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Bisphosphonates activate the 5-fluorouracil/uracil phosphoribosyltransferase activity present in *Saccharomyces cerevisiae* cell extracts
Implications for tumor treatments

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Dedicated to Amalia Valdenebro, for her bravery, on her 65th birthday.

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ABSTRACT

Most of the effects described for bisphosphonates (pC(R1)(R2)p) are related, directly or indirectly with a pyrophosphate moiety. Bisphosphonates are (i) analogs of pyrophosphate in the synthesis of ATP derivatives (AppC(R1)(R2)p) catalyzed by ligases and (ii) inhibitors of enzymes of the mevalonate pathway with substrates containing a terminal pyrophosphate. Searching for the role of bisphosphonates on other reactions involving pyrophosphate, we explored their effect on a phosphoribosyltransferase activity, present in *Saccharomyces cerevisiae* cell extracts, using 5-fluorouracil or uracil as substrates. Unexpectedly, bisphosphonates increased the initial rate of synthesis of 5-FUMP (from 5-fluorouracil and phosphoribosylpyrophosphate): etidronate (2.8 ± 0.3 times); pamidronate (2.6 ± 0.4 times); alendronate (2.5 ± 0.6 times) and clodronate (2.0 ± 0.1 times). Similar values for the synthesis of UMP (from uracil and phosphoribosylpyrophosphate) were obtained in the presence of bisphosphonates. The values of the activation constants determined for alendronate and clodronate for the synthesis of UMP were 0.05 ± 0.02 mM and 0.32 ± 0.22 mM, respectively. These results raise the possibility that bisphosphonates enhance the effect of 5-fluorouracil (or other uracil prodrugs) in the treatment of bone tumors or bone tumor metastases.

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Abbreviations: AppC(R_1)(R_2)p, adenosine 5′-triphosphate derivative of any bisphosphonate; AppCCl_2p, adenosine 5′-(**β**,**γ**-dichloromethylene)triphosphate; AppCH_2p, adenosine 5′-(**β**,**γ**-methylene)triphosphate; Apppl, (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester); BP(s), bisphosphonate(s); FU or 5-FU, 5-fluorouracil; N-BP, a bisphosphonate containing nitrogen; non-N-BP, a bisphosphonate not containing nitrogen; pC(OH)(CH_2–CH_2–CH_2–NH_3)p, alendronate or 4-amino-1-hydroxybutylidene-1,1-bisphosphonate; pC(OH)(CH_2–CH_2–NH_3)p, pamidronate or 3-amino-1-hydroxypropylidene-1,1-bisphosphonate; pC(OH)(CH_3)p (HEBP), etidronate or ethane-1-hydroxy-1,1-bisphosphonate or hydroxyethylidenebisphosphonate; pCCl_2p (Cl_2MBP), clodronate or methane-1-dichloro-1,1-bisphosphonate or dichloromethylenebisphosphonate; pCH_2p, methylenebisphosphonate or methyleneBP; PRPP, 5-phospho-**α**-D-ribose 1-pyrophosphate; R_f, the value traveled by the solvent front divided by the distance traveled by a given compound; TLC, thin layer chromatography.

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1. Introduction

Bisphosphonates (BPs), widely used for the treatment of osteoporosis and other bone diseases, are analogs of pyrophosphate in which the oxygen bridge between the two phosphates is replaced by a methylene group (–CH_2–) [1,2]. Substitution of one or both hydrogen atoms of the molecule by radicals brings about the possibility of chemical synthesis of a great variety of BPs (pC(R1)(R2)p).

Two main criteria have been adopted to classify BPs: their time of appearance (first, second or third generation) and absence or presence of nitrogen in their molecule, i.e. non-N-BPs and N-BPs, respectively. Bisphosphonates of the first generation were non-N-BPs (clodronate, etidronate, tiludronate) and those of the second and third generation were N-BPs (pamidronate, ibandronate, alendronate, risedronate, zoledronate).

BPs of the first generation act as analogs of pyrophosphate in the reverse reaction of some ligases, such as aminoacyl t-RNA synthetases [3,4], T4 RNA ligase [5], T4 DNA ligase, ubiquitin activating enzyme E1 and other ligases [6]. In these cases, ATP derivatives of bisphosphonates are synthesized according to the following reactions:

$$\mathrm{ATP} + \mathrm{X} + \mathrm{E} \leftrightarrow \mathrm{E{-}X{-}AMP} + \mathrm{PPi} \quad (1)$$

$$\mathrm{E{-}X{-}AMP} + \mathrm{pC(R1)(R2)p} \rightarrow \mathrm{AppC(R1)(R2)p} + \mathrm{X} + \mathrm{E} \quad (2)$$

where X stands for the substrate of the corresponding ligase.

Although in our view these ATP derivatives could be cytotoxic in any metabolic step involving ATP, their specific modes of action (if any) have not been pinpointed, except for the described inhibition of the mitochondrial ADP/ATP translocase by the ATP derivative of clodronate (AppCCl_2p) [7].

Bisphosphonates of the second generation interfere with the synthesis of geranyl pyrophosphate from isopentenyl pyrophosphate catalyzed by the sequential action of isopentenyl pyrophosphate isomerase (EC 5.3.3.2) and dimethylallyl-*trans*transferase (EC 2.5.1.1) [8]. The third generation of BPs interferes also with the synthesis of farnesyl pyrophosphate and geranylgeranyl pyrophosphate catalyzed by geranyl*trans*-transferase (EC 2.5.1.10) and farnesyl*trans*transferase (EC 2.5.1.29), respectively [9–11]. It is noteworthy that pyrophosphate or compounds containing a moiety of pyrophosphate in its structure (those of the mevalonate pathway) participate in the above reactions.

The aim of this work stems from the assumption that the effect of a BP could be due to its competition with pyrophosphate for the same active area of specific enzymes. Hence, we searched for other enzymes using substrates containing a moiety of pyrophosphate as potential candidates to be inhibited by bisphosphonates. Adenine/hypoxanthine/uracil phosphoribosyltransferase (EC 2.4.2.7; EC 2.4.2.8; EC 2.4.2.9), are enzymes which catalyze reactions of the type:

$$\mathrm{B} + \mathrm{PRPP} \leftrightarrow \mathrm{BRP} + \mathrm{PPi} \quad (3)$$

where B, PRPP and BRP are, respectively, a nitrogenous base, phospho-α-D-ribose 1-pyrophosphate and a nucleoside monophosphate.

Although we expected that bisphosphonates could act as inhibitors of this reaction (because of their similarity with PRPP and PPi), the effect of BPs was clearly stimulatory of the direct reaction. As a corollary, the potential use of BPs as enhancers of the antitumor effect of base analogs on bone tumors is considered here.

2. Materials and methods

2.1. Materials

Bisphosphonates: etidronate, pC(OH)(CH_3)p (HEBP) (Cat No: P5248) was obtained from Sigma–Aldrich; clodronate, pCCl_2p (Cl_2MBP) (Cat No: 233183), pamidronate, pC(OH)(CH_2–CH_2–NH_2)p (Cat No: 506600) and alendronate, pC(OH)(CH_2–CH_2–CH_2–NH_2)p (Cat No: 126855) were from Calbiochem (Merck Biosciences); 5-phospho-α-D-ribose 1-pyrophosphate pentasodium salt (Cat No: P8296) and 5-fluorouracil (Cat No: F6627) were from Sigma–Aldrich. TLC silica-gel fluorescent plates (Cat No: 1.05554.0001) were from Merck KgaA, Germany. [2-^{14}C]Uracil (59 mCi/mmol) and [2-^{14}C]fluorouracil (12.6 mCi/mmol) were from Amersham and MP Biomedicals, Inc., respectively. The scintillation cocktail used was ORTIPHASE 'HISAFE' 2 from PerkinElmer (Ref. 1200-436).

2.2. Preparation of cell extracts

All the procedures were carried out at 0–4 °C. Yeast cells (20 ml) grown to a density of around 1.5×10^7 cells/ml were collected by filtration and washed with extraction buffer (50 mM Hepes/KOH pH 7.2; 0.1 mM dithiothreitol). The cells (25 mg of wet weight) were disrupted in the presence of 0.5 ml of buffer plus 1 g of glass beads (0.5 mm diameter) by vortexing at top speed on a tabletop mixer for 10 periods of 0.5 min separated by 0.5-min periods of cooling on ice. The homogenate was centrifuged for 5 min at $750 \times g$ and the supernatant centrifuged further at $540{,}000 \times g$ for 30 min. Protein content was determined by the method of Bradford [12].

2.3. Enzyme activity assay

The reaction mixtures (0.02 ml) contained: 50 mM Hepes/KOH (pH 7.2); 6 mM $MgCl_2$; 0.27 mM [2-^{14}C]uracil (0.03 μCi), or 0.37 mM [2-^{14}C]fluorouracil (0.03 μCi); 1 mM PRPP, bisphosphonates and yeast supernatant as indicated. Incubation was performed at 30 °C. At time intervals, aliquots of 1.5 μl were applied onto TLC plates. Standards of uracil or fluorouracil, uridine or fluorouridine, and UMP were run in parallel. The chromatograms were developed for about 2 h in ethyl acetate:isopropanol:ammonia:water (27:23:5:3, v/v). In this chromatographic system, the R_f values (in parentheses) were for uracil (0.65); fluorouracil (0.42); uridine (0.31); fluorouridine (0.12) and UMP or FUMP (0.0). The radioactive spots were cut out, introduced into 4 ml of scintillation cocktail, and the radioactivity counted.

3. Results

We had previously observed that in the presence of 5-fluorouracil in the culture medium, *Saccharomyces cerevisiae*

cells accumulated FUDP-*N*-acetylglucosamine and FUDP-glucose [13]. These experiments, performed with the yeast strain W303-1A requiring uracil for growth, allowed studying the reciprocal influence of uracil and 5-FU on the synthesis of nucleotides. Concentrations as high as 7 mM FUDP-sugars were obtained when, in the absence of uracil, 50 mM 5-FU were added to the culture medium. Since FUMP is the first compound in the pathway for the synthesis of FUDP-sugars, the presence of these sugars denoted the occurrence in yeast of a phosphoribosyltransferase activity on 5-FU (see reaction (3)). Therefore, yeast extracts were used as a source of phosphoribosyltransferase to investigate the influence of bisphosphonates on this enzyme activity. The effect of alendronate (1 mM) was firstly tested in the presence of [2-^{14}C]FU, PRPP and yeast extract. Aliquots were taken at the indicated times (Fig. 1), and the synthesis of [2-^{14}C]FU derivatives, analyzed by thin layer chromatography (see Section 2). In the presence of alendronate a clear increase in the rate of synthesis of [2-^{14}C]FUMP was observed suggesting that alendronate, contrary to our expectations, behaved as a positive effector of the synthesis of [2-^{14}C]FUMP. To further clarify this finding, the effects of pamidronate (another N-BP), and etidronate and clodronate (non-N-BPs) were also tested on the synthesis of [2-^{14}C]FUMP or [2-^{14}C]UMP from 5-fluorouracil or uracil, respectively. All the bisphosphonates tested were activators of the synthesis of FUMP or UMP (Fig. 2). From the experiments shown in Fig. 2 and others (not shown), a standard deviation of the mean for the activation values was calculated (Table 1). In general, the degree of activation by each bisphosphonate was similar (varying from 2.0- to 2.8-fold), with a tendency to a slightly higher activation of the synthesis FUMP over that of UMP. Although we do not know the reason for this small difference, the atom of fluorine in the substrate could be a contributing factor. In our view, the important point is that all the bisphosphonates here studied activate the transferase activity on 5-fluorouracil.

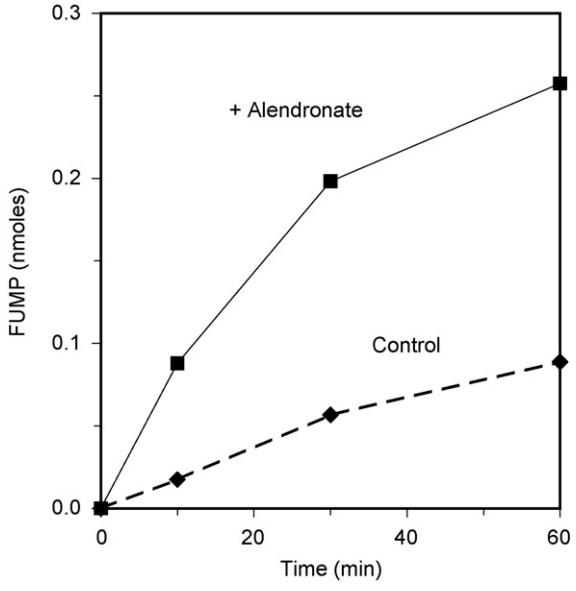

Fig. 1 – Effect of alendronate (a N-BP) on the synthesis of [2-^{14}C]FUMP catalyzed by a phosphoribosyltransferase activity present in yeast extracts. The reaction mixtures (0.02 ml) contained 0.37 mM [2-^{14}C]FU (0.03 μCi), 1 mM PRPP, yeast extract (5.4 μg), and 1 mM alendronate, when indicated; for other components, see Section 2. At the indicated times aliquots were taken and subjected to TLC. Spots corresponding to [2-^{14}C]FUMP were cut out, the radioactivity counted, the nmoles formed in the assay being represented.

Table 1 – Effect of bisphosphonates on the rate of synthesis of FUMP and UMP catalyzed by the phosphoribosyltransferase activity present in yeast extracts

Bisphosphonate	Times-fold increase in the rate of synthesis	
	[2-^{14}C]FUMP	[2-^{14}C]UMP
Etidronate	2.8 ± 0.3	2.1 ± 0.1
Pamidronate	2.6 ± 0.4	2.1 ± 0.2
Alendronate	2.5 ± 0.6	2.3 ± 0.1
Clodronate	2.0 ± 0.1	1.9 ± 0.2
MethyleneBP	n.d.	1.1

The reaction mixtures were as indicated in Fig. 2. The times-fold increase promoted by bisphosphonates were calculated dividing the rate of velocity obtained in the presence of bisphosphonate by that obtained in its absence. n.d., not determined.

3.1. *Activation constants (K_a) values for alendronate and clodronate*

These constants were determined in the presence of 0.27 mM uracil, 1 mM PRPP and in the absence or presence of three concentrations (0.01 mM, 0.1 mM and 1 mM) of alendronate or clodronate. The K_a values calculated from Hill plots of the experimental results (Fig. 3) were: 0.05 ± 0.02 mM and 0.32 ± 0.22 mM for alendronate and clodronate, respectively.

4. Discussion

The pharmacokinetics of bisphosphonates varies depending on their chemical nature, doses and modes of administration (oral, subcutaneous, intramuscular, venous, intraperitoneal) [14,15]. In general they are partly taken up by the bone, partially eliminated by the kidney, with rapid decay in plasma and in all non-calcified tissues after their administration [14]. The bisphosphonates accumulated in bone are slowly liberated to plasma when the bone is reabsorbed. These pharmacokinetics properties of bisphosphonates, together with the source of the phosphoribosyltransferase activity (*S. cerevisiae*) used to perform the above experiments, poses some caution regarding the clinical relevance of bisphosphonates as activators *in vivo* of the base-phosphoribosyltransferase activity here reported and, therefore as enhancers of the antitumoral potency of 5-FU or other nitrogenous base analogs. The possibility that a base analog may serve as an antitumor drug in an organ, might depend on the presence of the specific phosphoribosyltranferase activity in that tissue. As the activation constant values determined for clodronate and alendronate for the synthesis of UMP (taken as a general parameter) are in the order of 0.05–0.3 mM, such concentrations need to be attained for bisphosphonates to act as effectors of the reaction. These concentrations are not reached

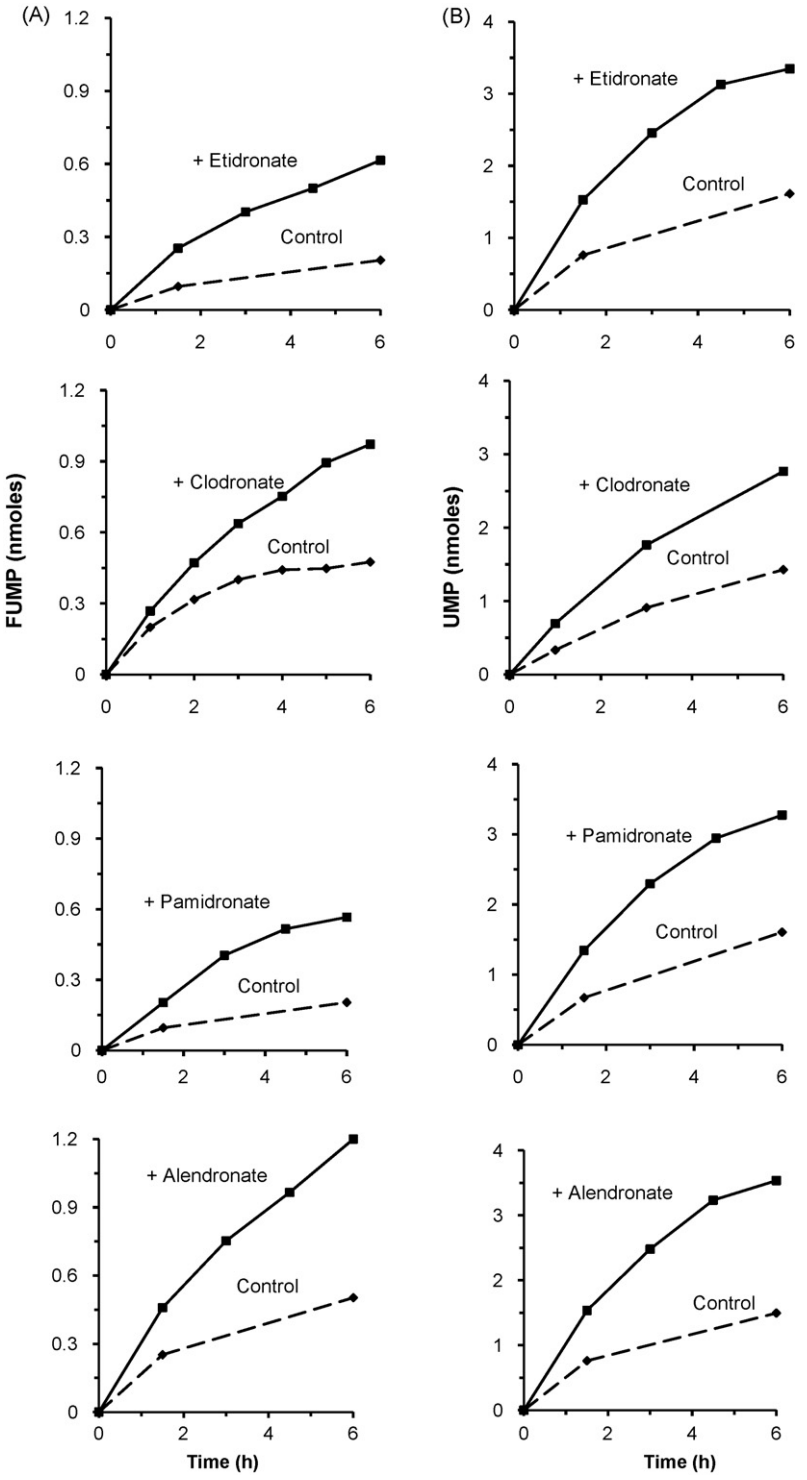

Fig. 2 – Effect of etidronate and clodronate (two non-N-BPs) and pamidronate and alendronate (two N-BPs) on the synthesis of [2-^{14}C]FUMP (A) or [2-^{14}C]UMP (B) catalyzed by a phosphoribosyltransferase activity present in yeast extracts. The reaction mixtures (0.02 ml) contained 0.37 mM [2-^{14}C]FU (0.03 μCi), or 0.27 mM [2-^{14}C]U (0.03 μCi), 1 mM PRPP, yeast extract (6.6 μg), and when indicated 1 mM each of the above bisphosphonates. For other components see Section 2. Aliquots of the reactions were taken at the times indicated. Spots corresponding to [2-^{14}C]FUMP or [2-^{14}C]UMP were counted as described in Fig. 1.

in non-calcified tissues but could be attained in bone; it is possible that bisphosphonates could be effective enhancers of the effect of 5-FU (and other base analogs?) in bone tumors or to prevent bone metastases.

Antitumor effects of bisphosphonates have been described: several BPs (at concentration ranges from 0.005 mM to 2 mM) inhibited proliferation and induced apoptosis of cancer cells from myeloma, breast, prostate, pancreas, lung and osteo-

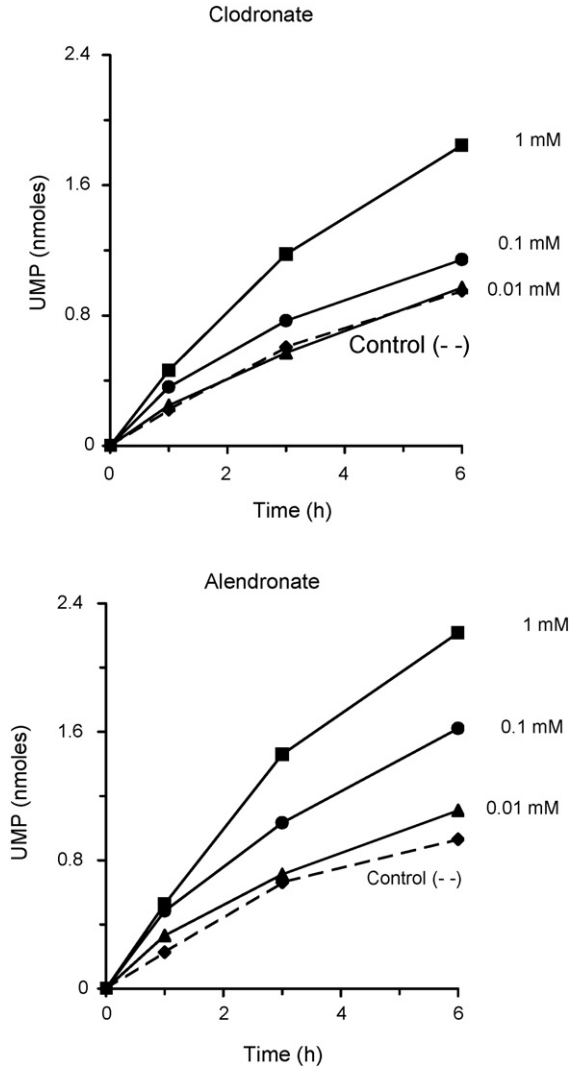

Fig. 3 – Activation by clodronate (a non-N-BPs) or alendronate (a N-BPs) of a phosphoribosyltransferase activity present in yeast extracts. The reaction mixtures (0.2 ml) contained 0.27 mM [2-^{14}C]UMP (0.03 μCi) and clodronate or alendronate at the indicated concentrations. Aliquots were taken after 1 h, 3 h and 6 h incubation. The [2-^{14}C]UMP formed was determined as described in Fig. 1.

sarcoma [16–18]. The postulated mechanisms of action of bisphosphonates as antitumor agents are similar to those reported for their damaging effect on osteoclasts, i.e.: synthesis of BPs derivatives of ATP and inhibition of the mevalonate pathway, the prenylation of important cell signaling proteins and the synthesis of cholesterol [8,10,19–21].

Bisphosphonates have also been described as enhancers of the antitumor effect of some drugs. Zoledronate increased synergistically (by unknown mechanisms) the effect of the following drugs *in vitro*: paclitaxel, etoposide, cisplatinum and irinotecan [18], gemcitabine, fluvastatin and doxorubicin [22,23]. Zoledronate also enhanced the cytotoxic effect of gemcitabine and fluvastatin in several cell lines originating from breast, lung, and prostate cancer. The possibility ought to be considered that in some of these cases BPs could act as activators of a phosphoribosyltransferase activity.

Finally, a clear projection of the results here reported is to test the effect of the combined treatment of 5-FU, alendronate, and 5-FU + alendronate on the development of tumors induced in nude mice, as planned in our laboratory with a tumorigenic cell line.

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