Bisphosphonate prodrugs: Synthesis and biological evaluation in HuH7 hepatocarcinoma cells

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · FEBRUARY 2014

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2014.02.054 · Source: PubMed

5 AUTHORS, INCLUDING:

Evelyne Migianu-Griffoni
Université Paris 13, France, Bobigny
19 PUBLICATIONS **258** CITATIONS
SEE PROFILE

Odile Sainte-Catherine
Université Paris 13 Nord
30 PUBLICATIONS **347** CITATIONS
SEE PROFILE

Mélanie Di Benedetto
Université Paris 13 Nord
43 PUBLICATIONS **621** CITATIONS
SEE PROFILE

Marc Lecouvey
Université Paris 13 Nord
119 PUBLICATIONS **1,080** CITATIONS
SEE PROFILE

European Journal of Medicinal Chemistry 77 (2014) 56–64

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Bisphosphonate prodrugs: Synthesis and biological evaluation in HuH7 hepatocarcinoma cells

Maelle Monteil, Evelyne Migianu-Griffoni, Odile Sainte-Catherine, Mélanie Di Benedetto, Marc Lecouvey*

Université Paris 13, Sorbonne Paris Cité, Laboratoire de Chimie, Structure, Propriétés de Biomatériaux et d'Agents Thérapeutiques (CSPBAT), CNRS UMR 7244, 74, Rue Marcel Cachin, F-93017 Bobigny, France

ARTICLE INFO

Article history:
Received 13 November 2013
Received in revised form
19 February 2014
Accepted 21 February 2014
Available online 23 February 2014

Keywords:
Bisphosphonates
Prodrugs
Phosphodiesterase inhibitor (IBMX)
Hepatocarcinoma

ABSTRACT

We investigated the biological effects of new synthesized bisphosphonates (BPs) on HuH7 hepatocarcinoma cells. BPs containing *p*-bromophenyl (R_1 = *p*-Br, Ph, **2**) in their side chain were the more potent to inhibit HuH7 cell viability. In addition, phenyl diesterified analogues ($R_2 = R_3$ = Ph, **2a**) were more potent than methyl ($R_2 = R_3$ = Me, **2b**) or non-esterified BPs (**2**) inducing more necrosis suggesting that they better entered into cells. Phosphodiesterase inhibitor (IBMX) reversed the effect of the esterified BPs and not that of non-esterified ones suggesting role of cell phosphodiesterases to release active BPs. BP analogues inhibited HuH7 cell migration but esterified ones had no effect on invasion due to the hiding of phosphonic groups. All together, these results indicated the therapeutic interest of these new BP prodrugs.

1. Introduction

Bisphosphonates (BPs) are currently the most widely used treatment of common skeletal disorders. They are used as inhibitors of bone resorption, in particular osteoporosis, solid tumour bone metastases and myeloma bone disease [1]. Most BPs contain a hydroxyl group on their P–C–P structure that confers high affinity binding to calcium phosphate (hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)) and is the basis for the bone-targeting properties of bisphosphonates [2,3]. The nature of their side chain (R_1, Fig. 1) gives rise to a variety of possible structures determining their different potencies.

Two classes of bisphosphonates depending on chemical structure can be distinguished: nitrogen-containing bisphosphonates, where the side chain possesses an amino function (i.e. alendronate) or a nitrogen heterocycle (i.e. zoledronate) and non-nitrogen-containing bisphosphonates corresponding to the others (i.e. clodronate, etidronate). It is clearly established that the mechanism of action on osteoclasts depends on the nature of bisphosphonates [4]. N-BPs in their side chain such as zoledronate, act on the mevalonate pathway inhibiting the farnesyl diphosphate synthase (FPPs) thereby depleting the cells of the farnesyl (FPP) or geranylgeranyl (GGPP) diphosphate isoprenoids. Isoprenoids are implicated in several important normal and tumour cellular pathways, via small G proteins like Rho or Ras. The inhibition of FPPS also results in the accumulation of its substrate, isopentenyl diphosphate (IPP), which can be converted to the isopentenyl ester of ATP (APPI), which is strongly pro-apoptotic and is thought to contribute to the overall efficacy of bisphosphonates, both *in vitro* and *in vivo* [2,5,6].

Non-N-BPs (e.g. etidronate or clodronate) cause the intracellular accumulation of non-hydrolysable cytotoxic ATP analogues that subsequently induce osteoclast apoptosis. The direct growth inhibitory effects have been attributed to cell cycle arrest and/or induction of apoptosis [4]. BPs also exhibit direct and indirect antitumour effects *in vitro* against a broad variety of tumour cell lines, including melanoma, mesothelioma, prostate, breast, lung and myeloma cancer cells [7–9]. Numerous studies have also described the ability of BPs to inhibit tumour cell adhesion and invasion, but the mechanisms of these effects are still unclear [8,10,11]. We have shown that some non-N-BPs had similar properties to N-BPs in particular in aromatic series [12,13]. However, *in vivo* efficacy of BPs on extra-osseous sites is still being debating. Only a small number of studies have demonstrated activity on soft tissues *in vivo* [14]. The reasons are the poor oral bioavailability (0.3–7% in humans) due to chelation of metal ions by phosphonic acid groups, poor membrane permeability due to poor BP lipophilicity as well as strong uptake by bone tissue [15]. In order to increase the pharmacological properties of bisphosphonates,

* Corresponding author.
E-mail address: marc.lecouvey@univ-paris13.fr (M. Lecouvey).

http://dx.doi.org/10.1016/j.ejmech.2014.02.054
0223-5234/

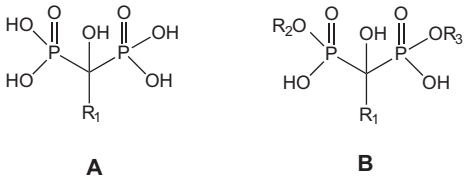


Fig. 1. General structures of BPs (**A**) or esterified BPs (**B**).

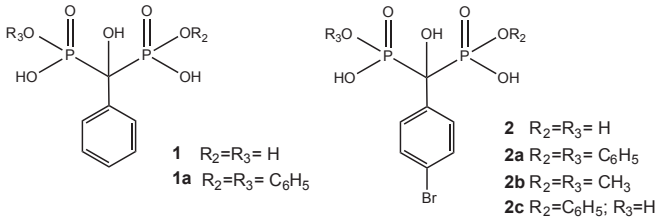


Fig. 2. General structures of BPs (**1**, **2**, **1a**, **2a**, **2b**, **2c**).

several drug delivery systems can be used, like nanoparticles [16] or liposomes [17]. Another strategy has been considered: the use of bisphosphonate prodrugs [18–21]. Few studies about the design of phosphonoester prodrugs have been reported in the literature [22,23], masking the phosphonic acid with organic functions [24,25]. We have demonstrated that partly esterified bisphosphonates display antitumour growth and antiangiogenic activities on A431 tumours and MDA-MB-231 tumours being more effective *in vivo* than *in vitro* [26,27]. In this last work, we have suggested that esterified BPs act like prodrug molecules being hydrolysed by cellular or serum environment containing phosphoesterase enzymes, leading to the release of the tetraacid active molecule.

Hepatocellular carcinoma (HCC) is one of the most common aggressive malignancies in the world [28]. More than 80% of patients have advanced or unresectable disease and when they do undergo resection, 50% of the patients present recurrence at the two years. Systemic chemical cytotoxic chemotherapy is used without satisfactory results and these therapies can induce significant toxicity due to the liver dysfunction [29]. Thus, new agents that target molecular abnormalities in HCC will improve the treatment of the patients. BPs could be effective in the treatment of HCC since FPP or GPP are crucial for the signalisation of small proteins G as Ras which can be mutated in HCC [30]. Accordingly, we have already demonstrated that BPs synthesized in our laboratory can inhibit the Ras processing [13]. Furthermore, another small G protein, Rho, has been proposed to play a crucial role in intrahepatic metastasis [31,32]. Only one work has demonstrated that N-BP (pamidronate) can inhibit several HCC cell line tumour growth and motility acting via the Ras and the Rho A pathway, respectively [33].

In this work, we study the efficacy of our non-nitrogen-containing BPs, the BPs containing phenyl ring without or with bromine (BP **1** and **2**, respectively) and esterified with different organic functions (**1a**, **2a**, **2b**, **2c**) on the viability, the migration and the invasion of the HuH7 HCC cell line (Fig. 2). We further evaluate the mechanism of action of these compounds by studying the effect of IBMX phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine) on the antiproliferative effect of esterified BPs.

2. Chemistry

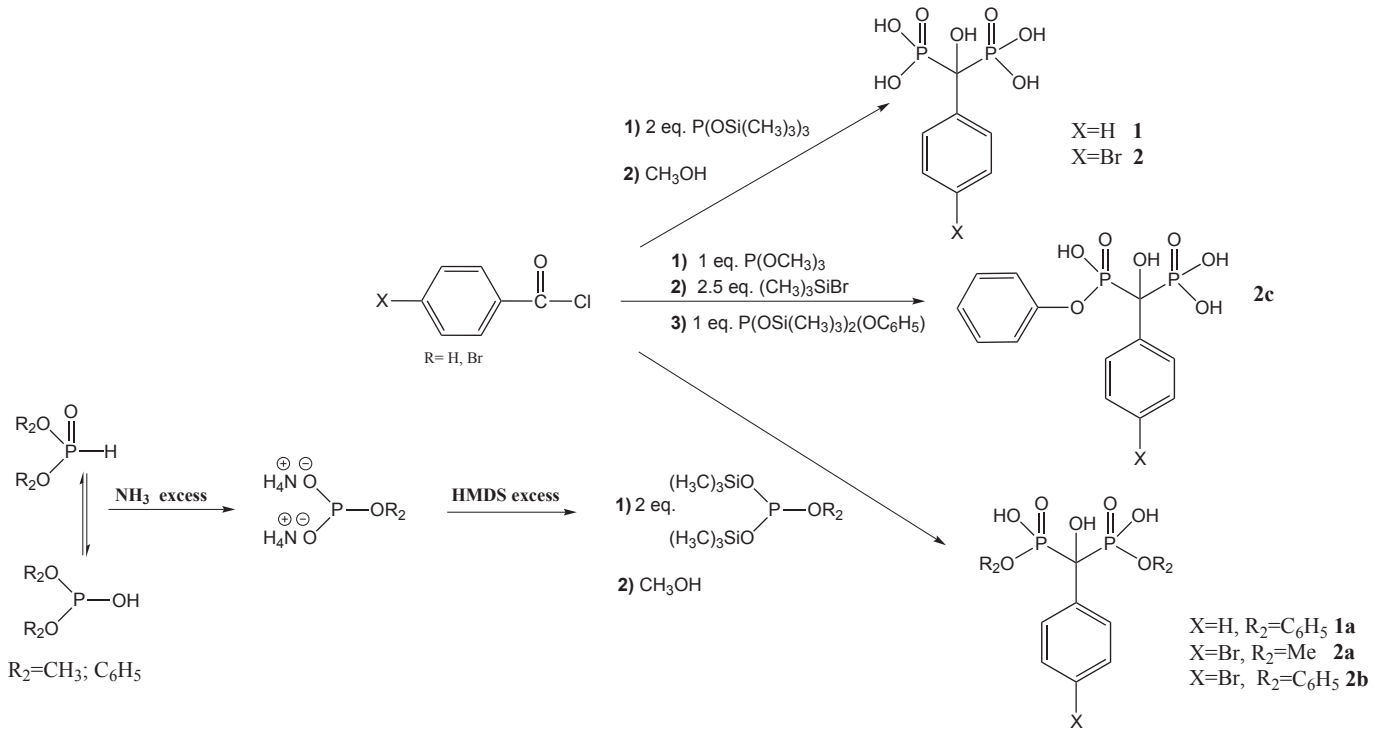
Synthesis of esterified bisphosphonates is not possible using standard procedure used for non esterified BPs. Most of the synthesis described up to nowadays usually goes through the making of the bisphosphonate tetraester which is then dealkylated [23]. The main drawback of this pathway is the thermal and basic instability of bisphosphonate tetraesters prone to isomerisation in phosphonate-phosphate [34]. Moreover the regioselective dealkylation to obtain partial esters is difficult and does not occur in good yields [23]. Our group has proposed a very mild and one-pot synthesis to obtain bisphosphonate methyl esters from methyl bis(trimethylsilyl) phosphite and acyl chloride [35]. We have further exemplified this synthesis to the obtaining of numerous bisphosphonate diesters varying both on the lateral chain and ester functions [24,25]. In this study, we chose to work with two aromatic bisphosphonates. Both compounds **1** (R_1 = Ph) and **2** (R_1 = *p*-BrPh) have already proved their biological activity on various tumour cell lines *in vitro* and/or *in vivo* [12,13]. The synthesis of **1** and **2** has been fully described earlier [36]. Products were obtained by reacting for 2 h, at room temperature, two equivalents of tris(-trimethylsilyl) phosphite or methyl (or phenyl) bis(trimethylsilyl) phosphite with the appropriate acid chloride (Scheme 1). After evaporation of the volatile fractions and treatment with methanol, products were purified by precipitation in a mixture of appropriate solvent. Products were obtained as powder in good yields from 70 to 95%. Product **2c** was obtained as described for BP monomethyl esters [37]. The successive addition of one equivalent of phenyl bis(trimethylsilyl) phosphite followed by another equivalent of tris(trimethylsilyl) phosphite on acid chloride only gave a mixture of BPs (non-esterified, mono and diesterified). Consequently the two step procedure going through the formation of the *p*-bromophenyl-α-ketophosphonate dimethyl ester was used. It was obtained by condensing at −10 °C one equivalent of trimethylphosphite with *p*-bromobenzoyl chloride. After the end of the addition, the reaction was kept 2 h at room temperature and evaporation under reduced pressure (0.1 Torr) gave quantitatively the *p*-bromophenyl-α-ketophosphonate dimethyl ester as a white powder. The α-ketophosphonate was then converted to its di(trimethylsilyl) ester in 5 h with 2.5 equivalents of bromotrimethylsilane. After vacuum evaporation of the volatile fractions, one equivalent of phenyl bis(trimethylsilyl) phosphite was added at 0 °C, under argon. The reaction was then let stirred 2 h at room temperature. The resulting mixture was evaporated and methanol treatment was done. Compound **2c** was then obtained by a simple wash with ether and was isolated as a white powder in 90% yield. All the different steps were followed by $^{31}P\{^1H\}$ NMR. **2c** shows the expected presence of two doublets in $^{31}P\{^1H\}$ NMR corresponding to the signal obtained for the two different phosphorus atom with a $^2J_{P-P}$ coupling constant of 28.6 Hz. Moreover the $^{13}C\{^1H\}$ NMR spectrum shows the characteristic signal at 77.6 ppm (dd) with a $^1J_{C-P}$ coupling constant of 142 Hz.

For biological evaluation, BP stock solution (1 mM) was prepared in sterilised distilled water using acid form. All the compounds were readily soluble at this concentration. The pHs were adjusted to 7.2 with a diluted potassium hydroxide solution.

3. Biological evaluation

3.1. BP effects on HuH7 cell viability

Two different classes of BPs regarding to their side chain (R_1 = Ph, compounds **1** or R_1 = *p*-BrPh, compounds **2**) were evaluated on the HuH7 cell viability (Fig. 3, Table 1). Cells were treated with esterified (**1a**, **2a**–**c**) or non-esterified BP (**1** and **2**) at concentrations ranging from 10 μM to 1 mM.



Scheme 1. Synthesis of Bisphosphonates (**1**, **2**, **1a**, **2a**, **2b**, **2c**).

As a positive control, the N-BP zoledronate was used. Zoledronate exhibited an IC_{50} of 60 μM on HuH7 cell viability. While the BPs **1** and **2b** only inhibited the HuH7 cell viability about 15% at each used concentration, the BPs **1a**, **2**, **2a** and **2c** inhibited the HuH7 cell viability in a dose-dependent manner. The IC_{50} were 550 μM for **1a**, 440 μM for **2**, 125 μM for **2a** and 100 μM for **2c** showing a better effect of PhBr-BPs esterified with Ph functions. Although zoledronate IC_{50} was better than phenyl esterified **2a** and **2c** BPs, it was interesting to note that the maximal effect (about 80%) of the three compounds was reached with the same concentration (250 μM).

3.2. BP and phosphodiesterase inhibitor (IBMX) combination effect on HuH7 cell viability

In order to state if the activity of phenyl esterified BP was related to cell phosphodiesterase activity on esterified phosphonic functions, we pretreated the cells with a phosphodiesterase inhibitor

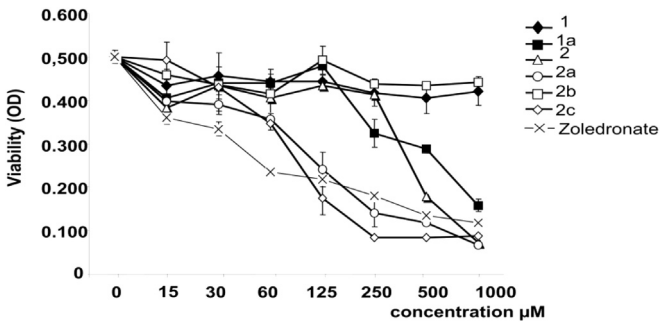


Fig. 3. Comparative effects of BPs on HuH7 cell proliferation. HuH7 cells were incubated with different concentrations of each compound. After 144 h, HuH7 cell proliferation was assessed as described in "Experimental protocols". Data represent the mean value ± SD of three independent experiments.

(3-isobutyl-1-methylxanthine, IBMX) (Fig. 4). We evaluated the effect of IBMX on the BP **1** and its esterified analogue **1a** as well as the compounds **2** and **2a**.

When HuH7 cells were treated with IBMX alone at 1 mM for 144 h, no significant inhibition of HuH7 cell viability was observed. Addition of IBMX to non-esterified BPs (**1** or **2**) increased their antiproliferative effects. In contrast, the addition of IBMX to esterified BPs (**1a** and **2a**) totally reversed the inhibitory effect of each compound suggesting that cell phosphodiesterase activities are essential for esterified BP activity on HuH7 viability.

To confirm that phosphodiesterase allowed the release of non-esterified BP into the cells, we tested the stability of diesterified bisphosphonates in different media over time (Table 2).

As shown in Table 2, diesterified bisphosphonate **2a** is very stable over time. After five days, no hydrolysis product has been observed in water, in cell culture medium and in human serum. The same stability study was carried out in presence of cellular extract. After five days, the ^{31}P NMR spectrum showed the presence of several phosphorylated species (Fig. 5).

Three major products have been identified. The structure of compound **2a** (12.09 ppm) is the main product. We also observed two other phosphorylated products, one singlet resonating at 15.84 ppm and one compound corresponding to two doublets (12.66 and 16.77 ppm). The structure of these compounds has been determined thanks to the synthesis of the pure compounds and the comparison of ^{31}P NMR data. The first product corresponds to the hydrolysis of the two phosphonic phenyl esters **2a** and the second was identified as the monodealkylation product **2c**. The presence of intracellular phosphodiesterases could explain this hydrolysis reaction.

3.3. BP effects on HuH7 cell apoptosis and necrosis

As the esterified BPs containing *p*-BrPh are more efficient that Ph analogues, we compared the effects of BP **2** and **2a** on HuH7 cell

Table 1
Structures and IC_{50} of BPs analogues.

Name	R_1	R_2	R_3	IC_{50}[b] (μM)
1	C_6H_5	H	H	[a]
1a	C_6H_5	C_6H_5	C_6H_5	555 ± 0.008
2	*p*-BrC_6H_4	H	H	440 ± 0.012
2a	*p*-BrC_6H_4	C_6H_5	C_6H_5	125 ± 0.04
2b	*p*-BrC_6H_4	CH_3	CH_3	[a]
2c	*p*-BrC_6H_4	C_6H_5	H	100 ± 0.03

[a] Major than the maximum concentration tested.
[b] Mean values of three independent experiments and standard deviation is given.

death (Fig. 6). Both **2** and **2a** induced apoptosis effect on the HuH7 cells. Results did not show significant differences between the BPs **2** and **2a** to induce the early or late HuH7 apoptosis phases. The only difference was observed regarding necrosis where the esterified BP **2a** increased about 10% the number of cells in the necrosis phase as compared to **2**. In addition, we found that compounds **2** and **2a** arrested the cells in the G1 phase of cell cycle with the same efficacy (58%, data not shown).

3.4. BP effects on HuH7 cell migration

As the hepatocarcinoma cells are highly invasive, we tested the effect of these new BPs on the motility of the HuH7 cells (Fig. 7). In the presence of HGF in the lower part of the Boyden migration chamber, the HuH7 cells migrated through the pores to the lower surface of the membrane (Fig. 7A). Compounds **2** and **2a** (100 μM) significantly reduced migration by 95% and 68% respectively ($p < 0.05$, Fig. 7B) while zoledronate at the same concentration inhibited the HuH7 cell migration by 72%.

3.5. BP effects on HuH7 cell invasion

In the presence of HGF in the lower part of the Boyden migration chamber, the HuH7 cells invaded the Matrigel and were visualised at lower surface of the membrane (Fig. 8A). BP **2** (100 μM) reduced the invasion of the cells by 40% as compared with untreated control cells (Fig. 8B). Zoledronate at this concentration inhibited the HuH7 cell invasion by 54%. In contrast, BP **2a** did not reduce HuH7 invasion (Fig. 8A, B).

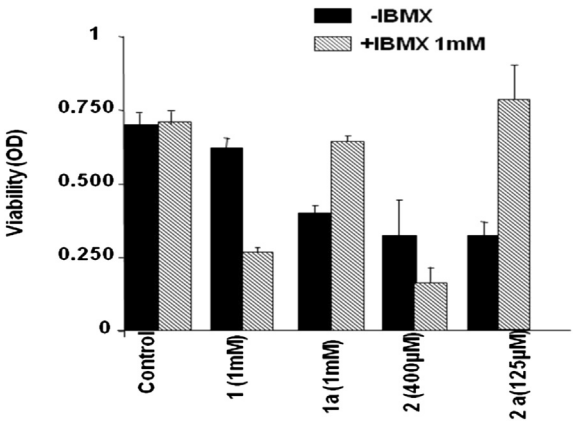


Fig. 4. Comparative effects of BPs in association with IBMX on HuH7 cell proliferation. HuH7 cells were pretreated with IBMX for 1 h prior to incubation with BPs. After 144 h, HuH7 cell proliferation was assessed as described in "Experimental protocols". Data represents the mean value ± SD of three independent experiments.

Table 2
Stability study of diesterified bisphosphonate **2a** in different media.

	^{31}P NMR chemical shift (ppm)			NMR signal multiplicity	Observation
	1 day	3 days	5 days		
D_2O	12.80	12.78	12.80	Singlet	No degradation
Cell culture medium	12.85	12.83	12.83	Singlet	No degradation
Human serum	12.85	12.65	12.80	Broad singlet	No degradation

4. Discussion

In this work, we demonstrated for the first time, the antiproliferative activity of BPs on HuH7 HCC cell line. Results showed the importance of esterification as well as the presence of *p*-bromophenyl (*p*-BrPh) as R1 side chain to inhibit HuH7 cancer cell viability. Indeed the p-bromophenyl (p-BrPh) containing BPs were more efficient than the non-bromine-containing ones (**2** versus **1**, respectively). These results were in agreement with previous data of our laboratory which showed that addition of bromine on the para position of the phenyl group in side chain exhibits an IC_{50} 10-fold better than non-substituted ones on A431 cell proliferation [12]. Herein, the antiproliferative improvement provided by bromine substituted phenyl ring is very important since the IC_{50} was about 440 μM for the compound 2 and whereas not reached for the compound **1** to inhibit HuH7 cells. Further, we demonstrated that bromine substituted compounds were non-toxic acting by inducing aponecrosis [38]. Accordingly, a crystallographic and computational investigation demonstrated that non nitrogen-containing BPs could interact with farnesyl enzyme thanks to the presence of benzyl ring in the side chain [39]. We could suppose that the presence of bromine on phenyl also let to a better arrangement of the BPs in the farnesyl enzyme pocket despite of the absence of benzyl. In another hand, the major improvement of our molecules was obtained by esterification of BPs. This result could be due to the higher lipophilicity of the phenyl group that could enhance entry into the cells. This observation was consistent with our hypothesis that the esterification of the phosphonic acid could help to increase lipophilicity and membrane permeability to BPs. Also, we thought that cell phosphodiesterases could liberate active BP into cells as well as in extracellular compartment. Indeed, to further evaluate the implication of phosphodiesterase in the activity of esterified BPs, we used IBMX which has been demonstrated to inhibit all phosphodiesterases [40]. Phosphodiesterases and more specifically the cyclic nucleotide phosphodiesterase 3 (PDE3) have been detected in HepG2, Hep3b and HuH7 HCC lines [41]. Only the esterified forms of BPs were reversed by IBMX showing the involvement of cell phosphodiesterases in the antiproliferative effect of esterified BPs. In contrast, the antiproliferative of non-esterified BPs was increased by IBMX in an additive manner. This additive effect is probably due to the association of the cell alteration induced by IBMX hydrolysing AMPc or GMPc [39] with the effect of BPs on small G proteins. Accordingly, we had previously described the inhibition of Ras processing by BPs [13]. It is noteworthy that non-N-BPs (as BPs) could also induce non-hydrolysable analogues of ATP [14]. Thus, this last effect could induce additive effects with that of IBMX. In contrast, reversion of esterified BP effects strongly suggested that these molecules acted like prodrugs by releasing the tetra-acid active form into the cells. Furthermore, we demonstrated that esterified BPs inhibited HuH7 cell migration but not invasion where metalloproteinases are important for the proteolytic degradation of the extracellular matrix. BPs could inhibit invasion by chelating zinc from the active site

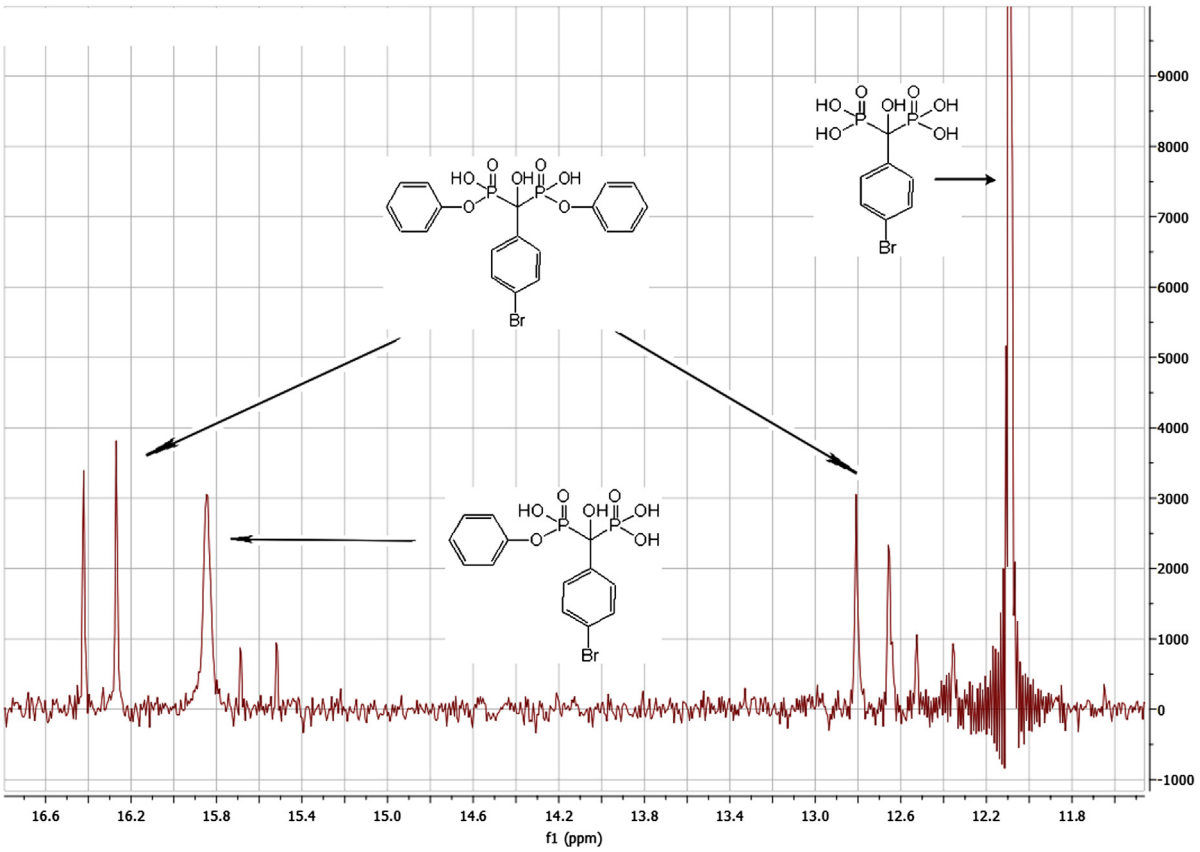


Fig. 5. ^{31}P NMR spectra of HuH7 cellular extract in the presence of **2a** (0.5 mM) during 5 days. The cellular extract concentrations are comparable (5×10^6 cells/mL) for each sample. The peak assignments are shown in figure. 50000 transients were accumulated for the spectrum.

of MMPs [7]. We confirmed this hypothesis with our non-esterified BPs which inhibited the activity of MMP-2 in MDA-MB-231 cancer cells (data not shown). In this work, the ineffective effect of esterified BPs suggested that the masking of phosphonic groups rendered the molecules enable to inhibit the active site of MMPs in our experimental condition. In the present study, we showed that all BPs were able to inhibit the motility of HuH7 HCC cells probably by inhibiting the anchorage of small G proteins such as Rho molecules like yet demonstrated for zoledronate [42]. In this work, the ineffective effect of esterified BPs suggested that the masking of phosphonic groups rendered the molecules enable to inhibit the active site of MMPs in our experimental conditions. Furthermore, esterified BPs were expected to give better results *in vivo* as compared to *in vitro* studies as well as to N-BPs since these compounds are chemically studied to improve their bioavailability to soft tissue as compared to bone.

5. Conclusion

In the present work, we demonstrated that diesterified BPs by phenyl groups, are better inhibitors of HuH7 HCC cell proliferation and migration as compared to non-esterified molecules. This improvement could be related to an increase of their lipophilicity inducing a better penetration into cells without toxicity. Furthermore, we confirmed the prodrug strategy of these compounds since cell phosphodiesterases can release active drug into cells.

All these results showed that *p*-bromophenyl and partly esterified BPs can be considered as lead compounds for the design of new BPs.

6. Experimental

6.1. BPs synthesis

6.1.1. General methods

Unless otherwise noted, all solvents and reagents were high-purity-grade materials and used without further purification. THF was distilled from benzophenone sodium. Diethylether was distilled from sodium. Trimethylphosphite and bromotrimethylsilane were distilled prior use. Solid acyl chlorides were used directly and liquid acid chlorides were distilled under reduced pressure. Boiling points are given in Torr ($B.p._{value}$). NMR spectra were recorded with a VARIAN Unity Inova 500 MHz (^{13}C: 125.9 MHz, ^{1}H: 500.6 MHz, ^{31}P: 200.7 MHz) spectrometer, a VARIAN Gemini 200 MHz (^{13}C: 50.3 MHz, ^{1}H: 200 MHz, ^{31}P: 80.9 MHz) spectrometer or a BRUKER Avance 400 MHz (^{13}C: 101 MHz, ^{1}H: 400 MHz, ^{31}P: 161.9 MHz) spectrometer in D_2O. Chemical shifts (δ) are given in ppm. ^{31}P and ^{13}C NMR spectra were recorded with phosphoric acid and methanol as external references, respectively. ^{1}H NMR spectra were recorded using HOD or trimethylsilane as internal standard in D_2O. Attribution of aromatic carbons and protons is given in the text by adding *o* for ortho, *m* for meta and *p* for para. IR spectra were recorded with a Perkin Elmer FT-IR model 2000 spectrophotometer in the 4000–500 cm^{-1} spectral domain. Spectral resolution was 2 cm^{-1} and 5 scans were usually accumulated. Multiple point baseline correction was performed using the PE Spectrum software. Samples were studied in D_2O solutions placed in cells closed by ZnSe windows.

Mass spectra were recorded in positive reflectron mode with DHB as a matrix on a MALDI-TOF-MS (Bruker). Microanalyses were

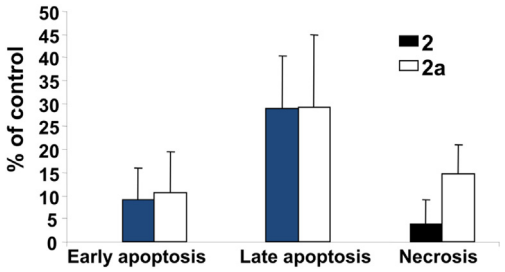


Fig. 6. Comparative effect of BPs on HuH7 cell death. HuH7 cells were treated with BPs for 144 h. Cells were stained with Annexin V and PI. Early and late apoptosis were determined by Ann V^+/PI^- and Ann V^+/PI^+ staining by flow cytometric analysis. Data represent the mean value ± SD of three independent experiments.

performed by the Service Central d'Analyse, CNRS, F-69390, Vernaison. France.

*6.1.2. General procedure for synthesis of bisphosphonic acid **1**, **2** and dialkyl or diaryl bisphosphonates **1a**, **2a**, **2b***

In a 50 mL round-bottom three-neck flask equipped with a thermometer and a condenser, acid chloride (2.5 mmol) in 5 mL THF was added under N_2 to tris(trimethylsilyl) phosphite or methyl (or phenyl) bis(trimethylsilyl) phosphite (5 mmol). When addition was completed, reaction mixture was allowed to stand at room temperature for 2 h. The end of the reaction was monitored by $^{31}P\{^1H\}$ NMR. Then, volatile fractions were evaporated under reduced pressure (0.1 Torr) before being hydrolysed with methanol. After evaporation, crude products were washed with diethylether and precipitated in an appropriate mixture of solvents. (tetrahydrofuran/dichloromethane 95/5).

*6.1.2.1. (Hydroxy-phenyl-phosphono-methyl)-phosphonic acid (**1**).* Precipitation in diethylether. Yield: 90%. M.p. 186 °C. IR (D_2O): 1645, 1598, 1495, 1450 (C═C), 1185, 1031, 1015, 970, 923, 775, 704, 667 cm^{-1}. ^{31}P NMR $\{^1H\}$ (200.7 MHz, D_2O) δ 15.9. ^1H NMR (500 MHz, D_2O) δ 7.33 (m, 1H, *p*-C_6H_5), 7.38 (dd, 2H, $^3J_{H-H}$ = 7.3 Hz, $^3J_{H-H}$ = 7.3 Hz, *m*-C_6H_5), 7.71 (d, 2H, $^3J_{H-H}$ = 7.3 Hz, *o*-C_6H_5). ^{13}C NMR $\{^1H\}$ (125.9 MHz, D_2O) δ 56.9 (OCH_3), 78.8 (t, $^1J_{P-C}$ = 145.7 Hz, P–*C*(OH)–P), 129.2 (*o*-C_6H_5), 131.0 (*p*-C_6H_5), 131.4 (*m*-C_6H_5), 140.0 (C_6H_5C(OH)).

*6.1.2.2. Disodium salt of [1-Hydroxy-(1-hydroxy-phenoxy-phosphoryl)-2-phenyl-methyl]-phosphonic acid monophenyl ester (**1a**).* Precipitation in diethylether. Yield: 78%. M.p. < 50 °C. ^{31}P NMR $\{^1H\}$

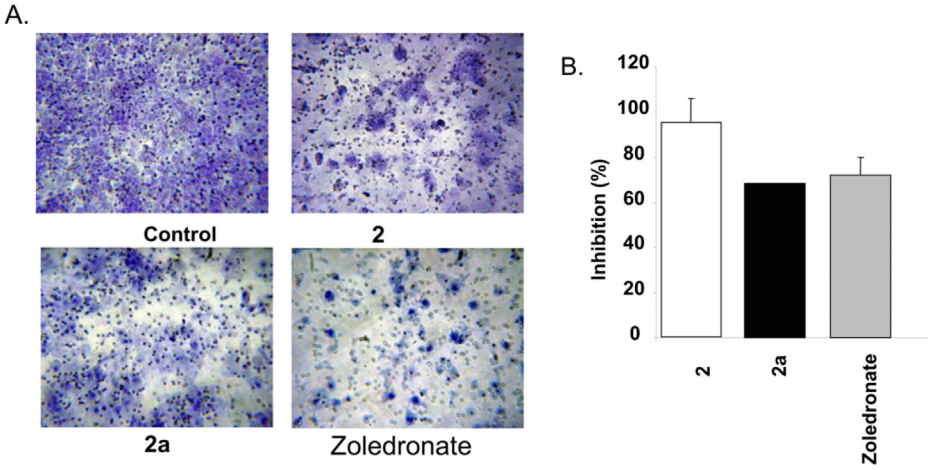


Fig. 7. Comparative effect of BPs on HuH7 cell migration. HuH7 cells treated with 100 μM of BPs or zoledronate were added to the upper chamber of fibronectin-coated inserts containing 8 μm diameter pore size. HGF (20 ng/mL) was used as a chemoattractant and placed in the lower chamber. After 24 h of incubation, cells at the lower side of the insert were fixed, stained and counted. Data represents the mean value ± SD of three independent experiments.

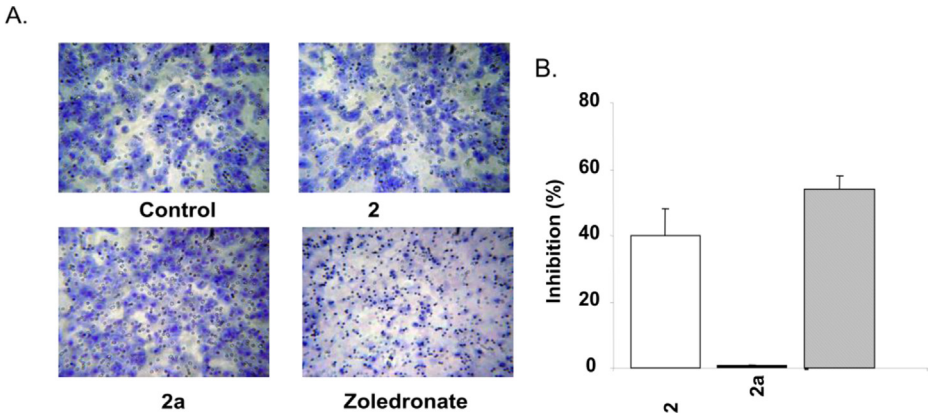


Fig. 8. Comparative effect of BPs on HuH7 cell invasion. HuH7 cells treated with 100 μM of BPs or Zoledronate were added to the upper chamber of Matrigel-coated inserts containing 8 μm diameter pore size. HGF (20 ng/mL) was used as a chemoattractant and placed in the lower chamber. After 24 h of incubation, cells at the lower side of the insert were fixed, stained and counted. Data represent the mean value ± SD of three independent experiments.

(200.7 MHz, D_2O) δ 11.6. ^1H NMR (500 MHz, D_2O) δ 6.75–7.89 (m, 15H, C_6H_5). ^{13}C NMR {^1H} (50.3 MHz, D_2O) δ 76.9 (t, $^1J_{P-C}$ = 143.1 Hz, P–*C*(OH)–P), 119.9–128.6 (*C*$_6$H$_5$; *C*$_6$H$_5$C(OH)), 136.8 (*C*$_6$H$_5$C(OH)), 150.7 (*C*$_6$H$_5$O). Anal. Calcd for $C_{19}H_{16}Na_2O_7P_2$: C, 49.15; H, 3.47; P, 13.34; Found: C, 49.22; H, 3.48; P, 13.39.

*6.1.2.3. [(4-Bromo-phenyl)-hydroxy-phosphono-methyl]-phosphonic acid (**2**).* Precipitation in diethylether. Yield: 90%. M.p. 210 °C. ^{31}P NMR {^1H} (200.7 MHz, D_2O) δ 16.4. ^1H NMR (500 MHz, D_2O) δ 7.54 (d, 2H, $^3J_{H-H}$ = 8.6 Hz, *m*-C$_6$*H*$_4$), 7.65 (d, 2H, $^3J_{H-H}$ = 8.6 Hz, *o*-C$_6$*H*$_4$). ^{13}C NMR {^1H} (125.9 MHz, D_2O) δ 75.6 (t, $^1J_{P-C}$ = 145.3 Hz, P–*C*(OH)–P), 123.3 (*p*-*C*$_6$H$_4$), 130.5 (*m*-*C*$_6$H$_4$), 132.9 (*o*-*C*$_6$H$_4$), 140.7 (*C*$_6$H$_4$C(OH)).

*6.1.2.4. [(4-Bromo-phenyl)-hydroxy-(hydroxy-phenoxy-phosphoryl)-methyl]-phosphonic acid monophenyl ester (**2a**).* Precipitation in diethylether. Yield: 70%. M.p. 92 °C. ^{31}P NMR {^1H} (200.7 MHz, D_2O) δ 12.8. ^1H NMR (500 MHz, D_2O) δ 6.93–7.80 (m, 14H, C$_6$*H*$_4$, C$_6$*H*$_5$). ^{13}C NMR (50.3 MHz, DMSO-*d*$_6$) δ 77.1 (t, $^1J_{P-C}$ = 145.4 Hz, P–*C*(OH)–P), 116.1 (*C*$_6$H$_5$), 121.5 (*C*$_6$H$_5$), 124.6 (*p*-*C*$_6$H$_4$), 129.9 (*C*$_6$H$_5$), 130.7 (*m*-*C*$_6$H$_4$), 130.8 (*o*-*C*$_6$H$_4$), 137.5 (*C*$_6$H$_4$C(OH)), 152.3 (O–*C*$_6$H$_5$). Anal. Calcd for $C_{19}H_{17}BrO_7P_2$: C, 45.72; H, 3.43; P, 12.41; Found: C, 45.62; H, 3.42; P, 12.36.

*6.1.2.5. [(4-Bromo-phenyl)-hydroxy-(hydroxy-methoxy-phosphoryl)-methyl]-phosphonic acid monomethyl ester (**2b**).* Precipitation in diethylether. Yield: 90%. M.p. 165 °C. ^{31}P NMR {^1H} (200.7 MHz, D_2O) δ 17.3. ^1H NMR (500 MHz, D_2O) δ 3.79 (d, 3H, $^3J_{P-H}$ = 3.0 Hz, OC*H*$_3$), 3.81 (d, 3H, $^3J_{P-H}$ = 3.0 Hz, OC*H*$_3$), 7.59 (d, 2H, $^3J_{H-H}$ = 8.0 Hz, *m*-C$_6$*H*$_4$), 7.66 (d, 2H, $^3J_{H-H}$ = 8.0 Hz, *o*-C$_6$*H*$_4$). ^{13}C NMR {^1H} (125.9 MHz, D_2O) δ 56.8 (OC*H*$_3$), 74.6 (t, $^1J_{P-C}$ = 146.2 Hz, P–*C*(OH)–P), 124.4 (*p*-*C*$_6$H$_4$), 131.1 (*m*-*C*$_6$H$_4$), 134.3 (*o*-*C*$_6$H$_4$), 138.9 (*C*$_6$H$_4$C(OH)). Anal. Calcd for $C_9H_{13}BrO_7P_2$: C, 28.82; H, 3.49; P, 16.52; Found: C, 28.92; H, 3.50; P, 16.57.

*6.1.3. General procedure for synthesis of bisphosphonate **2c***

p-Bromobenzoyl acid chloride (50 mmol) was added dropwise at −10 °C under argon to trimethylphosphite (5.9 mL, 50 mmol). The reaction mixture was then stirred at room temperature for 2 h (the end of the reaction was controlled by ^{31}P {^1H} NMR or IR spectroscopy). The crude product was purified by extraction with diethylether to furnish the corresponding α-ketophosphonate dimethyl ester (^{31}P {^1H} NMR ($CDCl_3$) = 0.4 ppm).

To α-ketophosphonate dimethyl ester (5 mmol) in 4 mL of distilled THF or dichloromethane at 0 °C under argon was added dropwise trimethylsilyl bromide (1.65 mL, 12.5 mmol). The reaction was exothermic and the temperature had to be maintained below 10 °C during the addition. The reaction mixture was stirred at room temperature for 5–6 h (the end of the reaction was controlled by ^{31}P {^1H} NMR) and evaporation of volatile fractions (0.01 Torr) at 50 °C gave bis(silylated) α-ketophosphonate. Phenyl bis(-trimethylsilyl) phosphite (5 mmol) was then added dropwise at 0 °C under argon. The reaction mixture was stirred overnight at room temperature and methanolysis for 2 h led to bisphosphonate monophenyl ester **2c**. After reduced pressure evaporation of volatile fractions, **2c** was purified by precipitation in a mixture of diethylether and hexane.

*[(4-Bromo-phenyl)-hydroxy-(hydroxy-phenoxy-phosphoryl)-methyl]-phosphonic acid (**2c**).* Precipitation in diethylether/hexane: 90/10. Yield: 64%. Decomposition 78 °C. ^{31}P NMR {^1H} (200.7 MHz, D_2O) δ 12.1 (d, 1P, $^2J_{P-P}$ = 28.6 Hz, *P*(O)(OH)(O–C$_6$H$_5$), 17.3 (d, 1P, $^2J_{P-P}$ = 28.6 Hz, *P*(O)(OH)$_2$). ^1H NMR (500 MHz, D_2O) δ 6.91 (d, 2H, $^3J_{H-H}$ = 7.5 Hz, o-C$_6$*H*$_5$), 7.12 (dd, 1H, $^3J_{H-H}$ = 7.5 Hz, p-C$_6$*H*$_5$), 7.27 (dd, 2H, $^3J_{H-H}$ = 7.5 Hz, m-C$_6$*H*$_5$), 7.56 (d, 2H, $^3J_{H-H}$ = 8.5 Hz, m-C$_6$*H*$_4$), 7.71 (d, 2H, $^3J_{H-H}$ = 8.5 Hz, o-C$_6$*H*$_4$). ^{13}C NMR {^1H} (125.9 MHz, D_2O) δ 77.6 (dd, $^1J_{P-C}$ = 142.3 Hz, $^1J_{P-C}$ = 142.3 Hz, P–*C*(OH)–P), 122.1 (*C*$_6$H$_5$), 122.4 (*C*$_6$H$_5$), 125.4 (p-C$_6$H$_4$), 129.4 (*C*$_6$H$_5$), 130.7 (m-*C*$_6$H$_4$), 132.2 (o-*C*$_6$H$_4$), 136.9 (*C*$_6$H$_4$C(OH)), 152.5 (O–*C*$_6$H$_5$). Anal. Calcd for $C_{13}H_{13}BrO_7P_2$: C, 36.90; H, 3.10; P, 14.64; Found: C, 37.00; H, 3.12; P, 14.58.

6.2. Chemical material

3-isobutyl-1-methylxanthine (IBMX) from Sigma (France) was dissolved in DMSO at a 500-fold concentration of the final concentration. The concentration of IBMX used was always kept at 0.2%.

6.3. NMR spectra of **2a** in different media

^{31}P NMR spectra of the diesterified bisphosphonate **2a** in the different media were obtained on a Bruker Avance 400 spectrometer operating at 161.943 MHz phosphorus frequencies at 20 °C. The data were recorded with a spectral width of 50 ppm and 8 K data point 50,000 scans were collected for each FID. The ^{31}P signals were referenced to an external standard of 85% phosphoric acid. For ^{31}P NMR experiment, the cellular extracts were prepared. HuH7 cells (5×10^6 cells/mL) were rinsed, trypsinized. The lysate was centrifuged at 1500 rpm for 5 min at 4 °C, and the pellet was resuspended in cold isotonic mixture of HEPES buffer. The sample vial was kept in an ice-water bath. This step is necessary to prevent significant heating in the sample during sonication (3 × 30 s). Bisphosphonate **2a** was added to the solution. After 5 days, 0.6 mL of solution was transferred to a 5 mm NMR tube, which was then placed in the magnet.

6.4. Cell line and cell culture

HuH7 hepatocarcinoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum at 37 °C in 5% CO_2 and 95% room air. Prior to the experiments, cells were cultured in the same seeding and growth conditions so as to achieve comparable growth behaviour.

6.5. Cell viability experiments

Cell viability was evaluated using the MTT microculture tetrazolium assay. HuH7 cells were seeded at a density of $5x10^4$ cells/well in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in complete culture medium for 24 h. Then, medium was removed and replaced by 2% FCS (Feotal Calf Serum)-medium containing increasing concentrations of BP (10^{-6}–10^{-3} M). After 144 h incubation, HuH7 cells were washed with phosphate buffered saline (PBS, Life Technologies) and incubated with 0.1 mL of MTT (2 mg/mL, Sigma–Aldrich) for additional 4 h at 37 °C. The insoluble product was then dissolved by addition of DMSO (Sigma–Aldrich). Optical density was measured at 570 nm using a Labsystems Multiskan MS microplate reader.

For IBMX experiments, a pretreatment (1 mM) of the cells was carried out for 30 min. The cells were then incubated with IBMX (1 mM) and the BPs, for 144 h at 37 °C in a 5% CO_2-incubator.

6.6. Cell death experiment

Apoptosis was analysed using the Annexin V-PI kit (R&D) following the manufacturer's instructions. HuH7 cells were treated with BPs for 144 h. Briefly, both BP-treated and untreated cells were stained with Annexin V and PI. Early and late apoptosis were

determined by Ann V^+/PI^- and Ann V^+/PI^+ staining, respectively, as determined by flow cytometric analysis with FACS.

6.7. Cell migration assay

Cell migration experiments were performed using migration chambers (Becton Dickinson), as described above. The undersides of the inserts were coated with 100 μL of fibronectin (100 μg/mL, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and were allowed to stand overnight at 4 °C. HGF (R&D) chemoattractant for HuH7 cells was added (20 ng/mL) to the lower chamber. HuH7 cells (2.5×10^5) with 100 μM of BPs or zoledronate were added to each insert (upper chamber). After 24 h incubation, non-migrated cells were removed by scraping and cells on the lower filter face were fixed, stained and counted. Results were expressed as a percentage, relative to controls normalised to 100%. Experiments were performed in triplicate.

6.8. Cell invasion assay

HuH7 cell invasion was investigated using Boyden invasion chambers with 8 μm pore size filters coated with Matrigel membrane matrix (Falcon, Becton Dickinson Labware, Bedford, MA, USA). 2.5×10^5 HuH7 cells with 100 μM of BPs or zoledronate were seeded in the upper well of the Boyden chamber. HGF (R&D) was added (20 ng/mL) to the lower chamber. After 24 h, non-migrated cells in the upper chamber were wiped with a cotton swab and the filters were fixed in methanol and stained with haematoxylin. Cells invading the lower surface of the filter were counted in 10-fields using a Zeiss microscope. Results were expressed as a percentage relative to controls and therefore normalised to 100%. Experiments were performed in triplicate.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.02.054.

References

[1] R.G. Russell, N.B. Watts, F.H. Ebetino, M.J. Rogers, Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy, Osteoporosis International 19 (2008) 733–759.
[2] G.H. Nancollas, R. Tang, R.J. Phipps, Z. Henneman, S. Gulde, W. Wu, A. Mangood, R.G.G. Russell, F.H. Ebetino, Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite, Bone 38 (2006) 617–627.
[3] R.G.G. Russell, Determinants of structure–function relationships among bisphosphonates, Bone 40 (2007) S21–S25.
[4] M.J. Rogers, J.C. Crockett, F.P. Coxon, J. Mönkkönen, Biochemical and molecular mechanisms of action of bisphosphonates, Bone 49 (2011) 34–41.
[5] J.E. Dunford, A.A. Kwaasi, M.J. Rogers, B.L. Barnett, F.H. Ebetino, R.G. Russell, U. Oppermann, K.L. Kavanagh, Structure-activity relationships among the nitrogen containing bisphosphonates in clinical use and other analogues: time-dependent inhibition of human farnesyl pyrophosphate synthase, Journal of Medicinal Chemistry 51 (2008) 2187–2195.
[6] H. Monkkonen, P.D. Ottewell, J. Kuokkanen, J. Monkkonen, S. Auriola, I. Holen, Zoledronic acid-induced IPP/ApppI production in vivo, Life Sciences 81 (2007) 1066–1070.
[7] P. Clézardin, Bisphosphonates' antitumor activity: an unravelled side of a multifaceted drug class, Bone 48 (2011) 71–79.
[8] M. Mongerard-Coulanges, E. Migianu-Griffoni, M. Lecouvey, B. Jolles, Impact of alendronate and VEGF-antisense combined treatment on highly VEGF-expressing A431 cells, Biochemical Pharmacology 77 (2009) 1580–1585.
[9] S. Muller, E. Migianu, M. Lecouvey, M. Kraemer, O. Oudar, Alendronate inhibits proliferation and invasion of human epidermoid carcinoma cells in vitro, Anticancer Research 25 (2005) 2655–2660.
[10] J. Green, P. Clézardin, The molecular basis of bisphosphonate activity: a pre-clinical perspective, Seminars in Oncology 37 (Suppl. 1) (2010) S3–S11.
[11] M. Gnant, P. Clézardin, Direct and indirect anticancer activity of bisphosphonates: a brief review of published literature, Cancer Treatment Reviews 38 (2012) 407–415.
[12] E. Guenin, D. Ledoux, O. Oudar, M. Lecouvey, M. Kraemer, Structure-activity relationships of a new class of aromatic bisphosphonates that inhibit tumor cell proliferation in vitro, Anticancer Research 25 (2005) 1139–1145.
[13] Y. Hamma-Kourbali, M. Di Benedetto, D. Ledoux, O. Oudar, Y. Leroux, M. Lecouvey, M. Kraemer, A novel non-containing-nitrogen bisphosphonate inhibits both in vitro and in vivo angiogenesis, Biochemical and Biophysical Research Communications 310 (2003) 816–823.
[14] V. Stresing, F. Daubiné, I. Benzaid, H. Mönkkönen, P. Clézardin, Bisphosphonates in cancer therapy, Cancer Letters 257 (2007) 16–35.
[15] A. Ezra, G. Golomb, Administration routes and delivery systems of bisphosphonates for the treatment of bone resorption, Advanced Drug Delivery Reviews 42 (2000) 175–195.
[16] F. Benyettou, Y. Lalatonne, I. Chebbi, M. Di Benedetto, J.M. Serfaty, M. Lecouvey, L. Motte, A multimodal magnetic resonance imaging nanoplatform for cancer theranostics, Physical Chemistry Chemical Physics 13 (2011) 10020–10027.
[17] I. Chebbi, E. Migianu-Griffoni, O. Sainte-Catherine, M. Lecouvey, O. Seksek, In vitro assessment of liposomal neridronate on MDA-MB-231 human breast cancer cells, International Journal of Pharmaceutics 383 (2010) 116–122.
[18] K. Hochdorffer, K. Abu Ajaj, C. Schafer-Obodozie, F. Kratz, Development of novel bisphosphonate prodrugs of doxorubicin for targeting bone metastases that are cleaved pH dependently or by cathepsin B: synthesis, cleavage properties, and binding properties to hydroxyapatite as well as bone matrix, Journal of Medicinal Chemistry 55 (2012) 7502–7515.
[19] M.R. Webster, M. Zhao, M.A. Rudek, C.L. Hann, C.L. Freel Meyers, Bisphosphonamidate clodronate prodrug exhibits potent anticancer activity in non-small-cell lung cancer cells, Journal of Medicinal Chemistry 54 (2011) 6647–6656.
[20] A. Ezra, A. Hoffman, E. Breuer, I.S. Alferiev, J. Monkkonen, N. El Hanany-Rozen, G. Weiss, D. Stepensky, I. Gati, H. Cohen, S. Tormalehto, G.L. Amidon, G. Golomb, A peptide prodrug approach for improving bisphosphonate oral absorption, Journal of Medicinal Chemistry 43 (2000) 3641–3652.
[21] J.R. Atack, A.M. Prior, S.R. Fletcher, K. Quirk, R. McKernan, C.I. Ragan, Effects of L-690,488, a prodrug of the bisphosphonate inositol monophosphatase inhibitor L-690,330, on phosphatidylinositol cycle markers, Journal of Pharmacology and Experimental Therapeutics 270 (1994) 70–76.
[22] M. Ahlmark, J. Vepsalainen, H. Taipale, R. Niemi, T. Jarvinen, Bisphosphonate prodrugs: synthesis and in vitro evaluation of novel clodronic acid dianhydrides as bioreversible prodrugs of clodronate, Journal of Medicinal Chemistry 42 (1999) 1473–1476.
[23] J.J. Vepsalainen, Bisphosphonate prodrugs, Current Medicinal Chemistry 9 (2002) 1201–1208.
[24] M. Monteil, E. Guenin, E. Migianu, D. Lutomski, M. Lecouvey, Bisphosphonate prodrugs: synthesis of new aromatic and aliphatic 1-hydroxy-1,1-bisphosphonate partial esters, Tetrahedron 61 (2005) 7528–7537.
[25] E. Guenin, M. Monteil, N. Bouchemal, T. Prange, M. Lecouvey, Syntheses of phosphonic esters of alendronate, pamidronate and neridronate, European Journal of Organic Chemistry (2007) 3380–3391.
[26] D. Ledoux, Y. Hamma-Kourbali, M. Di Benedetto, A. Foucault-Bertaud, O. Oudar, O. Sainte-Catherine, M. Lecouvey, M. Kraemer, A new dimethyl ester bisphosphonate inhibits angiogenesis and growth of human epidermoid carcinoma xenograft in nude mice, Anticancer Drugs 17 (2006) 479–485.
[27] M. Abdelkarim, E. Guenin, O. Sainte-Catherine, N. Vintonenko, N. Peyri, G.Y. Perret, M. Crepin, A.M. Khatib, M. Lecouvey, M. Di Benedetto, New symmetrically esterified m-bromobenzyl non-aminobisphosphonates inhibited breast cancer growth and metastases, PLoS One 4 (2009) e4685.
[28] R.G. Simonetti, C. Camma, F. Fiorello, F. Politi, G. D'Amico, L. Pagliaro, Hepatocellular carcinoma. A worldwide problem and the major risk factors, Digestive Diseases and Sciences 36 (1991) 962–972.
[29] M.B. Thomas, Systemic therapy for hepatocellular carcinoma, The Cancer Journal 14 (2008) 123–127.
[30] M. Barbacid, ras genes, Annual Review of Biochemistry 56 (1987) 779–827.
[31] T. Genda, M. Sakamoto, T. Ichida, H. Asakura, M. Kojiro, S. Narumiya, S. Hirohashi, Cell motility mediated by rho and rho-associated protein kinase plays a critical role in intrahepatic metastasis of human hepatocellular carcinoma, Hepatology 30 (1999) 1027–1036.
[32] K. Man, K.T. Ng, C.M. Lo, J.W. Ho, B.S. Sun, C.K. Sun, T.K. Lee, R.T. Poon, S.T. Fan, Ischemia-reperfusion of small liver remnant promotes liver tumor growth and metastases–activation of cell invasion and migration pathways, Liver Transplantation 13 (2007) 1669–1677.
[33] A. Wada, K. Fukui, Y. Sawai, K. Imanaka, S. Kiso, S. Tamura, I. Shimomura, N. Hayashi, Pamidronate induced anti-proliferative, apoptotic, and anti-migratory effects in hepatocellular carcinoma, Journal of Hepatology 44 (2006) 142–150.
[34] S.J. Fitch, K. Moedritzer, N.m.r. study of the P-C(OH)-P to P-C-O-P rearrangement: tetraethyl l-hydroxyalkylidenediphosphonates, Journal of the American Chemical Society 84 (1962) 1876–1879.
[35] E. Migianu, I. Mallard, N. Bouchemal, M. Lecouvey, One-pot synthesis of 1-hydroxymethylene-1,1-bisphosphonate partial esters, Tetrahedron Letters 45 (2004) 4511–4513.
[36] M. Lecouvey, I. Mallard, R. Burgada, Y. Leroux, A mild and efficient one-pot synthesis of 1-hydroxymethylene-1,1-bisphosphonic acids. Preparation of new tripod ligands, Tetrahedron Letters 42 (2001) 8475–8478.

[37] E. Migianu, E. Guenin, M. Lecouvey, New efficient synthesis of 1-hydroxymethylene-1,1-bisphosphonate monomethyl esters, Synlett (2005) 425–428.
[38] L. Formigli, L. Papucci, A. Tani, N. Schiavone, A. Tempestini, G.E. Orlandini, S. Capaccioli, S.Z. Orlandini, Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis, Journal of Cellular Physiology 182 (2000) 41–49.
[39] J. Mao, S. Mukherjee, Y. Zhang, R. Cao, J.M. Sanders, Y. Song, G.A. Meints, Y.G. Gao, D. Mukkamala, M.P. Hudock, E. Oldfield, Solid-state NMR, crystallographic, and computational investigation of bisphosphonates and farnesyl diphosphate synthase-bisphosphonate complexes, Journal of the American Chemical Society 128 (2006) 14485–14497.
[40] J. de Boer, A.J. Philpott, R.G. van Amsterdam, M. Shahid, J. Zaagsma, C.D. Nicholson, Human bronchial cyclic nucleotide phosphodiesterase isoenzymes: biochemical and pharmacological analysis using selective inhibitors, British Journal of Pharmacology 106 (1992) 1028–1034.
[41] T. Murata, M. Taira, V.C. Manganiello, Differential expression of cGMP-inhibited cyclic nucleotide phosphodiesterases in human hepatoma cell lines, FEBS Letters 390 (1996) 29–33.
[42] T. Yuasa, S. Kimura, E. Ashihara, T. Habuchi, T. Maekawa, Zoledronic acid - a multiplicity of anti-cancer action, Current Medicinal Chemistry 14 (2007) 2126–2135.