces différents points seront détaillés un peu plus loin.

Les différents objectifs de cette étude seront :

- Démontrer l'effet anti-tumoral direct de l'acide zolédronique sur les cellules d'ostéosarcome et de chondrosarcome *in vitro*.

- Eclaircir les mécanismes d'action intra-cellulaire de l'acide zolédronique sur les cellules tumorales.

- Démontrer l'efficacité de l'acide zolédronique seul ou associé à un agent de chimiothérapie dans le traitement du chondrosarcome, de l'ostéosarcome et des métastases pulmonaires associés ainsi que dans le traitement des métastases osseuses secondaires à un carcinome prostatique.

- Démontrer la possibilité d'une résistance des cellules tumorales à l'acide zolédronique et déterminer les mécanismes responsables de cette résistance afin de pouvoir établir le meilleur protocole thérapeutique possible.

PARTIE I

Traitement des ostéosarcomes par l'acide zolédronique

I. Les bisphosphonates, l'acide zolédronique et les tumeurs osseuses

Introduction

Les bisphosphonates constituent la classe d'agents anti-résorption la plus efficace et la plus utilisée dans le traitement des hypercalcémies associés aux pathologies osseuses métaboliques ou aux ostéolyses tumorales ou l'hypercalcémie (Coleman 2001).

Les bisphosphonates sont des analogues synthétiques du pyrophosphate endogène (PPi), leur structure P-C-P leurs confère la capacité de se fixer aux ions divalents tels que le Ca^{2+} (Rogers 2003). Pour cette raison, les bisphosphonates quittent rapidement la circulation sanguine pour se fixer à la surface osseuse *in vivo* aux sites de remodelage actif et en particulier aux sites de résorption ostéoclastique. Cette affinité particulière des bisphosphonates pour les zones de remodelages osseux peut expliquer leurs effets hautement spécifiques pour les ostéoclastes. Cependant, ceci n'exclut pas la possibilité d'un effet des molécules sur les cellules voisines telles que les ostéoblastes, les cellules de la moelle osseuse ou encore les cellules tumorales.

Les bisphosphonates de première génération (dont la structure est très proche de celle du PPi ; par exemple le clodronate et l'étidronate) sont métabolisés par les cellules de mammifères en analogue toxique de l'ATP (Frith et al. 1997). L'accumulation dans le cytoplasme des ostéoclastes de ce métabolite toxique inhibe la résorption osseuse en induisant l'apoptose des cellules ostéoclastiques (Frith et al. 2001), probablement en inhibant des enzymes dépendantes de l'ATP composant les pores de perméabilité mitochondriale (Lehenkari et al. 2002).

Les bisphosphonates contenant un atome d'azote (N-BPs : tel que l'acide zolédronique) quant à eux ne sont pas métabolisés en analogue toxique de l'ATP mais agissent en inhibant une enzyme clef de la voie du mévalonate : la farnésyl diphosphate synthase (FPPs). Des études ont montré qu'une légère modification conformationelle de la chaine latérale R^2, déjà connue pour ses effets sur le potentiel anti-résorption des ostéoclastes (Dunford et al. 2001), affecterait également l'inhibition de la FPP synthase (Rogers 2003). Ces études suggèrent que la FPP synthase est la cible pharmacologique majeure des N-BPs dans les ostéoclastes et permettraient d'expliquer la relation entre la structure des

bisphosphonates et leur potentiel anti-résorption. Récemment, grâce à la co-cristallisation de la FPPs humaine et de l'acide zolédronique, le mécanisme par lequel les N-BPs inhibent la FPPs a commencé à s'éclaircir (Kavanagh et al. 2006; Rondeau et al. 2006). En effet, ces études révèlent que dans un premier temps les N-BPs interagissent avec le site de liaison au géranyl diphosphate de l'enzyme, stabilisant ainsi l'interaction de la partie nitrogène du N-BP avec un résidu thréonine lysine. Les analyses de cinétique enzymatique indiquent que les interactions des N-BPs avec la FPPs peuvent être très complexes. En effet, les N-BPs entrent directement en compétition avec le diméthylallyl diphosphate ou le GPP pour la fixation à leur site de liaison à la FPPs (Kavanagh et al. 2006). Cette compétition est suivie par des interactions encore plus complexes modifiant la conformation et la structure tertiaire de l'enzyme permettant d'expliquer le fort potentiel inhibiteur des N-BPs pour cette enzyme.
En inhibant la FPPs, les N-BPs inhibent la synthèse du farnésyl diphosphate (FPP) et du géranylgéranyl diphosphate (GGPP). Or, ces deux métabolites sont essentiels à une modification post-traductionnelle des protéines : la prénylation. La principale conséquence est l'inhibition de prénylation des petites GTPase (Lane et al. 2006) ; cette inhibition a pu être démontrée dans les ostéoclastes aussi bien *in vitro* (Sato et al. 1990; Breuil et al. 1998) que *in vivo* (Frith et al. 2001). L'inhibition de la prénylation des petites GTPase telles que celles de la famille de Ras, Rho et Rab inhibe leur ancrage à la membrane et par conséquent perturbe d'importantes voies de signalisation essentielles aux ostéoclastes (Coxon et al. 2003). Une étude récente fait état d'un nouveau type de mécanisme par lequel les N-BPs pourraient atteindre les ostéoclastes, toujours en agissant sur la voie du mévalonate (Monkkonen et al. 2006). En effet, l'inhibition de la FPP synthase provoque l'accumulation de son substrat, l'IPP, qui semble s'associer à l'AMP pour former un nouvel analogue de l'ATP : L'ApppI. Ce métabolite, à l'instar de celui des bisphosphonates de première génération, inhiberait l'adénine nucléotide translocase (composant les pores de perméabilité mitochondriale) et induirait la mort par apoptose des ostéoclastes.

La FPP synthase étant une enzyme hautement conservée et ubiquitaire, les N-BPs peuvent potentiellement affecter n'importe quel type cellulaire *in vitro*, donc potentiellement les cellules tumorales. De nombreux effets de N-BPs ont déjà été démontrés *in vitro* sur la survie, la prolifération, l'adhérence, la migration et l'invasion des cellules tumorales (Green 2004).

Article 1

« Les bisphosphonates: nouveaux agents thérapeutiques pour le traitement des tumeurs osseuses. »

Trends in Mol Med, Vol.10 No.7 July 2004, Heymann D, Ory B, Gouin F, Green J R and Rédini F**.**

Les Bisphosphonates ont été utilisés avec succès depuis des années dans le traitement des tumeurs osseuses, bénignes ou malignes, caractérisées par une hyperactivité de la résorption osseuse de la part des ostéoclastes. Jusque récemment, il était admis que l'efficacité clinique des bisphosphonates dans le traitement des patients atteints de métastases osseuses n'était le fruit que de l'inhibition des ostéoclastes et de leur activité de résorption. Cependant, de récentes études ont démontré que les Bisphosphonates étaient capables d'agir directement, en culture, sur des cellules tumorales en inhibant leur prolifération, leur capacité d'adhérence et de migration, et même d'induire leur apoptose. Ces résultats suggèrent que les Bisphosphonates peuvent être des agents anti-tumoraux ayant la capacité d'inhiber la colonisation des organes viscéraux par les cellules tumorales. Cependant, les résultats issus de différentes études cliniques sont contradictoires et le potentiel anti tumoral des Bisphosphonates reste contesté.

Review TRENDS in Molecular Medicine Vol.10 No.7 July 2004

Bisphosphonates: new therapeutic agents for the treatment of bone tumors

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Bisphosphonates (BPs) have been used successfully for many years to reduce the skeletal complications associated with the benign and malignant bone diseases that are characterized by enhanced osteoclastic bone resorption. Until recently, it was thought that the clinical efficacy of BPs in the treatment of cancer patients with bone metastases was purely a result of the inhibition of osteoclast-mediated bone resorption. However, recent studies have demonstrated that BPs inhibit the growth, attachment and invasion of cancer cells in culture and promote their apoptosis. These results suggest that BPs are also anti-cancer agents, raising the possibility that BPs could inhibit cancer-cell colonization in visceral organs. However, results from clinical trials are conflicting, and whether BPs possess anti-cancer effects or not remains controversial.

The clinical use of bisphosphonates (BPs) has increased dramatically during the past ten years. The most common indicator for the use of these compounds is osteoporosis, but their use in osteolytic bone diseases, in which enhanced bone resorption occurs (including Paget's disease and hypercalcemia of malignancy), has increased rapidly. Initially, the efficacy of BPs, particularly for the treatment of hypercalcemia and for the reduction of the skeletal complications of metastases, was thought to be solely by the inhibition of osteoclast activity. In addition to having an apoptotic and anti-proliferative effect on osteoclasts, BPs might exert a similar influence on macrophages and tumor cells. BPs reduce proliferation and induce apoptosis of tumor cell lines, and they inhibit tumor-cell adhesion and invasion in the extracellular matrix *in vitro*. Furthermore, recent preclinical research has shown that BPs exert potent anti-tumor activity in bone sites. Although an anti-tumor action for BPs on bone metastasis seems to be accepted, animal data concerning the effect of BPs on the incidence and growth pattern of non-osseous metastases is less conclusive. In spite of considerable data, the evidence for a direct anti-tumor action of BPs *in vivo* is much debated, and appears to depend on the site of the tumor.

Bisphosphonate groups

BPs are simple chemical compounds that are based on a phosphorous–carbon–phosphorous template. They are similar to endogenous pyrophosphates, but have a carbon molecule replacing the central oxygen molecule, which enables the accommodation of two additional substituents, R1 and R2 (Figure 1). BPs form a three-dimensional; structure that is capable of binding to divalent metal ions, such as Ca^{2+}, Mg^{2+} and Fe^{2+}, in a bidentate manner through the coordination of one oxygen from each phosphate group with the divalent cation. The affinity for Ca^{2+} can be increased further if the R1 side chain is a hydroxyl (OH) or primary amino (NH_2) group, because this enables the formation of a tridentate conformation that is able to bind to Ca^{2+} more effectively [1]. Different chemical groups attached to the second free carbon atom account for the variability in anti-resorptive potency of the bisphosphonates. There are three main groups of BPs (Figure 1): (i) the first-generation compounds, such as clodronate and etidronate, which have been in clinical use for more than 30 years, possess simple substituents attached to the central carbon and exert comparatively weak actions on bone resorption [2]; (ii) the more potent second-generation BPs (for example, pamidronate, alendronate and ibandronate) are characterized by an aliphatic side chain containing a single nitrogen atom (N–BPs); (iii) the third-generation N–BPs are characterized by a heterocyclic substituent, containing one nitrogen atom in a pyridyl ring (risedronate) or two nitrogen atoms in an imidazole ring (zoledronic acid) [2]. These are the most potent BPs that are currently available, which, together with a recently developed third-generation bisphosphonate, named YH529 or YM529 {1-hydroxy-2-[imidazo (1,2-a) pyridin-3-yl] ethylidene}, exhibit the strongest bone-resorption-inhibiting action among the currently known BPs [3].

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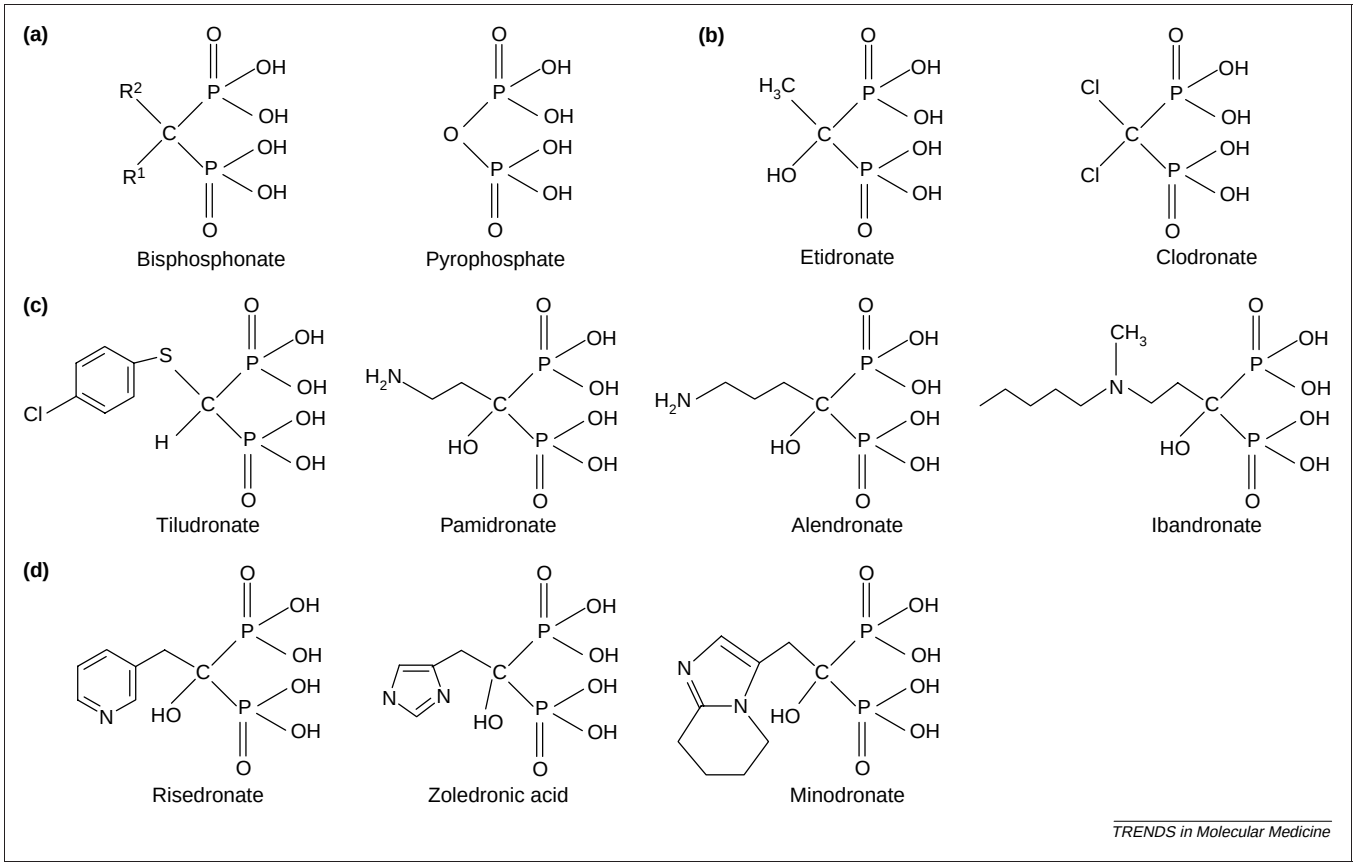

Figure 1. Generations of bisphosphonates: the structure of geminal bisphosphonates compared with pyrophosphate. **(a)** The general structures of bisphosphonate and pyrophosphate. **(b)** The first-generation bisphosphonates have short alkyl, halide or hydroxyl side chains. If etidronate has an *in vitro* antiresorptive potency of 1, clodronate has a relative potency of 10. **(c)** The second-generation bisphosphonates (with the exception of tiludronate) have a nitrogen atom in their side chain. Tiludronate has a relative potency of 10; pamidronate,100; alendronate, 100–1000; and ibandronate, 1000–10 000. **(d)** The third-generation bisphosphonates have a heterocyclic side chain. Risedronate has a relative potency of 1000–10 000; zoledronic acid, >10 000; and minodronate, >10 000.

Bisphosphonates inhibit osteoclast activity

BPs have became indispensable for the treatment of a wide spectrum of osteolytic bone diseases that are characterized by enhanced osteoclastic bone resorption. BPs selectively concentrate on the bone resorption surface, at the interface with the active osteoclast, where bone mineral is most exposed [4]. The local BP concentration at an active resorption site could be in the range 0.1–1 mM [5]. Initially, BPs were used as 'bone-seeking' agents that were widely used in skeletal scintigraphy for the delineation of bone metastases when labeled with ^{99}Technetium. Following internalization, the primary drug action is to inhibit osteoclast activity. Unlike their first-generation predecessors, which are metabolized into cytotoxic analogues of ATP, N–BPs inhibit the activity of farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) synthases, two key enzymes that are involved in the mevalonate pathway. Because FPP and GGPP are required for the post-translational lipid modification (prenylation) of small guanine triphosphatases, such as Ras, Rho and Rac [6], inhibiting the prenylation of these small intracellular GTPases could account for the majority, if not all, of the various effects on osteoclast function (Figure 2): (i) the loss of the ruffled border and disruption of the actin cytoskeleton, (ii) altered trafficking of membranes and intracellular proteins, such as the osteoclast proton ATPase, (iii) the disruption of integrin-induced intracellular signaling and (iv) the induction of osteoclast apoptosis [2,7].

All BPs appear to induce the apoptosis of osteoclasts by the activation of caspase-3-like proteases [8]. Although several studies have demonstrated that BPs can induce osteoclast apoptosis, it appears that their dominant effect is an early and rapid inhibition of osteoclast function resulting in reduced bone resorption, rather than through a decrease in cell viability or number [9]. Evidence from *in vitro* and animal models confirms that N–BPs also inhibit osteoclastogenesis and the recruitment of osteoclast progenitors into bone [10]. However, other mechanisms cannot be excluded. Recent *in vitro* studies have shown that BPs exert direct effects on osteoblasts, thereby affecting osteoblast proliferation, differentiation and gene expression [11]. Another potential mechanism by which N–BPs might inhibit osteoclastogenesis relates to the increased production of an anti-bone-resorption cytokine, termed osteoprotegerin (OPG), by pamidronate and zoledronic acid in primary human osteoblasts [12,13]. Accumulating evidence indicates that receptor activator of NF-κB ligand (RANKL), which is produced by bone marrow stromal cells and osteoblasts, is the final extracellular regulator of osteoclast development [14]. OPG is a soluble decoy receptor for RANKL that antagonizes its activities [15]. Therefore, the ratio RANKL to OPG regulates osteoclastogenesis, and a disturbance of this

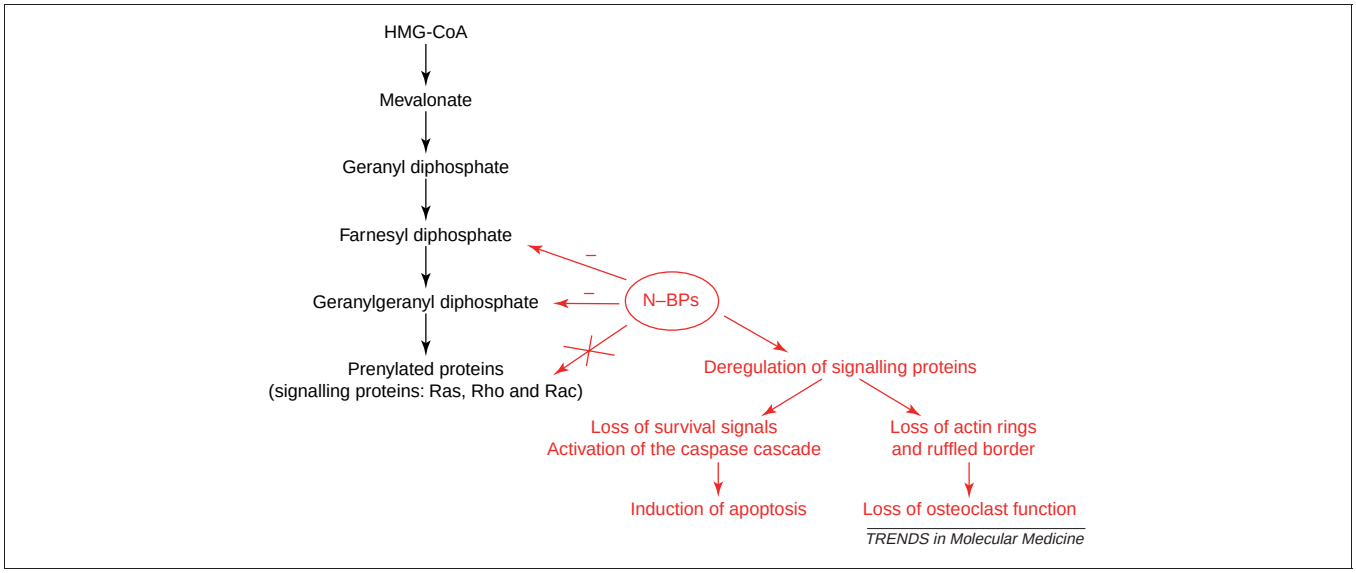

Figure 2. The mevalonate pathway and the proposed mechanism of action for N-containing bisphosphonates (N–BPs). Prenylated (especially geranylgeranylated) proteins are required for osteoclast formation and function. Cellular uptake of N–BPs by the osteoclast leads to inhibition of the mevalonate pathway and the loss of prenylated proteins, causing the inhibition of osteoclast activity and cell death by apoptosis. Abbreviation: HMG-CoA, hydroxymethylglutaryl-coenzyme A.

balance by BPs could explain part of the BP-induced inhibition of bone resorption. Moreover, alterations in the balance between RANKL and OPG are involved in pathological conditions in which elevated bone resorption occurs, such as the osteolysis that is associated with malignant bone diseases [16]. However, contradictory results have recently been published, reporting that the inhibitory action of alendronate and pamidronate on bone resorption does not involve the regulation of RANKL and OPG expression [17]. Taken together, these studies suggest numerous potential mechanisms by which BPs, and more specifically N–BPs, might inhibit bone resorption.

Anti-tumor effects of bisphosphonates

BPs have been successfully used to reduce the skeletal complications that are associated with bone metastases. Initially, it was thought that the specific inhibition of osteoclastic bone resorption was the only mechanism by which BPs were effective in the treatment of cancer patients with bone metastases. However, evidence is emerging from both preclinical and clinical studies to suggest that BPs also have antitumor activities that contribute to their therapeutic efficacy in malignant bone diseases. These antitumor effects seem to vary among the different compounds, in relation to their basic structure. The N–BPs have been suggested to be clinically superior to their first generation counterparts [18]. Some *in vitro* cell culture experiments have shown that BPs induce apoptosis in several human tumor cell lines, derived from breast, prostate, lung, renal, and pancreatic cancers, as well as in osteosarcoma, neuroblastoma and multiple myeloma cell lines. Caspase-dependent apoptosis appears to be the major mechanism that is responsible for the induction of tumor cell apoptosis (Figure 2) [19]. Recent data indicate that the induction of caspase-dependent apoptosis in breast and prostate-cancer cell lines by N–BPs is mediated by the impairment of Ras membrane localization, which is dependent on the prenylation of Ras [20]. In addition, the N–BP zoledronic acid has been shown to inhibit tumor-cell invasion by both RhoA-dependent and independent effects on the actin cytoskeleton and on the expression of CXCR-4, the stromal-derived factor-1 (SDF-1) receptor [21].

These *in vitro* findings are supported by data from several animal models showing that the newer N–BPs can reduce skeletal-tumor burden in myeloma, breast cancer, renal cancer and prostate cancer [22]. Interestingly, the N–BP zoledronic acid inhibited the development of osteoblastic, as well as osteolytic, bone lesions in a murine model of prostate cancer [23]. The precise mechanisms by which BPs inhibit the growth of bone tumors or bone metastasis, or whether the anti-tumor effects are direct or indirect remain unknown. A 'vicious cycle' has been described in osteolytic metastases, which consists of (i) the release of osteolytic mediators by tumor cells, (ii) bone degradation, (iii) the release of growth factors from degraded bone and (iv) enhanced tumor-cell growth, which is followed by the further release of osteolytic mediators [24]. The inhibitory effects of BPs on tumor cells might reflect either direct anti-tumor effects or indirect effects through osteoclast inhibition and an alteration of the bone microenvironment (the seed and soil hypothesis: Figure 3). The indirect mechanism of the suppressive action of BPs is thought to be the result of an inhibition of osteoclastic bone resorption, which, under normal circumstances, releases growth factors that are stored in the bone that can feed cancer cells that are colonizing bone.

Bisphosphonates can inhibit tumor growth through several mechanisms (Figure 4): the induction of tumor-cell apoptosis [19], the inhibition of cell growth [25], the inhibition of tumor-cell adhesion and spreading [26] and the inhibition of tumor-cell invasion [27]. These effects were observable with different ranges of BP concentrations: from 10^{-12}–10^{-8} M for the inhibition of tumor-cell invasion [24] to 10^{-5}–10^{-2} M for the inhibition of

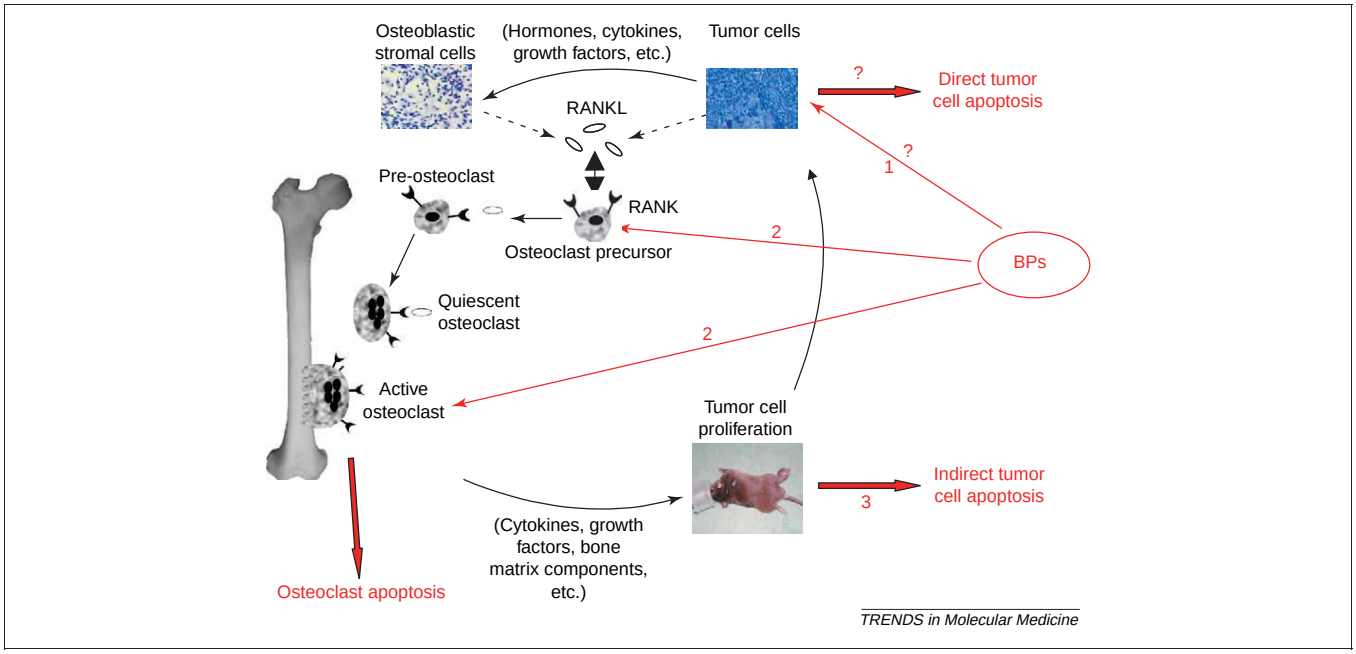

Figure 3. Schematic representation of tumor-cell induced osteolysis: the seed-and-soil hypothesis. Tumor cells might release soluble mediators, such as hormones, cytokines or growth factors, that act on osteoblastic stromal cells. The stromal cells and, occasionally, the tumor cells themselves produce RANKL (receptor activator of NF-κB Ligand), which binds to its cognate receptor, RANK, which is expressed on osteoclast precursors. The RANKL–RANK complex enhances the differentiation of pre-osteoclasts and their subsequent activation into mature osteoclasts that are capable of resorbing bone. Proposed mechanisms for the anti-tumor effects of bisphosphonates (BPs) include: (1) a direct effect on tumor growth and survival; (2) a direct inhibition of bone resorption through effects on osteoclast precursors and direct effects on mature osteoclasts; (3) indirect effects on primary tumor growth through the inhibition of bone resorption [through the effects described in (2)].

tumor-cell growth *in vitro* [25]. The specific mechanisms remain to be elucidated but it appears to involve an interaction with integrins [28] and the inhibition of the proteolytic activity of the matrix metalloproteinases 2, 9 and 12, through the chelation of zinc from the enzyme active site [27,29]. Another possible mode of action of N–BPs is the potent angiostatic effects on human endothelial cells demonstrated by *in vitro* and *in vivo*

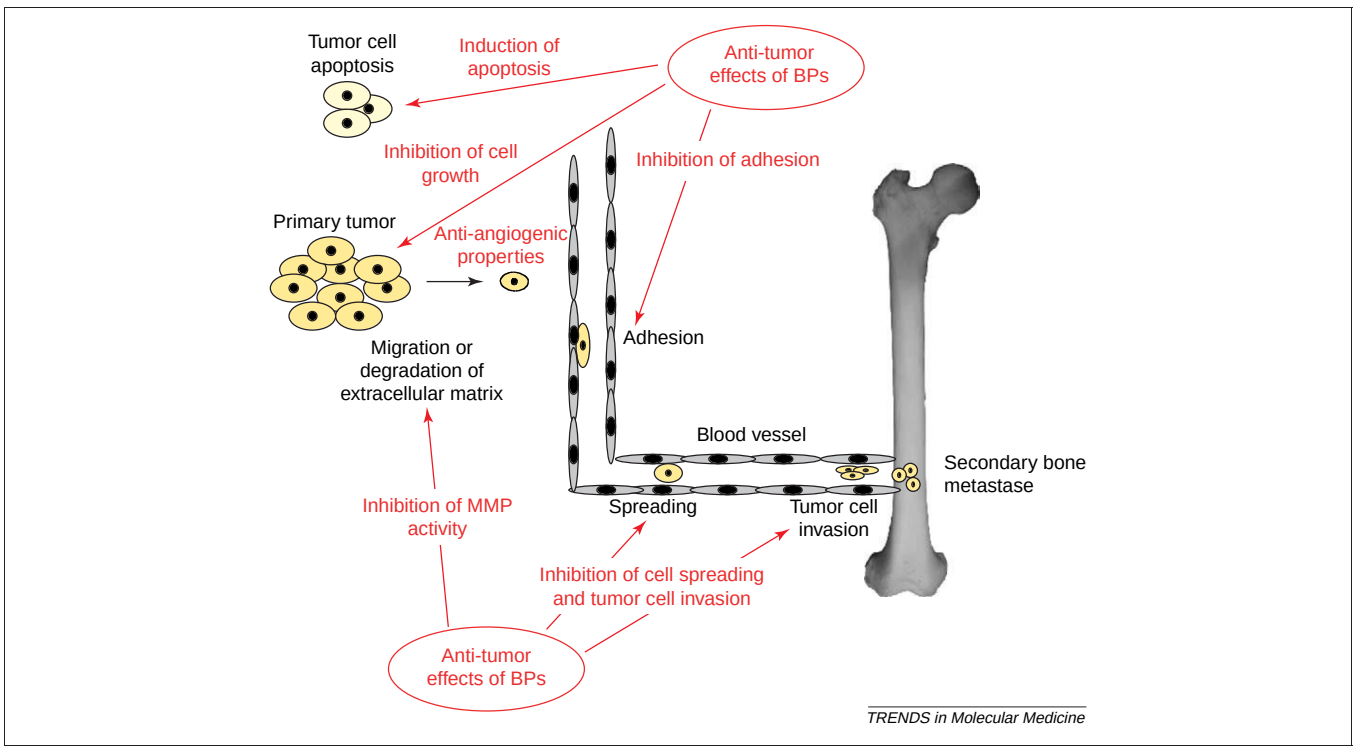

Figure 4. Hypothetical anti-tumor action of bisphosphonates *in vivo*. Tumor functions (black) are inhibited by a variety of effects of BPs (red). Evidence from *in vitro* models includes the induction of apoptosis, inhibition of tumor cell growth, inhibition of cell adhesion, cell spreading and tumor cell invasion, inhibition of matrix metalloproteinases (MMP) activity and anti-angiogenic properties.

studies [30]. Although the inhibition of angiogenesis in a soft-tissue tumor has yet to be demonstrated, zoledronic acid treatment has reduced tumor burden and angiogenesis in the bone lesions of mice bearing 5T2 multiple-myeloma cells [31].

New data indicate that bisphosphonates can also antagonize the stimulatory and anti-apoptotic effects of growth factors (insulin-like growth factors and fibroblast growth factor-2) on breast cancer cell lines, therefore interrupting the vicious cycle [32]. Another novel hypothesis for the antitumor action of BPs is that of immune system modulation, because N–BPs appear to have a variety of immunomodulatory effects that might contribute to their antitumor activity. N–BPs stimulate the proliferation of a specific γδ T-cell subset [33], which exhibits cytotoxic activity against several tumor cell lines *in vitro* [34]. It has also been reported that zoledronic acid inhibits the chemotactic effect that is induced by SDF-1, a key chemokine involved in cancer metastasis to bone [21]. This effect is related to reduced cell motility, decreased expression of the SDF-1 receptor CXCR-4 and a reduction of both Cox-2 expression and, consequently, prostaglandin E_2 (PGE_2) secretion. Because PGE_2 stimulates osteoclast-mediated bone resorption, this inhibition could block the vicious cycle that takes place in osteolytic tumors and contributes to bone protection in breast cancer.

Taken together, these data demonstrate that N–BPs might be considered as bi- (or multi-) functional molecules that exert both endogenous anti-tumor and anti-osteolytic activities.

Benefits of the clinical use of BPs

Several clinical trials have provided evidence in support of the concept that BPs can reduce the occurrence of skeletal metastases in patients with cancer and might provide a survival benefit in some patient populations. In patients with breast cancer, but no skeletal metastases at study entry, clodronate significantly reduced the incidence of skeletal and visceral metastases and conferred a survival advantage [35]. However, two similar studies, with the same BP used as adjuvant therapy in patients with breast cancer, produced different results. One trial demonstrated a significant reduction in the incidence of skeletal metastases but no survival benefit [36], whereas another found no significant difference in the incidence of skeletal metastases between the clodronate and placebo groups at five years, as well as an increase in visceral metastases and decreased survival in the clodronate-treated group [37]. More recently, Powles *et al.* [38] have reported that clodronate significantly inhibits bone metastases, whereas visceral metastases are not decreased, but survival is prolonged. These data question the potential survival benefit but suggest that BPs might reduce the occurrence of skeletal metastases in patients with breast cancer when used in the adjuvant setting (reviewed in [39]). To date, no study has demonstrated a statistically significant survival benefit associated with BP therapy in patients with established skeletal involvement of their cancer, although a survival benefit has been reported in subsets of patients with multiple myeloma or breast cancer treated with pamidronate [40,41]. However, these studies used first and second-generation BPs, the activities of which differ from those of the third-generation compounds. In a systematic review of the role of BPs in metastatic disease that was published recently [42], the authors concluded that BPs significantly reduce skeletal-related events (SRE) and delay the time to the first SRE in patients with bony metastatic disease, but that BPs do not affect survival. The greatest body of evidence supports the use of intravenous N–BPs.

The anti-tumor effect of BPs on visceral metastases has also been studied in animal models, but the results are controversial. It is possible that zoledronic acid treatment significantly reduces bone metastasis and tumor burden in the liver, and prolongs the survival of tumor-bearing animals (unpublished data*). However, two other experimental studies [with ibandronate and minodronate (YM529)] in animal models showed no effect on visceral metastasis [43,44]. Taken together, most of the results conclude that there is an anti-tumor effect of BPs on bone sites. It appears that the pharmacokinetic properties of BPs (the binding to bone and the rapid clearance from the blood and soft tissue) make it unlikely that they will exert a useful anti-tumor affect in soft tissues. Although an anti-tumor effect of BPs on visceral metastases is rarely observed, any effect that does occur in these tissues might be explained by any step in the metastatic process requiring stem cells or cytokines from bone marrow, because these might be influenced by the high local concentration of BPs.

Combined treatment with BPs and anticancer agents

Recent *in vitro* findings indicate that the combination of zoledronic acid with standard anticancer drugs, including dexamethasone and paclitaxel, results in synergistic apoptotic effects on myeloma cells and breast-cancer cell lines [45,46]. In two experimental models of bone metastases, Yoneda *et al.* [47] demonstrated that the BPs incandronate or zoledronic acid, combined with UFT (tegafur and uracil), inhibited not only bone metastases but also lung or liver metastases in an additive fashion and increased the survival of breast-cancer-bearing animals. These results are consistent with those of a previous clinical study by Diel *et al.* [34]. A recent study from Yano *et al.* [48] indicated that the new bisphosphonate YM529 suppressed the production of bone metastases but not visceral metastasis in a model of small-cell lung cancer. In the same model, etoposide inhibited both bone and visceral (lung and liver) metastasis. However, neither YM529 nor etoposide alone significantly prolonged the survival of cancer-bearing mice. Combined use of YM529 with etoposide significantly prolonged survival. Furthermore, a study by Kuroda *et al.* [49] showed a remarkable prolongation of survival with zoledronic acid and Glivec in a leukemia model. In an experimental model of rat osteosarcoma with lung metastases, histological studies revealed that the combination of zoledronic acid with the anti-cancer drug ifosfamide induced extensive fibrosis at

* Nobuyuki, N. *et al.* (2001) The bisphosphonate zoledronic acid inhibits metastases to bone and liver with suppression of osteopontin production in mouse mammary tumor. *J. Bone Miner. Res.* 16 (Suppl. 1), S191.

the graft site, suggesting that this therapeutic regimen improved tissue repair compared with ifosfamide or zoledronic acid alone (unpublished data†). This pro-fibrotic effect is in accordance with the increased expression of type I collagen that was observed in the presence of zoledronic acid in osteoblast cultures [10]. Overall, these data provide a rationale for clinical investigation to determine whether combinations of N–BPs with common antineoplastic drugs have synergistic anti-tumor activity.

Concluding remarks

The inhibitory effects of BPs on bone metastases are well established, whereas the effects of BPs on cancer cells in non-bone sites are still unclear. Although *in vitro* data indicating that BPs exert anti-cancer effects on various types of tumor cells are accumulating, the requirement for relatively high concentrations suggests that these effects might not be clinically relevant, at least for BP monotherapy.

In summary, none of the currently available clinical or *in vivo* data unequivocally supports the notion that BPs exert direct anti-cancer actions. The recent results obtained using the new generation BP zoledronic acid are promising and suggest that modification of the chemical structure might create BPs with additional anti-cancer properties. To resolve the question of a direct antitumor effect of the BPs, other experiments are necessary, in particular, the use of animal models of non-osseous tumors. The development of further models might be useful to differentiate the action of individual BPs, and well-controlled clinical trials are required to investigate whether the antitumor potential of bisphosphonates that is observed in preclinical studies translates into a significant clinical benefit for patients with cancer.

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References

1 Jung, A. *et al.* (1973) The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals. *Calcif. Tissue Res.* 11, 269–280
2 Rogers, M.J. *et al.* (2000) Cellular and molecular mechanisms of action of bisphosphonates. *Cancer* 88, 2961–2978
3 Sasaki, A. *et al.* (1998) Effect of a newly developed bisphosphonate, YH529, on osteolytic bone metastases in nude mice. *Int. J. Cancer* 77, 279–285
4 Marasachia, P. *et al.* (1996) Comparison of the distribution of ^{3}H-alendronate and ^{3}H-etidronate in rat and mouse bones. *Bone* 19, 281–290
5 Sato, M. *et al.* (1991) Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. *J. Clin. Invest.* 88, 2095–2105
6 Gibbs, J.B. and Oliff, A. (1997) The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. *Annu. Rev. Pharmacol. Toxicol.* 37, 143–166
7 Coxon, F.P. *et al.* (2000) Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. *J. Bone Miner. Res.* 15, 1467–1476
8 Benford, H.L. *et al.* (2001) Visualization of bisphosphonate-induced caspase-3 activity in apoptotic osteoclasts *in vitro*. *Bone* 28, 465–473
9 Hughes, D.E. *et al.* (1995) Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. *J. Bone Miner. Res.* 10, 1478–1487
10 Clohisy, D.R. *et al.* (2001) Pamidronate decreases tumor-induced osteoclastogenesis in osteopetrotic mice. *J. Orthop. Res.* 19, 554–558
11 Reinholz, G.G. *et al.* (2000) Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. *Cancer Res.* 60, 6001–6007
12 Viereck, V. *et al.* (2002) Bisphosphonates pamidronate and zoledronic acid stimulate osteoprotegerin production by primary human osteoblasts. *Biochem. Biophys. Res. Commun.* 291, 680–686
13 Pan, B. *et al.* (2004) The nitrogen-containing bisphosphonate, zoledronic acid, influences RANKL expression in human osteoblast-like cells by activating TNF-α converting enzyme (TACE). *J. Bone Miner. Res.* 19, 147–154
14 Lacey, D.L. *et al.* (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93, 165–176
15 Grimaud, E. *et al.* (2001) Osteoprotegerin: a new therapeutic agent for the treatment of bone disease. *Drug Discov. Today* 6, 1241–1242
16 Grimaud, E. *et al.* (2003) Receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis. *Am. J. Pathol.* 163, 2021–2031
17 Kim, Y.H. *et al.* (2002) Inhibitory action of bisphosphonates on bone resorption does not involve the regulation of RANKL and OPG expression. *Exp. Mol. Med.* 34, 145–151
18 Sietsema, W.K. *et al.* (1989) Antiresorptive dose-response relationships across three generations of bisphosphonates. *Drugs Exp. Clin. Res.* 15, 389–396
19 Senaratne, S.G. *et al.* (2000) Bisphosphonates induce apoptosis in human breast cancer cell lines. *Br. J. Cancer* 82, 1459–1468
20 Senaratne, S.G. *et al.* (2002) The bisphosphonate zoledronic acid impairs membrane localisation and induces cytochrome c release in breast cancer cells. *Br. J. Cancer* 86, 1479–1486
21 Denoyelle, C. *et al.* (2003) New insights into the actions of bisphosphonate zoledronic acid in breast cancer cells by dual RhoA-dependent and -independent effects. *Br. J. Cancer* 88, 1631–1640
22 Green, J.R. (2002) Bisphosphonates in cancer therapy. *Curr. Opin. Oncol.* 14, 609–615
23 Corey, E. *et al.* (2003) Zoledronic acid exhibits inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer. *Clin. Cancer Res.* 9, 295–306
24 Chirgwin, J.M. and Guise, T.A. (2000) Molecular mechanisms of tumor-bone interactions in osteolytic metastases. *Crit. Rev. Eukaryot. Gene Expr.* 10, 159–178
25 Lee, M.V. *et al.* (2001) Bisphosphonate treatment inhibits the growth of prostate cancer cells. *Cancer Res.* 61, 2602–2608
26 Boissier, S. *et al.* (1997) Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. *Cancer Res.* 57, 3890–3894
27 Boissier, S. *et al.* (2000) Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. *Cancer Res.* 60, 2949–2954
28 Bezzi, M. *et al.* (2003) Zoledronate sensitizes endothelial cells to tumor necrosis factor-induced programmed cell death: Evidence for the suppression of sustained activation of focal adhesion kinase and protein kinase B/Akt. *J. Biol. Chem.* 278, 43603–43614
29 Heikkila, P. *et al.* (2002) Bisphosphonates inhibit stromelysin-1 (MMP-3), matrix metalloelastase (MMP-12), collagenase-3 (MMP-13) and enamelysin (MMP-20), but not urokinase-type plasminogen activator, and diminish invasion and migration of human malignant and endothelial cell lines. *Anticancer Drugs* 13, 245–254
30 Wood, J. *et al.* (2002) Novel anti-angiogenic effects of the bisphosphonate compound zoledronic acid. *J. Pharmacol. Exp. Ther.* 302, 1055–1061
31 Croucher, P. *et al.* (2003) Zoledronic acid treatment of 5T2MM-bearing mice inhibits the development of myeloma bone disease: evidence for decreased osteolysis, tumor burden and angiogenesis, and increased survival. *J. Bone Miner. Res.* 18, 482–492

† Heymann, D. *et al.* (2003) Direct anti-tumor effects of zoledronate in rat osteosarcoma. Therapeutic relevance of its association with ifosfamide. *Calcif. Tissue Int.* 72, 338.

32 Fromigue, O. *et al.* (2003) Bisphosphonates antagonise bone growth factors' effects on human breast cancer cells survival. *Br. J. Cancer* 89, 178–184
33 Kunzmann, V. *et al.* (2000) Stimulation of γδ T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. *Blood* 96, 384–392
34 Das, H. *et al.* (2001) Vγ2Vδ2 T-cell receptor-mediated recognition of aminobisphosphonates. *Blood* 98, 1616–1618
35 Diel, I.J. *et al.* (1998) Reduction in new metastases in breast cancer with adjuvant clodronate treatment. *N. Engl. J. Med.* 339, 357–363
36 Kanis, J.A. *et al.* (1996) Clodronate decreases the frequency of skeletal metastases in women with breast cancer. *Bone* 19, 663–667
37 Saarto, T. *et al.* (2001) Adjuvant clodronate treatment does not reduce the frequency of skeletal metastases in node-positive breast cancer patients: 5-year results of a randomized controlled trial. *J. Clin. Oncol.* 19, 10–17
38 Powles, T. *et al.* (2002) Randomized, placebo-controlled trial of clodronate in patients with primary operable breast cancer. *J. Clin. Oncol.* 20, 3219–3224
39 Diel, I.J. (2000) antitumor effects of bisphosphonates: first evidence and possible mechanisms. *Drugs* 59, 391–399
40 Berenson, J.R. *et al.* (1998) Long-term pamidronate treatment of advanced multiple myeloma patients reduces skeletal events. *J. Clin. Oncol.* 16, 593–602
41 Lipton, A. *et al.* (2000) Pamidronate prevents skeletal complications and is effective palliative treatment in women with breast carcinoma and osteolytic bone metastases: long term follow-up of two randomized placebo-controlled trials. *Cancer* 88, 1082–1090
42 Ross, J.R. *et al.* (2004) A systematic review of the role of bisphosphonates in metastatic disease. *Health Technol. Assess.* 8, 1–176
43 Michigami, T. *et al.* (2002) The effect of the bisphosphonate ibandronate on breast cancer metastasis to visceral organs. *Breast Cancer Res. Treat.* 75, 249–258
44 Zhang, H. *et al.* (2003) A novel bisphosphonate minodronate (YM529) specifically inhibits osteolytic bone metastasis produced by human small-cell lung cancer cells in NK-cell depleted SCID mice. *Clin. Exp. Metastasis* 20, 153–159
45 Jagdev, S.P. *et al.* (2001) The bisphosphonate, zoledronic acid, induces apoptosis of breast cancer cells: evidence for synergy with paclitaxel. *Ann. Oncol.* 12, 1433–1438
46 Tassone, P. *et al.* (2000) Growth inhibition and synergistic induction of apoptosis by zoledronate and dexamethasone in human myeloma cell lines. *Leukemia* 14, 841–844
47 Yoneda, T. *et al.* (1999) Use of bisphosphonates for the treatment of bone metastasis in experimental animal models. *Cancer Treat. Rev.* 25, 293–299
48 Yano, S. *et al.* (2003) Combined therapy with a new bisphosphonate, minodronate (YM529), and chemotherapy for multiple organ metastases of small cell lung cancer cells in severe combined immunodeficient mice. *Clin. Cancer Res.* 9, 5380–5385
49 Kuroda, J. *et al.* (2003) The third generation zoledronate synergistically augments the anti-Ph + leukemia activity of imatinib mesylate. *Blood* 102, 2229–2235

Complément de discussion à l'article 1

Cette revue fait état de l'avancement des connaissances concernant les mécanismes d'actions directes des bisphosphonates sur les cellules tumorales. En effet de nombreuses études précliniques et cliniques décrivaient les bisphosphonates comme ayant une activité anti-tumorale en plus de leur activité anti-résorptive, contribuant à leur efficacité dans les pathologies osseuses malignes. De plus des études *in vitro* ont démontrées une induction de la mort cellulaire par apoptose dépendante des caspases dans de nombreuses lignées tumorales humaine dérivant de cancers du poumon, du sein (Senaratne et al. 2000), de la prostate, du pancréas, du rein mais aussi dans des cellules de myélome multiple, de neuroblastome et d'ostéosarcome (Mackie et al. 2001; Sonnemann et al. 2001). Cependant, les mécanismes exacts par lesquels les BPs inhibent la croissance des tumeurs osseuses ou le développement des métastases osseuses d'une part, et la voie directe ou indirecte empruntée par ces effets sont mal connus. En effet les effets anti-tumoraux des BPs pourraient être directes sur les cellules tumorales, mais également indirectes *via* l'inhibition des ostéoclastes et par conséquent la modification du microenvironnement osseux. C'est-à-dire que les BPs pourraient briser le cercle vicieux (figure 12) entre la prolifération tumorale et la résorption osseuse en inhibant le relargage des facteurs de croissance favorables au développement tumoral présent dans la matrice.

Qu'ils soient directs ou indirects, les effets des BPs sur la croissance tumorale s'opèrent par le biais de multiples mécanismes. On observe l'induction de l'apoptose des cellules tumorales (Senaratne et al. 2000), l'inhibition de la croissance cellulaire (Lee et al. 2001) ainsi que l'inhibition de l'adhérence (Boissier et al. 1997) et de la migration cellulaire (Bezzi et al. 2003).

L'objectif de mon étude a tout d'abord été de démontrer *in vitro* le potentiel anti tumoral des N-BPs et en particulier de l'acide zolédronique (Zol) sur des cellules d'ostéosarcomes humaines mais aussi de rat et de souris. Dans un deuxième temps, je me suis attaché à définir les mécanismes moléculaires sous jacents afin de déterminer la cinétique d'apparition des différents évènements cellulaires engendrés par le traitement au Zol.

II. Etude in vitro : mécanismes d'action de l'acide zolédronique sur les cellules d'ostéosarcomes.

Introduction

Comme nous l'avons vu précédemment, il a été démontré que les N-BPs, en plus de leurs effets anti-ostéoclastiques, pouvaient avoir des effets anti-tumoraux sur divers lignées cellulaires et de récentes études cliniques sur des patients atteints de métastases osseuses ont montré que le traitement au Zol était bien toléré pour une dose de 4 mg i.v à raison d'une injection toutes les 3 à 4 semaines.

De plus, l'effet direct du Zol à déjà été évoqué par Evdokiou et al (2003), d'après cette étude le Zol réduit le nombre de cellules humaines de sarcome ostéogène par un mécanisme ressemblant à l'anoikis, c'est-à-dire une mort cellulaire liée à la perte des signaux de survie provenant de la liaison de la cellule avec sont substrat (Evdokiou et al. 2003). Plus récemment, Kubista et al (2006), confirmaient cet effet direct du Zol et débutaient l'analyse des mécanismes moléculaires sous-jacents. Le Zol régulerait l'expression de p27 et des cyclines et induirait la mort des cellules par un mécanisme classique d'apoptose associé à une fragmentation de l'ADN. L'hypothèse des auteurs est que le Zol conduirait les cellules à une mitose catastrophique, Il s'agit de la mort cellulaire ayant lieu pendant la métaphase et qui peut être induite par un dommage à l'ADN ou une fusion de cellules asynchrones, à condition que le point de contrôle permettant de vérifier la bonne structure de l'ADN soit défaillant.

Ces résultats permettent d'envisager l'utilisation du Zol en tant qu'agent thérapeutique pour le traitement des ostéosarcomes. C'est pourquoi cette étude s'intéresse aux effets cellulaires et moléculaires du Zol sur différentes lignées d'ostéosarcomes ayant des statuts p53 et Rb différent : OSRGA (p53 et Rb sauvages), MG63 (p53 muté et Rb sauvage) et SAOS2 (p53 nul et Rb muté). En effet, un des problèmes majeurs rencontré dans le traitement du cancer est la très forte proportion de tumeurs mutées sur des anti-oncogènes essentiels tels que p53 et Rb ; ceci est particulièrement vrai pour les ostéosarcomes.

Article 2

« L'acide zolédronique active le checkpoint de phase S et induit la mort des cellules d'ostéosarcome par translocation de l'AIF et de l'EndoG indépendamment du statut de p53 et de Rb »

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L'ostéosarcome représente la plus fréquente des tumeurs osseuses primitives avec 200-250 nouveaux cas par an en France. Malgré les progrès réalisés dans les domaines chirurgicaux et le développement de nouveaux protocoles de polychimiothérapie, le taux de survie à 5 ans varie entre 50 et 70% selon les séries. Ce faible pronostique rend nécessaire le développement de nouvelles stratégies thérapeutiques. L'acide zolédronique (Zol) est un bisphosphonate de troisième génération possédant un atome d'azote dans sa chaine lattérale, (« nitrogen-containing bisphosphonate, N-BP ») utilisé en tant qu'inhibiteur de la différenciation et de l'activation des ostéoclastes dans des pathologies ostéolytiques, d'origine tumorale ou non. De plus, de nombreuses données montrent que les BPs peuvent directement agir sur les cellules tumorales en inhibant leur prolifération. Cependant leurs mécanismes d'action demeurent peu élucidés. Dans cette étude, les effets du ZOL ont été comparés *in vitro* entre plusieurs lignées d'ostéosarcome (OSRGA, MG63, SAOS2) ayant des statuts Rb et p53 différents et des ostéoblastes primaires de rat, en terme de prolifération, analyse du cycle cellulaire (cytométrie en flux, western blot), d'apoptose (analyse des caspases 1, 3 et 8, marquage au Hoechst, western blot, étude de la perméabilité mitochondriale, étude microscopique par Time Lapse) et d'adhérence cellulaire (test de migration, implication des intégrines, observation du cytosquelette par microscopie confocale).

Le ZOL inhibe la prolifération des cellules d'ostéosarcome (OSRGA) après 72 heures de traitement (IC50 de 0.2µM) alors que les ostéoblastes sont peu sensibles. Cette inhibition de prolifération est due à un blocage des cellules en phase S et G2/M du cycle cellulaire suite à une augmentation du niveau de P-ATR, P-chk1, Wee1 et P-cdc2 et une diminution de celui de cdc25c. Un marquage au Hoechst des lignées cellulaires d'ostéosarcome traitées par le ZOL révèle une altération de la morphologie nucléaire sans fragmentation des noyaux. De plus, le ZOL n'induit pas d'activation des caspases 1, 3 et 8. Cependant le marquage au bleu trypan et l'observation microscopique par la technique du time lapse mettent en évidence

l'induction sélective de la mort des cellules d'ostéosarcome et l'inhibition de leur prolifération par 10µM de ZOL. De plus le Zol active P-p53, Bax et inhibe Bcl-2, augmente la perméabilité mitochondriale associée à une libération de l'AIF et de l'EndoG. La cible principale du ZOL décrite dans la littérature est une enzyme de la voie du mévalonate, la farnésyl diphosphate synthase, mais d'autres voies impliquant les intégrines sont suggérées. Des analyses par western blot démontrent qu'un traitement avec 10µM de ZOL pendant 48 h inhibe la phosphorylation des kinases d'adhésion focale (FAK) impliquées dans la survie cellulaire, la prolifération, l'organisation du cytosquelette et la migration.

Les effets du Zol décrits ci-dessus sont observés quels que soient les statuts de Rb et p53 des lignées d'ostéosarcome utilisées ; le Zol représente ainsi un agent thérapeutique potentiel pour le traitement des tumeurs osseuses ainsi que d'autre types de tumeurs présentant des résistances au traitement de par leurs mutations sur des gènes clefs tels que Rb, p53 ou les gènes codant pour les caspases.

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Zoledronic Acid Activates the DNA S-Phase Checkpoint and Induces Osteosarcoma Cell Death Characterized by Apoptosis-Inducing Factor and Endonuclease-G Translocation Independently of p53 and Retinoblastoma Status

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ABSTRACT

The molecular mechanisms responsible for the cellular effects of the nitrogen-containing bisphosphonate zoledronic acid (Zol) were assessed on several osteosarcoma cell lines differing in their p53 and retinoblastoma (Rb) status. Zol inhibited cell proliferation and increased atypical apoptosis. The Zol effects on proliferation were due to cell cycle arrest in S and G_2/M phases subsequent to the activation of the intra-S DNA damage checkpoint with an increase in P-ATR, P-chk1, Wee1, and P-cdc2 levels and a decrease in cdc25c, regardless of the p53 and Rb status. In addition, the atypic apoptosis induced by Zol was independent of caspase activation, and it was characterized by nuclear alterations, increased Bax expression, and reduced Bcl-2 level. Furthermore, mitochondrial permeability was up-regulated by Zol independently of p53 in association with the translocation of apoptosis-inducing factor (AIF) and endonuclease-G (EndoG). Zol also disturbed cytoskeletal organization and cell junctions and inhibited cell migration and phosphorylation of focal adhesion kinases. The main difficulty encountered in treating cancer relates to mutations in key genes such as p53, Rb, or proteins affecting caspase signaling carried by many tumor cells. We have demonstrated for the first time that zoledronic acid activated the DNA damage S-phase checkpoint and the mitochondrial pathway via AIF and EndoG translocation, and it inhibited cell proliferation and induced cell death, bypassing these potentials mutations. Therefore, zoledronic acid may be considered as an effective therapeutic agent in clinical trials of osteosarcoma in which mutation for p53 and Rb very often occur, and where current treatment with traditional chemotherapeutic agents is ineffective.

Bisphosphonates (BPs) are stable synthetic analogs of the naturally occurring pyrophosphate (Heymann et al., 2004). Different side chains can be added to the central carbon atom, thus producing a range of BPs with varying clinical activity and potency (Rogers et al., 2000). Therefore, BPs can be grouped into two classes of non–nitrogen-containing and nitrogen-containing BPs. The clinical use of bisphosphonates has increased dramatically during the past decade. The most common indicator for the use of these compounds is osteoporosis, but their use has rapidly emerged in osteolytic bone diseases characterized by enhanced bone resorption (e.g., Paget's disease and hypercalcemia of malignancy). Indeed, BPs are currently the most effective class of antiresorptive drugs available, and their first targets identified were osteoclasts. Due to the high tropism of BPs for hydroxyapatite in bone and the ability of osteoclasts to release bone-bound bisphosphonate, a direct effect on mature osteoclasts seems to be the most important mechanism of action. BPs can be grouped into two classes of non–nitrogen-containing and nitrogen-containing BPs. The BPs that lack a nitrogen atom and are most closely related to pyrophosphate (such as clodronate, etidronate, and tiludronate) are metabolized intracellularly to cytotoxic analogs of ATP that reduce osteoclast

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ABBREVIATIONS: BP, bisphosphonate; FPP, farnesyl diphosphate; Zol, zoledrenic acid; Rb, retinoblastoma; AIF, apoptosis-inducing factor; EndoG, endonuclease G; XTT, sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate; FAK, focal adhesion kinase; RGD, Arg-Gly-Asp; PBS, phosphate-buffered saline; GGO, geranylgeraniol; cdk, cyclin-dependent kinase; Chk, checkpoint kinase; PARP1, poly(ADP-ribose) polymerase 1; ATR, ataxia-telangiectasia, mutated and Rad 3-related.

survival (Rogers et al., 2000). In contrast, the more potent nitrogen-containing bisphosphonates (such as pamidronate, alendronate, risedronate, ibandronate, and zoledronate) induce apoptosis in osteoclasts by inhibiting enzymes of the mevalonate pathway, mainly farnesyl diphosphate synthase (FPP) (Gibbs and Oliff, 1997). Inhibition of this enzyme in osteoclasts prevents the biosynthesis of cholesterol and isoprenoid lipids (FPP and geranylgeraniol diphosphate) that are essential for the post-translational farnesylation and geranylgeranylation of small GTPase signaling proteins. Loss of bone-resorptive activity and induction of osteoclast apoptosis is due primarily to loss of geranylgeranylated small GTPases (Coxon et al., 2000).

In addition to their potent antiosteoclastic effects, recent preclinical studies have shown that *N*-BPs induce apoptosis of cancer cells from several origins, including myeloma, breast, prostate carcinoma, and osteosarcoma cell lines (Mackie et al., 2001; Sonnemann et al., 2001). Recent clinical trials in patients with bone metastases or multiple myeloma demonstrated that zoledronic acid (Zol) was safe and well tolerated at the approved dose of 4 mg i.v. every 3 to 4 weeks (Heymann et al., 2004). Moreover, growing preclinical evidence shows that Zol also exhibits direct antitumor activity. Evdokiou et al. (2003) reported that Zol reduced the cell number of different human osteogenic sarcoma cell lines by a mechanism resembling anoikis. More recently, Kubista et al. (2006) confirmed these findings and showed that Zol regulates the cyclin and p27 expression. In this work, Zol induced cell death by a typical apoptotic pathway associated with nuclear fragmentation and Annexin-V staining. These authors suggested that the treatment of osteosarcoma cells by Zol might lead to mitotic catastrophes and consequently to the induction of cell death (Kubista et al., 2006). We also reported recently the enhancement of tumor regression and tissue repair when Zol is combined with ifosfamide in rat osteosarcoma (Heymann et al., 2005). Furthermore, Zol suppresses lung metastases and prolongs overall survival of osteosarcoma-bearing mice (Ory et al., 2005). However, the overall effects on osteosarcoma cells seem to be mediated via diverse and unclear pathways, such as apoptosis, proliferation, and metabolic events that need be clarified.

Because Zol represents a potential novel antineoplasic agent for the therapy of osteosarcoma, the present study investigated the cellular effects of Zol on several osteosarcoma cell lines possessing different p53 and Rb status, in particular OSRGA (p53 and Rb wild type), MG3 (p53 mutated and Rb wild type), and SaOS2 (p53 null and Rb-defective). We provide evidence that Zol exerts dual effects on osteosarcoma cell proliferation: high doses of Zol exert antiproliferative effects on osteosarcoma cells, resulting in their cell cycle arrest in S and G_2/M phases through the control of the intra-S DNA checkpoint. In contrast, low doses of Zol promote osteosarcoma cell proliferation through the control of the G_1/S DNA checkpoint. Furthermore, we report that Zol induces atypic apoptosis independently of caspase activation but involves the mitochondrial pathway, in particular AIF and EndoG translocation. Overall results demonstrate selective and original antitumor effects of Zol on several osteosarcoma cell lines independently of their p53 and Rb status, thus allowing these molecules to be considered as potential therapeutic agents in clinical trials of tumor bone pathologies, regardless of the p53 and Rb status of the patients.

Materials and Methods

Cells and Culture Conditions. The rat osteosarcoma OSRGA cell line was initially established from a radioinduced osteosarcoma (Klein et al., 1977; Thiéry et al., 1982). The rat ROS17/2.8 osteosarcoma cell line was kindly provided by Prof. H. J. Donahue (The Pennsylvania State University, University Park, PA), and the human MG63, SaOS2, U2OS, and MNNG-HOS cell lines were purchased from American Type Culture Collection (Manassas, VA). These cell lines were cultured in Dulbecco's modified Eagle's medium (Cambrex Bio Science Verriers S.p.r.l., Verviers, Belgium) supplemented with 5% fetal calf serum (Hyclone Laboratories, Brebières, France) and 2 mM L-glutamine (Cambrex Bio Science Verriers S.p.r.l.). Primary rat and human osteoblasts were isolated from bone explants and cultured in RPMI 1640 medium (Cambrex Bio Science Verriers S.p.r.l.) supplemented with 10% fetal calf serum and antibiotic mixture (100 IU/ml penicillin and 100 μg/ml streptomycin).

Cell Growth and Viability. Cell growth and viability were determined by a cell proliferation reagent assay kit using sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro-)benzene sulfonic acid hydrate (XTT) (Roche Molecular Biochemicals, Mannheim, Germany). Two thousand cells per well were plated into 96-well plates and cultured for 72 h in culture medium in the presence or absence of 10^{-12} to 10^{-4} M Zol and/or in the presence or absence of 1 and 10 mM RGD peptide (Arg-Gly-Asp consensus sequence for integrin interaction) (Sigma, St.-Quentin-Fallavier, France) diluted in the culture medium. Zol was provided by Pharma Novartis AG (Basel, Switzerland) as the disodium hydrate form. Additional experiments were performed in presence of geranylgeraniol (GGO) (Sigma). After the culture period, XTT reagent was added to each well and incubated for 5 h at 37°C; absorbance was then read at 490 nm using a 96-multiwell microplate reader. Cell viability was also assessed by trypan blue exclusion, and alive and dead cells were manually counted from trypsinized and floating cells. A minimum of 100 cells were counted in each culture condition. Cell death was also monitored microscopically after Hoechst 33258 (Sigma) staining. Cells were seeded at 10^4 cells/well in a 24-multiwell plate and treated or not with 10 μM Zol for 48 h or 100 nM staurosporine (Sigma) for 16 h, stained with 10 μg/ml Hoechst reagent for 30 min at 37°C, and then observed under UV microscopy (DMRXA; Leica, Wetzlar, Germany).

Western Blot Analysis. Zol-treated cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 5% Tris, pH 7.4, 1% Nonidet P-40, 0.25% Na deoxycholate, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Protein concentration was determined with a BCA kit (Pierce Chemical, Rockford, IL). Moreover anti-actin was used as loading control (Sigma-Aldrich, St. Louis, MO). Twenty micrograms of total cell lysate proteins was run on SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to Immobilon-P membrane (Millipore Corporation, Billerica, MA). The membrane was blotted with antibodies to P-p53 (Ser15); Bax, Bcl-2, and P-Rb (Ser795); P-Rb (Ser807/811); P-cdc2 (Tyr15); Wee1, cdc25c, and P-cdc25c (Ser216); P-chk1 (Ser345); P-ATR (Ser428); focal adhesion kinase (FAK) and P-FAK (Tyr925) (Cell Signaling Technology Inc., Danvers, MA); and $p21^{WAF1}$(BD Biosciences, San Jose, CA) in PBS, 0.05% Tween 20, and 3% bovine serum albumin. The membrane was washed and probed with the secondary antibody coupled to horseradish peroxidase. Antibody binding was visualized with the enhanced chemoluminescence system (ECL kit; Roche Molecular Biochemicals).

Caspase-1, -3, and -8 Activities. Caspase-1, -3, and -8 activities were assessed on 10 μl of total Zol-treated or not cell lysates using the kit CaspACE assay system, fluorometric (Promega, Madison, WI) following the manufacturer's recommendations. Cells treated with UV light for 30 s 24 h before harvesting were used as a positive control. Results are expressed in arbitrary units relative to the total protein content.

Cell Cycle Analysis and Mitochondrial Membrane Permeability Assay. Subconfluent cultures of OSRGA, MG63, and SAOS2 cells were incubated in the presence or absence of Zol for 48 h, trypsinized, washed twice, and incubated in PBS containing 0.12% Triton X-100, 0.12 mM EDTA, and 100 μg/ml ribonuclease A. Then, 50 μg/ml propidium iodide was added for each sample for 20 min at 4°C in the dark. Cell cycle distribution was analyzed by flow cytometry (FACScan; BD Biosciences), based on 2N and 4N DNA content.

The mitochondrial membrane potential of the cells treated with or without Zol was assessed using the MitoProbe JC-1 assay kit (Invitrogen, Carlsbad, CA) for flow cytometry after the manufacturer's recommendations. Mitochondrial depolarization is indicated by an increase in the green/red (FL1/FL2) fluorescence intensity ratio and allows detection of changes in membrane potential associated with the mitochondrial permeability transition. A mitochondrial membrane potential disrupter, carbonyl cyanine 3-chlorophenyldrazone, was used as a positive control. Fluorescence intensity of was measured by flow cytometry (FACScan; BD Biosciences).

Time-Lapse Microscopy. For time-lapse experiments, cells were seeded at 5×10^4 cells/well and cultured in six-multiwell plates in the presence or the absence of 10 μM Zol. Phase-contrast photographs (Leica) were taken every 10 min for 60 h and edited using MetaMorph software (Molecular Devices, Sunnyvale, CA). Cell divisions and apoptotic cells were then manually scored. To study the cell migration, cells were cultured in six-well plates until confluent and treated or not with 10 μM Zol for 24 h before a slit was made in the cell monolayer. Cell migration was then followed for the next 48 h.

Confocal Microscopy. For AIF and EndoG localization, cells were treated with or without Zol, fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated with primary polyclonal anti-AIF antibody (diluted 1:50; Cell Signaling Technology Inc.) or polyclonal anti-EndoG antibody (diluted 1:100; ProSci Incorporated, Lausen, Switzerland) in PBS, 1% bovine serum albumin, and 0.1% Triton for 1 h, washed, and incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG; 2 mg/ml; 1:200, Invitrogen) for 45 min. Nuclei were stained with 1 μg/ml Topro3 (Invitrogen) for 30 min. Coverglass fitting was achieved with the Long Pro Kit (Invitrogen). For actin filaments detection, cells were treated with or without Zol as indicated, fixed in 4% paraformaldehyde, and stained with 0.25 μg/ml fluorescein isothiocyanate-conjugated phalloidin (Sigma). Images were collected on a TCS-SP1 confocal microscope (Leica) with 63/1.4× oil immersion lens. The digital images were visualized with a 24-bit imaging system, including TCS-NT software (Leica), and projections were generated from z-stacks.

Electron Microscopy. OSRGA cells were treated for 72 h with 10 μM Zol in plastic Petri dishes, washed with 2% NaCl, 0.15 M sodium cacodylate buffer, pH 7.2, and fixed with cacodylate-buffered 3% osmium tetroxide at 4°C for 30 min. After being rinsed, cultures were dehydrated in a graded concentration of ethanol and immersed in ethanol/Epon (1:1) for 1 h. After evaporation, cells were rinsed three times with Epon and placed at 37°C for 12 h. Epon capsules were returned onto the cell monolayer in contact with the thin, partially polymerized Epon film and placed at 60°C until polymerization was complete (3 days). After detachment of the Epon capsules containing the cell monolayers from the Petri dishes, thin sections (70–80 nm) were stained with uranyl acetate and lead citrate, and sections were examined with a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan).

Results

Zoledronic Acid Induces Pro- and Antiproliferative Effects on Osteosarcoma Cells by an Integrin-Dependent Pathway. Consistent with previous results (Evdokiou et al., 2003; Heymann et al., 2005; Ory et al., 2005), Zol treatment of rat (Fig. 1A) and human (Fig. 1B) osteosarcoma cells strongly reduced their proliferation. Thus, 0.1 to 100 μM Zol decreased the viable cell number in a dose-dependent manner (IC_{50} = 1–8 μM) as revealed by the XTT assay. Similar experiments performed on primary human and rat osteoblasts showed that Zol was more potent against osteosarcoma cell lines than primary osteoblasts (IC_{50} = 10 μM) (Fig. 1, A and B). However, when lower concentrations of Zol (1–10^4 pM) were used, Zol exerted the opposite effect on the cell viability (Fig. 1C). The results demonstrated a 60% increase of viable rat OSRGA and human SaOS2 cells, and a 100% increase for MG63 osteosarcoma cells in the presence of 10 pM Zol after 72 h of treatment (Fig. 1C).

Because bisphosphonates modulate both the attachment of tumor cells to extracellular matrix proteins or to bone sections (Boissier et al., 1997) and the endothelial cell adhesion and migration on vitronectin (Bezzi et al., 2003), we raised the hypothesis that Zol effects may involve the integrin pathway. The integrins act as cell surface receptors, mediating cell functions (e.g., adhesion to extracellular matrix, migration, and cell death) through binding to an RGD motif (Ruoslahti, 1996; Salsmann et al., 2006). In this context, to determine whether zoledronic acid could exert its activities through integrin, the capacity of a recombinant RGD amino acid sequence to interfere with Zol activities was assessed. Thus, pretreatment of osteosarcoma cell lines with 1 mM RGD peptide prevented the inhibitory effects of 1 μM Zol previously observed on OSRGA (Fig. 1D) and SaOS2 and MG63 cell (Fig. 1, E and F) proliferation. These data demonstrate that Zol exerts differential effects on osteosarcoma cell proliferation depending on the concentrations used and that an integrin-dependent pathway is involved in Zol-induced inhibitory mechanisms.

Zoledronic Acid Induces Osteosarcoma Cell Death Independently of the p53 Status by a Caspase-Independent Mechanism but in Association with AIF/EndoG Translocation. To determine whether the inhibitory activity of Zol observed on osteosarcoma cell lines resulted from induction of cell death, we used time-lapse microscopy to monitor the apoptotic events in human and rat osteosarcoma cells treated with 10 μM Zol (Fig. 2A). The first apoptotic events occurred at an early time (5–10 h) in the Zol-treated OSRGA osteosarcoma culture, but they became significantly different compared with the control after 20 h of treatment. The observed cell death was accompanied by extensive plasma membrane blebbing characteristic of apoptotic cells (Fig. 2A, top photo). A similar phenomenon was observed on SaOS2 and MG63 human osteosarcoma cells (data not shown). To characterize the apoptotic mechanisms induced by Zol, nucleus fragmentation and caspase activations were analyzed in Zol treated-osteosarcoma cells. In contrast to 100 nM staurosporine for 16 h, which induced apoptosis associated with nucleus fragmentation, the nuclei of Zol-treated cells exhibited a characteristic kidney-like form with condensed chromatin clumps compared with control cells (Fig. 2B). Moreover, Western blot and enzymatic assays revealed no caspase-1, -3, or -8 activity in response to Zol treatment (data not shown), and the pan-caspase inhibitor Z-Vad-FMK did not inhibit the Zol-induced effects on osteosarcoma cell viability (data not shown). In the light of these data, we conclude that Zol induced atypic apoptosis of all analyzed osteosarcoma cell lines by a mechanism inde-

pendent of caspases but associated with membrane and nucleus alterations.

We next examined by Western blot the involvement of the apoptotic mitochondrial pathway in Zol-induced programmed cell death. In all osteosarcoma cell lines analyzed (OSRGA, SaOS2, and MG63), the ratio Bax/Blc2 was altered in favor of Bax, but with striking differences (Fig. 2C). Indeed, 10 μM Zol strongly up-regulated Bax expression in a time-dependent manner in OSRGA, whereas this modulation was not significant in SaOS2 and MG63 cells. Conversely, Zol decreased Bcl-2 expression in OSRGA cells, whereas this parameter remained stable in SaOS2 and MG63 cells (Fig. 2C). Zol also induced P-p53 (Ser15) in OSRGA cells, which possess a wild-type p53, in contrast to MG63 and SaOS2, which are mutated and null for p53, respectively.

To better define the role of mitochondria in Zol-induced cell death, the mitochondrial membrane potential and the mitochondrial effectors AIF and EndoG were studied. Figure 3 shows that the JC-1 mitochondrial membrane potential sensor of OSRGA, SaOS2, and MG63 was strongly increased in the presence of 10 μM Zol for 48 h as revealed by the FL1/FL2 fluorescence ratio (Fig. 3, left table). Moreover, confocal microscopy analysis revealed that 10 μM Zol treatment was followed by a time-dependent translocation of the two nucleases AIF and EndoG from a mitochondrial to a perinuclear location in the three osteosarcoma cell lines, in relation with a decrease of the Topro3 staining intensity, underlying potential DNA disruptions (Fig. 3).

Together, these results suggest that the Zol-induced atypic apoptotic in osteosarcoma cells involved apoptotic mitochondria pathways characterized by AIF/EndoG translocation independently of the p53 status.

Zoledronic Acid Induces Osteosarcoma Cell Cycle Arrest in S, G_2/M Phases Independently of the Rb Status. To determine whether the Zol-induced cell death was combined with an inhibition of cell proliferation, flow cytometry of osteosarcoma cell DNA content was performed after 24 and 48 h of 10 μM Zol treatment. Although 24 h of 10 μM Zol treatment did not modulate the cell cycle in OSRGA, SaOS2, and MG63 cells (data not shown), 48 h of Zol treatment induced a cell cycle arrest in S and G_2/M phases in all cell types (Fig. 4A). In OSRGA, SaOS2, and MG63 cells, Zol induced a cell cycle arrest in S and G_2/M phases. Indeed, the number of cells in S, G_2/M phases strongly increased from 45 to 75% for OSRGA cells, from 37 to 83% for MG63 cells, and from 38 to 60% for SaOS2 cells when treated with Zol (Fig. 4A). This observation was concomitant with a reduction of cells in G_0/G_1 phase: 51 versus 17% for OSRGA, 59 versus 6% for MG63, and 56 versus 9% for SaOS2. The cells in the apoptotic sub-G_0/G_1 peak also increased from 4 to 18 and 11% for OSRGA and MG63 cells, respectively, and from 6 to 31% for SaOS2 cells (Fig. 4A).

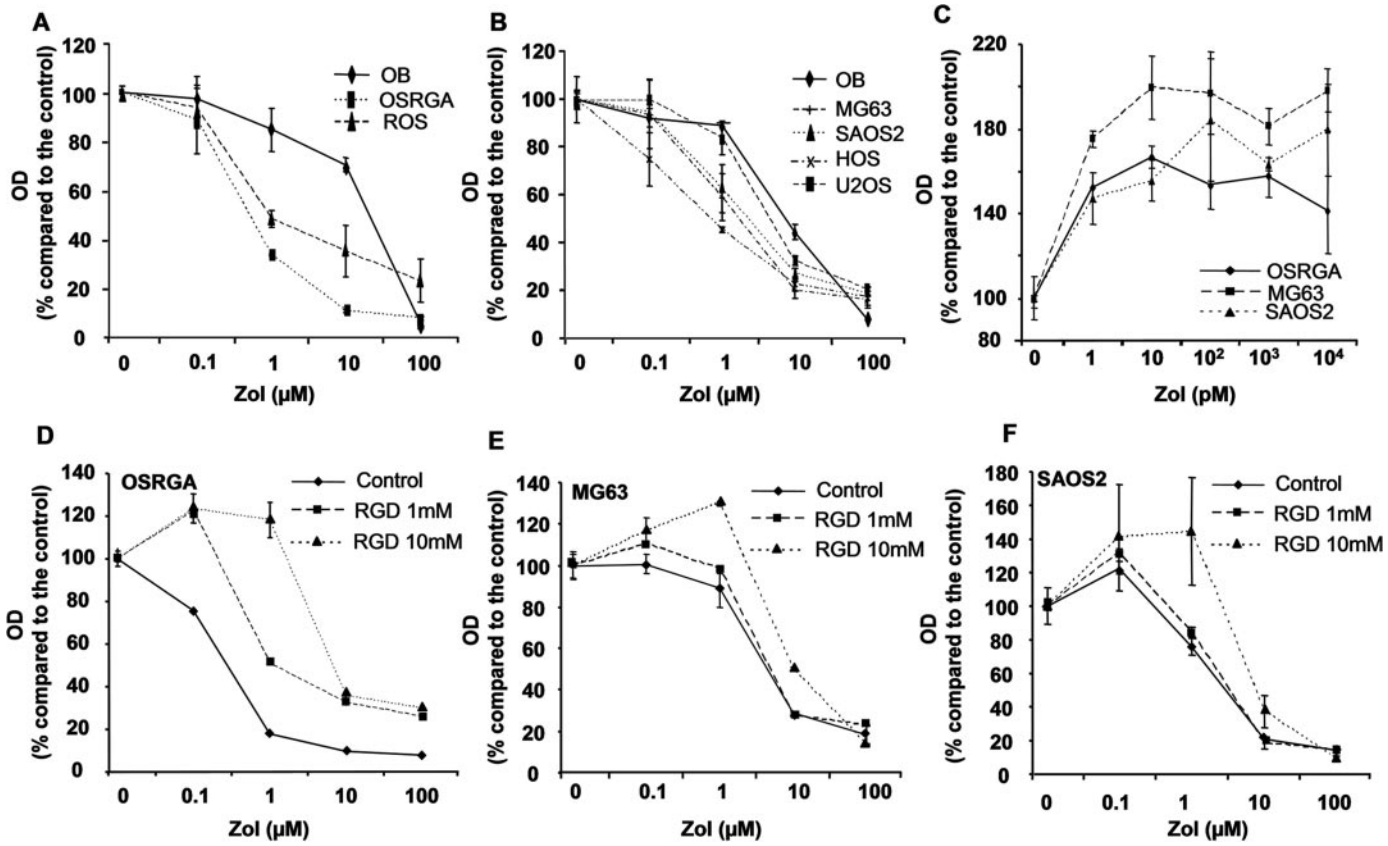

Fig. 1. Zoledronic acid treatment differentially affects the alive cell number through an integrin-dependent pathway. Several rat (A; OSRGA and ROS) and human (B; MG63, SaOS2, U2OS, and HOS) osteosarcoma cell lines were treated by increasing concentration of Zol (0.1–100 μM) for 72 h. The number of viable cells was then determined using an XTT assay and compared with primary culture of osteoblasts. C, viability assay of osteosarcoma cell lines was investigated in the presence of low concentrations of Zol (1–10^4 pM). D to F, similar experiments were performed in the presence of RGD peptide at 1 and 10 mM. Graphs represent the average values of three independent experiments performed in triplicate. Error bars represent the standard deviation.

We therefore investigated by Western blot which DNA checkpoints could be involved to delay the cell cycle progression observed in the presence of Zol. Thus, in the three osteosarcoma cell lines studied, high concentrations of Zol (10 μM) increased the inactive form of cdc2 (P-cdc2 Tyr15) after 72 h of treatment (Fig. 4B). The effect of Zol on the two regulators of cdc2 phosphorylation (cdc25c and Wee1) was further investigated. Zol promptly reduced the cdc25c phosphatase in a time-dependent manner (Fig. 4B), without affecting the phosphorylated cdc25c form, suggesting that cdc25c is predominantly present in its inactive phosphorylated form. In addition, Zol induced expression of Wee1 in MG63 an SAOS2 cells. The modulation of the effectors Wee1 and cdc25c coincided with an increase of their upstream transducers P-chk1 (Ser345) and ATR kinases (Fig. 4B). Thus, the phosphorylated form of cdc2, which is unable to interact with cyclin B, may block the cell cycle in G_2/M phase and then prevent the entry of osteosarcoma cells into mitosis, as observed by flow cytometry. In parallel with this phenomenon, 10 μM Zol strongly inhibited p21 in all osteosarcoma cell lines; transiently up-regulated Rb phosphorylation (Ser795, -807, and -81) at 24 and 48 h, respectively, in OSRGA and MG63 cells; but failed to modulate similarly P-Rb in SaOS2 cells (Fig. 4B). To understand the opposite effect of low Zol concentrations on cell proliferation, similar Western blot analyses were performed on these three osteosarcoma

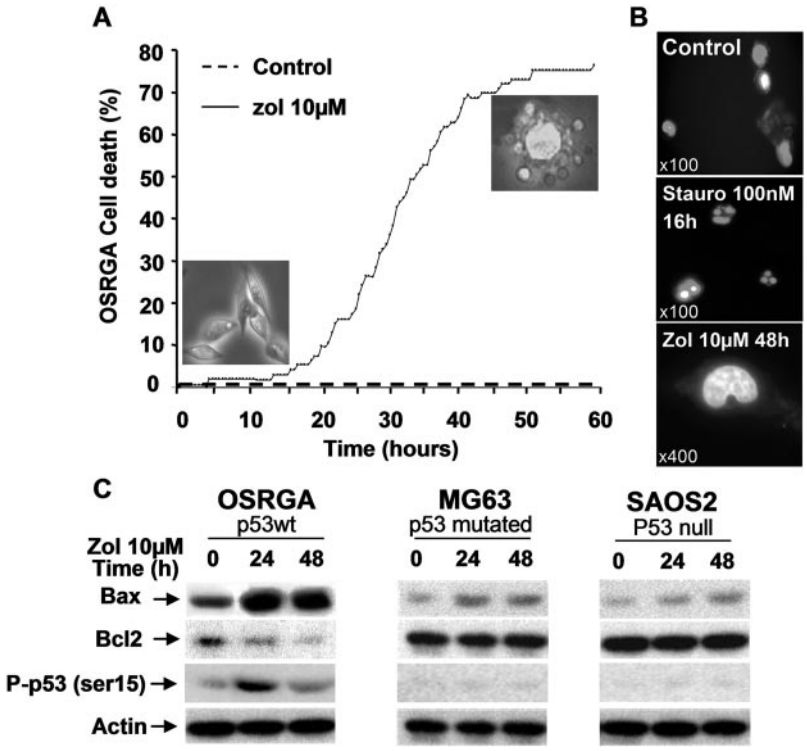

Fig. 2. Zoledronic acid induces atypic apoptosis of osteosarcoma cells independently of their p53 status. A, kinetic study of OSRGA cell death was analyzed by time-lapse microscopy in the presence or the absence of 10 μM Zol. The number of dead cells was manually scored every 10 min until 60 h. Characteristic extensive membrane blebbing of OSRGA cells in the presence (top inset) of 10 μM Zol compared with control cells (bottom inset) is shown. Zol induced similar profile on MG63 and SaOS2 cells. B, nuclear morphology was studied by Hoechst staining after Zol (10 μM; 48 h) treatment in OSRGA cell line. Staurosporine (100 nM; 16 h) was used as a positive control of nucleus fragmentation. Similar observations were obtained for MG63 and SaOS2 cells. C, implication of Bax, Bcl-2, and p53 in Zol-induced cell death was assessed by Western blot. All experiments were repeated three times, and a representative blot is shown. wt, wild type.

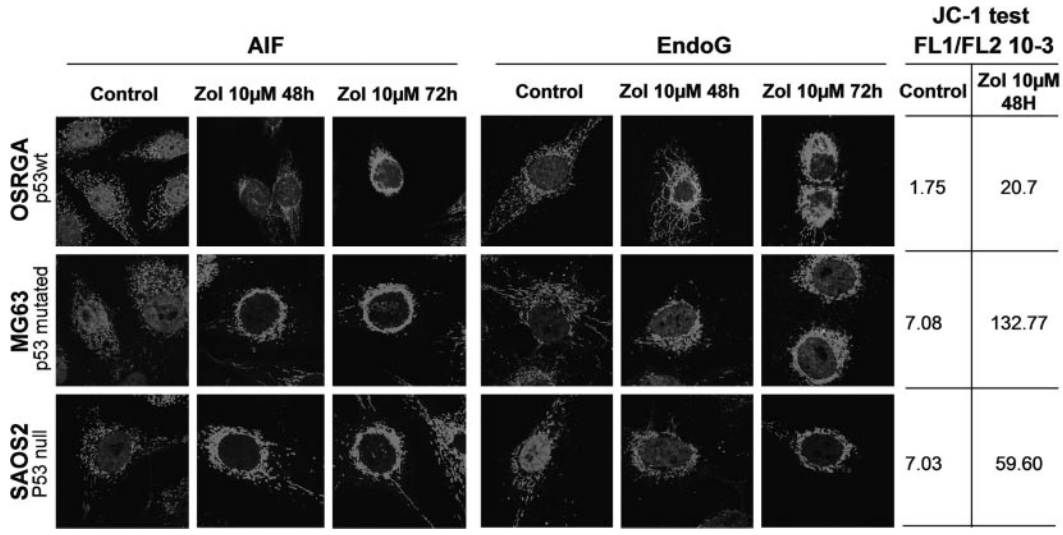

Fig. 3. Zoledronic acid stimulates mitochondrial permeability and AIF/EndoG translocation in OSRGA, MG63, and SaOS2 osteosarcoma cells. Osteosarcoma cell lines were treated by 10 μM Zol for 48 and 72 h. The mitochondrial permeability was studied by JC-1 mitochondrial membrane potential sensor as revealed by the FL1/FL2 ratio, and AIF/EndoG localization was followed by confocal microscopy using specific antibodies. Nuclei were stained by Topro3. Original magnification, 1000×.

cell lines in the presence of 10 pM and 10 nM of Zol (Fig. 4C). In contrast to high concentrations, 72-h treatment with low Zol concentrations strongly increased P-Rb in OSRGA and MG63 cells in a dose-dependent manner and not in SaOS2 cells. In the same conditions, Zol did not modulate P-cdc2 in the three cell lines. These results, in agreement with the proproliferative effects observed in the presence of low concentrations of Zol (Fig. 1C), demonstrate that low doses of Zol during 72 h of treatment had similar effects on Rb phosphorylation as high doses for shorter incubation time (24 h).

Because OSRGA and MG63 osteosarcoma cells are Rb wild type and SaOS2 is defective for Rb, our results also demonstrated that Zol blocks cell cycle and induces the cell death of osteosarcoma cells independently of their Rb status.

Zoledronic Acid Disturbs Cytoskeletal Organization and Cell Junctions and Inhibits Cell Migration. Because Zol alters osteosarcoma cell death and proliferation through an integrin-dependent pathway, we wondered whether Zol could disturb the cytoskeletal organization and cell migration. Confocal microscopic observations revealed a major disorganization of the actin stress fibers associated with membrane ruffling in the three osteosarcoma cell lines treated with 10 μM Zol for 72 h (Fig. 5A). Moreover, as shown by the time-lapse assay, 10 μM Zol totally blocked the migration of OSRGA and MG63 cells, and it strongly slowed down the migration of SaOS2 cells, which exhibit a higher proliferation rate (Fig. 5B). The effects of Zol on the cytoskeletal organization and cell migration were corroborated by the electron microscopic analysis, which showed that Zol induced striking morphological changes in cell shape, leading to the inhibition of cell interactions. Indeed, in the presence of Zol, OSRGA osteosarcoma cells were retracted, adopted a round

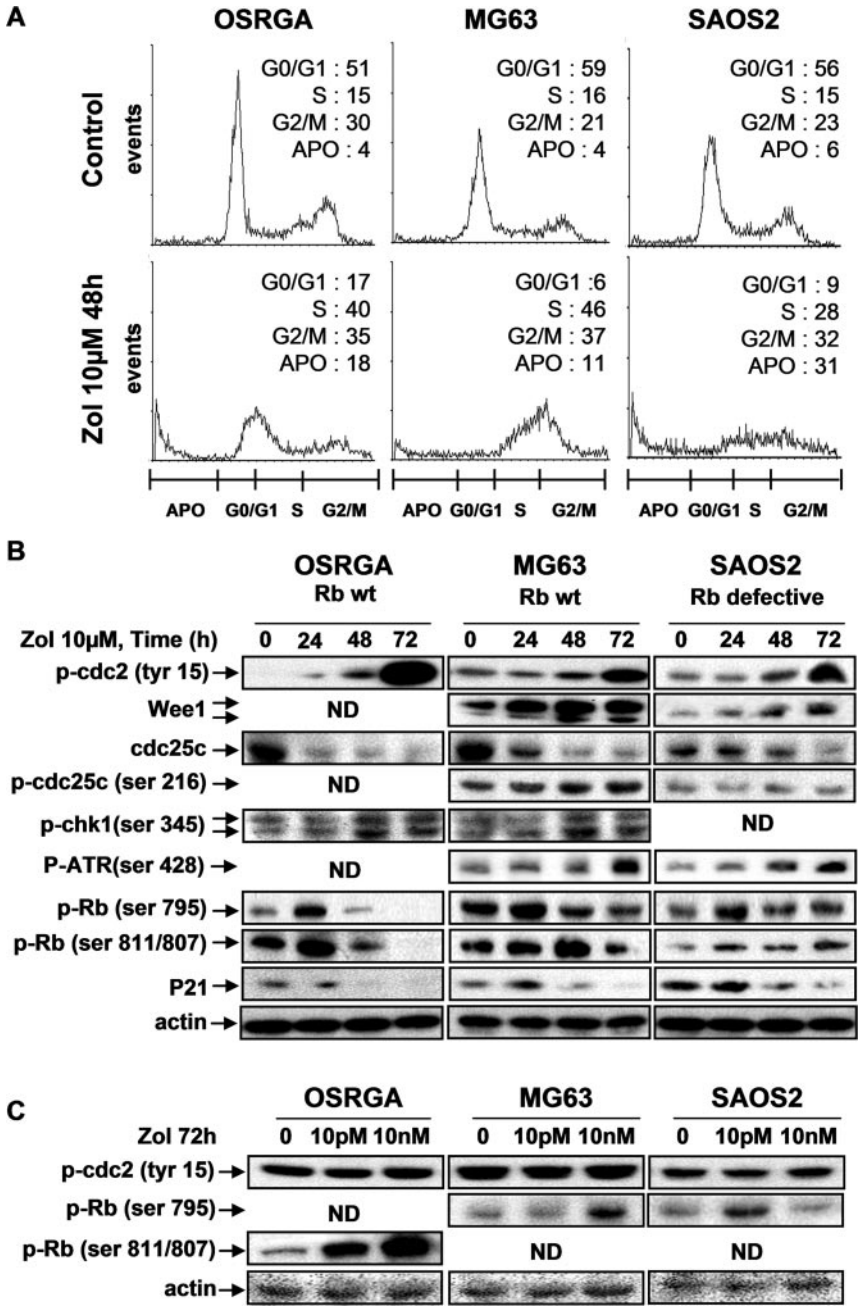

Fig. 4. Zoledronic acid blocks cell cycle of osteosarcoma cell lines in S, G_2/M phases independently of their Rb status. A, cell cycle distribution of osteosarcoma cell lines treated or not with 10 μM Zol for 48 h was analyzed by propidium iodide staining and fluorescence-activated cell sorting analysis. B, G_1/S and G_2/M DNA checkpoints were analyzed by Western blot on rat (OSRGA) and human (MG63 and SaOS2) osteosarcoma cell lines in the presence or absence of high Zol concentrations for 24, 48, and 72 h (B) or low Zol concentrations for 72 h (C). All experiments were repeated three times, and a representative blot is shown. N.D., not determined.

shape, and lost their cellular interactions. A disruption of the gap and desmosome-like junctions occurred in contrast to the control cells (Fig. 5C). These effects may be in part related to the time- and dose-dependent down-regulation of FAKs phosphorylation observed in the presence of Zol (Fig. 5D). Zol exerts the same effects on human osteosarcoma cell lines (data not shown).

GGO Partly Protects Osteosarcoma Cells from Zoledronic Acid Effects. To further determine the involvement of the mevalonate pathway in the Zol effects observed on human osteosarcoma cells, the effect of Zol on MG63 cell cycle was analyzed in the presence or the absence of 25 μM geranylgeraniol. Whereas 72 h of 10 and 25 μM Zol treatment blocked the MG63 cells in S G_2/M phases, 25 μM GGO restored a cell cycle profile similar to the control cells (Fig. 6A). The effect of 25 μM GGO was also determined by trypan blue exclusion on the MG63 cell death induced by Zol treatment. The results revealed that 25 μM GGO partly inhibited the effects of 10 and 25 μM Zol on cell death, even if the cell cycle was restored (Fig. 6, B and C). Similar data were obtained on SaOS2 human osteosarcoma and OSRGA rat osteosarcoma (data not shown).

Discussion

Despite recent improvements in surgery and the development of different regimens of multidrug chemotherapy over the past 25 years, survival of patients suffering from osteosarcoma remains around 55 to 70% after 5 years (Provisor et al., 1997). The prognosis is worse with nonextremities localization, advancing age, radioinduced osteosarcoma, and those arising from Paget's disease of bone, representing 40% of the entire osteosarcoma population. In addition, patients with metastatic osteosarcoma at the time of diagnosis have poor survival statistics (30% at 5 years). The major challenge in treating cancer relates to mutations in key genes such as p53, Rb, or proteins affecting caspase signaling carried by many tumor cells. Moreover, several mutations or inactivations of the antioncogenes p53 and Rb are detected in 50% patients suffering from osteosarcoma (Wadayama et al., 1994; Fuchs and Pritchard, 2002). In this context, p53 and Rb status become the major predictors of failure to respond to radiotherapy and chemotherapy in osteosarcomas. The poor prognosis of osteosarcoma warrants new therapeutic strategies to improve the overall rate of survival, especially in high-risk subgroups. In this context, one of the future ther-

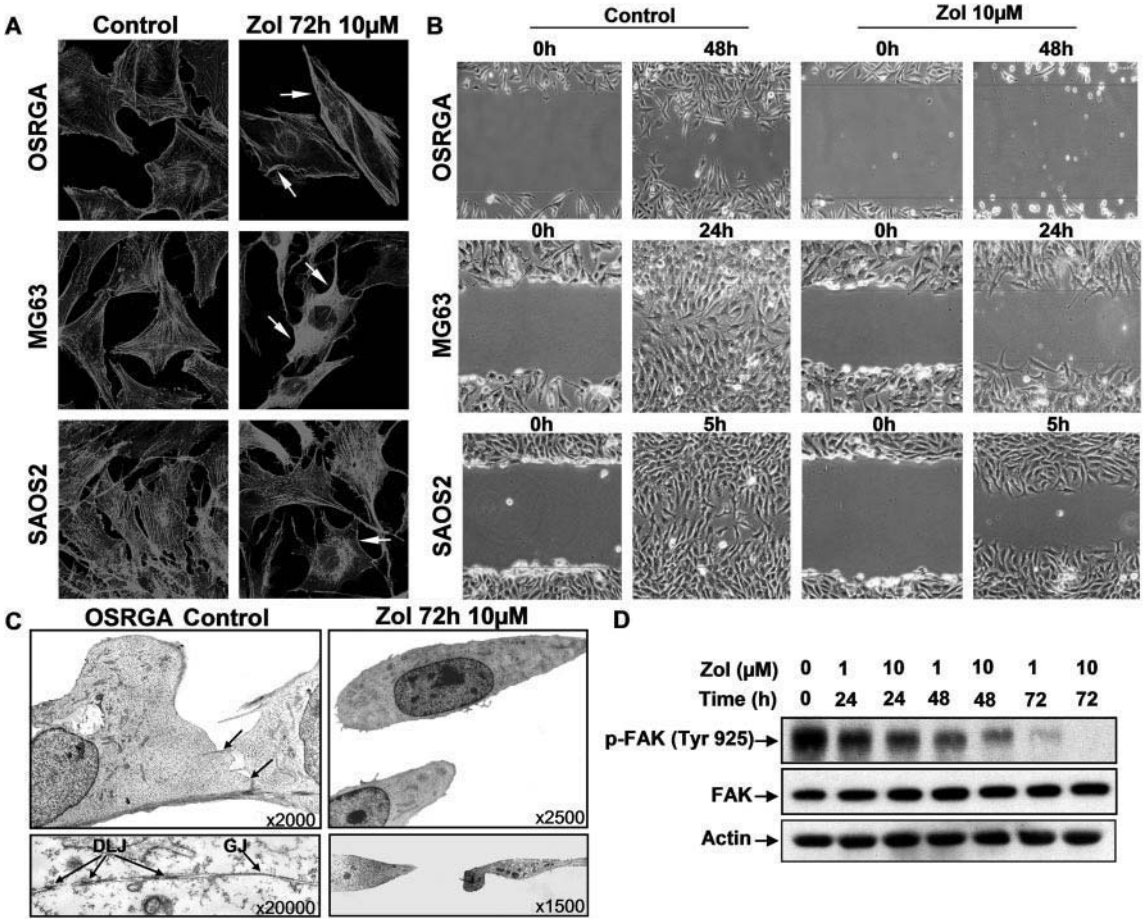

Fig. 5. Zoledronic acid disturbs cytoskeleton organization, disrupts cell junctions, and inhibits cell migration. A, Zol effects on actin stress fibers organization were observed by confocal microscopy after phalloidin staining. The actin network reorganization was associated with membrane ruffling (white arrow). Original magnification, 1000×. B, Zol effects on cell migration were observed by time-lapse microscopy. The horizontal red bars represent the limit of the slit performed on the cell monolayer at the start of the experiment. Original magnification, 100×. C, ultrastructural analysis of OSRGA cells treated or not by 10 μM Zol for 72 h. DLJ, desmosome-like junction; GJ, gap junction. D, FAK phosphorylation was assessed by Western blot on OSRGA cell line at the indicated condition of Zol treatment. All experiments were repeated three times, and a representative blot is shown.

apeutic challenges is based on therapeutic approaches bypassing p53, Rb, and caspase cascade.

Zol would exert one part of its activity on this G_1/S DNA checkpoint (Fig. 7). Indeed, Zol induces Rb phosphorylation and stimulates Rb wild-type osteosarcoma cell G_1/S progression. Rb protein belongs to the pocket protein family, which includes Rb, p107, and p130. Rb acts as a generic corepressor of the E2F family of transcription factors (Harbour and Dean, 2000). Hypophosphorylated Rb is recruited by E2F, which in turn recruits histone deacetylase, leading to active transcriptional repression (Harbour and Dean, 2000). In contrast, hyperphosphorylated Rb triggers the activity of E2F transcription factor, leading to enhanced cyclin E level. Increased cyclin E/cdk2 activity allows further Rb hyperphosphorylation, G_1 progression, and S-phase initiation (Pestell et al., 1999; Blagosklonny and Pardee, 2002). Zol exerts one part of its activity through this G_1/S DNA checkpoint (Fig. 7). Indeed, Zol induces Rb phosphorylation and stimulates Rb wild-type osteosarcoma cell G_1/S progression. The repression of p21 by Zol is in agreement with the known role of p21 to bind to and inhibit the S-phase-promoting cdk2-cyclin E complex, thus further enhancing G_1/S progression. p21 also binds to the cdk4-cyclin D complex and prevents it from phosphorylating Rb (Harper et al., 1993). Furthermore, if p21 repression reduces cell proliferation, it may also have an anticancer effect. For example, c-myc, which represses p21, sensitizes tumor cells simultaneously to apoptosis by anticancer drugs (Gartel and Radhakrishnan, 2005) However, low doses of Zol also stimulate the proliferation of the Rb-defective-SaOS2 osteosarcoma cell line, demonstrating that the Zol proproliferative activity bypasses Rb pathway. In this system, Rb phosphorylation must be discussed as a dynamic process. At early time of treatment or low Zol concentration, Rb is phosphorylated, and cells accumulate in S phase and become more sensitive to DNA damage. Because cells are not able to repair their DNA damage, they start a feedback process by inhibiting Rb phosphorylation at late time points by cyclin/cdk complex regulation (Krucher et al., 2006). Zol also stimulates proliferation of the Rb-defective SaOS2 osteosarcoma cell lines, demonstrating that the Zol proproliferative activity bypasses the Rb pathway. Indeed, the SaOS2 osteosarcoma cell line has been used frequently for the studies on the biological function of the p53 and Rb genes, because p53 is homozygously deleted and only truncated Rb protein was expressed in these cells (Huang et al., 1988). How could the Zol effect on G_1/S checkpoint be explained in the absence of

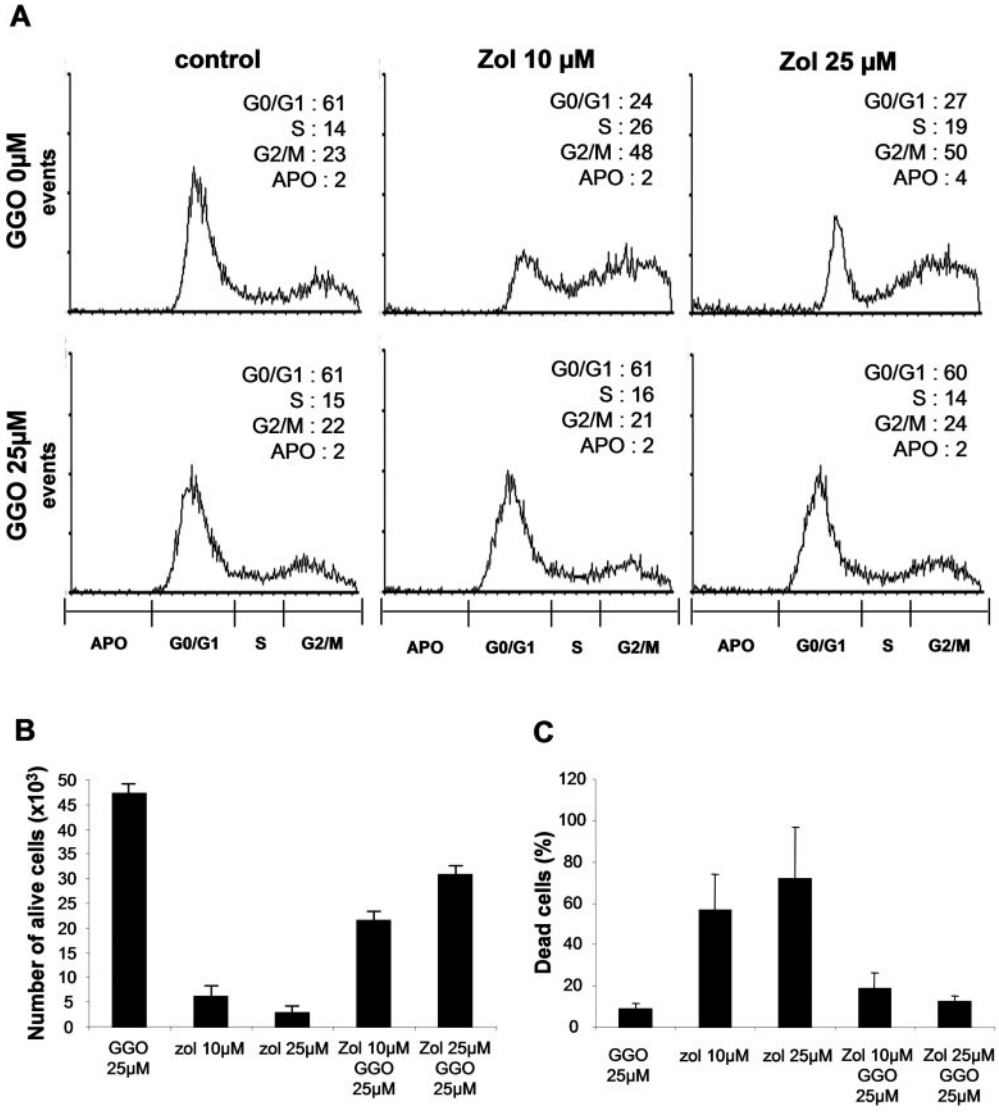

Fig. 6. GGO partly protects osteosarcoma cells from zoledronic acid effects. MG63 cells were cultured for 72 h as described above, before incubation with 10 and 25 μM Zol in the presence or the absence of 25 μM GGO. A, cell cycle distribution of MG63 cells analyzed by propidium iodide staining and fluorescence-activated cell sorting analysis. The alive (B) and dead (C) MG63 cell number (from trypsinized and floating cells) was manually scored after trypan blue exclusion.

an efficient Rb? Dimri et al. (1996) demonstrated that p21 suppresses cell growth and E2F activity in cells lacking a functional Rb protein. In this case, the p21 repression induced by Zol treatment may facilitate the S-phase entry of SaOS2 cells. More recently, Jori et al. (2005) demonstrated both specific and overlapping functions of Rb and p130 genes during the early stages of in vitro neural differentiation of marrow stromal stem cells (Jori et al., 2005), thus revealing a potential compensatory mechanism by the other Rb family genes in Rb null cells. Although p107, p130, and Rb are closely related members of the same family, they have different affinities for E2F family members, and they exhibit distinct temporal regulation during the cell cycle. Although the E2F-p130 complex is the most abundant in quiescent cells, the E2F-p107 and E2F-Rb complexes accumulate in G_1 cells but not in S, G_2, or M phases (Moberg et al., 1996). Thus, p107, which is expressed by SaOS2 cells (Gao et al., 2002), may bypass the Rb defect in these cells and could explain the observed Zol effects.

DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to DNA damage (Nyberg et al., 2002). The G_2/M checkpoint prevents cells from undergoing mitosis in the presence of DNA damage. Depending on the DNA damage, the ATM-Chk2-cdc25c signal transduction pathway and/or the ATR-Chk1-cd25c pathway is activated to arrest the cell cycle (Sancar et al., 2004). Checkpoint kinases inhibit the entry into mitosis by down-regulating cdc25c and up-regulating Wee1, which together control Cdc2/cyclin B complex activity (Yarden et al., 2002). According to these data from the literature, high con-

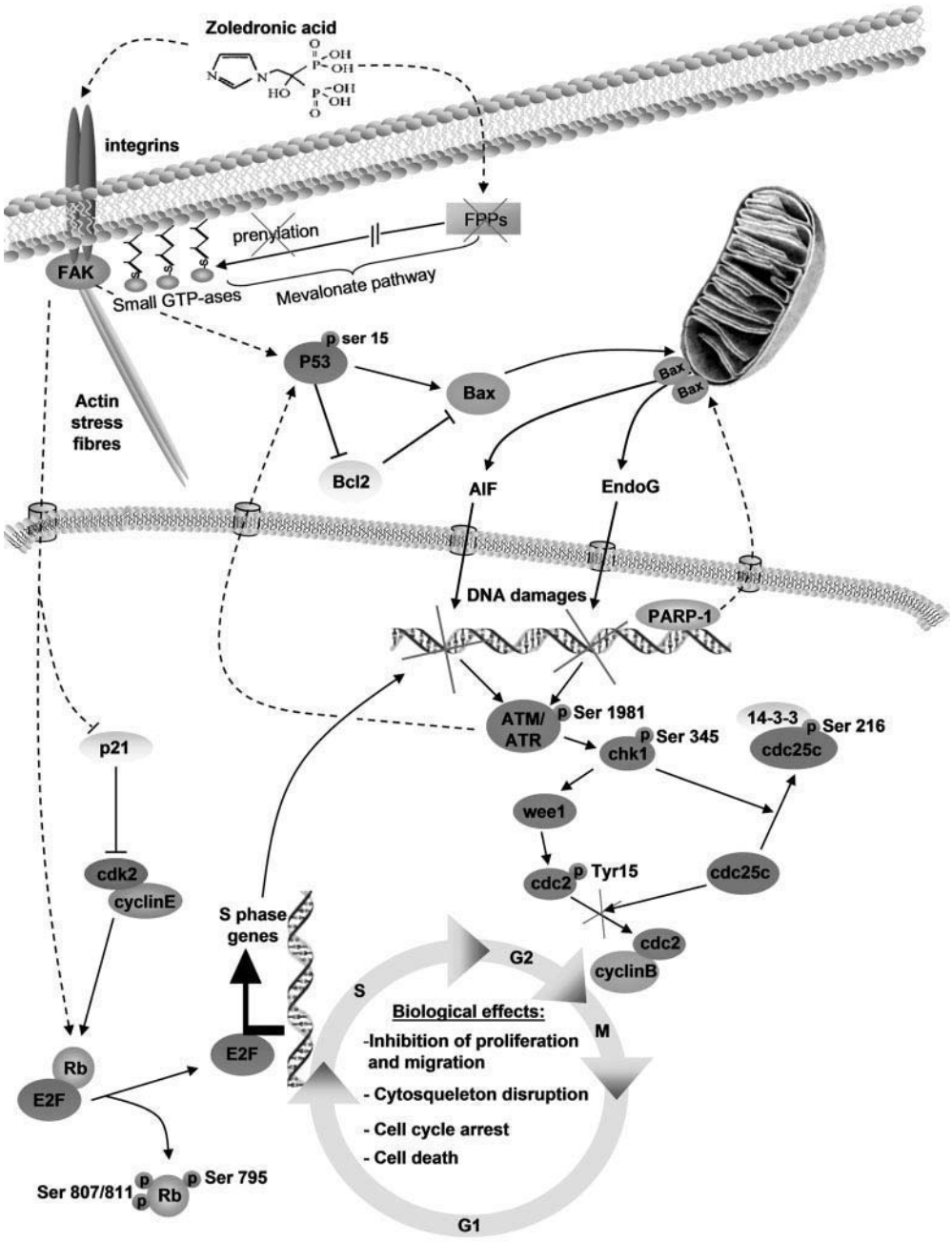

Fig. 7. Potential mechanism of action of zoledronic acid on osteosarcoma cell lines. Membrane molecules such as integrins participate in Zol activities. Low Zol concentrations facilitate the entry in S phase through a decrease of p21 and the phosphorylation of Rb and then liberate the E2F transcription factor activating the S-phase genes. High Zol concentration induced the phosphorylation of cdc2, through a decrease of cdc25c and an increase of Wee1 and the phosphorylation of chk1. P-cdc2 is then no more able to bind to the cyclin B, resulting to a cell cycle arrest in S, G_2/M phase. In tandem, Zol induces atypic apoptosis independently of caspase activation and involving the mitochondria pathway, in particular AIF and EndoG translocation, which may result in DNA damage. The Zol affects the cell cycle and cell death of osteosarcoma cell lines by an integrin-dependent mechanism but independently of the p53 and Rb status of the cells.

centrations of Zol strongly inhibit cell proliferation and induce a cell cycle arrest in S, G_2/M phase, presumably via the control of the intra-S and G_2/M checkpoint (Fig. 7). Indeed, this intra-S and G_2/M checkpoint delays transiently cell cycle progression through S phase to allow the repair of DNA damage. If the DNA damage is not repaired during this period, the intra-S and G_2/M checkpoint should block the cell cycle later in G_2/M phase to avoid "catastrophic mitosis" (Bartek et al., 2004) as observed in the presence of Zol, hypothesize in opposition with Kubista et al. (2006). Moreover, this checkpoint does not require p53, which is the main target of the sustained G_1/S checkpoint (Bartek et al., 2004).

Because these control checkpoints are usually activated in response to DNA damage, two hypotheses can be proposed to understand Zol effects on osteosarcoma cell lines. First, the Zol influence on the G_1/S checkpoint could facilitate and accelerate the entry of osteosarcoma cells into S phase (genetically, the most vulnerable period). The accelerated transcription of S-phase genes could result in numerous DNA damage at multiple points after transcription errors, resulting, for example, from base pair mismatches or limiting dNTP pools. Indeed, intracellular dNTP pools occur as a common feature involved in DNA repair, because limiting dNTP pools enhance damage sensitivity (Zhao et al., 1998). The DNA damage caused by such phenomenon is responsible for the activation of the checkpoint-specific sensors, in particular ataxia-telangiectasia, mutated and/or ATR members of the phosphoinositide 3-kinase-like kinase family (Helt et al., 2005). These sensors are key molecules in the G_2/M DNA checkpoint, as underlined by the consequences induced by their mutations and the induced cancer predispositions (Shiloh, 1997). Second, high concentrations of Zol increase mitochondrial permeability and AIF/EndoG translocation, which may contribute to apoptotic nuclear DNA damage in a caspase-independent way, as reported in other models (Cregan et al., 2004). Such DNA damage could activate the intra-S DNA checkpoint, which slows down the S-phase progression to allow DNA repair and the G_2/M cell cycle arrest to prevent cell mitosis in the presence of DNA damage. Moreover, nuclear and mitochondrial conversations in cell death have recently been pointed out (Hong et al., 2004). Poly(ADP-ribose) polymerase 1 (PARP1) is emerging as an important activator of caspase-independent cell death, and the overactivation of PARP1 or/and DNA damage initiates a nuclear signal that propagates to mitochondria and triggers the release of AIF. Thus, a vicious cycle takes place between mitochondria and nucleus via AIF and potentially PARP1 independently of p53 (Fig. 7). Similar p53-independent effects of Zol were also described on leukemia and colorectal carcinoma cell lines (Kuroda et al., 2004).

Zol also modulates strongly the mevalonate pathway. Indeed, the mevalonate pathway is responsible for the production of cholesterol and isoprenoid lipids such as isopentenyl diphosphate, FPP, and geranylgeranyl diphosphate. FPP and geranylgeranyl diphosphate are required for the posttranslational lipid modification (prenylation) of small GTPases such as Ras, Rho, and Rac. Prenylation involves the transfer of a farnesyl or geranylgeranyl lipid group onto a cysteine residue in characteristic carboxyl-terminal motifs, giving rise to farnesylated and geranylgeranylated proteins. To our knowledge, the majority of the prenylated proteins identified are small GTPases (preferentially geranylgeranylated), which are important signaling proteins regulating a variety of cell processes such as the control of cell morphology, integrin signaling, trafficking of endosomes, and apoptosis. Prenylation is then required for the correct function of these proteins because the lipid prenyl group allows the anchorage of the proteins in cell membranes and also may participate in protein-protein interactions (Rogers et al., 2000). Therefore, the inhibition of the mevalonate pathway by nitrogen-containing bisphosphonates and consequently the loss of prenylation of small GTPases such as Rho, Rac, cdc42, and Rab could account for the various effects observed on osteosarcoma cells (disruption of the actin cytoskeleton, disrupted intracellular signaling by integrins, and induction of cell death).

The anti-oncogene p53 plays a key role in the control of cell proliferation and/or of programmed cell death (Moll et al., 2005). Pivotal to the tumor-suppressor activity of p53 is its ability to activate apoptosis via multiple caspase-dependent or caspase-independent pathways (Lane, 1992). Phosphorylation of p53 represses Bcl-2 expression and up-modulates Bax expression, resulting in apoptosis via the mitochondrial pathway (Park et al., 2005), as observed in Zol-treated OS-RGA cells. However, the mechanism by which p53 is activated remains to be elucidated. For example, Zol-induced DNA damage may maintain p53 activation (Tibbetts et al., 1999). Furthermore, the FAKs may also participate in p53 activation. Indeed, FAK is a tyrosine kinase considered as a central molecule in integrin-mediated signaling, involved in cellular motility and protection against apoptosis (Mukhopadhyay et al., 2005). This characteristic is in agreement with the observed Zol effects, including an integrin-dependent mechanism, mediating a decreased of P-FAK, decreased motility and apoptosis. More recently, a direct interaction of the N-terminal domain of FAK with the N-terminal transactivation domain of p53 has been demonstrated previously (Goluboskaya et al., 2005), strengthening the potential role of p53 and FAK in Zol-induced cell death in wild-type p53 osteosarcoma cells. However, Zol induced the cell death of p53-mutated MG63 and of null p53 SaOS2 cells, thereby suggesting that Zol is able to bypass the p53 pathway to activate cell death mechanisms in osteosarcoma cells. In this case, FAK may represent a privileged place in the Zol mechanism of action. Indeed, Boissier et al. (1997) demonstrated that BPs inhibit carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrix, suggesting that BPs exert their activities via adhesion molecules. This observation has been more recently confirmed in endothelial cells (Bezzi et al., 2003). These authors demonstrated that Zol sensitizes endothelial cells to tumor necrosis factor-induced, caspase-independent programmed cell death and identified the FAK-protein kinase B/Akt pathway as a novel Zol target. Adhesion molecules must be considered as key factors for Zol activities, but other investigations are needed to determine whether FAK is the first target involved in Zol effects, via membranous integrins, or whether the effects are the consequence of Zol on cell death.

We previously demonstrated that Zol enhances osteosarcoma regression in vivo in rodent models (Heymann et al., 2005; Ory et al., 2005) and slows down rat primary chondrosarcoma development (Gouin et al., 2006). Skerjanec et al. (2003) showed that the peak levels of ZOL detected in plasma of patients suffering from cancer is around 1 μM, which is in

agreement with the IC_{50} of ZOL measured on osteosarcoma cells in the present study. Furthermore, because of its high affinity for bone matrix, the local concentration of Zol in this tissue is certainly higher than its plasma levels, thereby strengthening an in vivo potential effect of ZOL even at a low concentration. Taken together, these data demonstrate that treatment of osteosarcoma cells with an *N*-BP strongly inhibits cell proliferation through the induction of cell death via AIF and EndoG translocation and the cell cycle arrest in S, G_2/M phase independently of p53, Rb, and caspases. These observations open a new area in the field of therapeutic combinations for the treatment of osteosarcoma.

Acknowledgments

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References

Bartek J, Lukas C, and Lukas J (2004) Checking on DNA damage in S phase. *Nat Rev Mol Cell Biol* **5:**792–804.

Bezzi M, Hasmin M, Bieler G, Dormond O, and Ruegg C (2003) Zoledronate sensitizes endothelial cells to tumor necrosis factor-induced programmed cell death: evidence fort the suppression of sustained activation of focal adhesion kinase and protein B/Akt. *J Biol Chem* **278:**43603–43614.

Blagosklonny MV and Pardee AB (2002) The restriction point of the cell cycle. *Cell Cycle* **1:**103–110.

Boissier S, Magnetto S, Frappart L, Cuzin B, Ebetino FH, Delmas PD, and Clézardin P (1997) Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. *Cancer Res* **57:**3890–3894.

Coxon FP, Helfrich MH, Van't Hof R, Sebti S, Ralston SH, Hamilton A, Rogers MJ (2000) Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. *J Bone Min Res* **15:**1467–1476.

Cregan SP, Dawson VL, and Slack RS (2004) Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene* **23:**2785–2796.

Dimri GP, Nakanishi M, Desprez PY, Smith JR, and Campisi J (1996) Inhibition of E2F activity by the cyclin-dependent protein kinase inhibitor p21 in cells expressing or lacking a functional retinoblastoma protein. *Mol Cell Biol* **16:**2987–2997.

Evdokiou A, Labrinidis A, Bouralexis S, Hays S, and Findlay DM (2003) Induction of cell death of human osteogenic sarcoma cells by zoledronic acid resembles anoikis. *Bone* **33:**216–228.

Fuchs B and Pritchard DJ (2002) Etiology of osteosarcoma. *Clin Orthop Rel Res* **397:**40–52.

Gao CF, Ren S, Wang J, Zhang SL, Jin F, Nakajima T, Ikeda M, and Tsuchida N (2002) P130 and its truncated from mediated p53-induced cell cycle arrest in Rb−/− Saos2 cells. *Oncogene* **21:**7569–7579.

Gartel AL and Radhakrishnan SK (2005) Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res* **15:**3980–3985.

Gibbs JB and Oliff A (1997) The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. *Annu Rev Pharmacol Toxicol* **37:**143–166.

Goluboskaya VM, Finch R, and Cances WG (2005) Direct interaction of the N-terminal domain of focal adhesion kinase with the N-terminal transactivation domain of p53. *J Biol Chem* **280:**25008–25021.

Gouin F, Ory B, Redini F, and Heymann D (2006) Zoledronic acid slows down rat primary chondrosarcoma development, recurrent tumor progression after intralesional curettage and increases overall survival. *Int J Cancer* **119:**980–984.

Harbour JW and Dean DC (2000) The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev* **14:**2393–2409.

Harper JW, Adami GR, Wei N, Keyomarsi K, and Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75:**805–816.

Helt CE, Cliby WA, Keng PC, Bambara RA, and O'Reilly MA (2005) Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related protein exhibit selective target specificities in response to different forms of DNA damage. *J Biol Chem* **14:**1186–1192.

Heymann D, Ory B, Gouin F, Green J, and Rédini F (2004) Bisphosphonates: new therapeutic agents for the treatment of bone tumors. *Trends Mol Med* **10:**337–343.

Heymann D, Ory B, Blanchard F, Heymann MF, Coipeau P, Charrier C, Couillaud S, Thièry JP, Gouin F, and Rédini F (2005) Enhanced tumor regression and tissue repair when zoledronic acid is combined with ifosfamide in rat osteosarcoma. *Bone* **37:**74–86.

Hong SJ, Dawson TM, and Dawson VL (2004) Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signalling. *Trends Pharmacol Sci* **25:**259–264.

Huang HJ, Yee JK, Shew JY, Chen PL, Bookstein R, Friedmann T, Lee FY, and Lee WH (1988) Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science (Wash DC)* **242:**1563–1566.

Jori FP, Melone MAB, Napolitano MA, Cipollaro M, Cascino A, Giordano A, and Galderisi U (2005) Rb and Rb/p130 genes demonstrate both specific and overlapping functions during the early steps of in vitro neural differentiation of marrow stromal stem cells. *Cell Death Differ* **12:**65–77.

Klein B, Pals S, Masse R, Lafuma J, Morin M, Binart N, Jasmin JR, and Jasmin C (1977) Studies of bone and soft-tissue tumors induced in rats with radioactive cerium chloride. *Int J Cancer* **20:**112–119.

Krucher NA, Rubin E, Tedesco VC, Roberts MH, Sherry TC, and De Leon G (2006) Dephosphorylation of Rb (Thr-821) in response to cell stress. *Exp Cell Res* **312:**2757–2763.

Kubista B, Trieb K, Sevelda F, Toma C, Arrich F, Heffeter P, Elbling L, Suterluty H, Scotlandi K, Kotz R, et al. (2006) Anticancer effects of zoledronic acid against human osteosarcoma cells. *J Orthop Res* **24:**1145–1152.

Kuroda J, Kimura S, Segawa H, Sato K, Matsumoto S, Nogawa M, Yuasa T, Kobayashi Y, Yoshikawa T, Ottmann OG, et al. (2004) p53-Independent antitumor effects of the nitrogen-containing bisphosphonate zoledronic acid. *Cancer Sci* **95:**186–192.

Lane DP (1992) Cancer, p53, guardian of the genome. *Nature (Lond)* **358:**15–16.

Mackie PS, Fisher JL, Zhou H, and Choong PF (2001) Bisphosphonates regulate cell growth and gene expression in the UMR 106–01 clonal rat osteosarcoma cell line. *Br J Cancer* **84:**951–958.

Moberg K, Starz MA, and Lees JA (1996) E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Mol Cell Biol* **16:**1436–1449.

Moll UM, Wolff S, Speidel D, and Deppert W (2005) Transcription-independent pro-apoptotic functions of p53. *Curr Opin Cell Biol* **17:**631–636.

Mukhopadhyay NK, Gordon GJ, Chen CJ, Bueno R, Sugarbaker DJ, and Jaklitsch MT (2005) Activation of focal adhesion kinase in human lung cancer cells involves multiple and potentially parallel signaling events. *J Cell Mol Med* **9:**387–397.

Nyberg KA, Michelson RJ, Putnam CW, and Weinert TA (2002) Toward maintaining the genome: DNA damage and replication checkpoints. *Annu Rev Genet* **36:**617–656.

Ory B, Heymann MF, Blanchard F, Kamijo A, Gouin F, Heymann D, and Rédini F (2005) Zoledronic acid suppresses lung metastases and extends overall survival of osteosarcoma of osteosarcoma-bearing mice. *Cancer* **104:**2522–2529.

Park BS, Song YS, Yee SB, Lee BG, Seo SY, Park YC, Kim JM, Kim HM, and Yoo YH (2005) Phospho-Ser 15-p53 translocates into mitochondria and interacts with Bcl-2 and Bcl-xL in eugenol-induced apoptosis. *Apoptosis* **10:**193–200.

Pestell RG, Albanese C, Reutens AT, Segall JE, Lee RJ, and Arnold A (1999) The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. *Endocrin Rev* **20:**501–534.

Provisor AJ, Ettinger LJ, Nachman JB, Krailo MD, Makley JT, Yunis EJ, Huvos AG, Betcher DL, Baum ES, Kisker CT, et al. (1997) Treatment of non-metastatic osteosarcoma of the extremity with pre-operative chemotherapy: a report from the Children's Cancer Group. *J Clin Oncol* **15:**76–84.

Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Monkkonen J, and Frith JC (2000) Cellular and molecular mechanisms of action of bisphosphonates. *Cancer* **88:**2961–2978.

Ruoslahti E (1996) RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol* **12:**697–715.

Salsmann A, Schaffner-Reckinger E, and Kieffer N (2006) RGD, the Rho'd to cell spreading. *Eur J Cell Biol* **85:**249–254.

Sancar A, Lindsey-Boltz LA, Ünsal-Kaçmaz K, and Linn S (2004) Molecular mechanism of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* **73:**39–85.

Shiloh Y (1997) Ataxia-telangiectasia and the Nijmegen breakage syndrome: related disorders but genes apart. *Annu Rev Genet* **31:**635–662.

Skerjanec A, Berenson J, Hsu C, Major P, Miller WH Jr, Ravera C, Schran H, Seaman J, and Waldmeier F (2003) The pharmacokinetics and pharmacodynamics of zoledronic acid in cancer patients with varying degrees of renal function. *J Clin Pharmacol* **43:**154–162.

Sonnemann J, Eckervogt V, Truckenbrod B, Boos J, Winkelmann W, and van Valen F (2001) The bisphosphonate pamidronate is a potent inhibitor of human osteosarcoma cell growth in vitro. *Anticancer Drugs* **12:**459–465.

Thiéry JP, Perdereau B, Gongora R, Gongora G, and Mazabreaud A (1982) Un modèle d'ostéosarcome chez le rat. L'ostéosarcome greffable de rat. *Sem Hop Paris* **58:**1686–1689.

Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh SY, Taya Y, Prives C, and Abraham RT (1999) A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev* **13:**152–157.

Wadayama B, Toguchida J, Shimizu T, Ishizaki K, Sasaki MS, Kotoura Y, and Yamamuro T (1994) Mutation spectrum of the retinoblastoma gene in osteosarcomas. *Cancer Res* **54:**3042–3048.

Yarden RI, Pardo-Reovo S, Sgagias M, Cowan KH, and Brody LC (2002) BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. *Nat Genet* **30:**285–289.

Zhao X, Muller EG, and Rothstein R (1998) A suppressor of two essential ckeckpoint gene identifies a novel protein that negatively affects dNTP pools. *Mol Cell* **2:**329–340.

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Complément de discussion à l'article 2

Le tissu osseux est un dérivé du tissu conjonctif, complexe et en perpétuel remaniement, reposant sur l'équilibre de deux mécanismes: la formation et la résorption osseuse. Tout déséquilibre entre ces processus est à l'origine de pathologies qui peuvent être ostéocondensantes ou ostéolytiques, bénignes ou malignes. L'ostéosarcome dérive des ostéoblastes, et est très souvent associée à une ostéolyse. Les traitements actuels, qui consistent en une résection chirurgicale de la tumeur et en une polychimiothérapie (associant principalement le méthotrexate, le cisplatine, la doxorubicine et l'ifosfamide), ont certes permis d'améliorer les taux de survie, mais la forte toxicité des molécules de chimiothérapie utilisées est souvent mal tolérées chez les jeunes patients, dont la médiane d'âge est de 18 ans. Ainsi le mauvais pronostic associé à la détection de métastases pulmonaires, fait qu'il est nécessaire de développer de nouvelles approches thérapeutiques des ostéosarcomes.

Des mutations ou inactivations des antioncogènes p53 et Rb peuvent être détectées dans 50% des patients atteint d'ostéosarcomes (Wadayama et al. 1994; Fuchs et al. 2002), à tel point quelles constituent le facteur pronostique majeur de réponse à la radiothérapie et à la chimiothérapie des ostéosarcomes. Dans ce contexte, l'acide zolédronique semble être une approche thérapeutique pertinente puisse que comme nous l'avons vu, cette molécule n'est ni sensible aux mutations de p53 ni à celle de Rb ; de plus elle induit la mort cellulaire par des mécanismes indépendants des caspases.

Cette étude a permis de lever une partie du voile sur les mécanismes d'action directe de l'acide zolédronique sur les cellules d'ostéosarcomes.

En résumé, nous avons mis en évidence le caractère dual des effets de l'acide zolédronique. Pour bien les comprendre, ces effets doivent être considérés comme des phénomènes dynamiques. Pour des temps courts de traitement (<24h), ou pour de faibles concentrations (picoM), on observe une phosphorylation de Rb ; or, une hyperphosphorylation de Rb entraine une libération du facteur de transcription E2F conduisant entre autre à l'augmentation du niveau de transcription de la cycline E. Le complexe cycline E/cdk2 permet alors l'initiation de la phase S. Par conséquent, dans ces conditions, le traitement par l'acide zolédronique conduit à l'accumulation des cellules en phase S du cycle cellulaire, phase au cours de laquelle les cellules sont hautement sensibles aux dommages de l'ADN, étant vigilantes aux possibles erreurs commises pendant la phase de synthèse de l'ADN. La stimulation trop importante de la synthèse de l'ADN par l'acide zolédronique conduit

probablement la machinerie cellulaire à commettre des erreurs ou encore à une pénurie de dNTP. Les cellules n'étant plus capables de réparer les dommages de l'ADN, elles déclenchent un rétrocontrôle en inhibant la phosphorylation de Rb (*figure 18*), et en activant le point de contrôle situé en phase G_2/M. Ce point de contrôle empêche les cellules d'entrer en mitose en présence de dommages de l'ADN non réparés. Nous observons l'activation de la voie ATR-ChK1-cdc25c, caractéristique de ce point de contrôle du cycle cellulaire. Finalement la cellule déclenche un processus de mort cellulaire *via* la libération de l'AIF et l'EndoG de l'espace intermembranaire mitochondrial (*figure 17*). Très récemment, Kook et al ont également rapporté un cas de mort cellulaire indépendante des caspases et associée à une translocation de l'AIF et de l'EndoG dans un modèle cellulaire d'ostéosarcome humain suite à un traitement par des flavonoïdes (Kook et al. 2007).

La manière exacte par laquelle l'acide zolédronique pénètre dans les cellules d'ostéosarcome reste inconnue. Les travaux de Thompson et al suggèrent que les bisphosphonates pénètrent dans le cytoplasme des cellules macrophagiques par acidification des vésicules après endocytose en phase liquide (Thompson et al. 2006). Nos résultats suggèrent quant à eux que l'acide zolédronique interagit avec les intégrines, ou tout du moins que les mécanismes d'action de l'acide zolédroniques sont associés à une voie de transduction associée aux intégrines. Nous avons observé que les effets de l'acide zolédronique étaient réprimés par l'adjonction de peptides RGD dans le milieu de culture ; ce peptide se fixant avec une forte affinité aux intégrines présentes à la surface cellulaire, il joue probablement le role d'inhibiteur compétitif de l'acide zolédronique pour la fixation à ces intégrines. Nous avons également observé une déphosphorylation des kinases d'adhésions focales (FAK) suite au traitement par le Zol qui pourrait expliquer les effets observés sur la migration et la mort cellulaire. En effet, les FAK sont des tyrosines kinases considérées comme des molécules centrales des voies de signalisation impliquant les intégrines et régulant les phénomènes de migration cellulaire et d'apoptose. Ceci est en accord avec les travaux de Bezzi et al qui démontrent l'inhibition de l'adhérence et de la migration des cellules endothéliales en présence de bisphosphonates sur la vitronectine (Bezzi et al. 2003). Plus récemment, Bellahcène et al rapportent que l'acide zolédronique diminue significativement l'expression des intégrines alphaVbeta3 et alphaVbeta5 à la surface des cellules HUVECs et que ceci est lié à ses propriétés anti-angiogénes (Bellahcene et al. 2007). Par conséquent, les molécules d'adhésions doivent être considérées comme étant des facteurs clefs dans les mécanismes d'action du Zol, mais de plus amples investigations doivent être menées afin de déterminer si les FAK sont la première cible du Zol *via* l'interaction avec les intégrines ou si ce phénomène

n'est qu'une conséquence du Zol sur la mort cellulaire. Ce point sera détaillé dans la partie perspective de ce manuscrit.

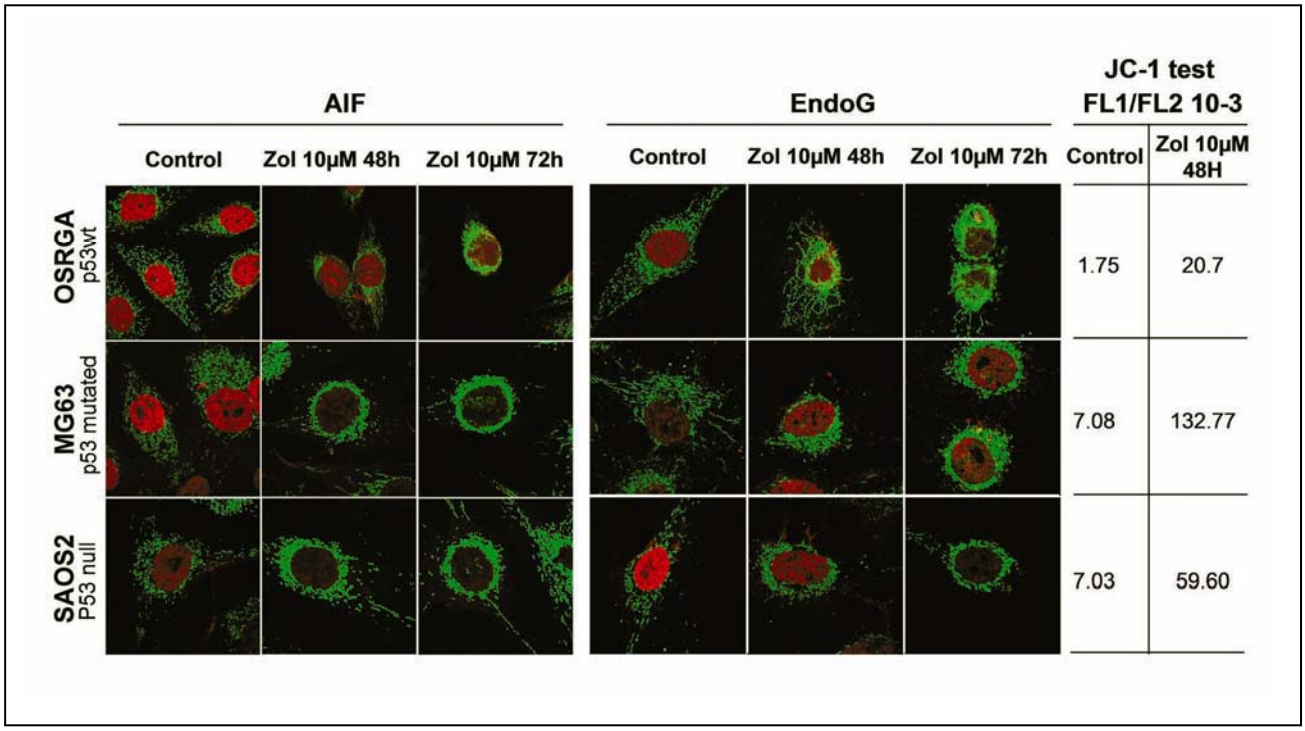

Figure 17 **: L'acide zolédronique augmente la perméabilité mitochondriale ainsi que la translocation de l'AIF et de l'EndoG.**

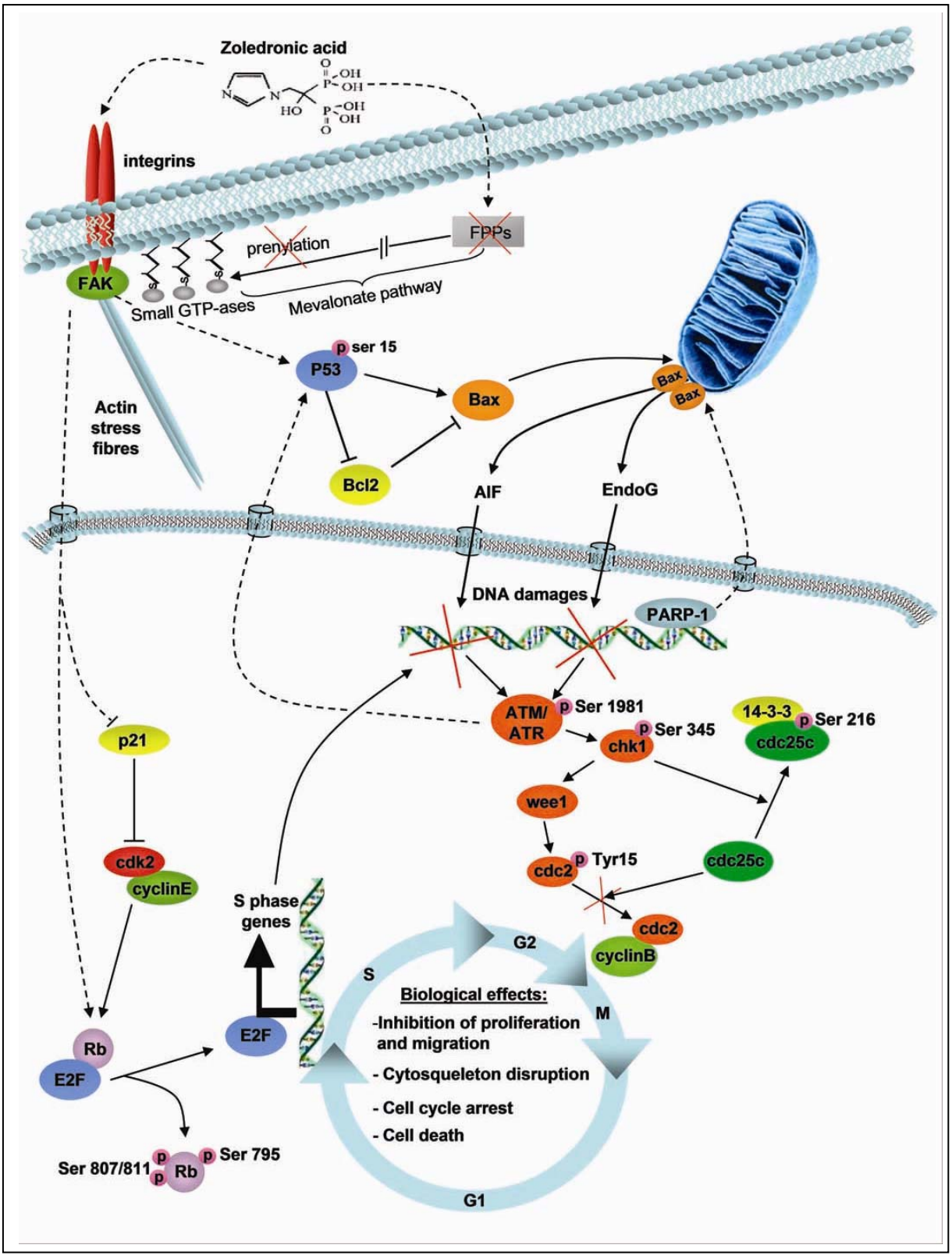

Figure 18 **: Hypothèse de mécanisme d'action de l'acide zolédronique sur les cellules d'ostéosarcomes.**

III. Etude *in vivo* des effets de l'acide zolédronique.

Introduction

Les résultats issus des études *in vitro* nous indiquent que l'acide zolédronique pourrait être un bon candidat dans le traitement des tumeurs osseuses primitives ; nos efforts se sont alors portés sur la validation de ces effets anti-tumoraux *in vivo*, et plus particulièrement, dans un premier temps, sur l'éventuel bénéfice d'une association avec un agent de chimiothérapie communément utilisé dans le traitement des ostéosarcomes.

Il a déjà été rapporté que l'acide zolédronique potentialisait les effets cytostatiques du paclitaxel *in vitro* sur des cellules de cancer du sein (Jagdev et al. 2001) ; de plus, la combinaison de bisphosphonates avec des agents anti-tumoraux tels que l'uracil et le tégafur améliore la réduction des métastases osseuses en comparaison du tégafur et de l'uracil seuls (Hiraga et al. 2003). A l'inverse, des travaux d'Evdokiou et al rapportent des effets de l'acide zolédronique sur la réduction du nombre de cellules osseuses tumorales viables mais aucun effet de synergie avec d'autres agents de chimiothérapie n'a pu être observé (Evdokiou et al. 2003). C'est pourquoi, l'effet synergique de la combinaison d'un agent anti résorption osseuse avec un agent conventionnel de chimiothérapie doit être étudié sur le développement des tumeurs osseuses primitives. L'accent sera mis sur l'étude des paramètres histo-morpho-métriques grâce à l'analyse radiographique, histologique et au micro-scanner.

Pour réaliser cette étude, un modèle transplantable d'ostéosarcome de rat a été utilisé, ce modèle mime le développement d'un ostéosarcome humain tant sur le plan temporel que biologique puisqu'il se caractérise par une prolifération tumoral associée à un de remodelage osseux excessif.

L'objectif de cette étude est l'évaluation de l'efficacité de la combinaison, acide zolédronique (agent anti-résorption osseuse) et ifosfamide (agent conventionnel de chimiothérapie) sur la croissance tumorale, la réponse histologique et la survie des animaux.

Article 3

« L'association de l'acide zolédronique et de l'ifosfamide augmente la régression tumorale et la réparation tissulaire dans les ostéosarcomes de rat »

Bone, Vol 37, 74-86, mai 2005**,** D. Heymann, B. Ory, F. Blanchard, M-F. Heymann, P. Coipeau, C. Charrier, S. Couillaud, J.P. Thiery, F. Gouin, F. Redini**.**

L'efficacité de l'acide zolédronique (Zol), associé ou non à l'ifosfamide (agent de chimiothérapie utilisé habituellement dans le traitement des ostéosarcomes), a été testé sur la croissance des tumeurs osseuses primitives à l'aide d'un modèle d'ostéosarcome transplantable chez le rat. Les effets sur le remodelage osseux et la croissance de la tumeur ont été analysés par radiographie, micro-scanner et coloration histologique. Les effets *in vitro* de l'acide zolédronique ont quant à eux été étudiés en comparant la prolifération, l'apoptose et la progression du cycle cellulaire, des cellules d'ostéosarcome de rat OSRGA et des ostéoblastes primaires de rat. *In vivo*, l'acide zolédronique prévient efficacement les lésions ostéolytiques et la diminution de la croissance tumorale. L'analyse architecturale de l'os trabéculaire indique que l'association de l'acide zolédronique et de l'ifosfamide est plus efficace que chaque agent utilisé individuellement pour ce qui est de la prévention de la récidive tumorale, la réparation tissulaire et la formation osseuse. Les études *in vitro* ont démontré que l'acide zolédronique était plus efficace sur les cellules d'ostéosarcome que sur les ostéoblastes primaires de rat (IC50=0,2 et 20µM respectivement sur la prolifération cellulaire), l'inhibition de prolifération des cellules OSRGA étant due à un blocage du cycle cellulaire en phase S. Aucune induction d'apoptose par l'acide zolédronique n'a pu être observée, aussi bien *in vitro* après coloration au hoechst et test d'activité caspase 1 et 3, qu'*in vivo* après marquage TUNEL. Cette étude décrit pour la première fois les effets anti-résorption et anti-tumoral de l'acide zolédronique dans un modèle d'ostéosarcome de rat, ainsi que son association bénéfique avec un agent anti-tumoral dans la prévention des récidives tumorales.

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Enhanced tumor regression and tissue repair when zoledronic acid is combined with ifosfamide in rat osteosarcoma

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Abstract

The efficacy of zoledronic acid (ZOL), with or without the anticancer drug ifosfamide (IFO), was tested on primary bone tumor growth using a rat-transplantable model of osteosarcoma. The effects on bone remodeling and tumor growth were analyzed by radiography, micro-computed tomography (micro-CT), and histological staining. The in vitro effects of ZOL were studied by proliferation, apoptosis, and cell cycle analyses on the osteosarcoma cells OSRGA compared to rat primary osteoblasts. Treatment with ZOL was effective in preventing the formation of osteolytic lesions that developed in bone sites and in reducing the local tumor growth, as compared to the untreated rats. The combination of ZOL and IFO was more effective than each agent alone in preventing tumor recurrence, improving tissue repair, and increasing bone formation as revealed by the analysis of trabecular architecture. In vitro studies demonstrated that ZOL was more potent against the OSRGA cell line than osteoblasts (with a half-maximal inhibitory effect on proliferation seen at 0.2 and 20 μM, respectively), the ZOL-induced inhibition of OSRGA proliferation being due to cell cycle arrest in S-phase. No effect on OSRGA apoptosis could be observed in vitro, as assessed by Hoechst staining and caspase-1 and -3 activation. In situ cell death was determined by TUNEL staining on tumor tissue sections. No significant difference in TUNEL-positive cells could be observed between ZOL-treated and -untreated rats. This is the first report of the anti-bone resorption and antitumoral activities of zoledronic acid in a rat model of osteosarcoma, and its beneficial association with an antitumoral chemotherapeutic drug in preventing tumor recurrence.

Keywords: Bone tumor; Bisphosphonate; Chemotherapy; Osteolysis

Introduction

Osteosarcoma is the most frequent primary bone tumor, it develops mainly in the young, the median age of diagnosis being 18 years. The current strategy for treatment of high-grade osteosarcoma is based on neo-adjuvant chemotherapy, delayed en-bloc wide resection, and adjuvant chemotherapy adapted to the histologic profile assessed on tumor tissue removed during surgery [1]. Despite recent improvements in surgery and the development of different regimens of multidrug chemotherapy over the past 25 years, survival remains around 55–70% after 5 years [2,3]. The prognosis is worse with non-extremities localization, advancing age, radio-induced osteosarcoma, and those arising from Paget's disease of bone, representing 40% of the entire osteosarcoma population. In addition, patients with metastatic osteosarcoma at the time of diagnosis have poor survival statistics (30% at 5 years). This poor

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prognosis of osteosarcoma warrants new therapeutic strategies to improve the overall rate of survival, especially in high-risk sub-groups.

As evidenced for bone metastases, a vicious cycle between osteoclasts, bone stromal cells/osteoblasts, and cancer cells has been hypothesized during the progression of primary bone tumors [4]. Accordingly, suppression of osteoclasts would be a primary approach to inhibit local cancer growth. Several investigators have enhanced the potential effect of osteoclast-regulating drugs on tumor growth. Among these drugs, bisphosphonates (BPs) are an important class of molecules for the treatment of bone diseases with different molecular mechanisms of action. Nitrogen-containing BPs, such as alendronate or zoledronic acid, inhibit bone resorption by preventing the prenylation of GTP binding proteins such as ras and raf in osteoclasts by inhibition of farnesyl diphosphate synthase in the HMG CoA mevalonate pathway [5], whereas non-nitrogen-containing BPs such as clodronate are metabolized to non-hydrolyzable analogs of ATP [6]. The final common result is the induction of osteoclast apoptosis. BPs act by inhibiting the recruitment, proliferation, and differentiation of preosteoclasts, or by impeding the resorptive activity of mature osteoclasts [7–10]. They also shorten the life span of osteoclasts by inducing their apoptosis [11]. Previous studies revealed that BPs have the ability to reduce the osteolytic bone resorption associated with multiple myeloma and breast cancer [12,13]. Zoledronic acid has also shown efficacy in cancer metastases to bone due to prostate cancer and other solid tumors, demonstrating that this BP can reduce skeletal morbidity in both osteolytic and osteoblastic diseases [14,15]. Moreover, recent preclinical data show that BPs can act on tumor cells by inhibiting tumor cell adhesion to mineralized bone as well as tumor cell invasion and proliferation [16,17]. BPs also induce tumor cell apoptosis and stimulate γδ T cell cytotoxicity against tumor cells [18]. For example, it has been reported that the BP zoledronic acid has a clear direct antitumor activity on breast cancer cells in vitro [19]. In the same study, the commonly used antineoplastic agent, paclitaxel, potentiated the antitumor effects of zoledronic acid, or vice versa, ZOL enhances the cytostatic effect of paclitaxel. Moreover, the combination of BP with anticancer agents such as uracil and tegafur caused an enhanced reduction of bone metastases compared to UFT (tegafur/uracil) alone [20]. Two in vitro studies on the effects of BPs alone on osteosarcoma have reported inhibition of human osteosarcoma cell growth by pamidronate and clodronate [21,22]. A recent work from Evdokiou et al. reported that ZOL reduced the cell number of different human osteogenic sarcoma cell lines, but no synergistic effect was evidenced when ZOL was combined with chemotherapeutic agents [23]. Thus, the synergistic effect of combining a bone regulating factor with conventional chemotherapy should be further investigated on the development of primary tumors at skeletal sites. To perform this study, a rat-transplantable osteosarcoma model was used that mimics human osteosarcoma development at the temporal and physiological levels, presenting aspects of both tumor proliferation and bone remodeling [24]. Hence, the purpose of this study was to evaluate the efficacy of the combination of zoledronic acid (ZOL: anti-bone resorption bisphosphonate) with ifosfamide (IFO: conventional chemotherapy) on local tumor growth, histological response, and animal survival using a rat-transplantable model of osteosarcoma.

Materials and methods

The osteosarcoma model

The osteosarcoma was initially induced by a local injection of colloidal radioactive ^{144}Cerium in rats [25]. The evolution of the tumor is comparable at the temporal (ratio 1:100 between rats and humans) and physiological levels to the development of human osteosarcoma. The tumor can be re-grafted as described below and maintained in vivo for many months, or fragments can be frozen until re-utilization. Lung metastases are observed in 75–90% of rats bearing advanced malignant bone tumors.

Treatment with zoledronic acid and/or ifosfamide: effect on local tumor growth and bone remodeling

Four-week-old male Sprague–Dawley rats (IFFA-CREDO, L'Arbresle, France) were housed under pathogen-free conditions at the Experimental Therapy Unit (Medicine Faculty of Nantes, France), in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators. For the implantation, the rats were anaesthetized by inhalation of a combination isoflurane/air (1.5%, 1 L/min) associated with an intra-muscular injection of Imalgene (100 mg/kg, Merial Lab., Lyon, France). Using a right tibial approach, the periostum of the diaphysis was opened and resected along a length of 5 mm, the underlying bone was intact. A 10-mm^3 fragment of osteosarcoma was placed contiguous to the exposed bone surface without periostum, and the cutaneous and muscular wounds were sutured. Tumors appeared at the graft site approximately 7–10 days later. Rats bearing growing tumors with a volume >1200 mm^3, which were considered as progressive tumors, were individually identified and assigned to the control or treatment group (6–8 animals/group) and treated with ZOL and/or IFO. ZOL, kindly provided as the disodium hydrate by Pharma Novartis AG (Basel, Switzerland), was administered at 100 μg/kg s.c. twice a week, starting at day 11 after tumor implantation. IFO was supplied by Baxter Lab. (France) and administered i.p. (15 mg/kg) to tumor-bearing rats at 24-h intervals on days 20, 21, and 22 after tumor implantation (Fig. 1). Rats in the control group received the same volume of the drug-formulating vehicle

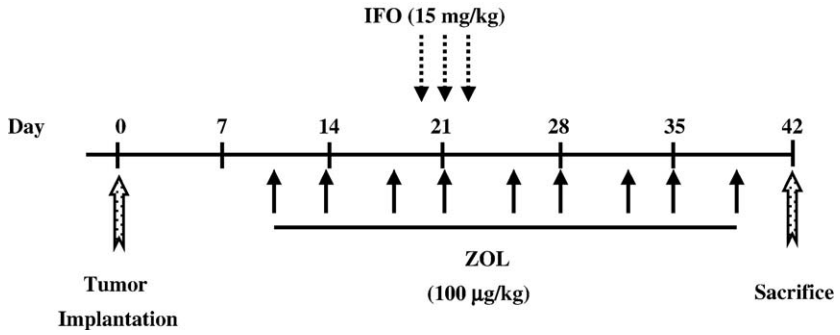

Fig. 1. Summary of the experimental protocol. Osteosarcoma tumors were implanted contiguous to the tibia of male Sprague–Dawley rats on day 0. Zoledronic acid (ZOL) was administered at 100 μg/kg s.c. twice a week, starting on day 11 after tumor implantation. IFO was administered i.p. (15 mg/kg) to tumor-bearing rats at 24-h intervals on days 20, 21, and 22 after implantation. All of the mice were sacrificed at 6 weeks.

with the same schedule as the treated animals. The animals were weighed twice a week. At that time, the tumor volume was calculated from the measurement of two perpendicular diameters using a caliper. Each tumor volume (V) was calculated according to the following formula: $0.5 \times L \times (S)^2$, where L and S are, respectively, the largest and smallest perpendicular tumor diameters. Relative tumor volumes (RTVs) were calculated from the formula: RTV = (V_{42}/V_{25}), where V_{42} is the tumor volume on day 42 and V_{25} the tumor volume at day 25. Radiographic analyses were performed once a week. The animals were sacrificed 6 weeks after tumor implantation, except for spontaneous death, or when the tumor became too bulky and when the life of the animal was threatened. At the time of autopsy, right legs were kept for radiographic, histological studies and micro-architectural parameter quantification. Lung tumor dissemination was assessed by analyzing the number and the size of tumor foci. In one experiment, animals were treated with ZOL for more than 4 weeks as mentioned above (from day 11 to day 42), then the rats were left for a further 6 weeks after the end of ZOL treatment to evaluate tumor recurrence.

Treatment of the rats with ZOL: effect on survival

To determine the effect of ZOL on survival in the osteosarcoma model, an experiment similar to that described above was performed. Sixteen rats were implanted with osteosarcoma tumor fragments: 8 were treated with vehicle and 8 with ZOL from day 11 after tumor implantation. Treatment continued until each animal showed signs of morbidity, which included cachexia, respiratory distress, or animals bearing too bulky tumors (one diameter >40 mm), at which point they were sacrificed.

Histological analysis

Right tibias and femora were fixed in 10% buffered formaldehyde, decalcified by electrolysis, and after embedding in paraffin, 5-μm-thick sections were mounted on glass slides. Sections were stained with hematoxylin-eosin-safran (HES) or with Masson trichrom. Analysis of necrotic and fibrotic areas was performed on each tumor section using a Leica Q500 image analysis system.

Micro-architectural quantification

Analysis of architectural parameters was performed using the high-resolution X-ray micro-CT system for small animal imaging SkyScan-1072 (SkyScan, Aartselaar, Belgium). Relative volume (BV/TV) and specific surface (BS/BV) of the tibiae [total bone (cortical + trabecular) or trabecular bone] were quantified for each group and compared to the tibia of control rats.

Cell proliferation

Rat osteosarcoma cells (OSRGA) and primary rat osteoblasts were cultured in DMEM supplemented with, respectively, 5% and 10% FBS, 1% glutamine, and maintained at 37°C in a humidified atmosphere with 5% CO_2. The rat OSRGA cell line was derived from the osteosarcoma used in the present study. Replicate subconfluent cell cultures in 96-well plates were treated for 1–3 days with increasing concentrations of ZOL (10^{-7} to 10^{-4} M, diluted in PBS). Cell viability was determined by a cell proliferation reagent assay kit using sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)-benzene sulfonic acid hydrate (XTT) (Roche Molecular Biomedicals, Mannheim, Germany). Cell viability was also assessed by trypan blue exclusion.

Induction of apoptosis

Programmed cell death was monitored microscopically following Hoechst and trypan blue staining. OSRGA or primary osteoblasts were seeded at 10^4 cells/well in a 24-well plate and cultured for 24 h as described above before being incubated with ZOL at indicated concentrations during 24, 48, and 72 h. Trypsinized cells were then resuspended in the presence of Hoechst no. 33258 staining (10 μg/ml; Sigma) for 30 min at 37°C. Cells

were then observed by UV microscopy (Leica, Wetzlar, Germany).

Induction of apoptosis was also investigated by cleavage of caspase-1 and -3 substrates in supernatants of cultures with or without ZOL treatment. OSRGA cells or osteoblasts were seeded at 15×10^3 cells/well (in a 24-well plate), then incubated with ZOL (1 and 10 μM) for 24, 48, and 72 h. Cells incubated with 1 μM staurosporine for 6 h were used as positive controls. At the end of the incubation period, the cells were lysed with 50 μl of RIPA buffer for 30 min. The cells were then scraped off and protein content was quantified in parallel samples using the BCA (bicinchominique acid + Copper II sulfate) assay. Caspase 1 and 3 activity was assessed on 10 μl of the cell lysate with the kit CaspACE™ Assay System (Fluorometric, Promega, Madison, USA) following the manufacturer's instructions.

TUNEL assay for apoptosis

Apoptotic cells in the osteosarcoma were detected with an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany), based on the terminal-deoxynucleotidyl transferase-mediated dUTP nick-end labeling method (TUNEL). After formaldehyde-fixed and paraffin-embedded tissue sections had been deparaffinized, specimens were digested with proteinase K at 37°C for 15 min and washed with PBS. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in PBS for 5 min, then the sections were immersed in the terminal deoxynucleotidyl transferase reaction mixture containing enzyme and biotin-labeled dUTP at 37°C for 1 h in the dark. Avidin peroxidase was applied to the sections to detect labeled nucleotides. Binding was localized with aminoethylcarbazole (AEC) and the sections were lightly counterstained with hematoxylin. Four to six sections per animal were prepared for staining of apoptotic cells. The number of TUNEL-positive cells was counted by microscopic examination with a 40× objective lens, and indices were determined as the mean percentages of positive cells among total cells.

Cell cycle analysis

Confluent cultures of OSRGA and osteoblasts were incubated for the indicated times (24, 48, or 72 h) with or without 10 μg/ml ZOL, trypsinized, washed twice, and lysed in PBS containing 0.12% Triton X-100, 0.12 mM EDTA, and 100 μg/ml ribonuclease A. Then 50 μg/ml propidium iodide was added for each sample for 20 min at 4°C in the dark. The intensity of propidium iodide labeling was measured by flow cytometry (FACScan, BD Biosciences) using the CellQuest software.

Statistics

The unpaired Mann–Whitney test was used to assess differences in tumor progression between IFO-, ZOL-, or IFO + ZOL-treated and vehicle-treated control groups. Results with $P < 0.05$ were considered significant. Statistical evaluation of the in vitro proliferation data was performed by Student's t test.

Results

Histological characterization of osteosarcoma

The tumor mass was characterized by large mesenchymal cells possessing nuclear chromatin condensations and clear cytoplasm (Figs. 2A and B). Large necrotic foci were observed inside the tumor mass and were surrounded by cells with nuclear chromatin condensation and dense cytoplasm which were considered as dead cells (Fig. 2A). Areas of proliferating tumor cells (presence of numerous mitoses) were detected at the periphery of the tumor mass or between necrotic foci (Fig. 2B). Where the tumor cells were weakly differentiated (osteoblastic type), they induced new bone span formation (Fig. 2B, insert) and recruited numerous osteoclasts in contact with the bone initially formed (Fig. 2C). Thus, a strong cortical reaction characterized by a great number of activated osteoclasts and an intense bone remodeling activity was observed in contact with the tumor tissue. Cancellous bone invaded by tumor cells was also completely remodeled (data not shown). Moreover, invasion of articular cartilage by tumor cells induced cartilage damage and new bone formation (Fig. 2C, insert). Sacrificed animals possessed lung metastasis (Fig. 2D). The pulmonary tissue was invaded by tumor foci characterized by central necrosis, high-grade proliferating tumor cells, and numerous venous emboli (Fig. 2D, insert).

Protocol design

ZOL treatment began 11 days after tumor implantation, when the tumor volume exceeded 1200 mm^3 to avoid spontaneous tumor regression. This treatment regimen was selected to establish whether inhibition of bone resorption by bisphosphonate could reduce local osteosarcoma growth. Preliminary experiments were conducted to determine the optimal dose of IFO to induce partial tumor regression: 1 × 15 mg/kg, 1 × 30 mg/kg, 3 × 15 mg/kg, 3 × 30 mg/kg, and 3 × 60 mg/kg. Doses of 3 × 30 and 3 × 60 mg/kg induced strong inhibition of tumor growth, with a decrease in the tumor volume of more than 90%. Below these doses (1 × 15, 1 × 30, and 3 × 15 mg/kg), tumor volume was reduced by IFO, with complete regression 12 days after IFO injection (3 × 15 mg/kg) in 50–75% cases, but tumor recurrence was observed 22 days after IFO injections (data not shown). Thus, a dose of 3 × 15 mg/kg was chosen in studies to investigate whether ZOL could reverse the tumor recurrence associated with IFO treatment.

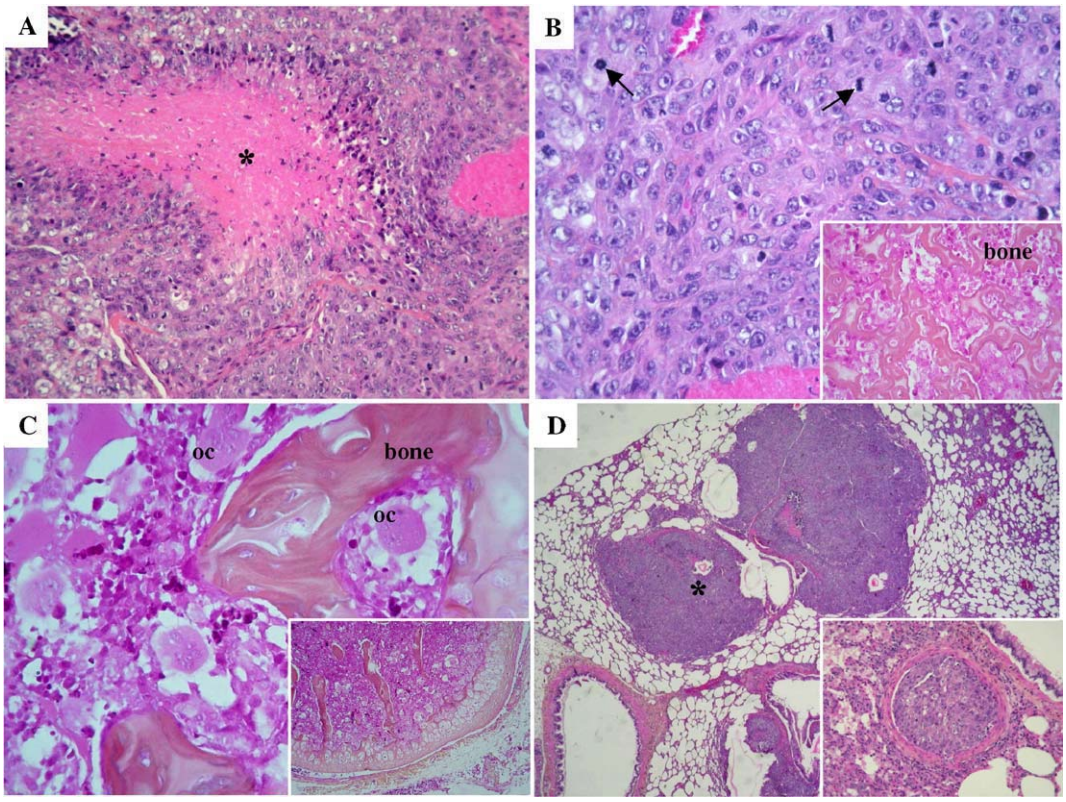

Fig. 2. Histological characterization of the osteosarcoma model after 6 weeks implantation (HES stain). Osteosarcoma fragments were implanted in contact to the tibia of male Sprague–Dawley rat. (A) Large necrotic foci (*) observed inside the tumor mass; (B) Area of proliferating tumor cells presenting numerous mitoses (arrows); new bone formation induced by tumor cells (insert). (C) Intense remodeling activity in contact with tumor tissue (oc: osteoclast); invasion of articular cartilage by tumor cells (insert). (D) Presence of lung metastasis (*) with numerous venous emboli (insert). Original magnification: ×5 (C insert and D), ×10 (D insert), ×20 (A and B insert), ×40 (B and C).

ZOL and IFO strongly increase bone formation in osteosarcoma-bearing rats

Radiographs taken at the time of sacrifice, i.e., 6 weeks after tumor implantation, are shown in Figs. 3A–D. In control rat tibiae, high bone remodeling was observed at the tumor implantation site, resulting in maximal cortical destruction and intensive interactions between altered bone tissue and tumor cells (Fig. 3A). In the ZOL-treated group, osteolytic lesions were rarely observed (Fig. 3B) and the metaphyses of long bones exhibited high bone density reflecting inhibition of bone resorption and retention of the primary spongiosa (arrows, Fig. 3B). Radiographs of the tibiae from IFO-responsive and IFO + ZOL-treated animals revealed no osteolytic lesions (Figs. 3C and D).

By combining micro-CT and 3D image registration, we could follow the bone remodeling associated with osteosarcoma development. The tibia from tumor-bearing rat was characterized by large remodeling activities as compared to control tibia: deep re-structuring of the cortical bone with a reduction of its thickness and paralleled enhancement of trabecular bone (Figs. 4A and B). Micro-architectural parameters [Bone Volume (BV)/Total Volume (TV) and Bone Surface (BS)/TV] were calculated and compared between each group of treated animals (Fig. 4 and Table 1). When animals were treated with IFO, ZOL, and IFO + ZOL, a significant increase in bone mass due to increase in bone formation was observed both at the cortical and trabecular levels (Figs. 4C–E), which was confirmed by the quantification of trabecular relative bone volume: respectively, +24, 48% and 76% as compared to tumor control tibia (Table 1). The same effects were observed when the relative total bone volume was considered: +12, 19% and 32%, respectively. It can be deduced from histological analysis that the increased bone mass upon treatment is woven bone.

IFO increased by itself the relative bone volume, but to a lesser extent than ZOL alone (+12 and 19%, respectively, for the total bone, and +24 and 48% for the trabecular bone), suggesting that IFO alone could block the vicious cycle and indirectly decreased bone resorption.

ZOL prevents the tumor recurrence observed with IFO alone

Treatment of tumor-bearing animals with IFO led to a partial response: an inhibition of tumor progression was observed in all animals, but 28% showed tumor recurrence 42 days after tumor implantation (Fig. 5A). The association of IFO with ZOL led to a complete regression of the tumor in 100% of cases, preventing the possible tumor recurrence observed with ifosfamide alone (Fig. 5A). The relative tumor volumes calculated between days 25 and 42 for the

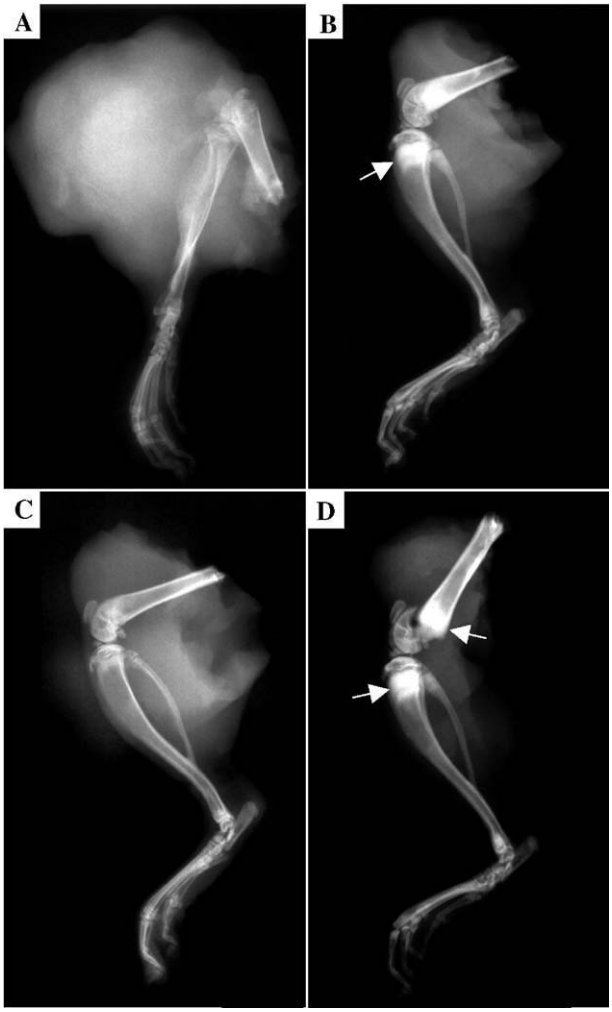

Fig. 3. Radiograph of Sprague–Dawley rat tibias 6 weeks after implantation with osteosarcoma. (A) Untreated group. (B) 100 μg zoledronate treatment group (ZOL, at 100 μg/kg s.c. twice a week, starting at day 11 after tumor implantation). (C) 15 mg ifosfamide treatment (IFO, i.p. to tumor-bearing rats at 24-h intervals on days 20, 21, and 22 after implantation), (D) Combined zoledronate + ifosfamide treatment group (arrow: zoledronate-induced high bone density).

whole of the series [control group (n = 35), ZOL-treated group (n = 15)] was significantly decreased in ZOL-treated animals as compared to the control group: respectively 2.46 + 3.19 vs. 3.44 + 5.2 (−29%, $P < 0.05$, Fig. 5B). IFO treatment strongly reduced the overall tumor progression with a relative tumor volume of 0.6 + 0.7 [−83% compared to the control, $P < 0.001$ (n = 14)], whereas IFO + ZOL totally inhibited tumor proliferation with a relative tumor volume of 0 + 2.61 [−100% compared to the control, $P < 0.001$; Fig. 5B (n = 15)].

ZOL markedly diminishes metastasis dissemination and enhances survival

At the time of autopsy, the number and size of lung metastases were assayed. No metastases were detected in animals treated with ZOL and/or IFO that present significant inhibition of tumor progression (not shown). Furthermore, tumor recurrence could not be observed up to 6 weeks after the end of ZOL treatment (not shown). To evaluate the effect of tumor burden on survival, osteosarcoma-bearing rats were treated with ZOL in separate experiments and the time of onset of morbidity was assessed. The results demonstrate a significant delay in morbidity onset in ZOL-treated rats as compared to the control group, as shown in Fig. 5C for one representative series.

The combination of ZOL with IFO improves tissue repair as compared to each agent alone

Histological analyses demonstrated that the residual bone mass of animals treated with the combination of ZOL and IFO was mainly composed of an extensive fibrosis with small foci of calcified necrosis compared to the other groups which were characterized by a greater necrotic area. As revealed by collagen Masson trichrom staining, untreated tumor tissues presented with around 50% necrosis without a detectable fibrotic component (Fig. 6A). IFO treatment resulted in both necrosis and fibrosis at the site of implantation (respectively 20% and 25%, Fig. 6B) and a strong macrophage reaction phagocyting necrotic tissues (data not shown), whereas ZOL-treated tissues exhibited extensive necrotic tissue associated with a weak fibrotic component (50% necrosis vs. 10% fibrosis, Fig. 6C). The combination IFO + ZOL showed extensive fibrosis associated with small calcified necrotic foci and residual macrophage polycaryons (40% fibrosis with 20% necrosis, Fig. 6D).

ZOL inhibits OSRGA cell proliferation in vitro

Osteosarcoma cells are more sensitive to ZOL than osteoblasts

To determine whether the antitumor activity of ZOL observed in vivo could be mediated by a direct antitumor effect on cell proliferation, the effect of ZOL was assessed in vitro on the proliferation of osteosarcoma cells OSRGA compared to that of primary osteoblasts (Fig. 7A). The XTT viability test showed that ZOL was more potent against the OSRGA cell line than primary osteoblasts, with a half-maximal inhibitory effect on proliferation seen at 0.2 and 20 μM, respectively (Fig. 7A). To determine whether these effects were due to inhibition of cell proliferation and/or induction of cell death, the time-dependent effects of ZOL were assessed by counting viable cells, as assessed by trypan blue exclusion, over a 72-h period in OSRGA cells cultured with or without 10 μM ZOL. The results demonstrated a 85% induction of cell death in the presence of 10 μM ZOL after 24 h of culture as compared to osteoblasts where no cell death was observed (Fig. 7B).

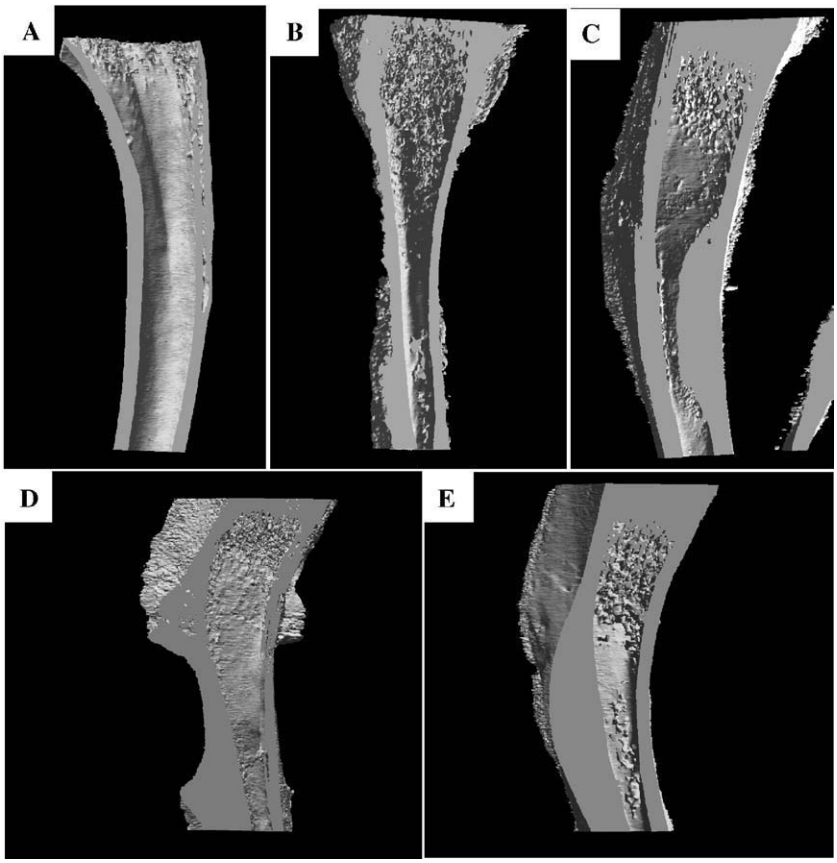

Fig. 4. Comparison of micro-CT scans of rat tibiae 6 weeks after implantation with osteosarcoma, treated with vehicle, IFO, ZOL, or both drugs. (A) Control tibia, (B) Control tumor (untreated group), (C) 15 mg ifosfamide treatment group (IFO, i.p. to tumor-bearing rats at 24-h intervals on days 20, 21, and 22 after implantation), (D) 100 μg zoledronate treatment group (ZOL, at 100 μg/kg s.c. twice a week, starting at day 11 after tumor implantation), (E) Combined zoledronate + ifosfamide treatment group. Hemi-sagittal sections are shown.

ZOL-induced cell death is not mediated by caspase activation

To determine whether the ZOL-induced death in OSRGA cells was caused by apoptosis, Hoechst staining and caspase-1 and -3 activation were investigated. Hoechst staining showed no modification of nuclear morphology in the presence of ZOL as compared to control cells (data not shown). Concerning the caspase-1 and -3 activity in OSRGA cells and osteoblasts, the results showed that ZOL does not induce any activation of caspases in either OSRGA cells or osteoblasts (not shown). TUNEL staining of tumor tissue sections showed no significant differences in TUNEL-positive cells from untreated and ZOL-treated rats (not shown).

ZOL induces S-phase arrest in OSRGA cells

Flow cytometry of DNA content was performed to identify cell cycle perturbations following treatment with ZOL over a 72-h period in OSRGA cells compared to primary rat osteoblasts. In osteoblasts, in which ZOL did not cause a loss of cell viability, the cell cycle profiles were not modified in the presence of 10 μM ZOL for 48 or 72 h of

Table 1
Quantification by micro-CT scan of the bone remodeling activity in tibiae of rats treated or not with zoledronic acid, ifosfamide, or both

	Tumor control (CT)	Ifosfamide (IFO)	Zoledronic acid (ZOL)	Ifosfamide + Zoledronic acid (I + Z)
Total bone				
BV/TV	0.57	0.64 (+12%)	0.68 (+19%)	0.75 (+32%)
BS/BV (1/mm)	7.72	4.98 (−36%)	4.89 (−37%)	4.51 (−42%)
Trabecular bone				
BV/TV	0.25	0.31 (+24%)	0.37 (+48%)	0.44 (+76%)
BS/BV (1/mm)	23.95	16.87 (−30%)	11.60 (−52%)	13.15 (−45%)

Rats were treated with zoledronic acid (ZOL: 100 μg/kg s.c. twice a week, starting at day 11 after tumor implantation), ifosfamide (IFO: 15 mg/kg administered i.p. to tumor-bearing rats at 24-h intervals on days 20, 21, and 22 after implantation), or both. The relative bone volume (BV/TV) and the specific surfaces (BS/BV) of tibiae from each group were calculated from 3D image registration data and compared to the vehicle-treated tumor control (CT). Two compartments were studied: trabecular bone and total bone (trabecular + cortical bone).

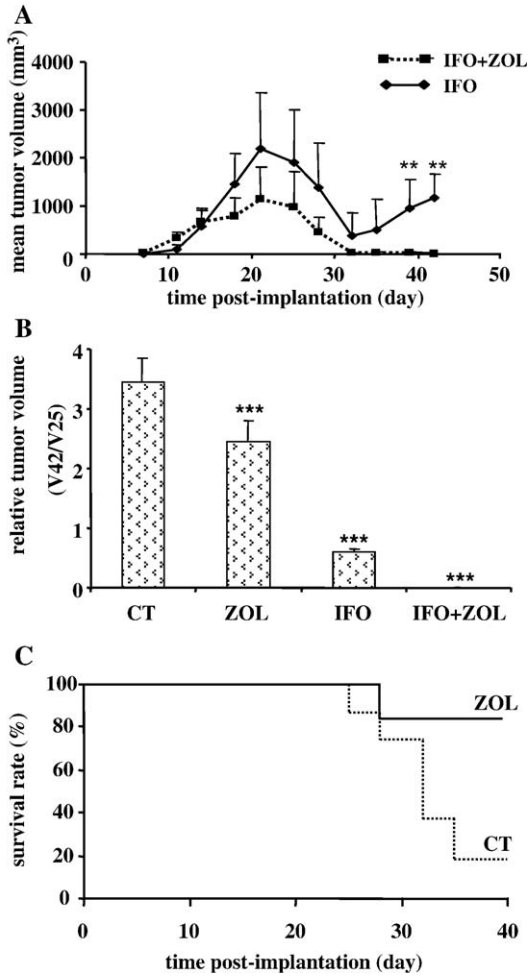

Fig. 5. Effect over time of IFO alone or combined with ZOL on tumor volume after osteosarcoma implantation. Rats were treated by zoledronic acid (ZOL, 100 μg/kg s.c. twice a week, starting at day 11 after tumor implantation) to ifosfamide (IFO administered i.p. at 15 mg/kg to tumor-bearing rats at 24-h intervals on days 20, 21, and 22 after implantation). (A) Time course of the mean tumor volume from a representative series (IFO: ifosfamide; IFO + ZOL: ifosfamide + zoledronate). $^{**}P < 0.05$. (B) Relative tumor volume (RTV) between day 25 and day 42 for the whole series. $^{***}P < 0.001$. (C) Survival rate of a representative series of 8 animals treated (ZOL) or not (CT) with 100 μg/kg ZOL, as described in Fig. 1, where day 0 represents the time of implantation.

incubation (Fig. 8). However, in OSRGA cells, ZOL caused a 3-fold increase in the number of cells arrested in S-phase: ZOL treatment resulted in S-phase arrest in the OSRGA cells, exerting its effects at 48 h, with the number of cells in S-phase increasing from 10% in control untreated cells to 25% or 32% in the corresponding 1 or 10 μM ZOL-treated cells. This observation was concomitant with a reduction of cells in G_0/G_1 and G_2/M phases: 37% vs. 48% and 17% vs. 33%, respectively (Fig. 8). The cells in the apoptotic sub-G_0/G_1 peak also increased from 9% to 14%.

It was previously suggested that a possible mechanism for the BP effects on cell growth was the ability of these compounds to act as calcium-chelating agents. We investigated this possibility by the addition of equimolar concentrations of EDTA with respect to the concentration of ZOL that reduced cell viability by 50%. We found no significant effect of EDTA on cell viability in both OSRGA and primary osteoblasts, suggesting that calcium chelation was not responsible for the observed effects (data not shown).

Discussion

The vicious cycle that has been described in osteolytic metastases consists of release of osteolytic mediators by tumor cells, bone degradation, release of growth factors from degraded bone, enhanced tumor cell growth, and further release of osteolytic mediators [4]. Inhibitors of bone resorption thus appear one of the more promising tools to manage skeletal metastases. One can speculate that this vicious cycle may also apply in the case of the primary bone tumors, and that inhibitors of bone resorption such as bisphosphonates may interfere with primary tumor development at a skeletal site. Zoledronic acid was used in the present study since its efficacy has been widely reported as a bone anti-resorptive agent and also as a potent inhibitor of skeletal complications associated with bone metastases. Its combination with ifosfamide has been also studied as a means to reinforce the anticancer potency of this drug, which could then be used at lower doses. ZOL doses used in the present study are justified as the clinical dose (4 mg IV every 3–4 weeks) is equivalent to approximately 100 μg/kg of the research grade disodium salt used in this study. However, even if dosing frequency of twice a week is greater, it could be justified by the very aggressive nature of the osteosarcoma and the short survival.

Initially, it was thought that the specific inhibition of osteoclastic bone resorption is the only mechanism of action of BPs, by which they are effective in the treatment of cancer patients bearing bone metastases. However, evidence is emerging from both preclinical and clinical studies to suggest that BPs also have direct antitumor properties that may contribute to their therapeutic efficacy in malignant bone diseases [26]. Interestingly, the N-BP zoledronic acid inhibited the development not only of osteolytic but also osteoblastic bone lesions in a murine model of prostate cancer [27,28]. Beyond induction of tumor cell apoptosis, bisphosphonates can inhibit tumor growth via other mechanisms: inhibition of cell growth, inhibition of tumor cell adhesion and spreading, and inhibition of tumor cell invasion [29]. However, the precise mechanism by which BPs inhibit the growth of bone tumors or bone metastases is not known: direct or indirect antitumor effect via osteoclast inhibition and alteration of the bone microenvironment (the seed-and-soil hypothesis)? Currently, the data are conflicting and whether or not BPs possess anticancer effects is still controversial. Using a rat-transplantable model of osteosarcoma, we demonstrated here the efficacy of zoledronic acid on primary bone tumor growth. Our results are in agreement

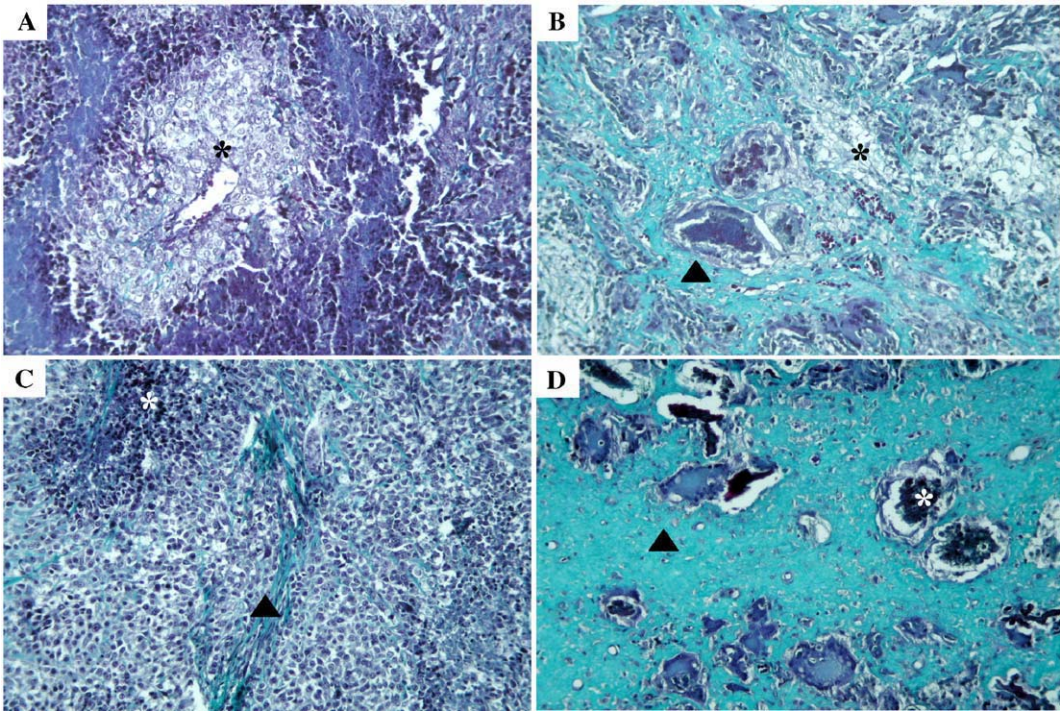

Fig. 6. Collagen Masson trichrom staining of osteosarcoma implanted in Sprague–Dawley rats treated with IFO or ZOL alone or combined. Rats were treated by zoledronic acid (ZOL, 100 μg/kg s.c. twice a week, starting at day 11 after tumor implantation), ifosfamide (IFO administered i.p. at 15 mg/kg to tumor-bearing rats at 24-h intervals on days 20, 21, and 22 after implantation), or both. (A) Untreated tumor tissue presented approximately 50% necrosis. (B) Ifosfamide treatment resulted in both necrosis (*) and fibrosis (arrowhead). (C) Zoledronate-treated tissues exhibited extended necrotic tissue (arrowhead). (D) The combination IFO + ZOL showed extensive fibrosis (arrowhead) with small calcified necrotic foci (*) and residual macrophage polycaryons. Original magnification: ×20.

with previous studies that reported the efficacy of zoledronic acid in the treatment of osteolytic lesions associated with other types of cancer: multiple myeloma [30] and bone metastases associated with prostate cancer [27,28] or breast carcinoma [31]. A single publication reported the use of the bisphosphonate alendronate for palliative management of osteosarcoma in two dogs [32]. The authors observed that both animals remained comfortable and survived for more that 10 months after diagnosis, despite the fact that neither primary tumor was resected. However, our present study is the first to reveal an in vivo inhibitory effect of N-BP on primary bone tumor and lung metastases development in a rodent model. The inhibitory effect of BPs on cancer cells in non-skeletal sites is still unclear in the literature. Diel et al. have reported that breast cancer patients treated with clodronate together with anticancer therapies show not only decreased bone metastases but also reduced visceral metastases and increased survival compared to patients treated with conventional anticancer therapies alone [33]. However, a few years later, Saarto et al. published contrasting data showing that clodronate increased visceral metastases and had little effect on bone metastases [34]. Powles et al. have reported that clodronate significantly inhibits bone metastases without affecting visceral metastases [35]. Using the 4T1 mouse breast cancer model, ibandronate was shown to reduce bone metastases but failed to inhibit lung and liver metastases [36]. More recently, using the same animal model, Yoneda et al. observed the suppression of bone metastases by zoledronic acid and also a significant reduction in tumor burden in lung and liver [37].

The antitumor effects of BPs observed in vivo can be partly explained by an inhibitory effect exerted by these compounds on the proliferation and survival of a variety of tumor cells themselves including those of breast [38–40], prostate [27], multiple myeloma [41–43], and osteosarcoma [21–23]. In our experimental model, we have also demonstrated that ZOL induced a dose- and time-dependent decrease in cell proliferation together with an induction of cell death in OSRGA cells, whereas primary osteoblasts were more resistant. Flow cytometry of OSRGA cells demonstrated that the underlying mechanism of the ZOL effect on cell proliferation involves inhibition of cell-cycle progression mainly due to S-phase arrest. Unexpectedly, no sign of apoptosis was observed in OSRGA cells as assessed by Hoechst staining and caspase activation. No clear evidence of in situ apoptosis could be observed by TUNEL staining of tumor tissue sections from untreated and ZOL-treated rats. However, cell cycle analysis did show a small proportion of hypodiploid OSRGA cells in the apoptotic sub-G0/G1 peak when the cells were treated with ZOL, consistent with the onset of apoptosis. Complementary time-lapse experiments showed a huge increase in OSRGA cell death from 20 h of treatment with 10 μM ZOL, whereas

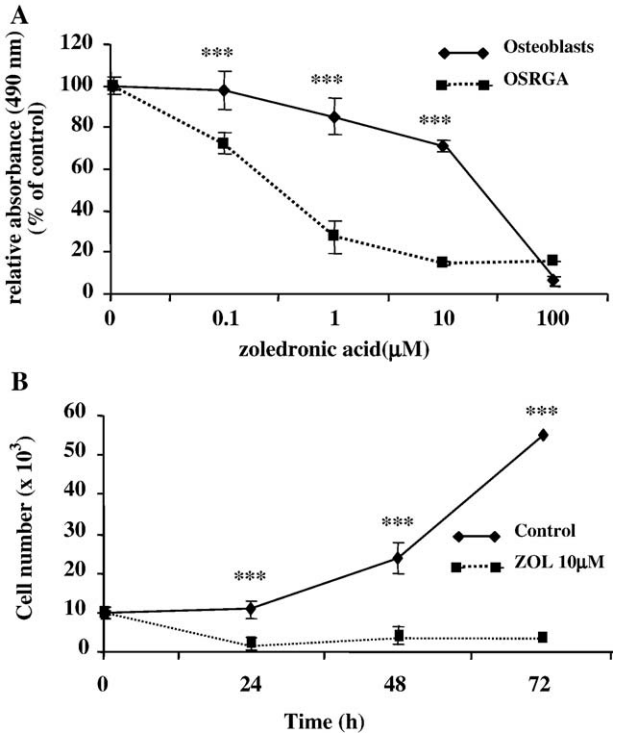

Fig. 7. In vitro effects of zoledronic acid on OSRGA and osteoblast cell proliferation and caspase activation. (A) Proliferation of OSRGA (dotted line) and osteoblastic (full line) cells was determined after exposure to zoledronic acid ($0-10^{-4}$ M) for 3 days with 10% SVF using the XTT quick proliferation kit as described in Materials and methods. Changes in absorbance at 490 nm were measured and results are plotted as percentage of untreated cell proliferation. (B) The viable cell number was determined after trypan blue exclusion on OSRGA cells treated (dotted line) or not (full line) with 10 μM ZOL for the indicated times. ***$P < 0.001$. Statistical evaluation of the data was performed by Student's t test.

osteoblasts were not affected (not shown). In these experiments, sensitive cells showed morphological changes characterized by the formation of dense rounded apoptotic bodies. We therefore hypothesize that the induction of cell death in OSRGA cells by ZOL resembled "anoikis", a special mode of apoptosis that occurs when adherent cells detach or lose the particular attachment contacts with the extracellular matrix that confer survival signals to the cells, a process independent of caspase activation. It is now becoming increasingly clear that apoptosis can occur in the absence of caspase activation, as has been documented in a number of recent studies [44]. Our results parallel those of Evdokiou et al. who reported a ZOL-induced reduction of cell number in a panel of human osteogenic sarcoma cell lines, due either to cell cycle arrest in S-phase or to the induction of apoptosis [23]. It seems that OSRGA cells behave like MG-63 cells in the presence of ZOL, which inhibits cell cycle progression and increases the proportion of cells arrested in S-phase. This effect has also been recently reported in another in vitro cancer model by Forsea et al. who described an accumulation of melanoma cells in the S-phase of the cycle after ZOL treatment [45]. Lee et al. demonstrated a reduction of the of growth prostate cancer cell lines by ZOL, with a major increase in cells present in the G0/G1 and S phase [46]. These results show that ZOL has direct effects on the proliferation and survival of osteosarcoma cells in vitro, suggesting a new therapeutic application for this compound in the case of primary bone tumors, where new therapeutic strategies are warranted. However, the precise molecular mechanisms implicated in the ZOL-induced cell death need further investigation.

The purpose of this study was also to check the possible synergistic effect between ZOL and the anticancer drug ifosfamide, which is currently used in the treatment of human osteosarcoma [47,48]. Considering the quantification of relative bone volume by micro-scanner analysis and the measurement of tumor volumes, only additive effects were demonstrated when ifosfamide and ZOL were combined, but not synergy. This is probably due to the strong inhibitory effect of ifosfamide alone that masks an eventual synergistic effect when associated with ZOL. A few studies have reported inconsistent results on the benefit of a combination of ZOL and anticancer agents in vitro: two studies in breast cancer cells [19] and more recently in leukemic cell lines [49] reported synergistic effects on tumor cell number and apoptosis when ZOL and paclitaxel were combined. Similarly, results from Witters et al. showed enhanced growth inhibition in human breast cancer cell lines in the presence of both ZOL and docetaxel [50]. However, the treatment of a panel of human osteogenic sarcoma cell lines with the combination of the chemotherapeutic agents doxorubicin or etoposide with ZOL did not significantly augment apoptosis in any of the cell lines tested [23]. Unfortunately, we cannot test the combination of ZOL with ifosfamide in vitro in the OSRGA cell line, as this chemotherapeutic agent needs prior hepatic enzymatic conversion to exert its cytostatic activity [51].

Concerning the in vivo combination of ZOL + ifosfamide in our experimental model of rat osteosarcoma, we demonstrated that this therapeutic combination induces a huge increase in bone mass as revealed by the quantification of micro-architecture parameters and an improvement of tissue repair. Few data have reported the association between a BP and an anticancer drug in vivo, and to our knowledge, only three of them described this combination in osteosarcoma and only with the aim of targeting anticancer drugs to bone via the BP moiety. Two of them analyzed the effects of new cisplatin-linked phosphonates (compounds that contain both an osteotropic and an anticancer moiety) in rat models of osteolytic bone metastasis and transplantable osteosarcoma, revealing that antineoplastic and osteotropic properties can be maintained after addition of a cisplatin moiety to a triphosphonate or bismethylenephosphonate [52,53]. The third paper published by Hosain et al. described a methotrexate-bisphosphonate conjugate containing a peptide bond that possessed over five times greater antineoplastic activity against osteosarcoma in experimental models compared to methotrexate alone, but the conjugate also appeared to be three to

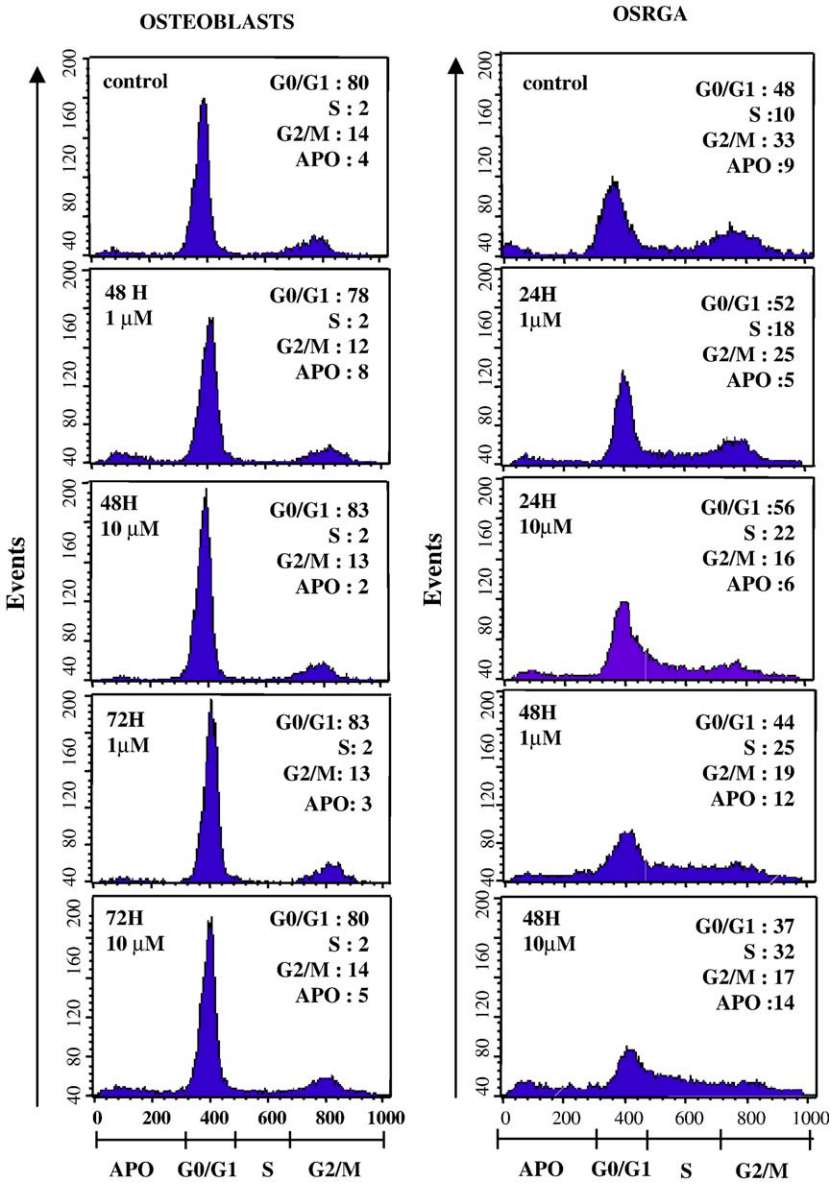

Fig. 8. Flow cytometry of ZOL-treated OSRGA cells compared to osteoblasts. OSRGA cells and osteoblasts were incubated for 24–72 h in the absence (control) or presence of 1 or 10 μM ZOL. At each point, cells were harvested, fixed, and stained with propidium iodide. The positions on the histograms of the hypodiploid sub-G_0/G_1, G_0/G_1, S, and G_2/M peaks, and the percentage of cells in each of the cycle phases, in a representative experiment are indicated.

four times more toxic than methotrexate, possibly due to a myelosuppressive effect from increased delivery of methotrexate to bone [54]. A recent case report revealed the successful management of widespread osteosarcoma with a combination of radio- and chemotherapy in a patient who received bisphosphonates regularly [55], indicating the benefit of tri-therapy in osteosarcoma. Beside osteosarcoma, a unique study has been recently published on the in vivo effects of the chemotherapeutic agent UFT (a combination of tegafur and uracil) with or without zoledronic acid, in the murine 4T1/luc model of breast cancer [20]. In this study, the combination of UFT with ZOL enhanced the reduction of bone metastases compared with UFT alone, suggesting that combination with BP increases the anti-metastatic effect of UFT. In our osteosarcoma model, the combination ifosfamide-bisphosphonate led to more qualitative than quantitative responses as this therapeutic regimen improves tissue repair, as compared to ifosfamide or ZOL alone, with a higher proportion of fibrotic tissue than necrosis at the graft site. Previous data have reported stimulatory effects of ZOL on osteoblast differentiation by enhancing type I collagen secretion [56] that could explain part of the fibrosis induced by the ZOL + IFO.

Taken together, these data demonstrate that treatment with ZOL after osteosarcoma implantation not only prevents the development of osteolytic lesions, but also inhibits primary bone tumor growth by a potential direct antitumor effect (as revealed by inhibitory effects on OSRGA cell

proliferation). The relevance of its combination with the standard antitumor drug IFO resides in the histological improvement of tissue repair and opens new areas in the field of therapeutic combinations for the treatment of primary bone tumors.

Acknowledgments

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References

[1] Rosen G, Murphy ML, Huvos AG, Gutierrez M, Marcove RC. Chemotherapy, en bloc resection, and prosthetic bone replacement in treatment of osteogenic sarcoma. Cancer 1976;37:1–11.
[2] Link MP, Goorin AM, Horowitz M, Meyer WH, Belasco J, Baker A, et al. Adjuvant chemotherapy of high-grade osteosarcoma of the extremity. Updated results of the multi-institutional osteosarcoma study. Clin Orthop 1991;270:8–14.
[3] Provisor AJ, Ettinger LJ, Nachman JB, Krailo MD, Makley JT, Yunis EJ, et al. Treatment of non-metastatic osteosarcoma of the extremity with pre-operative chemotherapy : a report from the Children's Cancer Group. J Clin Oncol 1997;15:76–84.
[4] Chirgwin JM, Guise TA. Molecular mechanisms of tumor-bone interactions in osteolytic metastases. Crit Rev Eukaryot Gene Expr 2000;10:159–78.
[5] Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, et al. Structure–activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates. J Pharmacol Exp Ther 2001;296:235–42.
[6] Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Monkkonen J, et al. Cellular and molecular mechanisms of action of bisphosphonates. Cancer 2000;88:2961–78.
[7] Body J. Zoledronate. Cancer 1997;80:1699–701.
[8] Cheer S, Noble S. Zoledronic acid. Drugs 2001;61:799–805.
[9] Gatti D, Adami S. New bisphosphonates in the treatment of bone diseases. Drugs Aging 1999;15:285–96.
[10] Pataki A, Muller K, Green J, Ma Y, Li Q, Jee W. Effects of short-term treatment with the bisphosphonate zoledronate and pamidronate on rat bone: a comparative histomorphometric study on the cancellous bone formed before, during, and after treatment. Anat Rec 1997;249:458–68.
[11] Benford HL, McGowan NWA, Helfrich MH, Nuttall M, Rogers MJ. Visualization of bisphosphonate-induced caspase-3 activity in apoptotic osteoclasts in vitro. Bone 2001;28:465–73.
[12] Berenson J, Rosen L, Howell A, Porter L, Coleman RE, Morley W, et al. Zoledronic acid reduces skeletal-related events in patients with osteolytic lesions. Cancer 2001;91:1191–200.
[13] Lipton A. Bisphosphonates and breast carcinoma. Cancer 2000; 8:3033–7.
[14] Saad F, Gleason DM, Murray R, Tchekmedyian S, Venner P, Lacombe L, et al. Zoledronic Acid Prostate Cancer Study Group. A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma. Natl Cancer Inst 2000;94:1458–68.
[15] Rosen LS, Gordon D, Tchekmedyian S, Yanagihara R, Hirsh V, Krzakowski M, et al. Zoledronic acid versus placebo in the treatment of skeletal metastases in patients with lung cancer and other solid tumors: a phase III, double-blind, randomized trial—The Zoledronic Acid Lung Cancer and Other Solid Tumors Study Group. J Clin Oncol 2003;21:3150–7.
[16] Boissier S, Magnetto S, Frappart L, Cuzin B, Ebetino FH, Delmas PD, et al. Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. Cancer Res 1997;57:3890–4.
[17] Boissier S, Ferreras M, Peyruchaud O, Magnetto S, Ebetino FH, Colombel M, et al. Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. Cancer Res 2000;60:2949–54.
[18] Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, De Libero G. Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 2003; 197:163–8.
[19] Jagdev SP, Coleman RE, Shipman CM, Rostami-H A, Croucher PI. The bisphosphonate zoledronic acid induces apoptosis of breast cancer cells: evidence for synergy with paclitaxel. Br J Cancer 2001;84: 1126–34.
[20] Hiraga T, Ueda A, Tamura D, Hata K, Ikeda F, Williams PJ, et al. Effects of oral UFT combined with or without zoledronic acid on bone metastasis in the 4T1/luc mouse breast cancer. Int J Cancer 2003; 106:973–9.
[21] Mackie PS, Fisher JL, Zhou H, Choong PF. Bisphosphonates regulate cell growth and gene expression in the UMR 106-01 clonal rat osteosarcoma cell line. Br J Cancer 2001;84:951–8.
[22] Sonnemann J, Eckervogt V, Truckenbrod B, Boos J, Winkelmann W, van Valen F. The bisphosphonate pamidronate is a potent inhibitor of human osteosarcoma cell growth in vitro. Anticancer Drugs 2001; 12:459–65.
[23] Evdokiou A, Labrinidis A, Bouralexis S, Hay S, Findlay DM. Induction of cell death of human osteogenic sarcoma cells by zoledronic acid resembles anoikis. Bone 2003;33:216–28.
[24] Thiéry J-P, Perdereau B, Gongora R, Gongora G, Mazabraud A. Un modèle experimental d'ostéosarcome chez le rat. II L'ostéosarcome greffable du rat. Sem Hop Paris 1982;58:1686–9.
[25] Klein B, Pals S, Masse R, Lafuma J, Morin M, Binart N, et al. Studies of bone and soft-tissue tumours induced in rats with radioactive cerium chloride. Int J Cancer 1977;20:112–9.
[26] Heymann D, Ory B, Gouin F, Green JR, Rédini F. Bisphosphonates: new therapeutic agents for the treatment of bone tumors. Trends Mol Med 2004;10:337–43.
[27] Lee YP, Schwarz EM, Davies M, Jo M, Gates J, Zhang X, et al. Use of zoledronate to treat osteoblastic versus osteolytic lesions in a severe-combined-immunodeficient mouse model. Cancer Res 2002;62: 5564–70.
[28] Corey E, Brown LG, Quinn JE, Poot M, Roudier MP, Higano CS, et al. Zoledronic acid exhibits inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer. Clin Cancer Res 2003;9: 295–306.
[29] Green JR. Antitumor effects of bisphosphonates. Cancer 2003;97: 840–7.
[30] Croucher PI, De Hendrik R, Perry MJ, Hijzen A, Shipman CM, Lippitt J, et al. Zoledronic acid treatment of 5T2MM-bearing mice inhibits the development of myeloma bone disease: evidence for decreased osteolysis, tumor burden and angiogenesis, and increased survival. J Bone Miner Res 2003;18:482–92.
[31] Yoneda T, Michigami T, Yi B, Williams PJ, Niewolna M, Hiraga T. Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma. Cancer 2000;88:2979–88.

[32] Tomlin JL, Sturgeon C, Pead MJ, Muir P. Use of the bisphosphonate drug alendronate for palliative management of osteosarcoma in two dogs. Vet Rec 2000;147:129–32.
[33] Diel IJ, Solomayer E-F, Costa SD, Gollan C, Goerner R, Wallwiener D, et al. Reduction in new metastases in breast cancer with adjuvant clodronate treatment. N Engl J Med 1998;339:357–63.
[34] Saarto T, Blomqvist C, Virkkunen P, Elomaa II. Adjuvant clodronate treatment does not reduce the frequency of skeletal metastases in node-positive breast cancer patients: 5-year results of a randomised controlled trial. J Clin Oncol 2001;9:10–7.
[35] Powles T, Paterson S, Kanis JA, McCloskey E, Ashley S, Tidy A, et al. Randomized, placebo-controlled trial of clodronate in patients with primary operable breast cancer. J Clin Oncol 2002;20:3219–24.
[36] Michimagi T, Hiraga T, Williams PJ, Nishimura R, Mundy GR, Yoneda T. The effect of the bisphosphonate ibandronate on breast cancer metastasis to visceral organs. Breast Cancer Res Treat 2002; 75:249–58.
[37] Yoneda T, Hashimoto N, Hiraga T. Bisphosphonate actions on cancer. Calcif Tissue Int 2003;73:315–8.
[38] Fromigue O, Lagneaux L, Body JJ. Bisphosphonates induce breast cancer cell death in vitro. J Bone Miner Res 2000;15: 2211–21.
[39] Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW. Bisphosphonates induce apoptosis in human breast cancer cell lines. Br J Cancer 2000;82:1459–68.
[40] Hiraga T, Williams PJ, Mundy GR, Yoneda T. The bisphosphonate ibandronate promotes apoptosis in MDA-MB-231 human breast cancer cells in bone metastases. Cancer Res 2001;61:4418–24.
[41] Shipman CM, Croucher PI, Russell RG, Helfrich MH, Rogers MJ. The bisphosphonate incadronate (YM175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway. Cancer Res 1998;58:5294–7.
[42] Tassone P, Forciniti S, Galea E, Morrone G, Turco MC, Martinelli V, et al. Growth inhibition and synergistic induction of apoptosis by zoledronate and dexamethasone in human myeloma cell lines. Leukemia 2000;14:841–4.
[43] Ito M, Amizuka N, Nakajima T, Ozawa H. Ultrastructural and cytochemical studies on cell death of osteoclasts induced by bisphosphonate treatment. Bone 1999;25:447–52.
[44] Mathiassen IS, Jaattela M. Triggering caspase-independent cell death to combat cancer. Trends Mol Med 2002;8:212–20.
[45] Forsea AM, Muller C, Riebeling C, Orfanos CE, Geilen CC. Nitrogen-containing bisphosphonates inhibit cell cycle progression in human melanoma cells. Br J Cancer 2004;91:803–10.
[46] Lee MV, Fong EM, Singer FR, Guenette RS. Bisphosphonate treatment inhibits the growth of prostate cancer cells. Cancer Res 2001;61:2602–8.
[47] Bruland OS, Pihl A. On the current management of osteosarcoma. A critical evaluation and a proposal for a modified treatment strategy. Eur J Cancer 1997;33:1725–33.
[48] Marti C, Kroner T, Remagen W, Berchtold W, Cserhati M, Varini M. High dose ifosfamide in advanced osteosarcoma. Cancer Treat Rep 1985;69:115–7.
[49] Kimura S, Kuroda J, Segawa H, Sato K, Nogawa M, Yuasa T, et al. Antiproliferative efficacy of the third-generation bisphosphonate, zoledronic acid, combined with other anticancer drugs in leukemic cell lines. Int J Hematol 2004;79:37–43.
[50] Witters LM, Crispino J, Fraterrigo T, Green J, Lipton A. Effect of the combination of docetaxel, zoledronic acid, and a COX-2 inhibitor on the growth of human breast cancer cell lines. Am J Clin Oncol 2003;26:S92–S97.
[51] Brock N, Hilgard P, Peukert M, Pöhl J, Sindermann H. Basis and new developments in the field of oxazaphosphorines. Cancer Invest 1988; 6:513–32.
[52] Klenner T, Wingen F, Keppler BK, Krempien B, Schmahl D. Anticancer-agent-linked phosphonates with antiosteolytic and anti-neoplastic properties: a promising perspective in the treatment of bone-related malignancies? J Cancer Res Clin Oncol 1990;116: 341–50.
[53] Klenner T, Wingen F, Keppler B, Valenzuela-Paz P, Amelung F, Schmahl D. Therapeutic efficacy of two different cytostatic-linked phosphonates in combination with razoxane in the transplantable osteosarcoma of the rat. Clin Exp Metastasis 1990;8:345–59.
[54] Hosain F, Spencer RP, Couthon HM, Sturtz GL. Targeted delivery of antineoplastic agent to bone: biodistribution studies of technetium-99m-labeled gem-bisphosphonate conjugate of methotrexate. J Nucl Med 1996;37:105–7.
[55] Grohn P, Uttamchandani A, Al-Haider Z, Adams B. Successful management of a widespread osteosarcoma. A case report. Med Princ Pract 2004;13:54–6.
[56] Viereck V, Emons G, Lauck V, Frosch KH, Blaschke S, Grundker C, et al. Bisphosphonates pamidronate and zoledronic acid stimulate osteoprotegerin production by primary human osteoblasts. Biochem Biophys Res Commun 2002;291:680–6.

Complément de discussion à l'article 3

A l'origine, l'inhibition de l'activité de résorption des ostéoclastes était la seule activité connue des bisphosphonates et la seule explication avancée pour expliquer l'efficacité de ce traitement chez les patients atteints de métastases osseuses. Comme nous l'avons montré dans notre étude *in vitro*, l'acide zolédronique est également pourvu d'une activité anti-tumorale directe sur les cellules d'ostéosarcome. C'est pourquoi, l'objectif était à la fois l'utilisation d'un agent anti-tumoral utilisé conventionellement (l'ifosfamide) en association avec un agent anti résorption osseuse (l'acide zolédronique) mais également la potentialisation des effets de l'ifosfamide sur les cellules tumorales par le Zol.

Au regard des mesures de volumes tumoraux et de volumes osseux relatifs réalisés par micro-scanner, nous n'avons pu observer que des effets additifs et non synergiques lors de l'association du Zol et de l'ifosfamide. Ceci est probablement dû au fort effet inhibiteur de l'ifosfamide qui pourrait masquer un éventuel effet synergique lorsqu'il est associé au Zol. Nous avons également observé l'augmentation de la formation de tissu de réparation lors de l'association de ces deux molécules. Peu de données sont disponibles sur l'association des bisphosphonates avec des agents anti-tumoraux *in vivo*, en particulier dans le traitement des ostéosarcomes ; de plus toutes ces études portent non pas sur des associations de deux molécules mais sur la combinaison chimique d'agent anti-tumoraux et de bisphosphonates, c'est-à-dire sur l'activité de molécules hybrides n'utilisant les bisphosphonates que pour leur fort tropisme pour l'os (Klenner et al. 1990; Klenner et al. 1990). Dans notre modèle d'ostéosarcome, l'association ifosfamide-bisphosphonate conduit à une augmentation de la qualité de la réponse anti-tumorale. En effet, nous observons un tissu cicatriciel de meilleure qualité, majoritairement fibreux et non nécrotique comme avec le Zol ou l'ifosfamide seul. Ceci pouvant être dû au fait que le Zol a la propriété d'accroitre la sécrétion de collagène de type I par les ostéoblastes (Viereck et al. 2002).

Une étude récente s'intéresse aux effets *in vivo* de l'association du Zol et de l'agent de chimiothérapie UFT (combinaison de tégafure et d'uracile) dans le modèle murin de cancer du sein 4T1/luc (Hiraga et al. 2003). Dans cette étude, la combinaison de l'UFT et du Zol a un effet synergique contre les métastases osseuses en comparaison de l'UFT seul, suggérant un effet bénéfique du Zol sur le développement métastatique. C'est pourquoi, dans la suite de notre travail, nous nous sommes intéressés aux effets de l'acide zolédronique sur le

développement des métastases, en particulier pulmonaire, les poumons étant le site privilégié de dissémination des cellules tumorales lors du développement d'un ostéosarcome.

De nouvelles expérimentations seront réalisées, en particulier sur l'amélioration du modèle *in vivo* d'ostéosarcome par implantation intra-osseuse. Nous étudierons également les mécanismes d'actions du Zol *in vivo* afin de démontrer un effet direct ou non sur les cellules tumorales.

En plus de ces effets anti-ostéoclastiques, de récentes études précliniques rapportent des effets pro-apoptotiques des bisphosphonates sur des cellules tumorales de diverses origines ainsi que des effets anti-angiogéniques et inhibiteurs sur l'invasion et la migration des cellules tumorales. En dépit de leurs effets largement reconnus sur les métastases osseuses, les effets des BPs sur les autres organes restent incertains et il est nécessaire d'évaluer l'efficacité des BPs sur le développement de métastases non-osseuses secondaires à un ostéosarcome par exemple. Le faible taux de survie faisant suite à la détection de métastases pulmonaires justifie pleinement le développement de nouveaux protocoles thérapeutiques s'intéressant particulièrement aux métastases pulmonaires et à leurs conséquences catastrophiques sur le pronostique vital.

Dans l'étude suivante, nous avons étudié les effets de l'acide zolédronique sur le développement de métastases pulmonaires induites par l'injection intraveineuse (i.v) de cellules d'ostéosarcomes POS-1. La lignée cellulaire POS-1 dérive d'un ostéosarcome apparu spontanément chez la souris C3H. La tumeur peut être transplantée à d'autres souris C3H ou encore les cellules POS-1 peuvent être injectées dans le coussinet plantaire de la souris. Dans tous les cas, le développement de la tumeur est macroscopiquement identifiable 2 semaines après l'inoculation et se développe dans 90% des souris inoculées, des métastases pulmonaires étant observées dans toutes les souris développant une tumeur. Pour cette étude nous avons développé à l'aide des cellules POS-1 un modèle de métastases pulmonaires sans tumeur osseuse primaire grâce à une inoculation cellulaire par i.v rétro-orbital. Ce modèle permet d'étudier l'efficacité du Zol sur la progression des métastases pulmonaire en s'affranchissant totalement de son interaction avec le microenvironnement osseux.

Article 4

« L'acide zolédronique supprime les métastases pulmonaires et augmente la survie globale des souris atteintes d'ostéosarcomes »

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Bien qu'il ne fasse aucun doute que les Bisphosphonates (BPs), inhibiteurs spécifiques des ostéoclastes, soient bénéfiques pour le traitement des métastases osseuses, leurs effets sur les métastases viscérales sont incertains. Dans cette étude, les effets de l'acide zolédronique (ZOL), sont étudiés sur la progression des métastases pulmonaires induites par inoculation i.v. de cellules d'ostéosarcome POS-1 chez des souris C3H/He. La présence de métastases pulmonaires est évaluée au moment de l'autopsie.

Les mécanismes d'action du ZOL sont également évalués *in vitro* sur la prolifération des cellules d'ostéosarcome POS-1, sur la progression du cycle cellulaire et sur l'activation des caspases-1 et -3. Le traitement par le ZOL (0.1 mg/kg, cinq fois par semaine) augmente significativement le taux de survie des animaux. Alors que toutes les souris contrôles meurent 23 jours après l'injection de cellules POS-1, 83 et 66% des animaux traités par le ZOL survivent respectivement 24 et 45 jours après l'injection des cellules POS-1. Le taux de survie global de cinq expériences indépendantes (deux séries traitées avec 0,1 mg/kg deux fois par semaine, et trois séries traitées avec 0,1 mg/kg cinq fois par semaine) montre une augmentation significative de la survie : 0,422 +/- 0.07 chez les animaux traités par le ZOL contre 0,167 +/- 0.07 dans les conditions contrôles (p=0,036). Des analyses histologiques ont démontré qu'aucune souris traitée par le ZOL n'avait de métastases pulmonaires. *In vitro*, une incubation de 48 heures en présence de 10 µM de ZOL inhibe la prolifération des cellules POS-1 avec un IC50 de 25 µM, ceci étant associé à un arrêt du cycle cellulaire en phase S : le nombre de cellules en phase S passe de 14% dans les conditions contrôles à 19% et 25%, respectivement en présence de 1 et 10 µM de ZOL. Cette observation est concomitante à une diminution du nombre de cellules en phase G0/G1 : 55% et 53% pour respectivement 1 et 10 µM ZOL (48 heures d'incubation) contre 63% pour les cellules POS-1 non traitées. Aucune modification de la morphologie nucléaire caractéristique de l'apoptose n'a pu être observée après coloration au Hoechst des cellules POS-1 traitées par le ZOL. Les résultats montrent

que le ZOL n'induit pas d'activation de la caspase-1 dans les cellules POS-1, mais induit une activation de la caspase-3 à la concentration de 10 μM (72 heures).

Nous avons démontré que le ZOL exerçait un effet anti tumoral direct sur les cellules POS-1 *in vitro* et qu'il diminuait significativement les métastases pulmonaires induits par les cellules d'ostéosarcome POS-1 *in vivo*, augmentant significativement la survie des animaux.

Zoledronic Acid Suppresses Lung Metastases and Prolongs Overall Survival of Osteosarcoma-Bearing Mice

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BACKGROUND. Although there is no doubt that bisphosphonates (BPs), specific inhibitors of osteoclasts, are beneficial for the treatment of bone metastases, their effects on visceral metastases are unclear. The effect of zoledronic acid (ZOL) was examined in vivo on lung metastasis progression and animal survival, and in vitro on the cellular mechanisms involved.

METHODS. An animal model of lung metastasis was developed in C3H/He mice inoculated intravenously with a spontaneous murine osteosarcoma POS-1 cell line. Lung metastasis was determined at the time of autopsy. ZOL was assessed in vitro on POS-1 cell proliferation, cell cycle progression, and caspase-1 and -3 activities.

RESULTS. The overall survival in five independent experiments (two series treated with ZOL 0.1 mg/kg twice a week, and three series with 0.1 mg/kg five times a week) showed a significant increase of the actuarial survival: 0.422 $\pm$ 0.07 in ZOL-treated animals versus 0.167 $\pm$ 0.07 in controls ($P = 0.036$). Lung metastases were absent in all ZOL-treated mice that survived more than 21 days postinjection as revealed by macroscopic and histologic analysis. In vitro, a 48-hour incubation with 10 μM ZOL inhibited POS-1 cell line proliferation associated with cell cycle arrest in S-phase. In addition, ZOL induced a weak increase of caspase-3 activity, but not caspase-1.

CONCLUSION. We demonstrate that ZOL exerts a direct antitumor effect on POS-1 cells in vitro, significantly diminishes osteosarcoma-induced lung metastasis in vivo, thereby prolonging survival of POS-1-inoculated animals. ***Cancer* 2005;104: 2522–9.** *© 2005 American Cancer Society.*

KEYWORDS: zoledronic acid, lung metastases, osteosarcoma.

Bisphosphonates (BPs) are currently the most important class of inhibitors of osteoclast-mediated bone resorption. They are widely and successfully used for the treatment of skeletal diseases, such as Paget disease, postmenopausal osteoporosis, and tumor-induced osteolysis.[1] BPs have a high affinity for hydroxyapatite mineral in bone and are taken up selectively and adsorbed to mineral surfaces at sites of increased bone turnover, where they inhibit osteoclast activity.[2] In addition to their potent antiosteoclast effects, recent preclinical studies have shown that BPs induce apoptosis of cancer cells from several origins, including human myeloma, breast, and prostate carcinoma cell lines.[3] It has also been demonstrated that BPs inhibit cancer cell invasion and angiogenesis.[3] In spite of the widely recognized beneficial effects of BPs on bone metastases, the effects of BPs on visceral organs are unclear.[4] In preclinical studies, the positive effects of zoledronic acid (ZOL) on nonskeletal metastases have been demonstrated in breast carcinoma models.[5,6] There-

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fore, it is necessary to extend these studies to nonosseous metastases secondary to cancers from other origins. Osteosarcoma (OS) is the most frequent primary bone tumor that develops mainly in the young, the median age of diagnosis being 18 years. A preference for pulmonary metastases compared with other metastatic sites is a distinct feature of OS and 5-year survival rates after the detection of lung metastasis are less than 30%.[7] Despite recent improvements in chemotherapy and surgery, the problem of nonresponse to chemotherapy remains and current strategies for the treatment of high-grade osteosarcoma fail to improve its prognosis.[8] Therefore, development of new therapies is needed.

In the present study, we investigated the effect of ZOL, an N-BP of the third generation, on the outcome of lung metastases induced by intravenous (i.v.) inoculation of POS-1 osteosarcoma cells. The POS-1 cell line is derived from an osteosarcoma which developed spontaneously in C3H mice. The tumor can be successfully transplanted in C3H mice or POS-1 cells inoculated into the hind footpad of mice and shows spontaneous metastasis to lung.[9] Tumors were first recognized macroscopically at 2 weeks after inoculation and developed in more than 90% of inoculated mice at 5 weeks, lung metastasis being observed in all mice that developed tumors. Using the POS-1 cells, we developed a model of pulmonary metastases without primary bone tumor by inoculating the cells i.v. into the tail vein or by a retro-orbital approach. Therefore, this model was used to test the efficacy of ZOL on the progression of pulmonary metastases.

MATERIALS AND METHODS

Animal Model

Four-week-old male C3H/He mice (Elevages Janvier, Le Genest St Isle, France) were housed under pathogen-free conditions at the Experimental Therapy Unit (Medicine Faculty of Nantes, France), in accordance with the institutional guidelines of the French Ethical Committee. The spontaneous murine osteosarcoma cell line POS-1 was kindly provided by the Kanagawa Cancer Centre (Kanagawa, Japan). The cells were cultured in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, Dominique Dutscher, Brumath, France) at 37 °C in a humidified atmosphere (5% CO_2/95% air). The mice were anesthetized by inhalation of a mixture of isoflurane/air (1.5%, 1 L/min) combined with an intramuscular injection of Imalgene (100 mg/kg, Merial Laboratories, Lyon, France) prior to i.v. injection of 50 μL of POS-1 cell suspension containing 1.5×10^5 cells. Under these conditions, pulmonary metastases developed rapidly, leading to the death of the animals in 3 weeks after POS-1 cell injection.

Treatment of Mice with Zoledronic Acid

To determine the effect of zoledronic acid (ZOL, kindly provided as the disodium salt by Pharma Novartis, Basel, Switzerland) on lung metastasis development and mouse survival in the POS-1 osteosarcoma model, 24 mice were injected with POS-1 osteosarcoma cells as described above. At Day 2 after tumor cell inoculation, 6 mice were treated with vehicle alone (phosphate buffered saline, PBS), and 18 with ZOL in PBS at 3 different concentrations and sequences: 1) ZOL 100 μg/kg, twice a week; 2) ZOL 100 μg/kg 5 times a week; and 3) ZOL 1 mg/kg, twice a week. Treatment continued until each animal showed signs of morbidity, which included cachexia or respiratory distress, at which point they were sacrificed by cervical dislocation. Lung tumor dissemination was assessed by analyzing the number of tumor foci. Five independent experiments were performed.

Histologic Analysis

Lungs were fixed in 10% buffered formaldehyde, then embedded in paraffin. Sections (5 μM thick) were mounted on glass slides and stained with hematoxylin-eosin-safran (HES) and picrosirius red for collagen.

In Vitro Analyses

Cell proliferation

Replicate subconfluent cell cultures of POS-1 cells in 96-well plates were treated for 1–3 days with increasing concentrations of ZOL (10^{-7} to 10^{-4} M, diluted in RPMI). Cell viability was determined by the sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) cell proliferation reagent assay kit (Roche Molecular Biomedicals, Mannheim, Germany).

Caspase activity

POS-1 cells (2×10^4) grown in 24-well plates were treated with 1 or 10 μM ZOL for the indicated times, washed once with PBS, and lysed with 50 μL of RIPA buffer for 30 minutes. The cells were then scraped off and the protein amount was quantified using the BCA (bicinchominic acid + Copper II sulfate) test (Pierce Chemical, Rockford, IL). Caspase-1 and -3 activity was assessed on 10 μL of cell lysate with the CaspACE assay kit (Promega, Madison, WI) following the manufacturer's recommendations. Cells treated with UV light for 30 seconds 24 hours before harvesting were used as a positive control for caspase activity.

Cell cycle analysis

Confluent POS-1 cells (treated with increasing concentrations of ZOL for 24 and 48 hr) were removed from culture dishes by trypsinization, washed twice in PBS, and incubated in PBS containing 0.12% Triton X-100, 0.12 mM EDTA, and 100 μg/mL DNase-free ribonuclease A (Sigma Chemical, St. Louis, MO). Then, 50 μg/mL propidium iodide (Sigma) were added for each sample for 20 minutes at 4 °C in the dark. The stained nuclei were analyzed by flow cytometry (FACScan, BD Biosciences, Franklin Lake, NJ) using CellQuest software. Cell cycle distribution was based on 2N and 4N DNA content.

Statistical Analysis

Cell proliferation data are expressed as mean ± SE. Comparison between groups was performed by the Mann-Whitney *U*-test. The effect of ZOL on disease-free survival was determined using the log rank test.

RESULTS

ZOL Increases Mice Survival by Inhibiting Pulmonary Metastases Development

Five independent experiments were performed in which mice received 1.5×10^5 POS-1 cells, leading to the development of lung metastasis in 80–90% of mice, with death occurring between Days 17 and 24 after cell inoculation. First, a dose–response of ZOL was performed to determine the optimal sequence and concentration able to affect animal survival. ZOL was well tolerated, without any overt clinical signs of adverse effects. The results presented in Figure 1A reveal that the three sequences improved survival: ZOL 0.1 and 1 mg/kg twice a week induced a survival rate of 40% 45 days after POS-1 cell inoculation. ZOL 0.1 mg/kg 5 times per week maintained survival at 83% up to day 31, and then 66% at 45 days after POS-1 cells injection. A representation of the overall survival rate of five independent experiments (two series treated with 0.1 mg/kg twice a week, and three series with 0.1 mg/kg five times a week) shows a significant increase of the actuarial survival: 0.422 ± 0.07 in ZOL-treated animals versus 0.167 ± 0.07 in controls ($P = 0.036$, Fig. 1B).

A high incidence of pulmonary metastases was observed in POS-1 cells-inoculated mice: 87% of mice were positive, with more than 50 tumor foci in each case.

The pulmonary tissue was invaded by tumor foci characterized by high-grade proliferating tumor cells and numerous venous emboli (Fig. 2A,B). Lung metastases were absent in all ZOL-treated mice that survived more than 21 days postinjection, as revealed by

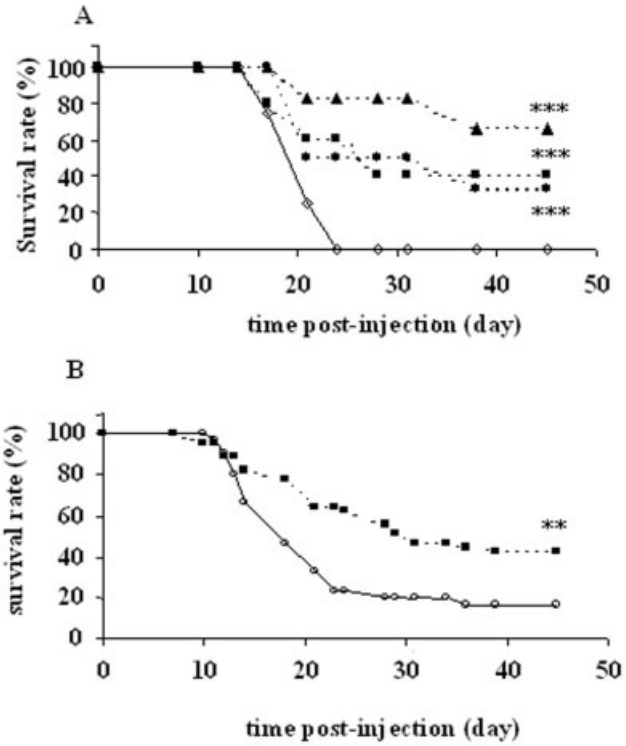

FIGURE 1. Zoledronic acid (ZOL) prolongs survival of POS-1 cell-inoculated mice. (A) A typical dose–response experiment for ZOL is shown. Four-week-old male C3H/He mice received 1.5×10^5 POS-1 cells intravenously. Two days after inoculation the mice were separated into 4 groups ($n = 6$) treated subcutaneously as follows: control mice received vehicle (PBS) alone (full line), ZOL 1 mg/kg, twice a week (solid squares, dotted line), 0.1 mg/kg, five times a week (solid triangles, dotted line), 0.1 mg/kg twice a week (solid circles, dotted line). $^{**}P < 0.05$, $^{***}P < 0.005$. (B) Overall survival in five independent series of mice treated with ZOL (solid squares, dotted line: 1 mg/kg, twice or five times a week) as compared with controls (full line). $^{**}P < 0.05$.

macroscopic observation. No metastases were observed at the histologic level 45 days after injection (Fig. 2C,D). The treated tissue is characterized by heterogeneous lung alveolar parenchyma and small fibrotic areas corresponding to regenerative healing tissue (Fig. 2C,D). The presence of collagen in this regenerative tissue was confirmed by picrosirius red staining (data not shown).

ZOL Inhibits POS-1 Cell Proliferation In Vitro and Induces Caspase-3 Activation

To determine whether the in vivo antitumor activity of ZOL could be mediated by a direct effect on POS-1 cell proliferation, ZOL effects were assessed in vitro. The XTT viability test showed that ZOL inhibited POS-1 cell proliferation, with an IC_{50} value of 44.28 μM (Fig. 3A). Hoechst 33258 staining and caspase activation were investigated to see whether the ZOL-induced

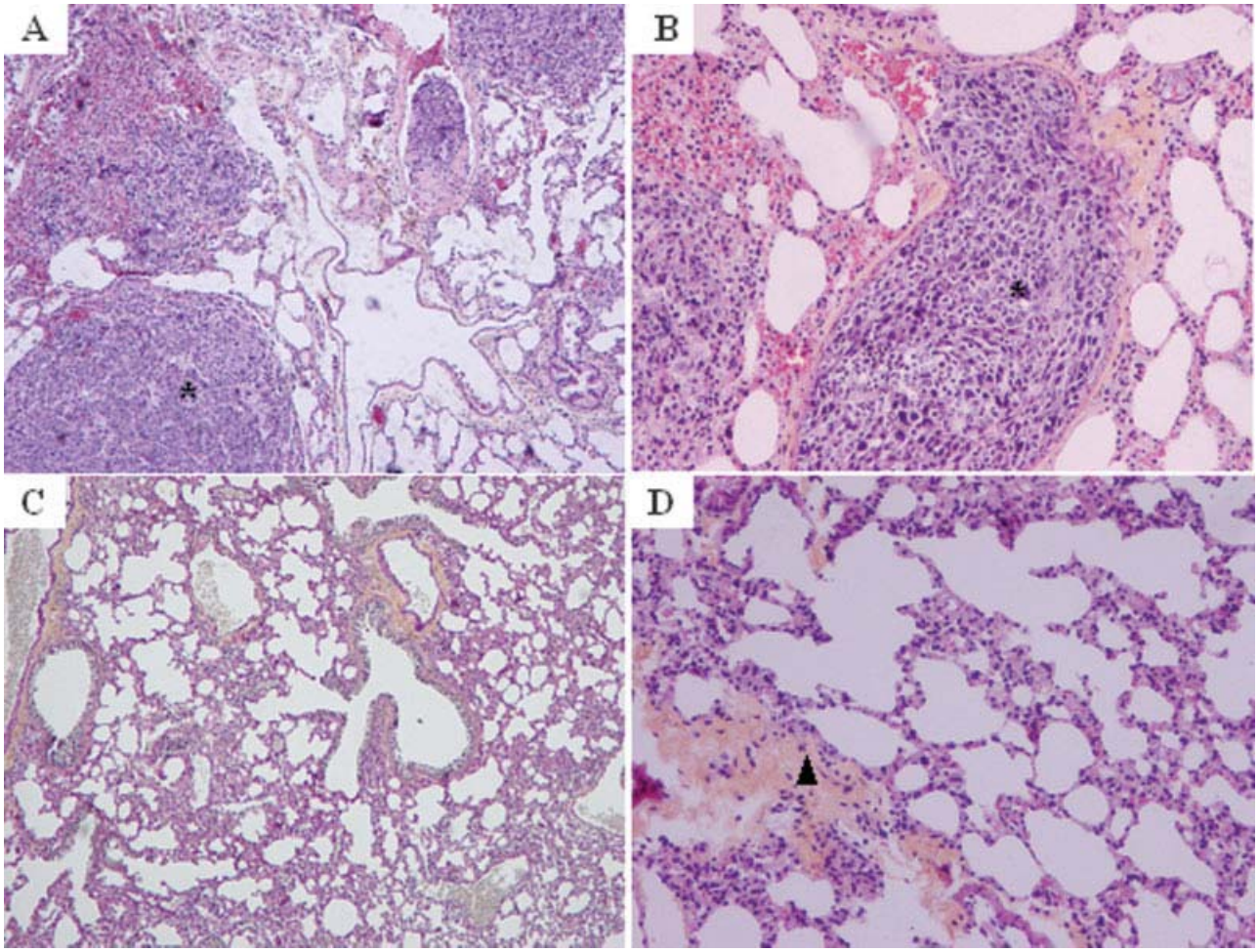

FIGURE 2. Zoledronic acid inhibits the development of lung metastasis in the mouse model of POS-1 cell-induced osteosarcoma. (A,B) Intravenously inoculated POS-1 cells induced numerous lung metastases. The pulmonary tissue is then invaded by high-grade proliferating cell foci (*) and venous emboli. (C,D) No lung metastatic foci were observed after zoledronic acid treatment and small areas of healing tissue can be observed (solid triangle, D). Animals were sacrificed at 45 days postinjection. Original magnification ×40 (A,C); ×200 (B,D).

inhibition of POS-1 cell proliferation was caused by apoptosis. No modification of nuclear morphology that is characteristic of apoptosis was observed in ZOL-treated POS-1 cells after Hoechst staining (not shown). The results showed that ZOL did not induce any activation of caspase-1 in POS-1 cells (not shown), but it did increase caspase-3 activity at a concentration of 10 μM (72 hr), as compared to positive (UV-treated cells) and negative controls (Fig. 3B).

ZOL Induces S-phase Arrest in POS-1 Cells

Flow cytometry analysis of DNA content was performed with osteosarcoma POS-1 cells to identify cell cycle perturbations after ZOL treatment for 48 hours. The results presented in Figure 4 show a 1.8-fold increase in the number of cells arrested in S-phase after ZOL treatment (the number of cells in S-phase increase from 14% in control cells to 19% and 25% in the presence of 1 and 10 μM ZOL, respectively). This observation was concomitant with a reduction of cells in the G_0/G_1 phase: 55% and 53% for 1 and 10 μM ZOL, respectively (48 hr incubation) versus 63% in the control untreated POS-1 cells (Fig. 4).

DISCUSSION

Using a rat model of osteosarcoma, we previously demonstrated that ZOL was able to reduce primary tumor growth and prolong rat survival by decreasing lung metastases dissemination associated with a primary bone tumor.[10] However, a direct effect on pulmonary metastases alone could not be identified in the rat model. Using a murine model of lung metastases induced by i.v. injection of osteosarcoma cells, we here demonstrate that ZOL signifi-

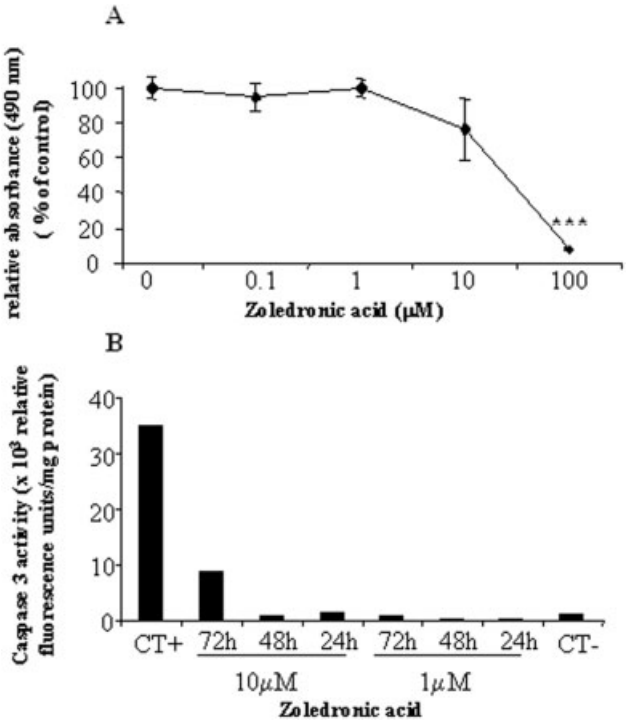

FIGURE 3. In vitro effects of zoledronic acid (ZOL) on POS-1 cell proliferation and caspase activation. (A) Proliferation of POS-1 cells was determined after exposure to ZOL ($0–10^{-4}$M) for 3 days with 10% fetal bovine serum, using the XTT quick proliferation kit as described in Materials and Methods. Changes in absorbance at 490 nm were measured and the results were plotted as percentage of untreated cell proliferation. $^{***}P < 0.005$. (B) POS-1 cells were treated with 1 or 10 μM ZOL for the indicated times. Caspase-3 activity was determined in cell lysates using a caspase-3-specific fluorogenic substrate as described in Materials and Methods. CT+ represents a positive control of cells treated with UV light for 30 seconds. CT– represents the POS-1 cells alone as a negative control. Results of three independent experiments are presented and the caspase activity is expressed as a change in absorbance at 460 nm per μg of protein.

cantly reduced lung metastasis progression, thus extending animal survival. The overall available data on the effects of BPs on visceral metastases in clinical and preclinical studies remain controversial. In a human study, Diel et al.[11] initially described that clodronate had adjuvant inhibitory effects on metastases in visceral organs in breast cancer. Later, however, they found no significant effects of clodronate on visceral metastases in the same populations of patients in the extended follow-up.[12] Moreover, McCloskey et al.[13] did not observe adjuvant effects of clodronate. In contrast, Saarto et al.[14] reported that adjuvant treatment with clodronate increased the development of nonskeletal metastases in breast carcinoma patients. Thus, the evidence from the completed clinical trials remain conflicting, as also revealed by preclinical studies in experimental animal models. Indeed, some data suggest that BPs may increase tumor burden and metastases in soft tissues.[15,16] In contrast, the experimental bisphosphonate YH529 reduced nonosseous metastases in the MDA-MB-231 model of breast carcinoma in nude mice.[17] More recently, Michigami et al.[5] reported that ibandronate reproducibly reduced bone metastases in two animal models of breast carcinoma, but in one of these models (the 4T1 mouse model), neither the preventive nor therapeutic administration of ibandronate caused any effects on lung metastases. In the MDA-MB-231 model of breast cancer, therapeutic administration of ibandronate showed no effects on adrenal metastases.[5] More recently, using the 4T1 mouse model, Hiraga et al.[6] demonstrated that zoledronic acid significantly suppressed lung and liver metastases and prolonged overall survival of tumor-bearing mice. Using the same model, Nobuyuki et al.[18] showed that i.v. ZOL decreased tumor burden not only in bone but also in the liver and lungs of treated mice. In another model of mammary carcinoma cells injected into the medullar space of the proximal tibia of Fisher rats, alendronate reduced lung nodule counts by 95%.[19] Here, we confirm the antitumor effect of ZOL on lung metastases progression using an experimental model different from breast carcinoma-derived lung metastases. In the present study, no metastases could be observed macroscopically 21 days after injection, and histologically 45 days postinjection. It can be suggested that micrometastases were present at 21 days, inducing the death of some animals between Days 21 and 45 (Fig. 1), and that at Day 45 the alive animals do not exhibit any further lung metastases. At that time, necrosis could not be observed in ZOL-treated animals. To explain why all of these treated animals do not survive, it can be suggested that in vivo some tumor cells escape to ZOL-induced inhibition of proliferation, probably by developing resistance to ZOL treatment. This phenomenon has been reported in the case of myeloma cells treated with alendronate, another N-BP.[20] In this study, although N-BP induced apoptosis of myeloma cells in vitro, most in vivo studies fail to demonstrate a corresponding antitumor effect. This discrepancy might reflect the development of a metabolic resistance to the antitumor effect of N-BP in myeloma cells when they are exposed to N-BP for a prolonged time. In our laboratory, we developed a rat model of osteosarcoma in vivo and used the corresponding cell lines for in vitro experiments.[10] When these cells were maintained for a long time in culture, part of these cells

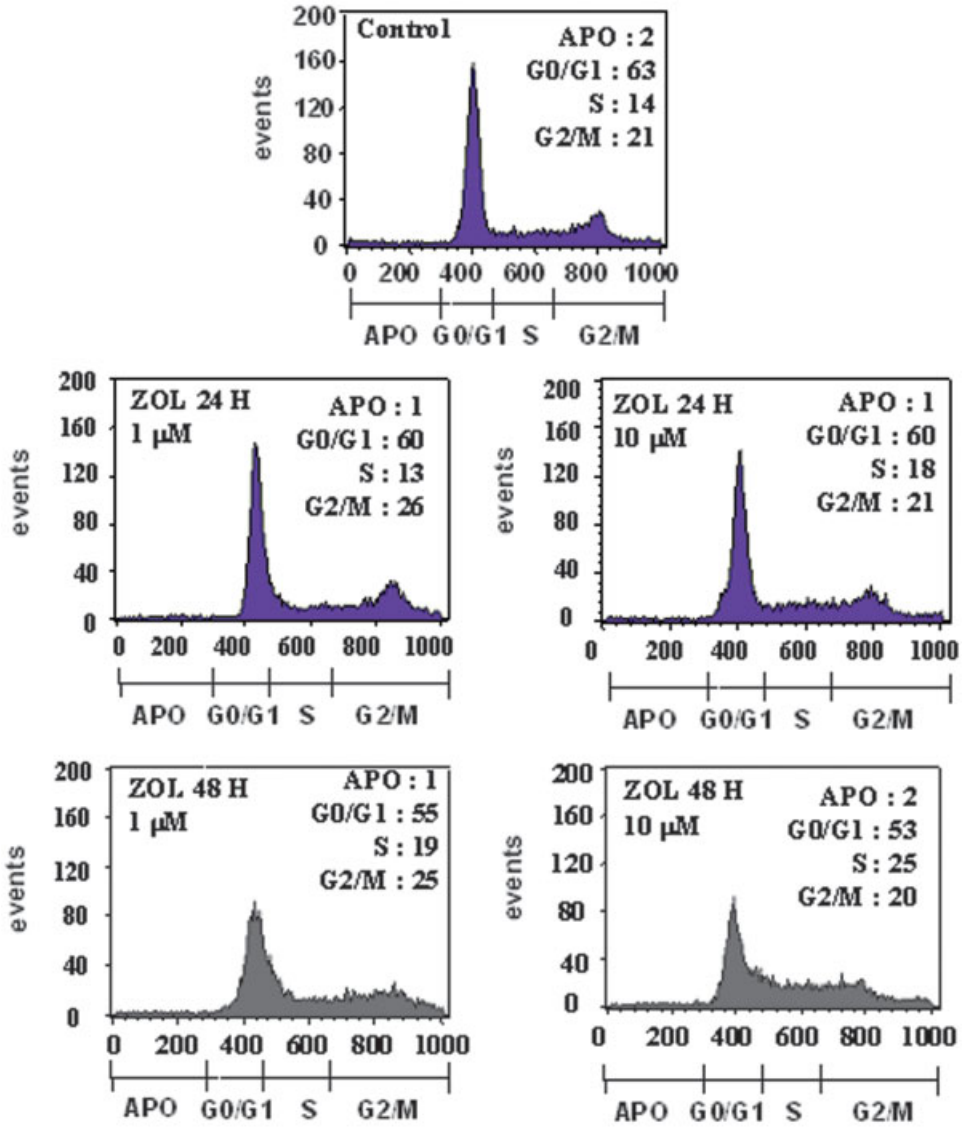

FIGURE 4. Flow cytometry of zoledronic acid (ZOL)-treated POS-1 cells. POS-1 cells were incubated for 24–48 hours in the absence (control) or presence of 1 or 10 μM ZOL. At each point cells were harvested, fixed, and stained with propidium iodide. The positions on the histograms of the hypodiploid sub-G_0/G_1 (APO), G_0/G_1, S and G_2/M peaks, and the percentage of cells in each of the cycle phases are indicated from a representative experiment.

became resistant to ZOL treatment, so we can suggest that the same phenomenon happened with POS-1 cells.

Thus, ZOL, which seems to be one of the most effective antiresorptive BPs in vitro, may also exert potent antitumor activity in vivo against the progression of visceral metastases. Inhibition of several cellular mechanisms such as vascularization, adhesion, invasion, and migration have been proposed to explain this phenomenon.[3] These mechanisms could be studied with the POS-1 cells used to induce pulmonary metastases in the present model. Indeed, the availability of the corresponding cells will allow testing the effects of ZOL on migration and invasion of POS-1 cells in vitro, and also integrin expression and cell adhesion to endothelial cells.

The ZOL doses used in the present study are justified, as 0.1 mg/kg is clearly equivalent to the clinical dose (4 mg i.v. every 3–4 weeks is equivalent to approximately 100 μg/kg of the research grade disodium salt). However, even if a dosing frequency of twice or five times a week is much greater, it could be justified by the very aggressive nature of lung metastases in the murine model and the short survival times. The doses and schedules of ZOL used in this preclinical study are in agreement with the subchronic and chronic toxicity data given by the Novartis Pharma Laboratories (pers. commun.). Therefore, ZOL doses used in the present study are related to the achievable, safe levels in humans. The observation that lung metastases were reduced suggests a direct effect of ZOL on tumor cells that was not dependent on its effect on the bone microenvironment. However, given the low transient levels of

ZOL in blood and soft tissues, we cannot discriminate between a direct effect on tumor cell dissemination from the primary injection site or growth inhibition of the secondary lung metastases. Among the mechanisms hypothesized for the BP antitumor activity, data from the literature report that caspase-dependent apoptosis appears to be the major mechanism responsible for BP-induced tumor cell apoptosis, and caspase-3 is certainly the major player in this response.[21] In our experimental model, ZOL caused a direct inhibition of POS-1 cell proliferation and an accumulation of cells in the S-phase of the cycle. Similar results were observed by Evdokiou et al.[22] Indeed, using a panel of human osteogenic sarcoma cell lines, those authors demonstrated that ZOL reduced cell numbers in a dose- and time-dependent manner, due either to cell cycle arrest in the S-phase or to the induction of apoptosis.[22] A comparable mechanism was also described for N-containing BPs such as ZOL in other cell types—for example, melanoma cells.[23] In our study, cell cycle arrest in the S-phase was accompanied by a weak activation of caspase-3 activity, a well-characterized effect of N-BPs.[21] However, it is difficult to conclude which is the exact mechanism involved in this model, as Hoescht staining did not reveal any modification of nuclear morphology in ZOL-treated cells (not shown). Moreover, in the cell cycle analysis (Fig. 4), no cells in the sub-G_0/G_1 phase were observed that could represent apoptotic cells. Therefore, the weak caspase-3 activation observed in our study may be nonspecific, associated with a general cytotoxic effect of the high concentration of ZOL. TUNEL staining, performed on rat osteosarcoma tumor samples, were negative for ZOL-treated animals. Another mechanism could be envisaged, such as anoikis, previously reported in human osteogenic sarcoma cells treated with ZOL by Evdokiou et al.[22]

In conclusion, using a model of lung metastases different in its origin from the well-studied breast carcinoma, we confirm the direct antitumor effect of ZOL both in vitro (antiproliferative and apoptotic effect) and in vivo on lung metastasis progression, leading to a significant prolongation of disease-free survival in animals. This result reveals that this compound could benefit patients with nonskeletal metastases, including osteosarcoma patients who remain at high risk of eventual relapse, with overt metastatic disease, with tumors that recur after treatment, or that show a low degree of necrosis after administration of chemotherapy and continue to have an unsatisfactory outcome.

REFERENCES

1. Fleish H. Development of bisphosphonates. *Breast Cancer Res.* 2002;4:30–34.
2. Sato M, Grasser W, Endo N, et al. Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. *J Clin Invest.* 1991;88:2095–2105.
3. Heymann D, Ory B, Gouin F, Green JR, Redini F. Bisphosphonates: new therapeutic agents for the treatment of bone tumors.*Trends Mol Med.* 2004;10:337–343.
4. Yoneda T, Hashimoto N, Hiraga T. Bisphosphonate actions on bone and visceral metastases. *Cancer Treat Res.* 2004;118: 213–229.
5. Michigami T, Hiraga T, Williams PJ, et al. The effect of the bisphosphonate ibandronate on breast cancer metastasis to visceral organs. *Breast Cancer Res Treat.* 2002;75:249–258.
6. Hiraga T, Williams PJ, Ueda A, Tamura D, Yoneda T. Zoledronic acid inhibits visceral metastases in the 4T1/luc mouse breast cancer model. *Clin Cancer Res.* 2004;10:4559–4567.
7. Tsuchiya H, Kanazawa Y, Abdel-Wanis ME, et al. Effect of timing of pulmonary metastases identification on prognosis of patients with osteosarcoma: the Japanese Musculoskeletal Oncology Group study. *J Clin Oncol.* 2002;20:3470–3477.
8. Scotlandi K, Serra M, Nicoletti G, et al. Multidrug resistance and malignancy in human osteosarcoma. *Cancer Res.* 1996; 56:2434–2439.
9. Nitto H, Koshino T, Mitsugi N, Hiruma T. Growth of a murine osteosarcoma-derived cell sarcoma increases serum immunosuppressive acidic protein levels. *Cancer J.* 2000;60: 5625–5629.
10. Heymann D, Ory B, Blanchard F, et al. Enhanced tumor regression and tissue repair when zoledronic acid is combined with ifosfamide in rat osteosarcoma. *Bone.* 2005;37: 74–86.
11. Diel IJ, Solomayer E-F, Costa SD, et al. Reduction in new metastases in breast cancer with adjuvant clodronate treatment. *N Engl J Med.* 1998;339:357–363.
12. Diel IJ, Solomayer E-F, Gollan C, Shutz F, Bastert G. Bisphosphonates in the reduction of metastases in breast cancer — results of the extended follow-up of the first study population. *Proc ASCO* 2001 (Abstr. 314).
13. McCloskey EV, Powles T, Paterson AHG, Ashley S, Kanis JA. Clodronate reduces incidence of skeletal metastases in women with primary breast cancer. *Bone.* 1998;23:S189.
14. Saarto T, Blomqvist C, Virkkunen P, Eloma II. Adjuvant clodronate treatment does not reduce the frequency of skeletal metastases in node-positive breast cancer patients: 5-year results of a randomised controlled trial. *J Clin Oncol.* 2001;19:10–17.
15. Sasaki A, Boyce BF, Story B, et al. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. *Cancer Res.* 1995;55:3551–3557.
16. Stearns ME, Wang M. Effects of alendronate and taxol on PC-3ML cell bone metastases in SCID mice. *Invasion Metastasis.* 1996;16:116–131.
17. Sasaki A, Kitamura K, Alcade RE et al. Effect of a newly developed bisphosphonate, YH529, on osteolytic bone metastases in nude mice. *Int J Cancer.* 1998;77:279–285.
18. Nobuyuki H, Hiraga T, Williams PJ, et al. The bisphosphonate zoledronic acid inhibits metastases to bone and liver with suppression of osteopontin production in mouse mammary tumor. *J Bone Miner Res.* 2001;16:S191.

19. Alvarez E, Galbreath EJ, Westmore M, et al. Properties of bisphosphonates in the 13762 syngenic rat mammary carcinoma model of tumor induced bone resorption. *Proc Am Assoc Cancer Res*. 2002;43:316.
20. Salomo M, Jurlander J, Nielsen LB, Gimsing P. How myeloma cells escape bisphosphonate-mediated killing: development of specific resistance with preserved sensitivity to conventional chemotherapeutics. *Br J Haematol*. 2003;122: 202–210.
21. Benford HL, McGowan NW, Helfrich MH, Nuttall ME, Rogers MJ. Visualization of bisphosphonate-induced caspase-3 activity in apoptotic osteoclasts in vitro. *Bone*. 2001;28:465–473.
22. Evdokiou A, Labrinidis A, Bouralexis S, Hay S, Findlay DM. Induction of cell death of human osteogenic sarcoma cells by zoledronic acid resembles anoikis. *Bone*. 2003;33:216–228.
23. Forsea AM, Muller C, Riebeling C, Orfanos CE, Geilen CC. Nitrogen-containing bisphosphonates inhibit cell cycle progression in human melanoma cells. *Br J Cancer*. 2004;91: 803–810.

Complément de discussion à l'article 4

Dans l'article précédent, nous avons démontré dans un modèle d'ostéosarcome de rat que le Zol était capable de réduire la croissance de la tumeur primaire et de prolonger la survie des animaux en réduisant la dissémination métastatique au niveau des poumons. Cependant, un effet direct de cet agent sur les métastases pulmonaires reste à démontrer. En utilisant un modèle murin de métastases pulmonaire induites par inoculation i.v de cellules d'ostéosarcomes, nous avons pu démontrer que le Zol réduisait significativement la progression des métastases pulmonaires et par conséquent augmentait la survie des animaux.

Les donnés cliniques et précliniques des effets des BPs sur les métastases viscérales restent incertaines et contradictoires. En effet, dans une étude sur le cancer du sein, Diel *et al* (Diel et al. 1998) décrivent le Clodronate comme ayant un effet inhibiteur sur le développement des métastases viscérales ; cependant, quelque temps plus tard, après un suivi plus poussé sur la même population, ils n'observent plus aucun effet bénéfique du Clodronate sur le développement métastatique (Diel 1998). Saarto *et al* (Saarto et al. 2001) quant à eux, observent des effets inverses et montrent qu'un traitement adjuvant avec le Clodronate chez des patientes atteintes de carcinome mammaire augmentait le développement de métastases viscérales. Les études précliniques réalisées chez l'animal sont elles aussi contradictoires. Certaines données suggèrent que les BPs pourraient accroitre le développement tumoral et métastatique dans les tissus mous (Sasaki et al. 1995; Stearns et al. 1996). A l'inverse, le nouveau BPs de troisième génération YH529 réduit les métastases non osseuses dans le modèle MDA-MB-231 de carcinome mammaire chez la souris nude (Sasaki et al. 1998). Plus récemment, Michigami *et al* (Michigami et al. 2002) montrèrent que l'Ibandronate réduisait les métastases osseuses dans deux modèles animaux de carcinome mammaire, cependant, dans un de ces deux modèles (le modèle murin 4T1), aucun effet sur les métastases pulmonaire n'a pu être observé. Higara *et al* (Hiraga et al. 2004) ont démontré dans le modèle murin 4T1 que le Zol supprimait significativement les métastases au niveau des poumons et du foie et par conséquent prolongeait la survie des animaux.

Dans cette étude nous confirmons l'effet anti-tumoral du Zol sur la progression des métastases pulmonaires en utilisant un modèle expérimental différent du modèle le plus décrit dans la littérature de métastases pulmonaires dérivées d'un carcinome mammaire. Macroscopiquement, aucune métastase n'a pu être observée 21 jours après l'injection des cellules et la recherche de métastases 45 jours après l'injection est restée infructueuse. Ceci

suggère que des micro-métastases étaient présentes à 21 jours, induisant la mort de certains animaux entre les jours 21 et 45, les animaux survivant n'ayant pas développé de métastases. Pour expliquer pourquoi certains animaux sont morts malgré le traitement une hypothèse peut être avancée : *in vivo*, certaines tumeurs échappent à l'inhibition de prolifération induite par le Zol grâce au développement d'une résistance à ce traitement. Ce phénomène à déjà été rapporté dans un cas de cellules de myélome traitées par de l'Alendronate (N-BPs) (Salomo et al. 2003), de même, nous avons développé au laboratoire un modèle de cellules d'ostéosarcome de rat résistant au traitement par le Zol. Ce modèle à été obtenu *in vitro* par un traitement de longue durée avec de faibles concentrations de Zol. Ces résultats suggèrent que le même phénomène pourrait intervenir avec les cellules POS-1 *in vivo*. Ces phénomènes seront décrits plus en détail dans la partie II de cette thèse.

La concentration de Zol utilisée dans cette étude est de 0,1 mg/kg (sous forme sel disodique) ; selon les données pharmacocinétiques fournis par Novartis, cette concentration est équivalente à 4 mg de Zol utilisée chez les patients i.v toutes les 3-4 semaines. La différence principale est que dans cette étude la fréquence de traitement de 2 ou 5 fois par semaine est supérieure à celle utilisée chez l'homme. Ceci peut être justifié par le fait que les métastases pulmonaires de notre modèle murin sont de nature plus agressive que la pathologie humaine et que le temps de survie des animaux contrôles est court.

En conclusion, la réduction de la dissémination métastatique que nous avons observée suggère un effet direct du Zol sur les cellules tumorales indépendamment de ses effets sur le microenvironnement osseux. Cependant, notre modèle ne nous permet pas de déterminer si l'effet du Zol sur les métastases pulmonaires est direct ou indirect, plusieurs hypothèses peuvent être envisagées : soit le Zol module le remodelage osseux et affecte indirectement le développement des métastases en modifiant le microenvironnement osseux, soit le Zol module le système immunitaire qui agit ensuite sur les cellules tumorales, soit le Zol agit directement sur les cellules tumorales.

L'acide zolédronique pourrait présenter un intérêt thérapeutique pour les patients atteints entre autres de métastases non osseuses tels que les patients souffrant d'un ostéosarcome chez qui le diagnostic de métastases pulmonaires est synonyme d'éventuelle rechute et de mauvais pronostic vital.

Introduction

Le chondrosarcome, identifié par Lichtenstein et Jaffe comme étant une tumeur osseuse maligne distincte de l'ostéosarcome, est couramment défini comme une tumeur maligne du cartilage succédant ou non à une tumeur bénigne préexistante (Lichtenstein L 1942). Les chondrosarcomes représentent 25% des sarcomes de l'os et leur grade est évalué à l'aide d'analyses histologiques et cytologiques (O'Neal et al. 1952). Le taux de récidive locale est de l'ordre de 24 à 33% et la survie à 10 ans varie entre 46 et 70% selon les séries (Pritchard et al. 1980). Ce faible pronostique est en partie dû à la pauvreté de l'arsenal thérapeutique ; en effet la résection chirurgicale est pratiquement la seule issue possible, la majorité des chondrosarcomes étant radio-résistants et chimio-résistants. Les bisphosphonates et plus particulièrement le Zol sont des molécules de choix dans le traitement des pathologies tumorales osseuses du fait de leur capacité à inhiber la résorption osseuse en induisant l'apoptose des ostéoclastes, et de leur activité directe sur les cellules tumorales. Dans ce contexte, cette étude s'intéresse à l'efficacité du Zol dans le traitement du chondrosarcome, *in vitro* et *in vivo* en termes de croissance tumorale et de survie des animaux dans un modèle transplantable de chondrosarcome de rat avant et après curetage intra lésionnel de la tumeur.

Article 5

« Applications thérapeutiques de l'acide zolédronique dans le chondrosarcome. »

Int J Cancer, Vol 119, 980-984, Sep 2006, Gouin F, Ory B, Redini F, Heymann D**.**

Les chondrosarcomes sont des tumeurs primitives malignes produisant une matrice extracellulaire de type cartilagineuse contenant du collagène de type I et II et autres protéoglycanes. Ces tumeurs étant chimio- et radio-résistantes, l'exérèse chirurgicale représente le traitement principal. Malgré un contrôle de la croissance tumorale dans 60 à 80% des cas, la dissémination métastatique et la récidive locale entraînent la mort des patients dans de nombreux cas. Les bisphosphonates, principalement ceux de dernière génération, sont des agents thérapeutiques prometteurs dans le contrôle des tumeurs osseuses en général. Cette étude s'est focalisée sur les effets de l'acide zolédronique (ZOL) dans un modèle de chondrosarcome de Swarm *in vivo* et sur des cellules tumorales extraites de cette tumeur *in vitro*.

Des fragments tumoraux ont été implantés dans trois séries de 12 rats mâles Sprague-Dawley : série A (n=6) : les rats ont été traités par 100 microg/kg ZOL deux fois par semaine à partir de J4 après implantation jusqu'à la mort ou l'euthanasie des animaux ; séries B (n=6) et C (n=6) : les rats ont été traités à partir de J4 avant exérèse de la tumeur et jusqu'à la mort ou l'euthanasie des animaux. Dans toutes les séries, des rats traités avec du PBS ont servi de contrôle. La croissance tumorale a été évaluée deux fois par semaine par la mesure du volume tumoral jusqu'à l'euthanasie. Les effets de ZOL ont également été étudiés *in vitro* sur la prolifération des cellules tumorales et l'apoptose (activation des caspases 1 et 3, fragmentation nucléaire).

Le ZOL diminue la progression tumorale dans toutes les séquences de traitement : dans la série A, le volume tumoral moyen est significativement réduit dans les groupes traités à J25 et J27 (p=0,046), alors que la progression tumorale entre J19 et J32 est significativement plus forte dans le groupe contrôle que chez les animaux traités (p=0,046). La probabilité de survie à J40 est de 0,3 pour le groupe contrôle comparativement à 0,667 pour les rats traités. Dans les séries B et C, bien qu'un traitement par le ZOL n'empêche pas la récurrence locale tumorale (dans 70% des rats traités), elle survient plus tard dans tous les cas. Le volume tumoral moyen est plus faible pour les rats traités dans les deux séries entre

J32 et J54, la progression du volume tumoral entre J39 et J49 est significativement plus élevée dans les contrôles que dans les rats traités (respectivement 15 691 mm^3 et 7396 mm^3, p=0,025). *In vitro*, une incubation de 72 heures avec 1 microM ZOL inhibe la prolifération des cellules tumorales de 40%, alors que 10 microM ZOL l'inhibe complètement. ZOL n'a pas d'effet sur la fragmentation nucléaire, n'induit pas d'activation de la caspase 1 mais augmente celle de la caspase 3 à la concentration de 10 microM (48 et 72 heures).

Ces résultats démontrent que le ZOL pourrait représenter un agent thérapeutique prometteur dans le traitement du chondrosarcome.

Int. J. Cancer: **119,** 980–984 (2006)

Zoledronic acid slows down rat primary chondrosarcoma development, recurrent tumor progression after intralesional curretage and increases overall survival

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Chondrosarcoma is a difficult musculoskeletal tumor to treat. Surgical treatment leads to severe disability, with high rates of local recurrence and life threat. No adjuvant therapy is effective in differentiated chondrosarcomas. Bisphosphonates (BPs) are a class of molecules which is effective in malignant bone diseases. The aim of the present study was to determine the effects of zoledronic acid (ZOL) on chondrosarcoma tumor progression. ZOL was tested *in vivo* (s.c. 100 μg/kg, twice a week) in a rat chondrosarcoma model and *in vitro* (10^{-7}–10^{-4} M) on cells derived from this model. Two types of animal models were assessed, the first simulated development after intralesional curettage, the second nonoperative development of the tumor. Cell proliferation, caspase-1, -3 activities and cell cycle analysis were studied. The results revealed that ZOL slows down primary tumor development, tumor progression after intralesional curretage and increases overall survival. ZOL inhibits cell proliferation and increases cell death, with no significant variation of caspase-1 and -3 activities and cell cycle profiles. The present study demonstrates for the first time that in addition to surgery, the therapy of chondrosarcoma with BPs might be beneficial. Because of these first results, new therapeutic approaches of chondrosarcoma must be considered, mainly for low grade chondrosarcoma when disabling operation is planned and when only intralesional resection can be undertaken.
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Key words: bisphosphonate; chondrosarcoma; therapy; primary bone tumors; adjuvant treatment

Chondrosarcoma, identified by Lichtenstein and Jaffe as a malignant bone tumor clearly distinct from osteosarcoma, is currently defined as malignant cartilage tumor arising *de novo* or within a preexisting benign cartilage tumor.[1] Human chondrosarcomas, which represent about 25% of all bone sarcoma,[2,3] are classified as low, intermediate or high grade on the basis of histological and cytological features.[4] Overall local recurrence and 10-year survival are 24–33% and 46–70%, respectively.[5,7] Risk factors for the former include inadequate surgical margins[5,8] and tumor size, and for the later include local recurrence, extracompartmental spread and high histological grade.[5] No effective adjuvant treatment (radiation therapy, chemotherapy) is available.[6]

As evidenced for bone metastases, a vicious cycle between osteoclasts, bone stromal cells/osteoblasts and cancer cells has been hypothesized during the progression of primary bone tumors.[9] Accordingly, suppression of osteoclasts would be a primary approach to inhibit local cancer growth. Among the potential drugs available, bisphosphonates (BPs) are an important class of molecules for the treatment of bone diseases with different molecular mechanisms of action. Nitrogen-containing BPs act by inhibiting the recruitment, proliferation and differentiation of preosteoclasts, or by impeding the resorptive activity of mature osteoclasts.[10–13] They also shorten the life span of osteoclasts by inducing their apoptosis.[14] Previous studies revealed that BPs have the ability to reduce the osteolytic bone resorption associated with multiple myeloma and breast cancer[15,16] and also show efficacy in cancer metastases to bone due to prostate cancer and other solid tumors, demonstrating that this BPs can reduce skeletal morbidity in both osteolytic and osteoblastic diseases.[17,18] A clear direct antitumor activity on breast cancer cells has been demonstrated *in vitro*.[19] In primary malignant bone tumor, inhibition of human osteosarcoma cell growth by pamidronate and clodronate[20,21] and zoledronic acid (ZOL)[22] have been reported *in vitro*. These results are consistent with our *in vitro* and *in vivo* studies demonstrated efficacy of ZOL on osteosarcoma tumor progression and metastatic spreading.[23,24]

Hence, the purpose of the present study was to determine the efficacy of ZOL on chondrosarcoma *in vitro* and *in vivo*, in term of local tumor growth, animal survival after and before intralesional curettage using a rat transplantable model of chondrosarcoma.

Material and methods

Materials

Dulbecco's modified eagle's medium (DMEM), L-glutamine and trypsin were obtained from Invitrogen (Eragny, France), fetal bovine serum (FBS) from Hyclone (Perbio, France). Rat swarm chondrosarcoma (RCS) was a generous gift from Dr. P.A. Guerne (Geneva, Switzerland).[25,26] The subline used in the present study expresses collagen II. Although RCS cells are not able to form mineralized nodules *in vitro*, they decrease the capacity of bone marrow to mineralize and do not modify the osteoclastic differentiation.[26] After subcutaneous or intramuscular development, RCS was well delimited, poorly vascularized, lobular in organisation and soft (unlike normal cartilage). Histological examination revealed chondroid tumor proliferation consisting of lobules of variable size containing chondroid stroma delimited by fine septa. This chondrosarcoma displayed the histological feature of grade II chondrosarcoma.[26] Zoledronic acid (ZOL) was kindly provided as the research grade disodium salt by Novartis Pharma AG (Basel, Switzerland).

In vivo *assessment of ZOL treatment*

Male Sprague-Dawley rats were purchased from the ''Centre d'Elevage Janvier'' (Le Genest Saint Isle, France). Four-week-old rats were housed under pathogen free conditions at the Experimental Therapy Unit (Medicine Faculty of Nantes, France), in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators. For the implantation, the rats were anaesthetized by inhalation of a combination isoflurane (Abbott, Rungis, France)/air (1.5%, 1 L/min) associated with an intramuscular injection of Imalgene (100 mg/kg, Merial Lab., Lyon, France). Allograft transplantations of tumor fragments were performed as follows: using a lateral approach, the cortical surface of the diaphysis was scarified laterally on 10 mm, a 10 mm^3 fragment of RCS was placed contiguous

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to the scarified surface, and the cutaneous and muscular wounds were sutured. Tumors appeared at the graft site 7–11 days later.

Two protocols were applied: (*i*) curative treatment: rats were individually identified and assigned to control or treatment group 4 days after implantation (6 animals/group). Each rat in treated group received s.c. 100 μg/kg ZOL twice a week, started at day 4 after implantation until euthanasia; (*ii*) in other series, rats bearing growing tumors with a volume >1200 mm^3, which were considered as progressive tumors, were individually identified at day 20 after implantation and assigned to the control or treatment group (6 animals/group). Treatment group received 100 μg/kg ZOL s.c. the same day, and twice a week until euthanasia. All animals were blindly operated at day 24 by intralesional curettage.

In all series ($n = 3$), control groups received the same volume of PBS s.c. at the same schedule as treated animals. The animals were weighed twice a week, at the same time the tumor volume was calculated from the measurement of 2 perpendicular diameters using a caliper. Each tumor volume (V) was calculated according to the following formula : $0.5 \times L \times (S)^2$, where L and S are the largest and smallest perpendicular tumor diameters, respectively. The animals were sacrificed, except spontaneous death, when tumor became too bulky and when life of the animal was threatened.

In vitro *analyses*

Cell proliferation. The RCS cells was derived from the Swarm tumor used in the present study. Briefly, RCS were isolated from small tumor fragments, treated 2 hr with 1 mg/mL collagenase A (Boehringer Manheim). The cell suspension was then washed 3 times with PBS, cultured in DMEM supplemented with 10% FCS, 1% glutamine and maintained at 37°C in a humidified atmosphere with 5% CO_2. The medium was replaced twice a week and the adherent RCS were replated using a 0.05% trypsine–0.02% EDTA solution. Replicate subconfluent cell cultures in 96-multiwell plates were treated for 1 to 3 days with increasing concentrations of ZOL (10^{-7}–10^{-4} M, diluted in PBS). Cell proliferation was determined by a cell proliferation reagent assay kit using sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) (Roche Molecular Biomedicals, Mannheim, Germany). Cell viability was also assessed by trypan blue exclusion and manual counting.

Induction of apoptosis. Programmed cell death was monitored microscopically following Hoechst staining and cell viability by trypan blue exclusion. RCS cells were seeded at 10^4 cells/well in a 24-multiwell plate and cultured for 24 hr as described above, before being incubated with ZOL at indicated concentrations during 24, 48 and 72 hr. Trypsinized cells were then resuspended in the presence of Hoechst n°33258 staining (10 μg/mL; Sigma) for 30 min at 37°C. Cells were then observed by UV microscopy (Leica, Wetzlar, Germany). Induction of apoptosis was also investigated by cleavage of caspase-1 and -3 substrates, in cell lysates with or without ZOL treatment. RCS cells were seeded at 15×10^3 cells/well (in a 24-multiwell plate), then incubated with ZOL (1 and 10 μM) for 24, 48 and 72 hr. Cells treated with UV for 30 sec, 24 hr before cell extraction, were used as positive controls. At the end of the incubation period, the cells (adherent and non adherent) were lysed with 50 μL of RIPA buffer for 30 min. The cells were then scraped off and protein content was quantified in parallel samples using the BCA (bicinchominique acid + Copper II sulfate) assay. Caspase 1 and 3 activities were assessed on 10 μL of the cell lysate with the kit CaspACE™ Assay System (Fluorometric, Promega, Madison, USA) following the manufacturer's instructions.

Cell cycle analysis. Confluent cultures of RCS cells were incubated for 24, 48 or 72 hr with or without 10 μg/mL ZOL, trypsinized, washed twice and lysed in PBS containing 0.12% Triton X-100, 0.12 mM EDTA and 100 μg/mL ribonuclease A. Then 50 μg/mL propidium iodide were added for each sample for 20 min at 4°C in the dark. The intensity of propidium iodide labelling was measured by flow cytometry (FACScan, BD Biosciences) using the CellQuest software.

Statistics

For *in vivo* experimentations, the nonparametric Willcoxon test was used to compare the tumor volume (quantitative data) between controls and ZOL-treated animals. The cumulative rate of overall survival was calculated according to actuarial method and the end point considered was either death of animals or tumor volume superior to 20,000 cm^3. The differences of actuarial survival were determined by the K^2 test. Statistical evaluation of the *in vitro* proliferation data was performed by Student's *t*-test. Results are given as mean + SD and results with $p < 0.05$ were considered significant.

Results

ZOL slows down the chondrosarcoma tumor progression and significantly enhances rat survival in curative treatment

In curative treatments, rats received s.c. 100 μg/kg ZOL twice a week, from day 4 after tumor implantation until death. The mean tumor volume was significantly lower in the ZOL-treated group than in control group at day 25 (4318 + 2278 and 10355 + 7414 mm^3 respectively, $p < 0.05$, Fig. 1*a*) and at day 27 (5253 + 4133 and 15092 + 10781 respectively, $p < 0.05$). The volume tumor progression calculated between day 14 and 27 was significantly higher in the control group than in treated animals (13986 + 10986 *versus* 4418 + 4059 mm^3, $p < 0.05$, Fig. 1*b*). As a consequence, ZOL significantly prolonged overall survival of chondrosarcoma-bearing rats, as the probability of survival at day 40 was 0.3 + 0.197 for the control group compared to 0.667 + 0.33 for treated animals ($p < 0.05$) with tumor volume superior to 20,000 mm^3 as the end point (Fig. 1*c*).

Recurrent tumors after intralesional curettage grow slower in ZOL-treated rats

In a second set of experiments, the effects of ZOL were examined on tumor growth that occurred after intralesional curettage. In these series, the rats were operated at day 24 after tumor implantation. The treatment group received s.c. 100 μg/kg ZOL, 4 days before intralesional curettage, then twice a week until euthanasia. In this protocol, even if ZOL treatment failed to prevent local growth, it occurred later in all cases. The mean tumor volume was smaller for ZOL-treated animals from day 32 to 54. For example, mean tumor volume was 2.6-fold higher in control group than in the treated group at day 54, 31204 + 10781 and 12066 + 5492 mm^3, respectively ($p < 0.05$, Fig. 2*a*). At all time points, the control value is always superior to ZOL-treated animals. In the same way, volume tumor progression between day 39 and 49 was significantly higher in control group than in the treated animals (15691 + 3173 *versus* 7396 + 5621 mm^3, $p < 0.03$, Fig. 2*b*). In another independent series, the mean tumor volumes were always much higher in controls than in ZOL-treated rats, even if the differences were not found significant (21821 + 22913 and 7171 + 11232 at day 46, and 12253 + 5479 *versus* 5547 + 9218 at day 42, respectively) (data not shown).

ZOL inhibits RCS cell proliferation and increases cell death

To determine whether the antitumor activity of ZOL observed *in vivo* could be mediated by a direct antitumor effect on cell proliferation, the effect of ZOL (0.1–100 μM for 72 hr) was assessed *in vitro* on the RCS cell proliferation (Fig. 3*a*). The XTT viability test showed that ZOL significantly decreased chondrosarcoma cell proliferation with an IC50 value of 1.7 μM. Thus, ZOL inhibited cell proliferation by 38% at 1 μM and by 100% at 10 and 100 μM ($p < 0.001$) (Fig. 3*a*). To determine whether these effects were due to inhibition of cell proliferation and/or induction of cell death, the effects of ZOL were assessed by counting viable cells based on trypan blue exclusion. The results confirmed the low proliferation rate of RCS cells in the control group (30% increase after 72 hr of culture, Fig. 3*b*) and a decrease of alive cell number over 72 hr in the presence of 1 μM ZOL compared to the control

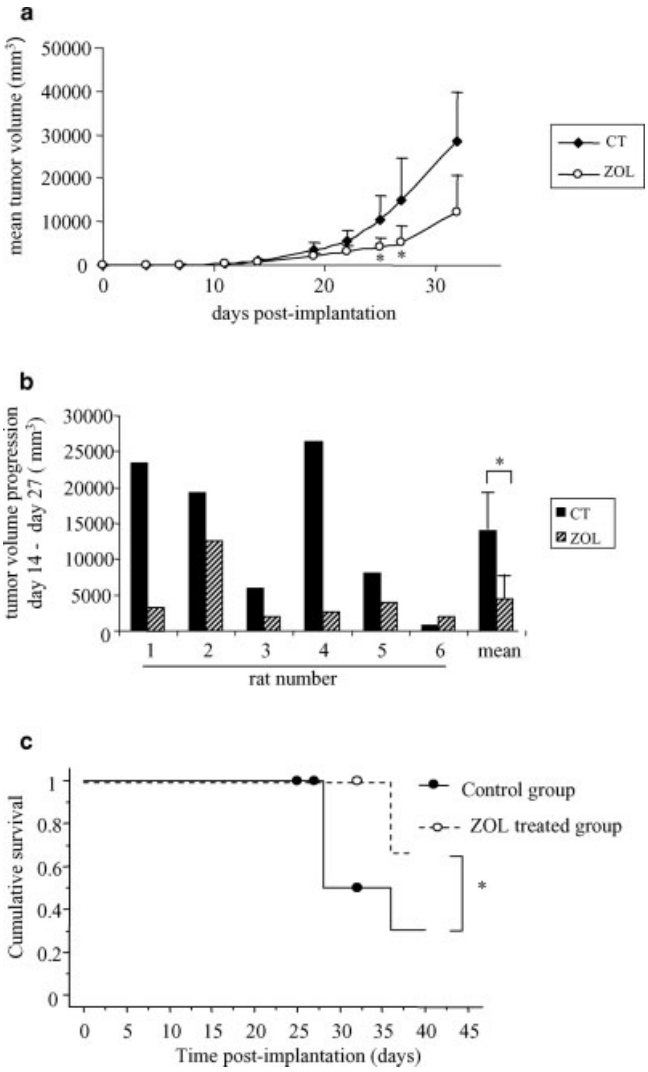

Figure 1 – ZOL slows down the chondrosarcoma tumor development associated with a significant increase of rat survival in curative treatment. Effect on tumor progression and rat survival of ZOL administered 4 days after tumor implantation twice a week. (*a*) Time course of the mean tumor volume (6 rats/group). (*b*) Tumor progression between day 14 and 27 after implantation for a representative series. (*c*) Survival rate (6 rats/group) with 100 μg/kg ZOL twice a week from day 4 after implantation. *$p < 0.05$, ZOL-treated compared to the control group.

group (38% decrease, $p < 0.01$). During the same period of culture, a marked increase of cell death was observed in the presence of ZOL (20% compared to 4.7% in the control group, $p < 0.01$), thus suggesting that no proliferation takes place in the ZOL-treated cell groups (Fig. 3*b*). All the RCS cells died after 72 hr in the presence of 10 μM ZOL. To determine whether the ZOL-induced death in RCS cells was caused by apoptosis, Hoechst staining and caspase-1 and -3 activation were investigated. Both tests gave negative results: Hoechst staining showed no modification of nuclear morphology in the presence of ZOL as compared to control cells (data not shown), and no significant activation of caspase 1 (data not shown) and 3 (Fig. 3*c*) activity could be detected after ZOL treatment in these cells.

Moreover, flow cytometric analysis of DNA content was performed to identify cell cycle perturbations in RCS cells following treatment with ZOL over a 48-hr period. Results showed no modification of the cell cycle profiles in the presence of 10 μM ZOL for 48 hr of incubation (Fig. 3*d*).

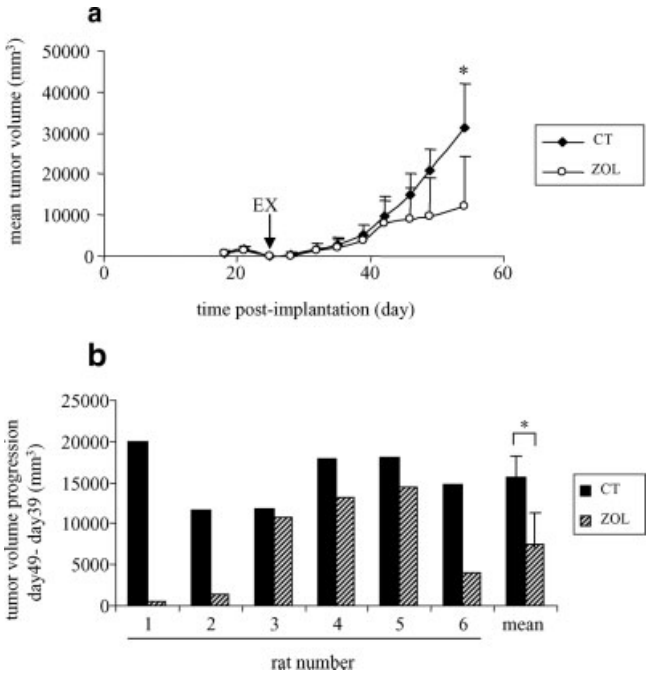

Figure 2 – ZOL postpones tumor growth after intralesional curettage. Effect of ZOL administered 4 days before intralesional curettage twice a week until euthanasia. (*a*) Time course of the mean tumor volume for the whole series (6 rats/group). Tumor recurred in all cases. (*b*) Tumor progression between day 39 and 49 after implantation for a representative series. EX: day of excision. *$p < 0.05$, ZOL-treated compared to the control group.

Discussion

The present study focuses on the potential effect of ZOL on tumor progression and residual disease after intralesional curettage in a rat model of chondrosarcoma, and demonstrates for the first time that bisphosphonate has a potent beneficial effect *in vivo* on chondrosarcoma tumor progression. Most of the literature on chondrosarcoma has confirmed that adequate surgery is the mainstay of treatment for local control which itself is a risk factor for survival.[5,6,27,28] Despite different definitions of adequate margins, this goal can be achieved in only 46–76% of patients.[5–7,28] Adequate surgery means wide margins of normal tissue, that leads to severe disability, mainly when tumor occurs on spine or pelvis, when possible. On the other hand, inadequate surgery is an independent risk factor for local recurrence (as well as tumor size greater than 10 cm). Adjuvant treatment is of great interest to improve local control of this tumor when wide margin is not possible to obtain metastatic spreading and even to consider intralesional treatment of low grade chondrosarcoma. Radiation therapy is not effective in local control of the tumor[7,27,29] but can be used in exceptional situations delaying local recurrence probably only in patients who have minimum residual microscopic disease.[30] Chemotherapy is recommended in high risk chondrosarcoma and dedifferentiated chondrosarcoma,[6,31–33] but it is not administered according to a specific protocol and no controlled study is available. Cryosurgery after intralesional curettage is much promising in grade 1 chondrosarcoma, with at least similar results of marginal excision in term of oncological control and mostly better than those of wide excisions in terms of functional results.[34–36]

A vicious cycle has been described in osteolytic metastasis; tumor cells release osteolytic mediators and bone resorption release growth factors which enhance tumor cell growth and further release of osteolytic mediators.[9] One can speculate that this vicious cycle may also occur in the case of the primary bone tumor. Thus, previous study with RCS pointed out the role of bone microenvironment on tumor aggressiveness, interactions between

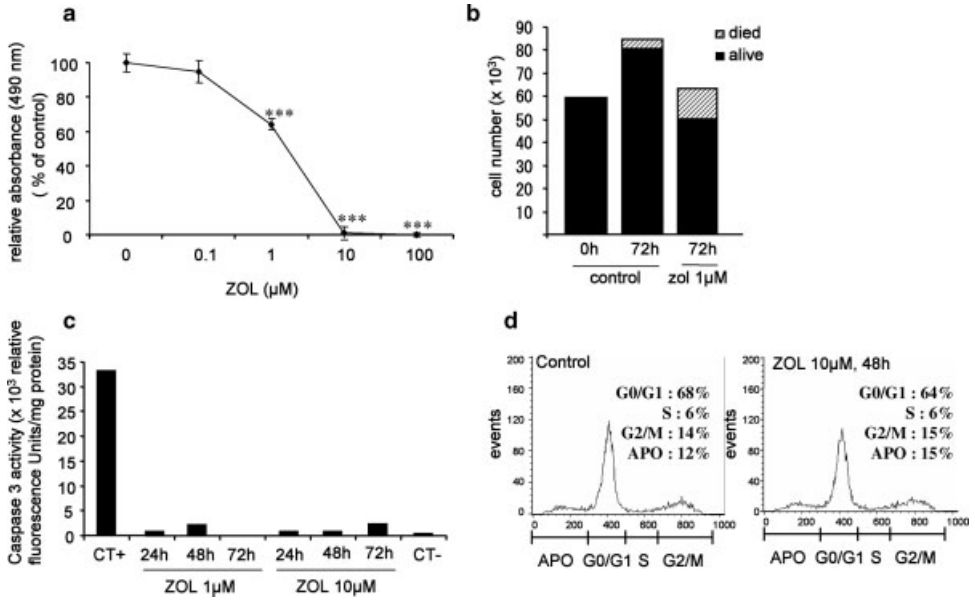

FIGURE 3 – ZOL decreases rat chondrosarcoma cell proliferation and induced cell death with no modification of cell cycle. *In vitro* effects of ZOL on chondrosarcoma cell proliferation, caspase activation and cell cycle. (*a*) Replicate subconfluent cell cultures in 96-multiwell plates were treated for 1–3 days with increasing concentrations of ZOL. Proliferation of chondrosarcoma cells was then determined after exposure to ZOL (0.1–100 μM) for 72 hr. (*b*) RCS cells were seeded at $\times 60\ 10^3$ cells/well in a 6-multiwell plate and cultured for 72 hr as described above, before being incubated with 1 μM ZOL. The alive and dead cell number (from trypsinized and floating cells) was determined after trypan blue exclusion on RCS cells treated or not treated with 1 μM ZOL for 72 hr and by manually counting. (*c*) Assess of caspase 3 activity of chondrosarcoma cells after 1 and 10 μM ZOL treatment for 24 to 72 hr. (*d*) Representative effect of ZOL on cell cycle of chondrosarcoma cells (10 μM, 48 hr), similar result was obtained for 24 hr and 1 μM ZOL (24–72 hr). $***p < 0.001$, ZOL-treated compared to the control cells. Each experiment was performed in triplicate, 3 times independently.

bone and tumor-induced bone remodelling and modification of the grade (grade II with foci of grade III, according to O'Neal and Ackerman grading) when tumor tissue is transplanted in close contact to the scarified bone.[26] Inhibitors of bone resorption such as BPs may interfere with primary tumor development at the skeletal site. To our knowledge, such hypothesis has never been tested *in vivo* and only one oral communication has been recently reported with chondrosarcoma.[37] RCS simulates the conditions of human chondrosarcoma development. It has been well characterized histologically, biochemically and structurally.[38,39] Kenan and Steiner[39] showed that RCS is a well-differentiated malignant tumor, histologically similar to well-differentiated human chondrosarcoma. In the present study, this RCS tumor tissue was transplanted in close contact to femur after mechanical scarifications to insure interaction between tumor progression and bone remodelling as previously described.[26] Human low grade chondrosarcomas induce 3–12% of metastasis appearing along time after diagnosis (mean 47 months).[5,6] As the present corresponds to a low grade differentiated chondrosarcoma (mostly grade II),[26] it is unsurprising that any metastasis was observed during this short experimental course (maximal 5 weeks postimplantation) with animals dying from local development of the tumor. Such condition is seldom observed in clinical situation.

In vivo results confirmed in all experiments of the present study, the efficacy of ZOL on RCS progression. On the other hand, ZOL treatment postponed local recurrence in treated group, but failed to prevent tumor recurrence after intralesional curettage. Because of extensive spreading of the tumor in the soft tissue, intralesional curettage in all cases simulated microscopic residual disease. Such situation is frequent in clinical conditions, and efficient medical adjuvant treatment such as bisphosphonate would be of greatest interest in potential microscopic residual disease, in order to reduce margins and improve functional results. Unfortunately, this condition is difficult to mimick with RCS. Indeed, intraosseous implantation, which could be a better model for an extensive curettage, leads to inconstant and small tumors in our experience. ZOL doses used in the present study are justified as the clinical dose (4 mg i.v. every 3–4 weeks) is equivalent to ~100 μg/kg of the research grade disodium salt used in this study. However, even if dosing frequency of twice a week is greater than in human, it could be justified by the aggressive nature of the chondrosarcoma.

Initially, it was thought that the specific inhibition of osteoclastic bone resorption is the only mechanism of action of BPs, by which they are effective in the treatment of cancer patients bearing bone metastasis. However, evidence is emerging from both preclinical and clinical studies to suggest that BPs also have direct antitumor properties that may contribute to their therapeutic efficacy in malignant bone disease.[40] Potential direct antitumor effect of pamidronate and clodronate have been suggested in 2 *in vitro* studies showing inhibition of osteosarcoma cell growth proliferation.[20,21] Morever, ZOL induced in a dose- and time-dependant decrease in cell proliferation in osteosarcoma cell lines *in vitro*, and reduced tumor progression and metastatic lung spreading *in vivo*.[23,24] The present study reports a significant decrease of chondrosarcoma cell proliferation and an increase of cell death at 1 and 10 μ*M in vitro* with an IC50 comparable to those observed on osteosarcoma and breast carcinoma cells.[1,19,24] ZOL appears to be a more potent inhibitor of cell proliferation compared to the other N-BPs.[40] Indeed, while several BPs exert antitumor activities, their effects seem to vary among the different compounds, in relation to their basic structure. The nitrogen-containing BPs such as ZOL have been suggested to be clinically superior to their first generation counterparts.[41] Moreover, the peak plasma levels of ZOL appear to be around 1 μM as shown by Skerjanec *et al.*[42] which is in agreement with the IC50 of ZOL measured on cancer cells, thus then strengthening an *in vivo* potential effect of ZOL even at a low concentration. ZOL has been reported to inhibit cell cycle progression and increase the proportion of cells arrested in S-phase in rat and human osteosarcoma,[21,23] that was not confirmed in the present study with RCS cells. This observation can be explained by the slow proliferation rate of the RCS cells as

shown in the control condition (Fig. 3*d*). Moreover, ZOL-induced RCS cell death is not mediated by activation of caspase 1 and 3 as in osteosarcoma cells.[21,22] Thus, our data support a cytotoxic effects of ZOL which could look like to an anoikis mechanism as already described in osteosarcoma cells.[21] However, the precise molecular mechanisms implicated in the ZOL-induced cell death need further investigation.

The present study demonstrated for the first time, that in addition to surgery, the therapy of chondrosarcoma with BPs might be beneficial. Such results open the way to the development of effective adjuvant treatment associated with surgical approach for treatment of chondrosarcoma.

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References

1. Lichtenstein L, Jaffe HL. Chondrosarcoma of bone. Am J Pathol 1942;19:553–74.
2. Dahlin DC, Unni KK. Bone tumors: general aspects and data on 8542 cases, 4th ed. Sprinfield, IL: Charles C Thomas, 1986.
3. Enneking WF. Musculoskeletal tumor surgery, vol. 2. New York: Churchill Livingstone, 1983. 875–997.
4. O'Neal LW, Ackerman LV. Chondrosarcoma of bone. Cancer 1952;5: 551–77.
5. Fiorenza F, Abudu A, Grimer RJ, Carter SR, Tillman RM, Ayoub K, Mangham DC, Davies AM. Risk factors for survival and local control in chondrosarcoma of bone. J Bone Joint Surg Br 2002;84:93–9.
6. Lee FY, Mankin HJ, Frondren G, Gebhardt MC, Springfield DS, Rosenberg AE, Jennings LC. Chondrosarcoma of bone: an assessment of outcome. J Bone Joint Surg Am 1999;81:326–38.
7. Pritchard DJ, Lunke RJ, Taylor WF, Dahlin DC, Medley BE. Chondrosarcoma: a clinicopathological and statistical analysis. Cancer 1980;45:149–57.
8. Donati D, El Ghoneimy A, Bertoni F, Di Bella C, Mercuri M. Surgical treatment and outcome of conventional pelvic chondosarcoma. J Bone Joint Surg Br 2005;87:1527–30.
9. Chirgwin JM, Guise TA. Molecular mechanisms of tumor-bone interactions in osteolytic metastases. Crit Rev Eukaryot Gene Expr 2000; 10:159–78.
10. Body J. Clinical research update: zoledronate. Cancer 1997;80:1699–1701.
11. Cheer S, Noble S. Zoledronic acid. Drugs 2001;61:799–805.
12. Gatti D, Adami S. New bisphosphonates in the treatment of bone diseases. Drugs Aging 1999;15:285–96.
13. Pataki A, Muller K, Green J, Ma Y, Li Q, Jee W. Effects of short-term treatment with the bisphosphonate zoledronate and pamidronate on rat bone: a comparative histomorphometric study on the cancellous bone formed before, during, and after treatment. Anat Rec 1997; 249:458–68.
14. Benford HL, McGowan NWA, Helfrich MH, Nuttall M, Roger MJ. Visualization of bisphosphonate-induced caspase-3 activity in apoptotic osteoclasts in vitro. Bone 2001;28:465–73.
15. Berenson J, Rosen L, Howell A, Porter L, Coleman R, Morley W, Dreicer R, Kuross S, Lipton A, Seaman J. Zoledronic acid reduces skeletal-related events in patients with osteolytic lesions. Cancer 2001;91:1191–1200.
16. Lipton A. Bisphosphonates and breast carcinoma. Cancer 2000;8:3033–7.
17. Saad F, Gleason DM, Murray R, Tchekmedyian S, Venner P, Lacombe L, Chin JL, Vinholes JJ, Goas JA, Zheng M. Zoledronic acid prostate cancer study group. Long term efficacy of zoledronic acid for the prevention of skeletal complications in patients with metastatic hormone-refractory prostate cancer. J Natl Cancer Inst 2004; 96:879–82.
18. Rosen LS, Gordon D, Tchekmedyian S, Yanagihara R, Hirsh V, Krzakowski M, Pawlicki M, de Souza P, Zheng M, Urbanowitz G, Reitsma D, Seaman JJ. Zoledronic acid versus placebo in the treatment of skeletal metastases in patients with lung cancer and other solid tumors: a phase III, double-blind, randomized trial—the zoledronic acid lung cancer and other solid tumors study group. J Clin Oncol 2003;2:3150–7.
19. Jagdev SP, Coleman RE, Shipman CM, Rostami -HA, Croucher PI. The bisphosphonate zoledronic acid induced apoptosis of breat cancer cells: evidence for synergy with paclitaxel. Br J Cancer 2001;84: 1126–34.
20. Mackie PS, Fisher JL, Zhou H, Choong PF. Bisphosphonates regulate cell growth and gene expression in the UMR 106–01 clonal rat osteosarcoma cell line. Br J Cancer 2001;84:951–8.
21. Sonnemann J, Eckervogt V, Truckenbrod B, Boos J, Winkelmann W, Van Valen F. The bisphosphonate pamidronate is a potent inhibitor of human osteosarcoma cell growth in vitro. Anticancer Drugs 2001; 12:459–65.
22. Evdokiou A, Labrinidis A, Bouralexis S, Hay S, Findlay DM. Induction of cell death of human osteogenic sarcoma cells by zoledronic acid resembles anoikis. Bone 2003;33:216–28.
23. Heymann D, Ory B, Blanchard F, Heymann MF, Coipeau P, Charrier C, Couillaud S, Thiery JP, Gouin F, Redini F. Enhanced tumor regression and tissue repair when zoledronic acid is combined with ifosfamide in rat osteosarcoma. Bone 2005;37:74–86.
24. Ory B, Heymann MF, Kamijo A, Gouin F, Heymann D, Rédini F. Zoledronic acid suppresses lung metastases and prolongs overall survival of osteosarcoma-bearing mice. Cancer 2005;104:2522–31.
25. Guerne PA, Lotz M. Interleukin-6 and transforming growth factor-b synergistically stimulate chondrosarcoma cell proliferation. J Cell Physiol 1991;149:117–24.
26. Grimaud E, Damiens C, Rousselle AV, Passuti N, Heymann D, Gouin F. Bone remodelling and tumor grade modifications induced by interactions between bone and swarm rat chondrosarcoma. Histol Histopathol 2002; 17:1103–11.
27. Gitelis S, Bertoni F, Picci P, Campannacci M. Chondrosarcoma of bone. The experience at the Instituto Ortopedico Rizzoli. J Bone Joint Surg Am 1981;63:1248–57.
28. Sheth DS, Yasko AW, Johnson ME, Ayala AG, Murray JA, Romsdahl MM. Chondrosarcoma of the pelvis. Prognostic factors for 67 patients treated with definitive surgery. Cancer 1996;78: 745–50.
29. Evans HL, Ayala AG, Romsdahl MM. Prognostic factors in chondrosarcoma of bone: a clinicopathologic analysis with emphasis on histologic grading. Cancer 1977;40:818–31.
30. Springfield DS, Gebhardt MC, McGuire MH. Chondrosarcoma: a review. Instr Course Lect 1996;45:417–24.
31. Anract P, Tomeno B, Forest M. Dedifferentiated chondrosarcoma. A study of 13 clinical cases and review of the literature. Rev Chir Orthop Reparatrice Appar Mot 1994;80:669–80.
32. Capanna R, Bertoni F, Betteli G, Picca P, Bacchini P, Present D, Guinti A, Campanacci M. Dedifferentiated chondrosarcoma. J Bone Joint Surg Am 1988;70:60–9.
33. Frassica FJ, Unni KK, Beabout JW, Sim FH. Dedifferentiated chondrosarcoma. A report of the clinico-pathological features and treatment of seventy-eight cases. J Bone Joint Surg Am 1986;68:1197–205.
34. Mittermayer F, Dominkus M, Krepler P, Schwameis E, Sluga M, Toma C, Lang S, Grampp S, Kotz R. Chondrosarcoma of the hand: is a wide surgical resection necessary? Clin Orthop Relat Res 2004; 424:211–5.
35. Van Der Geest IC, Servaes P, Schreuder HW, Bleijenberg G, Verhagen CA, Pruszczynski M, Lemmens JA, Veth RP. Chondrosarcoma of bone: functional outcome and quality of life. J Surg Oncol 2002; 81:70–4.
36. Veth R, Schreuder B, van Beem H, Pruszczynski M, de Rooy J. Cryosurgery in aggressive, benign, an low-grade malignant bone tumors. Lancet Oncol 2005;6:25–34.
37. Streibuerger A, Hardes J, Ahrens H, Gebert C, Boos J, Winkelmann W, Gosheger G. In vitro trial: cytotoxic effect of bisphosphonates on the chondrosarcoma cell line CAL 78. In: Eighteenth annual meeting of european musculoskeletal oncology society, Trieste, Italy, 2005. C24.
38. Breitkreutz D, Diaz de Leon L, Paglia L, Gay S. Swarm RL, Stern R. Histological and biochemical studies of a transplantable rat chondrosarcoma. Cancer Res 1979;39:5093–5100.
39. Kenan S, Steiner GC. Experimental transplantation of the swarm rat chondrosarcoma into bone: radiological and pathological studies. J Orthop Res 1991;9:445–51.
40. Heymann D, Ory B, Gouin F, Green JR, Rédini F. Bisphosphonates: new therapeutic agents for the treatment of bone tumors. Trends Mol Med 2004;10:337–43.
41. Sietsema WK. Antiresorptive dose-response relations across three generations of bisphosphonates. Drugs Exp Clin Res 1989;15: 389–96.
42. Skerjanec A, Berenson J, Hsu C, Major P, Miller WH, Jr, Ravera C, Schran H, Seaman J, Waldmeier F. The pharmacokinetics and pharmacodynamics of zoledronic acid in cancer patient with varying degrees of renal function. J Clin Pharmacol 2003;43: 154–62.

Complément de discussion à l'article 5

Cette étude montre pour la première fois un effet bénéfique potentiel *in vivo* du Zol sur la progression tumorale du chondrosarcome. La littérature est unanime sur le fait qu'une exérèse chirurgicale est la base du traitement local du chondrosarcome mais qu'elle est en même temps à l'origine d'une morbidité et d'une mortalité importantes pour le patient (Lee et al. 1999; Fiorenza et al. 2002). En effet, malgré une bonne définition de la résection, l'opération n'est un succès que pour 46 à 76% des patients selon les séries (Sheth et al. 1996; Lee et al. 1999; Fiorenza et al. 2002). La résection devant être large et comporter du tissu sain conduit à de sévères invalidités. Les traitements adjuvants sont d'un grand intérêt dans l'amélioration du traitement de ces tumeurs pour lesquelles la radiothérapie est inefficace (Pritchard et al. 1980; Gitelis et al. 1981) et la chimiothérapie peut convaincante. Un cercle vicieux peut s'instaurer entre la prolifération des cellules tumorales en site osseux et la résorption osseuse conséquente de l'activité ostéoclastique. Il est tout à fait envisageable qu'un tel cercle vicieux puisse exister lors du développement d'un chondrosarcome, en effet, des études antérieures ont mis en évidence les relations étroites existantes entre le microenvironnement osseux et l'agressivité de la tumeur (Grimaud et al. 2002). Des inhibiteurs de la résorption osseuse tels que les bisphosphonates pourraient alors interférer avec le développement de la tumeur primaire au niveau de l'os. Les ressources bibliographiques sont très pauvres concernant l'utilisation des BPs dans le traitement du chondrosarcome et cette étude est la première à tester cette hypothèse *in vivo*. Le modèle animal utilisé est très similaire au chondrosarcome humain différencié, aussi bien du point de vue histologique, biochimique que morphologique. La transplantation tumorale est effectuée au contact du fémur après l'avoir légèrement scarifié afin d'induire une réaction périostée favorable au développement tumoral.

Les résultats *in vivo* confirment l'efficacité du Zol sur la progression du chondrosarcome, cependant, le traitement par le Zol retarde la récidive locale dans le groupe traité mais ne parvient pas à prévenir totalement celle-ci après le curetage intra lésionnel. Le développement de la tumeur s'étendant aux tissus mous, le curetage intra lésionnel induit l'apparition de microscopiques foyers tumoraux. Le Zol pourrait être un bon moyen d'éviter cet écueil, malheureusement ces conditions sont difficiles à mimer avec le modèle utilisé. Une implantation intra osseuse pourrait être un meilleur modèle.

Les résultats *in vitro* démontrent une activité anti-prolifération du Zol sur les cellules de chondrosarcome pour des concentrations comparables à celles utilisées pour le traitement des cellules d'ostéosarcome. L'étude du cycle cellulaire ne nous a pas permis d'observer une influence du Zol sur la répartition des cellules dans les différentes phases du cycle, contrairement à ce que nous avons pu observer pour les cellules d'ostéosarcome. Ceci peut être expliqué par le fait que les cellules de chondrosarcome de rat utilisées dans cette étude ont un faible taux de prolifération ce qui ne permet pas dans les conditions expérimentales d'observer une modification significative de la répartition de cellules dans les différentes phases du cycle. De même que pour les ostéosarcomes, le Zol induit la mort des cellules de chondrosarcome par un mécanisme indépendant des caspases 1 et 3.

Cette étude démontre pour la première fois l'intérêt thérapeutique du Zol, en plus de l'intervention chirurgicale, dans le traitement du chondrosarcome, ouvrant ainsi la voie au développement de nouveaux traitements adjuvant associés à l'acte chirurgical.

Introduction

L'adénocarcinome prostatique est le deuxième cancer le plus fréquent chez l'homme en Amérique du nord, il est associé à une forte mortalité en raison du développement de métastases osseuses chez environ 80 à 100% des patients (Goltzman 1997; Carlin et al. 2000). Par conséquent, de nouvelles approches thérapeutiques sont nécessaires afin d'améliorer le pronostique de cette pathologie. Une particularité du cancer de la prostate est que les métastases osseuses sont dans la pluspart des cas ostéocondensantes contrairement à celles du myélome ou du cancer du sein qui présentent un profil radiologique ostéolytique (Shimazaki et al. 1992). Pour étudier de nouvelles cibles thérapeutiques, il est nécessaire de disposer d'un modèle expérimental qui reproduit la lésion osseuse ostéocondensante, malheureusement il existe peu de modèles précliniques reproduisant ce phénomène.

Les modèles animaux sont des outils importants pour le développement de stratégies thérapeutiques. Etant donné que les métastases osseuses spontanées sont rares chez les animaux, les modèles doivent être développés expérimentalement. Des modèles animaux ont déjà été développés utilisant divers techniques telles que des xénogreffes de tumeurs humaines sur des souris ou des rats immunodéficients, des inductions chimiques, des transplantations syngéniques (Tennant et al. 2000; Zhau et al. 2000). De tous ces modèles seuls quelques uns miment les métastases ostéocondensantes, et ce sont tous des modèles utilisant l'injection intra osseuse de cellules de carcinome prostatique (Corey et al. 2002). Dans cette étude nous décrivons un nouveau modèle de lésions ostéocondensantes induites par l'injection de cellules d'adénocarcinome prostatique de rat AT6-1 (Ke et al. 1998), , dans la cavité intramédulaire du fémur. La cinétique et l'intensité des lésions ostéocondensantes ainsi que l'apparition de métastases pulmonaires sont contrôlées par le nombre de cellules injectées. L'analyse radiologique a permis de confirmer la similarité avec la pathologie humaine et ainsi de légitimer son utilisation pour l'étude des effets du Zol en tant qu'agent thérapeutique dans le cadre de cette pathologie à la fois ostéolytique et ostéocondensante. Le Zol à démontré son intérêt thérapeutique en tant que traitement adjuvant dans des études précliniques (Lee et al. 2001; Corey et al. 2003) et cliniques (Saad et al. 2002; Michaelson et al. 2006) de métastases osseuses secondaires à un carcinome prostatique.

Article 6

« Intérêt thérapeutique de l'acide zolédronique dans le traitement des lésions ostéoblastiques secondaires à un carcinome prostatique chez le rat »

Int J of Cancer, in press**,** Lamoureux F, Ory B, Battaglia S, Pilet P, Heymann MF, Gouin F, Duteille F, Heymann D, Redini F

L'adénocarcinome prostatique est un cancer de l'homme vieillissant, 85 % des cas étant diagnostiqués chez l'homme de plus de 60 ans. Il est associé, dans la majorité des cas, au développement de métastases osseuses majoritairement ostéocondensantes, responsables des taux élevés de mortalité chez les patients. C'est pourquoi, il est nécessaire de développer de nouvelles stratégies thérapeutiques. Cependant, l'absence de modèles animaux reproduisant ce type de lésions osseuses rend cette recherche difficile. Nous avons développé un nouveau modèle expérimental de lésions ostéoblastiques comparables à la pathologie humaine, induites par l'injection de cellules tumorales prostatiques de rat AT6-1 dans la cavité médullaire de fémurs de rats Sprague-Dawley. *In vitro*, les cellules AT6-1 sont caractérisées au niveau phénotypique par l'expression de marqueurs ostéoblastiques (phosphatase alcaline, Cbfa1, collagène de type I, ostéocalcine et bone sialoprotein) et ostéoclastiques (TRAP, cathepsine K, récepteur à la calcitonine), ainsi que par la mise en évidence de nodules de minéralisation. Cependant aucune induction de l'activité phosphatase alcaline n'est démontrée *in vitro*. Suite à l'injection intra fémorale de cellules AT6-1, une désorganisation trabéculaire ainsi qu'une dégradation de la corticale sont observées, dont l'intensité peut être corrélée avec le nombre de cellules injectées. De plus, des analyses par radiologie, micro-scanner et microscopie électronique à balayage révèlent que la tumeur est calcifiée, avec induction de formation minérale par les cellules tumorales elles-mêmes, phénomène superposable aux lésions ostéocondensantes observées chez les patients atteints d'adénocarcinome prostatique.

Dans le cadre du remodelage osseux, la formation osseuse étant toujours précédée d'une phase de résorption osseuse, l'utilisation thérapeutique d'un agent anti-résorption osseuse comme l'acide zolédronique (ZOL), se trouve justifiée. L'objectif de ce travail a été d'étudier l'effet du ZOL dans le modèle AT6.1 de lésions ostéoblastiques afin d'évaluer son éventuelle capacité thérapeutique dans le traitement des métastases ostéoblastiques secondaires à un carcinome prostatique. Les rats ont été traités dès trois jours post-injection des cellules tumorales avec 100 µg/kg ZOL deux fois par semaine pendant 6 semaines. Les

rats traités par ZOL montrent une prévention significative des lésions osseuses induites par les cellules de carcinome prostatique, associée à une inhibition du développement tumoral comparativement aux animaux contrôles. Des études réalisées *in vitro* sur les cellules AT6.1 à l'origine de ce modèle révèlent que le ZOL exerce une action directe sur les cellules tumorales en inhibant leur prolifération par un mécanisme indépendant de la caspase-3 et induisant un arrêt du cycle cellulaire. Ces résultats démontrent l'intérêt thérapeutique du bisphosphonate ZOL dans l'indication des lésions ostéoblastiques secondaires à un adénocarcinome prostatique, en tant que molécule bi-fonctionnelle inhibant à la fois la formation des lésions osseuses et la prolifération des cellules tumorales.

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Relevance of a new rat model of osteoblastic metastases from prostate carcinoma for preclinical studies using zoledronic acid

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Animal models that mimic osteoblastic metastases associated with prostate carcinoma are required to improve the therapeutic options in humans. A new model was then developed and characterized in immunocompetent rats. The bisphosphonate zoledronic acid (ZOL) was tested to validate this model as a therapeutic application. Rat AT6-1 prostate tumor cells were characterized *in vitro* at the transcriptional (bone and epithelial markers) and functional (induction of mineralized nodules) levels. The bone lesions induced after their direct injection into the femur bone marrow were characterized by radiography, microscanner and histology analyses. ZOL effects were studied *in vivo* on bone lesion development and *in vitro* on AT6-1 cell proliferation, apoptosis and cell cycle analysis. Apart from epithelial markers, AT6-1 cells express an osteoblast phenotype as they express osteoblastic markers and are able to induce mineralized nodule formation in vitro. A disorganization of the trabecular bone at the growth zone level was observed *in vivo* after intraosseous AT6-1 cell injection as well as cortical erosion. The tumor itself is associated with bone formation as revealed by SEM analysis and polarized light microscopy. ZOL prevents the development of such osteoblastic lesions, related to a direct inhibitory effect on tumor cell proliferation independent of caspase 3 activation, but associated with cell cycle arrest. A new rat model of osteoblastic bone metastases was validated in immunocompetent rats and used to show the relevance of using ZOL in such lesions, as this compound shows bifunctional effects on both bone remodelling and tumor cell proliferation.

Key words: prostate carcinoma; osteoblastic metastases; experimental model; zoledronic acid

Prostate adenocarcinoma, the second leading cause of cancer in men of North America is associated with significant morbidity and mortality due to skeletal metastases that commonly occur in advanced disease, affecting 80–100% of patients.[1,2] Therefore, new therapeutic strategies are needed to improve the prostate cancer patient's outcome. In contrast to bone metastases of breast cancer and myeloma, which are mainly osteolytic, a high percentage of prostate cancer metastases exhibit the radiographic appearance of osteoblastic lesions.[3] To investigate new targets for the development of novel therapies, experimental models that reproduce these osteoblastic bone lesions are required. However, there is a paucity of good preclinical models to study osteoblastic lesions.

Animal models are important tools to investigate the pathogenesis and develop treatment strategies for bone metastases in humans. Because spontaneous bone metastasis in animals is uncommon, most animal models of bone metastasis must be derived experimentally. Experimental models of osteolytic, osteoblastic and mixed osteolytic/osteoblastic bone metastases include syngeneic transplantation of spontaneous occurring rodent cancers, chemical induction of cancers in selected strains of rats and mice, newly developed transgenic mouse models and xenografts of human tumors or cell lines derived from human cancers into immunodeficient rodents (*e.g.*, nude mice and rats, and severely compromised immunodeficient SCID mice).[4–7] Among these models, only a few mimic osteoblastic or mixed osteolytic/osteoblastic metastases, being all induced by intraosseous injections of prostate carcinoma cells.[8] For example, the PA-III cell line derived from a spontaneous prostate adenocarcinoma in a Lobund Wistar rat induced both osteolytic and osteoblastic lesions when transplanted adjacent to bone.[9] This tumor cell line may be useful to investigate the ability of prostate carcinoma to induce new woven bone formation. More recently, Liepe *et al.* investigated a new model of osteoblastic lesions in rats, induced by a direct intraosseous injection of R-3327 prostate cancer cells leading to osteoblastic lesions and no lung metastases.[10]

In the present article, we describe a new model of osteoblastic lesions induced by the direct injection of AT6-1 rat prostate adenocarcinoma cells in the femoral medullar cavity of immunocompetent rats. The rat androgen-independent AT6-1 prostatic cell line is derived from the original Dunning R3327 rat prostatic carcinoma exhibiting high metastatic phenotypes when grown in syngenic Copenhagen rats.[11] According to the cell number injected, we are able to control the kinetic of osteoblastic lesion development with pulmonary metastases for a high number of cells injected. Its reliability to human pathology has been confirmed by radiographic analysis, together with its relevance in therapeutic assays using zoledronic acid (ZOL), a bisphosphonate containing nitrogen atoms (N-BP). Bisphosphonates are currently the most important class of inhibitors of osteoclast-mediated bone resorption that are widely and successfully used for the treatment of skeletal diseases, from tumor origin or not such as skeletal complications from tumors that metastasize to bone (reviewed in Ref. 12). In the past, this compound has demonstrated therapeutic interest as adjuvant treatment both in preclinical[13,14] and clinical[15,16] settings of bone metastases secondary to prostate carcinoma. Among these studies, Corey *et al.* demonstrated that ZOL was able to inhibit the growth of not only osteolytic but also osteoblastic metastases *in vivo*.[14] Therefore, this model of osteoblastic lesions secondary to prostate carcinoma was validated for therapeutic application using ZOL.

Abbreviations: ALP, alkaline phosphatase; BSP, bone sialoprotein; CT, computed tomography; EDAX, energy dispersive X-ray microanalysis; GMA: glycolmethylmethacrylate; OC, osteocalcin; OP, osteopontin; PAP, prostatic acid phosphatase; PSA, prostate specific antigen; SEM, scan electron microscopy; ZOL, zoledronic acid.

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TABLE I – OLIGONUCLEOTIDE PRIMERS USED FOR RT-PCR

Primer's name	5′ forward 3′ 3′ reverse 5′	Product size	Tm	Cycle numbers
ALP	cctttgtggctctctccaag ctggccttctcatccagttc	451	60	40
BSP	atggagatggcgatagttcg gttccttctgcacctgcttc	439	58	40
OC	catgaggaccctctctctgc cctaaacggtggtgccatag	302	58	40
Coll I	atgaccaggttcacctttcg aagaggcgagagaggtttcc	469	60	30
Cbfa1	ggaccgtggttaccgtcat atgcgccctaaatcactgag	304	60	30
Cytokeratin 18	ttgagttgctccatctgcac attgccagttctgggttgac	335	60	30
E-cadherin	tgccccattcgttcagata accggaagtgattcgaaatg	263	60	30
18 S	tcaagaacgaaagtcggaggttcg ttattgctcaatctcgggtggctg	462	62	27

Primers are presented in a 5′ to 3′ orientation for the coding strand (forward) and 3′ to 5′ for the non coding strand (reverse). The product size generated by RT-PCR is indicated together with the experimental conditions used (Tm and cycle number corresponding to the linear part of the amplification curve used to quantify the messages *versus* the 18S signal, determined in the same way). All the primers are against rat molecules.

Material and methods

Cell lines

The prostate carcinoma cell line AT6-1 was kindly provided by Dr. J.-P. Thiéry (Villejuif, France; from European Collection of Cell Cultures) and cultured on plastic surface in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, Hyclone, Perbio, France). AT6-1 cells were compared to DU-145 cells (American Type Culture Collection, USA), derived from a human prostate carcinoma that induces osteolytic bone lesions when injected in the tibial metaphysis of immunosuppressed rats.[17]

RT-PCR analysis

Total RNA was isolated from cultured AT6-1 and DU 145 cells using TRIzol reagent (Invitrogen, Eragny, France). Total RNA were quantified by measuring the OD_{260} and integrity was checked by 1% agarose/formaldehyde gel electrophoresis. First, RNA was reversed-transcribed (RT), using 400 U MMLV-RT from Invitrogen, then 2 µl of the RT reaction mixture were subjected to PCR using 0.25 µl of 5 U/µl Taq polymerase (Eurobio, Les Ulis, France) and upstream and downstream primers (30 pmoles each, Table I) to determine the expression of rat epithelial markers [endothelin-1, PAP (prostatic acid phosphatase), cytokeratin 18, cadherin-E and prostate specific antigen (PSA)] and bone markers [Alkaline Phosphatase (ALP), Osteopontin (OP), Osteocalcin (OC), Bone Sialoprotein (BSP), Cbfa1]. After the number of PCR cycles was increased, a plot was done for each sample, the cycle values corresponding to the linear part of the amplification curve were determined (see Table I) and used to quantify the messages *versus* the 18S signal determined in the same way. The PCR products were electrophoresed in a 1% agarose gel containing ethidium bromide. The band densities were measured using the ImageQaNT computer software program (Molecular Dynamics). Three independent experiments were performed for each gene and a representative experiment is shown in the Results section.

Confocal microscopy analysis

Cytokeratin 18 expression was determined by confocal analysis. AT6-1 and H35 cells (2×10^4) were grown on glass during 48 hr. Rat H35 cells are used as epithelial positive control cells. AT6-1 cell morphology was similar when cultured on glass or plastic surface as observed by optic microscopy. After washing, the cells were fixed in methanol (3 min at 20°C) and incubated with primary polyclonal anticytokeratin 18 (1/50, Santa Cruz biotechnology, Heidelberg, Germany) in PBS/1% bovine serum albumin/0.1% Triton for 1 hr, washed, incubated with secondary biotinyled antibody (anti-mouse, diluted 1:300; Sigma) for 45 min, then with Streptavidin Exafluor 488 (diluted 1:500; Molecular Probes) for 60 min and washed. Coverglass fitting was achieved with the Long Pro Kit (Invitrogen). Images were collected on a TCS-SP1 confocal microscope (Leica, Wetzlar, Deutchland) with 63/1.4 oil immersion lens. The digital images were visualized with a 24-bit imaging system, including TCS-NT software (Leica), and projections were generated from stacks.

Mineralization assay

Mineralization assays were performed as previously described.[18] Briefly, bone marrow (positive control) cells and AT6-1 cells were seeded in a 24-well plate at a the density of respectively 2×10^4 cells and 500 cells/well, in DMEM medium supplemented with 10% FBS, in the absence or the presence of 50 µg/ml ascorbic acid (Sigma) and 10^{-8} M dexamethasone (Sigma). The medium was changed twice a week. After 7 days of culture, 10 mM of Na-β-glycerophosphate (Sigma) were added into each well in association with ascorbic acid and dexamethasone, and maintained in culture for 7 days. At the end of the incubation period, supernatant was removed and ice-cold ethanol was added into the wells for 1 hr. The cells were then washed twice with distilled water and the presence of mineralized nodes was revealed by microscopic observation (Leica, DM IRB) by a 40 mM alizarin red staining solution, pH 7.4.

Protein quantification and alkaline phosphatase assays

AT6-1 cells were seeded in a 6-well plate at the density of 10^5 cells/well and cultured as described earlier (§ Cell lines). After 72 hr of culture, AT6-1 cells were washed with PBS and lysed in ice-cold buffer [NaCl 150 mM, Tris 50 mM, Nonidet P-40 1%, sodium deoxycholate 0.25%, NaF 1 mM, leupeptine 10 mg/ml, aprotinin 10 mg/ml, phenymethylsulfonylfluoride (PMSF) 0.5 mM and glycerol 10%]. Total amount of proteins for each sample was determined using a bicinchominic acid (BCA; Sigma) based method. Ten microliters of cellular lysis or standard BSA solution were added to 200 µl of reagent (Copper II solution 1/50 diluted in bicinchominic acid) and incubated 30 min at 37°C. OD was determined at 570 nm as well as the protein concentration compared to the standard curve. The same amounts of proteins were used to perform alkaline phosphatase assays using Enzyline PAL kit according to the supplier's recommendations (BioMérieux, Marcy

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l'Etoile, France). At the end of the incubation period (10 min at room temperature), the reaction was stopped and OD was determined at 405 nm using a Wallac 1420 VICTOR 2^{TM} multilabel counter.

Cell proliferation

Replicate subconfluent cell cultures of AT6-1 cells in 96-well plates were treated for 12–72 hr with increasing concentrations of ZOL (kindly provided by Novartis Pharma, Bâle, Switzerland). Cell viability was determined by the sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) cell proliferation reagent assay kit (Roche Molecular Biomedicals, Mannheim, Germany).

Caspase activity

AT6-1 cells (2×10^4) grown in 24-well plates were treated with 50 μM ZOL for the indicated times, washed once with PBS and lysed with 50 μl of RIPA buffer for 30 min in the presence of protease inhibitors. The cells were then scraped off and the protein amount was quantified using the BCA based method as described. Caspase-3 activity was assessed on 10 μl of cell lysate with the CaspACE^{TM} assay kit (Promega, Madison, WI) following the manufacturer's recommendations. Cells treated with UV light for 30 sec, 24 hr before harvesting were used as a positive control for caspase activity.

Cell cycle analysis

Confluent AT6-1 cells (treated with 10 μM ZOL for 24 and 48 hr) were removed from culture dishes by trypsinisation, washed twice in PBS and incubated in PBS containing 0.12% Triton X-100, 0.12 mM EDTA and 100 μg/ml DNase-free ribonuclease A (Sigma). Then 50 μg/ml propidium iodide (Sigma) were added for each sample for 20 min at 4°C in the dark. The stained nuclei were analyzed by flow cytometry (FACScan, BD Biosciences) using the CellQuest software. Cell cycle distribution was based on 2*N* and 4*N* DNA content.

Experimental model

Four-week-old male Sprague Dawley rats (IFFA-CREDO, L'Arbresle, France) were housed under pathogen-free conditions at the Experimental Therapy Unit (Medicine Faculty of Nantes, France) in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators. Animals were anesthetized with isoflurane (2% in air atmosphere, flow rate of 2 l/min) associated with a s.c. injection of buprenorphine (0.05 mg/kg; Temgésic®, Schering-Plough, Levallois-Perret, France), and the distal femoral medullar cavity was injected with 50 μl of either alginate solution alone (1.2%, left femur) or alginate solution containing $2–10 \times 10^6$ tumor cells (right femur).

Experimental protocol

To determine the effect of ZOL, the rats were injected in the medullar cavity of the femora with 6×10^6 AT6-1 cells as described earlier. Groups of 8 rats were assigned respectively as controls (PBS) or ZOL (100 μg/kg s.c. twice a week, beginning at Day 3 after AT6-1 tumor cell injection). Twice a week, the animals were weighed and the tumor development was followed by clinical observations. Treatment continued until each animal showed signs of morbidity, which included cachexia or respiratory distress, at which point they were sacrificed by cervical dislocation. After the rats had been sacrificed and autopsied, the tumor-bearing femora were kept for histological studies and microarchitectural parameter quantification. Three independent experiments were performed.

Radiographic analysis

Once a week, animals were anesthesized (50 mg/kg, Nesdonal®, Merial, Lyon, France) and a flat plate radiograph was taken each week after tumor cell injection and at the time of sacrifice with a diagnostic mammography PLANMED Sophie apparatus (SN RAH 40710, Helsinki, Finland).

Quantitative computed tomography analysis

After sacrifice, cortical and trabecular bone mineral density as well as cortical geometry in rat tibiae were determined by peripheral quantitative CT (pQCT) on a SkyScan-1072 (SkyScan, Aartselaar, Belgium). The pixel size of 19.20 μm was chosen for the measurement. An identical bone portion in the femur was analyzed. Parameters of bone mineral density end points measured by pQCT were total crosssectional bone mineral density, volumetric trabecular bone mineral density, volumetric cortical bone mineral density (in mg/cm^3 for all), and mean cortical thickness (in millimetres), presented as relative volume (BV/TV) of the femora injected with AT6-1 tumor cells.

Scanning electron microscopy

At time of autopsy, tumor and corresponding bone samples were fixed in 10% phosphate-buffered formaldehyde (pH 7.4) for 2 weeks. Samples were then dehydrated through increasing ethanol gradients and embedded in glycolmethylmethacrylate (GMA). GMA-embedded samples were polished, gold-palladium-coated, and observed by Scanning Electron Microscopy (SEM) with back-scattered electrons (Jeol JSM-6300, Tokyo, Japan).

Histological analyses

Specimens were fixed in 10% buffered formaldehyde. Undecalcified tumor was embedded in GMA as previously described.[19] Briefly, tissue was dehydrated in acetone and infiltrated at −20°C for 2×4 days in an anhydrous mixture of MMA and polyethylene glycol 400, with benzoyle peroxide/N,N-dimethylanilin as catalyser-initiator. Then, the specimen was placed in fresh mixture of GMA and polymerized at 4°C in plastic molds during 3 days. Undecalcified sections of 80 μm were done using an Isomet low speed saw (Buehler, Lake Bluff, Il). For polarized light microscopy, unstained sections were observed with a Leica DMRX microscope (Leica) using 2 orthogonaly polarized light waves transversing the specimen. Decalcified tissues were embedded in paraffin and 4-mm sections were mounted on glass slides for routine Masson Trichrome staining.

Results

In vitro *characterization of AT6-1 cells*

Confirmation of epithelial phenotype. Endothelin-1, ACPP (prostatic acid phosphatase), cadherin-E, cytokeratin 18 and prostate specific antigen (PSA) were studied to confirm the epithelial character of the AT6-1 prostate adenocarcinoma cells. Among these markers, the expression of cytokeratin 18 could be only evidenced, H35 rat liver cells being used as positive control epithelial cells (Fig. 1*a*). Its expression was then studied and confirmed at the protein level by confocal microscopy (Fig. 1*b*). Fi

Bone markers. Prostate carcinoma AT6-1 cells were studied for their ability to express osteoblastic markers (ALP, BSP, type I collagen, OC, Cbfa1), as bone metastases secondary to prostate cancer exhibit osteoblastic lesions. The phenotype of these cells was compared with human DU145 cells, described as osteolytic bone metastases. Results presented in Figure 1*c* show that AT6-1 cells present a totally different gene expressing profile compared to the DU 145 cells. Indeed, AT6-1 cells express all the osteoblastic markers studied while the DU145 cells do not (Fig. 1*c*).

Functionality in vitro. In view of the gene expression profile previously observed, AT6-1 cells were assessed for their capacity to demonstrate alkaline phosphatase activity and to induce mineralization nodules *in vitro*. AT6-1 cells do not demonstrate any alkaline phosphatase activity as compared to primary osteoblasts

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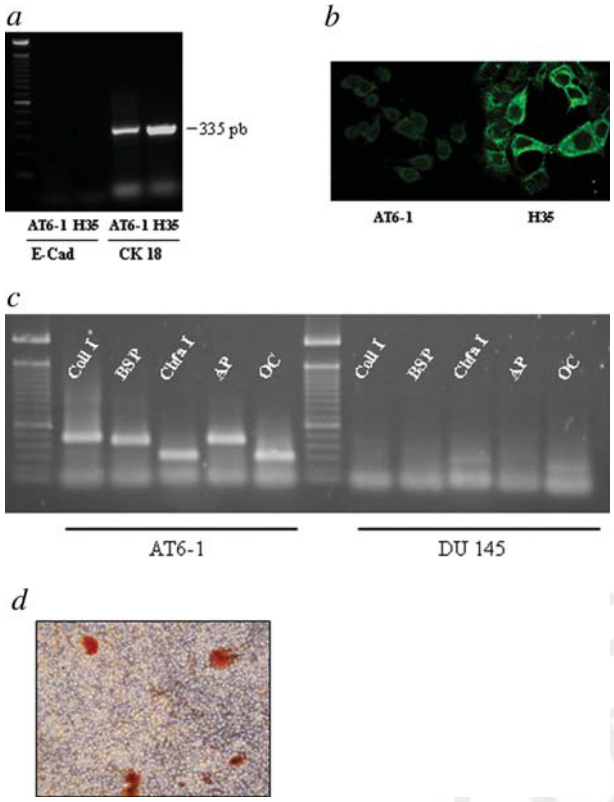

FIGURE 1 – *In vitro* characterization of AT6-1 cells. (*a*, *b*) Confirmation of epithelial marker expression by AT-61 cells. AT6-1 cells express cytokeratin 18 at the transcriptional (*a*; RT-PCR) and protein (*b*; confocal analysis) levels. The rat epithelial H35 cells were used as positive controls. (*c*) AT6.1 cells express osteoblastic marker as analyzed by RT-PCR as compared to the osteolytic DU145 cells. ALP, alkaline phosphatase; BSP, bone sialoprotein; coll I, type I collagen; OC, osteocalcin; Cbfa1, Core binding factor a1. (*d*) AT6-1 cells induced mineralized nodule formation. AT6-1 cells are cultured 21 days in a medium containing 50 μg/ml ascorbic acid, 10 mM Na β-glycerophosphate, 10^{-8} M dexamethasone. Typical alizarin red-S^{+} nodules are observed in phase contrast microscopy. Magnification ×100.

(not shown), though these cells express ALP at the transcriptional level (Fig. 1*c*). Bone marrow cells (BMC) isolated from C3H mice were used as positive controls for mineralization nodule tests, as they contain osteoblastic precursor cells that can differentiate into mature osteoblasts when cultured with dexamethasone. Both AT6-1 cells and BMC were incubated with ascorbic acid, dexamethasone and Na-β-glycerophosphate or not. After 14 days of culture, the results of alizarin red staining revealed that AT6-1 cells are able to induce mineralized nodule formation in the presence of β-glycerophosphate (Fig. 1*d*).

AT6-1 cells induce bone formation in vivo

The bone lesions induced by the injection of AT6-1 cells in the medullar cavity of the rat femora were characterized by complementary methods: histology, polarized light microscopy and scan electron microscopy.

Histology: Masson trichrome staining. Slices of the tumor that develops first inside the bone then in the neighbouring soft tissues 4–6 weeks after AT6-1 cell injection were analyzed by Masson trichrome staining to distinguish collagen fibres and the formation of calcified tissue (Fig. 2*a*). The presence of collagen fibres was evidenced within the tumor together with areas of calcified tissue, whereas only collagen fibres were detected in pulmonary metastases (Fig. 2*a*).

Polarized light microscopy. To evaluate the presence of woven bone tissue in calcified areas of tumor tissue, polarized light microscopy was performed. In regular light, the character of the substance composing the tumor could be described as fibrous resembling natural woven bone. However, only the presence of osteocytes could confirm this suggestion. By high magnification, bundles of collagen fibres are detected. Using the quartz plate red first order as compensator, the bundle of collagen fibres are clearly identified by their blue color (Fig. 2*b*).

Scan electron microscopy and energy dispersive X-ray microanalysis. Calcified material is accumulated in different areas of the AT6-1 cells induced tumor (Fig. 2*c*). Energy dispersive X-ray microanalysis (EDAX) was carried out to determine the element content in these calcified areas. Mineral deposits that were frequently observed in areas resembling bone-like tissue features contain calcium and phosphorus as demonstrated by characteristic EDAX patterns (Fig. 2*d*). The calculation of the ratio Ca/P revealed that these elements are present in a 1.64 ratio, representative of hydroxyapatite crystals. Therefore, this technique enables to reveal that calcified material observed in AT6-1 tumors is mineralized tissue characterized by the presence of hydroxyapatite crystals.

In vivo *behavior of AT6-1 cells as compared to osteolytic DU145 cells*

Radiographic analysis. Representative radiographs of AT6-1 and DU145 tumors in rat femora are compared with bone metastatic lesions observed in a patient bearing prostate carcinoma (Figs. 3*a* and 3*b*). The same number of AT6-1 and DU145 cells (6×10^6 cells) was injected into the intramedullary canal of rat femora. The centrifuged tumor cells were resuspended in an alginate viscous solution (1.2% in Hepes buffer) that facilitates the injection of tumor cells inside the medullar cavity of the femora. DU145 tumors show extensive osteolytic lesions with cortical degradation and trabecular disappearance as at the metaphysis/epiphysis of distal femur compared to the contralateral femur (Fig. 4*a*). In the AT6-1 tumor model, an alternation of osteolytic and osteoblastic lesions is observed, with a structural disorganization of the tissue which may lead to the loss of its mechanical properties and degenerate into fractures. These characteristics can be superimposed to those observed in prostate carcinoma patients bearing bone metastatic lesions (Fig. 3*b*).

Microarchitectural analysis. The same characteristic lesions were confirmed at the microarchitectural level by microcomputed tomography analysis (Fig. 4*a*): DU145 tumors show osteolytic lesions with cortical degradation, whereas AT6-1 cell injection induces a complete disorganization of both the cortical and cancellous bone structures, and the presence of bone formation is clearly evidenced. Three independent series of rats were injected with AT6-1 cells in the distal right femora, the number ranging from 2 to 10×10^6 cells/leg. Perturbations in the bone structure can be observed and quantified from 4×10^6 AT6-1 cells, and are more pronounced with 8–10×10^6 cells with a total disorganization of growth plate and trabecular structures (Fig. 4*b*). Therefore the further studies were conducted with the injection of 6×10^6 AT6-1 cells into the medullar cavity of rat femora.

ZOL treatment prevents AT6-1-induced bone lesions in rats

Rats injected with 6×10^6 AT6-1 cells were designed as control or ZOL groups. They received subcutaneous injections of 100 μg/kg ZOL twice a week beginning 3 days posttumor cell injection, during 6 weeks. Figure 5*a* shows radiographs of rats treated or not with ZOL. In the ZOL-treated group, bone lesions are rarely observed (less than 20%) and the metaphyses

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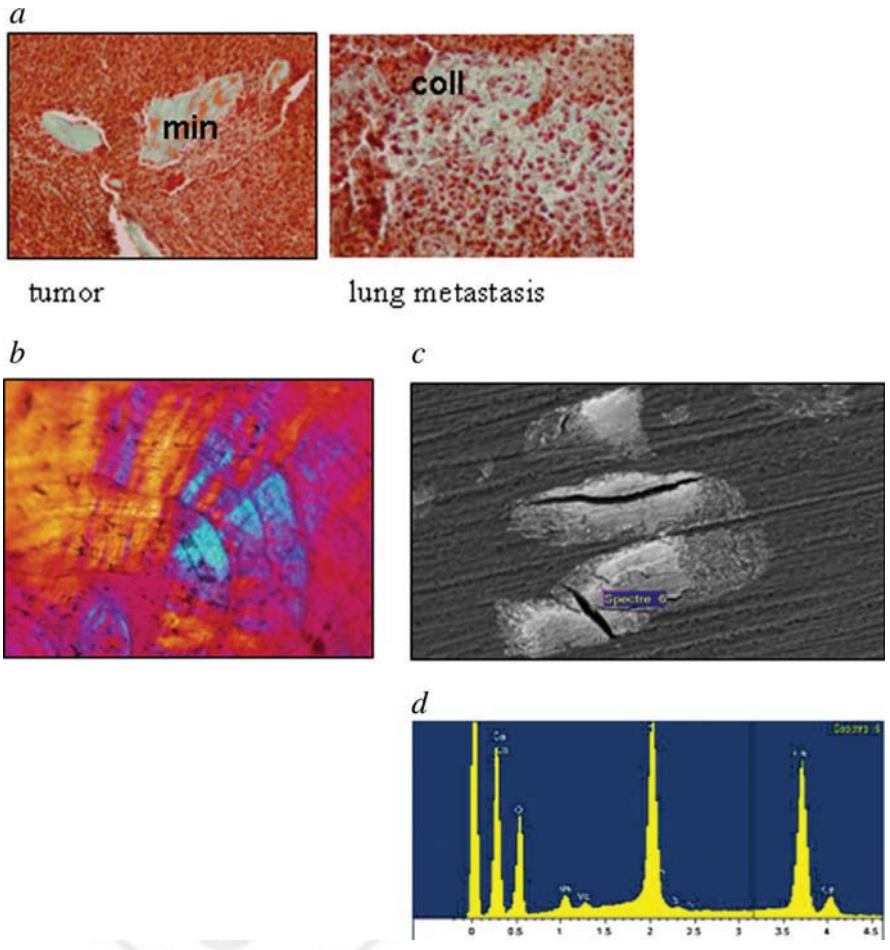

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FIGURE 2 – *In vivo* functional properties of AT6-1 cells: direct production of mineralized tissue. (*a*) Histological analyses (Masson's trichrome staining). The presence of bone matrix is revealed by collagen staining in green (coll). Mineralization areas are visualized both in the tumor in bone site and in lung metastasis nodules (min). Original magnification ×400 and ×200. (*b*) Polarized light microscopy of the tumor induced by AT6-1 cells injection (×40). (*c*) Scan Electron Microscopy. The presence of mineralized deposits is evidenced by SEM. (*d*) Energy dispersive X-ray microanalysis of the different elements composing the calcified tissue has been realized. Mineralized deposits contain calcium and phosphorous as demonstrated by EDAX pattern in a 1.68 ratio, characteristic of the presence of hydroxyapatite crystals.

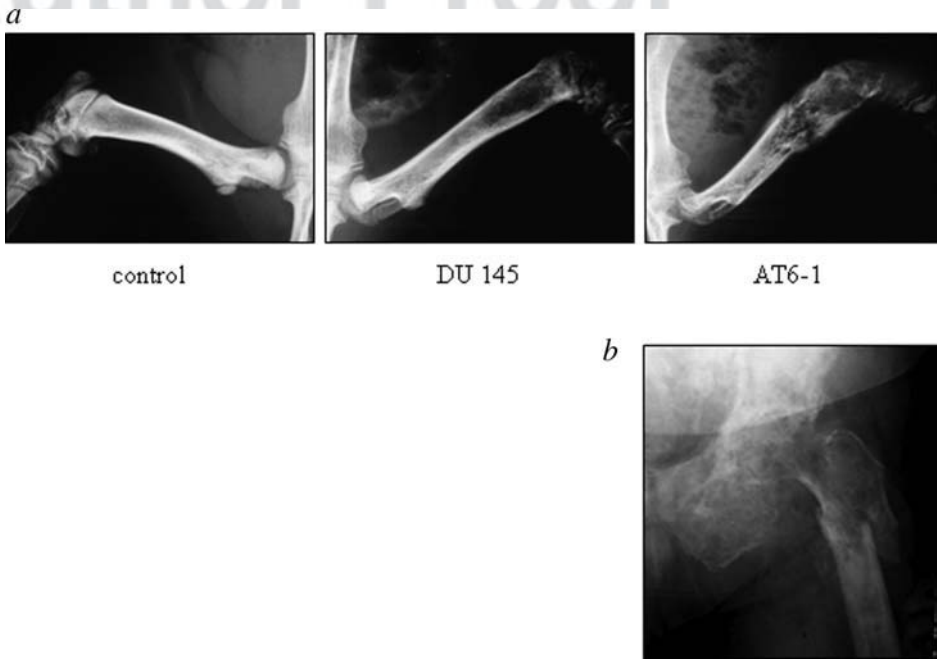

FIGURE 3 – Radiographic analysis of bone lesions induced after intraosseous inoculation of AT6-1 cells. Radiographs of Sprague Dawley rat femora 6 weeks after injection with 6×10^6 AT6-1 or DU145 cells (*a*), as compared to a representative radiograph of a patient bearing metastatic bone lesions secondary to prostate carcinoma (*b*).

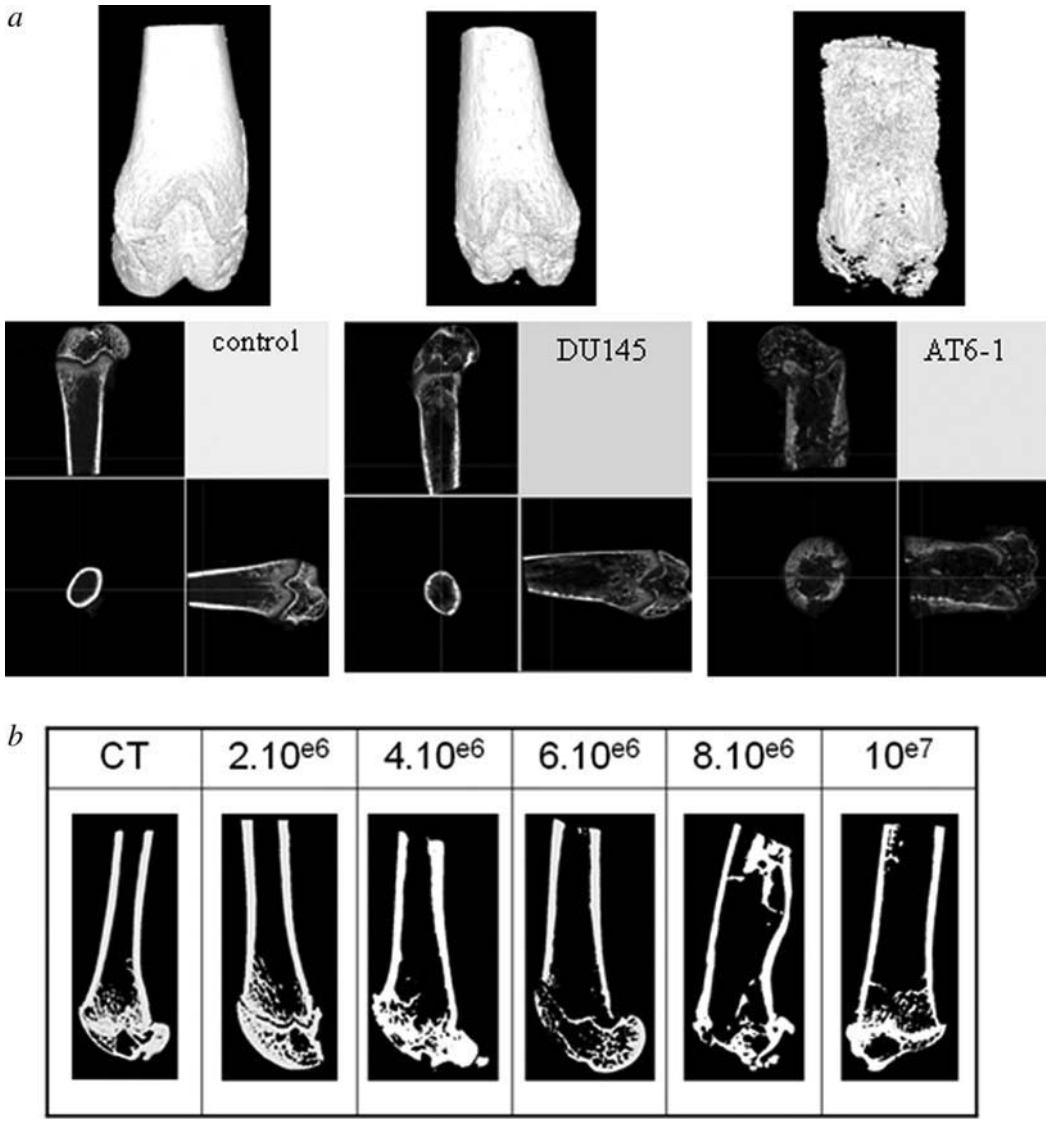

FIGURE 4 – Bone microarchitecture analysis by micro-QCT of the AT6-1 cell-induced bone lesions. (*a*) comparison of lesions induced 6 weeks earlier by the intraosseous injection of 6×10^6 AT6-1 and DU-145 cells in the medullar cavity of Sprague-Dawley rats, *versus* control femur. (*b*) Hemi-sagittal sections of femora injected with increasing number of AT6-1 cells, from 2×10^6 to 10^7 cells.

of long bones exhibited high bone density reflecting inhibition of bone resorption and retention of the primary spongiosa. In the control group, more than 80% of animals exhibited a structural disorganization of the bone tissue with both osteolytic and osteoblastic lesions. Microcomputed tomography analysis shows a huge increase of bone formation at the trabecular level that can be quantified by the specific bone volume that increased from 12.54 to 74.37% (Fig. 5*b*).

Mechanisms of action of ZOL on AT6-1 cells

Cell proliferation. AT6-1 cells were cultured for 24, 48 and 72 hr in the presence of increasing concentrations of ZOL (1, 10, 50 and 100 μM). The results show a significant time- and dose-dependent inhibition of cell proliferation as measured by a XTT-based method in the presence of ZOL (Fig. 6*a*).

Apoptosis induction. To see whether the inhibition of AT6-1 cell proliferation was due to apoptosis, the potential activation of caspase-3 was measured in AT6-1 cells incubated during 24, 48 or 72 hr with 50 μM ZOL. Figure 6*b* shows that ZOL is not able to induce caspase-3 activation at any time of incubation.

Cell cycle distribution. In an attempt to characterize the mechanisms involved in inhibition of tumor cell proliferation, the distribution of AT6-1 cells in the different phases of the cell cycle was examined. The results revealed a blockade of the AT6-1 cells in the S phase, consistent with a huge decrease of cells in the G0/G1 phases after 24 hr incubation in the presence of ZOL, this effect being more pronounced at 48 hr (Fig. 6*c*).

Discussion

Animal models of prostate cancer are critically important for defining the molecular basis of the disease and are also required to accelerate the development of new chemopreventive approaches and therapies for prostate cancer. Bone is a common site of pros-

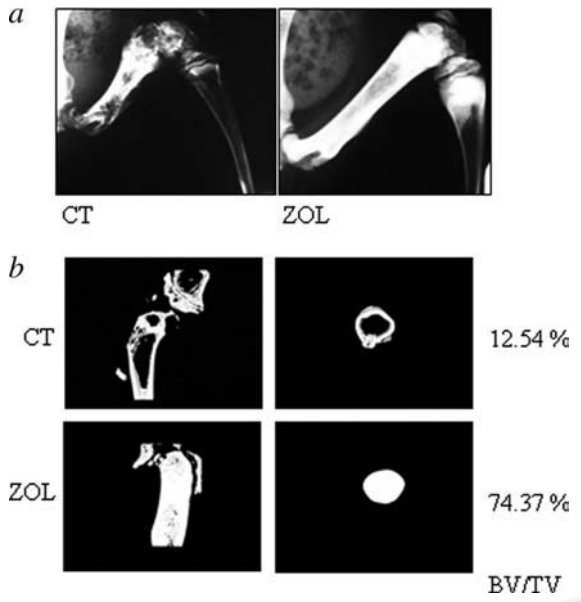

FIGURE 5 – Zoledronic acid (ZOL) effects on bone remodelling induced by AT6-1 cells. (*a*) Radiographs of representative bone lesions observed in control rats bearing AT6-1 tumor (5/6 positive) and in ZOL-treated rats (100 μg/kg, twice a week, beginning 3 days posttumor cell injection, 1/6 positive). (*b*) quantification of the micro-architectural parameters of the bone lesions induced by injection of AT6-1 cells are assessed by Micro-CT analysis at the time of sacrifice, compared between control rats bearing AT6-1 tumor and ZOL-treated rats.

tate cancer metastases and bone metastases constitute the major source of morbidity and mortality in prostate cancer.[20,21] As metastatic prostate cancer is unique in its ability to induce the osteoblastic reaction in skeleton, representative animal models of these particular bone lesions are warranted. Unfortunately, although there are a lot of animal models for osteolytic bone metastases, there is a paucity of good models to study osteoblastic lesions.[22]

In the present study, we investigated a new model of osteoblastic lesions in immunocompetent rats, induced by intraosseous inoculation of AT6-1 prostate cancer cells. In the first part of this study, bone lesions were characterized by complementary methods providing evidence that these lesions are osteoblastic. The AT6-1 cell line was characterized *in vitro* at the transcriptional and protein level for the confirmation of its epithelial origin by the expression of cytokeratin 18, and also for bone markers. The gene expression pattern of AT6-1 cells has been compared to that of the osteolytic DU145 cells, and showed extensive differences as DU145 cells express no osteoblast markers at all, this result being in accordance with the main osteolytic character of this model.[17] However, one limit of the experiment could be the presence of growth factors and hormones contained in the fetal calf serum that could influence the phenotypic data. However, the differences observed between DU145 and AT6-1 cells at the gene expression level were confirmed by the *in vivo* behavior of the cells (respectively osteolytic and mixed osteoblastic/osteolytic). Bone metastases with a bone-forming phenotype are reported to be the result of osteoblasts stimulation.[23] Indeed, prostate cancer cells influence bone homeostasis by secreting paracrine factors that regulate osteoblast proliferation or differentiation. These factors include BMP, TGF-β, IGF, PDGF, VEGF, endothelin-1 (ET-1), the bone metastatic factor MDA-BF-1, urokinase-type plasminogen activator (uPA) and Prostate Specific Antigen (PSA). These factors have been shown to support osteoblast proliferation by exerting direct effects on osteoblasts (BMP, TGF-β, IGF, PDGF, VEGF, ET-1, MDA-BF-1)[24–30] or influence osteoblast proliferation by modifying growth factors present in the bone microenvironment (uPA, PSA).[31,32] However, it was rather difficult to compare the factors produced by AT6-1 cells with those expressed by human prostate carcinoma cells. Indeed, a large number of tools are not available in the rat species. What is comparable is the *in vivo* behavior of the tumor cells as the radiographic data between rat and humans could be superimposed, that has never been done for other published prostate carcinoma models. In addition, these factors modulate osteoblast function to promote deposition of new bone matrix. The newly formed bone has features of immature bone (woven bone) with collagen fibres arranged in irregular random arrays, except that osteocytes were not detected at that level. Woven bone is eventually converted into lamellae. In the present study, the presence of woven bone was demonstrated by scan electron microscopy and EDAX, and the measurement of the calcium/phosphorous ratio revealed the presence of hydroxyapatite crystals, characteristic of mineralized tissue.

As the AT6-1 cells themselves express osteoblastic markers *in vitro*, we wonder whether these tumor cells were able to induce mineralized nodule formation *in vitro* and to form mineralized woven bone *in vivo*. Our results show direct mineralized nodule induction *in vitro* even in the absence of bone marrow cells, demonstrating the osteoblastic character of the cells. However, AT6-1 cells were unable to demonstrate any alkaline phosphatase activity *in vitro* although it was evidenced at the transcript level. It could be hypothesized that one parameter was missing or insufficient, such as inorganic phosphate, β-glycerophosphate, *etc*. However, even if *in vivo* characterization of the tumor demonstrated mineralized tissue formation, one cannot precisely tell if it is directly producted by the tumor cells or by osteoblasts, through induction by factors produced by tumor cells, or both. In addition to histology and microscopy, bone lesions were also analyzed by radiography and microcomputed tomography. The radiographs clearly evidenced the superimposition of the lesions obtained in this new model and those observed in prostate cancer patients with bone metastases. Microscanner data give complementary information about the quantification of the bone microarchitecture parameters, such as specific bone volume, bone surface, *etc*.

Therefore, we provide evidence for the development of a new model of osteoblastic bone metastases in immunocompetent rats. The development of such models is useful for the development of novel therapies. There has been a large number of published data in the literature on human xenograft models in nude mice.[7,8] Among these models, some had been induced by intratibia or intrafemora injection of human tumor cells in nude mice, leading to osteoblastic lesions comparable to those observed in the present model.[8] Many syngenic models of prostate carcinoma in rats and mice do not metastasize readily to bone. However, sublines of the cancers can be selected *in vivo* that have an increased incidence of bone metastasis after orthotopic or intracardiac administration. For example, the MatLyLu androgen-insensitive subline of the rat Dunning prostate carcinoma (R3327) has been used as reliable models for bone metastasis *in vivo*.[4] However, in this model, the osteolytic bone metastases do not mimic the osteoblastic or mixed osteolytic/osteoblastic metastases of human prostate carcinoma.[33] The PA-III cell line derived from a spontaneous prostate adenocarcinoma in a Lobund Wistar rat induced both osteolytic and osteoblastic reactions when transplanted adjacent to bone.[9] Another approach relies to the chemical induction of prostate carcinoma in rats. These can be induced in Noble rats with testosterone/estradiol or NMU (N-methyl-N-nitrosoUrea)/testosterone combinations. An increase incidence of prostate adenocarcinoma can be induced in Lobund Wistar rats with NMU and testosterone.[34] However, these tumors uncommonly metastasize to the lymph nodes and lungs but do not metastasize to bone. A third approach is the transgenic induction of prostate carcinoma in mice. Oncogene expression can be targeted to the prostate gland using the selective probasin, C(3)1 and PSA promoters.[35] The disadvantage of the transgenic models is the low incidence of metas-

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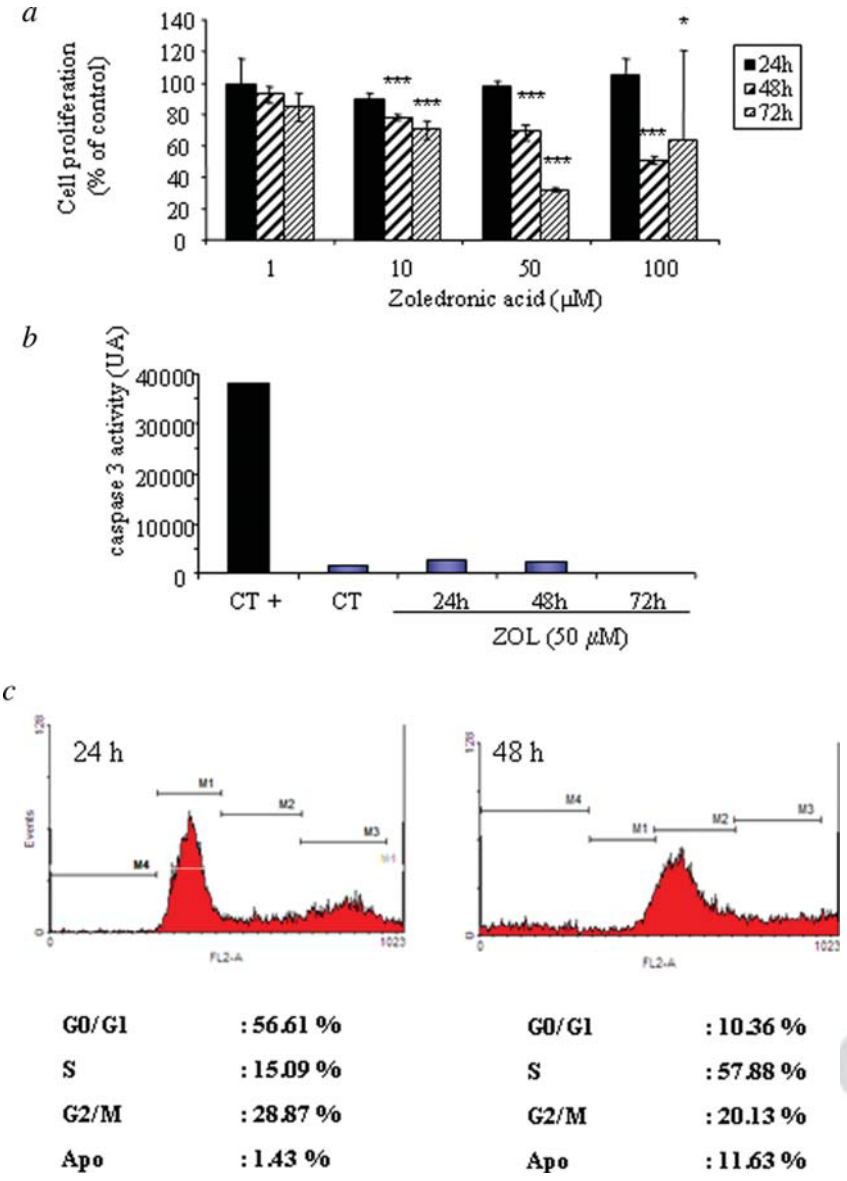

FIGURE 6 – Mechanisms of action of zoledronic acid on AT6-1 cells. (*a*) Proliferation of AT6-1 cells was determined after exposure to zoledronic acid ($0–10^{-4}$ M) for 3 days with 10% fetal bovine serum, using the XTT quick proliferation kit as described in "Material and methods." Changes in absorbance at 490 nm were measured and results are presented as percentage of untreated cell proliferation. *: $p < 0.05$; ***: $p < 0.001$. (*b*) AT6-1 cells were treated with 1 or 10 μM ZOL for the indicated times. Caspase-3 activity was determined in cell lysates using a caspase-3-specific fluorogenic substrate, as described in the Materials and Methods section. CT+ represents a positive control of cells treated with UV light for 30 sec. CT− represents the AT6-1 cells alone as a negative control. Results of 3 independent experiments are presented and the caspase activity is expressed as change in absorbance at 460 nm per μg of protein. (*c*) Flow cytometry of ZOL-treated AT6-1 cells. AT6-1 cells were incubated for 24, 48 and 72 hr in the absence (control) or the presence of 10 μM ZOL. At each point, cells were harvested, fixed, and stained with propidium iodide. The positions on the histograms of the hypodiploid sub-G_0/G_1 (APO), G_0/G_1, S and G_2/M peaks, and the percentage of cells in each of the cycle phases are indicated from a representative experiment.

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tasis, especially bone metastases, often due to rapid progression of the primary neoplasm. Several models of human xenografts by injection of human cancer cell lines into the left ventricle of nude mice induce bone metastases *in vivo*.[36] These models have many advantages and have enabled testing of Paget's "seed and soil" hypothesis with cancer cells serving as the seeds and the bone marrow microenvironment serving as the soil.[37] However, the disadvantage of these models is the uncertain pathogenesis of bone metastasis after left ventricular injection of tumor cells. Human cancer cells or tumor tissue can also be xenografted into orthotopic sites to model bone metastasis *in vivo*. Orthotopic injection of cells into the prostate gland usually results in a low incidence of bone metastasis from late-stage cancers. Human prostate carcinoma cell lines have been transplanted orthotopically in mice and utilized to successfully represent different stages of cancer progression *in vivo* including bone metastases[38]

As ZOL was shown to exhibit inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer,[8] this therapeutic compound has been used to validate our model. BPs are nonhydrolyzable pyrophosphate analogs, which have inhibitory effects on osteoclast generation, maturation and activity, thereby reducing osteoclastic bone resorption. BPs are used to treat Paget's disease, hypercalcemia associated with cancer, and lytic bone metastases of breast cancer and myeloma.[12] Even when the overall character of prostate cancer appears to be osteoblastic, preponderance of evidence indicates that osteolysis is present.[39,40] This process justifies the use of BPs in prostate cancer treatment.[41] On our new model of osteoblastic metastases, ZOL exerts a preventing effect on bone lesions development, as revealed by radiography analyses, leading to an augmentation of animal survival. The mechanisms underlying BP effects on cancer cells are not fully understood, but involve in several cases apoptosis *via* mevalonate pathway and activation of caspase.[42–44] However, few reports indicate that ZOL may directly acts on tumor cells, inhibiting cell proliferation but inducing apoptosis in a caspase-independent way.[45] In the context of AT6-1 cells, a direct effect of ZOL on prostate carcinoma cells was demonstrated *in vitro* with the inhibition of cell proliferation, no caspase activation but cell cycle arrest in S phase. However, we cannot conclude whether the effects of ZOL against osteoblastic prostate cancer are direct or indirect

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in vivo and what will be its effects in a nonosseous environment. In another model of non osseous tumor (pulmonary metastases secondary to osteosarcoma), we previously demonstrated that ZOL exerts an inhibitory effect on tumor progression.[46] When AT6-1 cells were injected subcutaneously, they induce the local development of an osteoblastic tumor, even in the absence of bone microenvironment. ZOL exerts the same inhibitory effect on this calcified tumor induced by subcutaneous injection of the AT6-1 cells (not shown).

Therefore, the use of ZOL seems promising in the case of osteoblastic lesions, as determined in a rat model that mimicks the osteoblastic metastases observed in patients with prostate carcinoma.

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References

1. Goltzman D. Mechanisms of the development of osteoblastic metastases. Cancer 1997;80:1581–7.
2. Carlin BI, Andriole GL. The natural histology, skeletal complications, and management of bone metastases in patients with prostate carcinoma. Cancer 2000;88:2989–94.
3. Shimazaki J, Higa T, Akimoto S, Masai M, Isaka S. Clinical course of bone metastasis from prostatic cancer following endocrine therapy: examination with bone x-ray. Adv Exp Med Biol 1992;324:269–75.
4. Tennant TR, Kim H, Sokoloff M, Rinker S. The Dunning model. Prostate 2000;43:295–302.
5. Huss WJ, Maddison LA, Greenberg NM. Autochthonous mouse models for prostate cancer: past, present and future. Semin Cancer Biol 2001;11:245–60.
6. Yoneda T. Cellular and molecular basis of preferential metastasis of breast cancer to bone. J Orthop Sci 2000;5:75–81.
7. Zhau HE, Li CL, Chung LW. Establishment of human prostate carcinoma skeletal metastasis models. Cancer 2000;88:2995–3001.
8. Corey E, Quinn JE, Bladou F, Brown LG, Roudier MP, Brown JM, Buhler KR, Vessella RL. Establishment and characterization of osseous prostate cancer models: intra-tibial injection of human prostate cancer cells. Prostate 2002;52:20–33.
9. Koutsilieri M. PA-III rat prostate adenocarcinoma cells. In Vivo 1992;6:199–203.
10. Liepe K, Geidel H, Haase M, Hakenberg OW, Runge R, Kotzerke J. New model for the induction of osteoblastic bone metastases in rat. Anticancer Res 2005;25:1067–73.
11. Ke Y, Beesley C, Smith P, Barraclough R, Rudland P, Foster CS. Generation of metastatic variants by transfection of a rat non-metastatic epithelial cell line with genomic DNA from rat prostatic carcinoma cells. Br J Cancer 1998;77:287–96.
12. Heymann D, Ory B, Gouin F, Green J, Rédini F. Bisphosphonates: new therapeutic agents for the treatment of bone tumors. Trends Mol Med 2004;10:337–43.
13. Lee MV, Fong EM, Singer FR, Guenette RS. Bisphosphonate treatment inhibits the growth of prostate cancer cells. Cancer Res 2001;61:2602–8.
14. Corey E, Brown LG, Quinn JE, Poot M, Roudier MP, Higano CS, Vessella RL. Zoledronic acid exhibits inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer. Clin Cancer Res 2003;9:295–306.
15. Saad F, Gleason DM, Murray R, Tchekmedyian S, Venner P, Lacombe L, Chin JL, Vinholes JJ, Goas JA, Chen B, for Zoledronic Acid Prostate Cancer Study Group. A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma. J Clin Cancer Inst 2002;92:1458–68.
16. Michaelson MD, Kaufman DS, Kantoff P, Oh WK, Smith MR. Randomized phase II study of atrasentan alone or in combination with zoledronic acid in men with metastatic prostate cancer. Cancer 2006;107:530–5.
17. Nemeth JA, Harb JF, Barroso U, Jr, Grignon DJ, Cher ML. Severe combined immunodeficient-hu model of human prostate cancer metastasis to human bone. Cancer Res 1999;59:1987–93.
18. Chipoy C, Berreur M, Couillaud S, Pradal G, Vallette F, Colombeix C, Rédini F, Heymann D, Blanchard F. Down-regulation of osteoblast markers and induction of the glial fibrillary acidic protein by oncostatin M in osteosarcoma cells required PKCd and STAT3. J Bone Miner Res 2004;19:1850–61.
19. Chappard D, Palle S, Alexandre C, Vico L, Riffat G. Bone embedding in pure methyl methacrylate at low temperature preserves enzyme activities. Acta Histochem 1987;81:183–90.
20. Karayi, MK, Markham AF. Molecular biology of prostate cancer. Prostate Cancer Prostatic Dis 2004;7:6–20.
21. Bubendorf L, Schopfer A, Wagner U, Sauter G, Moch H, Willi N, Gasser TC, Mihatsch MJ. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Hum Pathol 2000;31:578–83.
22. Goltzman D. Extending preclinical models of skeletal manifestations of malignancy to the clinical setting. Semin Oncol 2001;28:9–14.
23. Logothetis CJ, Lin S-H. Osteoblasts in prostate cancer metastasis to bone. Nat Rev Cancer 2005;5:21–8.
24. Autzen P, Robson CN, Bjartell A, Malcolm AJ, Johnson MI, Neal DE, Hamdy FC. Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies. Br J Cancer 1998;78:1219–23.
25. Shariat SF, Shalev M, Menesses-Diaz A, Kim IY, Kattan MW, Wheeler TM, Slawin KM. Preoperative plasma levels of transforming growth factor beta(1) (TGF-beta(1)) strongly predict progression in patients undergoing radical prostatectomy. J Clin Oncol 2001;19:2856–64.
26. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, Hennekens CH, Pollak M. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science 1998;279:563–6.
27. Fudge K, Wang C, Stearns ME. Immunohistochemistry analysis of platelet-derived growth factor A and B chains and platelet-derived growth factor alpha and beta receptor expression in benign hyperplasias and Gleason-graded human prostate adenocarcinomas. Mod Pathol 1994;7:549–54.
28. Dai J, Kitagawa Y, Zhang J, Yao Z, Mizokami A, Cheng S, Nor J, McCauley LK, Taichman RS, Keller ET. Vascular endothelial growth factor contributes to the prostate cancer-induced osteoblast differentiation mediated by bone morphogenetic protein. Cancer Res 2004;64:994–9.
29. Nelson JB, Hedican SP, George DJ, Reddi AH, Piantadosi S, Eisenberger MA, Simons JW. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. Nat Med 1995;1:944–9.
30. Vakar-Lopez F, Cheng CJ, Kim J, Shi GG, Troncoso P, Tu SM, Yu-Lee LY, Lin SH. Up-regulation of MDA-BF-1, a secreted isoform of ErbB3, in metastatic prostate cancer cells and activated osteoblasts in bone marrow. J Pathol 2004;203:688–95.
31. Rabbani SA, Desjardins J, Bell AW, Banville D, Mazar A, Goltzman D. An amino-terminal fragment of urokinase isolated from a prostate cancer cell line (PC-3) is mitogenic for osteoblast-like cells. Biochem Biophys Res Commun 1990;173:1058–64.
32. Iwamura M, Hellman J, Cockett AT, Lilja H, Gershagen S. Alteration of the hormonal bioactivity of parathyroid hormone-related protein (PTHrP) as a result of limited proteolysis by prostate-specific antigen. Urology 1996;48:317–25.
33. Blomme EAG, Dougherty KM, Pienta KJ, Capen CC, Rosol TJ, McCauley LK. Skeletal metastasis of prostate adenocarcinoma in rats: morphometric analysis and role of parathyroid hormone-related protein. Prostate 1999;39:187–97.
34. Pollard M, Wolter WR, Sun L. Prostate-seminal vesicle cancers induced in noble rats. Prostate 2000;43:71–4.
35. Huss WJ, Maddison LA, Greenberg NM. Autochthonous mouse models for prostate cancer: past, present and future. Semin Cancer Biol 2001;11:245–60.
36. Yoneda T. Cellular and molecular basis of preferential metastasis of breast cancer to bone. J Orthop Sci 2000;5:75–81.
37. Paget S. The distrubution of secondary growths in cancer of the breast. Lancet 1889;1:571–2.
38. Zhau HE, Li CL, Chung LW. Establishment of human prostate carcinoma skeletal metastasis models. Cancer 2000;88:2995–3000.
39. Coleman RE, Houston S, James I, Rodger A, Rubens RD, Leonard RC, Ford J. Preliminary results of the use of urinary excretion of pyridinium crosslinks for monitoring metastatic bone disease. Br J Cancer 1992;65:766–8.

40. Ikeda I, Miura T, Kondo I. Purridinium cross-links as urinary markers of bone metastases in patients with bone cancer. Br J Cancer 1996;77:102–6.
41. Berruti A, Dogliotti L, Ducci M, Tarabuzzi M, Fontana D, Angeli A. Metabolic bone disease induced by prostate cancer: rationale for the use of bisphosphonates. J Urol 2001;166:2023–31.
42. Luckman SP, Hughes DE, Coxon FP, Graham R, Russell G, Rogers MJ. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. J Bone Miner Res 1998;13:581–9.
43. Coxon FP, Benford HL, Russell RG, Rogers MJ. Protein synthesis is required for caspase activation and induction of apoptosis by bisphosphonate drugs. Mol Pharmacol 1998;54:631–8.
44. Shipman CM, Croucher PI, Russel RGG, Helfrich MH, Rogers MJ. The bisphosphonate Incadronate (YM175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway. Cancer Res 1998;58:5294–7.
45. Ory B, Blanchard F, Battaglia S, Gouin F, Rédini F, Heymann D. Zoledronic acid activates the DNA S-phase checkpoint and induces osteosarcoma cell death characterized by apoptosis-inducing factor and endonuclease-G translocation independently of p53 and retinoblastoma status. Mol Pharmacol 2007;71:333–43.
46. Ory B, Heymann MF, Blanchard F, Kamijo A, Gouin F, Heymann D, Rédini F. Zoledronic acid suppresses lung metastases and extends overall survival of osteosarcoma of osteosarcoma-bearing mice. Cancer 2005;104:2522–29.

Complément de discussion à l'article 6

Dans la première partie de cette étude nous nous sommes attachés à démontrer le caractère ostéoblastique des lésions osseuses, puis, la lignée cellulaire AT6-1 a été caractérisée *in vitro* au niveau transcriptionnel et traductionnel. L'expression de la cytokératine 18 confirme son origine épithéliale. Le panel d'expression de gènes étudiés chez les cellules AT6-1 a été comparé à celui des cellules DU145 à l'origine d'un modèle animal ostéolytique. Contrairement aux AT6-1, les cellules DU145 n'expriment aucun marqueur ostéblastique en cohérence avec le caractère exclusivement ostéolytique de ce modèle (Nemeth et al. 1999). La formation de tissu calcifié accompagnant les métastases osseuses est décrite comme étant la conséquence d'une stimulation des ostéoblastes (Logothetis et al. 2005). En effet, les cellules de carcinome prostatique influencent l'homéostasie osseuse en sécrétant des facteurs de la prolifération et de la différenciation des ostéoblastes. Ces facteurs sont, BMP, TGF-β, IGF, PDGF, VEGF, endotheline-1 (ET-1), le facteur métastatique osseux MDA-BF-1 et l'antigène spécifique de la prostate (PSA). Ces facteurs sont impliqués dans l'induction de la prolifération des ostéoblastes en exerçant leurs effets directement sur les ostéoblastes (BMP, TGF-β, IGF, PDGF, VEGF, ET-1, MDA-BF-1) (Nelson et al. 1995; Chan et al. 1998) ou en influençant leur prolifération *via* des facteurs de croissance présents dans le microenvironnement osseux (PSA) (Rabbani et al. 1990; Iwamura et al. 1996). Dans ces conditions, l'os néoformé est immature avec des fibres de collagène désordonnées ne contenant pas d'ostéocyte. La présence de cet os néoformé immature a été confirmée par microscopie électronique à balayage, de plus la mesure du ratio calcium/phosphore révèle la présence de cristaux d'hydroxyapatite caractéristiques du tissu osseux.

Sachant que les cellules AT6-1 expriment elles mêmes des marqueurs ostéoblastiques *in vitro*, nous nous sommes interrogés sur leur capacité à former des nodules de minéralisation *in vitro* et de l'os immature *in vivo*. Nos résultats montrent une induction directe de nodules de minéralisation *in vitro*, démontrant ainsi le caractère ostéoblastique de ces cellules. Cependant les cellules AT6-1 ne présentent aucune activité phosphatase alcaline *in vitro* pourtant indispensable au phénotype ostéoblastique. Par conséquent, nous ne pouvons pas conclure quant à l'origine de la formation du tissu minéralisé observé *in vivo*, celle-ci étant due soit directement aux cellules tumorales, soit à l'induction des ostéoblastes par des facteurs produits par les cellules tumorales, soit les deux. Différentes approches ont déjà été testées utilisant diverses lignées cellulaires mais aucune ne mime parfaitement la pathologie

humaine, en effet soit les métastases sont uniquement ostéolytiques (Tennant et al. 2000), soit à la place des classiques métastases osseuses ostéolytiques et ostéocondancentes, apparaissent des métastases des poumons et des ganglions lymphatiques (Pollard et al. 2000).

Dans ce nouveau modèle de métastases osseuses ostéocondensantes, les analyses radiologiques révèlent que le Zol exerce un effet préventif sur le développement des lésions osseuses, augmentant ainsi la survie des animaux. In vitro nous avons également pu confirmer que le Zol, comme pour les cellules d'ostéosarcome, inhibait la prolifération des cellules AT6-1 par un arrêt du cycle cellulaire en phase S et induisait l'apoptose par un mécanisme indépendant des caspases. Notre modèle mimant les lésions ostéocondensantes observées chez les patients atteins de carcinome prostatique nous permet de constater que le Zol semble être un traitement prometteur des lésions osseuses secondaires à une tumeur de la prostate.

PARTIE II

Mécanismes de résistance des ostéosarcomes à l'acide zolédronique

I. Mécanismes généraux de résistance

Au cours des dernières années le traitement des cancers est devenu de plus en plus spécifique et cible désormais des molécules issues d'études sur les oncogènes et les anti-oncogènes impliqués dans le développement des cancers. Cependant, aucun traitement n'est efficace à 100% en raison de résistances aux traitements observées dans les tumeurs. L'origine de ces résistances est multifactorielle et liée à la variabilité inter-individuelle des patients mais aussi à la variabilité génétique des cellules au sein d'une même tumeur. Dans la majorité des cas la résistance est intrinsèque à la tumeur ; elle survient rapidement lors de l'administration des premières séances de chimiothérapie, sans phase de sensibilité initiale ; en effet, il y a 28 ans, Goldie et Coldman (Goldie et al. 1979) proposaient un modèle mathématique pour expliquer l'apparition de clones malins résistants dans une tumeur, ce modèle postulait l'apparition statistiquement probable de mutations donnant une moindre sensibilité à un ou plusieurs agents cytostatiques. La théorie de Goldie et Coldman repose sur le fait qu'au moment du diagnostic la plupart des tumeurs possèdent des clones résistants. En effet, pour un gramme de tumeur, soit 10^9 cellules, le taux de mutation par gène est probablement de 10^{-5} donc 10^4 clones sont potentiellement résistants à une drogue donnée dans cette tumeur. La résistance à deux drogues survient alors dans moins de une cellule sur $10^5 x 10^5$ soit 10^{10} cellules. Ceci est la base de l'intérêt d'utilisation de plusieurs drogues dans un protocole de chimiothérapie. Cependant, avec l'augmentation en très forte croissance de l'utilisation de la chimiothérapie, l'acquisition de résistance devient de plus en plus commune, après une phase initiale de grande chimio-sensibilité, apparaît secondairement une progression de la maladie témoignant d'une résistance acquise.

L'origine la plus fréquente de l'acquisition de résistance à une large variété de molécules anti-tumorales est l'expression d'un ou plusieurs transporteurs membranaires détectant et rejetant ces molécules hors de la cellule, ceci ayant pour conséquence une diminution de la concentration intracellulaire ainsi qu'une augmentation de l'efflux du médicament anti-tumoral. Il s'agit d'un phénomène aujourd'hui appelé MDR (multidrug resistance) mis en évidence par Biedler et Riehm, en 1970, dans des lignées tumorales : ils ont observé qu'en rendant ces cellules résistantes à un cytostatique, celles-ci devenaient simultanément résistantes à d'autres cytostatiques de mode d'action et de structure totalement différents (Biedler et al. 1970). Cette résistance croisée, retrouvée dans de nombreuses lignées tumorales d'espèce et d'origine tissulaire différentes, s'observe vis-à-vis de nombreux agents

d'origine naturelle tels que les anthracyclines (daunorubicine, doxorubicine) des alcaloïdes (vinblastine, vincristine), des taxanes, des épipodophyllotoxines (étoposide). Le phénotype MDR se caractérise par un efflux actif (adénosine triphosphate [ATP]-dépendant) des cytostatiques (*Figure 17*). A l'origine de cet efflux se trouvent plusieurs molécules de la même famille des transporteurs à cassettes ATP également appelés transporteurs ABC (ATP-*binding cassette*), c'est-à-dire que cette molécule utilise l'énergie fournie par l'ATP pour expulser à l'extérieur de la cellule un grand nombre d'agents de chimiothérapie. Dans cette famille on trouve la P-glycoprotéine (*permeability glycoprotein*) (Nielsen et al. 1996), codée par le gène MDR1 localisé sur le chromosome 7 (Kantharidis et al. 2000), les MRP (multidrug resistance associated protein) caractérisés par la présence dans la membrane plasmique d'autres protéines canal codées entre autre par les gènes MRP_1 et MRP_2 (Leslie et al. 2001) ; ce phénotype est caractérisé par un efflux dépendent de l'ATP des médicaments sous forme conjuguée au glutathion (GSH). Enfin on observe les MXR (Mitoxantrone Resistance associated transporter) et les BCRP (breast cancer resistance protein). Une autre protéine canal peut être distinguée : la protéine LRP (lung resistant protein), identifiée dans une lignée résistante de cancer du poumon, cette protéine régulerait l'entrée nucléaire des médicaments concernés. Il ne s'agit pas d'une protéine de la famille ABC. Sa palette de résistance est plus large que celle des protéines ABC, et s'étend en particulier aux alkylants et aux sels de platine. Cette protéine est l'homologue de la *major vault protein* de rat qui constitue un composant majoritaire d'une organelle intracytoplasmique ayant probablement un rôle dans le trafic cellulaire (Scheffer et al. 2000).

La grande difficulté rencontrée dans le traitement des cancers par les agents chimio-thérapeutiques est la diversité des réponses des cellules tumorales au traitement. En effet, chaque cellule tumorale d'un patient donné a un profil génétique qui lui est propre. Par conséquent, chaque tumeur présente un profil de résistance différent, et les cellules au sein d'une même tumeur présentent une forte hétérogénéité vis-à-vis des mécanismes de résistance, même si un certain nombre d'entre elles sont issues d'un même clone. De plus, même si une tumeur n'est pas intrinsèquement résistante à un agent de chimiothérapie en particulier, son hétérogénéité génétique pourra la conduire à acquérir une résistance par sélection clonale face à une drogue active imposant une forte sélection.

Il existe bien d'autres mécanismes de résistance ne mettant pas en jeu d'efflux ou de pompes moléculaires (*Figure 19*). Un certain nombre d'entre eux n'induisent de résistance qu'à une faible quantité de médicaments ayant des mécanismes d'action semblables. Parmi cette catégorie les mécanismes de résistance sont peu nombreux : perte des récepteurs

membranaires ou encore augmentation ou altération de la cible spécifique du médicament. Dans de telles situations, l'utilisation de plusieurs agents thérapeutiques aux mécanismes d'actions différents suffit dans la majorité des cas à obtenir de bons taux survie. Malheureusement, le plus souvent les cellules présentent des mécanismes qui confèrent une résistance simultanée à des médicaments ayant des structures et des mécanismes différents. Ce phénomène est nommé « Multidrug resistance ». Parmi ces mécanismes on compte : une augmentation de l'efflux (décrit précédemment), une diminution de l'assimilation du médicament ou une modification de la structure lipidique de la membrane plasmique (Liu et al. 2001), des modifications génétiques bloquant la mort cellulaire (Lowe et al. 1993), une activation des mécanismes de réparation de l'ADN (Synold et al. 2001), une altération de la régulation du cycle cellulaire et enfin une inhibition de l'internalisation nucléaire *via* les LRP (Lung Resistance Protein) (Dalton et al. 1999).

De manière générale, les lésions induites par les agents cytostatiques, si elles sont irréparables, ont pour conséquence l'induction de la mort cellulaire programmée ou apoptose. Par conséquent tous les facteurs pouvant diminuer cette mort cellulaire chimio-induite vont augmenter la survie cellulaire des cellules tumorales, et donc la résistance à tous les agents cytostatiques. A titre d'exemple la protéine p53 est activée en réponse aux dommages de l'ADN, et stoppe le cycle cellulaire en phase G1 (*via* p21), permettant soit la réparation de l'ADN lésé, soit l'induction de l'apoptose si les lésions sont irréparables, jouant ainsi son rôle de gardien de l'intégrité du génome. Dans le cas d'une mutation de p53, le seuil de lésion de l'ADN conduisant à l'apoptose augmente, responsable de survie cellulaire et d'aggravation des lésions géniques transmises aux cellules tumorales filles.

Des altérations géniques des inhibiteurs de cycle cellulaire sont également observées, par exemple dans la famille INK4 (p15 et p16), qui contrôlent la progression des cellules de la phase G1 à la phase S. En effet, un taux élevé de p27, inhibiteur universel des cyclines, dans les lymphocytes tumoraux est corrélé à une résistance relative à la fludarabine *in vitro* et une évolutivité plus rapide de la maladie (Vrhovac et al. 1998).

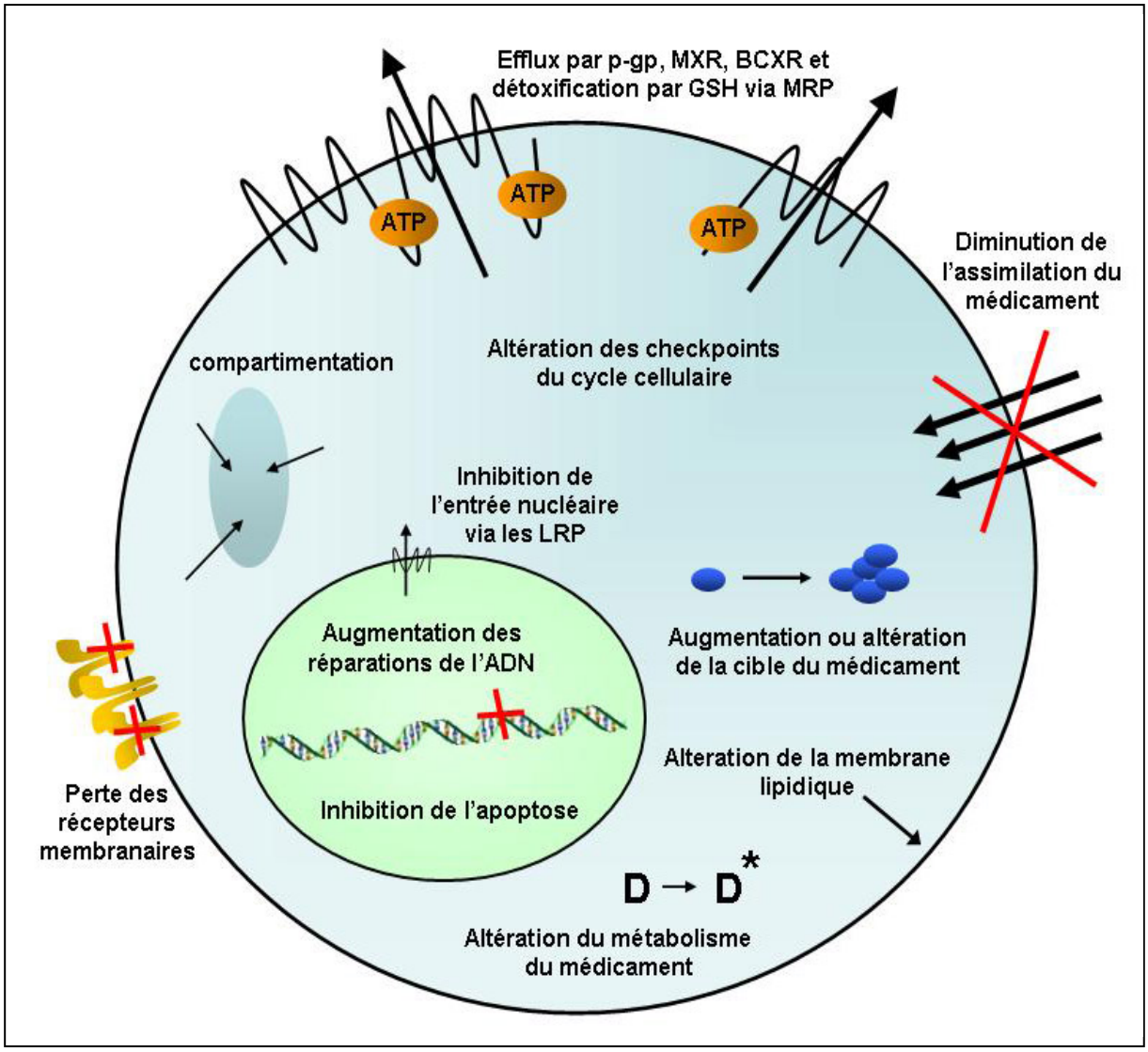

Figure 19 **: Principaux mécanismes de résistances aux agents anti tumoraux observés dans les cellules cancéreuses.**

II. Ostéosarcome et résistance

L'utilisation de la chimiothérapie dans le traitement des ostéosarcomes a permis l'augmentation du taux de survie de 11% en 1960 (avec seulement une résection chirurgicale) à 70% au milieu des années 1980 grâce à la polychimiothérapie (Bertermann et al. 1985). Ce protocole thérapeutique repose sur des cures alternées combinant différents agents pharmacologiques et alternant une chimiothérapie d'induction, chirurgie et chimiothérapie adjuvante. Cependant, le taux de survie peine à être améliorée et ce malgré l'apparition de nouvelles thérapies anti-tumorales. C'est pourquoi l'étude des mécanismes de résistance impliqués dans l'échappement thérapeutique des ostéosarcomes est une étape primordiale de l'amélioration du taux de survie.

Les mécanismes de résistance des ostéosarcomes vis-à-vis des agents anti tumoraux sont bien documentés (Chou et al. 2006). Les études de cytogénétique réalisées dans l'ostéosarcome montrent l'existence d'une grande instabilité génétique avec la présence d'anomalies chromosomiques multiples, de nombre et de structure avec des variations importantes d'une tumeur à l'autre et au sein d'une même tumeur, d'une cellule à l'autre. Les anomalies les plus fréquentes sont l'amplification des régions 6p12-p21, 17p11.2 et 12q13-q14 et la perte de régions chromosomiques en particulier 2q ,3p ,9, 10p, 12q, 13q, 14q, 15q , 16, 17p et 18q (Wang 2005). Ces modifications génétiques affectent des voies de signalisations essentielles à la cellule telles que : des altérations des voies de régulation de l'apoptose et du cycle cellulaires régulées par p53 et Rb (Arndt et al. 1999; Sandberg et al. 2003), des modifications d'oncogènes telles que la délétion de $p16^{INK4A}$ (inhibiteur des cyclines dépendante des kinase 2A) ainsi qu'une surexpression de c-fos et une amplification de la kinase dépendante des cyclines 4 (cdk4) (Benassi et al. 1999; Wei et al. 1999; David et al. 2005). Cette instabilité génétique conduit à l'existence d'une population cellulaire hétérogène au sein d'une même tumeur et à l'émergence de sous populations résistantes. L'ostéosarcome est une tumeur décrite comme étant relativement résistante aux traitements chimiothérapeutiques, en effet des agents habituellement actifs dans les autres tumeurs solides pédiatriques tels que la vincristine et le 5-fluorouracil s'avèrent inefficaces dans le traitement des ostéosarcomes (Pratt et al. 1994). Les résistances les plus fréquemment décrites sont les résistances aux agents chimiothérapeutiques habituellement employés tels que le cisplatine, la doxorubicine et le methotrexate. Ces résistances ont alors pour origine les mécanismes

suivants: mutation de la cible du médicament, surexpression ou sous expression de la cible du médicament, diminution de l'assimilation du médicament, inactivation du médicament, augmentation de l'élimination du médicament et augmentation des réparations de l'ADN (Grem et al. 1988; Siddik 2003; Beretta et al. 2004). Cependant, le mécanisme de résistance le plus cité pour les ostéosarcomes reste le phenotype MDR (MultiDrug Resistance phenotype) dû à la surexpression à la membrane de la P-gp ou autres protéines associées. En effet les niveaux d'expression de MDR1 (Gomes et al. 2006) ou de la P-gp (Baldini et al. 1999; Serra et al. 2006) peuvent être utilisés en tant que facteurs pronostiques. Plus récemment, d'autres facteurs pronostiques ont été décrits tels que le niveau d'expression des Clusterin/Apolipoprotein J (Lourda et al. 2007), l'expression de PXR (Pregnate Xenobiotic Receptor) un inducteur majeur du cytochrome P450 3A4 (Mensah-Osman et al. 2007) ou encore l'expression de la glutathion S-transferase P1, une enzyme de détoxification cellulaire fréquemment impliquée dans les mécanismes de résistance (Huang et al. 2007). Ces facteurs pronostiques de la sensibilité aux agents chimiothérapeutiques permettent de personnaliser les traitements aux patients et ainsi d'améliorer leur prise en charge.

III. Résistance des ostéosarcomes à l'acide zolédronique

Article 7

« Implication de la Farnesyl Diphosphate synthase dans les mécanismes de résistance à l'acide zolédronique dans les cellules d'ostéosarcome »

Journal of cellular and molecular medicine. Ory B, Moriceau G, Rédini F, Rogers M.J, Heymann D. In press

Nous avons récemment démontré un effet anti tumoral original et sélectif de l'acide zoledronique (Zol) sur divers lignées d'ostéosarcomes indépendamment de leur statut p53 et Rb. Le Zol inhibe la prolifération cellulaire, la migration, perturbe l'organisation du cytosquelette et induit l'apoptose indépendante des caspases. Cette étude traite de l'éventuel développement d'une résistance au Zol après un traitement prolongé des ostéosarcomes.

En effet un traitement de 1µM de Zol pendant 4 à 8 semaines réduit significativement la sensibilité des cellules d'ostéosarcomes aux fortes concentrations de Zol (10 à 100µM) aussi bien en termes d'inhibition de prolifération, d'effet sur le cycle cellulaire que d'effet sur la migration et le cycle cellulaire. Des tests de prolifération aux XTT ont démontré d'une part que les cellules résistantes au Zol étaient toujours sensibles aux agents conventionnels tels que le methotrexate, le mafosfamide et la doxorubicine et d'autre part que le processus de résistance n'était pas associé avec un phénotype de type multidrug resistance (MDR). De plus des expériences complémentaires réalisées en présence de clodronate et de pamidronate indiquent que ce phénomène de résistance est restreint à la famille de bisphosphonates contenant un atome d'azote (N-BPs).

C'est pourquoi notre étude s'est portée sur l'implication de la Farnésyl Diphosphate Synthase (FPPs), enzyme clef de la voie d'action des N-BPs. Une relation entre le niveau de transcription de la FPPs, son activité enzymatique et le niveau de résistance a pu être établie. Pour démontrer l'implication de la FPPs dans les mécanismes de résistance au Zol, des cellules résistantes ont été transfectées avec des siRNA FPPs, ce qui accroît fortement la sensibilité des ces cellules au Zol. L'origine innée ou acquise de cette résistance à été étudiée d'une part par clonage de la lignée cellulaire OSRGA et d'autre part par traitement avec de très faibles concentrations de Zol. Il s'avère que les cellules d'ostéosarcomes présentent à la fois une hétérogénéité pour l'expression de la FPPs corrélée au niveau de sensibilité au Zol, et

une propension à la surexpression de la FPPs en réponse à un traitement de Zol à faible dose également corrélée au niveau de sensibilité au Zol .

Cette étude confirme le potentiel thérapeutique du Zol dans le traitement des tumeurs osseuses et révèle que la durée du traitement ainsi que la concentration employée sont des facteurs importants à prendre en compte afin d'éviter le développement de résistance métabolique.

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Farnesyl diphosphate synthase is involved in the resistance to zoledronic acid of osteosarcoma cells

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Abstract

We recently demonstrated original anti-tumor effects of zoledronic acid (Zol) on osteosarcoma cell lines independently of their p53 and Rb status. The present study investigated the potential Zol-resistance acquired by osteosarcoma cells after prolonged treatment. After 12 weeks of culture in the presence of 1 μm Zol, the effects of high doses of Zol (10–100 μm) were compared between the untreated rat (OSRGA, ROS) and human (MG63, SAOS2) osteosarcoma cells and Zol-pretreated cells in terms of cell proliferation, cell cycle analysis, migration assay and cytoskeleton organization. Long-term treatment with 1 μm Zol reduced the sensitivity of osteosarcoma cells to high concentrations of Zol. Furthermore, the Zol-resistant cells were sensitive to conventional anti-cancer agents demonstrating that this resistance process is independent of the multidrug resistance phenotype. However, as similar experiments performed in the presence of clodronate and pamidronate evidenced that this drug resistance was restricted to the nitrogen-containing bisphosphonates, we then hypothesized that this resistance could be associated with a differential expression of farnesyl diphosphate synthase (FPPS) also observed in human osteosarcoma samples. The transfection of Zol-resistant cells with FPPS siRNA strongly increased their sensitivity to Zol. This study demonstrates for the first time the induction of metabolic resistance after prolonged Zol treatment of osteosarcoma cells confirming the therapeutic potential of Zol for the treatment of bone malignant pathologies, but points out the importance of the treatment regimen may be important in terms of duration and dose to avoid the development of drug metabolic resistance.

Key words: osteosarcoma • bisphosphonate • zoledronic acid • metabolic resistance • farnesyl diphosphate synthase

Introduction

Osteosarcoma is the most frequent malignant primary bone tumor that develops mainly in the young, the median age of diagnosis being 18 years [1]. Despite recent improvements in chemotherapy and surgery, the problem of non-response to chemotherapy remains. Thus, current strategies for the treatment

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of high-grade osteosarcoma fail to improve its prognosis [2, 3], mainly because of chemotherapy resistance. This poor prognosis of osteosarcoma warrants new therapeutic strategies to improve the overall rate of survival.

Bisphosphonates (BPs) are stable synthetic analogues deriving from endogenous pyrophosphate (PPi) [4]. Various side chains can be added to the central carbon atom, thus producing a range of BPs with differential clinical activity and potency [5]. The most common therapeutic application of BPs is osteoporosis, and their use has been extended to the treatment of malignant osteolysis and hypercalcemia. Two groups of BPs can be identified including non-nitrogen-containing and nitrogen-containing BPs. The BPs that lack a nitrogen atom, closely related to PPi (such as clodronate, etidronate and tiludronate) are metabolized intracellularly to cytotoxic analogues of ATP and decrease osteoclast survival [5]. In contrast, nitrogen-containing BPs (such as pamidronate, alendronate, risedronate, ibandronate and zoledronate) induce apoptosis of osteoclasts by inhibiting enzymes of the mevalonate pathway, especially farnesyl diphosphate synthase (FPPS) [6, 7]. FPPS prevents the biosynthesis of cholesterol and isoprenoid lipids (FPP and geranylgeraniol diphosphate) which are required for the prenylation of small GTPases (i.e. Ras, Rho and Rac), a biochemical reaction essential for the anchorage of small GTPases to cell membranes and to protein-protein interactions [8]. In addition to their powerful anti-bone resorption effects, recent *in vitro* studies evidenced a direct anti-tumor activity exerted by zoledronic acid (Zol) on several cancer cells (myeloma, carcinoma and sarcoma) [9, 10]. Preclinical data confirmed the Zol anti-tumor activity in experimental models of bone tumors. Among these studies, we reported recently the enhancement of tumor regression and tissue repair when Zol is combined with ifosfamide in rat osteosarcoma [11] and that Zol suppresses lung metastases and prolongs overall survival of osteosarcoma-bearing mice [12]. Furthermore, recent clinical trials in patients suffering from malignant bone diseases demonstrated that Zol was safe and well tolerated at the approved dose of 4 mg i.v. every 3–4 weeks [4]. Because the main difficulty encountered in treating cancer relates to mutations carried by many tumor cells in key genes such as p53, Rb or proteins affecting caspase signalling, we demonstrated selective and original anti-tumor effects of Zol on several osteosarcoma cell lines independently of their p53 and Rb status [13]. Indeed, Zol inhibited osteosarcoma cell proliferation through a cell cycle arrest in S and G2/M phases and induced atypical apoptosis independent of caspase activation, characterized by the translocation of Apoptosis Inducing Factor and Endonuclease-G [13]. These data now allow to consider these molecules as potential therapeutic agents in clinical trials of tumor bone pathologies independently of the p53 and Rb status of the tumor.

The optimization and increase in specificity of cancer treatments has improved their efficacy and reduced the associated adverse effects, but unfortunately has not yet resulted in a cure for the majority of patients. Studies of the mechanisms by which tumor cells escape treatment is essential to circumvent drug resistance in cancer cells and to design new therapeutic protocols that are not subject to these drug-resistances [14]. Two types of resistance mechanism have been identified [15]. The first one results in resistance restricted to a specific drug or limited to a small number of related drugs, which can be bypassed by modification of the chemotherapeutic agent. The second mechanism confering multiresistance to many unrelated drugs, is called multidrug resistance (MDR) and is responsible for many failures of cancer treatment [16]. The most common mechanisms responsible for the various forms of resistance are the overexpression of efflux pumps, inhibition of apoptosis, increased repair of DNA damage, mutations in key cell cycle checkpoint genes and increased or altered drug targets [14]. Similar to non-osseous malignancies, osteosarcomas frequently exhibit a MDR phenotype explaining why patient survival has not improved since the mid-1980s despite advances in anticancer therapies. Because Zol represents a potential novel anti-neoplastic agent for the therapy of osteosarcoma, the present study investigated the potential development of innate and/or acquired resistance to Zol and the molecular mechanisms involved in this phenomenon.

Material and Methods

Patients

This study included seven patients (three females aged 41–93 years, four males aged 16–79 years) that were referred to our institution for the treatment of osteosarcoma.

All cases were diagnosed as osteogenic osteosarcoma based on histological samples obtained by open biopsies. The experimental procedures followed in the present study were in accordance with the ethical standards of the responsible institutional committee on human experimentation and with Helsinki Declaration of 1975, revised in 1983. The study was approved by the institutional ethic committee.

Cells, culture conditions and establishment of Zol-resistant cell lines

The rat osteosarcoma OSRGA cell line was initially established from a radio-induced osteosarcoma [17, 18]. The rat ROS17/2.8 cell line was kindly provided by Prof. H.J. Donahue (Penn State University, USA), and the human MG63 and SAOS2 cell lines were purchased from ATCC (USA). These cell lines were cultured in DMEM (BioWhittaker, Belgium) supplemented with 5% Fetal Calf Serum (Hyclone, France) and 2 mM L-glutamine (BioWhittaker). Rat and human osteosarcoma cell lines resistant to Zol (MG53res, SAOS2res, ROSres, OSRGAres) were established by 3 months of continuous treatment with 1 µm Zol.

Cell growth and viability

Cell growth and viability were determined by a cell proliferation reagent assay kit using sodium 3'[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) (Roche Molecular Biomedicals, Germany). Two thousand cells/well were plated into 96-well plates and cultured for 72 hrs in culture medium in the presence or the absence of 10^{-12}–10^{-4} M Zol. Zol was provided by Novartis Pharma AG (Basel, Switzerland) as the disodium hydrate form. In another set of experiments, cells were treated for 72 hrs in the presence or the absence of 10(9–10(6 M methotrexate (Sigma, France), doxorubicine (Sigma) and 1–50 µg/ml mafosfamide (Baxter, France), 10–1000 µm clodronate (Sigma), 1–500 µm pamidronate (Sigma) and 5 µm verapamil (Sigma). After the culture period, XTT reagent was added to each well and incubated for 5 hrs at 37°C, the corresponding absorbance was then determined at 490 nm. Cell viability was also assessed by trypan blue exclusion and live and dead cells were scored manually. Cell death was also monitored microscopically after Hoechst n°33258 staining (Sigma). In this experiment, cells were seeded at 10^4 cells/well in a 24-well plate and treated or not with 10 µm Zol for 48 hrs or 100 nm staurosporine (Sigma) for 16 hrs, stained by 10 µg/ml Hoechst reagent for 30 min at 37°C and then observed under UV microscopy (DMRXA, Leica, Germany). Statistical evaluation of the data was performed using the ANOVA test.

Western blot analysis

Zol-treated cells were lysed in RIPA buffer (150 mM NaCl, 5% Tris pH 7.4, 1% NP-40, 0.25% Na deoxycholate, 1 mM Na_3VO_4, 0.5 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin). Protein concentration was determined by the BCA kit (Pierce Chemical, USA). A total of 50 µg of total cell lysate protein were run on SDS-PAGE, electrophoretically transferred to Immobilon-P membrane (Millipore, MA, USA). The membrane was blotted with antibodies anti-p-Rb (Ser 807/811), -p-cdc2 (tyr15), -actin (Cell Signaling Technologies, USA), -$p21^{WAF1}$(BD Biosciences, USA) and the unprenylated form of Rap1A (Santa Cruz, USA) to indirectly quantified FPPS enzymatic activity, in PBS, 0.05% Tween 20 and 3% bovine serum albumin (BSA). The membrane was washed and probed with the secondary antibody coupled to horseradish peroxidase. Antibody binding was then visualized with the enhanced chemoluminescence system (ECL Kit; Roche Molecular Biomedicals). The band densities were measured using the GeneTools computer software program (SynGene).

Caspase -1, -3 and -8 activities

Caspase -1, -3 and -8 activities were assessed on 10 µl of total Zol-treated or not cell lysates using the kit CaspACE^{TM} Assay System, 'Fluorometric' (Promega, USA) following the manufacturer's recommendations. Cells treated with UV light for 30 sec 24 hrs before harvesting were used as a positive control. Results were expressed in arbitrary units referred to the total protein content.

Cell cycle analysis

OSRGA, MG63 and SAOS2 cells were incubated in the absence or the presence of 10 µm Zol for 48 hrs, trypsinized, washed twice and incubated in PBS containing 0.12% Triton X-100, 0.12 mM EDTA and 100 µg/ml ribonuclease A. Then 50 µg/ml propidium iodide were added to each sample for 20 min at 4°C. Cell cycle distribution was analyzed by flow cytometry (FAC Scan), based on 2N and 4N DNA content.

Time-lapse microscopy and confocal microscopic analysis

For time-lapse experiments, cells were seeded at 5 x 104 cells/well and cultured in 6-multiwell plates in the absence or the presence of 10 µm Zol. Phase-contrast photographs (Leica) were taken every 10 min during 60 hrs and edited

using the Metamorph™ software. Cell divisions and apoptotic cells were then manually scored. To study cell migration, cells plated in 6-well plates and cultured until confluence were treated or not with 10 μm Zol for 24 hrs before a slit was made in the cell monolayer. Actin filament detection was performed after cell treatment with or without 10 μm Zol fixed in 4% paraformaldehyde and stained with FITC-conjugated phalloidin (0.25 μg/ml; Sigma). Cover glasses were fitted with the Long Pro Kit (Molecular probes). Images were collected on a Leica TCS-SP1 confocal microscope with 63/1.4x oil immersion lens. The digital images were visualized with a 24-bit imaging system including Leica's TCS-NT software and projections were generated from z-stacks.

siRNA gene silencer

The FPPS gene expression was knocked down using specific human and rat FPPS siRNA (Ambion, France) and the INTERFERin™ transfection reagent (Polyplus transfection, France). Cells were seeded at 40% confluency in a 24-well plate 1 day before transfection. In each well 10 nm siRNA duplexes diluted in serum-free medium were incubated with 2 μl of INTERFERin™ for 30 min at room temperature. Then, 100 μl mixture per well were added onto the cells and incubated at 37°C. The 72 hrs-Zol treatment started 24 hrs after siRNA transfection. For each condition tested, a negative siRNA control was used (Santa Cruz biotechnology, Germany). Additional experiments were performed in the presence of geranylgeraniol (GGO) (Sigma, France).

RT-PCR analysis

Total RNA was isolated from cultured OSRGA, MG63 and SAOS2 cells using the TRIzol reagent (Invitrogen, France). First, RNA was reversed-transcribed (RT), using 400 U MMLV-RT from Invitrogen, then 2 μl of the RT reaction mixture were subjected to PCR using upstream and downstream primers to determine the expression of rat and human FPPS [Human FPPS sense: AGATCTGTGGGGGTCTTCCT, anti sense: TCCCGGAATGCTACTACCAC; Rat FPPS sense: AGTACAATCGGGGTCTGACG, anti sense: CGCGATAGGCAGGTAGAAAG] and 0.25 μl of 5 U/μl Taq polymerase (Eurobio, France). After the number of PCR cycles was increased, a plot was done for each sample, the cycle values corresponding to the linear part of the amplification curve were then determined (28 cycles, Tm = 58°C) and used to quantify the message *versus* the 18S signal determined in the same way. The PCR products were electrophoresed in 1% agarose gel-containing ethidium bromide. The band densities were measured using the GeneTools computer software program. Three independent experiments were performed for each gene and a representative experiment is shown in the Results section.

Results

Osteosarcoma cell lines develop Zol-resistance after long-term continuous treatment with low-dose Zol

Consistent with previous results [11, 12, 19], Zol treatment of Zol-sensitive rat ROS, OSRGA (Fig. 1A) and human MG63, SAOS2 (Fig. 1B) osteosarcoma cells strongly reduced their proliferation and induced the cell death without any caspase 1, 3 or 8 activation (data not shown). Thus, 0.1–100 μM Zol decreased the viable cell number in a dose-dependent manner (IC50: 1–8 μM) as revealed by the XTT assay. After 3 month continuous treatment with 1 μm Zol, rat and human osteosarcoma cells became less sensitive to Zol and resistant cell lines (OSRGAres, ROSres, MG63res, SAOS2res) were then progressively established (Fig. 1A). Indeed, the potency of Zol to affect cell proliferation was strongly reduced on human resistant cell lines and Zol was ineffective on rat resistant cell lines (Fig. 1A).

The influence of this resistance process was also assessed on the other known activities of Zol on tumor cells [cell cycle (Fig. 1B), DNA checkpoints (Fig. 1B), cytoskeleton (Fig. 1C), cell migration (Fig. 1C) [13]). Cell cycle analysis was performed after 48 hrs of 10 μm Zol-treatment. The results obtained confirmed that 48 hrs of Zol-treatment induced a strong cell cycle arrest in S and G2/M phases in Zol-sensitive OSRGA cells (Fig. 1B, [13]) and showed that Zol-treatment did not modulate the cell cycle in OSRGAres cells (Fig. 1B). Indeed, the number of cells in S, G2/M phases strongly increased from 35% to 53% for OSRGA cells in the presence of 10 μm Zol concomitantly with a decrease of cells in G_0/G_1 phase: 42% *versus* 64% (Fig. 1B). A similar phenomenon was observed in human osteosarcoma cell lines (data not shown). We therefore investigated by western blot whether the DNA checkpoint proteins were involved in the cell cycle blockade observed in the presence of Zol. Thus, the treatment of sensitive

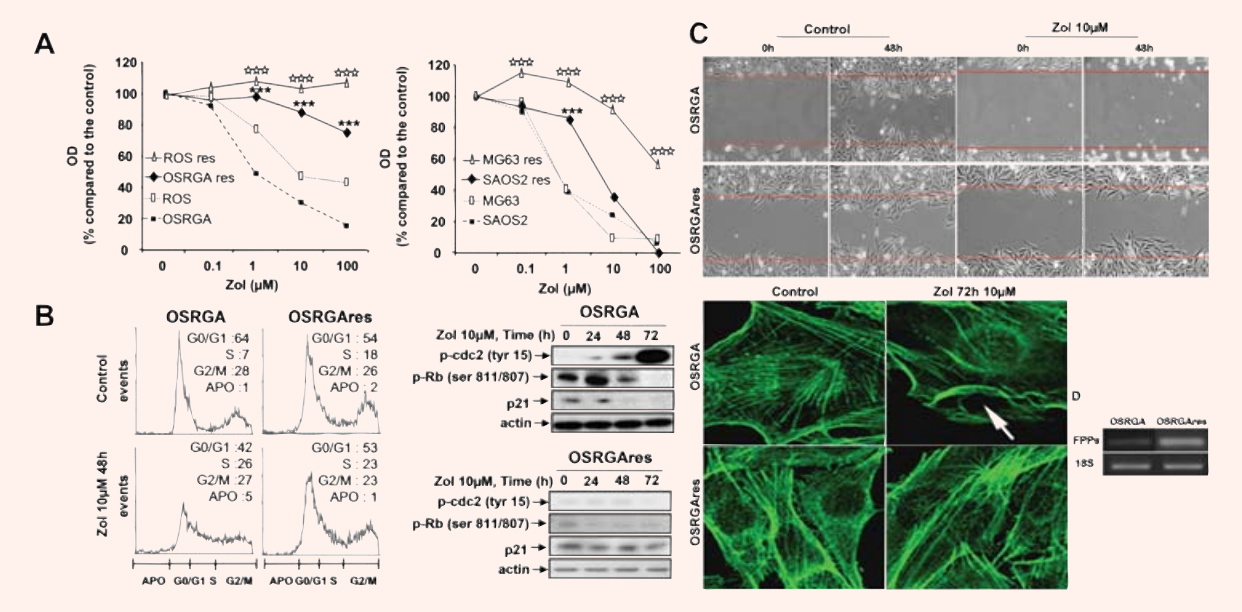

Fig. 1 Osteosarcoma cell lines develop Zol-resistance after long-term of continuous treatment with low doses of Zol. (**A**) rat (OSRGA, ROS) and human (MG63, SAOS2) sensitive and resistant (corresponding cell Name-res) osteosarcoma cell lines were treated with increasing concentrations of Zol (0.1--100 μm) for 72 hrs. The number of viable cells was then measured using the XTT assay. Graphs represent the mean values of three independent experiments performed in triplicate. *** $P < 0.001$. Statistical evaluation of the data was performed using the ANOVA test. (**B**) Cell cycle distribution of OSRGA and OSRGAres, treated or not with 10 μm Zol for 48 hrs was analyzed by propidium iodide staining and FACS analysis. G1/S and G2/M DNA checkpoints were analyzed by western blot and compared between sensitive and resistant OSRGA osteosarcoma cell lines in the presence or absence of 10 μm Zol for 24, 48 and 72 hrs. All experiments were repeated three times and a representative blot is shown. (**C**) Zol effects on organization of actin stress fibres were observed by confocal microscopy after phalloïdine staining. The actin network reorganization was associated with membrane ruffling (white arrow) in Zol-sensitive OSRGA cell line (Original magnification: x1000). Zol effects on cell migration were also analyzed by time-lapse microscopy. The horizontal bars represent the limit of the slit cut performed on the cell monolayer at the start of the experiment (Original magnification: x100). (**D**) Farnesyl diphosphate synthase (FPPS) transcription level was determined by semi quantitative RT-PCR in OSRGA sensitive and resistant cell lines. The 18S was used as a control.

OSRGA cells by 10 μm Zol increased the inactive form of cdc2 (p-cdc2, Tyr15) after 72 hrs of treatment. Simultaneously, Zol strongly reduced p21 expression and transiently upregulated Rb phosphorylation (Ser 807 and 811) after 24 hrs of Zol treatment (Fig. 1B). No modulation of p-cdc2, Rb and p21 was observed in OSRGAres cells regardless of the duration of Zol treatment (Fig. 1B).

As Zol has been shown to disturb cytoskeletal organization and to inhibit cell migration [13], we wondered whether Zol could alter such parameters in OSRGAres cells. Confocal microscopy revealed a major disorganization of the actin stress fibres associated with membrane ruffling in sensitive OSRGA cells treated with 10 μm Zol for 72 hrs, this was never observed in OSRGAres cells (Fig. 1C). Moreover, as shown by the time-lapse assay, 10 μm Zol totally blocked the migration of sensitive OSRGA cells but was not able to abolish migration of OSRGAres cells (Fig. 1C).

The molecular mechanism involved in the reduced-Zol sensitivity is not associated with a multidrug resistance (MDR) phenotype and is restricted to the nitrogen-containing bisphosphonates

The potential role of the MDR phenotype in the Zol resistance phenomenon was assessed by XTT assays. The MDR phenotype is conventionally

defined as the resistance of cells to conventional chemotherapeutic agents such as mafosfamide, methotrexate and doxorubicin [20, 21]. The XTT assays revealed that OSRGAres cells were still always sensitive to increasing doses of mafosfamide, methotrexate and doxorubicin (Fig. 2). Furthermore, 5 μm verapamil, a P-gp pump inhibitor [22] was not able to abolish the Zol resistance (Fig. 2). Overall, these data demonstrate that the Zol resistance was not associated with MDR phenotype. In addition, similar experiments performed in the presence of clodronate, a non-nitrogen containing-BP [4], revealed that OSRGAres are as sensitive to clodronate as they are to lower concentrations of Zol (Fig. 2). When, osteosarcoma cells were treated with another nitrogen-containing BP, pamidronate that also targets FPPS, it significantly reduced Zol-sensitive OSRGA proliferation in contrast to OSRGAres cells, which are also resistant to pamidronate (Fig. 2). Similar results have been obtained with the osteosarcoma cell lines MG63 and SAOS2 (data not shown). These experiments demonstrated that the Zol-resistance phenomenon in osteosarcoma cells appears to be MDR-independent and is apparently restricted to nitrogen-containing BPs.

Farnesyl diphosphate synthase (FPPS) is implicated in the Zol-resistance mechanism of osteosarcoma cell lines

FPPS being the main molecular target of nitrogen containing BPs [23], the FPPS transcript expression was analyzed by RT-PCR and compared in sensitive OSRGA and OSRGAres cells (Fig. 1D). Thus, the Zol-resistant cells expressed a higher level of FPPS mRNA than the sensitive cells. To further determine the involvement of FPPS in the Zol-resistance mechanism of human and rat osteosarcoma cells, the effect of Zol on OSRGA, OSRGAres, MG63 and SAOS2 was analyzed after transfection with FPPS siRNA. Semi-quantitative RT-PCR analysis was used to evaluate the efficacy of FPPS siRNA on FPPS mRNA expression. In all experiments, FPPS mRNA levels were significantly decreased in FPPS siRNA-transfected cell lines compared to the siRNA control (Fig. 3A). Inhibition of FPPS activity was then assessed indirectly by the expression of the unprenylated form of the small GTPase Rap1A (unRAP1A) that is expressed after inhibition of FPPS [24, 25] (Fig. 3B). The transfection of Zol-sensitive cells with FPPS siRNA strongly increased their sensitivity to Zol in all osteosarcoma cell lines studied. Indeed, FPPS siRNA transfection modified the unRAP1A expression kinetic in OSRGA, MG63 and SAOS2 cells. In the presence of FPPS siRNA, unRAP1A expression was strongly induced by 1 μm Zol treatment for 24 hrs whereas its expression was only observed with 10 μm Zol treatment for 48 hrs in control siRNA transfected cells (Fig. 3B). In OSRGAres cells, a very weak expression of unRAP1A was observed after Zol treatment. Interestingly, FPPS siRNA re-induced the sensitivity to Zol treatment in these resistant cells to a level comparable to parental OSRGA cells transfected with FPPS siRNA. Thus, the unRAP1A expression was observed after 24 hrs treatment with 1 μm Zol in FPPS siRNA-OSRGAres transfected (Fig. 3B). Similarly, micoscopic observations confirmed the FPPS siRNA effects on the sensitization of osteosarcoma cells to Zol treatment (Fig. 3C). Thus, an increase of floating cell number associated with an inhibition of cell proliferation was observed after transfection of all osteosarcoma cell lines with FPPS siRNA (Fig. 3C).

XTT analyses were performed to determine the impact of FPPS siRNA on Zol activity (Fig. 3D). Transfection with FPPS siRNA significantly increased the sensitivity to Zol treatment of all osteosarcoma cell lines analyzed (Fig. 3D). The sensitivity to 10 μm Zol was up-modulated by 22%, 31%, 53% and 42% in OSRGA, OSRGAres MG63 and SAOS2, respectively, in the presence of FPPS siRNA compared to the control siRNA (Fig. 3D). Furthermore, the efficacy of FPPS siRNA occured for lower doses of Zol in OSRGAres compared to OSRGA cells (respectively 22% and 1% increase of sensitivity in the presence of 0.1 μm Zol) (Fig. 3D).

siRNA FPPS increases the Zol-induced blockade of the cell cycle in S, G2/M phases in osteosarcoma cell lines

We previously demonstrated that Zol induces osteosarcoma cell cycle arrest in S, G2/M phases in OSRGA sensitive cells [13]. To determine whether FPPS siRNA could modulate this sensitivity, the cell cycle of FPPS siRNA-transfected osteosarcoma cells was analyzed by flow cytometry. Figure 4 reveals that FPPS siRNA accentuates the Zol-induced effects

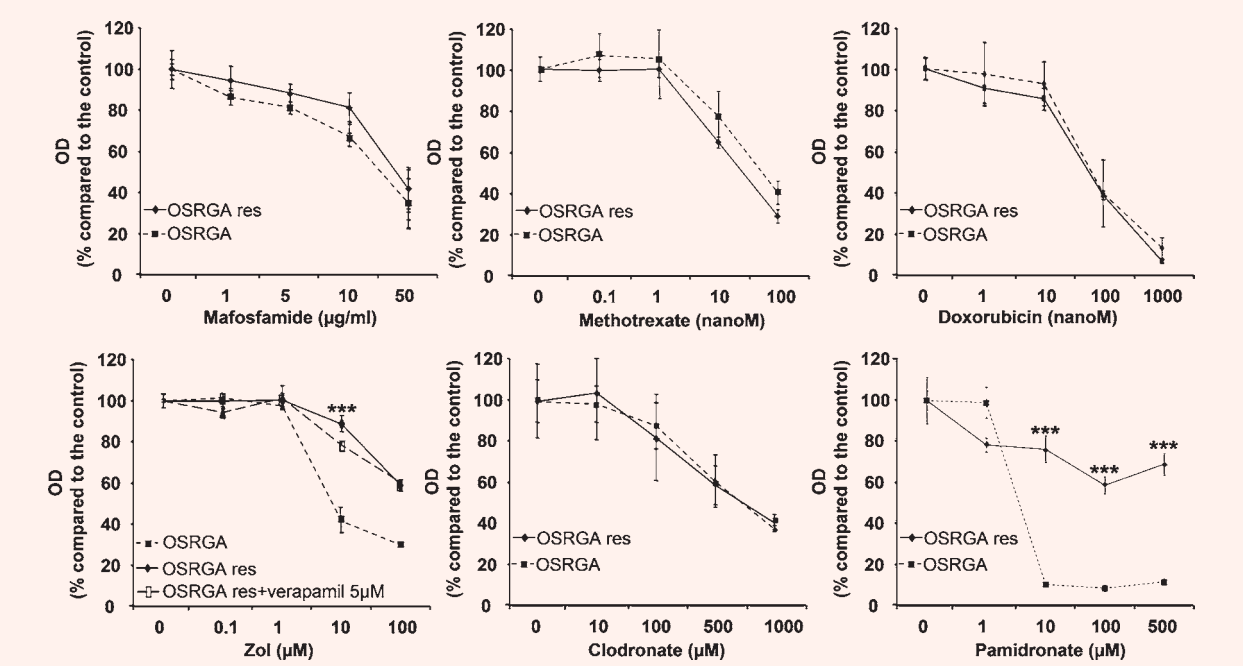

Fig. 2 The molecular mechanism involved in the reduced-Zol sensitivity is not associated with a multidrug resistance (MDR) phenotype and is restricted to the nitrogen-containing bisphosphonates. OSRGA and OSRGAres sensitivity to conventional anti-cancer agents mafosfamide, methotrexate, doxorubicin and sensitivity to Zol in the presence or absence of a P-gp pump inhibitor (5 µm verapamil) was analyzed by the XTT assay. Similar experiments were performed in the presence of clodronate and pamidronate. Graphs represent the mean values of three independent experiments performed in triplicate. Error bars represent the standard deviation. ***$P < 0.001$. Statistical evaluation of the data was performed using the ANOVA test.

observed on cell-cycle distribution, leading to a significant increase of cells blocked in S phase compared to the control siRNA. Indeed, the number of cells in S phase increased from 26% to 30% for OSRGA, from 20% to 26% for MG63, from 34% to 46% for SAOS2 and from 23% to 38% for OSRGAres cells in the presence of FPPS siRNA compared to the control siRNA after 48 hrs of treatment with 10 µm Zol (Fig. 4). Furthermore, these observations were concomitant with a significant reduction of the cell number in G_0/G_1 phase: 35% *versus* 42% for OSRGA, 61% *versus* 69% for MG63, 36% *versus* 57% for SAOS2 and 41% *versus* 53% for OSRGAres.

Geranyl geraniol (GGO) reversed the FPPS siRNA effects in osteosarcoma cell lines

To determine whether the effects previously demonstrated for the FPPS siRNA in osteosarcoma cells are reversible, FPPS siRNA transfected cells treated with increasing doses of Zol were cultured in the presence of 25 µm geranylgeraniol, the FPPS metabolic product (Fig. 5). GGO protected rat and human osteosarcoma cell lines from the effects of Zol in the FFPS siRNA-transfected cells and totally reversed FPPS siRNA effects (Fig. 5A). We therefore investigated by western blot the expression kinetic of unRAP1A in the presence of 25 µm GGO in FPPS siRNA-transfected cells (Fig. 5B). GGO totally abolished unRAP1A expression similar to what had been observed in Zol-resistant cell lines (Figure 3B). Overall, these data then strengthen our conclusion that FPPS is involved in the Zol-resistance mechanism.

Dual origin of Zol resistance: innate and/or acquired

To explain the origin of the Zol-resistance observed in osteosarcoma cell lines, two hypotheses can be

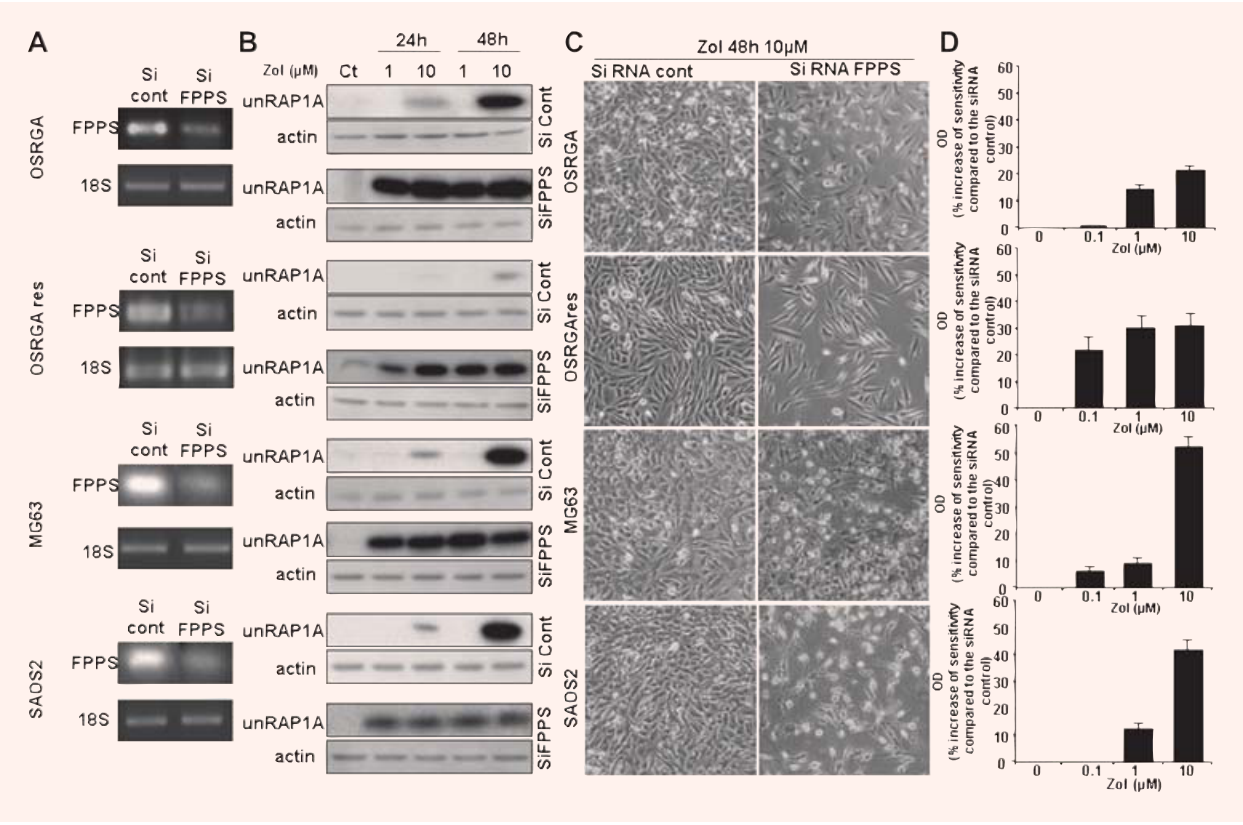

Fig. 3 Involvement of farnesyl diphosphate synthase (FPPS) in the Zol-induced resistance mechanism in osteosarcoma. (**A**) Farnesyl diphosphate synthase (FPPS) transcription level was determined by semi quantitative RT-PCR in FPPS siRNA transfected cell lines compared to the siRNA control cells. The 18S was used as a control. (**B**) Western blot analysis of unprenylated RAP1A (unRAP1A) from OSRGA cell lines transfected with FPPS siRNA and control siRNA, treated 24 and 48 hrs with 1 and 10 μm Zol. All experiments were repeated three times, and a representative blot is shown. (**C**) Photomicrographs of FPPS siRNA transfected cells after 48 hrs with 10 μm Zol compared to control siRNA. Original magnification: ×100. (**D**) Rat (OSRGA, OSRGAres) and human (MG63, SAOS2) osteosarcoma cell lines were transfected with FPPS siRNA and treated after 24 hrs of culture by increasing concentrations of Zol (0.1–10 μm) for 72 hrs. The number of viable cells was then determined using the XTT assay. Histograms represent the percentage of the increased sensitivity to Zol in the presence of FPPS siRNA compared to control siRNA. Values are mean of three independent experiments performed in triplicate. Error bars represent the standard deviation.

proposed: (*i*) an innate resistance mechanism linked to differential levels of FPPS expression and associated with selection of a sub-population of cells expressing a higher FPPS activity, (*ii*) an acquired resistance mechanism linked to an increased FPPS transcription level as a feedback response to long-term, low-dose Zol treatment. To distinguish between these two hypotheses, OSRGA osteosarcoma cell lines were treated with low Zol concentrations (1–10^4 pM) for 72 hrs (Fig. 6A). Low concentrations of Zol induced a 60% increase of viable cells and up-modulated the expression of FPPS mRNA in a dose-dependent manner (Fig. 6A), these results support acquired resistance to Zol. Since a potential mechanism of innate resistance could be also envisaged, OSRGA cell line was cloned by limiting dilution and the expression of FPPS was analyzed by semi-quantitative RT-PCR (Fig. 6B). Several clones were isolated with heterogenous sensitivity to Zol treatment (Fig. 6B). Furthermore, the isolated clones expressed differential levels of FPPS related to their sensitivity to Zol treatment, these results support innate resistance to Zol (Fig. 6B). Similarly, we analyzed the transcriptional expression of FFPS in seven human osteosarcoma samples analyzed by semi-quantitative RT-PCR before any chemotherapy (Fig. 6C).

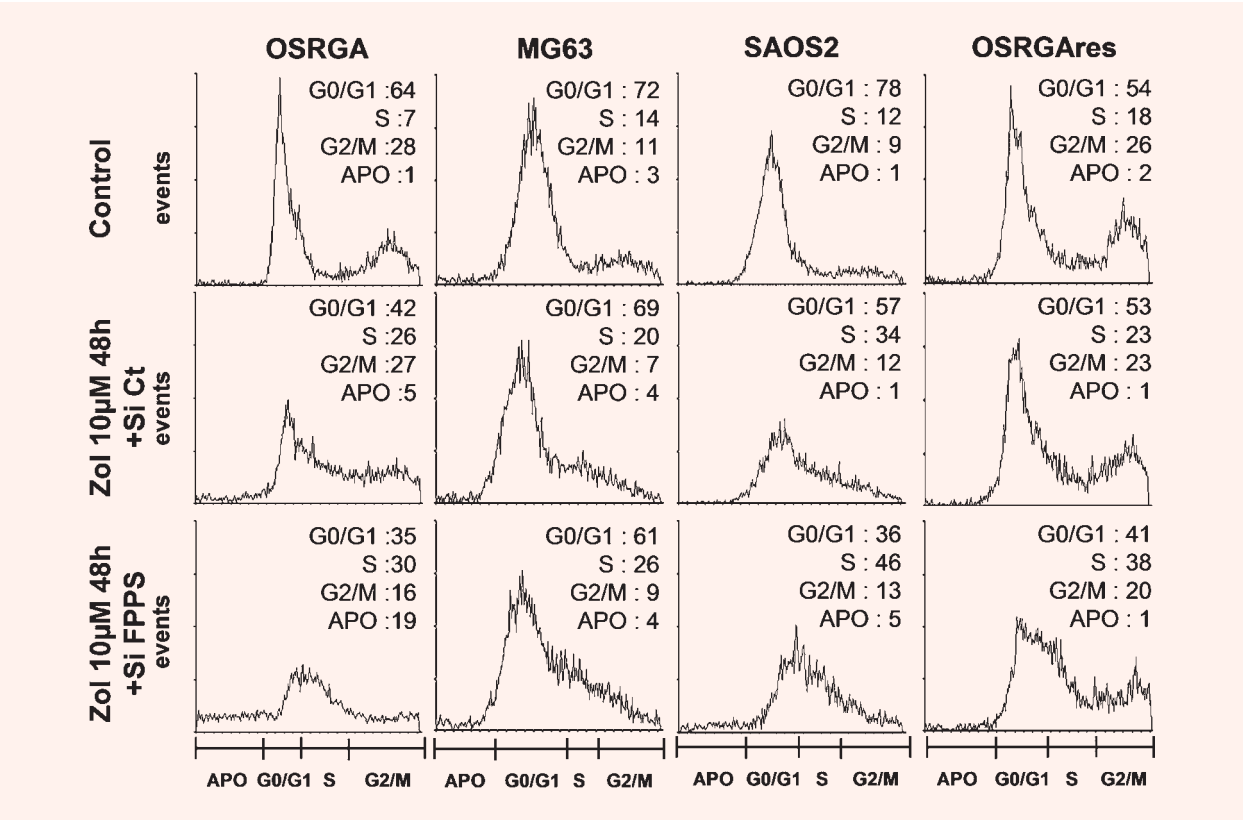

Fig. 4 FPPS siRNA increases the Zol-induced blockade of the cell cycle in S phases in osteosarcoma cell lines. Cell cycle distribution of osteosarcoma cell lines (FPPS siRNA versus control siRNA) treated or not treated with 10 µm Zol for 48 hrs were analyzed by propidium iodide staining and FACS analysis.

The results revealed that a very high heterogeneity of FPPS expression in these patients strengthening the hypothesis of innate resistance to Zol.

Discussion

The first effects of BPs on calcium metabolism were discovered over 30 years ago, and these drugs have become the most widely used agents in the treatment of bone diseases associated with excessive resorption (osteoporosis, malignant osteolysis, etc). The recent evidences of an anti-tumor effect of nitrogen-containing BPs have led to investigation of the potential-acquired resistance mechanism. Indeed, failure of anti-cancer therapies often occur from innate or acquired drug resistance of the tumor cells to the chemotherapeutic agents [26]. In this context, the elucidation of potential resistance mechanisms to the Zol will allow adaptation of the treatment regimen in terms of duration and dose to avoid the development of drug resistance. The present study demonstrated that after 3 months of continuous treatment with 1 µm Zol, osteosarcoma cell lines became less sensitive to Zol inhibition and resistant cell lines were then progressively established. Furthermore, this resistance appeared to be independent of the MDR phenotype and was clearly related to a differential expression of FPPS.

To exert its activities, Zol must be internalized by cells. Although the mode of Zol internalization is still controversial, two mechanisms have been proposed: first, cellular uptake of Zol may require fluid-phase endocytosis in osteoclasts [27]; in the second case, integrins located at the cell membrane could represent a binding site for Zol which could explain why Zol is able to inhibit cell adhesion and that RGD peptide prevents the Zol effects on osteosarcoma cell lines [13]. However, it remains unclear whether cell

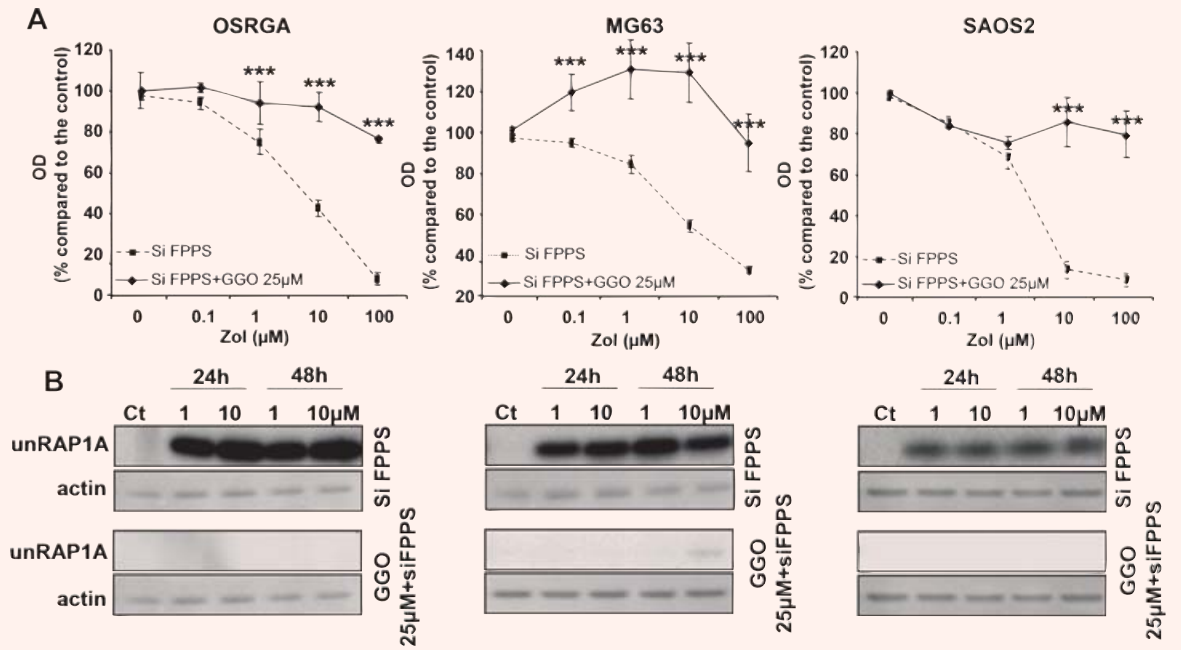

Fig. 5 Geranylgeraniol (GGO) reverses FPPS siRNA effects in osteosarcoma cell lines. (**A**) Rat (OSRGA) and human (MG63, SAOS2) osteosarcoma cell lines were transfected with FPPS siRNA and treated 24 hrs after with increasing concentrations of Zol (0.1–100 µm) for 72 hrs in the presence or not of 25 µm GGO. The number of viable cells was then determined using the XTT assay. Graphs represent the mean values of three independent experiments performed in triplicate. Error bars represent the standard deviation. ***$P < 0.001$. Statistical evaluation of the data was performed using the ANOVA test. (**B**) Western blot analysis of unprenylated RAP1A (unRAP1A) form. Cells transfected with control siRNA or with FPPS siRNA combined with 25 µm GGO were treated with 1 and 10 µm Zol for 24 and 48 hrs. All experiments were repeated three times, and a representative blot is shown.

types other than osteoclasts can internalize BPs [27]. Recently, Notarnicola *et al.* demonstrated that high FPPS activity level correlates to a stronger inhibition of cellular apoptosis in colorectal cancer cells [28]. Similarly, Ortiz-Gomez *et al.* demonstrated that overexpression of FPPS confers resistance to risedronate in *Leishmania major* and that the degree of resistance was correlated with an increase in this enzymatic activity [29]. These data strongly support our present results and strengthen the pivotal function of FPPS in the Zol-resistance mechanism. Although FPPS is considered as the main target of nitrogen-containing-BPs, the inhibition of prenylation being the most likely explanation for their biological effects, van Beek *et al.* evidenced that undetermined additional mechanisms could be involved which may be also proposed for specific resistance mechanisms in certain specific cell types [30].

In the present study, we wondered what could be the origin of the Zol-induced resistance mechanism: an innate or an acquired resistance mechanism? In fact, the results did not allow us to distinguish between these two hypotheses. The main argument in favor of an innate resistance mechanism is the differential FPPS expression of OSRGA osteosarcoma sub-clones composing the heterogenous 'parental' OSRGA cell line. Indeed, Zol treatment exerts a selective inhibitory effect on cancer cells expressing less FPPS and after several weeks of culture, FPPS overexpressing cells become predominant and emerge from the parental population (Fig. 6B) [28]. Interestingly, we observe the same kind of heterogeneity in patients. We haven't yet correlated this with sensitivity to Zol treatment but it will be performed in a clinical trial and we could expect using FPPs expression level as a prognosis factor of Zol efficacy. On the other hand, the effect of Zol treatment on FPPS expression is in favor of an acquired resistance mechanism. Indeed, 72 hrs treatment with low doses of Zol ($1–10^4$ pM) increased FPPS

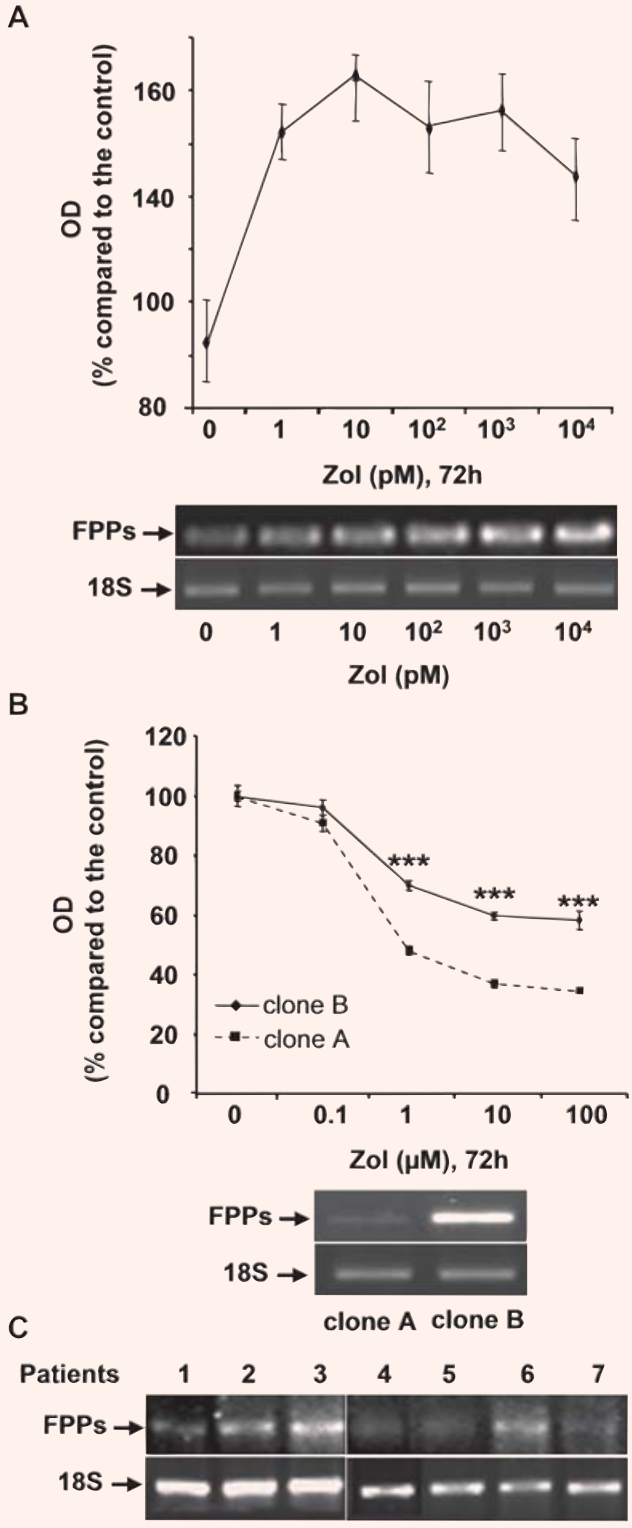

Fig. 6 Dual origin of Zol-resistance: innate and/or acquired. (**A**) OSRGA osteosarcoma cell lines were treated with increasing low concentration of Zol (1–10^4 pM) for 72 hrs. The number of viable cells was then determined using a XTT assay. Graphs represent the average values of three independent experiments performed in triplicate. Error bars represent the standard deviation. Farnesyl diphosphate synthase (FPPS) transcription level was determined by semi-quantitative RT PCR under the same conditions of Zol treatment. The 18S was used as a control. (**B**) Similar experiments were performed with higher concentrations of Zol (0.1–100 μM) in two OSRGA clones obtained by limiting dilution. ***$P < 0.001$. Statistical evaluation of the data was performed using the ANOVA test. (**C**) Transcriptional analysis of FFPS in seven human osteosarcoma samples analyzed by semi-quantitative RT-PCR.

expression in OSRGA cells (Fig. 6A) inducing the development of FPPS overexpressing tumor cells (Fig. 1D). Similar involvement has been envisaged in myeloma cells [31]. This hypothesis was also strengthened by Ortiz-Gomez *et al.* who obtained resistant cell lines by stepwise selection in the presence of risedronate, resulting in the development of resistant promastigotes exhibiting increased levels of FPPS at the transcriptional and the translational levels [29]. These authors considered that as a result of drug pressure, cells overcame the effects of risedronate by overexpressing the target protein. Such modification has been already observed in osteosarcoma patients treated with chemotherapy. Indeed, after comparison of primary biopsy tissue with that removed after metastasectomy, genetic changes acquired by the tumors have been demonstrated [32, 33]. An acquired resistance to BPs was also reported by Papapoulos *et al.* in Paget's disease [34]. These authors argued that resistance to the action of BPs in Paget's disease is caused by disease-related factors rather than decreased responsiveness of the molecular target in contrast to the present data. They supported this hypothesis with studies using statins that target the same intracellular biochemical pathway upstream of FPPS, these studies showed no evidence of development of resistance to their action [35, 36]. They also presented data suggesting that acquired resistance is specific for pamidronate and does not extend to other nitrogen-containing BPs. In summary, various and concomitant resistance mechanisms cannot be excluded: direct or indirect effects on FPPS, innate and/or acquired mechanisms.

Chemotherapy resistance in osteosarcoma is well documented [37]. Osteosarcoma cells are subjected to genetic disturbances such as alterations in the tumor suppressor pathways centered on p53 and Rb [38, 39], changes in oncogenes/anti-oncogenes such as deletions in $p16^{INK4A}$ (cyclin-dependent kinase inhibitor 2A), c-fos overexpression and amplification of cyclin-dependent kinase 4 [40–42]. These genetics instabilities lead to heterogenic cell populations within the same tumor and to the emergence of resistant tumor cells. The most described resistance phenomena concern widely used chemotherapeutic agents such as cisplatin, doxorubicin or methotrexate. In these cases, the resistance mechanisms involved are mutation of the drug target, up- or down-regulation of the drug target, decreased drug uptake, drug inactivation, increased drug elimination and increased DNA repair [43–45]. Multidrug resistance phenotype (MDR), due to P-gp or related protein overexpression is the most reported resistance mechanism. In osteosarcoma, MDR1 [46] or P-gp [47] expression could be used as a prognostic marker for sensitivity to chemotherapy, allowing the selection of patients for whom alternative treatments may be considered. Recently, other prognostic factors have been described, such as the expression level of clusterin/apolipoprotein J [48] or expression of a pregnane xenobiotic receptor (PXR), a major inducer of cytochrome P450 3A4 [49]. Therefore, these factors may also represent predictive markers correlating with the response of cancer cells to chemotherapy.

We described in ostesarcoma a Zol-resistance mechanism specific to nitrogen-containing BPs which did not confer simultaneous resistance to other unrelated drugs. In this context, drug resistance could be circumvented using multiple drugs with different cellular targets and different mechanisms of action. For instance, when Zol is associated with ifosfamide in rat osteosarcoma, enhanced tumor regression and tissue repair have been observed [11]. In the future, Zol could be combined with other chemotherapeutic agent to increase therapeutic efficacy and avoid the emergence of resistance mechanism [50].

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References

1. **Link MP, Goorin AM, Horowitz M, Meyer WH, Belasco J, Baker A, Ayala A, Shuster J.** Adjuvant chemotherapy of high-grade osteosarcoma of the extremity. Updated results of the Multi-Institutional Osteosarcoma Study. *Clin Orthop Relat Res.* 1991: 8–14.

2. **Rosen G, Murphy ML, Huvos AG, Gutierrez M, Marcove RC.** Chemotherapy, en bloc resection, and prosthetic bone replacement in the treatment of osteogenic sarcoma. *Cancer.* 1976; 37: 1–11.
3. **Provisor AJ, Ettinger LJ, Nachman JB, Krailo MD, Makley JT, Yunis EJ, Huvos AG, Betcher DL, Baum ES, Kisker CT, Miser JS.** Treatment of nonmetastatic osteosarcoma of the extremity with preoperative and postoperative chemotherapy: a report from the Children's Cancer Group. *J Clin Oncol.* 1997; 15: 76–84.
4. **Heymann D, Ory B, Gouin F, Green JR, Redini F.** Bisphosphonates: new therapeutic agents for the treatment of bone tumors. *Trends Mol Med.* 2004; 10: 337–43.
5. **Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Monkkonen J, Frith JC.** Cellular and molecular mechanisms of action of bisphosphonates. *Cancer.* 2000; 88: 2961–78.
6. **Rogers MJ.** New insights into the molecular mechanisms of action of bisphosphonates. *Curr Pharm Des.* 2003; 9: 2643–58.
7. **Russell RG.** Bisphosphonates: mode of action and pharmacology. *Pediatrics.* 2007; 119 Suppl 2: S150–62.
8. **Coxon FP, Helfrich MH, Van't Hof R, Sebti S, Ralston SH, Hamilton A, Rogers MJ.** Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. *J Bone Miner Res.* 2000; 15: 1467–76.
9. **Mackie PS, Fisher JL, Zhou H, Choong PF.** Bisphosphonates regulate cell growth and gene expression in the UMR 106-01 clonal rat osteosarcoma cell line. *Br J Cancer.* 2001; 84: 951–8.
10. **Sonnemann J, Eckervogt V, Truckenbrod B, Boos J, Winkelmann Wvan Valen F.** The bisphosphonate pamidronate is a potent inhibitor of human osteosarcoma cell growth *in vitro*. *Anticancer Drugs.* 2001; 12: 459–65.
11. **Heymann D, Ory B, Blanchard F, Heymann MF, Coipeau P, Charrier C, Couillaud S, Thiery JP, Gouin F, Redini F.** Enhanced tumor regression and tissue repair when zoledronic acid is combined with ifosfamide in rat osteosarcoma. *Bone.* 2005; 37: 74–86.
12. **Ory B, Heymann MF, Kamijo A, Gouin F, Heymann D, Redini F.** Zoledronic acid suppresses lung metastases and prolongs overall survival of osteosarcoma-bearing mice. *Cancer.* 2005; 104: 2522–9.
13. **Ory B, Blanchard F, Battaglia S, Gouin F, Redini F, Heymann D.** Zoledronic acid activates the DNA S-phase checkpoint and induces osteosarcoma cell death characterized by apoptosis-inducing factor and endonuclease-G translocation independently of p53 and retinoblastoma status. *Mol Pharmacol.* 2007; 71: 333–43.
14. **Gottesman MM.** Mechanisms of cancer drug resistance. *Annu Rev Med.* 2002; 53: 615–27.
15. **Gottesman MM, Ambudkar SV, Ni B, Aran JM, Sugimoto Y, Cardarelli CO, Pastan I.** Exploiting multidrug resistance to treat cancer. *Cold Spring Harb Symp Quant Biol.* 1994; 59: 677–83.
16. **Ozben T.** Mechanisms and strategies to overcome multiple drug resistance in cancer. *FEBS Lett.* 2006; 580: 2903–9.
17. **Jasmin C, Allouche M, Jude JG, Klein B, Thiery JP, Perdereau B, Gongora R, Gongora G, Mazabraud A.** [An experimental model of osteosarcomas in rats]. *Sem Hop.* 1982; 58: 1684–9.
18. **Klein B, Pals S, Masse R, Lafuma J, Morin M, Binart N, Jasmin JR, Jasmin C.** Studies of bone and soft-tissue tumours induced in rats with radioactive cerium chloride. *Int J Cancer.* 1977; 20: 112–9.
19. **Evdokiou A, Labrinidis A, Bouralexis S, Hay S, Findlay DM.** Induction of cell death of human osteogenic sarcoma cells by zoledronic acid resembles anoikis. *Bone.* 2003; 33: 216–28.
20. **Biedler JL, Riehm H.** Cellular resistance to actinomycin D in Chinese hamster cells *in vitro*: cross-resistance, radioautographic, and cytogenetic studies. *Cancer Res.* 1970; 30: 1174–84.
21. **Gottesman MM, Pastan I.** Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem.* 1993; 62: 385–427.
22. **Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y.** Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.* 1981; 41: 1967–72.
23. **Gibbs JB, Oliff A.** The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. *Annu Rev Pharmacol Toxicol.* 1997; 37: 143–66.
24. **Suri S, Monkkonen J, Taskinen M, Pesonen J, Blank MA, Phipps RJ, Rogers MJ.** Nitrogen-containing bisphosphonates induce apoptosis of Caco-2 cells *in vitro* by inhibiting the mevalonate pathway: a model of bisphosphonate-induced gastrointestinal toxicity. *Bone.* 2001; 29: 336–43.
25. **Reszka AA, Halasy-Nagy J, Rodan GA.** Nitrogen-bisphosphonates block retinoblastoma phosphorylation and cell growth by inhibiting the cholesterol biosynthetic pathway in a keratinocyte model for esophageal irritation. *Mol Pharmacol.* 2001; 59: 193–202.
26. **Kruh GD.** Introduction to resistance to anticancer agents. *Oncogene.* 2003; 22: 7262–4.
27. **Coxon FP, Thompson K, Rogers MJ.** Recent advances in understanding the mechanism of action

of bisphosphonates. *Curr Opin Pharmacol.* 2006; 6: 307–12.

28. **Notarnicola M, Messa C, Cavallini A, Bifulco M, Tecce MF, Eletto D, Di Leo A, Montemurro S, Laezza C, Caruso MG.** Higher farnesyl diphosphate synthase activity in human colorectal cancer inhibition of cellular apoptosis. *Oncology.* 2004; 67: 351–8.
29. **Ortiz-Gomez A, Jimenez C, Estevez AM, Carrero-Lerida J, Ruiz-Perez LM, Gonzalez-Pacanowska D.** Farnesyl diphosphate synthase is a cytosolic enzyme in Leishmania major promastigotes and its overexpression confers resistance to risedronate. *Eukaryot Cell.* 2006; 5: 1057–64.
30. **van Beek ER, Cohen LH, Leroy IM, Ebetino FH, Lowik CW, Papapoulos SE.** Differentiating the mechanisms of antiresorptive action of nitrogen containing bisphosphonates. *Bone.* 2003; 33: 805–11.
31. **Salomo M, Jurlander J, Nielsen LB, Gimsing P.** How myeloma cells escape bisphosphonate-mediated killing: development of specific resistance with preserved sensitivity to conventional chemotherapeutics. *Br J Haematol.* 2003; 122: 202–10.
32. **Ifergan I, Meller I, Issakov J, Assaraf YG.** Reduced folate carrier protein expression in osteosarcoma: implications for the prediction of tumor chemosensitivity. *Cancer.* 2003; 98: 1958–66.
33. **Zhou H, Randall RL, Brothman AR, Maxwell T, Coffin CM, Goldsby RE.** Her-2/neu expression in osteosarcoma increases risk of lung metastasis and can be associated with gene amplification. *J Pediatr Hematol Oncol.* 2003; 25: 27–32.
34. **Papapoulos SE, Eekhoff EM, Zwinderman AH.** Acquired Resistance to Bisphosphonates in Paget's Disease of Bone. *J Bone Miner Res.* 2006; 21 Suppl 2: P88–91.
35. **Endo A.** The discovery and development of HMG-CoA reductase inhibitors. *J Lipid Res.* 1992; 33: 1569–82.
36. **Doggrell SA.** Statins in the 21st century: end of the simple story? *Expert Opin Investig Drugs.* 2001; 10: 1755–66.
37. **Chou AJ, Gorlick R.** Chemotherapy resistance in osteosarcoma: current challenges and future directions. *Expert Rev Anticancer Ther.* 2006; 6: 1075–85.
38. **Arndt CA, Crist WM.** Common musculoskeletal tumors of childhood and adolescence. *N Engl J Med.* 1999; 341: 342–52.
39. **Sandberg AA, Bridge JA.** Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. *Cancer Genet Cytogenet.* 2003; 145: 1–30.
40. **Benassi MS, Molendini L, Gamberi G, Ragazzini P, Sollazzo MR, Merli M, Asp J, Magagnoli G, Balladelli A, Bertoni F, Picci P.** Alteration of pRb/p16/cdk4 regulation in human osteosarcoma. *Int J Cancer.* 1999; 84: 489–93.
41. **David JP, Mehic D, Bakiri L, Schilling AF, Mandic V, Priemel M, Idarraga MH, Reschke MO, Hoffmann O, Amling M, Wagner EF.** Essential role of RSK2 in c-Fos-dependent osteosarcoma development. *J Clin Invest.* 2005; 115: 664–72.
42. **Wei G, Lonardo F, Ueda T, Kim T, Huvos AG, Healey JH, Ladanyi M.** CDK4 gene amplification in osteosarcoma: reciprocal relationship with INK4A gene alterations and mapping of 12q13 amplicons. *Int J Cancer.* 1999; 80: 199–204.
43. **Grem JL, King SA, Wittes RE, Leyland-Jones B.** The role of methotrexate in osteosarcoma. *J Natl Cancer Inst.* 1988; 80: 626–55.
44. **Siddik ZH.** Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene.* 2003; 22: 7265–79.
45. **Beretta GL, Gatti L, Tinelli S, Corna E, Colangelo D, Zunino F, Perego P.** Cellular pharmacology of cisplatin in relation to the expression of human copper transporter CTR1 in different pairs of cisplatin-sensitive and -resistant cells. *Biochem Pharmacol.* 2004; 68: 283–91.
46. **Gomes CM, van Paassen H, Romeo S, Welling MM, Feitsma RI, Abrunhosa AJ, Botelho MF, Hogendoorn PC, Pauwels E, Cleton-Jansen AM.** Multidrug resistance mediated by ABC transporters in osteosarcoma cell lines: mRNA analysis and functional radiotracer studies. *Nucl Med Biol.* 2006; 33: 831–40.
47. **Serra M, Pasello M, Manara MC, Scotlandi K, Ferrari S, Bertoni F, Mercuri M, Alvegard TA, Picci P, Bacci G, Smeland S.** May P-glycoprotein status be used to stratify high-grade osteosarcoma patients? Results from the Italian/Scandinavian Sarcoma Group 1 treatment protocol. *Int J Oncol.* 2006; 29: 1459–68.
48. **Lourda M, Trougakos IP, Gonos ES.** Development of resistance to chemotherapeutic drugs in human osteosarcoma cell lines largely depends on up-regulation of Clusterin/Apolipoprotein J. *Int J Cancer.* 2007; 120: 611–22.
49. **Mensah-Osman EJ, Thomas DG, Tabb MM, Larios JM, Hughes DP, Giordano TJ, Lizyness ML, Rae JM, Blumberg B, Hollenberg PF, Baker LH.** Expression levels and activation of a PXR variant are directly related to drug resistance in osteosarcoma cell lines. *Cancer.* 2007; 109: 957–65.
50. **Ory B, Moriceau, G, Redini, F, Heymann, D.** mTOR inhibitors (rapamycin and their derivatives) and nitrogen bisphosphonates: bi-functional conpounds for the treatment of bone tumors. *Curr Med Chem.* 2007; 14: 1381–87.

Complément de discussion à l'article 7

Les premiers effets des bisphosphonates sur le métabolisme phospho-calcique ont été découverts il y a plus de 30 ans ; depuis, ces molécules sont devenues les plus utilisées dans le traitement des pathologies osseuses associées à une résorption excessive telle que l'ostéoporose, les ostéolyses malignes, etc… La récente démonstration des effets anti tumoraux du Zol a conduit à l'étude des potentiels mécanismes de résistances associés au traitement. En effet, l'échec des traitements anti tumoraux résulte fréquemment d'une résistance innée ou acquise des cellules tumorales aux agents de chimiothérapies (Kruh 2003). Dans ce contexte, l'élucidation des mécanismes de résistance au Zol permettrait l'adaptation du traitement en termes de dose et de durée.

Dans ce travail, après 3 mois de traitement continu avec de faibles concentrations de Zol, les cellules d'ostéosarcome apparaissent de moins en moins sensibles au Zol et des lignées cellulaires résistantes sont progressivement établies. De plus, cette résistance est indépendante du phénotype MDR (multidrug resistant) et est corrélée au niveau d'expression de la farnesyl diphosphate synthase (FPPs). Récemment, Notarnicola *et al* ont démontré qu'une forte activité FPPs était corrélée à une plus forte inhibition de l'apoptose dans les cellules de cancer colorectal (Notarnicola et al. 2004). De même, Ortiz-Gomez *et al* montrent que la surexpression de la FPPs confère une résistance au risédronate chez *leishmania major* et que le degré de résistance est corrélé à l'augmentation de l'activité enzymatique de la FPPs (Ortiz-Gomez et al. 2006). L'ensemble de ces données met en évidence le rôle essentiel de la FPPs dans les mécanismes de résistance au Zol.

Une des questions posées par ces observations est : quelle peut être l'origine des mécanismes de résistance observés : innée ou acquise ? Les résultats obtenus ne nous ont pas permis de trancher entre ces deux hypothèses. Le principal argument en faveur d'une résistance innée est la différence de niveau d'expression de la FPPs entre les différents sous clones cellulaires composant la population parentale OSRGA. En effet, le traitement par le Zol exercerait une pression de sélection sur la population cellulaire, les clones exprimant le plus faiblement la FPPs étant affectés les premiers. Après plusieurs semaines de culture dans ces conditions, les clones surexprimant la FPPs deviennent prédominants et émergent de la population parentale (Notarnicola et al. 2004). Il est intéressant de constater que les patients atteints d'ostéosarcome présentent eux aussi une forte hétérogénéité inter-individuelle de l'expression de la FPPs au sein de leur tumeur. Dans une étude clinique future, le niveau

d'expression de la FPPs au sein de la tumeur des patients sera corrélé avec l'efficacité du traitement par le Zol. D'autres expériences quant à elles laissent envisager un mécanisme de résistance acquis. En effet, après 72h de traitement par le Zol à faible concentration, on observe une augmentation de la prolifération des cellules tumorales liée à une augmentation du niveau d'expression de la FPPs. Cette hypothèse est également avancée par Ortiz-Gomez *et al* ; en effets ces auteurs obtiennent des lignées cellulaires résistantes par le même protocole de traitement au risédronate sur des cellules promastigotes (Ortiz-Gomez et al. 2006), et considèrent ce résultat comme un mécanisme de pression de sélection, les cellules s'opposant au risédronate en surexprimant sa protéine cible. Un tel phénomène a déjà été rapporté chez des patients atteints d'ostéosarcome à la suite d'un traitement chimiothérapeutique. En effet, en comparant les biopsies effectuées avant et après le traitement chimiothérapeutique, des modifications génétiques acquises par les tumeurs ont pu être démontrées (Ifergan et al. 2003; Zhou et al. 2003). Une résistance acquise aux bisphosphonates a également été rapportée par Papapoulos *et al* dans la maladie de Paget (Papapoulos et al. 2006) ; les auteurs pensent que la résistance observée n'est due qu'à des facteurs liés à la maladie et non à une diminution de la réponse de la cible moléculaire. En résumé, le mécanisme de résistance des cellules d'ostéosarcomes à l'encontre du Zol est lié au niveau d'expression de la FPPs mais aucune hypothèse ne peut être exclue concernant le caractère inné ou acquis de la résistance.

La résistance à la chimiothérapie dans les ostéosarcomes est un sujet bien documenté (Chou et al. 2006). Les cellules d'ostéosarcome sont sujettes à des altérations génétiques de gènes clefs de la prolifération cellulaire tels que p53 et Rb (Arndt et al. 1999; Sandberg et al. 2003), de modifications d'oncogènes et d'anti-oncogènes telles qu'une délétion du gène $p16^{INK4}$ (inhibiteur de kinase dépendante des cyclines), une surexpression de c-fos et une amplification de la kinase dépendante des cyclines 4 (Benassi et al. 1999; Wei et al. 1999; David et al. 2005). Cette instabilité génétique est responsable du développement de populations cellulaires hétérogènes au sein d'une seule et même tumeur ainsi que de l'émergence de cellules tumorales résistantes. Les cas de résistance les plus décrits concernent les agents de chimiothérapie les plus employés tels que le cisplatine, la doxorubicine ou le métotrexate. Le phénotype MDR (multidrug resistance) est le mécanisme de résistance le plus décrit pour les ostéosarcomes, à tel point que les niveaux d'expression des gènes MDR1 et P-gp peuvent être utilisés à des fin diagnostique pour présager de l'efficacité du traitement chimiothérapeutique (Serra et al. 2006). Ces facteurs pronostiques permettent la sélection des patients pour qui un traitement alternatif devra être considéré.

Cette étude fait état d'un mécanisme de résistance à l'acide zolédronique de la part des cellules d'ostéosarcome spécifique des N-BPs. Ce mécanisme ne conférant pas de résistances croisées à d'autres molécules n'ayant aucun point commun, structurel ou fonctionnel avec le Zol il pourrait être contourné en utilisant plusieurs molécules ayant des cibles moléculaires et des mécanismes d'action différents. Par exemple, lorsque le Zol est associé à l'ifosfamide dans un modèle d'ostéosarcome de rat, une augmentation de la régression tumorale et de la réparation tissulaire peut être observée (Heymann et al. 2005). Dans le futur, le Zol pourra être combiné avec d'autres agents chimiothérapeutiques afin d'accroitre l'efficacité thérapeutique et éviter l'émergence de mécanismes de résistance (Ory et al. 2007).

Conclusions
Et
Perspectives

I. Mécanismes d'action de l'acide zolédronique et phénomène de résistance dans le traitement des ostéosarcomes.

Le ZOL réduit la viabilité des cellules d'ostéosarcome (OSRGA) après 72 heures de traitement alors que les ostéoblastes sont peu sensibles. Cette réduction de viabilité est due à une inhibition de prolifération suite à un blocage des cellules en phase S et G2/M du cycle cellulaire, mais également à une induction de la mort cellulaire. En effet, l'acide zolédronique induit la mort des cellules d'ostéosarcome par un mécanisme atypique d'apoptose indépendamment de l'activation des caspases et caractérisé par une activation de P-p53, de Bax et une inhibition de Bcl-2. Une augmentation de la perméabilité mitochondriale associée à une libération de l'Apoptosis inducing factor (AIF) et de l'Endonucléase G (EndoG) est également observée.

Afin de déterminer les rôles respectifs de l'AIF et de l'EndoG dans les mécanismes de mort cellulaire induit par l'acide zolédronique, leurs expressions seront réprimées par la transfection de shRNA dans les cellules d'ostéosarcome. Chaque shRNA (contre l'AIF et contre l'EndoG) sera contenu dans un plasmide spécifique ce qui permettra la sélection de populations cellulaires doublement transfectées, c'est-à-dire dont l'expression des deux facteurs est réprimée, mais également la sélection de cellules transfectées uniquement par un des deux plasmides, ceci permettant l'étude de ces deux facteurs indépendamment l'un de l'autre. La mort cellulaire induite par l'acide zolédronique sera alors étudiée *in vitro* en utilisant les techniques décrites dans l'article 2. Afin de déterminer les rôles de l'AIF et de l'EndoG dans cette mort cellulaire *in vivo* l'efficacité de l'acide zolédronique sera comparée dans notre modèle de rat (voir article 3) réalisé à partir de cellules tumorales contrôles ou transfectées stablement avec les shRNA pour l'AIF et/ou l'EndoG.

Comme nous l'avons vu précédemment, la façon précise par laquelle l'acide zolédronique pénètre dans les cellules d'ostéosarcome reste inconnue. Les travaux de Thompson et al suggèrent que les bisphosphonates pénètrent dans le cytoplasme des cellules macrophagiques par acidification des vésicules après endocytose en phase liquide (Thompson et al. 2006). Nos résultats (voir article 2) suggèrent quant à eux que l'acide zolédronique interagit avec les intégrines, ou tout du moins que les mécanismes d'action de l'acide zolédronique sont associés à une voie de transduction associée aux intégrines. Ceci est en accord avec les travaux de Bezzi et al qui démontrent l'inhibition de l'adhérence et de la

migration des cellules endothéliales en présence de bisphosphonates sur la vitronectine (Bezzi et al. 2003). Afin d'approfondir le mécanisme et la cinétique de pénétration des bisphosphonates dans les cellules d'ostéosarcomes, du risédronate fluorescent (risédronate-FAM) sera utilisé pour traiter nos cellules ; l'étude sera réalisée par vidéo microscopie à fluorescence. Des cellules transfectées avec des FAK fluorescentes et/ou des intégines fluorescentes seront utilisées afin de permettre une localisation relative de ces différents partenaires au cours de la cinétique de traitement, ceci en comparaison avec des cellules traitées avec un placebo. Différents marqueurs seront également utilisés afin de distinguer les différents types d'endocytose : l'agglutinin-633 (marqueur de l'endocytose d'adsorption, non spécifique), la transferrine-633 (marqueur de l'endocytose médiée par un récepteur) et le TAMRA-dextran (marqueur de l'endocytose en phase liquide)

Nous avons démontré, *in vitro*, l'activité directe de l'acide zolédronique sur les cellules d'ostéosarcome, en effet, il est possible par Western blot, après traitement au Zol, d'observer l'apparition de la forme non prénylée de Rap1A (voir article 7) témoignant de l'inhibition de la Farnésyl diphosphate synthase par le Zol. Afin de démontrer l'activité directe du Zol *in vivo* nous utiliserons notre modèle de rat (voir article 3) réalisé à partir de cellules tumorales contrôles ou transfectées stablement avec le plasmide PCMV-TAG2B-flag-Rap1A. Ce plasmide code pour une forme de Rap1A ayant un poids moléculaire plus élevé que le Rap1A physiologique. Ainsi, après traitement des rats par le Zol, il sera possible par Western Blot de distinguer l'apparition de la forme non prénylée de Rap1a présente dans toutes les cellules du rat de celle présente uniquement dans les cellules d'ostéosarcome. De cette façon nous serons en mesure de démontrer l'effet direct *in vivo* du Zol sur les cellules d'ostéosarcomes.

Nous avons également démontré *in vitro* le développement d'une résistance métabolique dans les cellules d'ostéosarcomes suite au traitement par le Zol, cette résistance étant liée à une surexpression de la FPPs. Les effets *in vivo* de cette surexpression seront étudiés dans notre modèle d'ostéosarcome de rat réalisé à partir de cellules contrôles et de cellules résistantes développées précédemment *in vitro* par sélection ou par surexpression de la FPPs.

II. Traitement des chondrosarcomes par l'acide zolédronique après curetage intra-lésionnel.

Le traitement chirurgical représente le traitement exclusif des chondrosarcomes, en effet, le seul traitement efficace reste la résection chirurgicale large. La chimiothérapie n'a aucune efficacité sur les chondrosarcomes. La radiothérapie quant à elle est inefficace, de plus, le chondrosarcome est considéré comme une tumeur radio-résistante (Springfield et al. 1996). La résection tumorale réalisée est large, c'est-à-dire qu'elle retire une grande partie de tissu sain, de plus, ce geste est suivi d'un curetage intra-lésionnel. Malgré cela, le taux de récidive reste très élevé.

L'efficacité du Zol dans le traitement du chondrosarcome *in vivo* sera évaluée, en termes de croissance tumorale et de survie, avant et après curetage intra-lésionnel mais surtout en termes de récidive dans un modèle de chondrosarcome de rat où le geste chirurgical utilisé est identique à celui de la pratique clinique.

III. Développement d'études cliniques du traitement par l'acide zolédronique des tumeurs osseuses primitives.

Les donnés que nous avons obtenues associées à celles de la littérature permettent de considérer l'acide zolédronique comme un agent potentiel pour le traitement des maladies osseuses malignes. C'est pourquoi une première étude clinique a été mise en place en 2005 : « Etude de phase 2 des effets de l'acide zolédronique sur la récidive locale des tumeurs osseuses à cellules géantes après intervention chirurgicale ». Les tumeurs à cellules géantes sont des lésions ostéolytiques de siège habituellement épiphysaire : pourvues d'une abondante vascularisation cette tumeur récidivante est majoritairement bénigne. Ce programme hospitalier multicentrique de recherche clinique (PHRC) concerne les établissements de Paris, Tours, Toulouse, Strasbourg et Limoges. L'investigateur principal est le Pr Gouin (hôpital de Nantes). Cette étude envisage l'inclusion de 23 patients avec un suivi pendant 3 ans. L'objectif principal de cette étude est la réduction du taux de récidive des tumeurs osseuses à cellules géantes après l'intervention chirurgicale. Le deuxième objectif est d'étudier la tolérance générale et locale au traitement par le Zol.

Un deuxième programme hospitalier multicentrique de recherche clinique est actuellement en cours, en collaboration avec l'institut Gustave Roussy. Cette étude s'intéresse à l'association de l'acide zolédronique avec les agents conventionnels de chimiothérapie utilisés pour le traitement de l'ostéosarcome chez l'enfant et l'adulte. L'investigateur principal est le Dr Brugières (IGR) ; notre laboratoire comme le service d'orthopédie de l'hôpital de Nantes en sont co-investigateurs. Cette étude envisage l'inclusion de 470 patients sur une période de 6 ans avec un suivi de 3 ans. En plus d'analyser les effets bénéfiques de l'association d'agents de chimiothérapies avec le Zol, cette étude nous permettra d'éclaircir les éventuels mécanismes de résistances développés par les tumeurs lors du traitement. Notamment, nous étudierons le niveau d'expression de la Farnésyl diphosphate synthase (FPPs) au sein des tumeurs des patients résistants au traitement.

IV. Intérêt de l'association de l'acide zolédronique et des inhibiteurs de mTOR dans le traitement des ostéosarcomes.

Introduction

L'association de deux ou de plusieurs agents de chimiothérapie repose sur les principales règles suivantes :

- La théorie de Goldie et Coldman, permettant de réduire le nombre de clones résistants.
- L'utilisation de drogues à mécanismes d'action distincts permettant de créer des lésions sur des cibles cellulaires différentes, réduisant ainsi l'acquisition de phénomènes de résistance.
- L'utilisation de drogues à mécanisme de résistance cellulaire non croisée.
- Un profil de toxicité différent, permettant d'éviter des effets secondaires sévères et limitant.

La revue suivante nous rappelle les mécanismes d'action et de résistance respectifs de l'acide zolédronique et de la rapamycine (et de ses dérivés). Elle nous permet ainsi de constater que leurs cibles cellulaires étant aussi différentes que leurs mécanismes de résistance, ces deux molécules constituent de ce point de vu de bonnes candidates pour une association thérapeutique.

De plus, le fait que ces deux molécules présentent une activité aussi bien sur les ostéoclastes que sur les cellules tumorales fait d'elles des candidates de choix dans le traitement des ostéosarcomes.

Article 8

« Inhibiteurs de mTOR (rapamycin et ses dérivés) et nitrogène-bisphosphonate : agents bi fonctionnel pour le traitement des tumeurs osseuses »

Ory B, Moriceau G, Redini F, Heymann D. Fev 2007. Cur Med Chem. Vol 14: 1381-1387

La rapamycine et ses dérivés ont été à l'origine développés comme agents anti-fongiques. Les bisphosphonates quant à eux ont originellement été développés comme agent anti-résorption osseuse. En fait, des études *in vitro* et *in vivo* ont démontré que ces agents étaient des molécules multi-fonctionnelles ayant des effets sur la croissance tumorale ainsi que sur le remodelage osseux. Cette revue décrit ces deux familles de molécules et discute leur intérêt thérapeutique dans le traitement des tumeurs osseuses primitives et des métastases osseuses.

La difficulté principale dans le traitement des cancers est le contournement de mutations affectant des gènes clés tels que p53, Rb et les protéines impliquées dans la cascade de signalisation des caspases, ces mutations étant portées par une majorité de cellules tumorales. Les N-BPs ont la capacité d'inhiber la prolifération cellulaire et d'induire la mort des cellules d'ostéosarcome, et ce quel que soit le statut p53 et RB des cellules. De plus la mort cellulaire induite est indépendante des caspases et est caractérisée par la translocation de deux molécules mitochondriales : l'AIF (apoptosis inducing factor) et l'EndoG (endonuclease G). Par conséquent les N-BPs peuvent être considérés comme des agents thérapeutiques potentiels des ostéosarcomes, ces tumeurs présentant fréquemment des mutations de p53 et de Rb. De plus, les dérivés de la rapamycine sont capables d'inhiber la traduction protéique *via* l'inhibition de la voie mTOR à la fois dans les ostéoclastes et dans les cellules tumorales. Les mécanismes cellulaires physiologiques régulés par mTOR sont sous l'influence de nombreux facteurs environnementaux tels que les facteurs de croissances, les hormones, les cytokines, les acides aminés, l'énergie disponible et le stress cellulaire. La régulation de mTOR est couplée avec la progression du cycle cellulaire et la croissance cellulaire. L'inhibition de la voie PI3K/Akt/mTOR est une approche thérapeutique prometteuse pour le traitement des tumeurs osseuses primitives et des métastases osseuses étant donné son importance dans le métabolisme des ostéoclastes et des cellules tumorales. L'association des ces deux familles

d’agents bi-fonctionnels pourrait permettre d’éviter le développement de résistances et représente le traitement du futur pour les tumeurs ostéolytiques.

mTOR Inhibitors (Rapamycin and its Derivatives) and Nitrogen Containing Bisphosphonates: Bi-Functional Compounds for the Treatment of Bone Tumours

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Abstract: N-BP, rapamycin and its derivatives have been originally developed respectively as anti-resorptive and anti-fungal agents. In fact, *in vitro* and *in vivo* experiments demonstrated that these compounds are multi-functional molecules exerting their effects on tumour cell growth and bone remodelling. The major challenge in treating cancer relates to mutations in key genes such as p53, Rb or proteins affecting caspase signalling carried by many tumour cells. Whether nitrogen containing bisphosphonates (N-BP) are potent bone inhibitors, they also inhibit tumour cell proliferation and increase atypical apoptosis of bone tumour cells regardless of the p53 and Rb status. N-BP may be then considered as effective therapeutic agents in clinical trials of bone tumours. Rapamycin and its derivatives inhibit mTOR dependent mRNA translation both in osteoclasts and tumour cells. Cellular physiological mechanisms regulated by mTOR integrate many environmental parameters including growth factors, hormones, cytokines, amino acids, energy availability and cellular stresses that are coupled with cell cycle progression and cell growth. Rapamycin and its derivatives as well as N-BP must be considered as bi-(multi) functional molecules affecting simultaneously bone and tumour metabolisms. The present survey describes these two molecular families and discusses their therapeutic interests for primary bone tumours and bone metastases.

Keywords: Rapamycin, bisphosphonate, bone, osteolysis, bone remodelling, primary bone tumours, metastases.

I. INTRODUCTION

Bone tumours can be divided into primary and secondary bone diseases. Malignant primary bone tumours are rare pathologies, among them osteosarcoma being the most frequent tumour in children (56% of all malignant tumours of bone) and adults with a poor prognosis due to its propensity to metastasis. Current therapeutic strategies of osteosarcoma are based on tumour resection associated with highly toxic chemotherapy [1]. Survival is closely related to the response of the tumour cells to anti-mitotic drugs, reaching 70% at 5 years in the best series and only 30% when pulmonary metastases are detected at the time of diagnostic. These data demonstrate that the response to the conventional treatment (chemotherapy) is disappointing, showing questionable benefit in terms of improvement in disease-free survival. The second type of bone tumours is composed by bone metastases from non osseous primary origin and unfortunately, the development of bone metastatic disorders are very often associated with a fatal outcome. In all series reported, the most common site of first cancer relapse is bone as more than 50% of patients will develop bone metastases at the time of first recurrence [2]. Bone metastases are usually osteolytic tumours, and may be expected to complicate the course of up to 80% of patients with disseminated disease, with pathologic fracture and/or hypercalcemia.

The combinatorial chemistry allows the emergence of new molecular families targeting specifically key enzymes of cellular metabolism and signalling pathways. The present survey focuses on two molecular families derived from rapamycin and endogenous pyrophosphate, which represent future therapeutic approaches of tumour osteolytic diseases.

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II. COMPOUNDS DERIVED FROM ENDOGENOUS PYROPHOSPHATE AND RAPAMYCIN: TWO CLASSES OF THERAPEUTIC AGENTS TARGETING BONE RESORPTION AND TUMOUR GROWTH

Histological analyses of osteolytic primary and secondary bone tumours reveal that bone destruction is mediated by osteoclasts rather than by tumour cells themselves. The interactions between tumour cells, soluble tumour-derived factors and bone microenvironment are crucial for the initiation and promotion of skeletal malignancies. These observations suggest the existence of a vicious cycle driving the formation of osteolytic bone tumours. In short, tumour cells and modified bone microenvironment induce the recruitment of osteoclast progenitors which in turn destroy bone tissue and release growth factors favourable to the proliferation of tumour cells [3]. As the activators of bone resorption and tumour growth are involved in a common vicious cycle, the modulation of bone resorption by biological and chemical agents may interfere with the tumour growth and reciprocally. To block this vicious cycle that takes place during tumour development in bone site, molecules exerting a bi-functional activity (anti-bone resorption and anti-tumor cell proliferation) are now envisaged.

1. The Nitrogen Containing Bisphosphonates Derived from Endogenous Pyrophosphate

Bisphosphonates are simple chemical compounds which structure is based on a phosphorus-carbon-phosphorus template. These compounds are characterized by their strong affinity for bone hydroxyapatite crystals and their anti-resorptive potency [4]. Three main groups of bisphosphonates are described (see review in [1]): (i) the first generation is constituted by simple substituents attached to the central carbon and weakly inhibits the bone resorption; (ii) the second generation possesses an aliphatic side chain containing a single nitrogen atom and exerts a more potent anti-resorptive activity compared to the first generation; (iii) the compounds of the third generation contain a heterocyclic substituent with one or two nitrogen atoms and are powerful bone resorption inhibitors

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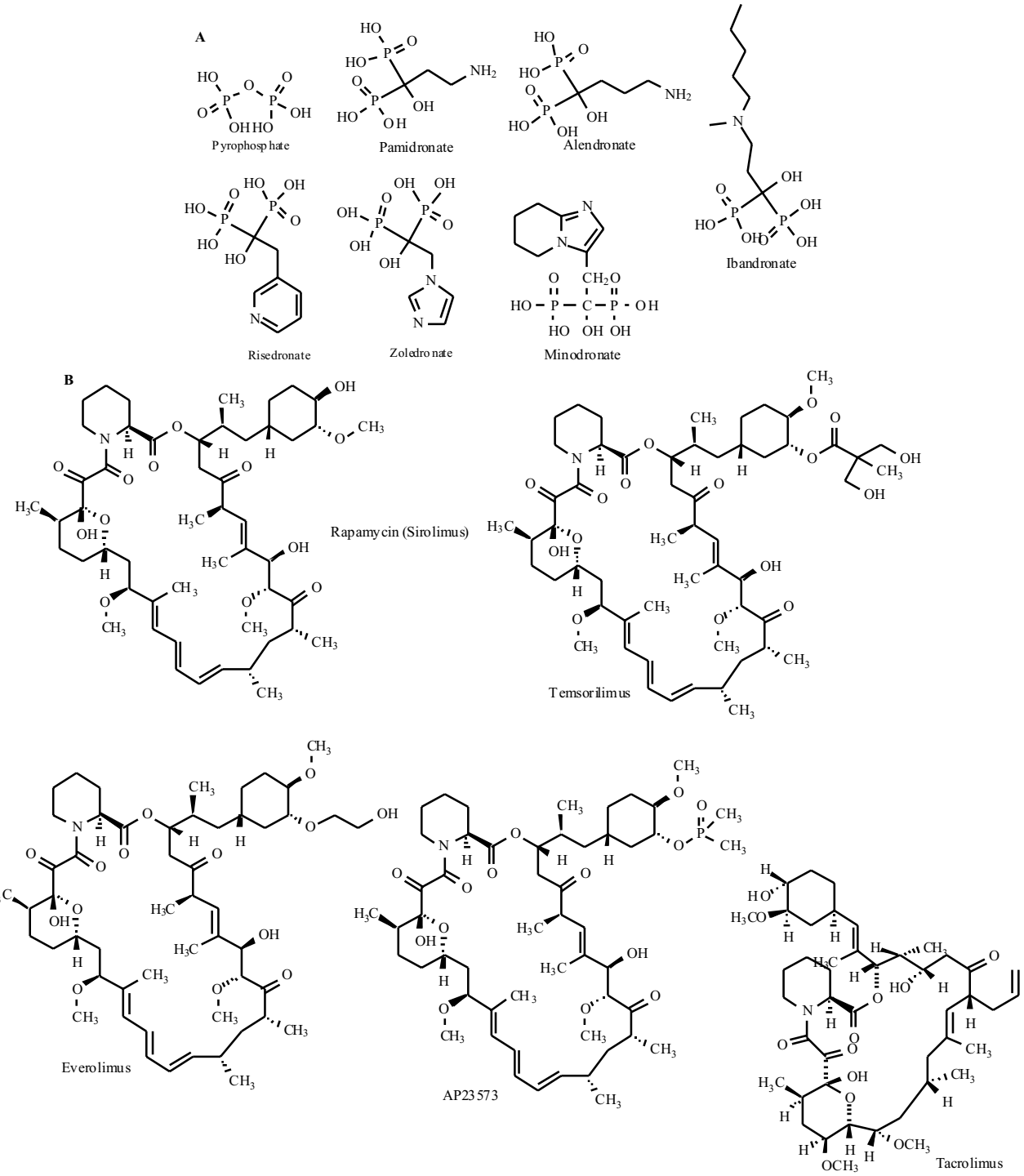

Fig. (1). Chemical structures of mTOR inhibitors (rapamycin and derivatives) and N-BP.
(**A**) N-BP structure compared to pyrophosphate. Pamidronate, alendronate and ibandronate belong to the second-generation of bisphosphonate characterized by a nitrogen atom in their side chain. Pamidronate has a relative potency of 100; alendronate, 100–1000 and ibandronate, 1000–10 000. Risedronate, minodronate and zoledronate belong to the third-generation of bisphosphonates characterized by a heterocyclic side chain with one or two nitrogen atoms. Risedronate has a relative potency of 1000–10 000; zoledronic acid and minodronate >10 000.
(**B**) chemical structures of rapamycin and their derivatives. Despites its similar name and chemical structure, tacrolimus is not a mTOR inhibitor, but binds to FKBP12 to inhibit calcineurin similarly to cyclosporine.

and anti-tumour agents (Fig. **1A**). Although all bisphosphonates induce the apoptosis of osteoclasts, they precociously affect osteoclast function (Fig. **2A**). Thus, in contrast to the first generation of bisphosphonates which are metabolised in cytotoxic analogues of ATP, the N-BP inhibit the activity of two enzymes involved in the mevalonate pathway: farnesyl diphosphate synthase (FPP) and geranylgeranyl diphosphate synthase (GGPP) [5] (Fig. **2B**). The inhibition of FPP and GGPP is responsible to the loss of the ruffled border and modifications of the cytoplasmic actin ring.

These alterations then induce the apoptosis of osteoclasts primarily due to loss of geranylgeranylated small GTPases [6]. FPP and GGPP are required for the posttranslational lipid modification (prenylation) of small GTPases (i.e. Ras, Rho, and Rac). Prenylation involves the transfer of a farnesyl or geranylgeranyl lipid group onto a cysteine residue in carboxy-terminal domains, giving rise to farnesylated and geranylgeranylated proteins. Such biochemical reactions are essential for the anchorage of small GTPases to cell membranes and to protein-protein interactions. Therefore, the inhibition of the mevalonate pathway is associated with the loss of prenylation of small GTPases including Rho, Rac, cdc42 and Rab which could account for the numerous effects observed on cancer cells (see following paragraph). Although the molecular mechanism by which bisphosphonates affect osteoclasts are now evident, the potential route by which they are internalised by cells has been very recently elucidated [7]. Using a novel fluorescently labelled analog of alendronate, Thompson *et al.* revealed that labelled bisphosphonate was rapidly internalised into intracellular vesicles in J774 macrophages and rabbit osteoclasts [7]. These results demonstrated that cellular uptake of nitrogen bisphosphonates requires fluid-phase endocytosis whereas transfer from endocytic vesicles into the cytosol requires endosomal acidification [7]. More recently, Saltel *et al.* described a new property of mature multinucleated osteoclasts to transmigrate through various cell types of the bone microenvironment. Such activity can be activated by bone metastatic breast cancer cells and is abolished by bisphosphonate treatment. In this context, the blockade of osteoclast transmigration by bisphosphonates represents a new therapeutic strategy for bone diseases associated with an excessive osteoclast resorption [8].

Although bisphosphonates are powerful agents to treat degenerative bone diseases such as osteoporosis, recent *in vitro*, pre-clinical and clinical data demonstrated that N-BP exert anti-tumour activities especially in breast, prostate, lung renal, osteosarcoma and chondrosarcoma [4, 9, 10]. This phenomenon can be explained by induction of tumour-cell apoptosis [10], inhibition of cell proliferation [11], modulation of tumour-cell adhesion and inhibition of tumour-cell dissemination [12]. In fact, even if these anti-tumour activities

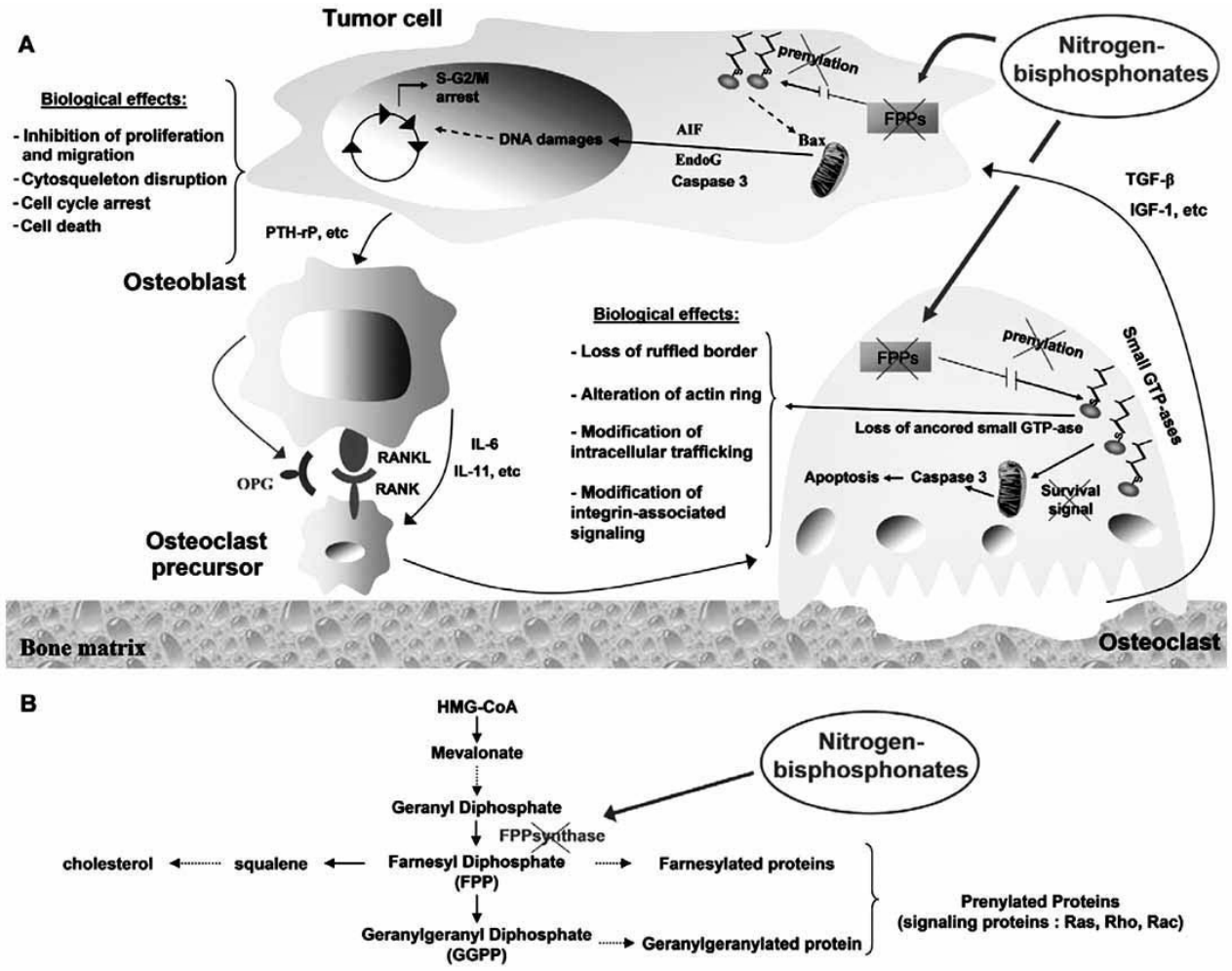

Fig. (2). N-BP: bi-functional compounds in osteolytic bone tumours context.

The main mechanism of action of N-BP is to inhibit the mevalonate pathway, especially farnesyl diphosphate (FPP) synthase and geranylgeranyl diphosphate (GGPP) synthase activities (**B**). Consequently the posttranslational lipid modification (prenylation) of small GTPase is abolished, then inhibiting their cell membrane anchorage. Several cellular pathways are affected by N-BP that induce lots of biological effects on osteoclast including apoptosis (**A**). The inhibition of the mevalonate pathway inhibition also affects the tumour cell growth characterized by cycle arrest and induction of apoptosis. IGF1: Insulin Growth Factor-1; TGF-b: Transforming Growth Factor-b; IL-6, -11: Interleukin-6, -11; PTH-rP: ParaThyroïd Hormone related Protein.

are not fully understood, the data of the literature reveal that N-BP such as zoledronic acid induce tumour cell death, block the cell cycle in S-G2/M phases and modify the cyclin expression [13-15]. Furthermore, very recent data revealed that zoledronic acid induces atypic apoptosis of ostesarcoma cells characterised by nuclear alterations and modification of the Bax/Bcl2 equilibrium in favour of Bax [15]. Cell death induced by zoledronic acid is also associated with increased mitochondrial permeability and translocation of Apoptosis Inducing Factor (AIF) and Endonuclease-G (Endo-G), independently of caspase activation [16]. Furthermore, zoledronic acid also disturbs cytoskeletal organization and cell junctions, down regulates phosphorylation of focal adhesion kinases resulting in a slow down of cell migration, cell shape alterations and inhibition of cell spreading [15, 16]. Interestingly, zoledronic acid associated cell death bypasses the mutations of p53 and Rb, two key genes frequently mutated in tumour cells [16, 17]. Thus, the p53-, Rb-independent anti-tumour activities of zoledronic acid suggest that it may be an attractive agent for treating cancers, especially those with chemoresistance resulting from the loss of p53 or Rb function. Indeed, several mutations or inactivations of the anti-oncogenes p53 and Rb are detected in 50% of patients suffering from osteosarcoma [17, 18].

Preclinical [4, 9, 10, 16] and clinical [19-21] data demonstrated the therapeutic interest to associate N-BP and conventional chemotherapeutic drugs in terms of survival, tumour progression and tissue repair. Indeed, the N-BP increase the effects of anti-cancer drugs in various cell lines [22-25] and *in vivo* combination of bisphosphonates with anti-cancer agents such as uracil and tegafur causes an enhanced reduction of bone metastases compared to UFT (tegafur/uracil) alone [26]. Similarly, we reported recently the enhancement of tumour regression and tissue repair when zoledronic acid is combined with ifosfamide in rat osteosarcoma [9]. Furthermore, zoledronic acid suppresses lung metastases and prolongs overall survival of osteosarcoma lung metastase-bearing mice [27]. Synergistic effect of the combination of zoledronic acid and radiation has been also revealed recently *in vitro* on breast cancer cells [28]. These observations led to investigate the effects of a N-BP alone or in combination with anti-cancer drugs on tumor cells in clinical context. Thus, clinical trials established the benefit to use such therapeutic approach in cancer diseases [19-21, 29, 30] as well as in the treatment of osteoporosis [31, 32] and. However, clinical trials using zoledronic acid also pointed out the potential risk of osteonecrosis of the jaw in treated patients, length of exposure appearing to be the most important risk factor for this complication [33, 34]. This question is still open, as the patients diagnosed with jaw osteonecrosis probably had a concurrent infection, being aggressively treated with antibiotics [34]. Further clinical evaluations are needed before stating definitively on the adverse effects of N-BP.

Overall, as the literature clearly demonstrated that the N-BP can be used as very efficient anti-resorption agents and as anti-tumour molecules with some limits, these compounds can be then considered as bi-functional agents useful for the treatment of tumour bone diseases.

2. The Compounds Derived from Rapamycin

Sirolimus is a macrolide antibiotic first discovered as a product of the bacterium *Streptomyces hygroscopicus* in a soil sample from an island called Rapa Nui, better known as Easter Island [35] (Fig. **1B**). Sirolimus is also known as rapamycin and has been originally developed as an antifungal agent. However, this application was abandoned when it was discovered that rapamycin had potent immunosuppressive and anti-proliferative properties. Rapamycin is a specific inhibitor of the target of rapamycin (TOR), initially identified in screening assay for rapamycin-resistant yeast mutant. The mammalian orthologue called mTOR, is a conserved ser/thr kinase, a member of the phosphoinositide -3-kinase-related kinase (PIKK) family such as other central protein like Ataxia Telengiectasia Mutated (ATM) and Ataxia Telengiectasia Related (ATR) proteins which are involved in DNA repair [36]. This ser/thr kinase has a broad impact on cellular function including control of cell growth [37, 38], cytoskeletal organization [39, 40], autophagy [41, 42], transcription activities and initiation and regulation of translation [43, 44].

To regulate these cellular physiological mechanisms, mTOR integrates many environmental parameters including growth factors, hormones, cytokines, amino acids, energy availability and cellular stresses. The knowledge of mTOR signalling has been greatly improved by studying the insulin receptor/Akt signalling network. Indeed, the insulin/insulin-like growth factor (IGF) system control cell and organismal growth similarly to nutrients [45] (Fig. **3**). Insulin and IGF bind to the insulin receptor, inducing Insulin Receptor Subtrate 1 (IRS-1) and class I PhosphoInositide 3 Kinase (PI3K) activation. PI3K then converts PIP2 to PIP3 (phosphatidylinositol-3, 4, 5-triphosphate) in contrast to the phosphatase PTEN (Phosphatase and Tensin Homologue deleted on chromosome 10) which is considered as a tumour suppressor gene mutated in many human cancers involving the mTOR pathway [46-47-50]. PIP3 recruits both Phosphoinositide Dependent Kinase 1 (PDK1) and Akt to the plasma membrane and subsequently PDK1 activates Akt by Thr 308 phosphorylation [46]. Thereafter, phospho-AKt phosphorylates lots of targets to increase cell survival and growth, including Tuberous Sclerosis Complex 2 (TSC2) [51]. Akt disrupts the TSC complexes (composed by TSC1 and TSC2) inactivating TSC2 GTPase-activity which enables the conversion of active GTP-Rheb into inactive GDP-Rheb (mTOR activator) [52].

mTOR is also strongly linked to the energy cell status by the 5'AMP-activated protein kinase (AMPK) pathway [53]. Indeed, changes in the intracellular ATP/AMP ratio activate Leukotoxin translocation ATP-binding protein-1 (LKBP1) and AMPK that is able to activate TSC2 or not, thereby inhibiting mTOR activation or not in response to energy status. Moreover, amino acids are also able to directly regulate mTOR activity [54]. Subsequently, protein synthesis is regulated by mTOR complex 1 [composed by mTOR, Regulatory Associated Protein of mTOR (raptor) and G-protein β subunit-like (GβL)] which phosphorylates several substrates including ribosomal S6 kinase (S6K) and the eukaryote initiation factor 4E binding protein-1 (4EBP-1) [55]. Once activated, S6K phosphorylates the ribosomal protein S6, resulting in the translation of a subset of mRNAs encoding for essential ribosome proteins, including eukaryotic initiation factor-4B (eIF4B) and increasing translation mechanisms. The second target of mTOR is the translational repressor 4EBP-1. After phosphorylation, 4EBP-1 becomes unable to inhibit the cap-dependent initiation factor eIF-4E which then directly increases cell growth through the regulation of key oncogenic proteins such as c-Myc, Cyclin D1, FGF and VEGF [56]. Therefore, mTOR plays a key role in cell metabolism and is able to regulate cell entry into G1 phase which depends on numerous environmental factors. Another mTOR complex called mTOR complex 2, insensitive to Rapamycin has been also evidenced and is composed by mTOR, Rapamycin-Insensitive Companion of mTOR (rictor) and GβL. This second complex may control the cytoskeletal organization [57].

In light of these data, rapamycin exerts its activities on cellular metabolism and inhibits the growth of a broad spectrum of cancer including breast and colon carcinomas, rhabdomyosarcoma and osteosarcoma [58-63]. Rapamycin (Rapamune®, Wyeth Ayerst) and its analogues (Fig. **1B**)

temserolimus (CCI-779, Wyeth Ayerts, PA, USA), everolimus (RAD001, Novartis, Switzerland) or AP23573 (Ariad, Pharmaceuticals, MA, USA) do not directly inhibit mTOR but bind to the cytosolic protein FK-binding protein 12 (FKBP12) to form a complex inhibiting mTOR [64].

These compounds have shown promise in preclinical models and clinical trials including patients suffering from carcinomas, solid malignancies, hematologic disorders and sarcomas such as osteosarcoma [65-69]. Thus, rapamycin reduces tumour cell metastases in a murine model of osteosarcoma [67]. Furthermore, immuno-suppression maintenance with the mTOR inhibitors, sirolimus and everolimus, is associated with a significantly reduced risk of developing any posttransplant de novo malignancy and non-skin solid malignancy [70]. Despites its similar name and chemical structure, tacrolimus (FK506, Prograf®, Fujisawa, Japan) (Fig. **1B**) is not a mTOR inhibitor like sirolimus and everolimus, but binds to FKBP12 to inhibit calcineurin similarly to cyclosporine. However, it has a similar suppressive effect on the immune system *via* different pathways. Sirolimus inhibits the response to interleukin-2 thereby blocking T- and B-cell activation, while tacrolimus and cyclosporine inhibit the production of interleulin-2. Although most immuno-suppressive treatments are known to potentiate neoplastic diseases, data of the literature support the use of rapamycin derivatives in the face of prior malignancy [71]. Similarly to N-BP, inhibitors of rapamycin have been already envisaged in therapeutic combination with anti-cancer drugs. Thus, recently, Treeck *et al.* studied the *in vitro* effects of combined treatment of RAD001 and tamoxifen on growth and apoptosis of human cancer cells [72]. They demonstrated that mTOR inhibition is able to restore tamoxifen response in tamoxifen-resistant breast cancer cells and moreover that these therapeutic agents exert an additive anti-tumour effect on ovarian and breast cancer cells. Similar results have been observed in xenograft models of human pancreatic cancer based on a combination of gemcitabine and CCI-779 [73] which has achieved clinical response in a patient with metastatic leiomyosarcoma [74]. Combination of rapamycin, or sirolimus with HER1/epidermal growth factor receptor inhibitors has been assessed *in vitro* an *in vivo* with success [75-77]. Such combinatory therapies reduce the potential risk of acquired resistance mechanisms already observed with mTOR inhibitors [78].

Rapamycin and derivatives (excepted tacrolimus) also influence bone tissues. Indeed, mTOR appears as an essential signalling pathway engaged in the stimulation of osteoclast survival [79] and affects osteoblast differentiation [80]. Tumour Necrosis Factor-α (TNF-α), Receptor Activator of Nuclear Factor κB Ligand (RANKL) and Macrophage-Colony Stimulating Factor (M-CSF) promote osteoclast survival by signalling through mTOR/S6K [70]. Furthermore, the signalling intermediates for mTOR/S6K activation include

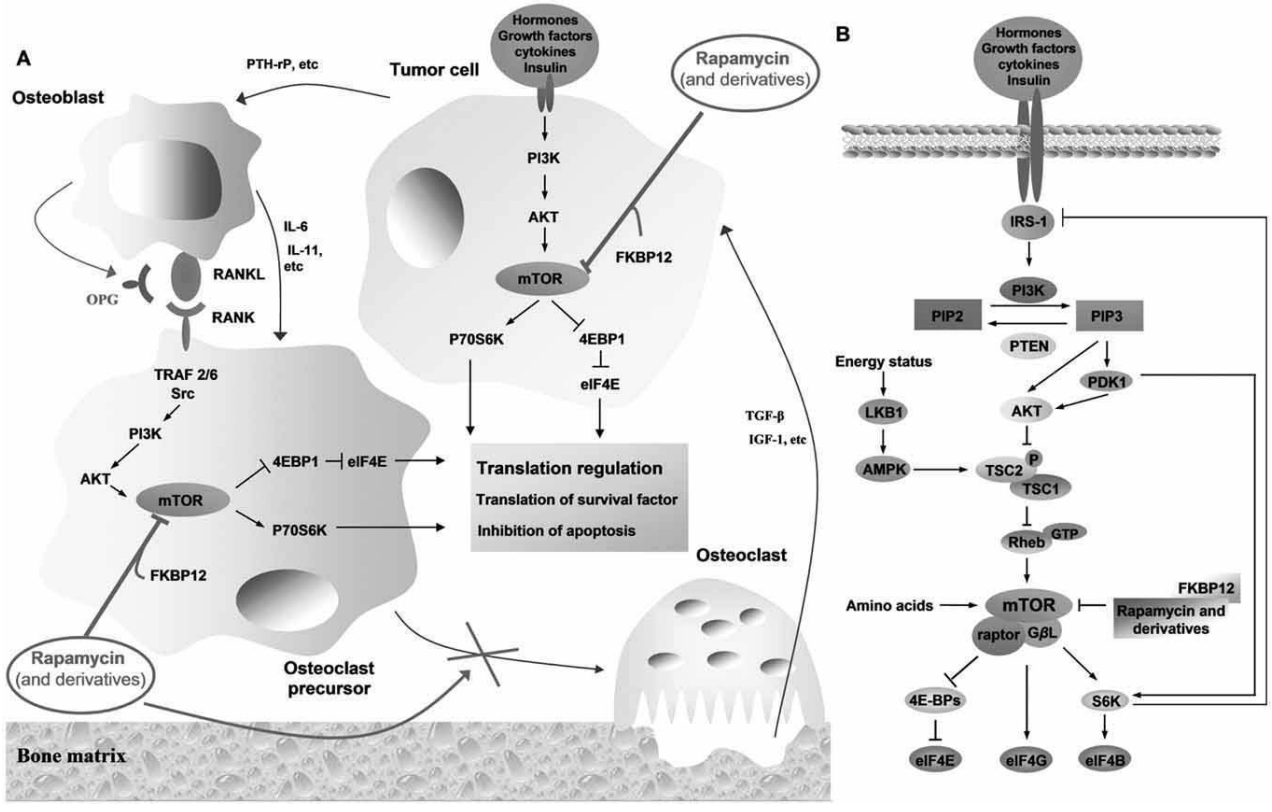

Fig. (3). Rapamycin and their derivatives: bi-functional compounds in osteolytic bone tumours context.

(**A**) Rapamycin derivatives (mTOR inibitors) affect tumour cell biology and bone remodelling. Indeed, rapamycin derivatives disturb osteoclast differentiation and survival in agreement with the involvement of mTOR in RANKL, TNFα and M-CSF signalling pathways. They also induce tumour cell apoptosis by disrupting cell cycle progression and cell growth. **(B)** Rapamycin and their derivatives bind to the cytosolic protein FK-binding protein 12 (FKBP12). mTOR pathway regulates cell growth, cytoskeletal organization, autophagy, transcription, translation initiation and regulation. To regulate these cellular mechanisms, mTOR integrates many environmental parameters: growth factors, hormones and cytokines by PI3K/Akt pathway, energy status by LKB1/AMPK pathway and amino acid availability. Consequently, mTOR regulates the translation of essential ribosome proteins, including eIF4B and translational repressor, 4EBP-1.

phosphatidylinositol-3 kinase, Akt, Erk and geranylgeranylated proteins, and inhibitors of these intermediates suppress S6K activation associated with osteoclast apoptosis. In this context, Rapamycin derivatives disturb osteoclast differentiation and survival. Thus, everolimus is a potent inhibitor of bone resorption and reduces osteoclastic differentiation [79]. Similarly, Sugutani and Hruska [81] demonstrated that mTOR stimulation by M-CSF leading to Bim inhibition plays a pivot function for cell survival in isolated osteoclast precursors.

Although further experiments are necessary to determine the real impact of mTOR inhibitors in bone metabolism (appropriate dose, period of administration) [82], they appear as bi-functional molecules able to target tumour cells and bone-resorptive cells. Similarly to bisphosphonates, first evidence emerge and reveal the interest to associate mTOR inhibitors with conventional chemotherapeutic drugs which can synergize together [83, 84].

III. CONCLUSIONS

Primary and secondary bone tumours frequently associate tumour growth and alterations of bone remodelling process. New therapeutic strategies of bone tumours target on the two main cellular protagonists strongly involved in these pathologies, tumour cells and osteoclasts. Taken together, the data presented above demonstrate that N-BP derived from endogenous pyrophosphate and rapamycin derivatives might be considered as bi (multi)-functional molecules that exert both endogenous anti-tumour and anti-osteolytic activies through distinct mechanisms and can be envisaged for the treatment of tumour bone diseases. However, if N-BP are powerful anti-resorptive agents with secondary activities on cancer cells, rapamycin derivatives are powerful anti-tumour compounds with complementary activities on bone tissue. Although both therapeutic agents are bi-functional, recent clinical data point out the interest to associate these molecules together or with other chemotherapeutic agents. Such associations demonstrated the synergistic effects of these compounds which target the tumour cells and osteoclasts through distinct biological pathways. These associations have also other interests, especially to limit the risk of therapeutic resistance developed by tumour cells. Indeed, several tumour cells develop resistance to many chemotherapeutic agents, mainly associated with the multidrug resistant phenotype. For instance, some rapamycin derivatives are substrates for cytochrome 3A4 and P-gp, known to influence the pharmacokinetics and bioavailability of many drugs that become ineffective on tumour cells presenting this multidrug resistant phenotype. In this context, the association of these two bi-functional compounds targeting tumour cells and bone cells through distinct pathways may limit the risk of drug resistances and represent a future therapeutic approach of tumour osteolytic diseases.

REFERENCES

[1] Rosen, G.; Murphy, M.L.; Huvos, A.G.; Gutierrez, M.; Marcove, R.C. *Cancer,* **1976**, *37, 1*.

[2] Harris, J.; Hellman, S.; Henderson, I.C.; Kinne, D.W. *Bone diseases*. Lippincott JB, Philadelphia, **1987.**

[3] Wittrant, Y.; Théoleyre, S.; Chipoy, C.; Padrines, M.; Blanchard, F.; Heymann, D.; Rédini, F. *Biochim. Biophys. Acta*, **2004**, *1704*, 49.

[4] Heymann, D.; Ory, B.; Gouin, F.; Green, J.R.; Rédini, F. *Trends Mol. Med.,* **2004**, *10*, 337.

[5] Kavanagh, K.L.; Guo, K.; Dunford, J.E.; Wu, X.; Knapp, S.; Ebetino, F.H.; Rogers, M.J.; Russell, R.G.; Oppermann, U. *Proc. Natl. Acad. Sci. USA*, **2006,** *103*, 7829.

[6] Coxon, F.P.; Helfrich, M.H.; Van't Hof, R.; Sebti, S.; Ralston, S.H.; Hamilton, A.; Rogers, M. *J. Bone Miner. Res.,* **2000**, *15*, 1467.

[7] Thompson, K.; Rogers, M.J.; Coxon, F.P.; Crockett, J.C. *Mol. Pharmacol.,* **2006**, *69*, 1624.

[8] Saltel, F.; Chabanel, A.; Zhao, Y.; Lafage-Proust, M.H.; Clézardin, P.; Jurdic, P.; Bonnelye, E. *J. Bone Miner. Res.*, *2006*, *21*, 1913.

[9] Heymann, D.; Ory, B.; Blanchard, F.; Heymann, M.F.; Coipeau, P.; Charrier, C.; Couillaud, S.; Thiery, J.P.; Gouin, F.; Redini, F. *Bone,* **2005**, *37*, 74.

[10] Gouin, F.; Ory, B.; Rédini, F.; Heymann, D. *Int. J. Cancer,* **2006**, *119*, 980.

[11] Senaratne, S.G.; Pirianov, G.; Mansi, J.L.; Arnett, T.R.; Colston, K.W. *Br. J. Cancer*, **2000**, *82*, 1479.

[12] Lee, M.V.; Fong, E.M.; Singer, F.R.; Guenette, R.S. *Cancer Res*., **2001**, *61*, 2602.

[13] Boissier, S.; Ferreras, M.; Peyruchaud, O.; Magnetto, S.; Ebetino, F.H.; Colombel, M.; Delmas, P.; Delaisse, J.M.; Clezardin, P. *Cancer Res.*, **2000**, *60*, 2949.

[14] Evdokiou, A.; Labrinidis, A.; Bouralexis, S.; Hay, S.; Findlay, D.M. *Bone,* **2003,** *33*, 216.

[15] Kubista, B.; Trieb, K.; Sevelda, F.; Toma, C.; Arrich, F.; Heffeter, P.; Elbling, L.; Sutterluty, H.; Scotlandi, K.; Kotz, R.; Micksche, M.; Berger, W. *J. Orthop. Res.,* **2006,** *24*, 1145.

[16] Ory, B.; Blanchard, F.; Battaglia, S.; Gouin, F.; Rédini, F.; Heymann, D. *Mol. Pharmacol*., **2006**, *71*, 333**.**

[17] Fuchs, B.; Pritchard, D.J. *Clin. Orthop. Rel. Res*., **2002**, *397*, 40.

[18] Wadayama, B.; Toguchida, J.; Shimizu, T.; Ishizaki, K.; Sasaki, M.S.; Kotoura, Y.; Yamamuro, T. *Cancer Res.*, **1994**, *54*, 3042.

[19] Kuroda, J.; Kimura, S.; Segawa, H.; Sato, K.; Matsumoto, S.; Nogawa, M.; Yuasa, T.; Kobayashi, Y.; Yoshikawa, T.; Ottmann, O.G.; Maekawa, T. *Cancer Sci*., **2004**, *95*, 186.

[20] Carteni, G.; Bordonaro, R.; Giotta, F.; Lorusso, V.; Scalone, S.; Vinaccia, V.; Rondena, R.; Amadori, D. *Oncologist*, **2006**, *11*, 841.

[21] Michaelson, M.D.; Kaufman, D.S.; Kantoff, P.; Oh, W.K.; Smith, M.R. *Cancer*, **2006**, *107*, 530.

[22] Kuroda, J.; Kimura, S.; Segawa, H.; Kobayashi, Y.; Yoshikawa, T.; Urasaki, Y.; Ueda, T.; Enjo, F.; Tokuda, H.; Ottmann, O.G.; Maekawa, T. *Blood*, **2003**, *102*, 2229.

[23] Matsumoto, S.; Kimura, S.; Segawa, H.; Kuroda, J.; Yuasa, T.; Sato, K.; Nogawa, M.; Tanaka, F.; Maekawa, T.; Wada, H. *Lung Cancer*, **2005**, *47*, 31.

[24] Yuasa, T.; Nogawa, M.; Kimura, S.; Yokota, A.; Sato, K.; Segawa, H.; Kuroda, J.; Maekawa, T. *Clin. Cancer Res*., **2005**, *11*, 853.

[25] Neville-Webe, H.L.; Evans, C.A.; Coleman, R.E.; Holen, I. *Tumour Biol.,* **2006**, *27*, 92.

[26] Hiraga, T.; Ueda, A.; Tamura, D.; Hata, K.; Ikeda, F.; Williams, P.J.; Yoneda, T. *Int. J. Cancer*, **2003**, *106*, 973.

[27] Ory, B.; Heymann, M.F.; Blanchard, F.; Kamijo, A.; Gouin, F.; Heymann, D.; Rédini, F. *Cancer*, **2005**, *104,* 2522.

[28] Tannir, N.; Jonasch, E., Pagliaro, L.C.; Mathew, P.; Siefker-Radtke, A.; Rhines, L.; Lin, P.; Tibbs, R.; Do, K.A.; Lin, S.H.; Tu, S.M. *Cancer*, **2006**, *107*, 497.

[29] Kiagia, M.; Karapanagiotou, E.; Charpidou, A.; Dilana, K.; Dionellis, G.; Dannos, I..; Georgiou, E.; Syrigos, K.N. *Anticancer Res.*, **2006**, *26*, 3133.

[30] Andrizky, B.; Schuch, G.; Thoem, I.; Goern, M.; Brandl, S.; Bokemeyer, C.; Laack, E. *Onkologie*, **2006**, *29*, 206.

[31] Kishimoto, H.; Fukunaga, M.; Kushida, M.; Shiraki, M.; Itabashi, A.; Nawata, H.; Nakamura, T.; Ohta, H., Takaoka, K., Oahshi, Y.; Risedronate Phase III research group. *J. Bone Miner. Res.*, **2006**, *24*, 405.

[32] De Nijs, R.N.; Jacobs, J.W.; Lems, W.F.; Laan, R.F.; Algra, A.; Huisman, A.M., Buskens, E.; De Laet, C.E.; Oostveen, A.C., Geusen, P.P.; Bruyn, G.A.; Dijkmans, B.A.; Bijlsma, J.W.; STOP investigators. *N. Engl. J. Med.*, **2006**, *355*, 675.

[33] Ural, A.U.; Avcu, F.; Candir, M.; Guden, M., Ozcan, M.A. *Breast Cancer Res.*, **2006**, *8*, R52.

[34] Bamias, A.; Kastritis, E.; Bamia, C.; Moulopoulos, L.A.; Melakopoulos, I.; Kozas, G.; Koutsoukou, V.; Gika, D.; Anagnostopoulos, A.; Papdimitriou, C.; Terpos, E.; Dimopoulos, M.A. *J. Clin. Oncol.*, **2005**, *23*, 8580.

[35] Thakkar, S.G.; Isada, C.; Smith, J.; Karam, M.A.; Reed, J.; Tomford, J.W.; Englund, K.; Richmond, M.; Licata, M.; Hatch, C.; Hussein, M.A. *Med. Oncol.,* **2006**, *23*, 51.

[36] Vezina, C.; Kudelski, A.; Sehgal, S.N. *J. Antibiot. (Tokyo),* **1975***, 28*, 721.

[37] Wullschleger, S.; Loewith, R.; Hall, M.N. *Cell,* **2006**, *124*, 471.

[38] De Virgillio, C.; Loewith, R. *Int. J. Biochem. Cell Biol.,* **2006**, *38*, 1476.

[39] Reiling, J.H.; Sabatini, D.M. *Oncogene,* **2006**, *25*, 6373.

[40] Jiang, X.; Yeung, R.S. *Cancer Res*., **2006**, *66*, 5258.

[41] Petiot, A.; Pattingre, S.; Arico, S.; Meley, D.; Codgno, P. *Cell Struct. Funt.*, **2002**, *27*, 431.

[42] Heymann, D. *Curr. Opin. Investig. Drugs*, **2006**, *7*, 443.

[43] Mamame,Y.; Petroulakis, E.; LeBacquer, O.; Sonenberg, N. *Oncogene*, **2006**, *25*, 6416.

[44] Averous, J., Proud, C.G. *Oncogene*, **2006**, *25*, 6423.

[45] Avruch, J.; Hara, K.; Lin, Y.; Liu, M.; Long, X.; Ortiz-Vega, S.; Yonezawa, K. *Oncogene*, **2006**, *25*, 6361.

[46] Vivanco, I.; Sawyers, CL. *Nat. Rev. Cancer*, **2002**, *2*, 489

[47] Tsuruta, H.; Kishimoto, H.; Sasaki, T.; Horie, Y.; Natsui, M.; Shibata, Y.; Hamada, K.; Yajima, N.. Kawahara, K.; ;Sasaki, M.; Tsuchiya, N.; Enomoto, K.; Mak, T.W.; Nakano, T.; Habuchi, T.; Suzuki, A. *Cancer Res*., **2006**, *66*, 8389.
[48] Anai, S.; Goodison, S.; Shiverick, K.; Iczkowski, K.; Tanaka, M.; Rosser, C.J. *Hum. Gene Ther*., **2006**, *17*, 975.
[49] Cao, C.; Sbhawong, T.; Albert, J.M.; Kim, K.W.; Geng, L.; Sekhar, K.R.; Gi, Y.J.; Lu, B. *Cancer Res*., **2006**, *66*, 10040.
[50] Schmidt, H.; DeAngelis, G.; Eltze, E.; Gockel, I.; Semjonow, A.; Brandt, B. *Cancer Res*., **2006**, *66*, 8959.
[51] Inoki, K.; Li, Y.; Zhu, T.; Wu, J.; Guan, KL. *Nat. Cell Biol*., **2002**, *4*, 648.
[52] Zhang, Y.; Gao, X.; Saucedo, LJ.; Ru, B.; Edgar, BA.; Pan, D. *Nat. Cell Biol*., **2003**, *5*, 578.
[53] Inoki, K.; Ouyang, H.; Zhu, T.; Lindvall, C.; Wang, Y.; Zhang, X.; Yang, Q.; Bennett, C.; Harada, Y.; Stankunas, K.; Wang, CY.; He, X.; MacDougald, OA.; You, M.; Williams, BO.; Guan, KL. *Cell*, **2006**, *126*, 955.
[54] Avruch, J.; Hara, K.; Lin, Y.; Liu, M.; Long, X.; Ortiz-Vega, S.; Yonezawa, K. *Oncogene*, **2006**, *25*, 6361.
[55] Hara, K.; Yonezawa, K.; Weng, QP.; Kozlowski, MT.; Belham, C.; Avruch, J. *J. Biol. Chem*., **1998**, *273*, 14484.
[56] Mamane, Y.; Petroulakis, E.; LeBacquer, O.; Sonenberg, N. *Oncogene*, **2006**, *25*, 6416.
[57] Jacinto, E.; Loewith, R.; Schmidt, A.; Lin, S.; Ruegg, MA.; Hall, A.; Hall, MN. *Nat. Cell Biol*., **2004**, *6*, 1122.
[58] Huang, S.; Houghton, P.J. *Curr. Opin. Invest. Drugs,* **2002***, 3,* 295.
[59] Rowinsky, E.K. *Curr. Opin. Oncol*., **2004**, *16*, 564.
[60] Thomas, G.V. *Curr. Opin. Genet. Dev*., **2006**, *16*, 78.
[61] Young, D.A.; Nickerson-Nutter, C.L. *Curr. Opin. Pharmacol*., **2005**, *5*, 418.
[62] Rubio-Viqueira, B.; Hidalgo, M. *Curr. Opin. Invest. Drugs*, **2006**, *7*, 501.
[63] Witzig, T.E.; Kaufmann, S.H. *Curr. Treat. Options Oncol*., **2006**, *7*, 285.
[64] Cruz, M.C.; Cavallo, L..M; Gorlach, J.M.; Cox, G.; Perfect, J.R.; Cardenas, M.E.; Heitman, J. *Mol. Cell Biol*., **1999**, *19*, 4101.
[65] Khariwala, S.S.; Kjaergaard, J.; Lorenz, R.; Van Lente, F.; Shu, S.; Strome, M. *Laryngoscope,* **2006,** *116*, 814.
[66] Wan, X.; Mendoza, A.; Khanna, C.; Helman, L.J. *Cancer Res.,* **2005,** *65,* 2406.
[67] Cutler, C.; Antin, J.H. *Cancer,* **2004**, *100*, 657.
[68] Kauffman, H.M.; Cherikh, W.S.; Cheng, Y.; Hanto, D.W., Kahan, B.D. *Transplantation,* **2005**, *80*, 883.
[69] Reddy, G.K.; Mughal, T.I.; Rini; B.I. *Clin. Genitourin Cancer*, **2006**, *5*, 110.
[70] Glantschig, H.; Fisher, J.E.; Wesolowski, G.; Rodan, G.A., Reszka, A.A. *Cell Death Diff.,* **2003**, *10*, 1165.
[71] Easton, J.B.; Houghton, P.J. *Oncogene*, **2006**, *25*, 6436.
[72] Treeck, O.; Wackwitz, B.; Haus, U., Ortmann, O. *Gynecol. Oncol*., **2006**, *102*, 292.
[73] Ito, D.; Fujimoto, K.; Mori, T.; Kami, K.; Koizumi, M.; Toyoda, E.; Kawaguchi, Y.; Doi, R. *Int. J. Cancer*, **2006**, *118*, 2337.
[74] Merimski, O. *Int. J. Mol. Med*., **2004**, *14*, 931.
[75] Adjei, A.A. *Clin. Cancer Res*., **2006**, *12*, 4446s.
[76] Birle, D.C.; Hedley, D.W. Mol. *Cancer Ther*., **2006**, *5*, 2494.
[77] Reardon, D.A.; Quinn, J.A.; Vredenburgh, J.J.; Gururangan, S.; Friedman, A.H.; Desjardins, A.; Sathornsumetee, S.; Herndon, J.E. 2nd; Dowell, J.M.; McLendon, R.E; Provenzale, J.M.; Sampson, J.H.; Smith, R.P.; Swaisland, A.J.; Ochs, J.S.; Lyons, P.; Tourt-Uhlig, S.; Bigner, DD; Friedman, H.S.; Rich, J.N. *Clin. Cancer Res*., **2006**, *12*, 860.
[78] Kurmasheva, R.T.; Huang, S.; Houghton, PJ. *Br. J. Cancer*, **2006**, *95*, 955.
[79] Kneissel, M.; Luong-Nguyen, N.H.; Baptist, M.; Cortesi, R.; Zumstein-Mecker, S.; Kossida, S.; O'Reilly, T.; Lane, H.; Susa, M. *Bone,* **2004**, *35*, 1144.
[80] Krocker, D.; Perka, C.; Tuischer, J.; Funk, J.; Tohtz, S.; Buttgereit, F.; Matziolis, G. *Transpl. Int.,* **2006**, *19,* 563.
[81] Sugatani, T.; Hruska, K.A. *J. Biol. Chem*., **2005**, *280*, 3583.
[82] Kaihara, S.; Bessho, K.; Okubo, Y., Sonobe, J.; Kusumoto, K.; Ogawa, Y.; Iizuka, T. *Life Sci.,* **2002**, *72*, 247.
[83] Pacey, S.; Rea, D.; Steven, N.; Brock, C.; Knowlton, N.; Shand, N.; Hazell, K.; Zoellner, U.; O'Donnell, A.; Judson I. *J. Clin. Oncol.,* **2004**, *16S*, 3007
[84] Avellino, R.; Romano, S.; Parasole, R.; Bisogni, R.; Lamberti, A.; Poggi, V.; Venuta, S.; Romano, M.F. *Blood,* **2005**, *106*, 1400.

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A/ Effets *in vitro* d'un inhibiteur de mTOR (RAD001) sur des lignées cellulaires d'ostéosarcomes et de chondrosarcomes ainsi que sur la différenciation ostéoclastique.

mTOR est fortement impliqué dans de nombreuses fonctions cellulaires, en particulier dans la croissance et la prolifération (Petroulakis et al. 2006). Par conséquent, RAD001 représente un bon agent anti-prolifératif dans le traitement des tumeurs osseuses primitives. Les effets de RAD001 seront étudiés sur différentes lignées de chondrosarcomes et d'ostéosarcomes. Une comparaison des effets *in vitro* sera effectuée entre les cellules tumorales et les cultures primaires d'ostéoblastes et de chondrocytes en termes de prolifération (test XTT et incorporation de thymidine), signalisation intracellulaire (Western Blot), analyse du cycle cellulaire (cytométrie en flux, analyse des cyclines et des checkpoint par Western Blot), d'apoptose (activité caspase 1, 3, 8, Bax/Bcl2, marquage au Hoechst, étude vidéo microscopique par time lapse, analyse de la perméabilité mitochondriale), d'adhérence et d'organisation du cytosquelette (cytométrie en flux et microscopie confocale). Une analyse des effets de RAD001 associé à d'autres agents cytotoxiques tels que le mafosfamide et le métotrexate sera réalisée. La sensibilité des différentes lignées cellulaire sera corrélée aux éventuelles mutations de PTEN, PI3K, Akt…. Les différentes modifications génétiques seront déterminées par micro puce à ADN.

RAD001 est un inhibiteur de la résorption osseuse et réduit la différenciation ostéoclastique (Kneissel et al. 2004). De même, Sugutani et Hruska ont démontré que la stimulation de mTOR suite au traitement par le M-CSF conduisait à l'inhibition de Bim ce qui est une condition essentielle à la survie des précurseurs ostéoclastiques. Cependant, la Rapamycine favorise la différenciation des cellules RAW264.7 en ostéoclastes (Shui et al. 2002). Ce paradoxe peut s'expliquer par la présence d'ostéoblastes dans la culture ou par la différence de modèle utilisé. Nous étudierons les effets du RAD001 en comparaison avec ceux de la rapamycine sur des cellules de moelle de souris, des cellules RAW264.7 ainsi que sur des cellules humaines CD_{14} en termes de différenciation ostéoclastique, de signalisation cellulaire et de résorption.

B/ Approche thérapeutique des ostéosarcomes combinant RAD001 à l'acide zolédronique ou à tout autre agent de chimiothérapie conventionnel.

L'intérêt thérapeutique de l'association d'un agent anti-résorption osseuse et anti-tumoral dans le traitement des ostéosarcomes a déjà été démontré au laboratoire, cet intérêt étant basé sur l'hypothèse thérapeutique du blocage du cercle vicieux développé dans ces pathologies. Les objectifs de notre future étude sont : étudier les effets du RAD001 seul sur nos modèles d'ostéosarcomes de rat (Heymann et al. 2005) et de souris (Ory et al. 2005), et comparer les effets de l'association RAD001+acide zolédronique à l'association RAD001+ifosfamide.

L'efficacité de ces traitements sur le développement tumoral sera analysée selon divers paramètres : survie des animaux, développement local de la tumeur, apparition de métastases pulmonaires, analyse histologique et suivi clinique par analyse radiographique et au microscanner ainsi qu'étude des paramètres biologiques (marqueurs de la résorption osseuse et de l'angiogenèse présents dans le sérum).

C/ Approche thérapeutique des chondrosarcomes utilisant un inhibiteur de mTOR (RAD001)

Comme nous l'avons vu précédemment, le chondrosarcome est une tumeur particulièrement difficile à traiter. En effet, le seul traitement efficace reste la résection chirurgicale large, la chimiothérapie et la radiothérapie n'ayant aucune efficacité sur les chondrosarcomes.

L'efficacité du RAD001 dans le traitement du chondrosarcome *in vivo* sera évaluée, en termes de croissance tumorale et de survie, avant et après curetage intra-lésionnel, dans un modèle de chondrosarcome de rat où le geste chirurgical utilisé est identique à celui de la pratique clinique.

Aarden, E. M., Burger, E. H. and Nijweide, P. J. (1994). "Function of osteocytes in bone." J Cell Biochem **55**(3): 287-99.

Alapetite, C. and Blay, J. (2001). "Traitement non chirurgicaux des tumeurs des os: chimiothérapie et radiothérapie." Encycl Méd Chir **7p**: 14-702.

Anract, P., de Pinieux, G. and Tomeno, B. (2001). "Chondrosarcomes intraosseux." Encycl Méd Chir: 14-716.

Arndt, C. A. and Crist, W. M. (1999). "Common musculoskeletal tumors of childhood and adolescence." N Engl J Med **341**(5): 342-52.

Baldini, N., Scotlandi, K., Serra, M., Picci, P., Bacci, G., Sottili, S. and Campanacci, M. (1999). "P-glycoprotein expression in osteosarcoma: a basis for risk-adapted adjuvant chemotherapy." J Orthop Res **17**(5): 629-32.

Bartek, J., Lukas, C. and Lukas, J. (2004). "Checking on DNA damage in S phase." Nat Rev Mol Cell Biol **5**(10): 792-804.

Bellahcene, A., Chaplet, M., Bonjean, K. and Castronovo, V. (2007). "Zoledronate inhibits alphavbeta3 and alphavbeta5 integrin cell surface expression in endothelial cells." Endothelium **14**(2): 123-30.

Benassi, M. S., Molendini, L., Gamberi, G., Ragazzini, P., Sollazzo, M. R., Merli, M., Asp, J., Magagnoli, G., Balladelli, A., Bertoni, F. and Picci, P. (1999). "Alteration of pRb/p16/cdk4 regulation in human osteosarcoma." Int J Cancer **84**(5): 489-93.

Beretta, G. L., Gatti, L., Tinelli, S., Corna, E., Colangelo, D., Zunino, F. and Perego, P. (2004). "Cellular pharmacology of cisplatin in relation to the expression of human copper transporter CTR1 in different pairs of cisplatin-sensitive and -resistant cells." Biochem Pharmacol **68**(2): 283-91.

Bertermann, O., Marcove, R. C. and Rosen, G. (1985). "Effect of intensive adjuvant chemotherapy on wound healing in 69 patients with osteogenic sarcomas of the lower extremities." Recent Results Cancer Res **98**: 135-41.

Bezzi, M., Hasmim, M., Bieler, G., Dormond, O. and Ruegg, C. (2003). "Zoledronate sensitizes endothelial cells to tumor necrosis factor-induced programmed cell death: evidence for the suppression of sustained activation of focal adhesion kinase and protein kinase B/Akt." J Biol Chem **278**(44): 43603-14.

Biedler, J. L. and Riehm, H. (1970). "Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies." Cancer Res **30**(4): 1174-84.

Blackwell, A. (1995). "Sarcome des tissus mous et ostéosarcomes." Standard-Options-Recommandations.

Blair, H. C., Kahn, A. J., Crouch, E. C., Jeffrey, J. J. and Teitelbaum, S. L. (1986). "Isolated osteoclasts resorb the organic and inorganic components of bone." J Cell Biol **102**(4): 1164-72.

Boissier, S., Magnetto, S., Frappart, L., Cuzin, B., Ebetino, F. H., Delmas, P. D. and Clezardin, P. (1997). "Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices." Cancer Res **57**(18): 3890-4.

Breuil, V., Cosman, F., Stein, L., Horbert, W., Nieves, J., Shen, V., Lindsay, R. and Dempster, D. W. (1998). "Human osteoclast formation and activity in vitro: effects of alendronate." J Bone Miner Res **13**(11): 1721-9.

Capasso, L. L. (2005). "Antiquity of cancer." Int J Cancer **113**(1): 2-13.

Carlin, B. I. and Andriole, G. L. (2000). "The natural history, skeletal complications, and management of bone metastases in patients with prostate carcinoma." Cancer **88**(12 Suppl): 2989-94.

Chambers, T. J. and Fuller, K. (1985). "Bone cells predispose bone surfaces to resorption by exposure of mineral to osteoclastic contact." J Cell Sci **76**: 155-65.

Chan, J. M., Stampfer, M. J., Giovannucci, E., Gann, P. H., Ma, J., Wilkinson, P., Hennekens, C. H. and Pollak, M. (1998). "Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study." Science **279**(5350): 563-6.

Chou, A. J. and Gorlick, R. (2006). "Chemotherapy resistance in osteosarcoma: current challenges and future directions." Expert Rev Anticancer Ther **6**(7): 1075-85.

Coleman, R. E. (2001). "Metastatic bone disease: clinical features, pathophysiology and treatment strategies." Cancer Treat Rev **27**(3): 165-76.

Corey, E., Brown, L. G., Quinn, J. E., Poot, M., Roudier, M. P., Higano, C. S. and Vessella, R. L. (2003). "Zoledronic acid exhibits inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer." Clin Cancer Res **9**(1): 295-306.

Corey, E., Quinn, J. E., Bladou, F., Brown, L. G., Roudier, M. P., Brown, J. M., Buhler, K. R. and Vessella, R. L. (2002). "Establishment and characterization of osseous prostate cancer models: intra-tibial injection of human prostate cancer cells." Prostate **52**(1): 20-33.

Coxon, F. P. and Rogers, M. J. (2003). "The role of prenylated small GTP-binding proteins in the regulation of osteoclast function." Calcif Tissue Int **72**(1): 80-4.

Cvitkovic E, D. J., Armand JP, Khoury S (1993). "Handbook of chemotherapyin clinicaloncology." ScientificCommunicationInternational: 221-345.

Dahlin, D. (1978). "Bone tumors: general aspect and data on 6 221 cases." Springfield : CC Thomas: 190-225

Dalton, W. S. and Scheper, R. J. (1999). "Lung resistance-related protein: determining its role in multidrug resistance." J Natl Cancer Inst **91**(19): 1604-5.

David, J. P., Mehic, D., Bakiri, L., Schilling, A. F., Mandic, V., Priemel, M., Idarraga, M. H., Reschke, M. O., Hoffmann, O., Amling, M. and Wagner, E. F. (2005). "Essential role of RSK2 in c-Fos-dependent osteosarcoma development." J Clin Invest **115**(3): 664-72.

Desandes, E., Clavel, J., Berger, C., Bernard, J. L., Blouin, P., de Lumley, L., Demeocq, F., Freycon, F., Gembara, P., Goubin, A., Le Gall, E., Pillon, P., Sommelet, D., Tron, I. and Lacour, B. (2004). "Cancer incidence among children in France, 1990-1999." Pediatr Blood Cancer **43**(7): 749-57.

Diel, I. J. (1998). "[Bisphosphonates in the adjuvant therapy of breast cancer--pathophysiology, animal models, and clinical experiences]." Gynakol Geburtshilfliche Rundsch **38**(2): 64-71.

Diel, I. J., Solomayer, E. F., Costa, S. D., Gollan, C., Goerner, R., Wallwiener, D., Kaufmann, M. and Bastert, G. (1998). "Reduction in new metastases in breast cancer with adjuvant clodronate treatment." N Engl J Med **339**(6): 357-63.

Dunford, J. E., Thompson, K., Coxon, F. P., Luckman, S. P., Hahn, F. M., Poulter, C. D., Ebetino, F. H. and Rogers, M. J. (2001). "Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates." J Pharmacol Exp Ther **296**(2): 235-42.

Evdokiou, A., Labrinidis, A., Bouralexis, S., Hay, S. and Findlay, D. M. (2003). "Induction of cell death of human osteogenic sarcoma cells by zoledronic acid resembles anoikis." Bone **33**(2): 216-28.

Feugeas, O., Guriec, N., Babin-Boilletot, A., Marcellin, L., Simon, P., Babin, S., Thyss, A., Hofman, P., Terrier, P., Kalifa, C., Brunat-Mentigny, M., Patricot, L. M. and Oberling, F. (1996). "Loss of heterozygosity of the RB gene is a poor prognostic factor in patients with osteosarcoma." J Clin Oncol **14**(2): 467-72.

Fiorenza, F., Abudu, A., Grimer, R. J., Carter, S. R., Tillman, R. M., Ayoub, K., Mangham, D. C. and Davies, A. M. (2002). "Risk factors for survival and local control in chondrosarcoma of bone." J Bone Joint Surg Br **84**(1): 93-9.

Frank, S. A. and Nowak, M. A. (2004). "Problems of somatic mutation and cancer." Bioessays **26**(3): 291-9.

Frith, J. C., Monkkonen, J., Auriola, S., Monkkonen, H. and Rogers, M. J. (2001). "The molecular mechanism of action of the antiresorptive and antiinflammatory drug clodronate: evidence for the formation in vivo of a metabolite that inhibits bone resorption and causes osteoclast and macrophage apoptosis." Arthritis Rheum **44**(9): 2201-10.

Frith, J. C., Monkkonen, J., Blackburn, G. M., Russell, R. G. and Rogers, M. J. (1997). "Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5'-(beta, gamma-dichloromethylene) triphosphate, by mammalian cells in vitro." J Bone Miner Res **12**(9): 1358-67.

Frost, H. M. (1969). "Tetracycline-based histological analysis of bone remodeling." Calcif Tissue Res **3**(3): 211-37.

Fuchs, B. and Pritchard, D. J. (2002). "Etiology of osteosarcoma." Clin Orthop Relat Res(397): 40-52.

Gitelis, S., Bertoni, F., Picci, P. and Campanacci, M. (1981). "Chondrosarcoma of bone. The experience at the Istituto Ortopedico Rizzoli." J Bone Joint Surg Am **63**(8): 1248-57.

Glimcher, M. (1992). "The nature of the mineral component of bone and the mechanism of calcification." Disorders of bone and mineral metabolism .NewYork :Raven Press: 265-286.

Gokhale JA, B. A., Robey PG (2001). "The biochemistry of bone." Osteoporosis, Academic Press: 107-188.

Goldie, J. H. and Coldman, A. J. (1979). "A mathematic model for relating the drug sensitivity of tumors to their spontaneous mutation rate." Cancer Treat Rep **63**(11-12): 1727-33.

Goltzman, D. (1997). "Mechanisms of the development of osteoblastic metastases." Cancer **80**(8 Suppl): 1581-7.

Gomes, C. M., van Paassen, H., Romeo, S., Welling, M. M., Feitsma, R. I., Abrunhosa, A. J., Botelho, M. F., Hogendoorn, P. C., Pauwels, E. and Cleton-Jansen, A. M. (2006). "Multidrug resistance mediated by ABC transporters in osteosarcoma cell lines: mRNA analysis and functional radiotracer studies." Nucl Med Biol **33**(7): 831-40.

Green, J. R. (2004). "Bisphosphonates: preclinical review." Oncologist **9 Suppl 4**: 3-13.

Grem, J. L., King, S. A., Wittes, R. E. and Leyland-Jones, B. (1988). "The role of methotrexate in osteosarcoma." J Natl Cancer Inst **80**(9): 626-55.

Grigoriadis, A. E., Schellander, K., Wang, Z. Q. and Wagner, E. F. (1993). "Osteoblasts are target cells for transformation in c-fos transgenic mice." J Cell Biol **122**(3): 685-701.

Grimaud, E., Damiens, C., Rousselle, A. V., Passuti, N., Heymann, D. and Gouin, F. (2002). "Bone remodelling and tumour grade modifications induced by interactions between bone and swarm rat chondrosarcoma." Histol Histopathol **17**(4): 1103-11.

Guinebretière JM, L. C. A., Le péchoux C, Missenard G, Bonvalot S, Terrier P and Vanel D (2001). "Ostéosarcome de forme commune." Encycl Méd Chir **13p**: 14-704.

Harbour, J. W. and Dean, D. C. (2000). "The Rb/E2F pathway: expanding roles and emerging paradigms." Genes Dev **14**(19): 2393-409.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. and Elledge, S. J. (1993). "The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases." Cell **75**(4): 805-16.

Heymann, D., Ory, B., Blanchard, F., Heymann, M. F., Coipeau, P., Charrier, C., Couillaud, S., Thiery, J. P., Gouin, F. and Redini, F. (2005). "Enhanced tumor regression and tissue repair when zoledronic acid is combined with ifosfamide in rat osteosarcoma." Bone **37**(1): 74-86.

Hill, P. A. (1998). "Bone remodelling." Br J Orthod **25**(2): 101-7.

Hiraga, T., Ueda, A., Tamura, D., Hata, K., Ikeda, F., Williams, P. J. and Yoneda, T. (2003). "Effects of oral UFT combined with or without zoledronic acid on bone metastasis in the 4T1/luc mouse breast cancer." Int J Cancer **106**(6): 973-9.

Hiraga, T., Williams, P. J., Ueda, A., Tamura, D. and Yoneda, T. (2004). "Zoledronic acid inhibits visceral metastases in the 4T1/luc mouse breast cancer model." Clin Cancer Res **10**(13): 4559-67.

Huang, G., Mills, L. and Worth, L. L. (2007). "Expression of human glutathione S-transferase P1 mediates the chemosensitivity of osteosarcoma cells." Mol Cancer Ther **6**(5): 1610-9.

Ifergan, I., Meller, I., Issakov, J. and Assaraf, Y. G. (2003). "Reduced folate carrier protein expression in osteosarcoma: implications for the prediction of tumor chemosensitivity." Cancer **98**(9): 1958-66.

Iwamura, M., Hellman, J., Cockett, A. T., Lilja, H. and Gershagen, S. (1996). "Alteration of the hormonal bioactivity of parathyroid hormone-related protein (PTHrP) as a result of limited proteolysis by prostate-specific antigen." Urology **48**(2): 317-25.

Jagdev, S. P., Coleman, R. E., Shipman, C. M., Rostami, H. A. and Croucher, P. I. (2001). "The bisphosphonate, zoledronic acid, induces apoptosis of breast cancer cells: evidence for synergy with paclitaxel." Br J Cancer **84**(8): 1126-34.

JM Guinebretière, A. L. C., C Le Péchoux, G Missenard, S Bonvalot, P Terrier, D Vanel (2001). "Ostéosarcome de forme commune. (Editions Scientifiques et Médicales Elsevier SAS, Paris, tous droits réservés), Appareil locomoteur." Encycl Méd Chir: 14-704.

Kantharidis, P., El-Osta, S., Silva, M., Lee, G., Hu, X. F. and Zalcberg, J. (2000). "Regulation of MDR1 gene expression: emerging concepts." Drug Resist Updat **3**(2): 99-108.

Kavanagh, K. L., Guo, K., Dunford, J. E., Wu, X., Knapp, S., Ebetino, F. H., Rogers, M. J., Russell, R. G. and Oppermann, U. (2006). "The molecular mechanism of nitrogen-containing bisphosphonates as antiosteoporosis drugs." Proc Natl Acad Sci U S A **103**(20): 7829-34.

Ke, Y., Beesley, C., Smith, P., Barraclough, R., Rudland, P. and Foster, C. S. (1998). "Generation of metastatic variants by transfection of a rat non-metastatic epithelial cell line with genomic DNA from rat prostatic carcinoma cells." Br J Cancer **77**(2): 287-96.

Klenner, T., Wingen, F., Keppler, B., Valenzuela-Paz, P., Amelung, F. and Schmahl, D. (1990). "Therapeutic efficacy of two different cytostatic-linked phosphonates in combination with razoxane in the transplantable osteosarcoma of the rat." Clin Exp Metastasis **8**(4): 345-59.

Klenner, T., Wingen, F., Keppler, B. K., Krempien, B. and Schmahl, D. (1990). "Anticancer-agent-linked phosphonates with antiosteolytic and antineoplastic properties: a promising perspective in the treatment of bone-related malignancies?" J Cancer Res Clin Oncol **116**(4): 341-50.

Kneissel, M., Luong-Nguyen, N. H., Baptist, M., Cortesi, R., Zumstein-Mecker, S., Kossida, S., O'Reilly, T., Lane, H. and Susa, M. (2004). "Everolimus suppresses cancellous bone loss, bone resorption, and cathepsin K expression by osteoclasts." Bone **35**(5): 1144-56.

Kook, S. H., Son, Y. O., Chung, S. W., Lee, S. A., Kim, J. G., Jeon, Y. M. and Lee, J. C. (2007). "Caspase-independent death of human osteosarcoma cells by flavonoids is driven by p53-mediated mitochondrial stress and nuclear translocation of AIF and endonuclease G." Apoptosis **12**(7): 1289-98.

Kruh, G. D. (2003). "Introduction to resistance to anticancer agents." Oncogene **22**(47): 7262-4.

Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J. and Boyle, W. J. (1998). "Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation." Cell **93**(2): 165-76.

Lane, D. P. (1992). "Cancer. p53, guardian of the genome." Nature **358**(6381): 15-6.

Lane, K. T. and Beese, L. S. (2006). "Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I." J Lipid Res **47**(4): 681-99.

Lee, F. Y., Mankin, H. J., Fondren, G., Gebhardt, M. C., Springfield, D. S., Rosenberg, A. E. and Jennings, L. C. (1999). "Chondrosarcoma of bone: an assessment of outcome." J Bone Joint Surg Am **81**(3): 326-38.

Lee, M. V., Fong, E. M., Singer, F. R. and Guenette, R. S. (2001). "Bisphosphonate treatment inhibits the growth of prostate cancer cells." Cancer Res **61**(6): 2602-8.

Lehenkari, P. P., Kellinsalmi, M., Napankangas, J. P., Ylitalo, K. V., Monkkonen, J., Rogers, M. J., Azhayev, A., Vaananen, H. K. and Hassinen, I. E. (2002). "Further insight into mechanism of action of clodronate: inhibition of mitochondrial ADP/ATP translocase by a nonhydrolyzable, adenine-containing metabolite." Mol Pharmacol **61**(5): 1255-62.

Leslie, E. M., Deeley, R. G. and Cole, S. P. (2001). "Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters." Toxicology **167**(1): 3-23.

Lichtenstein L, J. H. (1943). "Chondrosarcoma of bone." Am J Pathol **19**: 553-589.

Linet, M. S., Ries, L. A., Smith, M. A., Tarone, R. E. and Devesa, S. S. (1999). "Cancer surveillance series: recent trends in childhood cancer incidence and mortality in the United States." J Natl Cancer Inst **91**(12): 1051-8.

Link, M. P., Goorin, A. M., Miser, A. W., Green, A. A., Pratt, C. B., Belasco, J. B., Pritchard, J., Malpas, J. S., Baker, A. R., Kirkpatrick, J. A. and et al. (1986). "The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity." N Engl J Med **314**(25): 1600-6.

Liu, Y. Y., Han, T. Y., Giuliano, A. E. and Cabot, M. C. (2001). "Ceramide glycosylation potentiates cellular multidrug resistance." Faseb J **15**(3): 719-30.

Logothetis, C. J. and Lin, S. H. (2005). "Osteoblasts in prostate cancer metastasis to bone." Nat Rev Cancer **5**(1): 21-8.

Lopez-Guerrero, J. A., Lopez-Gines, C., Pellin, A., Carda, C. and Llombart-Bosch, A. (2004). "Deregulation of the G1 to S-phase cell cycle checkpoint is involved in the pathogenesis of human osteosarcoma." Diagn Mol Pathol **13**(2): 81-91.

Lourda, M., Trougakos, I. P. and Gonos, E. S. (2007). "Development of resistance to chemotherapeutic drugs in human osteosarcoma cell lines largely depends on up-regulation of Clusterin/Apolipoprotein J." Int J Cancer **120**(3): 611-22.

Lowe, S. W., Ruley, H. E., Jacks, T. and Housman, D. E. (1993). "p53-dependent apoptosis modulates the cytotoxicity of anticancer agents." Cell **74**(6): 957-67.

Mackie, P. S., Fisher, J. L., Zhou, H. and Choong, P. F. (2001). "Bisphosphonates regulate cell growth and gene expression in the UMR 106-01 clonal rat osteosarcoma cell line." Br J Cancer **84**(7): 951-8.

Manolagas, S. C. (2000). "Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis." Endocr Rev **21**(2): 115-37.

Mensah-Osman, E. J., Thomas, D. G., Tabb, M. M., Larios, J. M., Hughes, D. P., Giordano, T. J., Lizyness, M. L., Rae, J. M., Blumberg, B., Hollenberg, P. F. and Baker, L. H. (2007). "Expression levels and activation of a PXR variant are directly related to drug resistance in osteosarcoma cell lines." Cancer **109**(5): 957-65.

Michaelson, M. D., Kaufman, D. S., Kantoff, P., Oh, W. K. and Smith, M. R. (2006). "Randomized phase II study of atrasentan alone or in combination with zoledronic acid in men with metastatic prostate cancer." Cancer **107**(3): 530-5.

Michigami, T., Hiraga, T., Williams, P. J., Niewolna, M., Nishimura, R., Mundy, G. R. and Yoneda, T. (2002). "The effect of the bisphosphonate ibandronate on breast cancer metastasis to visceral organs." Breast Cancer Res Treat **75**(3): 249-58.

Miller, C. W., Aslo, A., Won, A., Tan, M., Lampkin, B. and Koeffler, H. P. (1996). "Alterations of the p53, Rb and MDM2 genes in osteosarcoma." J Cancer Res Clin Oncol **122**(9): 559-65.

Monkkonen, H., Auriola, S., Lehenkari, P., Kellinsalmi, M., Hassinen, I. E., Vepsalainen, J. and Monkkonen, J. (2006). "A new endogenous ATP analog (ApppI) inhibits the mitochondrial adenine nucleotide translocase (ANT) and is responsible for the apoptosis induced by nitrogen-containing bisphosphonates." Br J Pharmacol **147**(4): 437-45.

Mosende, C., Gutierrez, M., Caparros, B. and Rosen, G. (1977). "Combination chemotherapy with bleomycin, cyclophosphamide and dactinomycin for the treatment of osteogenic sarcoma." Cancer **40**(6): 2779-86.

Moussavi-Harami, F., Mollano, A., Martin, J. A., Ayoob, A., Domann, F. E., Gitelis, S. and Buckwalter, J. A. (2006). "Intrinsic radiation resistance in human chondrosarcoma cells." Biochem Biophys Res Commun **346**(2): 379-85.

Nelson, J. B., Hedican, S. P., George, D. J., Reddi, A. H., Piantadosi, S., Eisenberger, M. A. and Simons, J. W. (1995). "Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate." Nat Med **1**(9): 944-9.

Nemeth, J. A., Harb, J. F., Barroso, U., Jr., He, Z., Grignon, D. J. and Cher, M. L. (1999). "Severe combined immunodeficient-hu model of human prostate cancer metastasis to human bone." Cancer Res **59**(8): 1987-93.

Nielsen, D., Maare, C. and Skovsgaard, T. (1996). "Cellular resistance to anthracyclines." Gen Pharmacol **27**(2): 251-5.

Notarnicola, M., Messa, C., Cavallini, A., Bifulco, M., Tecce, M. F., Eletto, D., Di Leo, A., Montemurro, S., Laezza, C. and Caruso, M. G. (2004). "Higher farnesyl diphosphate synthase activity in human colorectal cancer inhibition of cellular apoptosis." Oncology **67**(5-6): 351-8.

Nyberg, K. A., Michelson, R. J., Putnam, C. W. and Weinert, T. A. (2002). "Toward maintaining the genome: DNA damage and replication checkpoints." Annu Rev Genet **36**: 617-56.

O'Neal, L. W. and Ackerman, L. V. (1952). "Chondrosarcoma of bone." Cancer **5**(3): 551-77.

Ortiz-Gomez, A., Jimenez, C., Estevez, A. M., Carrero-Lerida, J., Ruiz-Perez, L. M. and Gonzalez-Pacanowska, D. (2006). "Farnesyl diphosphate synthase is a cytosolic enzyme in Leishmania major promastigotes and its overexpression confers resistance to risedronate." Eukaryot Cell **5**(7): 1057-64.

Ory, B., Heymann, M. F., Kamijo, A., Gouin, F., Heymann, D. and Redini, F. (2005). "Zoledronic acid suppresses lung metastases and prolongs overall survival of osteosarcoma-bearing mice." Cancer **104**(11): 2522-9.

Ory, B., Moriceau, G., Redini, F. and Heymann, D. (2007). "mTOR inhibitors (rapamycin and its derivatives) and nitrogen containing bisphosphonates: bi-functional compounds for the treatment of bone tumours." Curr Med Chem **14**(13): 1381-7.

Owen, M. (1988). "Marrow stromal stem cells." J Cell Sci Suppl **10**: 63-76.

Pacifici, R. (1998). "Cytokines, estrogen, and postmenopausal osteoporosis--the second decade." Endocrinology **139**(6): 2659-61.

Pantel, K. and Brakenhoff, R. H. (2004). "Dissecting the metastatic cascade." Nat Rev Cancer **4**(6): 448-56.

Papachristou, D. J., Batistatou, A., Sykiotis, G. P., Varakis, I. and Papavassiliou, A. G. (2003). "Activation of the JNK-AP-1 signal transduction pathway is associated with pathogenesis and progression of human osteosarcomas." Bone **32**(4): 364-71.

Papapoulos, S. E., Eekhoff, E. M. and Zwinderman, A. H. (2006). "Acquired resistance to bisphosphonates in Paget's disease of bone." J Bone Miner Res **21 Suppl 2**: P88-91.

Parfitt, A. (1992). "The physiologic and pathogenetic significance of bone histomorphometric data.Disorders of bone and mineral metabolism." New York : Raven Press: 475-489.

Pestell, R. G., Albanese, C., Reutens, A. T., Segall, J. E., Lee, R. J. and Arnold, A. (1999). "The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation." Endocr Rev **20**(4): 501-34.

Petroulakis, E., Mamane, Y., Le Bacquer, O., Shahbazian, D. and Sonenberg, N. (2006). "mTOR signaling: implications for cancer and anticancer therapy." Br J Cancer **94**(2): 195-9.

Philip T, B. J., Brunat-Mentigny M, Carrie C, Chauvot P, and Farsi F, F. B. e. a. (2000). "Standards, options, recommandations (SOR) pour le diagnostic, le traitement et la surveillance de l'ostéosarcome." Bull Cancer **86**: 159-176

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S. and Marshak, D. R. (1999). "Multilineage potential of adult human mesenchymal stem cells." Science **284**(5411): 143-7.

Pollard, M., Wolter, W. R. and Sun, L. (2000). "Prostate-seminal vesicle cancers induced in noble rats." Prostate **43**(1): 71-4.

Poole, K. E., van Bezooijen, R. L., Loveridge, N., Hamersma, H., Papapoulos, S. E., Lowik, C. W. and Reeve, J. (2005). "Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation." Faseb J **19**(13): 1842-4.

Pratt, C. B., Meyer, W. H., Howlett, N., Douglass, E. C., Bowman, L. C., Poe, D., Mounce, K., Kun, L. E. and Houghton, J. A. (1994). "Phase II study of 5-fluorouracil/leucovorin for pediatric patients with malignant solid tumors." Cancer **74**(9): 2593-8.

Pritchard, D. J., Lunke, R. J., Taylor, W. F., Dahlin, D. C. and Medley, B. E. (1980). "Chondrosarcoma: a clinicopathologic and statistical analysis." Cancer **45**(1): 149-57.

Rabbani, S. A., Desjardins, J., Bell, A. W., Banville, D., Mazar, A., Henkin, J. and Goltzman, D. (1990). "An amino-terminal fragment of urokinase isolated from a prostate cancer cell line (PC-3) is mitogenic for osteoblast-like cells." Biochem Biophys Res Commun **173**(3): 1058-64.

Rogers, M. J. (2003). "New insights into the molecular mechanisms of action of bisphosphonates." Curr Pharm Des **9**(32): 2643-58.

Rondeau, J. M., Bitsch, F., Bourgier, E., Geiser, M., Hemmig, R., Kroemer, M., Lehmann, S., Ramage, P., Rieffel, S., Strauss, A., Green, J. R. and Jahnke, W. (2006). "Structural basis for the exceptional in vivo efficacy of bisphosphonate drugs." ChemMedChem **1**(2): 267-73.

Ruoslahti, E. (1991). "Integrins." J Clin Invest **87**(1): 1-5.

Saad, F., Gleason, D. M., Murray, R., Tchekmedyian, S., Venner, P., Lacombe, L., Chin, J. L., Vinholes, J. J., Goas, J. A. and Chen, B. (2002). "A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma." J Natl Cancer Inst **94**(19): 1458-68.

Saarto, T., Blomqvist, C., Virkkunen, P. and Elomaa, I. (2001). "Adjuvant clodronate treatment does not reduce the frequency of skeletal metastases in node-positive breast cancer patients: 5-year results of a randomized controlled trial." J Clin Oncol **19**(1): 10-7.

Salomo, M., Jurlander, J., Nielsen, L. B. and Gimsing, P. (2003). "How myeloma cells escape bisphosphonate-mediated killing: development of specific resistance with preserved sensitivity to conventional chemotherapeutics." Br J Haematol **122**(2): 202-10.

Sandberg, A. A. and Bridge, J. A. (2003). "Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors." Cancer Genet Cytogenet **145**(1): 1-30.

Sasaki, A., Boyce, B. F., Story, B., Wright, K. R., Chapman, M., Boyce, R., Mundy, G. R. and Yoneda, T. (1995). "Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice." Cancer Res **55**(16): 3551-7.

Sasaki, A., Kitamura, K., Alcalde, R. E., Tanaka, T., Suzuki, A., Etoh, Y. and Matsumura, T. (1998). "Effect of a newly developed bisphosphonate, YH529, on osteolytic bone metastases in nude mice." Int J Cancer **77**(2): 279-85.

Sato, M. and Grasser, W. (1990). "Effects of bisphosphonates on isolated rat osteoclasts as examined by reflected light microscopy." J Bone Miner Res **5**(1): 31-40.

Schajowicz, F. (1993). "Histological typing of bone tumor." Springer-Verlag(2nd ed): 10-13.

Scheffer, G. L., Schroeijers, A. B., Izquierdo, M. A., Wiemer, E. A. and Scheper, R. J. (2000). "Lung resistance-related protein/major vault protein and vaults in multidrug-resistant cancer." Curr Opin Oncol **12**(6): 550-6.

Senaratne, S. G., Pirianov, G., Mansi, J. L., Arnett, T. R. and Colston, K. W. (2000). "Bisphosphonates induce apoptosis in human breast cancer cell lines." Br J Cancer **82**(8): 1459-68.

Serra, M., Pasello, M., Manara, M. C., Scotlandi, K., Ferrari, S., Bertoni, F., Mercuri, M., Alvegard, T. A., Picci, P., Bacci, G. and Smeland, S. (2006). "May P-glycoprotein status be used to stratify high-grade osteosarcoma patients? Results from the Italian/Scandinavian Sarcoma Group 1 treatment protocol." Int J Oncol **29**(6): 1459-68.

Sheth, D. S., Yasko, A. W., Johnson, M. E., Ayala, A. G., Murray, J. A. and Romsdahl, M. M. (1996). "Chondrosarcoma of the pelvis. Prognostic factors for 67 patients treated with definitive surgery." Cancer **78**(4): 745-50.

Shimazaki, J., Higa, T., Akimoto, S., Masai, M. and Isaka, S. (1992). "Clinical course of bone metastasis from prostatic cancer following endocrine therapy: examination with bone x-ray." Adv Exp Med Biol **324**: 269-75.

Shui, C., Riggs, B. L. and Khosla, S. (2002). "The immunosuppressant rapamycin, alone or with transforming growth factor-beta, enhances osteoclast differentiation of RAW264.7 monocyte-macrophage cells in the presence of RANK-ligand." Calcif Tissue Int **71**(5): 437-46.

Siddik, Z. H. (2003). "Cisplatin: mode of cytotoxic action and molecular basis of resistance." Oncogene **22**(47): 7265-79.

Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M. S., Luthy, R., Nguyen, H. Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliott, R., Colombero, A., Tan, H. L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw-Gegg, L., Hughes, T. M., Hill, D., Pattison, W., Campbell, P., Sander, S., Van, G., Tarpley, J., Derby, P., Lee, R. and Boyle, W. J. (1997). "Osteoprotegerin: a novel secreted protein involved in the regulation of bone density." Cell **89**(2): 309-19.

Sonnemann, J., Eckervogt, V., Truckenbrod, B., Boos, J., Winkelmann, W. and van Valen, F. (2001). "The bisphosphonate pamidronate is a potent inhibitor of human osteosarcoma cell growth in vitro." Anticancer Drugs **12**(5): 459-65.

Springfield, D. S., Gebhardt, M. C. and McGuire, M. H. (1996). "Chondrosarcoma: a review." Instr Course Lect **45**: 417-24.

Stearns, M. E. and Wang, M. (1996). "Effects of alendronate and taxol on PC-3 ML cell bone metastases in SCID mice." Invasion Metastasis **16**(3): 116-31.

Synold, T. W., Dussault, I. and Forman, B. M. (2001). "The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux." Nat Med **7**(5): 584-90.

Tennant, T. R., Kim, H., Sokoloff, M. and Rinker-Schaeffer, C. W. (2000). "The Dunning model." Prostate **43**(4): 295-302.

Thompson, K., Rogers, M. J., Coxon, F. P. and Crockett, J. C. (2006). "Cytosolic entry of bisphosphonate drugs requires acidification of vesicles after fluid-phase endocytosis." Mol Pharmacol **69**(5): 1624-32.

Turner, C. H. and Pavalko, F. M. (1998). "Mechanotransduction and functional response of the skeleton to physical stress: the mechanisms and mechanics of bone adaptation." J Orthop Sci **3**(6): 346-55.

van Bezooijen, R. L., ten Dijke, P., Papapoulos, S. E. and Lowik, C. W. (2005). "SOST/sclerostin, an osteocyte-derived negative regulator of bone formation." Cytokine Growth Factor Rev **16**(3): 319-27.

van Oosterom, A. T. and Taminiau, A. H. (1992). "Present trends on osteosarcoma and adult soft tissue sarcomas." Ann Oncol **3 Suppl 2**: S1-2.

Viereck, V., Emons, G., Lauck, V., Frosch, K. H., Blaschke, S., Grundker, C. and Hofbauer, L. C. (2002). "Bisphosphonates pamidronate and zoledronic acid stimulate osteoprotegerin production by primary human osteoblasts." Biochem Biophys Res Commun **291**(3): 680-6.

Vrhovac, R., Delmer, A., Tang, R., Marie, J. P., Zittoun, R. and Ajchenbaum-Cymbalista, F. (1998). "Prognostic significance of the cell cycle inhibitor p27Kip1 in chronic B-cell lymphocytic leukemia." Blood **91**(12): 4694-700.

Wadayama, B., Toguchida, J., Shimizu, T., Ishizaki, K., Sasaki, M. S., Kotoura, Y. and Yamamuro, T. (1994). "Mutation spectrum of the retinoblastoma gene in osteosarcomas." Cancer Res **54**(11): 3042-8.

Wang, L. L. (2005). "Biology of osteogenic sarcoma." Cancer J **11**(4): 294-305.

Wei, G., Lonardo, F., Ueda, T., Kim, T., Huvos, A. G., Healey, J. H. and Ladanyi, M. (1999). "CDK4 gene amplification in osteosarcoma: reciprocal relationship with INK4A gene alterations and mapping of 12q13 amplicons." Int J Cancer **80**(2): 199-204.

Wyman, J. J., Hornstein, A. M., Meitner, P. A., Mak, S., Verdier, P., Block, J. A., Pan, J. and Terek, R. M. (1999). "Multidrug resistance-1 and p-glycoprotein in human chondrosarcoma cell lines: expression correlates with decreased intracellular doxorubicin and in vitro chemoresistance." J Orthop Res **17**(6): 935-40.

Yarden, R. I., Pardo-Reoyo, S., Sgagias, M., Cowan, K. H. and Brody, L. C. (2002). "BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage." Nat Genet **30**(3): 285-9.

Zhau, H. E., Li, C. L. and Chung, L. W. (2000). "Establishment of human prostate carcinoma skeletal metastasis models." Cancer **88**(12 Suppl): 2995-3001.

Zhou, H., Randall, R. L., Brothman, A. R., Maxwell, T., Coffin, C. M. and Goldsby, R. E. (2003). "Her-2/neu expression in osteosarcoma increases risk of lung metastasis and can be associated with gene amplification." J Pediatr Hematol Oncol **25**(1): 27-32.

TRAITEMENT DES TUMEURS OSSEUSES PRIMITIVES PAR L'ACIDE ZOLEDRONIQUE

Mots-clés : acide zolédronique, ostéosarcome, résistance, cycle cellulaire

L'ostéosarcome est la plus fréquente des tumeurs osseuses primitives malignes et est souvent associée à une ostéolyse. Les traitements actuels ont largement contribué à la progression du taux de survie des patients, mais la forte toxicité des molécules de chimiothérapie utilisées est souvent mal tolérée chez les jeunes patients. De plus, le mauvais pronostic associé à la détection de métastases pulmonaires, fait qu'il est nécessaire de développer de nouvelles approches thérapeutiques des ostéosarcomes. Lors du développement d'un ostéosarcome, un cercle vicieux s'installe entre la prolifération tumorale et l'ostéolyse associée. Dans ce contexte, notre hypothèse est le développement de protocoles thérapeutiques utilisant une molécule anti-tumorale et une molécule anti-résorption osseuse afin de briser le cercle vicieux en ses deux points les plus sensibles. L'acide zolédronique représente un candidat de choix du fait de sa très forte activité anti-résorption osseuse et de son tropisme pour les sites d'ostéolyse. De plus un certain nombre d'études évoquent un potentiel effet anti-tumoral de l'acide zolédronique. Les objectifs de cette étude sont de démontrer et d'approfondir les activités anti-tumorales de l'acide zolédronique sur les cellules d'ostéosarcome et de chondrosarcome *in vitro* et *in vivo* et de démontrer l'efficacité de l'acide zolédronique seul ou associé à un agent de chimiothérapie. Enfin les phénomènes de résistance des cellules tumorales à l'acide zolédronique seront étudiés et les mécanismes responsables de cette résistance seront déterminés afin de pouvoir établir le meilleur protocole thérapeutique possible.

ZOLEDRONIC ACID TREATMENT OF PRIMARY BONE TUMORS

Key words: zoledronic acid, osteosaroma, resitance, cell cycle

Primary bone tumours, mainly represented by osteosarcoma are rare pathologies that affect young patients. Current therapeutic strategies are based on tumour resection associated with highly toxic chemotherapy. Unfortunately, a recurrence associated with osteolytic processes as well as an absence of response to anti-tumour drugs are observed in many cases, leading to the development of pulmonary metastases and to patient death. These data demonstrate that the response to the conventional treatment (chemotherapy) is disappointing. It is thus necessary to develop new therapeutic strategies and to improve the medical answer of these tumours. As the activators of bone resorption and tumour growth are involved in a common vicious cycle, we hypothesized that the modulation of bone resorption by Zoledronic acid could interfere with the growth of the primary tumour. Several experimental models are used: models of transplantable osteosarcoma in rat and mice that are able to induce pulmonary metastases and a model of rat chondrosarcoma. The aim of this study is to define the action mechanisms of Zoledronic acid *in vitro* and *in vivo* on osteosarcoma and chondrosarcoma cells and to test its association with others chemotherapeutic agents. We also tried to demonstrate the possibility of a tumor resistance development during the Zoledronic acid treatment of the osteosarcoma in order to improve the therapeutic protocol.

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