

Clodronate acts on human osteoclastic cell proliferation, differentiation and function in a bioreversible manner

Raffaella Recenti^a
 Giuseppe Leone^b
 Lisa Simi^c
 Marco Orfei^b
 Pamela Pinzani^c
 Giuseppe Pieraccini^d
 Gloriano Moneti^d
 Anna Maria Carossino^a
 Alessandro Franchi^e
 Gianluca Bartolucci^f
 Silvia Carbonell Sala^a
 Mauro Ginanneschi^b
 Annalisa Tanini^{a,g}
 Maria Luisa Brandi^{a,g}

Departments of ^aInternal Medicine, ^bOrganic Chemistry, ^cClinical Physiopathology, ^dMass Spectrometry Centre, ^eHuman Pathology and Oncology, ^fPharmaceutical Science, and ^gDe Gene Spin-off, University of Florence, Florence, Italy

Address for correspondence:
 Maria Luisa Brandi, M.D., Ph.D.
 Regional Center for Hereditary Endocrine Tumors
 Department of Internal Medicine
 University of Florence
 Viale Pieraccini 6, 50139 Florence, Italy
 Ph. +39 055 4296586
 Fax +39 055 4296585
 E-mail: m.brandi@dmi.unifi.it

Summary

Background. Clodronate is used in high bone resorption diseases. Its action was defined as “cytotoxic” based on the induced cellular ATP loss, without any experimental verification of reversibility. In the present report the reversibility of clodronate action was tested on cultured human osteoclastic cell cultures. As “in vitro” bioeffects of clodronate are reversible, this compound should not be defined as “cytotoxic”.

Introduction. Bisphosphonates are pyrophosphate analogs able to inhibit osteoclast-mediated bone resorption widely used in the treatment of diseases with high bone turnover. Several evidences have shown that bisphosphonates can be divided into two groups with distinct molecular mechanisms of action depending on the nature of the R² side chain. The nitrogen-containing bisphosphonates act on osteoclasts by preventing protein prenylation, while non-nitrogen-containing bisphosphonates, like clodronate, are metabolized intracellularly to a β-γ-methylene analog of ATP that induces inhibition of the ADP/ATP translocase.

Materials and Methods. In order to evaluate clodronate effects on osteoclastic cells and the bioreversibility of its action, we have used a human preosteoclastic (FLG 29.1) cell line and primary cultures of human osteoclast-like (HOC) cells. Functional and differentiative modifications were evaluated with immunocytochemical tartrate-resistant acid phosphatase activity (TRAcP) assay and with rapid quantitative detection of

the complex “matrix metalloproteinase 9/tissue inhibitor of metalloproteinase” (MMP9/TIMP1) by RT-PCR analysis based on “TaqMan” technology. The apoptosis phenomenon were detected by DNA ladder analysis and quantified by counting apoptotic cells with Transmission Electron Microscopy (TEM) analysis. Adenosine-5'-[β-γ-dichloromethylene] triphosphate (AppCCl₂p) was detected and identified in cell extract by HPLC-ESI-MS-MS Mass Spectrometry. Intracellular ATP modulation in the presence of clodronate was evaluated by luciferin-luciferase assay. The Mann-Whitney “U” test was conducted for statistical analysis.

Results. We found that clodronate inhibited both proliferation and differentiative features of cells of the osteoclastic lineage. Furthermore, treatment of both cell types with clodronate caused apoptosis, generation of measurable levels of AppCCl₂p, and reduction of intracellular ATP levels. Addition of ATP to the culture medium caused an inhibition of the biological actions of clodronate on the human osteoclastic cell lineage.

Conclusions. These data indicate that intracellular accumulation of the metabolite AppCCl₂p is the likely route by which clodronate inhibits osteoclastic function and this effect is reversed by ATP.

KEY WORDS: bisphosphonates, clodronate, osteoclasts, ATP, bone resorption.

Introduction

Bisphosphonates are synthetic analogs of pyrophosphate which bind strongly to bone hydroxyapatite and inhibit osteoclast function. This explains the wide use of these compounds for the treatment of disorders characterized by accelerated bone resorption such as osteoporosis, Paget's disease of bone, and osteolysis associated with metastatic bone disease (1-3).

The chemical structure of bisphosphonates is similar to the inorganic pyrophosphate structure (PPi), but the two phosphate groups are linked by phosphorus-carbon bonds to a central carbon atom (P-C-P structure). The P-C-P structure is required for binding bone mineral and for interacting with molecular targets in the osteoclast (4, 5), while the antiresorptive potency is determined by the chemical structure of the two geminal substituents, R¹ and R², attached to the central carbon atom (6, 7). The R² side chain appears to play an important role in the interaction of bisphosphonates with a pharmacological target (4, 8-10). Bisphosphonates that contain an amino group in the R² side chain (nitrogen bisphosphonates), were shown to inhibit bone resorption through the control of the farnesyl-diphosphate synthetase, an enzyme in the mevalonate pathway central for the post-translational prenylation (10-13). Conversely, non nitrogen bisphosphonates, such as clodronate, do not affect the mevalonate pathway (12, 14-16). Clodronate contains a -Cl in R² and is metabolized “in vitro” by macrophages and osteoclasts into a non-hydrolysable analog of ATP, AppCCl₂p, in a reaction catalyzed by class II aminoacyl-tRNA synthetases (17-19). Inhibition of the ADP/ATP translocase by the metabolite AppCCl₂p is a likely route by

which clodronate causes inhibition of osteoclast function (19). The different mechanism of action of the two classes of molecules explains the competition observed in the combined treatment with clodronate and nitrogen-bisphosphonates (20). As the intracellular accumulation of AppCCl₂p appears to account for the pharmacological effects of clodronate on osteoclasts and macrophages (21-23), incorporation of clodronate into AppCCl₂p could lead to depletion of intracellular ATP and accumulation of a toxic ATP analog (21). However, despite the accumulated experimental data, the way in which clodronate inhibits osteoclast functions and the possible reversibility of its effects have not been fully elucidated. Here we report that human osteoclastic cells exposed to clodronate undergo apoptosis "in vitro" and we confirm that these cells accumulate the ATP analog AppCCl₂p, with a measurable decrease of intracellular ATP. The bioreversibility of clodronate action on human osteoclastic cells was, therefore, investigated by adding ATP into the culture medium.

Materials and Methods

Chemicals and equipments

Clodronate was provided by Società Prodotti Antibiotici Spa (Milan, Italy). Stock solutions of free clodronate at 10 mM were prepared by dissolving the bisphosphonate in mQ-bi-distilled water (Millipore, Molsheim, France), the pH was adjusted to 7.4 and the solution was filter-sterilized using a 0.22 µm filter. HPLC (high-performance liquid chromatography) grade acetonitrile and formic acid were from Carlo Erba (Milan, Italy). Dimethylhexylamine (DMH) was from Across (Milwaukee, WI, USA). Adenosine-2,8-³H 5' triphosphate (³H ATP) and all other chemicals were purchased by Sigma (Milan, Italy) unless otherwise stated.

The amount of radioactivity from ³H-DNA and ³H-ATP was measured by liquid scintillation using a Kontron Betamatic V counter (Kontron Instrument, Montigny le Bretonneux, France). ¹H NMR spectra were recorded on a Varian 300 MHz spectrometer and ³¹P NMR spectra on the same apparatus at 121.4 MHz for DMSO-*d*₆ solutions. The elemental analyses were performed on a Perkin Elmer 240 C Elemental Analyzer. Routinely HPLCs were acquired using a Beckman System Gold Nouveau apparatus equipped with a diode array detector. The RP-C18 columns were from Phenomenex (Chemtek Analytica, Anzola Emilia, Bologna, Italy). Eluents for ion-pairing HPLC: (A), 10 mM DMH formate (pH 5.0); (B), 50% acetonitrile and 50% of 20 mM DMH formate solution (pH 5.0) (30). On line HPLC ESI-MS measurements were carried out on a Thermo (Thermo Italia, Rodano, Milan, Italy) instrument, composed by a HPLC Surveyor or MS pump, equipped with a Surveyor autosampler, and coupled to a LTQ linear quadrupole ion trap mass spectrometer. The RP column was a Luna C18 (2) (3 µm, 50 x 2 mm I.D.) operating at a flow rate of 200 µl min⁻¹ at a temperature of 25°C. Eluents for ion pairing were the same above indicated. Total RNA was determined with the GeneQuant spectrophotometer (Pharmacia Biotechnologies, NJ, USA). The measurement of intracellular ATP was performed on Orion Microplate Luminometer (Berthold Detection Systems, Pforzheim, Germany).

Cell cultures

FLG 29.1 cell line was established from a 38-year-old female suffering from acute monoclonal leukaemia (24). FLG 29.1 cells displayed an osteoclastic phenotype (24-26) in the presence of 10⁻⁷ M TPA (FLG 29.1-TPA) (26). The cells were

grown at 37°C in RPMI-1640 medium (Biowhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS; Gibco Grand Island, NY) and gentamycin (100 µg/ml) in 5% CO₂ atmosphere. In this paper, we refer to the undifferentiated FLG 29.1 cells as preosteoclastic cells and to the TPA-treated cells as osteoclast-like cells.

HOC cells were obtained from human bone giant cell tumor according to previous published method (27). Briefly, cells from a surgically removed giant cell tumor tissue were mechanically disaggregated by pipetting fragments or, alternatively, were released by digestion for 30 minutes with 2 mg/ml of *C. histolyticum* neutral collagenase (Serva Electrophoresis GmbH, Heidelberg, Germany), in phosphate-buffered saline (PBS), pH 7.3 at 37°C. Cell obtained by both treatments were washed, plated in 25 cm² flasks at a density of 10⁴ cells/cm², and cultured in Iscove's Modified Dulbecco's Medium (IMDM; Gibco) supplemented with 10% heat-inactivated FCS, 100 IU/ml of penicillin (Biowhittaker), 100 µg/ml of streptomycin (Biowhittaker), 2.5 µg/ml of amphotericin B (Eurobio, Paris, France), and 50 IU/ml of mycostatin (Eurobio) at 37°C in 5% CO₂ atmosphere. Cells were fed by medium replacement every 2-3 days and osteoclast phenotype was evaluated according the typical osteoclastic markers.

J774 cells were obtained from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy). They were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker) containing 10% heat inactivated FCS, 1 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 µg/ml) at 37°C in 5% CO₂ atmosphere.

Cell morphology was observed by a phase-contrast microscopy and by transmission electron microscopy (TEM).

Cell viability

Cell viability was evaluated in FLG 29.1 and HOC cells by Trypan blue dye exclusion. Fifty µl of cell suspension was added to 50 µl of Trypan blue (0.25%), than viable and nonviable cells were evaluated by counting with a hemocytometer. Each experiment was repeated at least 3 times in triplicate and results were expressed as percentage of control (mean ± SD).

³H-ATP uptake

ATP is a small water soluble molecule that cells of the monocyte-macrophagic lineage, as HOC and FLG 29.1 cells, could readily take up from the medium. ATP uptake was evaluated in undifferentiated FLG 29.1 and in HOC cells by measuring ³H-ATP with a β counter.

Cells were plated at a density of 4 x 10⁴ cells/well in 24-well plates. After 24 hours the cells were maintained overnight in medium without FCS, then replaced with fresh medium containing 1% FCS (control) plus various concentration of ³H-ATP (0.1-5 nM for FLG 29.1 cells and 0.5-500 nM for HOC cells) in replicates of three wells. Forty-eight hours later the cells were washed in PBS and fixed with 4% TCA. After treatment with 0.5 M NaOH and 0.5 M HCl radioactivity was measured with a β counter.

Each experiment was repeated at least 3 times in triplicate and results were normalized vs ng DNA (evaluated with a fluorescent DNA quantitation kit; Bio-Rad, Milan, Italy) and expressed as mean ± SD.

³H-thymidine uptake

In order to choose the non-mitogenic ATP concentrations we performed the ³H-thymidine uptake in FLG 29.1 and HOC cells.

Cells were plated at a density of 4×10^4 cells/well in 24-well plates. After 24 hours the cells were cultured overnight in medium without FCS, then was replaced with fresh medium containing 1% of FCS (control) and different ranges of ATP concentrations in replicates of three wells. Forty-four hours later a 4 hours ^3H -thymidine pulse was performed and then the cells were exposed to 4% trichloroacetic acid (TCA). After treatment with 0.5M NaOH and 0.5M HCl the ^3H -labelled DNA was evaluated with a β counter.

Each experiment was repeated at least 3 times in triplicate and results were expressed as mean \pm SD.

Cell counting

FLG 29.1 and HOC cells were plated at a density of 10^5 cells/well in 6-well plates. Twenty four hours later the medium was replaced with fresh medium containing 1% FCS (control) plus clodronate in a concentration ranging from 1 μM to 1 mM in replicates of three wells. Moreover two experimental point were introduced in order to evaluate the effects of ATP on the action of the highest dose clodronate on FLG 29.1 and HOC cells. A non mitogenic ATP concentration was choice for each cell type, 1 nM for FLG 29.1 cells and 50 μM for HOC cells, according to dose response curve, as reported in previous paragraph. Cell growth was evaluated after 2 days of incubation by counting with a hemocytometer.

Each experiment was repeated at least 3 times in triplicate and results were expressed as mean \pm SD.

Measurement of tartrate-resistant acid phosphatase

Tartrate-resistant acid phosphatase (TRAcP) was evaluated by histochemistry. The staining was performed in undifferentiated FLG 29.1 cells, in TPA-treated FLG 29.1 cells and in HOC cells maintained for 2 days in 1% FCS medium with and without 1 mM clodronate. In the same conditions TRAcP activity was evaluated also in the presence of ATP with and without 1 mM clodronate.

Cell suspensions were cytopinned on slides and, after fixation in 80% ethanol, they were incubated with a solution containing naphthol AS-BI phosphate as a substrate for the reaction, 0.225% sodium tartrate and 4% pararosaniline in 2 N HCl as a coupler for 2 hours. The cultures were then counterstained with hematoxylin and TRAcP positive FLG 29.1 cells were counted. Experiments were carried out in triplicate and repeated three times. Results were expressed as mean \pm SD of three experimental points.

Quantitative RT-PCR

Undifferentiated (1×10^5 cells/ml) and TPA-treated (1×10^5 cells/ml) were incubated in culture medium containing 1% FCS in the absence or in the presence of 1 μM -1 mM clodronate. After 6 days of incubation FLG 29.1 cells were disrupted in 600 μl of guanidine isothiocyanate-containing lysis Qiagen buffer (QIAGEN, Milan, Italy) added with β -mercaptoethanol. Total RNA was extracted with Rneasy MiniKit Qiagen[®] columns and treated with RNase free DNase Set QIAGEN[®]. RNA was then eluted from columns with 50 μl RNase-free water. Total RNA was determined with the GeneQuant spectrophotometer.

Rapid quantitative detection of matrix metalloproteinase 9 (MMP9) and tissue inhibitor of metalloproteinase (TIMP1) transcripts in FLG 29.1 were evaluated by real-time reverse transcriptase polymerase-chain reaction (RT-PCR), based on "TaqMan" technology as previously reported (28).

For the measurement of both MMP9 and TIMP-1 mRNA ex-

pression, RNA extracted from HT1080 fibrosarcoma cell lines was used as reference standard curve. Standard curve consists of serial 1/10 dilution obtained from a starting quantity of 2.5×10^4 pg of total HT1080 RNA.

All the results are reported as pg HT1080 RNA/ μg of total RNA and were carried out in triplicate and expressed as the mean \pm SD of pg HT1080 RNA/ μg of total RNA.

Ultrastructural analysis

The percentage of apoptotic cells was determined by TEM. Five random fields, each of 100 cells, were counted in each slide.

FLG 29.1 cells and HOC cells (1×10^6 cells) were incubated for 2 days in 1% FCS medium (control) with 100 μM or 1 mM clodronate. In order to evaluate the apoptotic influence of ATP on clodronate treated cells 1×10^6 of both FLG 29.1 cells and HOC cells in 1% FCS medium were incubated with 1 mM clodronate in the presence of 1 nM or 50 μM ATP. At the end of the incubation time samples were fixed in 4% cold glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature and postfixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, at 4°C. The samples were then dehydrated in acetone series, passed through propylene oxide, and embedded in Epon 812. Semi-thin sections (1-2 μm thick) were cut and stained with toluidine blue-sodium tetraborate.

Analysis of DNA fragmentation

FLG 29.1 cells and HOC cells (1×10^6 cells) were incubated for 2 days in 1% FCS medium (control), 100 μM clodronate, 1 mM clodronate, 100 μM clodronate plus ATP (1 nM or 50 μM), 1 mM clodronate plus ATP (1 nM or 50 μM). Positive control of apoptosis was represented by HL60 cell line treated with 2.5 μM camptothecin for 4 hours.

The selective extraction of fragmented DNA was obtained by a DNA extraction kit according to manufacturer's instructions (ApopLadder EXTM; Takara Bio Inc., Otsu, Shiga, Japan). Briefly, a 15 μl DNA sample was loaded onto 2% horizontal agarose gels containing ethidium bromide. Gels were run at 60 V for 1 hour and DNA fragments were visualized using UV illumination.

Synthesis of AppCCl₂p metabolite and its identification on the cell lines

AppCCl₂p was obtained following the method of Blackburn and coworkers with minor modifications (29). The crude product was purified by CC on DEAE Sephagel using a linear salt gradient (LiCl, 0-0.5 M, pH 7.0, 0.5 L) and the isolated compound was repeatedly treated with methanol/acetone affording a white, hygroscopic powder. ESI-MS: *m/z* calcd 574.1 [M-H]⁻, 572.1 [M-2H]⁻; found: 573.9 [M-H]⁻, 571.8 [M-2]⁻; ¹H NMR, δ (from Me₄Si): 8.36 (s, 1H), 8.13 (s, 1H), 5.91 (d, 1H), 4.56 (m, 1H), 4.23 (m, 1H), 3.98 (m, 1H), 3.76 (m, 2H); ³¹P NMR 300 MHz, δ (from 85% H₃PO₄): -11.86 (d, P _{α} , ²J _{$\alpha\beta$} 28.1 Hz), -3.87 (dd, P _{β} , ²J _{$\alpha\beta$} 28.1 Hz, ²J _{$\beta\gamma$} 22.6 Hz), +5.86 (d, P _{γ} , ²J _{$\beta\gamma$} 22.6 Hz). Elemental analysis: calcd. for C₁₁H₁₂Cl₂Li₄N₅O₁₂P₃·6H₂O·19LiCl: C, 8.74; H, 1.60; N, 4.63. Found: C, 8.87; H, 1.60; N, 4.35. The purity of ATP and AppCCl₂p standards (200 μM) was checked just before the use, by reverse phase HPLC on a Jupiter C18 column (5 mm, 250 \times 4.6 mm I.D.) using the same eluents as for HPLC-ESI (gradient: 20% of B in 10 min and then to 80% of B in 30 min; tR: ATP 24.01 min; AppCCl₂p 24.08 min).

Cell extracts were obtained following the method of Frith et al. with some modifications (21). FLG 29.1 and J774 cells were

grown in 162 cm² flask. A total of approximately 1×10^7 FLG 29.1 cells were incubated for 2 days with 1 mM clodronate or with an equivalent volume of growth medium supplemented with 1% FCS. The same number of J774 cells were incubated with 250 μ M clodronate for 2 days in medium 10% FCS. At the end time cells were rinsed twice with PBS and the adherent cells were scraped from the flask. Each sample was then incubated with 10 ml of ice-cold 7% (v/v) perchloric acid and left at 4°C for 1 hour. The extracts were neutralized with a saturated solution of KHCO₃, left for 30 minutes on ice and then centrifuged twice (5 minutes, 220 g) to remove insoluble potassium perchlorate. Supernatants were then lyophilized. The ion-pair chromatographic separation was strongly influenced, in terms of retention time reproducibility and peak shape, by the high saline content of the samples, requiring a step of purification to eliminate part of it. This was accomplished by adding about 2 ml of distilled water (Milli-Q) to 100 mg of the lyophilized extracts from untreated and clodronate-treated cells. The suspension was centrifuged and the operation was repeated. The combined extracts were lyophilized and the residue was re-dissolved in 50 mM DMH formate, pH 5.0, as suggested by Auriola et al. (30). A 5% reproducibility in retention time (9.33 ± 0.42 min) was tolerated, considering the chromatographic technique applied and the high selectivity of the MS/MS detection.

Following the paper of Auriola et al. (30), ion-pair liquid chromatography was used for HPLC analysis of ATP and AppCCl₂p. Samples were eluted using an RP column using the following gradient elution: 10 to 75% B in 14 min; B was held at 75% for 2 min and then brought back to the starting value in 1.0 min). The equilibration time before the next injection was 12 min. The sample injection volume was 10 mL. The eluate was directly introduced into the LC/MS interface. The compounds of interest were ionised by negative ion electrospray and analysed by full-scan product ion MS/MS of their quasi-molecular ions. The spray needle capillary, heated capillary and tube lens potentials were -4000, -27 and -20 V, respectively. The capillary temperature was set at 265°C. Nitrogen was used both as sheath and auxiliary gas, respectively at flow rates of 26 and 6 (in arbitrary units). The CID parameters were optimised for each analyte by infusion and alternately applied to detect the two compounds during the HPLC run. For ATP detection, the precursor ion isolation width was 5 m/z units, the collision energy was 27%, and the product ion scan range was from m/z 170 to 550, while for AppCCl₂p detection, the precursor ion isolation width was 7 m/z units, the collision energy was 22%, and the product ion scan range was from m/z 170 to 630. The maximum ion injection time was 200 msec; each scan was composed of two microscans.

ATP measurement

Effect of 1 mM clodronate was evaluated on undifferentiated FLG 29.1 cells during 24 hours exposure. Cells were counted and washed once with PBS, then they were transferred in 96 well microplates for intracellular ATP measurement by an ATP bioluminescence assay kit (DCS Innovative Diagnostik-Systeme, Hamburg, Germany). Briefly, cellular ATP was extracted by adding 0.05 ml of extraction reagent to 0.1 ml of cell suspension. After 15 minutes incubation, the assay was measured in a Orion Microplate Luminometer using 0.05 ml of cellular extract injected with 0.1 ml of luciferin-luciferase reagent. A count integration time of 10 seconds with a 0,04 seconds delay was used. Relative Light Unit (RLU) results from unknown samples were extrapolated on a standard curve (concentration range: 83,33-0,012 ng ATP/ml) obtained by measuring nine 3-fold serial dilutions of an ATP-standard

solution of 250 ng ATP/ml in triplicate. Linear regression analysis of mean RLUs for each ATP standard concentration vs ATP standard concentration was evaluated to determine standard curve linearity. Results from a typical standard curve for ATP measurement were: $y = 2367,7x + 117,91$; $R^2 = 1$.

Statistical analysis

Data were expressed as the mean \pm SD of triplicate experimental points and are representative of three different experiments. Statistical differences were analyzed by using the Mann-Whitney U-test and were considered significant at the 0.05 p level.

Results

Cell morphology

FLG 29.1 cells stained with May-Grunwald-Giemsa had a typical aspect of undifferentiated leukemic blast, with large round nuclei, prominent nucleoli, dispersed nuclear chromatin and basophilic cytoplasm (24). Less than 3% of the cells were multinucleated, the majority were mononucleated. The diameter of the cells ranged from 15 to 20 μ m and had a round shape. Undifferentiated FLG 29.1 cells grew in suspension as small floating clusters. After 72 hours of treatment with 0.1 μ M TPA 36% of the cells adhered to the dishes and over 45% were multinucleated with size ranging from 50 to 100 μ m. Multinucleated cells were found attached or in suspension and lacked characteristic peripheral ruffling. TEM showed that most of the untreated cells had cytoplasmic features of undifferentiated blasts, namely abundant free ribosomes, scarce organelles, large and irregularly shaped nucleus with dispersed chromatin and large nucleoli. After treatment with 0.1 μ M TPA for 72 hours FLG 29.1 cells appeared to be in a more advanced stage of differentiation, containing rather numerous rod-shaped mitochondria and well developed RER, large Golgi areas, and several dense bodies resembling lysosomes.

HOC cells cultured from giant cell tumors of bone presented two main cell populations, one characterized by larger polymorphic cells with a variable distribution of nuclei and another, mainly composed of smaller irregularly shaped mononucleated cells. All the giant cells had an abundant finely granulated cytoplasm. The overall aspect of these cells was mostly like osteoclasts and macrophages with numerous pleomorphic mitochondria and lysosomes, dense bodies, and a wide Golgi areas, as previously described (30, 31). At the ultrastructural level, mononuclear cells revealed a diverse morphology, displaying features of fibroblast-like cells with scarce granules and numerous microvilli and desmosomes (data not shown).

Effects of clodronate on cell viability

Analysis of viable cells demonstrated that treating FLG 29.1 and HOC cells with clodronate concentrations ranging from 1 μ M to 1 mM had no effect on cell viability (Table I).

Measurement of ³H-ATP uptake in FLG 29.1 and HOC cells

Uptake of exogenous ³H-ATP was evaluated both in undifferentiated FLG 29.1 and in HOC cells with a β counter. Extracellular ATP was incorporated in a dose dependent manner (Table II).

Table I - Viability of undifferentiated FLG 29.1 cells and of HOC cells in the presence of clodronate.

Cell type	Clodronate (M)	Percent of control ^a	p ^b
FLG 29.1	0	100 ± 9	–
	1	100 ± 12	NS
	10	100 ± 15	NS
	100	100 ± 8	NS
	1000	92.6 ± 1.6	NS
HOC	0	100 ± 16	–
	1	91.1 ± 1.4	NS
	10	92.2 ± 1.5	NS
	100	90.0 ± 0.9	NS
	1000	85.2 ± 1.2	NS

^a Controls are represented by untreated FLG 29.1 and HOC cells. Results are expressed as mean ± SD of triplicate experimental points and are representative of three different experiments.

^b Data were considered Not Significant (NS) when p > 0.05.

Table II - Measurement of ³H-ATP uptake in undifferentiated FLG 29.1 and in HOC cells.

Cell type	³ H-ATP (nM)	³ H-ATP uptake [Mean ± SD (dpm/ng DNA)]
Undifferentiated FLG 29.1	0.01	117.67 ± 0.49
	0.05	516.96 ± 0.76
	1	1023.65 ± 5.25
	3	3030.02 ± 23.17
	5	4932.57 ± 114.81
HOC	0.05	8.5 ± 0.40
	5	40.33 ± 0.39
	50	361.55 ± 0.39
	100	719.43 ± 1.26
	250	1760.06 ± 60.87
500	3550.51 ± 67.05	

The experiments were repeated three times in triplicate and expressed as mean ± SD.

Effects of ATP on FLG 29.1 and HOC ³H-thymidine cellular uptake

The concentration of ATP added to the culture medium was chosen on the basis of the effect of a dose response curve of ATP on ³H-thymidine incorporation and Trypan blue exclusion test in undifferentiated FLG 29.1 and in HOC cells. Neither 1 nM nor 50 μM ATP affected ³H-thymidine incorporation or viability of respectively undifferentiated FLG 29.1 and HOC cells (data not shown).

The effects of clodronate on FLG 29.1 and HOC cell proliferation is bioreversible

Undifferentiated FLG 29.1 and HOC cell proliferation was evaluated by cell counting. Cells were incubated for 2 days in the presence of 1% FCS with clodronate added in a range of concentrations from 1 μM to 1 mM. Moreover, the possibility of re-

verting the effect of clodronate on cell proliferation both in undifferentiated FLG 29.1 and in HOC cells was evaluated after adding ATP into the culture medium.

Clodronate significantly inhibited FLG 29.1 cell proliferation from 10 mM dose (Fig. 1). Proliferation in HOC cells was significantly inhibited by clodronate at each dose considered (Fig. 1). One nM and 50 μM ATP reversed the inhibitory effect of simultaneously added 1 mM clodronate on proliferation of respectively undifferentiated FLG 29.1 cells and HOC cells (Fig. 1).

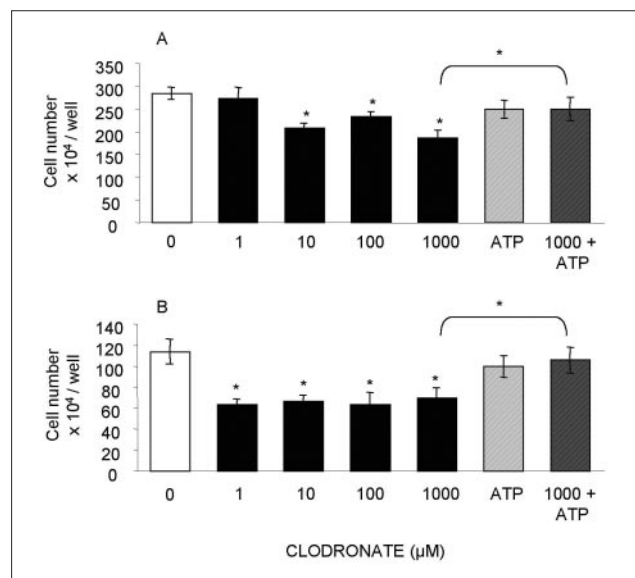


Figure 1 - Effects of clodronate on proliferation of undifferentiated FLG 29.1 (A) and of HOC (B) cells with and without respectively 1 nM and 50 μM ATP added to the culture medium. Cell proliferation was evaluated after incubation for 2 days by counting with a hemocytometer. Control (□); clodronate (■); ATP (▨); clodronate (1000 μM + ATP (▩)). Each experiment was repeated at least three times in triplicate and results were expressed as mean ± SD. *p < 0.05.

The effects of clodronate on TRAcP activity in FLG 29.1 and HOC cells is bioreversible

The histochemical assay for TRAcP activity was performed in undifferentiated and TPA-treated FLG 29.1 cells and in HOC cells.

High levels of TRAcP activity were present in TPA-treated FLG 29.1 cells and in HOC cells. Low doses of clodronate (1 μM-10 μM) not always interfered with TRAcP activity in three different experiments (data not shown), while the addition of 1 mM clodronate resulted in a significant inhibition of TRAcP activity (57 % and 62 % respectively) in each cell type considered (Fig. 2).

The decrease of TRAcP-positive cells in TPA-treated FLG 29.1 and HOC lineages was reversed in the presence of respectively 1 nM and 50 μM ATP concentrations (Fig. 2).

Effects of clodronate on MMP9 and TIMP-1 mRNA expression in FLG 29.1 cells

MMP-9 and TIMP-1, evaluated by RT-PCR, were expressed in both undifferentiated and TPA-treated FLG 29.1 cells. The incubation with 1 μM and 10 μM clodronate caused a not significant inhibition of MMP-9/TIMP-1 complex (data not shown), while 100 μM and 1 mM clodronate caused a dose-dependent inhibition compared with the respective controls (Fig. 3). Ex-

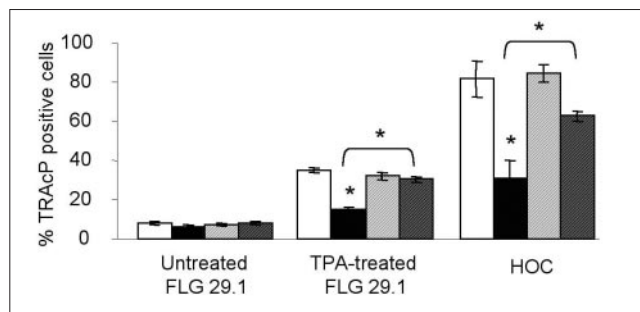


Figure 2 - Cells cultures were evaluated for TRAcP activity by a histochemical assay. Effects of 1 mM clodronate on TRAcP activity in undifferentiated and TPA-treated FLG 29.1 cells and in HOC cells was evaluated with and without respectively 1 nM, 1 nM and 50 μM ATP added to the culture medium. Control (□); clodronate (μM) (■); ATP (□); clodronate 1000 μM + ATP (■). Experiments were carried out in triplicate and repeated three times and results were expressed as mean ± SD. *p< 0.05 vs cells not exposed to clodronate.

cept for the 1 mM dose on undifferentiated FLG 29.1 cells, clodronate inhibitory action on TIMP-1 and MMP-9 expression preserved the physiological cellular equilibrium of the two enzymes, with significantly higher values of TIMP-1 when compared to those of MMP-9 (Fig. 3).

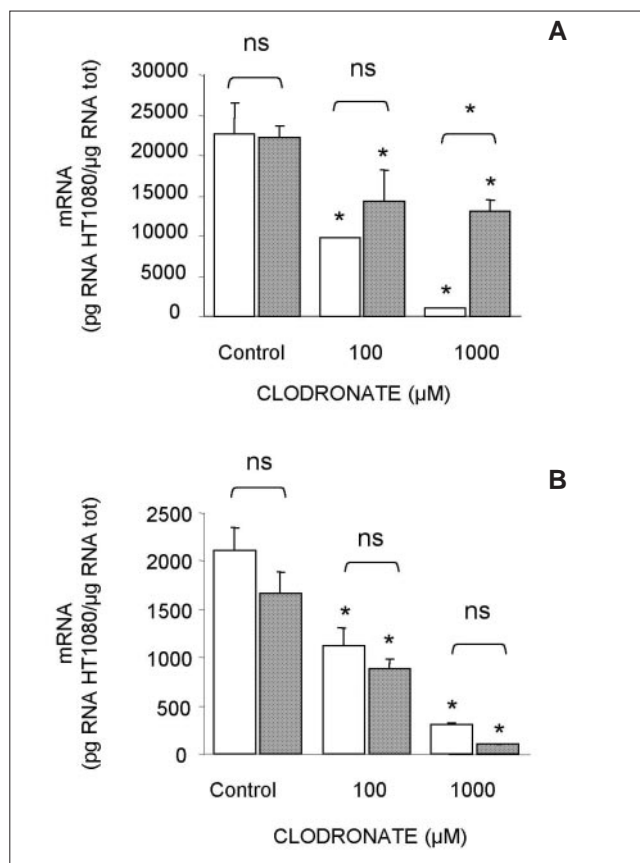


Figure 3 - Effects of clodronate on MMP9 and TIMP-1 mRNA expression in undifferentiated (A) and TPA-treated (B) FLG 29.1 cells. RT-PCR showed modulation of MMP9 (□) and TIMP-1 (■) mRNA expression by 100 μM and 1 mM clodronate. Concentrations were derived by comparison with values obtained with the HT1080 reference cell line. Experiments were carried out in triplicate and results were expressed as mean ± SD. *p<0.05; ns: not significative.

Effects of clodronate on apoptosis of undifferentiated FLG 29.1 cells and of HOC cells

Both undifferentiated FLG 29.1 cells and HOC cells showed typical apoptotic morphological events after exposure for 2 days to 100 μM and 1 mM clodronate. The cells were characterized by reduced cell volume, chromatin condensation, aggregation of the nuclear membrane, with either a condensate or a pale cytosol, without signs of necrosis (Fig. 4). The phenomenon was quantified by counting apoptotic cells in TEM random fields. In both cell lines apoptotic elements increased in a dose-dependent manner in response to clodronate (Table III).

Moreover, the contemporary treatment of undifferentiated FLG 29.1 and HOC cells respectively with 1 nM and 50 μM ATP prevented the apoptotic effect of 1 mM clodronate (Table III). No reversibility of clodronate effects was observed when cells already showing recognizable morphological signs of apoptosis were exposed to ATP (data not shown).

To confirm that 100 μM and 1 mM clodronate-induced apopto-

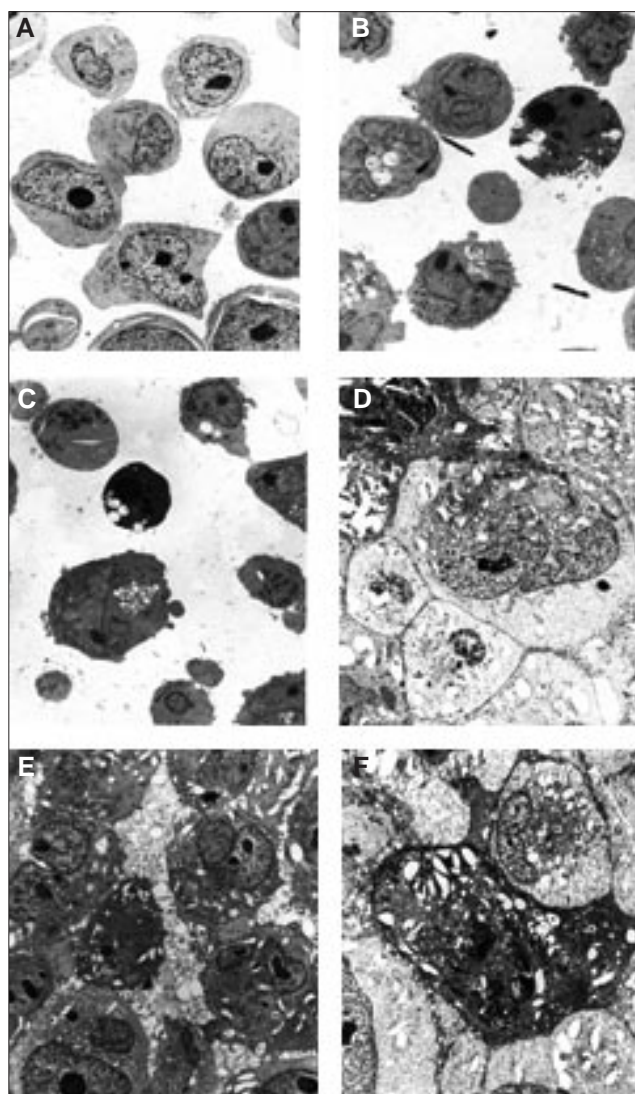


Figure 4 - TEM photographs (1600X) of apoptotic cellular morphology of undifferentiated FLG 29.1 cells (A, B, C) and of HOC cells (D, E, F). (A) and (D) defined controls, (B) and (E) are cells that have been treated for 2 days with 100 μM clodronate and (C) and (F) represent cells that have been treated for 2 days with 1 mM clodronate.

Table III - Effects of ATP on clodronate-induced apoptosis in undifferentiated FLG 29.1 cells and in HOC cells.

Treatment	Apoptotic cells (percent) ^a	
	Undifferentiated FLG 29.1	HOC
Control	3	5
Clodronate (100 μM)	10	11
Clodronate (1 mM)	13	15
ATP	3	6
ATP + Clodronate	5	4

^a The percentage of apoptotic cells was evaluated after 2 days of clodronate exposure by TEM counting random fields (100 cells per slide).

sis was reversed by ATP in undifferentiated FLG 29.1 cells and in HOC cells, the DNA ladder formation by agarose gel electrophoresis was examined. Cells treated with 100 μM and 1 mM clodronate showed a prominent DNA ladder characteristic of apoptosis which disappeared in the presence of 1 nM ATP for undifferentiated FLG 29.1 cells and of 50 μM ATP for HOC cells (Fig. 5).

Metabolism of clodronate to the ATP analogue AppCCI₂p in FLG 29.1 and J774 cells

The product ion spectra recorded from the standards of AppC-Cl₂p and ATP, together with their relevant MS/MS spectra are reported in Figure 6. The MS/MS spectrum of the metabolite

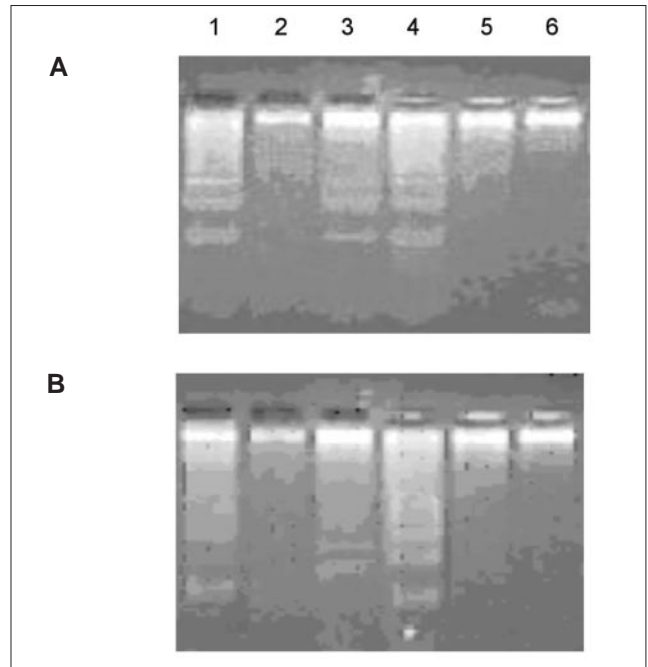


Figure 5 - Electrophoretic analysis of DNA extracted from undifferentiated FLG 29.1 cells (A) and HOC cells (B). Line 1 defined apoptosis positive controls, Lines 2 defined untreated cells, Lines 3 and 4 depict cells that have been treated for 2 days respectively with 100 μM and 1 mM clodronate. Lines 5 and 6 represent cells that have been treated for 2 days respectively with 100 μM clodronate plus 1 nM or 50 μM ATP and with 1 mM clodronate plus 1 nM or 50 μM ATP.

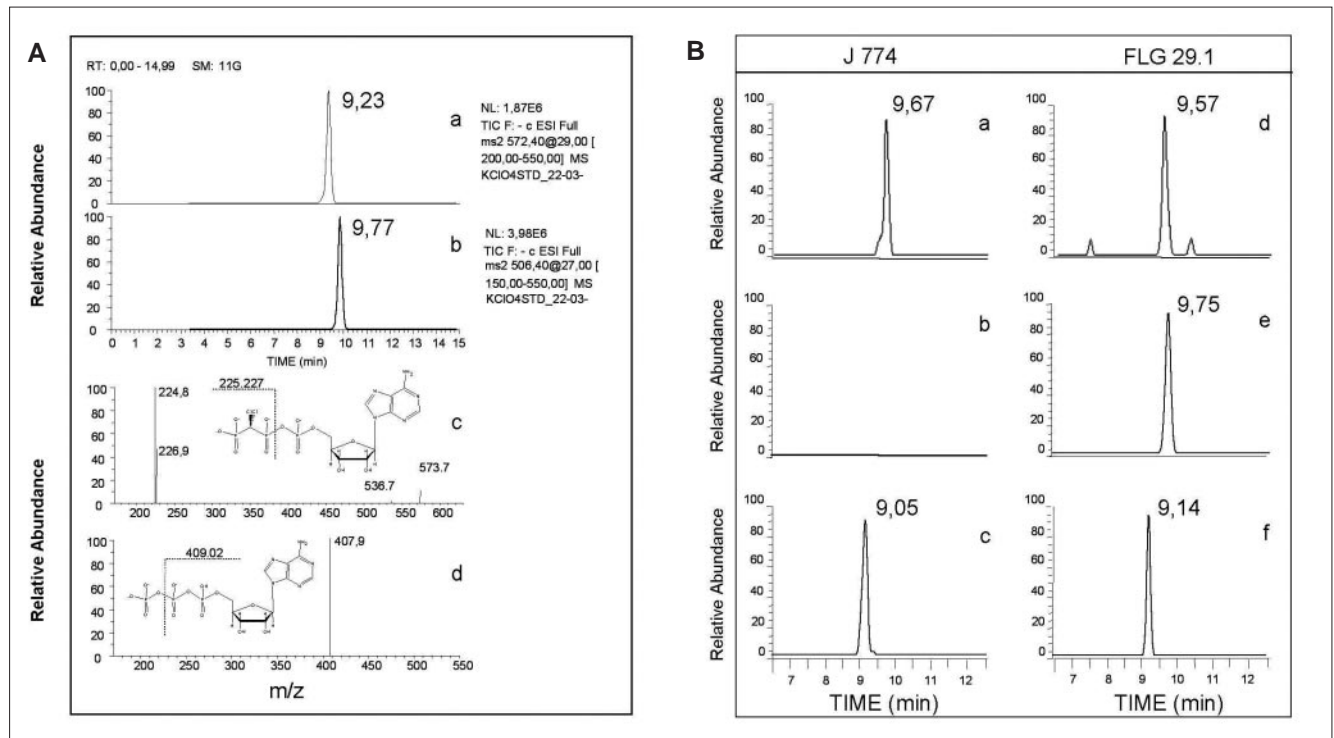


Figure 6 - A. Extracted ion chromatograms from product ion full scan negative ion HPLC-ESI-MS/MS of standard solutions of AppCCI₂p (a) and ATP (b). The ion range from m/z 224 to m/z 228 is shown in a (precursor ion 572,3) while the ion range from m/z 405 to m/z 410 is shown in b (precursor ion 506,4). Full scan negative ion MS/MS spectra of AppCCI₂p (c) and of ATP (d) showing the fragmentation patterns. B. Ion chromatograms from product ion full scan negative ion HPLC-ESI-MS/MS recorded from FLG 29.1 cell (figures on the left hand) and J774 cell (figures on the right hand) extracts. The ATP signal was present in both untreated cell (a and d traces) and in clodronate-treated FLG 29.1 cell (e trace), while was absent in J774 cell (b trace). The AppCCI₂p signal was present in both the treated cell lines (c and f traces). The ion range from m/z 224 to m/z 228 is shown for AppCCI₂p (precursor ion 572,3) while the ion range from m/z 405 to m/z 410 is shown for ATP (precursor ion 506,4).

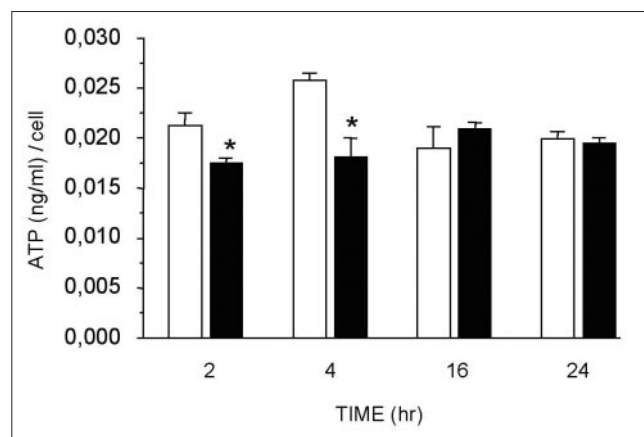


Figure 7 - Intracellular ATP levels in undifferentiated FLG 29.1 cells was measured in the absence (□) and in the presence (■) of 1 mM clodronate and monitored over 24 hr by a luciferase luminometric assay. Values are the mean \pm SD of three independent experiments carried out in triplicate. * $p < 0.05$.

was characterized by two principal diagnostic ions at m/z 225 and 227, due to the cleavage of the bisphosphonate moiety (Fig. 6). The presence of these two compounds in the analyzed samples was tested by comparing the spectral analysis of the treated and untreated cells with the standards profiles, in the expected retention time window (Fig. 6).

The signal due to the metabolite was absent in both the untreated cell lines, while it was present in the treated FLG 29.1 cell line; in this cell line, the ATP signal was also still detectable (Fig. 6). We have compared the obtained results with those for the J774 cells (Fig. 6), a cell line for which in the presence of clodronate AppCCl₂p metabolite was detected also by Auriola et al. (30). In the same analytical conditions, for the two cell lines investigated it was possible to make a raw quantitative evaluation of the yield of metabolite. J774 cell line incubated with 250 μ M clodronate for 2 days in medium containing 10% FCS, showed a strong signal of the metabolite, while ATP was not detectable under the same conditions (Fig. 6). The ATP signal in the FLG 29.1 cells incubated with 1 mM clodronate for 2 days in medium with 1% FCS was greatly emerging (Fig. 6) and it is possible to affirm that, in this case, the metabolite is present in lower amount respect to the remaining ATP, if we compare this situation with the behaviour of the J774 cell line. For these later cells we have chosen the protocol of clodronate incubation time and concentration indicated by Frith et al. (21), because in the conditions we adopted for FLG 29.1 (1 mM clodronate in 1% FCS medium for 2 days of culture), J774 cells did not survive, with 0% viability and characteristic sign of necrosis (data not shown).

Clodronate modulates intracellular ATP levels

In order to evaluate clodronate effect on intracellular ATP levels, FLG 29.1 cells were monitored over 24 hours with a luciferin-luciferase detection system. The results clearly indicated that 1 mM clodronate significantly down-regulated intracellular ATP in the early exposure time (2-4 hours) with a full recovery within 16 hours (Fig. 7).

Discussion

Clodronate (dichloromethylenebisphosphonate) is a synthetic analog of pyrophosphate (31) widely used in the treatment of

metabolic bone diseases that involve excessive bone resorption (32-34). The use of clodronate in this wide spectrum of disease states depends critically upon its ability to inhibit osteoclast-mediated bone resorption as supported by "in vitro" and "in vivo" studies (19, 20, 31-35). Differently than nitrogen-bisphosphonates, known to prevent protein prenylation, clodronate, like other non-nitrogen bisphosphonates, acts through the formation of an intracellular non-hydrolyzable ATP analog, AppCCl₂p, previously defined as a "cytotoxic" metabolite (7, 21). Despite the accumulated experimental data, the way in which clodronate exerts its putative "cytotoxic" function has not been fully elucidated.

Original studies describing the effect of clodronate on "in vitro" cellular models showed that clodronate was able to inhibit the growth and the glycolysis of fibroblasts and osteoclasts in a reversible manner (36). In 1982, Reitsma et al. used the term "cytotoxic" for the "in vitro" clodronate action on rat macrophages (37). However, in long-term cultures of bone marrow cells clodronate was unable to inhibit preosteoclastic cell proliferation, whereas it inhibited the differentiation of these cells into mature osteoclasts (38). Moreover, in various studies clodronate was not affecting the viability of cells of the osteoclastic lineage (38-40), while being able to induce apoptosis (40). Interestingly, the recent demonstration that combined treatment with clodronate could enhance or antagonize some of the molecular effects of nitrogen bisphosphonates suggested a competitive action not simply based on "cytotoxicity" (20).

The demonstration of the metabolism of clodronate to a methylene-analogue which could not be hydrolyzed, led to the suggestion that in clodronate-treated cells there is an accumulation of a non-physiological and non-hydrolyzable nucleotide metabolite (17, 18), reinforcing the concept of its "cytotoxic" action. However, before talking of "cytotoxicity" it would be necessary to demonstrate lack of bioreversibility of such effects either by measuring the clearance of the accumulated metabolite or by the supplementation of exogenous substrata capable to counteract the accumulation of the non-hydrolyzable metabolite.

In order to evaluate the bioreversibility of clodronate action on the bone resorption cycle we examined the ability of clodronate to modulate functional and proliferative properties of FLG 29.1 cells, a human preosteoclastic cell line (24-26) and of human osteoclastoma (HOC) cells in primary culture (27).

Previous studies with clodronate in non human osteoclastic cell lineages suggested that nitrogen bisphosphonates can affect "in vitro" both mature osteoclasts and precursors, whereas clodronate actions seemed limited to mature osteoclasts (41-43). In this report clodronate exhibits an inhibitory effect both on proliferative properties and differentiative features of human preosteoclasts and mature osteoclastic cells, without acutely influencing their viability, but inducing cell apoptosis, as shown in previous studies (19, 35, 40).

Interestingly, this study demonstrated for the first time that high concentrations of clodronate regulate the expression of MMP-9 and TIMP1 genes in both human preosteoclastic cells and human osteoclastoma cells. These observations reinforce the hypothesis of a role of clodronate on osteoclast recruitment, being the complex of metalloproteinase/metalloproteinase tissue inhibitor pivotal for cell migration and trafficking of bone cells, including osteoclasts (44-46).

The actions of clodronate on the human models explored in this report made possible to evaluate the bioreversibility of its effects. This is not a novel concept for bisphosphonates, as previous studies demonstrated that alendronate inhibited normal human epidermal keratinocytes growth in a reversible manner (47). The present data clearly indicate that the clodronate actions on human osteoclastic cells are reversed by adding exogenous ATP, which is incorporated by these cells. Indeed, we

were able to demonstrate the formation of intracellular AppC-Cl₂p in human preosteoclastic FLG 29.1 cells exposed to clodronate, with consequent inhibition of mitochondrial oxygen consumption by a mechanism that involves competitive inhibition of the ADP/ATP translocase, in agreement with previous findings in human osteoclasts and rat liver cells (19, 48). Interestingly, even though clodronate appears to acutely inhibit the intracellular levels of ATP in FLG 29.1 cells, the ATP synthesis was still present at intracellular concentrations exceeding those of the AppCCl₂p metabolite. Conversely, in the murine macrophage-like cell line J774, originally used to characterize the metabolism of clodronate to AppCCl₂p (21), ATP levels were very low when compared to the clodronate-derived metabolite, supporting the possibility of a "cytotoxic" effect in the latter cells (21). These findings altogether open the possibility of a cell-specific action of clodronate, depending on the metabolic pathways of the ATP/AppCCl₂p complex in a given cell type. In this report we demonstrated that clodronate affects the resorption cycle, by acting both on preosteoclastic and on mature osteoclastic cells. The lack of acute toxicity of clodronate on cells of the osteoclastic lineage together with its bioreversibility by ATP make unlikely the recognition of clodronate as a compound "cytotoxic" for human osteoclastic cells. Therefore, it is reasonable to assume that clodronate can be categorized as a drug capable to modulate in a bioreversible fashion the differentiative and functional processes of cells of the osteoclastic lineage.

Acknowledgements

This work was supported through the European Research Program, Fifth Framework Program "Quality of Life and Management of Living Resources Research and Technological Development Program" on "Genetic Markers for Osteoporosis", by the Cofin M.I.U.R., PNR 2001- 2003 (FIRB), and by the Fondazione Ente Cassa di Risparmio di Firenze [to M.L.B.]. R.R. was recipient of a post-doctoral fellowship of DeGene Spin-off of the University of Florence.

Conflict of interests

All authors have no conflict of interests.

Manufacturer names

Gibco (Grand Island, NY); Biowhittaker (Verviers, Belgium); SERVA Electrophoresis GmbH (Heidelberg, Germany); Eurobio (Paris, France); Società Prodotti Antibiotici Spa (Milan, Italy); Millipore (Molsheim, France); Takara Bio Inc (Otsu, Shiga, Japan); Carlo Erba (Milan, Italy); Across (Milwaukee, WI, USA); Sigma; Bio-Rad (Milan, Italy); DCS Innovative Diagnostik-Systeme (Hamburg, Germany).

References

1. Compston JE. The therapeutic use of bisphosphonates. *Br Med J*. 1994;309:711-715.
2. Russell RG, Rogers MJ. Bisphosphonates: from the laboratory to the clinic and back again. *Bone*. 1999;25:97-106.
3. Rodan GA, Martin TJ. Therapeutic approaches to bone diseases. *Science*. 2000;289:1508-1514.
4. Rogers MJ, Xiong X, Brown RJ, Watts DJ, Russell RG, Bayless AV, Ebetino FH. Structure-activity relationships of new heterocycle-containing bisphosphonates as inhibitors of bone resorption and as inhibitors of growth of *Dictyostelium discoideum* amoebae. *Mol Pharmacol*. 1995;47:398-402.
5. Van Beek ER, Lowik CW, Papapoulos SE. Bisphosphonates suppress bone resorption by a direct effect on early osteoclast precursors without affecting the osteoclastogenic capacity of osteogenic cells: the role of protein geranylgeranylation in the action of nitrogen-containing bisphosphonates on osteoclast precursors. *Bone*. 2002;30:64-70.
6. Geddes AD, D'Souza SM, Ebetino FH, Ibbotson KJ. Bisphosphonates: Structure-activity relationships and therapeutic implications. In: Heersche JNM and Kanis JA (ed), *Bone and Mineral Research*, Volume 8, Elsevier Science BV, Amsterdam. 1994:265-306.
7. Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Monkkonen J, Frith JC. Cellular and molecular mechanisms of action of bisphosphonates. *Cancer*. 2000;15(Suppl 12):2961-2978.
8. Sietsema WK, Ebetino FH, Salvagno AM, Bevan JA. Antiresorptive dose-response relationships across three generations of bisphosphonates. *Drugs Exp Clin Res*. 1989;15:389-396.
9. Ebetino FH, Bayless AV, Amburgery J, Ibbotson KJ, Dansereau S, Ebrahimpour A. Elucidation of a pharmacophore for the bisphosphonate mechanism of bone resorptive activity. *Phosphorus, Sulfur and Silicon*. 1996;109-110:217-220.
10. Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, Ebetino FH, Rogers MJ. Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates. *J Pharmacol Exp Ther*. 2001;296:235-242.
11. Bergstrom JD, Bostedor RG, Masarachia PJ, Reszka AA, Rodan G. Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. *Arch Biochem Biophys*. 2000;373:231-241.
12. Luckman SP, Hughes DE, Coxon FP, Graham R, Russell G, Rogers MJ. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. *J Bone Miner Res*. 1998;13:581-589.
13. Van Beek E, Pieterman E, Cohen L, Lowik C, Papapoulos S. Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. *Biochem Biophys Res Commun*. 1999;64:108-111.
14. Fisher JE, Rogers MJ, Halasy JM, Luckman SP, Hughes DE, Masarachia PJ, Wesolowski G, Russell RG, Rodan GA, Reszka AA. 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 U S A*. 1999;96:133-138.
15. Van Beek E, Lowik C, van der Pluijm G, Papapoulos S. The role of geranylgeranylation in bone resorption and its suppression by bisphosphonates in fetal bone explants in vitro: A clue to the mechanism of action of nitrogen-containing bisphosphonates. *J Bone Miner Res*. 1999;14:722-729.
16. Coxon FP, Helfrich MH, Van't Hof R, Sebti S, Ralston SH, Hamilton A, Rogers MJ. Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. *J Bone Miner Res*. 2000;15:1467-1476.
17. 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;224:863-869.
18. Rogers MJ, Ji X, Russell RG, Blackburn GM, Williamson MP, Bayless AV, Ebetino FH, Watts DJ. Incorporation of bisphosphonates into adenine nucleotides by amoebae of the cellular slime mould *Dictyostelium discoideum*. *Biochem J*. 1994;303 (Pt 1):303-311.
19. Lehenkari PP, Kellinsalmi M, Napankangas JP, Ylitalo KV, Monkkonen J, Rogers MJ, Azhaye A, Vaananen HK, Hassinen IE. Further insight into mechanism of action of clodronate: inhibition of mitochondrial ADP/ATP translocase by a nonhydrolyzable, adenine-containing metabolite. *Mol Pharmacol*. 2002;61:1255-1262.
20. Frith JC, Rogers MJ. Antagonistic effects of different classes of bisphosphonates in osteoclasts and macrophages in vitro. *J Bone Miner Res* 2003;18:204-212.
21. Frith JC, Monkkonen J, Russell RGG, Blackburn GM, Watts DJ, Rogers MJ. Clodronate and liposome-encapsulated clodronate are

- metabolized to a toxic ATP analogue, adenosine 5'- (β,γ -dichloromethylene triphosphate), by mammalian cells in vitro. *J Bone Miner Res.* 1997;12:1358-1367.
22. Frith JC, Monkkonen J, Auriola S, Monkkonen H, Rogers MJ. The molecular mechanism of action of the antiresorptive and anti-inflammatory drug clodronate: evidence for the formation in vivo of a metabolite that inhibits bone resorption and causes osteoclast and macrophage apoptosis. *Arthritis Rheum.* 2001;44:2201-2210.
 23. Makkonen N, Salminen A, Rogers MJ, Frith JC, Urtti A, Azhayaeva E, Monkkonen J. Contrasting effects of alendronate and clodronate on RAW 264 macrophages: the role of a bisphosphonate metabolite. *Eur J Pharm Sci.* 1999;8:109-118.
 24. Gattei V, Bernabei PA, Pinto A, Bezzini R, Ringressi A, Formigli L, Tanini A, Attadia V, Brandi ML. Phorbol ester induced osteoclast-like differentiation of a novel human leukemic cell line (FLG 29.1). *J Cell Biol.* 1992;116:437-447.
 25. Fiorelli G, Ballock RT, Wakefield LM, Sporn MB, Gori F, Masi L, Frediani U, Tanini A, Bernabei PA, Brandi ML. Role for autocrine TGF-beta 1 in regulating differentiation of a human leukemic cell line toward osteoclast-like cells. *J Cell Physiol.* 1994;160:482-490.
 26. Pegoraro L, Abrahm J, Cooper RA, Levis A, Lange B, Meo P, Rovera G. Differentiation of human leukemias in response to 12-O-tetradecanoylphorbol-13-acetate in vitro. *Blood.* 1980;55:859-862.
 27. Grano M, Colucci S, De Bellis M, Zigrino P, Argentino L, Zambonin G, Serra M, Scotlandi K, Teti A, Zambonin Zallone. A new model for bone resorption study in vitro: human osteoclast-like cells from giant cell tumors of bone. *J Bone Miner Res.* 1994;9:1013-1020.
 28. Simi L, Andreani M, Davini F, Janni A, Pazzagli M, Serio M, Orlando C. Simultaneous measurement of MMP9 and TIMP1 mRNA in human non small cell lung cancers by multiplex real time RT-PCR. *Lung Cancer.* 2004;45:171-179.
 29. Blackburn GM, Kent DE, Kolkman F. The Synthesis and Metal Binding Characteristics of Novel, Isopolar Phosphonate Analogues of Nucleotides. *J Chem Soc Perkin Trans.* 1984;1:1119-1125.
 30. Auriola S, Frith J, Rogers MJ, Koivuniemi A, Monkkonen J. Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography-electrospray mass spectrometry. *J Chromatogr B.* 1997;704:187-195.
 31. Fleisch H. Bisphosphonates. Pharmacology and use in the treatment of tumour-induced hypercalcaemic and metastatic bone disease. *Drugs* 1991;42:919-944.
 32. Plosker GL, Goa KL. Clodronate. A review of its pharmacological properties and therapeutic efficacy in resorptive bone disease. *Drugs.* 1994;47:945-982.
 33. Kanis JA, McCloskey EV, Sirtori P, Khan S, Fern D, Eyres K, Aaron J, Beneton MN. Rationale for the use of clodronate in osteoporosis. *Osteoporos Int.* 1993;3 (Suppl 2):23-28.
 34. Ghinai V, Brandi ML. Clodronate: mechanisms of action on bone remodelling and clinical use in osteometabolic disorders. *Expert Opin Pharmacother.* 2002;3:1643-1656.
 35. Selander KS, Monkkonen J, Karhukorpi EK, Harkonen P, Hannuniemi R, Vaananen HK. Characteristics of clodronate-induced apoptosis in osteoclasts and macrophages. *Mol Pharmacol.* 1996; 50:1127-1138.
 36. Fast DK, Felix R, Dowse C, Neuman WF, Fleisch H. The effects of diphosphonates on the growth and glycolysis of connective-tissue cells in culture. *Biochem J.* 1978;172:97-107.
 37. Reitsma PH, Teitelbaum SL, Bijvoet OL, Kahn AJ. Differential action of the bisphosphonates (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate (APD) and disodium dichloromethylidene bisphosphonate (Cl2MDP) on rat macrophage-mediated bone resorption in vitro. *J Clin Invest.* 1982;70:927-933.
 38. Hughes DE, MacDonald BR, Russell RG, Gowen M. Inhibition of osteoclast-like cell formation by bisphosphonates in long-term cultures of human bone marrow. *J Clin Invest.* 1989;83:1930-1935.
 39. Carano A, Teitelbaum SL, Konsek JD, Schlesinger PH, Blair HC. Bisphosphonates directly inhibit the bone resorption activity of isolated avian osteoclasts in vitro. *J Clin Invest.* 1990;85:456-461.
 40. Hughes DE, Wright KR, Uy HL, Sasaki A, Yoneda T, Roodman GD, Mundy GR, Boyce BF. Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo. *J Bone Miner Res.* 1995; 10:1478-1487.
 41. Boonekamp PM, van der Wee-Pals LJ, van Wijk-van Lennep MM, Thesing CW, Bijvoet OL. Two modes of action of bisphosphonates on osteoclastic resorption of mineralized matrix. *Bone Miner.* 1986;1:27-39.
 42. Boonekamp PM, Lowik CW, van der Wee-Pals LJ, van Wijk-van Lennep ML, Bijvoet OL. Enhancement of the inhibitory action of APD on the transformation of osteoclast precursors into resorbing cells after dimethylation of the amino group. *Bone Miner.* 1987; 2:29-42.
 43. Lowik CW, van der Pluijm G, van der Wee-Pals LJ, van Treslong-De Groot HB, Bijvoet OL. Migration and phenotypic transformation of osteoclast precursors into mature osteoclasts: the effect of a bisphosphonate. *J Bone Miner Res.* 1988;3:185-192.
 44. Peled A, Kollet O, Ponomaryov T, Petit I, Franitza S, Grabovsky V, Slav MM, Nagler A, Lider O, Alon R, Zipori D, Lapidot T. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood.* 2000; 1:3289-3296.
 45. Lane WJ, Dias S, Hattori K, Heissig B, Choy M, Rabbany SY, Wood J, Moore MA, Rafii S. Stromal-derived factor 1-induced megakaryocyte migration and platelet production is dependent on matrix metalloproteinases. *Blood.* 2000;15:4152-4159.
 46. Yu X, Huang Y, Collin-Osdoby P, Osdoby P. Stromal cell-derived factor-1 (SDF-1) recruits osteoclast precursors by inducing chemotaxis, matrix metalloproteinase-9 (MMP-9) activity, and collagen transmigration. *J Bone Miner Res.* 2003;18:1404-1418.
 47. Reszka AA, Halasy-Nagy J, Rodan GA. Nitrogen-bisphosphonates block retinoblastoma phosphorylation and cell growth by inhibiting the cholesterol biosynthetic pathway in a keratinocyte model for esophageal irritation. *Mol Pharmacol.* 2001;59:193-202.
 48. Halestrap AP, Woodfield KY, Connern CP. Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. *J Biol Chem.* 1997;7:3346-3354.