# Further Insight into Mechanism of Action of Clodronate: Inhibition of Mitochondrial ADP/ATP Translocase by a Nonhydrolyzable, Adenine-Containing Metabolite

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This article is available online at http://molpharm.aspetjournals.org

## ABSTRACT

Bisphosphonates are currently the most important class of antiresorptive drugs used for the treatment of diseases with excess bone resorption. Recent studies have shown that bisphosphonates can be divided into two groups with distinct molecular mechanisms of action depending on the nature of the $R^2$ side chain. Alendronate, like other nitrogen-containing bisphosphonates, inhibits bone resorption and causes apoptosis of osteoclasts and other cells in vitro by preventing post-translational modification of GTP-binding proteins with isoprenoid lipids. Clodronate, a bisphosphonate that lacks a nitrogen, does not inhibit protein isoprenylation but can be metabolized intracellularly to a $\beta$-$\gamma$-methylene (AppCp-type) analog of ATP, which is cytotoxic to macrophages in vitro. The detailed molecular basis for the cytotoxic effects of adenosine-5'-[$\beta,\gamma$-dichloromethylene]triphosphate ($AppCCl_2p$) has not been determined yet. We addressed this question by studying the effects of alendronate, clodronate, and the clodronate metabolite $AppCCl_2p$ on isolated mitochondria, mitochondrial fractions, and mitochondrial membrane potential in isolated human osteoclasts. We found that $AppCCl_2p$ inhibits mitochondrial oxygen consumption by a mechanism that involves competitive inhibition of the ADP/ATP translocase. Alendronate or the native form of clodronate did not have any immediate effect on mitochondria. However, longer treatment with liposome-encapsulated clodronate caused collapse of the mitochondrial membrane potential, although prominent apoptosis was a late event. Hence, inhibition of the ADP/ATP translocase by the metabolite $AppCCl_2p$ is a likely route by which clodronate causes osteoclast apoptosis and inhibits bone resorption.

Bone resorption is an essential process of bone modeling and remodeling in physiological and pathological conditions and is accomplished by osteoclasts that dissolve the mineral matrix through proton secretion and the organic matrix through proteolysis followed by endocytosis and transcytosis (Nesbitt and Horton, 1997; Salo et al., 1997). Ever since the discovery that bisphosphonates could inhibit bone resorption (Fleisch et al., 1969; Smith et al., 1971) more than 30 years ago, considerable efforts have been made to understand the molecular mechanisms involved in the mechanisms of action of these compounds. Bisphosphonates are used widely for the treatment of osteoporosis, Paget's disease, and osteolysis associated with metastatic bone disease. Bisphosphonates are synthetic analogs of pyrophosphate that contain a phosphate-carbon-phosphate backbone. The two side chains attached to the central carbon, $R^1$ and $R^2$, determine the anti-

resorptive potency of bisphosphonates. The $R^1$ chain is usually a hydroxyl group, which increases the affinity of the compounds for bone mineral. The $R^2$ chain in the first-generation bisphosphonate drugs is a simple halogen or alkyl group ($—CH_3$ in etidronate, $—Cl$ in clodronate). In the second-generation drugs, the $R^2$ side chain contains a primary amino group, which makes these compounds 10 to 1000 times more potent (Lin, 1996). The third generation of bisphosphonates contains a secondary, tertiary, or quaternary amino group, within an alkyl chain or a heterocyclic group (Rogers et al., 2000).

It is generally accepted that the most important route by which bisphosphonates inhibit bone resorption is by directly affecting the bone-resorbing osteoclasts (Rodan, 1998; Rogers et al., 2000). Bisphosphonates cause disruption of the ruffled border and actin cytoskeleton of osteoclasts (Sato et al., 1991; Rogers et al., 2000) and can cause osteoclast apoptosis (Hughes et al., 1995; Selander et al., 1996). The exact molec-

**ABBREVIATIONS:** $AppCCl_2p$, adenosine-5'-[$\beta,\gamma$-dichloromethylene]triphosphate; ANT, adenine nucleotide translocase (ADP/ATP translocase); AppCp, adenosine-[5'-$\beta,\gamma$-methylene]triphosphate; PT, (mitochondrial) permeability transition.

ular mechanisms by which bisphosphonates affect osteoclasts seem to be different for the first generation of bisphosphonates and the second and third generation of bisphosphonates that contain a nitrogen moiety. Alendronate and other nitrogen-containing bisphosphonates have recently been shown to induce apoptosis and inhibit bone resorption by osteoclasts by inhibiting farnesyl diphosphate synthase (van Beek et al., 1999b; Bergström et al., 2000; Dunford et al., 2001), an enzyme in the mevalonate pathway of cholesterol synthesis. Inhibition of this enzyme prevents the synthesis of isoprenoid lipids required for the prenylation of small GTP-binding proteins such as Rho and Rac, necessary for osteoclast function and survival (Luckman et al., 1998; Fisher et al., 1999; van Beek et al., 1999a; Reszka et al., 1999; Coxon et al., 2000). However, the mevalonate pathway does not seem to be affected by the bisphosphonates that lack a nitrogen, such as clodronate (Luckman et al., 1998; Benford et al., 1999; van Beek et al., 1999a; Coxon et al., 2000).). Clodronate can be metabolized by cells in vitro to a cytotoxic analog of ATP, adenosine-5′-[β,γ-dichloromethylene]triphosphate (AppCCl$_2$p) (Rogers et al., 1994; Auriola et al., 1997; Frith et al., 1997; Makkonen et al., 1999). The incorporation of clodronate into an analog of ATP seems to be catalyzed by a back reaction of class II aminoacyl-tRNA synthetases (Pelorgeas et al., 1992; Rogers et al., 1994, 1996).

The intracellular accumulation of AppCCl$_2$p may account for the pharmacological effects of clodronate on osteoclasts and macrophages, because AppCCl$_2$p is just as potent as clodronate itself at reducing the viability of macrophages and preventing cytokine release (Frith et al., 1997; Makkonen et al., 1999) in vitro. Furthermore, AppCCl$_2$p has recently been found to cause osteoclast apoptosis and inhibit bone resorption in vitro (Frith et al., 2001). However, the exact molecular mechanism by which AppCCl$_2$p affects osteoclast cell viability has not been clarified.

Osteoclasts contain large numbers of mitochondria, suggesting that these cells depend on high rates of ATP synthesis (Karhukorpi et al., 1992). Alterations in mitochondrial membrane permeability have recently been shown to be an early event in pathways leading to caspase activation and apoptosis (Crompton, 1999; Heiskanen et al., 1999; Kroemer, 1999).

In this study, we examined whether clodronate or its metabolite AppCCl$_2$p may affect osteoclast viability by inhibiting mitochondrial function. We therefore studied the effects of clodronate and AppCCl$_2$p on mitochondrial respiration, membrane potential, ATP synthase (F$_1$F$_o$-ATPase), and ADP/ATP translocase. AppCCl$_2$p is shown to inhibit the ADP/ATP translocase in the mitochondrial inner membrane, an effect that could lead to osteoclast apoptosis and hence inhibition of bone resorption.

We used liposomes for effective delivery of clodronate to osteoclasts. This has proved to be an effective means of conveying bisphosphonates to cells, which do not readily internalize these very hydrophilic and negatively charged compounds in their free form. When clodronate is used clinically to inhibit osteoclasts, liposomes are not required, because osteoclasts take up bisphosphonates attached to bone mineral during the resorption, but in vitro there is no bone mineral present, and therefore liposomes are needed for effective delivery (Mönkkönen et al., 1994).
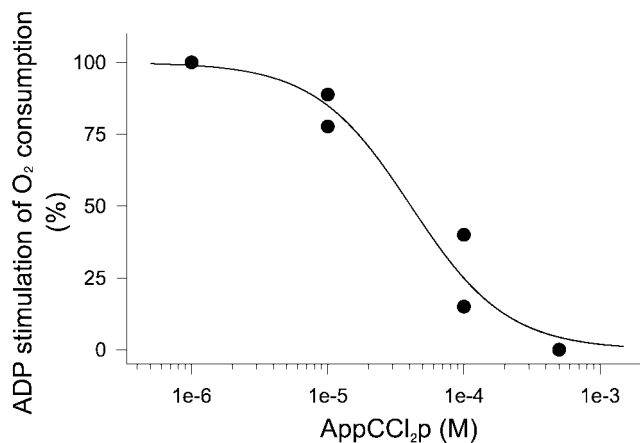
## Materials and Methods

**Chemicals.** Clodronate (dichloromethylene-1,1-bisphosphonate) and alendronate (4-amino-1-hydroxybutylidene-1,1-bisphosphonate) were kindly provided by Leiras Pharmaceutical (Turku, Finland) and Merck (Whitehouse Station, NJ), respectively. The synthesis of the clodronate metabolite AppCCl$_2$p was performed according to a slightly modified method (Blackburn et al., 1984; A. Azhayev and J. Vepsäläinen, unpublished data). Rhodamine 123 was obtained from Molecular Probes (Eugene, OR), bonkrekic acid (triammonium salt) from Calbiochem (La Jolla, CA), and [8-$^{14}$C]adenosine triphosphate (NH$_4$ salt) from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK).

**Preparation of Clodronate-Loaded Liposomes.** Clodronate-containing liposomes and nonloaded control liposomes were prepared by the reverse phase evaporation method as described previously (Mönkkönen et al., 1994; Makkonen et al., 1999).

**Animals.** The work was approved by the Laboratory Animals Committee of the University of Oulu (Oulu, Finland) and conducted according to the standards given in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85.23). Three-month-old male Sprague-Dawley rats from the Laboratory Animal Center of the University of Oulu were used for isolation of rat liver mitochondria.

**Mitochondrial Preparations.** After decapitation, rat liver was homogenized with a Potter-Elvehjem homogenizer in ice-cold isolation buffer containing 300 mM sucrose, 0.1 mM EGTA, and 10 mM HEPES, pH 7.4. The homogenate was centrifuged at 750$g$ for 3 min and the supernatant centrifuged at 7800$g$ for 10 min. The pellet was resuspended in the isolation buffer that was at the ratio of 1 ml/g of liver wet weight. Submitochondrial particles were prepared by sonication of liver mitochondria for a total of 25 s in five 5-s bursts separated by 7-s intervals in an ice bath.

**Measurement of Oxygen Consumption.** Oxygen consumption was measured in a thermostat-equipped (25°C), sealed chamber with a Clark-type oxygen electrode by adding mitochondria or submitochondrial particles into 0.5 ml of medium consisting of 140 mM KCl, 20 mM HEPES, 0.3 mM dithiothreitol, 10 mM EGTA, 3 mM CaCl$_2$ (free Ca$^{2+}$ concentration 0.1 μM), 3 mM magnesium acetate, 5 mM KH$_2$PO$_4$, 5 mM glutamate, and 5 mM malate. ADP was added to stimulate mitochondrial respiration (state 3), as depicted in Fig. 1A.

**Measurement of Adenine Nucleotide Translocation.** Rat liver mitochondria were prepared in 0.25 M sucrose, 2 mM HEPES, 0.5 mM EDTA, pH 7.4. The "forward exchange" catalyzed by the adenine nucleotide translocator (ANT) was measured (Paulson and Shug, 1984) in the presence of 50 or 150 μM ATP labeled with $^{14}$C in

**Fig. 1.** Effect of the clodronate metabolite AppCCl$_2$p on ADP stimulation of oxygen consumption of isolated mitochondria. ADP stimulation of oxygen consumption (state 3 minus state 2) before the addition of AppCCl$_2$p is taken as 100%. The curve is a logistic dose-response fit to the data.

a medium consisting of 48 mM sucrose, 80 mM KCl, 38 mM Tris, 0.32 mM HEPES, and 0.88 mM EDTA, pH 7.4. After 10 or 20 s the translocation was stopped by addition of 50 $\mu$M atractyloside. The mitochondria were sedimented by centrifugation at 8000$g$, suspended, and washed by centrifugation in the presence of 50 $\mu$M atractyloside. The mitochondria were solubilized in 2% SDS, and radioactivity was determined in a liquid scintillation counter.

**Measurement of $F_1F_o$-ATPase.** Oligomycin-sensitive ATPase was measured in submitochondrial particles spectrophotometrically in 33 mM Tris acetate, 83 mM sucrose 10 mM MgCl$_2$, 1 mM KCN, 1 mM EDTA, 2 mM ATP, 1.5 mM phosphoenolpyruvate, 0.17 mM NADH, 4 U/ml pyruvate kinase, and 20 U/ml lactate dehydrogenase, pH 7.4 (Rosing et al., 1975). NADH oxidation was monitored by changes in the relative absorbance at 340 and 385 nm by using a UV-3000 dual wavelength spectrophotometer (Shimadzu, Kyoto, Japan) at 22°C with an absorption coefficient of 5.33 mM$^{-1}$ cm$^{-1}$. The activity was determined in the absence and presence of oligomycin and the difference was attributed to $F_1F_o$-ATPase.

**Human Osteoclasts.** Human osteoclasts were prepared from osteoclastoma by using a method described by Nesbitt and Horton (1997). Frozen cells were kindly provided by Dr. Nesbitt. After thawing, the osteoclasts were allowed to attach to 30-mm-diameter glass coverslips for 1 h in in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), pH 7.0, buffered with 20 mM HEPES and containing 0.84 g/l sodium bicarbonate, 2 mM glutamine, 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin, and 10% heat-inactivated fetal calf serum. Unattached cells were rinsed away, and the osteoclasts were cultured in the above-described medium in 5% CO$_2$, 95% air at 37°C.

**Osteoclast Culture for Resorption Activity and Apoptosis Measurements.** A mixed rat bone cell population was cultured on clodronate-coated or control bovine bone slices as described previously (Boyde et al., 1984; Lakkakorpi et al., 1989). For detection of apoptosis, the cells were stained for tartrate-resistant acid phosphatase (kit 386A; Sigma-Aldrich, St. Louis, MO), a marker for osteoclast phenotype; the nuclei were stained with the DNA-binding fluorochrome Hoechst 33258 (Sigma-Aldrich); and osteoclasts with fragmented nuclei and shrunken cytoplasm were counted as apoptotic (Selander at al., 1994). The percentage of apoptotic cells of total osteoclasts identified was defined as apoptosis index.

**Mitochondrial Membrane Potential in Osteoclasts.** Osteoclasts were loaded with 10 $\mu$M rhodamine 123 for 15 min in incubation buffer containing 127 mM NaCl, 5 mM KCl, 2 mM MgCl$_2$, 0.5 mM NaH$_2$PO$_4$, 2 mM CaCl$_2$, 5 mM NaHCO$_3$, 10 mM glucose, 10 mM HEPES, and 0.1% bovine serum albumin (pH 7.0 in equilibrium with 5% CO$_2$).

Rhodamine 123 fluorescence was measured with a digital image analyzer (MCID/M2; Imaging Research, Brock University, St. Catharines, ON, Canada) consisting of an Intel 403 E microcomputer linked to an Image 1280 image processor (Matrox Dorral, QB, Canada). The cells were kept at 37°C under a thermostat-equipped hood (Nikon, Tokyo, Japan). A Sony charge-coupled device 72E camera (Dage-MTI, Michigan City, IN) and a KS-1381 signal amplifier (VideoScope, Washington, DC) were used to collect the data. Fluorescence was excited at 495 nm for 500-ms (15 frames for averaging per excitation) periods at 5-s intervals by using a computer-driven filter wheel (MAC2000; Ludl Electronic Products, Hawthorn, NY). Emitted light was collected through a dichroic mirror (450–490 nm reflecting) and a 590-nm interference filter (Nikon B2A).

Under the conditions used the mitochondrial rhodamine 123 concentration rises above the aggregation threshold, leading to fluorescence quenching so that fluorescence increases upon a decrease in mitochondrial membrane potential (Emaus et al., 1986). The zero potential reference level was achieved by treating the loaded cells with the uncoupler carbonylcyanide $m$-chlorophenylhydrazine.

**Statistical Analysis.** The apoptosis and rhodamine 123 fluorescence data were tested by analysis of variance followed by the Bonferroni post hoc test.

## Results

### Effects of Clodronate and AppCCl$_2$p on Rat Liver Mitochondria

**Oxygen Consumption.** Neither clodronate nor alendronate had any effect on mitochondrial oxygen consumption at concentrations up to 10$^{-4}$ M (data not shown). However, the clodronate metabolite AppCCl$_2$p caused a complete inhibition of the ADP stimulation (state 2 to state 3 transition) of oxygen consumption at a concentration of 5 × 10$^{-4}$ M, with half-maximal inhibition occurring at 50 $\mu$M (Fig. 1). The inhibition appeared after a delay of 30 s. Concentrations of 10$^{-6}$ M or less had no effect.

In state 3, mitochondrial respiration is mainly limited by components of the respiratory chain itself, the ADP/ATP translocase, and the P$_i$ translocase in the mitochondrial inner membrane. Therefore, the potential inhibitory effects of bisphosphonates or AppCCl$_2$p were tested in submitochondrial particles, which represent a vesicular, inside-out preparation of the mitochondrial inner membrane in which the ATP synthase ($F_1F_o$-ATPase) faces outward, and has substrate access without the requirement for translocases in the mitochondrial membrane. Under these conditions, respiration (oxygen consumption) is limited mainly by the respiratory chain. In submitochondrial particles AppCCl$_2$p at concentrations up to 10$^{-4}$ M did not alter oxygen consumption (data not shown).

**Effect of AppCCl$_2$p on Adenine Nucleotide Translocation.** The dose-response curve of the AppCCl$_2$p inhibition of ANT is depicted in Fig. 2A. In the presence of 50 $\mu$M ATP, half-maximal inhibition was obtained at 52 $\mu$M AppCCl$_2$p. Dixon plots (Fig. 2B) indicate that the inhibition is competitive and fitting the data of Fig. 4A into the kinetic equation of competitive inhibition gives a $K_i$ value of 72 $\mu$M, when the $K_m$ value for ATP influx is 50 $\mu$M (Chan and Barbour, 1983).

**$F_1F_o$-ATPase.** In leaky submitochondrial particles, an ATP-synthase (complex V)-related effect of AppCCl$_2$p on oxygen consumption could remain undetected. We therefore examined the effect of AppCCl$_2$p on the last component of the respiratory chain, complex V ($F_1F_o$-ATPase) and found no effect (data not shown).

### Effect of AppCCl$_2$p on Mitochondrial Membrane Potential in Human Osteoclasts

The basal fluorescence intensity of rhodamine 123, an indicator of the mitochondrial membrane potential, varied by 20% within the same culture of osteoclasts, possibly due to differences in mitochondrial or cellular uptake.

When the osteoclasts were exposed to liposome-encapsulated clodronate at concentrations of 10$^{-6}$ to 10$^{-4}$ M the mitochondrial membrane potential was not affected, even after 2 h. Liposome-encapsulated AppCCl$_2$p, however, had a biphasic effect on the mitochondrial membrane potential of osteoclasts. Initially, the membrane potential increased, as observed by a decrease in rhodamine 123 fluorescence (Fig. 4). Then a return to the basal value occurred.

The functionality of the optical readout of membrane potential was tested by the addition of an uncoupler (carbonylcyanide $m$-chlorophenylhydrazine), which depolarizes the mitochondria. An immediate increase in rhodamine 123 fluorescence occurred, confirming the operation of the mitochondrial rhodamine 123 uptake within the range of intra-

mitochondrial aggregation and fluorescence quenching under these experimental conditions (Emaus et al., 1986). Of the multinucleated osteoclasts, 60% responded this way, displaying an initial 10% decrease in rhodamine 123 fluorescence. An AppCCl$_2$p concentration of $10^{-4}$ M or higher was needed to observe this effect. At lower concentrations, rhodamine 123 fluorescence remained unaffected even after relatively long exposures (30 min) (Fig. 3). The effect of AppCCl$_2$p on ANT and mitochondrial membrane potential is an immediate increase due to slowing of potential discharge by the electrogenic ANT. It was, however, of interest to test the effects of AppCCl$_2$p on a longer time span, because it is principally harmful for the mitochondria and cells. To ensure cell targeting of the polar AppCCl$_2$p molecule it was administered in liposomes. Figure 4 shows that there is a time-dependent increase in the cellular rhodamine 123 fluorescence, indicating mitochondrial depolarization.

### Effect of Clodronate on Osteoclast Viability, Morphology, and Apoptosis

Because liposome-encapsulated clodronate failed to have any effect on the mitochondrial membrane potential of hu-

man osteoclasts even after 2 h, cultures were treated with $10^{-6}$ M liposome-encapsulated clodronate for 16 h to increase the cellular uptake of clodronate and hence allow sufficient time for the metabolism of clodronate to occur, leading to accumulation of the AppCCl$_2$p metabolite in the cells, and then loaded with rhodamine 123. This treatment caused an 80% decrease in the number of osteoclasts in culture. The remaining osteoclasts had condensed cytoplasm and increased rhodamine 123 fluorescence levels, indicative of the onset of apoptosis and loss of mitochondrial membrane potential (Fig. 4). To evaluate the magnitude of the effect we determined the average fluorescence intensities of 30 randomly selected osteoclasts from both control and clodronate-treated groups. The selected cells had normal nuclei and did not demonstrate any other hallmarks of apoptosis. We ob-
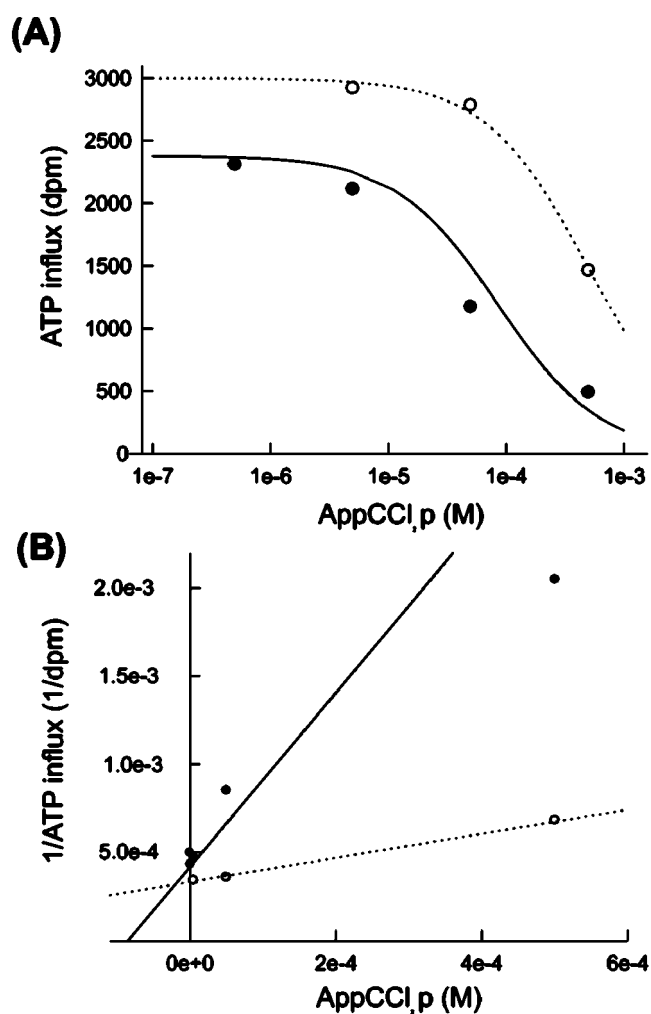


**Fig. 2.** Effect of AppCCl$_2$p on ADP/ATP translocation in rat liver mitochondria. A, fit of the equation $v = V_{max} / \{(1 + [K_m / [s]) \times [1 + ([i] / K_i)]\}$ to the data. Filled symbols and solid curve, 50 $\mu$M ATP; open symbols and dotted curve, 150 $\mu$M ATP. B, Dixon plots. The nonlinear regression curves become linearized, and the intercept of the lines is compatible with competitive inhibition.
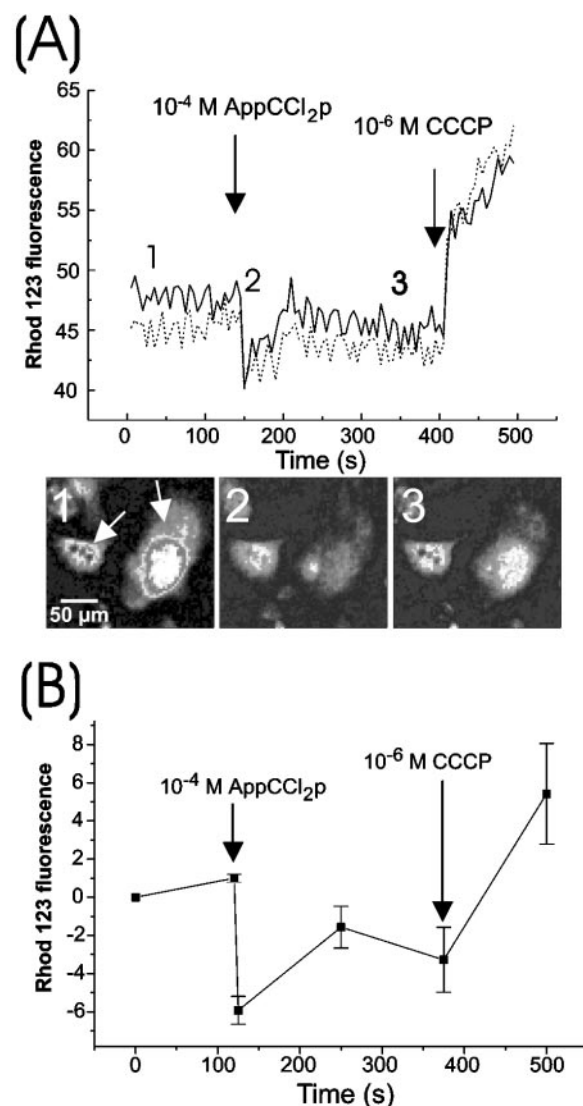


**Fig. 3.** Effects of AppCCl$_2$p on mitochondrial membrane potential in live osteoclastoma-derived osteoclasts. A, typical time course of mitochondrial membrane potential changes in two individual osteoclasts. The photomicrographs (1–3) represent a time series taken at time points shown by the corresponding number above the time course curves from the two cells indicated by arrows. The decrease in rhodamine 123 fluorescence indicates increased membrane potential. B, summary of nine similar experiments. Due to the variation in resting membrane potential in different osteoclasts, the values in B were normalized and the absolute changes from baseline shown (mean ± S.D.).

served a 40% increase in the average rhodamine fluorescence, indicative of a decrease in mitochondrial membrane potential. Although the variation was large, the difference in the fluorescence of the mitochondrial membrane potential probe between untreated cells and cells treated with liposome-encapsulated clodronate (Fig. 4) was statistically significant ($p < 0.0001$).

AppCCl$_2$p has immediate effects on mitochondrial membrane potential (Fig. 3). It has been recently shown that clodronate is metabolized in osteoclasts, and intracellular AppCCl$_2$p has been positively identified (Frith et al., 2001). AppCCl$_2$p also causes apoptosis (Frith et al., 2001), and to obtain further evidence that the apoptotic cascade would probably be initiated by mitochondrial effects of AppCCl$_2$p, the time courses of the mitochondrial membrane potential changes were compared with apoptosis in cells growing on bone slices coated with clodronate, which is taken up by the cells during resorption. Figure 5 shows that clodronate-induced apoptosis is a late effect that is prominent after 24 h, although it is known that bone resorption is initiated in 4 to 12 h after seeding osteoclasts on clodronate-coated or control bone slices (Lakkakorpi et al., 1989; Selander et al., 1994).
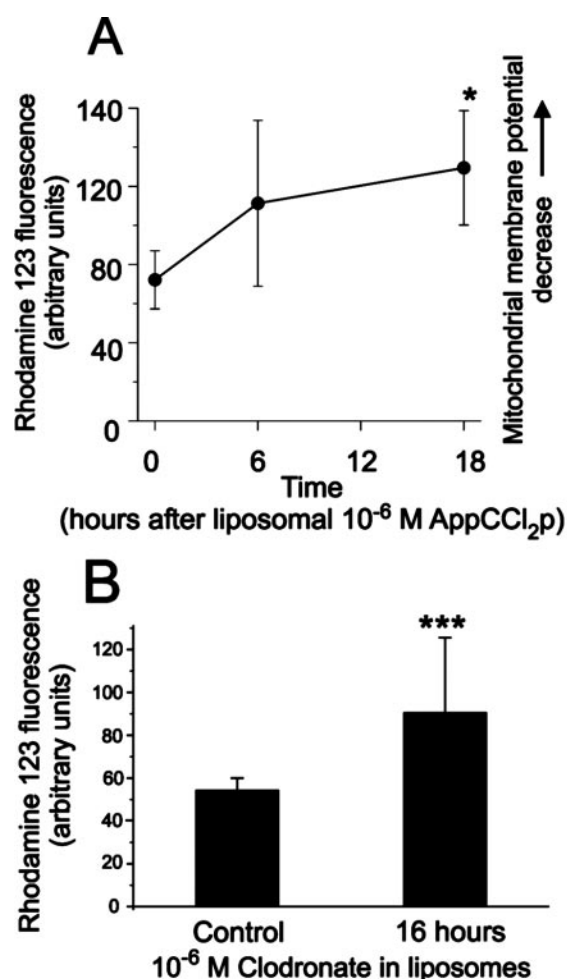
## Discussion

Bisphosphonates inhibit bone resorption via a route that probably involves a direct effect on mature osteoclasts (Hughes et al., 1991; Rodan 1998; Rogers et al., 2000). However, the molecular mechanisms by which the nitrogen-containing and the non-nitrogen-containing bisphosphonates affect osteoclasts seem to be different. Recently, it has been shown that nitrogen-containing bisphosphonates such as alendronate are inhibitors of farnesyl diphosphate synthase, an enzyme of the mevalonate pathway (van Beek et al., 1999b; Bergström et al., 2000; Dunford et al., 2001). Inhibition of this enzyme leads to loss of the isoprenoid intermediates farnesyl diphosphate and geranylgeranyl diphosphate, which are essential for the post-translational modification (prenylation) and function of GTP-binding proteins (Luckman et al., 1998). Inhibition of protein prenylation, and especially the loss of geranylgeranylated small GTP-binding proteins, inhibits the formation and activity of osteoclasts (Fisher et al., 1999; van Beek et al., 1999a; Coxon et al., 2000) and can cause activation of caspase-3-like proteases and apoptosis in osteoclasts and other cell types in vitro (Luckman et al., 1998; Benford et al., 1999; Reszka et al., 1999). The



**Fig. 4.** Probing of mitochondrial membrane potential with rhodamine 123 in rat and human osteoclasts cultured in the presence of $10^{-6}$ M liposomal AppCCl$_2$p or clodronate. The cells were loaded with 10 $\mu$M rhodamine 123 and cellular fluorescence measured as described under *Materials and Methods*. Fluorescence increase denotes membrane potential decrease. A, time course of the effect of liposome-encapsulated AppCCl$_2$p on mitochondrial membrane potential in rat osteoclasts. The values are means ± S.D. for 6 to 12 independent experiments. B, rhodamine 123 fluorescence from 30 live osteoclastoma-derived human osteoclasts treated with control or clodronate-containing liposomes. The error bars indicate S.D. C, typical multinucleated human osteoclast exposed for 16 h to empty liposomes. D, representative human osteoclast exposed for 16 h to $10^{-6}$ M clodronate encapsulated in liposomes. *, $p < 0.05$ and **, $p < 0.001$ compared with control cells.

non-nitrogen-containing bisphosphonates such as clodronate, however, do not inhibit the mevalonate pathway and have no effect on protein prenylation (Luckman et al., 1998; Benford et al., 1999; Fisher et al., 1999; van Beek et al., 1999a; Coxon et al., 2000). Furthermore, some studies have suggested that nitrogen-containing bisphosphonates (at high concentrations) can affect both mature osteoclasts and precursors in vitro, whereas clodronate affects mainly mature osteoclasts (Boonekamp et al., 1986, 1987; Lowik et al., 1988) and can cause osteoclast apoptosis (Hughes et al., 1995; Selander et al., 1996). The mechanism by which clodronate affects mature osteoclasts and causes osteoclast apoptosis remains unknown. However, one route could involve the formation of an adenosine-5′-[β,γ-methylene] triphosphate (AppCp-type) metabolite, which is not formed from any of the nitrogen-containing bisphosphonates studied to date (Auriola et al., 1997; Frith et al., 1997; Benford et al., 1999). The formation of AppCCl$_2$p could account for the cytotoxic effects of clodronate (Reitsma et al., 1982; Flanagan and Chambers, 1989), because AppCCl$_2$p is as effective as clodronate at reducing the viability of macrophages in vitro (Frith et al., 1997).

We describe in this report an inhibitory effect of AppCCl$_2$p, but not clodronate, on mitochondrial respiration by intact mitochondria. The effect is not detectable using submitochondrial particles, which represent an inside-out preparation of the mitochondrial inner membrane. We could not demonstrate any effect on any of the components of the mitochondrial respiratory complex. Instead, we observed a biphasic effect on mitochon-
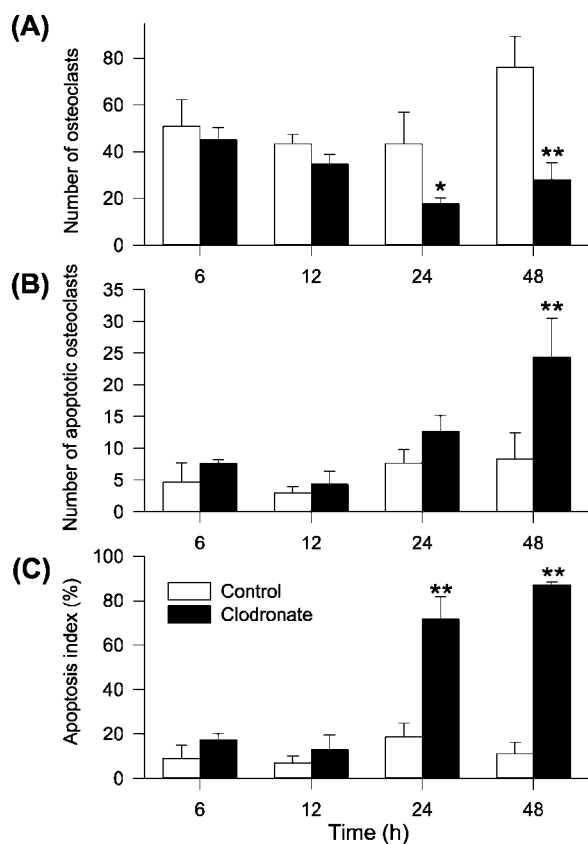


**Fig. 5.** Time course of clodronate-induced osteoclast apoptosis. Osteoclasts were cultured on clodronate-coated or uncoated bovine bone slices for times indicated, and number of viable osteoclasts (A), number of apoptotic osteoclasts (B), and apoptosis index determined (C). Values are means ± S.D., $n = 3$. *, $p < 0.01$ compared with the corresponding control value, and **, $p < 0.001$ compared with the corresponding control value.

drial membrane potential. The direction of the initial change (an increase in membrane potential) suggests that AppCCl$_2$p somehow inhibits the dissipation of the membrane potential. One consumer of the membrane potential is the electrogenic ADP/ATP translocase (ANT). Inhibition of the ANT would therefore account for the initial rise in mitochondrial membrane potential. The subsequent decrease of the membrane potential is probably due to some secondary adverse effect on the mitochondrial energy state. By studying the exchange of radiolabeled ATP into isolated mitochondria, we confirmed that the ANT is a target of AppCCl$_2$p, which is a competitive inhibitor with respect to ATP.

Because bisphosphonates are nonhydrolyzable analogs of pyrophosphate and their adenosine-containing metabolites (such as AppCCl$_2$p) are nonhydrolyzable ATP analogs, these compounds could interfere with several aspects of mitochondrial metabolism involving ATP. However, our data showed no effect on F$_1$F$_o$ATPase or the respiratory chain. In mitochondria, the translocation of pyrophosphate across the inner membrane is mediated by the ANT, not by the phosphate carrier (Kramer, 1985; Woldegiorgis et al., 1985). The nonhydrolyzable ATP analog AppCp has also been reported to be translocated into mitochondria by means of the ANT (Watanabe et al., 1985). Although no reports exit about the transport of its dichloromethylene analog AppCCl$_2$p, it is possible that bisphosphonates or their adenosine-containing metabolites are transported into mitochondria, or act as modulators of the adenylate translocase as our data strongly suggest.

Both nitrogen-containing bisphosphonates and non-nitrogen-containing bisphosphonates have been found to cause apoptosis of osteoclasts and macrophages in vitro (Hughes et al., 1995; Rogers et al., 1996; Selander et al., 1996). Early mitochondrial events in apoptosis include the dissipation of mitochondrial membrane potential, an increase in mitochondrial Ca$^{2+}$, extensive oxidation of mitochondrial NADPH, a decrease in cellular ATP, a burst of reactive oxygen species, increased opening of the mitochondrial permeability transition (PT) pore, and release of cytochrome $c$ (Heiskanen et al., 1999) and other pro-apoptotic factors into the extramitochondrial space (for a recent review, see Crompton, 1999). ANT forms the PT pore when associated with cyclophilin-D in the mitochondrial matrix and the nonselective voltage-dependent anion channel in the mitochondrial outer membrane (voltage-dependent anion channel is located at the contact points between the inner membrane cristae and the outer membrane). Cyclosporin-A (a ligand of with cyclophilin-D) and some inhibitors of the ANT inhibit PT pore formation, whereas other ANT inhibitors increase the probability of pore opening (Zamzami et al., 1996; Chavez et al., 1999).

Formation of the PT pore also is linked to cell death upon ischemia reperfusion. It has been reported that anoxic damage of mitochondria is prevented by ATP, ADP, and nonhydrolyzable AppCp, which is translocated by ANT (Watanabe et al., 1985). ATP and ADP are known to inhibit PT pore opening (Halestrap et al., 1997). It is assumed that this occurs by ATP binding to the outside of the inner mitochondrial membrane and ADP on the inner side of the membrane. Because the ANT is clearly involved in controlling the PT pore and hence in the regulation of apoptosis, it could therefore be a target of AppCp-type metabolites of the non-nitrogen-bisphosphonates such as clodronate. Our data indicate that the ANT is indeed inhibited by AppCCl$_2$p, a metabolite of clodronate. By inhibiting the ANT, AppCCl$_2$p could also cause opening of the PT pore and

hence cause apoptosis. In its translocating function, ANT alternates between two conformations with the adenylate binding site either on the mitochondrial matrix side (m-state) or on the cytosolic side (c-state) (Schultheiss and Klingenberg, 1984). The effect of ANT inhibition on PT pore formation depends on whether the ligands bind to the m-state or c-state. Ligands binding to the m-state inhibit the formation of the PT pore and ligands binding to the c-state induce the formation of the PT pore (Zamzami et al., 1996; Chavez et al., 1999). If AppCCl$_2$p acts as an enhancer of PT pore formation, it would be expected to do this by acting as a ligand binding to the c-state of the ANT (i.e., acting on the cytosolic side of the mitochondrial membrane). Thus, the proapoptotic effects of AppCCl$_2$p may be similar to that of the other ANT inhibitors.

The concentrations of AppCCl$_2$p that were needed to affect the ANT (IC$_{50}$ = 50 $\mu$M) and disrupt mitochondrial membrane potential in our studies were reasonably low. Mönkkönen et al. (2001) found that AppCCl$_2$p can reach concentrations as high as 1 mM in clodronate-treated cells in vitro. Furthermore, owing to the targeting of bisphosphonates to sites of resorption and selective uptake by osteoclasts (for review, see Rogers et al., 2000), high intracellular concentrations of clodronate and its metabolite could be achieved in osteoclasts in vivo. Moreover, if the translocation of bisphosphonate would occur in the protonated form, the high proton gradient across the basal membrane would force an intracellular enrichment of the compound. The

accumulation rate of AppCCl$_2$p is rather slow, so that its maximum concentration is reached after 12 h of exposure of macrophages in culture to liposomal clodronate (Mönkkönen et al., 2001). In a long-term experiment with a gradual increase of the inhibitor the acute effect (i.e., an initial increase in mitochondrial membrane potential due to lowered dissipation of the membrane energy charge by ANT and F$_1$F$_o$-ATPase would be difficult to observe).

Our present data clarify the likely molecular mechanism by which clodronate causes osteoclast apoptosis, owing to the formation of the metabolite AppCCl$_2$p. The latter, by inhibiting the mitochondrial ANT, causes mitochondrial membrane depolarization and subsequent events such as cytochrome *c* release and caspase activation (Heiskanen et al., 1999; Benford et al., 2001), leading to cell death. The previous observation that clodronate causes apoptosis more rapidly than the nitrogen-containing bisphosphonates can thus be explained as follows. The nitrogen-containing bisphosphonates act primarily by affecting the proton pumping and intracellular vesicular trafficking in the osteoclast. This leads to a subsequent change in osteoclast metabolism resulting from the inhibition of cell function. Consequently, the apoptotic mechanism is initiated (Selander et al., 1996). The faster action of clodronate is compatible with the present results, indicating binding of the clodronate metabolite to the c-state of ANT and direct apoptotic signaling via mitochondria. Thus, based on evidence presented herein and else-
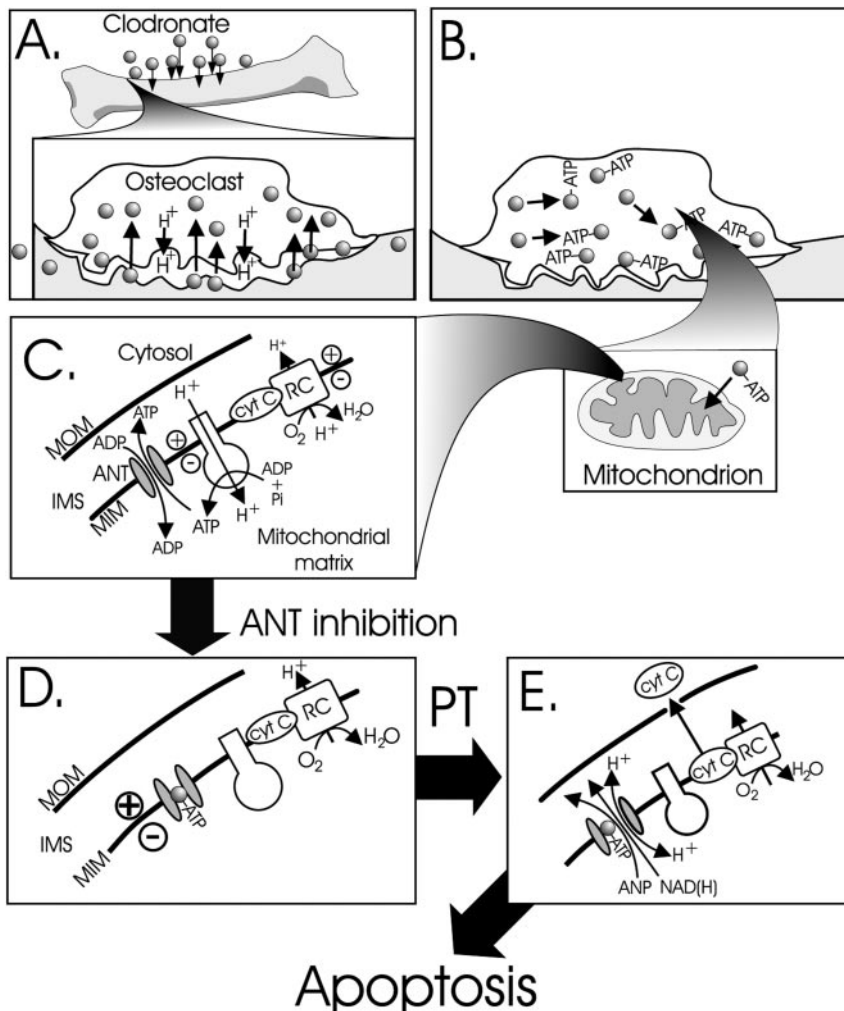


Fig. 6. Hypothetical mechanism of apoptosis induction by means of mitochondrial effects of clodronate in osteoclast. A, clodronate is bound to bone and taken up by osteoclasts, and a pH gradient across the basal membrane causes intracellular enrichment of the drug. B, clodronate is converted to AppCCl$_2$p, a nonhydrolyzable ATP analog (ATP marked with a sphere), which is taken up by mitochondria. C, schematic diagram of the main functions of the mitochondrial inner membrane. The respiratory chain (RC) builds an electrochemical proton gradient, including membrane potential. This is discharged by the ATP synthase and ADP/ATP translocator and converted to ATP energy. D, inhibition of ADP/ATP translocator interferes with the membrane potential conversion leading to an initial hyperpolarization. E, inhibition of the ADP/ATP translocator in the c-mode causes subsequent opening of the mitochondrial PT pore, leading to loss of mitochondrial matrix constituents and disappearance of the chemical and electric gradients. These processes lead to release of mitochondrial proapoptotic factors, including cytochrome *c* (cyt c) and commencement of apoptosis. MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; IMS, intermembrane space.

where we present a hypothesis of mechanism of action of clodronate on osteoclast apoptosis (Fig. 6).

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