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Synthesis of bisphosphonate derivatives of ATP by T4 DNA ligase, ubiquitin activating enzyme (E1) and other ligases

María A. Günther Sillero, Anabel de Diego, Francisco J. Pérez-Zúñiga, Antonio Sillero *

Departamento de Bioquímica, Instituto de Investigaciones Biomédicas Alberto Sols UAM/CSIC, Facultad de Medicina, 28029 Madrid, Spain

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ABSTRACT

T4 DNA ligase and the ubiquitin activating enzyme (E1), catalyze the synthesis of ATP β,γ-bisphosphonate derivatives. Concerning T4 DNA ligase: (i) etidronate ($pC(OH)(CH_3)p$) displaced the AMP moiety of the complex E-AMP in a concentration dependent manner; (ii) the K_m values and the rate of synthesis k_{cat} (s^{-1}), determined for the following compounds were, respectively: etidronate, 0.73 ± 0.09 mM and $(70 \pm 10) \times 10^{-3}$ s^{-1}; clodronate ($pCCl_2p$), 0.08 ± 0.01 mM and $(4.1 \pm 0.3) \times 10^{-3}$ s^{-1}; methylenebisphosphonate (pCH_2p), 0.024 ± 0.001 mM and $(0.6 \pm 0.1) \times 10^{-3}$ s^{-1}; tripolyphosphate (P_3) (in the synthesis of adenosine 5′-tetraphosphate, p_4A), 1.30 ± 0.30 mM and $(6.2 \pm 1.1) \times 10^{-3}$ s^{-1}; (iii) in the presence of GTP and ATP, inhibition of the synthesis of Ap_4G was observed with clodronate but not with pamidronate ($pC(OH)(CH_2–CH_2–NH_3)p$). Concerning the ubiquitin activating enzyme (E1): methylenebisphosphonate was the only bisphosphonate, out of the ones tested, that served as substrate for the synthesis of an ATP derivative ($K_m = 0.36 \pm 0.09$ mM and $k_{cat} = 0.15 \pm 0.02$ s^{-1}). None of the above bisphosphonates were substrates of the reaction catalyzed by luciferase or by acyl-CoA synthetase. The ability of acetyl-CoA synthetase to use methylenebisphosphonate as substrate depended on the commercial source of the enzyme. In our view this report widens our knowledge of the enzymes able to metabolize bisphosphonates, a therapeutic tool widely used in the treatment of osteoporosis.

1. Introduction

We have recently reported on some aspects of the biochemical action of bisphosphonates [1]. These compounds are analogs of pyrophosphate in which the oxygen bridge between the two phosphates is replaced by a methylene group (–CH_2–) [2]. Substitution of one or both hydrogen atoms of the molecule by radicals generates a great variety of bisphosphonates [3,4]. Although some of these derivatives are widely used in the treatment of osteoporosis, their mechanism of action presents many unknowns [4–7]. Two distinct mechanisms have been reported for bisphosphonates, depending on the absence (non-N-BPs) or presence (N-BPs) of nitrogen in their molecules. In the first case, bisphosphonates are metabolized to cytotoxic

* *Corresponding author*. Fax: +34 91 585 4401.
E-mail address: antonio.sillero@uam.es (A. Sillero).

Abbreviations: Aa, amino acid; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; Ap_4G, adenosine 5′,5‴-P^1,P^4-tetraphosphoguanosine; ApppI, triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester; $AppCCl_2p$, adenosine 5′-(β,γ-dichloromethylenetriphosphate); $AppCH_2p$, adenosine 5′-(β,γ-methylenetriphosphate); $AppC(R_1)(R_2)p$, adenosine 5′-triphosphate derivative of any bisphosphonate; BSA, bovine serum albumin; DTT, dithiothreitol; P_3, tripolyphosphate; p_4A, adenosine 5′-tetraphosphate; $pC(OH)(CH_2–CH_2–NH_3)p$, pamidronate, or 3-amino-1-hydroxypropylidene-1,1-bisphosphonate; $pC(OH)(CH_2–CH_2–CH_2–NH_3)p$, alendronate or 4-amino-1-hydroxybutylidene-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; $pC(R_1)(R_2)p$, any bisphosphonate; Ub, ubiquitin.

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ATP analogs (AppC(R_1)(R_2)p) [7–13] resulting in induction of apoptosis by inhibiting the mitochondrial ADP/ATP translocase [14]. The nitrogen containing bisphosphonates (N-BPs) inhibit farnesyl pyrophosphate synthetase in the mevalonate pathway. Inhibition of this enzyme prevents the modification of important signaling proteins with isoprenoid lipids, and the subsequent lack of prenylated proteins leads to a loss of osteoclasts function and, consequently, indirect apoptotic cell death; it may also affect the synthesis of cholesterol [4,6,7,15,16]. N-BPs are not metabolized to ATP analogs [12,17] but they can induce formation of a novel ATP analog, ApppI (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester), as a consequence of the inhibition of the mevalonate pathway in cells [18]. Similarly to AppC(R_1)(R_2)p-type metabolites of non-N-BPs [14], ApppI is able to induce apoptosis through blockade of the mitochondrial ADP/ATP translocase [18].

It was commonly thought that the synthesis of ATP bisphosphonate derivatives of the AppC(R_1)(R_2)p type took place exclusively by the reaction in reverse of aminoacyl tRNA synthetases [7–11]

$$E + Aa + ATP \leftrightarrow E \cdot Aa\text{-}AMP + PPi \quad (1)$$

$$E \cdot Aa\text{-}AMP + pC(R_1)(R_2)p \rightarrow AppC(R_1)(R_2)p + Aa + E \quad (2)$$

However, based on our previous experience on the mechanism of action of ligases [19], the possibility that this synthesis could be carried out by others ligases different to aminoacyl tRNA synthetases was tested [1]. In a previous paper, we showed that T4 RNA ligase [1] catalyzed reaction (3).

$$E + ATP + pC(R_1)(R_2)p \rightarrow E + AppC(R_1)(R_2)p + PPi \quad (3)$$

Here we explored the capacity of other ligases (T4 DNA ligase, ubiquitin activating enzyme (E1), luciferase, acetyl-CoA and acyl-CoA synthetases) to catalyze the synthesis of bisphosphonates derivatives of ATP. The results obtained showed that each bisphosphonate behaved differently as substrate of these ligases.

2. Materials and methods

2.1. Materials

Bisphosphonates: etidronate, pC(OH)(CH_3)p (Cat. No. P-5248) was from Sigma; alendronate, pC(OH)(CH_2–CH_2–CH_2–NH_2)p (Cat. No. 126855); clodronate, pCCl_2p (Cat. No. 233183), and pamidronate, pC(OH)(CH_2–CH_2–NH_2)p, (Cat. No. 506600) were from Calbiochem; methylenebisphosphonate, pCH_2p (Cat. No. 274291) was from Aldrich.

Enzymes: cloned T4 DNA ligase (EC 6.5.1.1), with a specific activity of 36,000 Weiss units/mg, and 2.5 mg/ml, was from Amersham Biosciences (Ref. 27-0870; lot 109222-012); one unit (U) is the enzyme activity catalyzing the conversion of 1 nmol of [^{32}P]PPi into a Norit-adsorbable form in 20 min at 37 °C; electrophoresis on polyacrylamide gels, in the presence of SDS showed a major band with an apparent molecular mass of 57 kDa. T4 RNA ligase (EC 6.5.1.3) was from BioLabs (M0204S, lot No. 34), with a specific activity of 12,000 U/mg) [1]. Ubiquitin activating enzyme (E1) (EC 6.3.2.19) (110 kDa) Ref. U-1758 (lot 085K0498); acyl-CoA synthetase (EC 6.2.1.3), Ref. A-3352 (lot 115K11611), acetyl-CoA synthetase (EC 6.2.1.1), Ref. A-5269 (lot 82H8045) and Ref. A-1765 (lot 045K1431) and luciferase (EC 1.13.12.7), Ref. L-9506 (lot 66H8275) were from Sigma. Yeast inorganic pyrophosphatase (EC 3.6.1.1) was from Roche Molecular Biochemicals.

Other materials: [α-^{32}P]ATP 3000 Ci/mmol) was from Amersham or Perkin Elmer; electrophoresis molecular weight standards (Ref. LS 1610304) was from Bio-Rad; sodium tripolyphosphate (P_3) (Ref. L-5633) (lot 87C50061) was from Sigma; TLC silica gel fluorescent plates were from Merck.

2.2. T4 DNA ligase: formation of E-AMP complex

The reaction mixtures (0.02 ml) contained 50 mM HEPES/KOH (pH 6.7), 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 10 μM (0.1 μCi) [α-^{32}P] ATP, 0.02 U desalted pyrophosphatase and etidronate at concentrations of: 0, 0.1, 0.2, 0.4, 1 and 2 mM, as indicated. The formation of the E-AMP complex was initiated by the addition of 0.5 μg of enzyme. After 15 min incubation at 25 °C, reactions were stopped with 6.5 μl of concentrated SDS sample buffer (0.25 M Tris–HCl (pH 6.8), 8% SDS, 40% glycerol, 240 mM dithiothreitol, 0.005% bromophenol blue). The mixtures were heated at 90 °C for 3 min and 6-μl aliquots of the reaction mixtures (6), and of molecular weight markers (4), were loaded onto a 12% denaturing polyacrylamide gel in an alternate way (Fig. 1). The gel was stained with Coomassie blue, dried down and the labeled enzyme–adenylate complex detected by autoradiography.

2.3. Synthesis of bisphosphonates derivatives of ATP

T4 DNA ligase: The reaction mixtures (0.02 ml) contained 50 mM HEPES/KOH (pH 7.2), 1 mM DTT, 5 mM $MgCl_2$, 1 U/ml desalted pyrophosphatase, 0.02 mM [α-^{32}P]ATP (0.4 μCi), bisphosphonates, P_3 and enzyme as indicated. When required, the enzyme was diluted in 50 mM Tris–HCl (pH 7.4), 10 mM DTT and 0.05% bovine serum albumin (BSA). A molecular mass of 57 kDa was considered to calculate the concentration of the enzyme. After incubation at 30 °C, the reaction mixtures were analyzed by thin layer chromatography (TLC) [20].

Ubiquitin activating enzyme (E1): The reaction mixtures (0.02 ml) contained 50 mM Tris–HCl (pH 7.5), 12 mM $MgCl_2$, 1 U/ml desalted pyrophosphatase, 0.02 mM [α-^{32}P]ATP (0.4 μCi), 6 μM Ub, bisphosphonates, P_3 and enzyme as indicated. When required the enzyme was diluted in 0.1 M Tris–HCl (pH 7.5)/0.5% BSA. A molecular mass of 110 kDa was considered to calculate the concentration of the enzyme. After incubation at 37 °C the reaction mixtures were analyzed by TLC [21].

Other enzymes: Luciferase [22], acyl-CoA synthetase [23] and acetyl-CoA synthetase [24] were assayed as previously described.

TLC: Aliquots of 1.5 μl of the reaction mixtures were spotted on silica gel plates and developed for 2 h in dioxane:ammonium hydroxide:water (6:1:6 or 6:1:7 by volume, as indicated). Radioactively labeled nucleotides were quantified with the help of an Instant Imager (Packard Instrument Co.), or a Typhoon Trio (GE Healthcare).

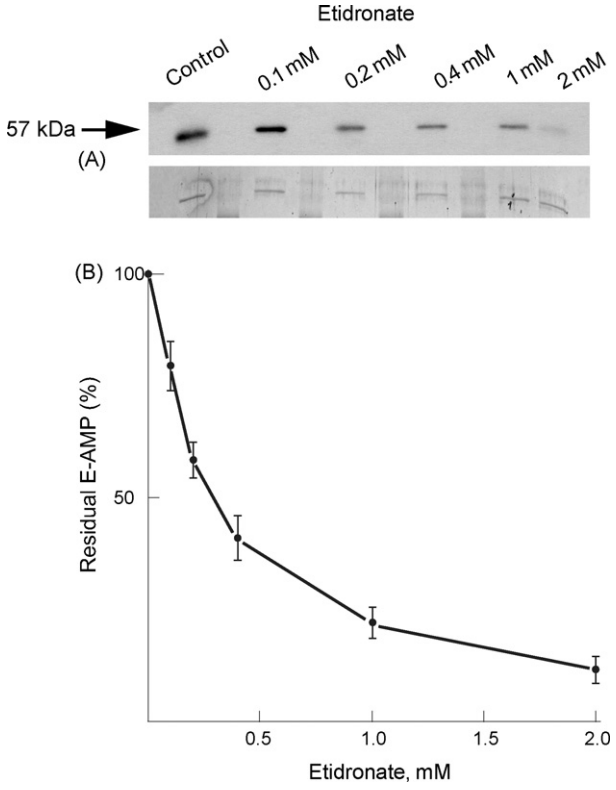

Fig. 1 – Displacement of the AMP moiety of the T4 DNA ligase-adenylyl complex by etidronate. The enzyme was incubated with [α-^{32}P]ATP in the absence or presence of increasing concentrations of etidronate as indicated, and subjected to SDS-PAGE as described in Section 2. Molecular weight markers were run in parallel. (A) The relevant portion of the gel is shown: the upper part shows the E-[α-^{32}P]AMP complex formed, whose size (in kDa) calculated from the coelectrophoresed molecular weight markers is indicated by an arrow at the left; the lower part shows the corresponding protein bands stained with Coomassie blue. Part B: the residual (%) adenylylated enzyme (E-AMP) complex, was quantified in an Instant Imager.

3. Results and discussion

3.1. T4 DNA ligase

T4 DNA ligase, one of the most widely enzymes used in genetic engineering, belongs to the group of ATP-dependent ligases, in which the mammalian DNA ligases are included [25]. It catalyzes the formation of phosphodiester bonds between neighboring 3′-hydroxyl and 5′-phosphate ends in double-stranded DNA (reactions (4)–(6)):

$$E + ATP \leftrightarrow E\text{-}AMP + PP_i \quad (4)$$

$$E\text{-}AMP + DNA\text{-}5'\text{-}P \rightarrow E \cdot DNA\text{-}5'\text{-}PP\text{-}'5A \quad (5)$$

$$E \cdot DNA\text{-}5'\text{-}PP\text{-}'5A + HO\text{-}3'\text{-}DNA \rightarrow E + DNA\text{-}5'\text{-}P\text{-}3'\text{-}DNA + AMP \quad (6)$$

3.1.1. Formation of the E-AMP complex and its reaction with etidronate (pC(OH)(CH$_3$)p)

It is here assumed that the AMP moiety of the E-AMP complex (reaction (4)) reacts with a bisphosphonate (pC(R_1)(R_2)p) with formation of the corresponding ATP derivative.

$$E\text{-}AMP + pC(R_1)(R_2)p \rightarrow AppC(R_1)(R_2)p + E \quad (7)$$

An E-AMP complex, migrating on SDS/PAGE in a position corresponding to a molecular weight of around 57 kDa, was observed when T4 DNA ligase was incubated with [α-^{32}P]ATP (Fig. 1A). When the reaction mixtures were supplemented with etidronate, the AMP moiety of the complex was displaced by this bisphosphonate (Fig. 1B), in a concentration dependent manner, similarly as occurred with P_3 [20]. Care was taken to load the same amount of enzyme in every lane (Fig. 1A). When the E-AMP complex was preformed in the absence of etidronate, the subsequent addition of this bisphosphonate displaced the AMP moiety of the complex similarly as above (result not shown). The possibility that etidronate could bind to the enzyme and impaired the formation of the E-AMP complex was also approached in an experiment based on that described in Section 3.1.2 (Fig. 2) (see below).

3.1.2. Relative efficiency of etidronate (pC(OH)(CH$_3$)p), clodronate (pCCl$_2$p), methylenebisphosphonate (pCH$_2$p), pamidronate (pC(OH)(CH$_2$–CH$_2$–NH$_2$)p) and tripolyphosphate (P_3) as substrates for the formation of ATP derivatives

Clodronate, methylenebisphosphonate, etidronate, pamidronate or P_3 were added to a final concentration of 1 mM to

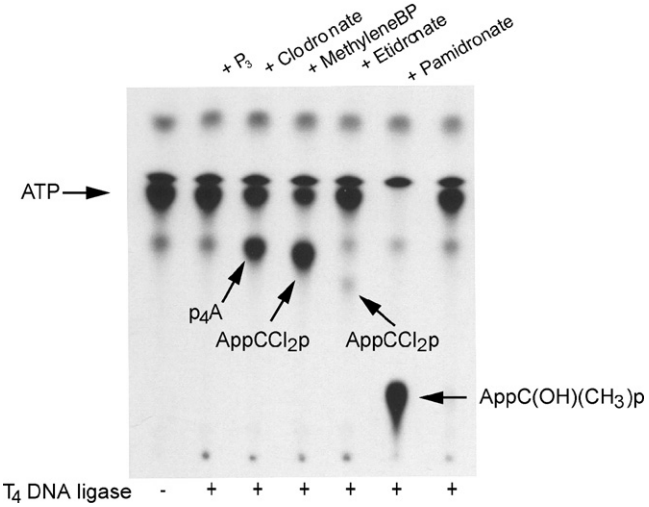

Fig. 2 – Tripolyphosphate (P_3), clodronate (pCCl$_2$p), methylenebisphosphonate (pCH$_2$p), etidronate, (pC(OH)(CH$_3$)p) and pamidronate (pC(OH)(CH$_2$–CH$_2$–NH$_3$)p) as substrates for the formation of ATP derivatives. The reaction mixtures contained 0.02 mM [α-^{32}P]ATP (0.4 μCi), 1 mM bisphosphonates or P_3 as indicated, and 1.7 μg of enzyme; other components as in Section 2. At different times of incubation (15, 30 and 60 min) aliquots were spotted on TLC plates, developed with dioxane:ammonium hydroxide:water (6:1:7) and subjected to autoradiography. The figure shows the result obtained after 15 min incubation.

reaction mixtures containing 0.02 mM [α-^{32}P]ATP. Aliquots of the reaction were taken at different times of incubation and subjected to TLC. From the experiment shown in Fig. 2, and others not shown, the following relative rates (indicated between brackets) were determined: etidronate (100); clodronate (21); P_3 (13) and methylenebisphosphonate (3). Pamidronate was not substrate of the reaction (Fig. 2).

In a similar experiment, T4 DNA ligase was incubated in the absence (assay 1) or presence of 1 mM etidronate (assay 2) for 10 min at 25 °C; thereafter labeled ATP was added to both reaction mixtures, and etidronate, at 1 mM final concentration, was added only to assay 1. After 5 min incubation the reaction mixtures were analyzed by TLC as in Fig. 2. The rate of synthesis of AppC(OH)(CH_3)p, was essentially the same in both cases (result not shown), demonstrating that etidronate did not block the formation of the E-AMP complex, and the derivative is formed according to reaction (7).

Concerning the synthesis of ATP derivatives, etidronate was the best of the bisphosphonates tested as substrates for T4 DNA ligase (Fig. 2), and the worst substrate for T4 RNA ligase (Fig. 2 in [1]). This peculiarity was further assessed in the experiment presented in Fig. 3, where the efficiency of both enzymes (T4 DNA and T4 RNA ligases) to catalyze the synthesis of ATP derivatives of etidronate and methylenebisphosphonate was tested in parallel. The result obtained confirmed previous results and showed the different specificity of two related enzymes when using bisphosphonates as substrates.

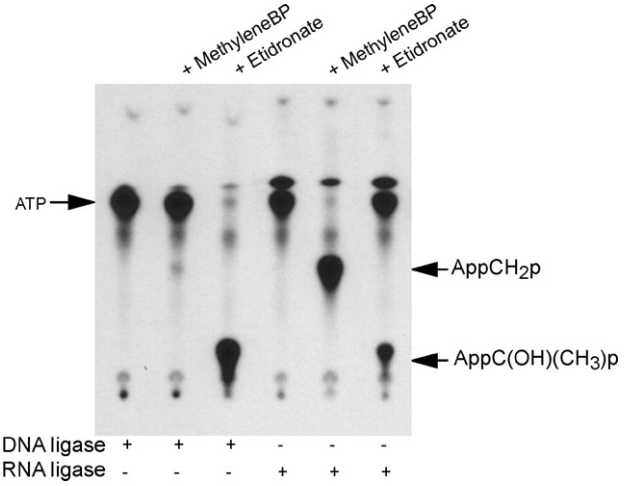

Fig. 3 – Etidronate (pC(OH)(CH_3)p) and methylenebisphosphonate (pCH_2p) as substrates of the reaction catalyzed by T4 DNA and T4 RNA ligases. The reaction mixtures contained 0.02 mM [α-^{32}P] ATP (0.4 μCi), 1 mM of the indicated bisphosphonates and 1.7 μg of T4 DNA ligase or 0.67 μg T4 RNA ligase; other components as in Section 2. At different times of incubation (6 and 12 min) aliquots were spotted on TLC plates, developed with dioxane:ammonium hydroxide:water (6:1:7) and subjected to autoradiography. The figure shows the result obtained after 6 min incubation.

3.1.3. *K_m values and rate of synthesis (k_{cat}) determination for bisphosphonates*

The determination of the K_m and k_{cat} values for clodronate, etidronate and methylenebisphosphonate in reaction (7), was performed in the presence of a fixed concentration of [α-^{32}P] ATP (0.02 mM) (see Section 2). The values obtained and the range of concentrations of bisphosphonates used in each assay, are specified in Table 1. For comparison, the K_m and k_{cat} values determined for P_3 in the synthesis of p_4A are also included in Table 1.

3.1.4. *Bisphosphonates as inhibitors of some reactions catalyzed by T4 DNA ligase*

It was previously known that T4 DNA ligase catalyzed the transfer of AMP from the E-AMP complex to NTP or NDP, forming dinucleotides of the type Ap_4N and Ap_3N, respectively [20]. The potential effect of some bisphosphonates (clodronate or pamidronate) on these reactions was assayed using GTP and labeled ATP as substrates. In the absence of bisphosphonates, the enzyme catalyzed the synthesis of Ap_4G [20]. In the presence of clodronate, both GTP and clodronate competed for the AMP moiety of the E-AMP complex and, hence, synthesis of $AppCCl_2p$ and inhibition of the synthesis of Ap_4G was observed. Pamidronate, a bisphosphonate unable to react with the E-AMP complex, did not affect the synthesis of Ap_4G (Fig. 4).

3.2. *Ubiquitin activating enzyme (E1)*

This enzyme is a part of the ubiquitin proteasome system, in which two main processes can be considered: ubiquitylation of proteins and proteolytic cleavage of the tagged proteins by the 26S proteasome complex with liberation of ubiquitin (Ub). In the first process participate the enzymes E1, E2s and E3s [26,27].

Here we paid attention only to E1, or ubiquitin activating enzyme. E1 catalyzes the reaction:

$$E1 + ATP + Ub \leftrightarrow E1 \cdot AMP\text{-}Ub + PPi \quad (8)$$

Table 1 – Kinetic constants for bisphosphonates as substrates of T4 DNA ligase

Compound	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)
Etidronate	0.73 ± 0.09	(70 ± 10) × 10^{-3}	0.10
Clodronate	0.08 ± 0.01	(4.1 ± 0.3) × 10^{-3}	0.05
Methylenebisphosphonate	0.024 ± 0.001	(0.6 ± 0.1) × 10^{-3}	0.025
Tripolyphosphate	1.30 ± 0.30	(6.2 ± 1.1) × 10^{-3}	0.005

Those values were determined in triplicate as indicated in Section 2, using the following range of bisphosphonate concentrations: etidronate (0.2–1.4 mM); clodronate (0.025–0.4 mM) methylenebisphosphonate (0.02–0.2 mM) and tripolyphosphate (0.2–1.5 mM).

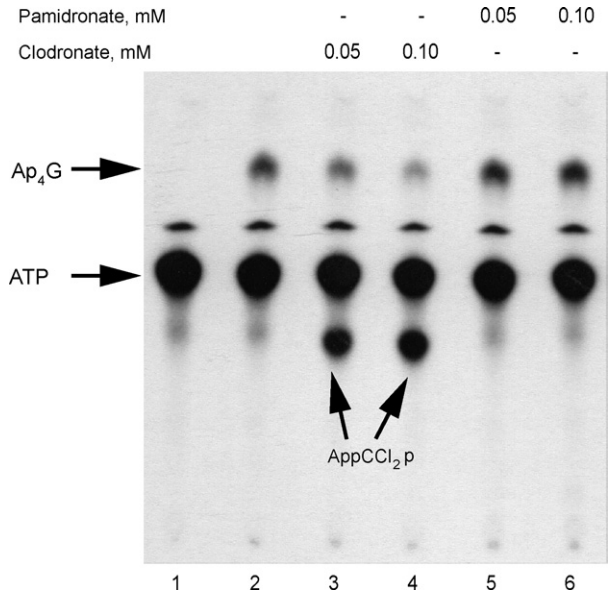

Fig. 4 – Effect of clodronate ($pCCl_2p$) and pamidronate ($pC(OH)(CH_2–CH_2–NH_3)p$) on the synthesis of Ap_4G catalyzed by T4 DNA ligase. T4 DNA ligase (0.64 μg) was incubated for 1 h in the presence of 0.02 mM [α-^{32}P] ATP (0.4 μCi), 1 mM GTP, and clodronate or pamidronate, as indicated in the upper part of the figure (lanes 3–6); controls without enzyme (lane 1) and without bisphosphonates (lane 2) were carried out in parallel. Analysis of the reaction mixtures was performed by TLC and developed with dioxane:ammonium hydroxide:water (6:1:6) as indicated in Section 2.

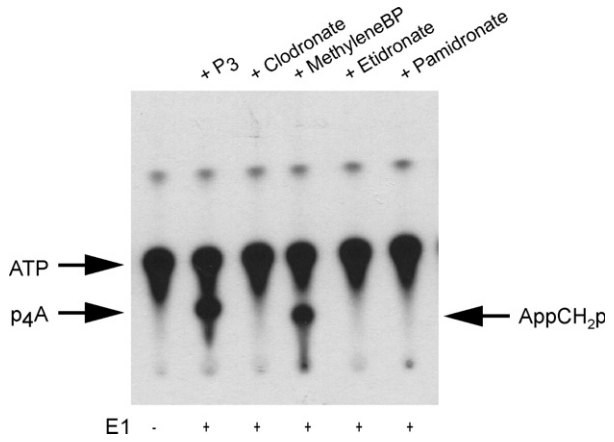

Fig. 5 – Tripolyphosphate (P_3), clodronate ($pCCl_2p$), methylenebisphosphonate (pCH_2p), etidronate ($pC(OH)(CH_3)p$) and pamidronate ($pC(OH)(CH_2–CH_2–NH_3)p$) as substrates of the reaction catalyzed by the ubiquitin activating enzyme E1. The reaction mixtures contained 0.02 mM [α-^{32}P] ATP, 1 mM bisphosphonates or P_3 as indicated, and 0.11 μg of enzyme; other components as in Section 2. At different times of incubation (8, 15 and 30 min) aliquots were spotted on TLC plates, developed with dioxane:ammonium hydroxide:water (6:1:6) and subjected to autoradiography. The figure shows the result obtained after 15 min incubation.

The AMP moiety of the above complex can be transferred to PPi with formation of ATP (the reverse reaction) or to P_3 with formation of p_4A (reaction (9)), a reaction favored in the presence of pyrophosphatase [21].

$$E1 \cdot AMP\text{-}Ub + P_3 \leftrightarrow p_4A + Ub \quad (9)$$

To explore the capacity of E1 to use bisphosphonates as substrates or inhibitors of reaction (9), the following experiments were performed.

The enzyme E1 was incubated in the presence of labeled ATP, ubiquitin and a bisphosphonate (clodronate, methylenebisphosphonate, etidronate or pamidronate). A control with P_3 was carried out in parallel. Methylenebisphosphonate was the unique bisphosphonate out of the ones tested that accepted AMP from the E1·AMP-Ub complex, generating $AppCH_2p$ (Fig. 5). Preincubation of E1 with methylenebisphosphonate did not affect the rate of synthesis of the corresponding derivative (results not shown). As already known [21], P_3 was also acceptor of AMP, yielding p_4A (Fig. 5). The K_m and k_{cat} values (determined in triplicate) for methylenebisphosphonate were 0.36 ± 0.09 mM and $0.15 \pm 0.02\ s^{-1}$, and for P_3, 2.8 ± 0.6 mM and $0.29 \pm 0.05\ s^{-1}$ [21].

In another set of experiments it was observed that: (i) clodronate in spite of not being a substrate of reaction (9), inhibited the synthesis of p_4A formed in the presence of 0.8 mM P_3, with an IC_{50} value of about 0.4 mM (Fig. 6A); at present we are unable to explain this unexpected behavior of clodronate, (ii) methylenebisphosphonate, substrate of the reaction, inhibited the synthesis of p_4A (reaction (9)) (Fig. 6B) by competing with P_3 for the AMP moiety of the E1·AMP-Ub complex, and (iii) etidronate and pamidronate were neither substrates nor inhibitors of the synthesis of p_4A (results not shown).

3.3. *Luciferase, acetyl-CoA and acyl-CoA synthetases*

Luciferase, acetyl-CoA and acyl-CoA synthetases are ligases previously investigated in our laboratory [19].

In the presence of luciferin (LH_2) and ATP, luciferase catalyzes the formation of E-LH_2-AMP and the transfer of the AMP moiety of the complex to ATP with formation of Ap_4A [19]. Clodronate, methylenebisphosphonate, etidronate and alendronate (1 mM each) were neither substrates for the synthesis of the corresponding ATP derivatives, nor inhibitors of the synthesis of Ap_4A (not shown).

The effect of bisphosphonates on the reactions catalyzed by acetyl-CoA and acyl-CoA synthetases were also assayed for the following reasons: (i) as stated in the introduction the deleterious effect of some bisphosphonates on osteoclasts is due, in part, to their inhibitory effect on the synthesis of cholesterol (ii) acetyl-CoA synthetase is in the forefront of this pathway, and (iii) this enzyme, as well as acyl-CoA synthetase catalyze the transfer of AMP from the complex E-AMP to P_3 or P_4 with formation of p_4A and p_5A, respectively [19]. In the case of acyl-CoA synthetase, none of the above-mentioned bisphosphonates, were substrates for the synthesis of ATP derivatives, or inhibitors of the synthesis of p_4A formed in the presence of P_3.

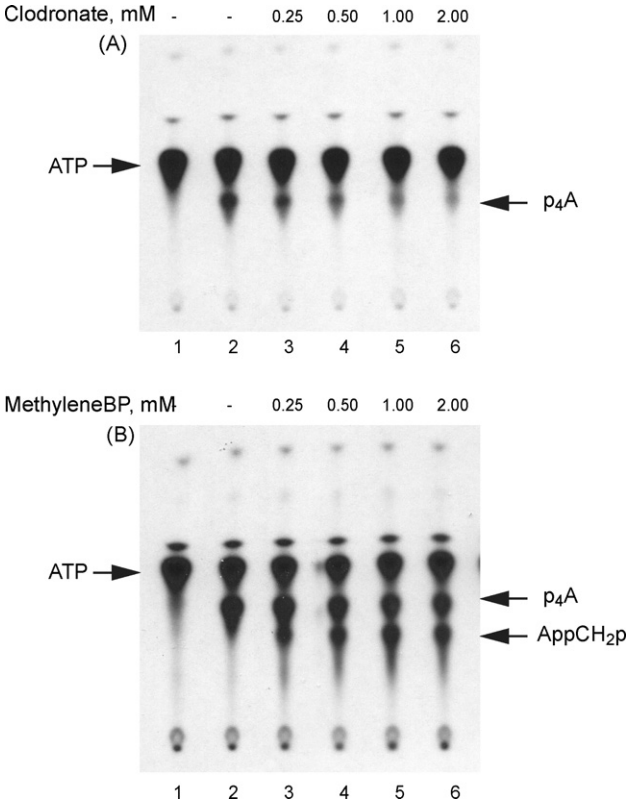

Fig. 6 – Effect of increasing concentrations of clodronate ($pCCl_2p$) and methylenebisphosphonate (pCH_2p) on the synthesis of p_4A (formed in the presence of P_3) catalyzed by the ubiquitin activating enzyme E1. The enzyme E1 (0.11 μg) was incubated for 15 min in the presence of 0.02 mM [α-^{32}P] ATP, 0.8 mM P_3, and the indicated concentrations (lanes 3–6) of clodronate (A) or methylenebisphosphonate (B). Controls without enzyme (lanes 1) or without bisphosphonates (lanes 2) were carried out in parallel. Aliquots were spotted on TLC plates, and developed with dioxane:ammonium hydroxide:water (6:1:6) as indicated in Section 2.

The ability of acetyl CoA synthetase to synthesize ATP derivatives was batch dependent. Yeast acetyl-CoA synthetase from Sigma Cat. No. A-5269 (not longer available), which we had used previously [24] to synthesize p_4A in the presence of P_3, synthesized the ATP derivative of methylenebisphosphonate at a rate of 0.02 s^{-1}; other bisphosphonates tested were not substrates of the reaction. But, a recent batch of yeast acetyl-CoA synthetase (Sigma, Cat. No. A 1765) that synthesized p_4A in the presence of P_3, with a k_{cat} of 0.019 ± 0.03 s^{-1}, did not use bisphosphonates (clodronate, methylenebisphosphonate and etidronate) neither as substrates nor as inhibitors of the reaction. The experiments were performed in the assay conditions described in [24].

3.4. *Concluding remarks*

The therapeutic use of bisphosphonates in humans is currently authorized for the treatment of bone diseases such as: postmenopausal or glucocorticoids induced osteoporosis; Paget's disease; heterotopic ossification, hypercalcemia of malignancy, bone metastases and osteolyses in multiple myeloma ([28] for an excellent, clinically oriented, monograph on bisphosphonates). Few pharmaceuticals agents are actually subjected to such number of expensive clinical studies as bisphosphonates [28]. Bisphosphonates are adsorbed to the bone hydroxyapatite crystal where they exert: (i) a harmful, although not intensive, negative effect on the crystallization and mineralization of bone and (ii) a beneficial effect on the course of osteoporosis through their deleterious action on osteoclasts, when these cells dissolve the bisphosphonate containing bone.

The difficulty in understanding the mechanism of action of bisphosphonates is due in part to the diversity of their chemical structures. Because of their analogy with pyrophosphate, they have affinity for the hydroxyapatite part of the bone; on the other side the potential diversity of chemical structures of the bisphosphonates is so large that each of them may, or may not, interact (as substrate, inhibitor or effector) with a particular enzyme. As reported here, the effect of a particular bisphosphonate on each ligase tested was different. Perhaps the take home message from the results reported here, and in other laboratories [4,6,7,15,29], is that each bisphosphonate to be tested in basic or in clinical research deserves an individualized approach to understand its way of action.

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REFERENCES

[1] Günther Sillero MA, de Diego A, Silles E, Pérez-Zúñiga F, Sillero A. Synthesis of bisphosphonate derivatives of ATP by T4 RNA ligase. FEBS Lett 2006;580:5723–7.
[2] Fleisch H. Diphosphonates: history and mechanisms of action. Metab Bone Dis Relat Res 1981;3:279–87.
[3] Fleisch H. Development of bisphosphonates. Breast Cancer Res 2002;4:30–4.
[4] Reszka AA, Rodan GA. Bisphosphonate mechanism of action. Curr Rheumatol Rep 2003;5:65–74.
[5] Epstein S, Zaidi M. Biological properties and mechanism of action of ibandronate: application to the treatment of osteoporosis. Bone 2005;37:433–40.
[6] Bergstrom JD, Bostedor RG, Masarachia PJ, Reszka AA, Rodan G. Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. Arch Biochem Biophys 2000;373:231–41.
[7] Rogers MJ. New insights into the molecular mechanisms of action of bisphosphonates. Curr Pharm Des 2003;9:2643–58.
[8] Zamecnik PC, Stephenson ML, Janeway CM, Randerath K. Enzymatic synthesis of diadenosine tetraphosphate and diadenosine triphosphate with a purified lysyl-sRNA synthetase. Biochem Biophys Res Commun 1966;24:91–7.
[9] Klein G, Martin JB, Satre M. Methylenediphosphonates, a metabolite poison in *Dictyostelium discoideum*. ^{31}P NMR

evidence for accumulation of adenosine 5′-(β,γ-Methylenetriphosphate) and diadenosine 5′-5‴-(P^2, P^3-methylenetetraphosphate). Biochemistry 1988;27:1897–901.
[10] Rogers MJ, Ji X, Russell RG, Blackburn GM, Williamson MP, Bayless AV, et al. Incorporation of bisphosphonates into adenine nucleotides by amoebae of the cellular slime mould *Dictyostelium discoideum*. Biochem J 1994;303(Pt 1):303–11.
[11] Rogers MJ, Brown RJ, Hodkin V, Blackburn GM, Russell RG, Watts DJ. Bisphosphonates are incorporated into adenine nucleotides by human aminoacyl-tRNA synthetase enzymes. Biochem Biophys Res Commun 1996;224:863–9.
[12] Auriola S, Frith J, Rogers MJ, Koivuniemi A, Mönkkönen J. Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography-electrospray mass spectrometry. J Chromatogr 1997;704:187–95.
[13] Frith JC, Mönkkönen J, Blackburn GM, Russell RG, Rogers MJ. Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5′-(β,γ-dichloromethylene) triphosphate, by mammalian cells *in vitro*. J Bone Miner Res 1997;12:1358–67.
[14] Lehenkari PP, Kellinsalmi M, Napankangas JP, Ylitalo KV, Mönkkönen J, Rogers MJ, et al. Further insight into mechanism of action of clodronate: inhibition of mitochondrial ADP/ATP translocase by a nonhydrolyzable, adenine-containing metabolite. Mol Pharmacol 2002;61:1255–62.
[15] Amin D, Cornell SA, Gustafson SK, Needle SJ, Ullrich JW, Bilder GE, et al. Bisphosphonates used for the treatment of bone disorders inhibit squalene synthase and cholesterol biosynthesis. J Lipid Res 1992;33:1657–63.
[16] Fisher JE, Rogers MJ, Halasy JM, Luckman SP, Hughes DE, Masarachia PJ, et al. Alendronate mechanism of action: geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation *in vitro*. Proc Natl Acad Sci USA 1999;96:133–8.
[17] Benford HL, Frith JC, Auriola S, Mönkkönen J, Rogers MJ. Farnesol and geranylgeraniol prevent activation of caspases by aminobisphosphonates: biochemical evidence for two distinct pharmacological classes of bisphosphonate drugs. Mol Pharmacol 1999;56:131–40.
[18] Mönkkönen H, Auriola S, Lehenkari P, Kellinsalmi M, Hassinen IE, Vepsalainen J, et al. A new endogenous ATP analog (ApppI) inhibits the mitochondrial adenine nucleotide translocase (ANT) and is responsible for the apoptosis induced by nitrogen-containing bisphosphonates. Br J Pharmacol 2006;147:437–45.
[19] Sillero A, Günther Sillero MA. Synthesis of dinucleoside polyphosphates catalyzed by firefly luciferase and several ligases. Pharmacol Ther 2000;87:91–102.
[20] Madrid O, Martin D, Atencia EA, Sillero A, Günther Sillero MA. T4 DNA ligase synthesizes dinucleoside polyphosphates. FEBS Lett 1998;433:283–6.
[21] Günther Sillero MA, de Diego A, Silles E, Sillero A. Synthesis of (di)nucleoside polyphosphates by the ubiquitin activating enzyme E1. FEBS Lett 2005;579:6223–9.
[22] Ortiz B, Sillero A, Günther Sillero MA. Specific synthesis of adenosine(5′)tetraphospho(5′)nucleoside and adenosine(5′)oligophospho(5′)adenosine ($n > 4$) catalyzed by firefly luciferase. Eur J Biochem 1993;212:263–70.
[23] Fontes R, Günther Sillero MA, Sillero A. Acyl coenzyme A synthetase from *Pseudomonas fragi* catalyzes the synthesis of adenosine 5′-polyphosphates and dinucleoside polyphosphates. J Bacteriol 1998;180:3152–8.
[24] Guranowski A, Günther Sillero MA, Sillero A. Adenosine 5′-tetraphosphate and adenosine 5′-pentaphosphate are synthesized by yeast acetyl coenzyme A synthetase. J Bacteriol 1994;176:2986–90.
[25] Lindahl T, Barnes DE. Mammalian DNA ligases. Annu Rev Biochem 1992;61:251–81.
[26] Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998;67:425–79.
[27] Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002;82:373–428.
[28] Bartl R, Frisch B, von Tresckow E, Bartl C. Bisphosphonates in medical practice. Berlin: Springer-Verlag; 2007.
[29] Moreau MF, Guillet C, Massin P, Chevalier S, Gascan H, Basle MF, et al. Comparative effects of five bisphosphonates on apoptosis of macrophage cells *in vitro*. Biochem Pharmacol 2007;73:718–23.