A new dimethyl ester bisphosphonate inhibits angiogenesis and growth of human epidermoid carcinoma xenograft in nude mice

Dominique Ledoux[a], Yamina Hamma-Kourbali[e], Mélanie Di Benedetto[b], Alexandrine Foucault-Bertaud[f], Olivier Oudar[c], Odile Sainte-Catherine[c], Marc Lecouvey[d] and Michel Kraemer[c]

Bisphosphonates are extensively used in the treatment of patients with metastasis-induced osteolysis. The major drawback in the efficacy of all bisphosphonates lies in their high hydrophilic nature, which results in poor membrane permeability and low availability for soft tissues. A reasonable approach to overcome these problems consists in masking one or more ionizable groups of bisphosphonates, notably by esterification of the hydroxyl functions. We have previously shown that the novel non-nitrogen-containing bisphosphonate BP7033 inhibited angiogenesis and growth of primary tumors in nude mice. The present study focuses on the dimethyl-esterified analog of this compound (Me-BP7033). *In-vitro*, Me-BP7033 inhibited proliferation of human carcinoma A431 cells as well as their invasive activity based on a transwell invasion assay. In-vivo, administration of Me-BP7033 (0.3 mg/kg) twice a week for 5 weeks inhibited the tumor growth of A431 cells xenografted in nude mice by 65%. Immunostaining of endothelial cells (ECs) in tumor sections revealed that Me-BP7033 inhibited the intratumor ECs density by 60%. The in-vivo anti-angiogenic properties of Me-BP7033 were also demonstrated in an in-vivo angiogenesis assay showing that Me-BP7033 reduced the vascular endothelial growth factor-stimulated infiltration of ECs in a Matrigel plug by 70%. In summary, we demonstrated for the first time that a diesterified bisphosphonate exhibited in vivo both anti-tumoral and anti-angiogenic activities with no apparent sign of toxic effects. These new diesterified compounds, which could display enhanced bioavailability and pharmacokinetics, thus represent interesting candidates for therapeutic applications such as cancer treatment. *Anti-Cancer Drugs* 17:479–485 © 2006 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2006, **17**:479–485

Keywords: A431 cells, angiogenesis, diesterified bisphosphonate, tumor growth

[a]EA3406, [b]INSERM U698, Laboratoire de Pharmacologie, [c]EA3410, [d]UMR CNRS 7033, Laboratoire de Chimie Structurale Biomoléculaire, UFR SMBH, Université Paris 13, Bobigny, France, [e]UMR CNRS 7149, Université Paris 12, Créteil, France and [f]INSERM U608, Laboratoire de Biochimie Fondamentale Moléculaire et Clinique, UFR de Pharmacie, Marseille, France.

Correspondence to D. Ledoux, EA3406, Service de Biochimie, Hopital Avicenne, 125 route de Stalingrad, 93000 Bobigny, France.
Tel: +331 48 95 59 28; fax: +331 48 95 56 27;
e-mail: dominique.ledoux@avc.ap-hop-paris.fr

Sponsorship: This work was supported by grants from the Association de Recherche contre le Cancer, the Ligue Nationale contre le Cancer and the Agence Nationale de Valorisation de la Recherche.

Received 10 November 2005 **Accepted** 20 December 2005

Introduction

Bisphosphonates are extensively used to treat a wide variety of pathologies characterized by abnormal calcium metabolism. Bisphosphonates are powerful inhibitors of bone resorption and are therefore used to treat bone metastases [1]. Furthermore, other important effects of bisphosphonates have been described, including induction of apoptosis in a large variety of cancer cells [2], inhibition of tumor cell proliferation and invasion in-vitro [3,4] as well as inhibition of tumor cell adhesion to bone matrix [5]. Recently, anti-angiogenic effects of bisphosphonates were demonstrated in tumoral [6] as well as non-tumoral angiogenesis in-vivo models [7]. Since primary and metastatic tumor growth are dependent on angiogenesis, bisphosphonates are promising therapeutic drugs for the treatment of cancer.

The major drawback in the use of bisphosphonate as an anti-tumoral agent is in its poor oral bioavailability (i.e. 0.3–7% in humans), poor membrane permeability and strong uptake for bone tissue [8]. These characteristics result from poor lipophilicity due to ionization of the phosphonic acid functions at physiological pH, and from their strong complexation with calcium and other ions in bone and intestinal lumen [9]. In order to overcome these problems, several strategies have been reported [10–12] and one led to a significant increase of drug efficiency in-vivo by introducing a dipeptide as the side-chain [13]. Another interesting approach is the modification of the phosphonic acid function itself by introducing ester groups. The presence of suitable hydrophobic groups should be able to decrease both the hydrophilicity and charge of the drug, and consequently increase the

0959-4973

bioavailability [14]. In order to develop such a strategy, we have previously described a new and original one-pot procedure to introduce one or several methyl group(s) on each acid function of the two phosphonic acid groups [15]. According to this procedure, we then elaborated a dimethyl ester bisphosphonate (Me-BP7033) corresponding to the diesterified derivative of the BP7033 molecule, whose anti-tumoral and anti-angiogenic properties have been previously reported [6]. In the present study, we demonstrated for the first time the anti-tumoral effect of a new esterified bisphosphonate on tumoral growth and angiogenesis using an in-vivo model of a primary xenografted solid tumor.

Material and methods

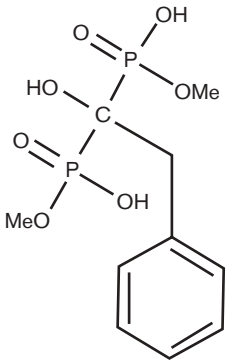
Chemicals

The novel bisphosphonate molecule evaluated in this study corresponds to a [1-hydroxy-1-(hydroxy-methoxy-phosphoryl)-2-phenyl-ethyl]-phosphonic acid monomethyl ester (Fig. 1). This compound, Me-BP7033, was synthesized as previously described [15] and corresponds to the partially diesterified derivative of the BP7033 molecule, whose anti-tumoral properties have been previously reported [6].

Cell proliferation assay

A431 cells (ATCC, Rockville, Maryland, USA) were seeded at a density of 5×10^4 cells/well in Dulbecco's Modified Eagle Medium (DMEM)/10% Fetal Calf Serum (FCS) and allowed to adhere to the plastic for 24 h. After washing with DMEM, the cells were incubated with Me-BP7033 at the indicated concentrations in DMEM/2% FCS. At different times, cells were washed with Phosphate Buffered Saline (PBS), dissociated with 0.025% trypsin–EDTA (Life Technologies, Gaithersburg, Maryland, USA) and counted using a Coulter counter (Coultronics, Margency, France).

Fig. 1



Structure of the Me-BP7033 molecule. Synthesis protocol and chemical characteristics of this diesterified bisphosphonate were described in detail in [15].

Cell invasion assay on a Matrigel-coated membrane in a transwell

A431 cells were pre-treated with the indicated concentration of Me-BP7033 in DMEM/2% FCS for 24 h or with Taxol (1 μmol/l) in the same medium for 60 min. Commercial cell culture invasion inserts with 8-μm pores (Corning, Acton, Massachusetts, USA) were coated in the upper surface with Matrigel (30 μg/insert = 100 μg/cm^2; BD Biosciences, San Jose, California, USA) for 16 h to prepare an in-vivo basement membrane. The assay was started by adding 3×10^4 pre-treated cells in 250 μl of 0.1% Bovine Serum Albumin (BSA)/DMEM on the upper chamber and 500 μl of DMEM/10% FCS in the lower chamber as a chemoattractant to induce invasion. After incubation at 37°C for 48 h the filters were fixed with paraformaldehyde 4% for 10 min. The upper surface of the filter was gently wiped with a cotton swab to remove non-migratory cells and the migrant cells on the under surface of the filter were stained with Mayer's hematoxylin. The membranes were cut from the inserts, mounted on glass slides and all the cells present on the under surface were counted (×20 magnification).

Tumor cell inoculation in nude mice

All in-vivo experiments were carried out with ethical committee approval and met the standards required by the UKCCCR guidelines [16]. A431 cells (5×10^5) were injected s.c. in the right flank of female athymic nude mice (Charles River, St Aubin-les-Elbeuf, France). All mice developed single s.c. palpable tumors of approximately 100 mm^3 1 week after cell inoculation. Mice were arbitrarily placed in control ($n = 6$) and Me-BP7033-treated groups ($n = 6$), and treated by s.c. injection close to the tumor of 0.1 ml PBS alone (control), or containing Me-BP7033 at 0.3 (0.006) or 3 mg/kg (0.06 mg/injection) twice a week. Their volumes were measured once a week along two major axes with calipers. Tumor volume (mm^3) was calculated as follows: $V = (4/3)\pi R_1^2 R_2$, where R_1 is radius 1, R_2 is radius 2 and $R_1 < R_2$.

Animal toxicity tests were performed according to the same protocol by s.c. injection of 300 mg/kg (6 mg) of Me-BP7033 on tumor-free healthy mice ($n = 5$). Each animal was weighed before, during and after treatments.

Tissue preparation, immunohistochemical staining and image analysis

Immediately after surgical resection, A431 cell-induced tumors were fixed in 4% paraformaldehyde and processed to paraffin inclusion. To visualize intratumor endothelial cells (ECs), sections of 5 μm were specifically stained with GSL-1 lectin (Vector, Burlingame, California, USA) as previously described [17–19]. The GSL-1 lectin binds specifically to galactosyl residues present on vascular endothelium in mice [20].

For each GSL-1-labeled section of control and Me-BP7033-treated tumors, three microscopic fields (× 200 magnification) containing exclusively viable tumor cells, as indicated by hematoxylin staining, were selected randomly for analysis. Image analysis was performed using the Image analysis NIH program as previously described [17–19]. The EC density in each field was expressed as the ratio of EC area/total area examined × 100 (%). These values were then averaged for untreated (control) and Me-7033-treated tumors.

In-vivo Matrigel plug assay model

The in-vivo angiogenesis assay using the Matrigel injection model (Matrigel plug assay) was carried out according to Passaniti *et al.* [21]. Batches of three nude mice (Charles River) were s.c. injected with 0.3 ml of Matrigel (BD Biosciences) alone (control) or containing 1 ng heparin supplemented with 4 nmol/l (30 ng) $VEGF_{165}$ (R & D Systems, Abingdon, UK) or with a mix of $VEGF_{165}$ (4 nmol/l) and Me-BP7033 (0.6 mg). Heparin was added because it improves vascular endothelial growth factor (VEGF)-induced EC penetration in the Matrigel. Mice were killed after 10 days, and the Matrigel plug was excised, frozen and fixed with acetone. Sample sections 5-μm thick were made with a cryostat and stained with hematoxylin for microscopic observations. The EC density was quantified by analyzing 10 Matrigel plug sections per mouse using the NIH Image software and expressed as the ratio of EC area/total area examined × 100 (%) as previously described [19].

Statistical analysis

Multiple statistical comparisons were performed using ANOVA in a multivariable linear model. Statistical comparisons were conducted using the Mann–Whitney *t*-test. $P < 0.05$ was considered statistically significant.

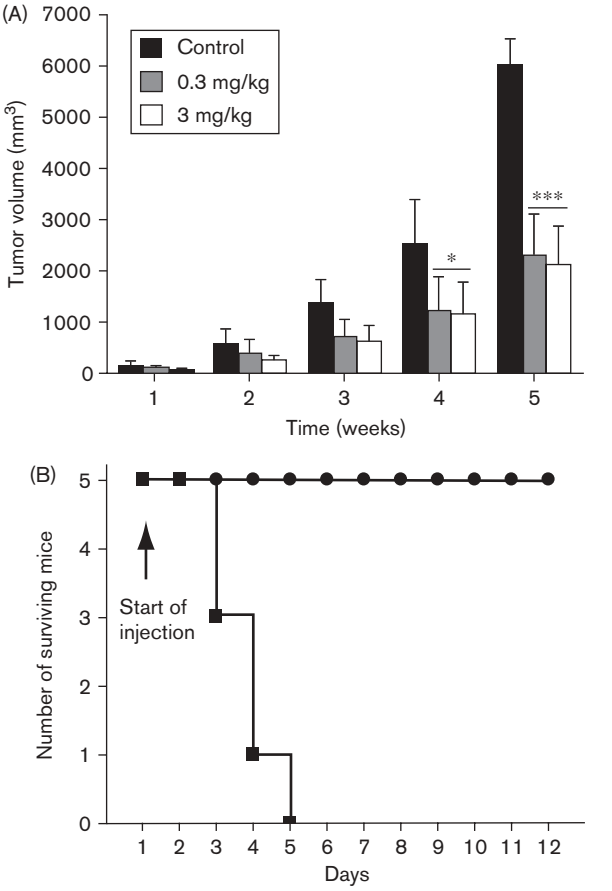
Results

Me-BP7033 inhibits the growth of A431 cells xenografted in nude mice with no signs of toxicity

All the mice developed tumors within 6 days after A431 cell inoculation and Me-BP7033 treatment was initiated at the end of the first week, when the tumors were well established. Me-BP7033 treatment (0.3 and 3 mg/kg twice a week s.c.) significantly affected primary tumor growth from 4 weeks of treatment as compared to control (Fig. 2A). After 5 weeks of treatment, Me-BP7033 inhibited the growth of A431 tumors by 65% ($P < 0.001$) and this effect is maximal even at the lowest dose tested (0.3 mg/kg).

All mice treated with the efficient doses of Me-BP7033 (0.3 and 3 mg/kg) were alive at the end of the 5 weeks of treatment ($n = 5$). No signs of toxicity such as diarrhea, infection, weakness or lethargy were observed, and the body weight of the inoculated mice was not affected by Me-BP7033 during the treatment. Me-BP7033 toxicity was also evaluated by daily s.c. administrating to healthy mice a dose 1000-fold higher (300 mg/kg) than the effective dose in vivo. As shown in Fig. 2(B), all treated mice were alive at the end of treatment (12 days) with no apparent sign of toxicity, whereas similar treatment with the parental tetra-acidic analog of Me-BP7033 (BP7033 compound) showed a marked toxicity characterized by a high level of morbidity from the third day after the start of injection.

Fig. 2

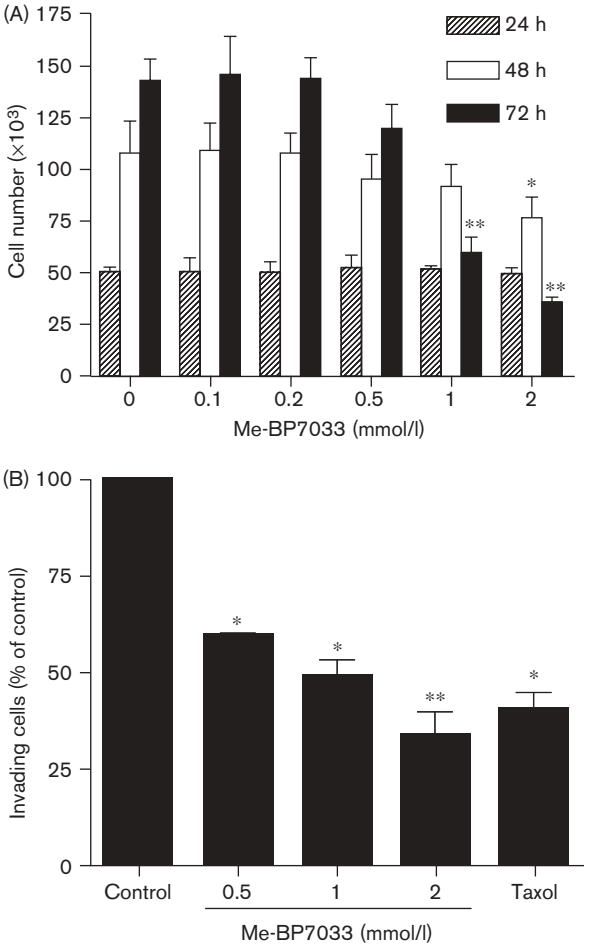


Inhibition of primary tumor growth by Me-BP7033. (A) A431 carcinoma cells (5×10^6) were inoculated s.c. into the right flank of female nude mice. When tumors reached about 100 mm^3 (6 days), Me-BP7033 (0.3 or 3 mg/kg) was administrated s.c. twice a week for 5 weeks. Tumors were measured and the results are presented as the mean tumor volume ± SE obtained from six mice in each group. ***$P < 0.001$; *$P < 0.05$ versus control. (B) A high dose (300 mg/kg) of Me-BP7033 (●) or its non-esterified analog (BP7033, ■) was s.c. inoculated daily for 12 days in the right flank of tumor-free nude mice ($n = 5$). Each point represents the number of surviving mice.

In-vitro effects of Me-BP7033 on A431 cell proliferation and invasion

As Me-BP7033 inhibited tumor growth, we first investigated if this effect was explained by an inhibition of A431 tumor cell proliferation. We demonstrated that treatment with Me-BP7033 at increasing concentrations, ranging

Fig. 3



In-vitro eects of Me-BP7033 on A431 cell proliferation (A) and invasion (B). (A) A431 cells were incubated with increasing concentrations of Me-BP7033, ranging from 0.1 to 2 mmol/l. At the indicated time, cells were trypsinized and counted. Mean of cell number ± SE from three independent experiments run in triplicate. $^{**}P<0.01$; $^{*}P<0.05$ versus control. (B) Cells were treated with various concentrations of Me-BP7033 for 24 h or with Taxol (1 μmol/l) for 60 min. After drug exposure, cells were harvested with trypsin/EDTA, washed and added to the upper chamber of Matrigel-coated inserts containing an 8-μm diameter pore size. FCS (10%), used as a chemoattractant, was placed in the lower chamber. After 48 h of incubation, cells that had traversed the filter were fixed, stained and counted. Data are expressed as the percentage (as compared to control) of the mean ± SE of three independent experiments. $^{**}P<0.01$; $^{*}P<0.05$ versus control.

from 0.1 to 2 mmol/l, resulted in a time- and concentration-dependent inhibition of A431 cell number (Fig. 3A). Me-BP7033 did not induce cell toxicity up to 72 h exposure of A431 cells, as assayed by Trypan blue dye exclusion, even at a high dose (5 mmol/l) (data not shown). The in-vitro anti-tumoral activity of Me-BP7033 was next investigated through its capacity to affect A431 cell invasion using an in-vitro transwell invasion assay (Boyden chamber). Under our conditions, as shown in Fig. 3(B), A431 cells were invasive in Matrigel-coated chambers. Pre-treatment of A431 cells for 24 h with Me-BP7033 inhibited A431 cell invasion in a dose-dependent manner (Fig. 3B). The number of invading cells was decreased by 65% ($P < 0.01$) following treatment with 2 mmol/l of Me-BP7033. Complete inhibition of this process was not achieved with this compound or with Taxol (1 μmol/l), used here as a positive control of inhibition of cell invasion [22]. It is important to note that the inhibitory effect of Me-BP7033 on A431 cell invasion is not due to an artifact effect on cellular adhesion on the Matrigel-coated membrane. Indeed, a 24-h pre-treatment of A431 cells with 2 mmol/l of Me-BP7033 did not affect their adhesion capacities as compared to untreated cells when this process was evaluated in Me-BP7033-free conditions and using a classical adhesion assay on Matrigel [23] (data not shown).

Me-BP7033 inhibits the intratumoral EC density

GSL-1 selectively labeled ECs and thus enabled us to determine the relative density of ECs (percentage of area occupied by ECs) in A431 tumor xenografted in nude mice. As shown in Fig. 4, Me-BP7033 treatment reduced the EC quantity in tumor tissues (Fig. 4b) as compared with control (Fig. 4a). The mean percentage of EC area in viable fields of tumors treated with 3 mg/kg of Me-BP7033 (2.8 ± 0.3; 18 fields in six tumors) was inhibited by 60% ($P < 0.01$) as compared to the control tumor value (7 ± 0.6; 18 fields in six tumors) (Fig. 4c).

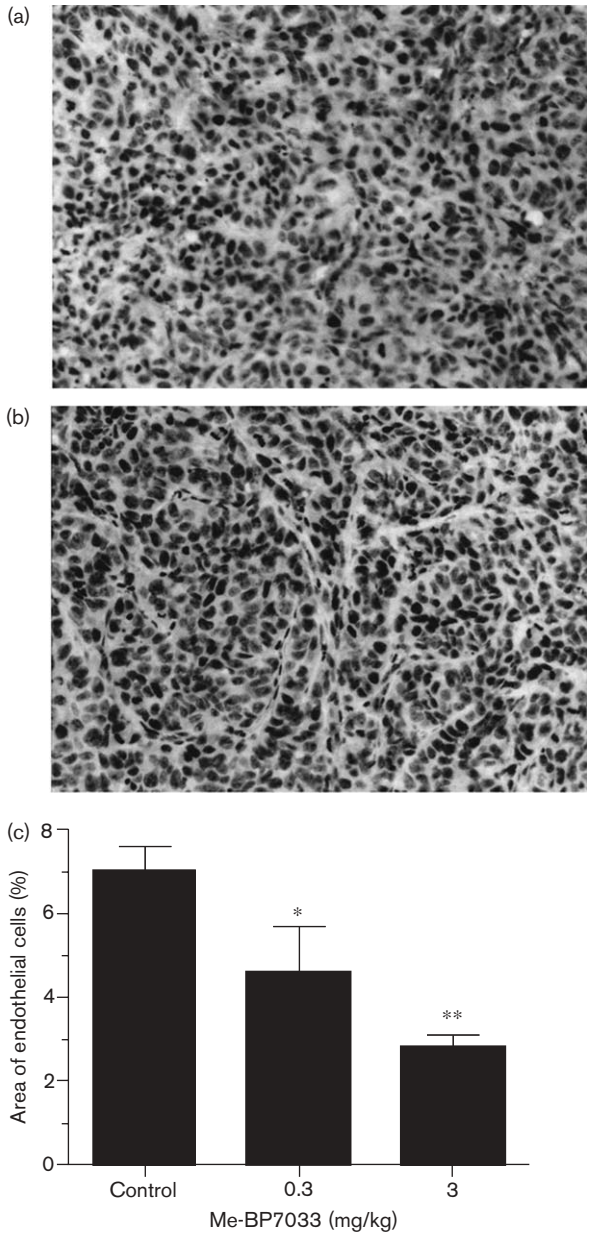
Me-BP7033 inhibits in-vivo angiogenesis in a Matrigel plug model

The inhibitory effect of Me-BP7033 on VEGF-induced angiogenesis in vivo was investigated in the Matrigel plug assay (Fig. 5). This method was previously developed for assessing effects of anti-angiogenic agents [21,24]. As compared to control, a 2.5-fold increase ($P < 0.05$) in the infiltration of ECs was observed when 4 nmol/l of VEGF was added to the Matrigel. Addition of Me-BP7033 (0.6 mg per plug) to the VEGF-containing Matrigel induced an inhibition of about 70% of the VEGF-stimulated EC infiltration ($P < 0.01$).

Discussion

Bisphosphonates are stable pyrophosphate analogs, potently inhibiting osteoclast-mediated bone resorption and widely used in a variety of human diseases including osteoporosis or complications associated with cancers that cause osteolysis. Once administrated, these drugs rapidly distribute to the bone, bind strongly to the hydroxyapatite bone mineral surface and internalize into osteoclasts. In addition to their potent anti-resorptive activity, there is now a growing body of evidence from in-vitro and in-vivo studies showing that bisphosphonates also exhibit anti-tumor activity [25,26]. These anti-tumor activities could result from a combination of direct and indirect effects such as inhibition of tumor cell invasion, proliferation and adhesion, as well as inhibition of

Fig. 4



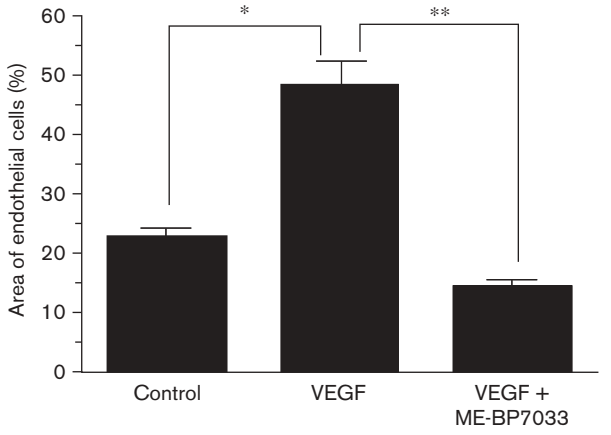
Immunohistochemical analysis of vascularization of xenografted A431 tumors in control and Me-BP7033-treated mice (original magnification $\times$ 400). Tumor vascularization was analyzed immunohistologically with the EC-specific marker GSL-1 in untreated tumors (a) and tumors treated with 3 mg/kg twice a week (s.c.) with Me-BP7033 (b). EC density was quantified (c) by Image analysis of GSL-1-labeled ECs as described in Materials and methods. The results are presented as the mean area ± SE (bars) of ECs in the Me-BP7033-treated and control tumor sections obtained from six mice in each group. $^{**}P<0.01$; $^{*}P<0.05$ versus control.

osteoclast activity. Recently, we have reported that a novel non-nitrogen-containing bisphosphonate (BP7033) inhibited both in-vitro and in-vivo tumoral angiogenesis [6]. Furthermore, we proposed that in addition to a direct effect on tumoral cells, inhibition of angiogenesis might be another likely mechanism by which BP7033 exerts its anti-tumoral activity in vivo.

The major drawback in using bisphosphonates as anti-tumor agents that target soft tissues lies in their strong uptake for bone tissue and poor oral bioavailability [8]. Bisphosphonate's poor oral bioavailability is often attributed to high ionization of phosphonic acid functions at physiological pH, which makes them very hydrophilic and poorly absorbed from the gastrointestinal tract. In addition, bisphosphonates are capable of binding divalent metal ions such as Ca^{2+}, Mg^{2+} and Fe^{2+} with high affinity, further diminishing intestinal absorption [9]. Thus, one of the major research targets has been to achieve better oral bioavailability by increasing lipophilicity of the bisphosphonate and one satisfying approach consists in masking one or more phosphonic acid functions by introducing ester groups [10,14]. These chemical modifications could further diminish the high avidity of bisphosphonates for Ca^{2+} [27], and consequently decrease their bone uptake and increase their soft tissue-targeting properties. According to an original procedure [15], we have synthesized from the parent BP7033 molecule a dimethyl ester bisphosphonate derivative (Me-BP7033), and we report in this study the first demonstration that a partially diesterified compound is able to inhibit tumor growth and angiogenesis in an in-vivo tumoral model.

Our results demonstrated that Me-BP7033 caused a decrease of tumor volume by 65% in A431 cell tumors xenografted in nude mice treated with 0.3 mg/kg. Interestingly, this inhibition of tumor growth is correlated with inhibition of tumor angiogenesis, since the Me-BP7033 treatment also led to a reduction of EC density in the tumor by 60%. The anti-angiogenic potency of Me-BP7033 was further supported by the results of experiments using the model of tumor-free angiogenesis, i.e. VEGF-enriched Matrigel. Indeed, inclusion of Me-BP7033 in VEGF-containing implants led to a decrease in the EC numbers that penetrate into the Matrigel plug. Thus, and as previously described for other anti-tumor drugs [17,18], it is likely that inhibition of angiogenesis could represent a mechanism by which Me-BP7033 prevents tumor growth. Direct effects could also contribute to the anti-tumor properties of Me-BP7033 since this compound prevented *in vitro* A431 cell proliferation and invasion – two well-known processes involved in tumoral progression. Importantly, animal toxicity studies indicated that in contrast to the tetra-acidic analog, Me-BP7033 was not toxic not only at the efficient dose, but also at a 1000-fold higher concentration. While this later result comes from animal studies, we believe that it is of particular interest for clinical applications since oral administration of classical bisphosphonates, especially nitrogen-containing compounds, is often accompanied

Fig. 5



In-vivo inhibition of VEGF-induced Matrigel infiltration of ECs by Me-BP7033. Liquid Matrigel was s.c. injected in nude mice alone (control) or in the presence of 1 ng heparin supplemented with 4 nmol/l $VEGF_{165}$ (VEGF) or supplemented with a mix of $VEGF_{165}$ (4 nmol/l) and Me-BP7033 (0.6 mg) (VEGF + Me-BP7033). Ten days after inoculation, Matrigel was removed and the number of infiltrated ECs was determined by averaging 10 Matrigel plug sections per mouse as described in Materials and methods. The results are presented as the mean area ± SE (bars) of ECs from triplicate experiments. $^{**}P < 0.01$ versus the VEGF-stimulated control; $^{*}P < 0.05$ versus control.

with esophageal and gastrointestinal disturbances, and even severe esophageal erosion and ulceration [8].

The molecular mechanisms by which Me-BP7033 inhibits tumor growth and angiogenesis remain to be determined. We have previously shown that BP7033, the non-esterified parental analog of Me-BP7033, prevented *in vitro* secretion of VEGF and matrix metalloproteinase 2 (MMP-2) from A431 cells, thus suggesting that VEGF- and/or MMP-2-related mechanisms may be involved in the anti-angiogenic effects of BP7033 observed in-vivo [6]. Intriguingly, Me-BP7033 did not affect VEGF and MMP-2 secretion by A431 cells when tested under the same experimental conditions (data not shown). It is then tempting to speculate that either Me-BP7033 and BP7033 inhibit tumor growth according to different in-vivo mechanisms or that Me-BP7033 functions as a pro-drug-like molecule. Pro-drugs are chemically modified inactive drug derivatives that contain a biologically active molecule which is covalently linked to a pro-moiety that can be released by either chemical or enzymatic hydrolysis. If such a mechanism takes place in vivo, activation of Me-BP7033 could require cleavage of the methyl ester pro-moieties covalently linked to the phosphorus atoms by serum- and/or cellular environment-containing (phospho)esterase enzymes, leading to the formation of the tetra-acidic, active molecule BP7033. We have investigated the relevance of such an enzymatic process by evaluating Me-BP7033 as a bioreversible pro-drug. Using conventional in-vitro tests where enzymatic hydrolysis is tested in serum, liver homogenates [11,28] or in whole A431 cell extracts, we do not detect significant amounts of BP7033 after a 72-h incubation of Me-BP7033, whatever the hydrolysis solution tested (data not shown). Accordingly, methyl esters of clodronic and etidronic acid were shown to be too chemically and enzymatically stable to release the parental drug [11,28]. The above results thus indicate that methyl ester derivatives of bisphosphonates, including Me-BP7033, cannot fulfill the criteria of a pro-drug and suggest the possibility that Me-BP7033 represents an anti-tumoral agent 'in its own right'. Whether this compound exerts its in-vitro (anti-proliferative, anti-invasive) and in-vivo anti-tumoral activities through mechanisms that differ from BP7033 needs further investigation. In particular, identification of the major metabolites of Me-BP7033 from the serum, bile and/or urine of rats is clearly necessary to investigate its in-vivo evolution and putative activation process. Interestingly, when phenyl groups were substituted instead of methyl groups, at least 50% of the diphenyl-esterified derivative was transformed to the monophenyl or tetra-acidic forms when assessed in enzymatic hydrolysis tests (data not shown). This indicates that, as previously suggested for diphenyl phosphonate [29], diphenyl bisphosphonic acids may be converted enzymatically into the tetra-acidic form by yet unidentified esterase(s) and thus represent good candidates in developing pro-drug-like molecules.

In conclusion, we report in this study the first demonstration that a dimethyl ester bisphosphonate (Me-BP7033) displays both in-vivo anti-tumoral and anti-angiogenic properties. Interestingly, Me-BP7033 exhibits similar efficacy in inhibiting tumor growth and angiogenesis compared with its non-esterified counterpart (BP7033) [6]; in contrast to BP7033, however, it is devoid of any apparent signs of toxicity. Moreover, and parallel to this work, we have recently performed and reported [3] a structure–activity study of a new class of aromatic non-esterified bisphosphonates with a view to further develop esterified bisphosphonates displaying a greater anti-tumor potency compared to Me-BP7033. Among the various non-esterified compounds tested, we identified the [(4-bromo-phenyl)-hydroxy-phosphono-methyl]-phosphonic acid (HMBP7) as the most potent anti-proliferative molecule toward A431 cells. Considering these overall results as well as the preliminary results showing the potential interest of phenyl ester groups as pro-moieties in developing pro-drug-like molecules, we decided to focus on the diphenyl-esterified derivative of HMBP7. Evaluation of its anti-tumoral and anti-angiogenic activities as well as its activation process is now in progress, and constitutes one of our major research goals in developing more efficient bisphosphonates for tumor and metastasis therapy.

References

1 Clezardin P, Fournier P, Boissier S, Peyruchaud O. *In vitro* and *in vivo* antitumor effects of bisphosphonates. *Curr Med Chem* 2003; **10**:173–180.

2 Green JR. Antitumor effects of bisphosphonates. *Cancer* 2003; **97**:840–847.

3 Guenin E, Ledoux D, Oudar O, Lecouvey M, Kraemer M. Structure–activity relationships of a new class of aromatic bisphosphonates that inhibit tumor cell proliferation *in vitro*. *Anticancer Res* 2005; **25**:1139–1145.

4 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**:2949–2954.

5 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**:3890–3894.

6 Hamma-Kourbali Y, Di Benedetto M, Ledoux D, Oudar O, Leroux Y, Lecouvey M, *et al.* A novel non-containing-nitrogen bisphosphonate inhibits both *in vitro* and *in vivo* angiogenesis. *Biochem Biophys Res Commun* 2003; **310**:816–823.

7 Wood J, Bonjean K, Ruetz S, Bellahcene A, Devy L, Foidart JM, *et al.* Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. *J Pharmacol Exp Ther* 2002; **302**:1055–1061.

8 Ezra A, Golomb G. Administration routes and delivery systems of bisphosphonates for the treatment of bone resorption. *Adv Drug Deliv Rev* 2000; **42**:175–195.

9 Lin JH. Bisphosphonates: a review of their pharmacokinetic properties. *Bone* 1996; **18**:75–85.

10 Vepsalainen JJ. Bisphosphonate prodrugs. *Curr Med Chem* 2002; **9**:1201–1208.

11 Niemi R, Turhanen P, Vepsalainen J, Taipale H, Jarvinen T. Bisphosphonate prodrugs: synthesis and *in vitro* evaluation of alkyl and acyloxymethyl esters of etidronic acid as bioreversible prodrugs of etidronate. *Eur J Pharm Sci* 2000; **11**:173–180.

12 Ahlmark M, Vepsalainen J, Taipale H, Niemi R, Jarvinen T. Bisphosphonate prodrugs: synthesis and *in vitro* evaluation of novel clodronic acid dianhydrides as bioreversible prodrugs of clodronate. *J Med Chem* 1999; **42**:1473–1476.

13 Ezra A, Hoffman A, Breuer E, Alferiev IS, Monkkonen J, El Hanany-Rozen N, *et al.* A peptide prodrug approach for improving bisphosphonate oral absorption. *J Med Chem* 2000; **43**:3641–3652.

14 Schultz C. Prodrugs of biologically active phosphate esters. *Bioorg Med Chem* 2003; **11**:885–898.

15 Migianu E, Mallard I, Bouchemal N, Lecouvey M. One-pot synthesis of 1-hydroxymethylene-1,1-bisphosphonate partial esters. *Tetrahedron Lett* 2004; **45**:4511–4513.

16 Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double J, *et al.* United Kingdom Coordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (second edition). *Br J Cancer* 1998; **77**:1–10.

17 Di Benedetto M, Starzec A, Vassy R, Perret GY, Crepin M, Kraemer M. Inhibition of epidermoid carcinoma A431 cell growth and angiogenesis in nude mice by early and late treatment with a novel dextran derivative. *Br J Cancer* 2003; **88**:1987–1994.

18 Hamma-Kourbali Y, Starzec A, Vassy R, Martin A, Kraemer M, Perret G, *et al.* Carboxymethyl benzylamide dextran inhibits angiogenesis and growth of VEGF-overexpressing human epidermoid carcinoma xenograft in nude mice. *Br J Cancer* 2003; **89**:215–221.

19 Bagheri-Yarmand R, Kourbali Y, Rath AM, Vassy R, Martin A, Jozefonvicz J, *et al.* Carboxymethyl benzylamide dextran blocks angiogenesis of MDA-MB435 breast carcinoma xenografted in fat pad and its lung metastases in nude mice. *Cancer Res* 1999; **59**:507–510.

20 Alroy J, Goyal V, Skutelsky E. Lectin histochemistry of mammalian endothelium. *Histochemistry* 1987; **86**:603–607.

21 Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, *et al.* A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest* 1992; **67**:519–528.

22 Magnetto S, Boissier S, Delmas PD, Clezardin P. Additive antitumor activities of taxoids in combination with the bisphosphonate ibandronate against invasion and adhesion of human breast carcinoma cells to bone. *Int J Cancer* 1999; **83**:263–269.

23 Virtanen SS, Vaananen HK, Harkonen PL, Lakkakorpi PT. Alendronate inhibits invasion of PC-3 prostate cancer cells by affecting the mevalonate pathway. *Cancer Res* 2002; **62**:2708–2714.

24 Inoki I, Shiomi T, Hashimoto G, Enomoto H, Nakamura H, Makino K, *et al.* Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. *FASEB J* 2002; **16**:219–221.

25 Clezardin P, Ebetino FH, Fournier PG. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. *Cancer Res* 2005; **65**:4971–4974.

26 Woodward JK, Coleman RE, Holen I. Preclinical evidence for the effect of bisphosphonates and cytotoxic drugs on tumor cell invasion. *Anticancer Drugs* 2005; **16**:11–19.

27 Raiman J, Niemi R, Vepsalainen J, Yritys K, Jarvinen T, Monkkonen J. Effects of calcium and lipophilicity on transport of clodronate and its esters through Caco-2 cells. *Int J Pharm* 2001; **213**:135–142.

28 Niemi R, Taipale H, Ahlmark M, Vepsalainen J, Jarvinen T. Simultaneous determination of clodronate and its partial ester derivatives by ion-pair reversed-phase high-performance liquid chromatography coupled with evaporative light-scattering detection. *J Chromatogr B Biomed Sci Appl* 1997; **701**:97–102.

29 DeLombaert S, Erion MD, Tan J, Blanchard L, el-Chehabi L, Ghai RD, *et al.* *N*-Phosphonomethyl dipeptides and their phosphonate prodrugs, a new generation of neutral endopeptidase (NEP, EC 3.4.24.11) inhibitors. *J Med Chem* 1994; **37**:498–511.