The Cellular Uptake and Metabolism of Clodronate in RAW 264 Macrophages

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Purpose. Non-nitrogen-containing bisphosphonates, such as clodronate (dichloromethylene bisphosphonate), appear to act as prodrugs, their active form being the AppCp-type analogues of ATP. To further elucidate this, we examined the cellular uptake of clodronate and intracellular accumulation of the metabolite of clodronate ($AppCCl_2p$) in RAW 264 macrophages, the influence of clodronate metabolism on the intracellular ATP concentration, and the time course of clodronate metabolism and the effects of clodronate on cytokine secretion from macrophages.

Methods. The cellular uptake of clodronate was measured using ^{14}C-labeled clodronate. $AppCCl_2p$ was determined in cell extracts by using an ion-pairing HPLC-ESI-MS. The cytokine concentrations in the culture supernatants were measured with time-resolved fluoroimmunoassay. Intracellular ATP concentration was measured with a luminometer using a luciferin-luciferase assay.

Results. Of the clodronate internalized by macrophages *in vitro,* 30–55% is metabolized to $AppCCl_2p$, which accumulates to high intracellular concentrations during the first 12 h of exposure. This accumulation does not affect the ATP levels in the cells. The time course of metabolite appearance in the cells and the inhibition of cytokine secretion were very similar.

Conclusions. These results strongly support the idea that clodronate acts as a prodrug, the active form being its intracellular $AppCCl_2p$ metabolite.

KEY WORDS: clodronate; metabolism; cellular uptake; macrophage.

INTRODUCTION

Clodronate (dichloromethylene bisphosphonate) belongs to the family of bisphosphonates (BPs), which are currently the most important class of antiresorptive drugs. BPs are widely used in the treatment of bone diseases that involve excessive osteoclast-mediated bone resorption, such as Paget's disease, postmenopausal osteoporosis, tumor-induced osteolysis, and hypercalcemia (1).

BPs are synthetic analogues of pyrophosphate (PPi) (Fig. 1). The high avidity of BPs for Ca^{2+} ions and hydroxyapatite is the basis of the bone targeting property of these compounds. The antiresorptive effect could not be accounted for simply by adsorption of BPs to bone mineral and prevention of hydroxyapatite dissolution, and it is now clear that BPs inhibit bone resorption by cellular effects on osteoclasts (1). BPs can be divided into two classes in accordance with their structure and mechanism of action. In contrast to non-nitrogen-containing BPs (non-N-BPs), such as clodronate and etidronate, nitrogen-containing bisphosphonates (N-BPs), such as alendronate and ibandronate, contain a nitrogen atom in the R^2 side chain. These two BP groups have different cellular mechanism of action (1). Until recently, it was generally believed that BPs were not metabolized. However, Rogers *et al.* (2,3) and others (4) have shown that clodronate and other BPs that closely resemble PPi in structure can be metabolized by amoebae of the slime mold *Dictyostelium discoideum* into non-hydrolyzable, adenine-containing analogues of adenosine triphosphate (ATP). The metabolite of clodronate is adenosine 5'(β,γ-dichloromethylene) triphosphate ($AppCCl_2p$) (Fig. 1). The AppCp-type metabolites of BPs are formed by a back reaction catalyzed by class II aminoacyl-tRNA-synthetase enzymes. These enzymes can probably bind bisphosphonate instead of pyrophosphate in the ATP binding site (2–5). The BPs with larger, bulkier R^2 side chains (such as N-BPs) are not metabolized. More recently, it was demonstrated that clodronate is also metabolized *in vitro* by intact mammalian cells, such as J774 and RAW 264 macrophages (6–8). Owing to the nonhydrolyzable nature of the ATP analogues, their accumulation is likely to inhibit numerous intracellular metabolic enzymes, thus having detrimental effects on cell function and survival (1).

In addition to the ability of non-N-BPs to inhibit bone resorption, these compounds (particularly clodronate) have been shown to have anti-inflammatory effects. Clodronate inhibits the LPS-induced release of proinflammatory cytokines [interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)α] (9) and nitric oxide (10) from RAW 264 macrophages. Macrophages play a key role in inflammatory diseases, such as rheumatoid arthritis, and liposome-encapsulated clodronate has been found to eliminate macrophages and reduce inflammation in both rat adjuvant arthritis (11) and antigen-induced arthritis (12). The anti-inflammatory effect of clodronate on macrophages appears to be due to the $AppCCl_2p$ metabolite because Frith *et al.* (6) found that chemically synthesised $AppCCl_2p$ encapsulated in liposomes has the same potency for reducing macrophage cell viability and affects cell morphology in a similar manner to clodronate itself. Furthermore, the metabolite also seems to underlie the effects of clodronate on cellular functions, such as cytokine release and nuclear localisation of NF-κB (8). This strongly suggests that non-N-BPs (clodronate, etidronate, and tiludronate) may act as prodrugs, being converted to active metabolites after intracellular uptake.

Although earlier data strongly suggest that clodronate and other non-N-BPs act as the prodrugs, the active form being the metabolite $AppCCl_2pp$, nothing is known on the time course and efficacy of clodronate metabolism in the cells. These data would be of particular interest because of rapidly increasing interest in manipulation of macrophage activities by liposome-encapsulated clodronate e.g., in rheumatoid arthritis (11–14). Thus, in this article, we studied the cellular uptake of clodronate and intracellular accumulation of the

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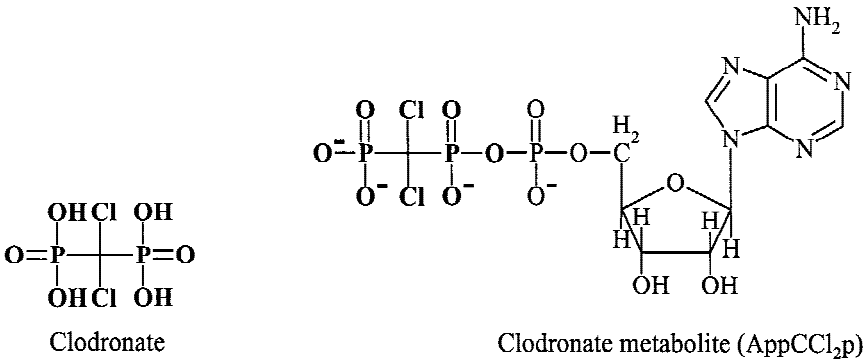

Fig. 1. The structure of clodronate and clodronate metabolite ($AppCCl_2p$).

clodronate metabolite ($AppCCl_2p$) in RAW 264 macrophage cells. Furthemore, the influence of clodronate metabolism on the intracellular ATP concentration, the time course of clodronate metabolism, and the effects of clodronate on cytokine secretion from macrophages were also evaluated.

MATERIALS AND METHODS

Chemicals

Unlabelled and ^{14}C-labeled clodronate (dichloromethylene-1,1-bisphosphonate) were kindly provided by Leiras Pharmaceutical Co., (Turku, Finland). Distearoylphosphatidylglycerol was obtained from Orion Farmos Co., (Turku, Finland) and cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO). Water was deionized using a Milli-Q system from Millipore (Bedford, MA). Sodium dodecyl sulphate was from Merck (Darmstadt, Germany) and *N,N*-dimethylformamid (DMF) from Riedel-de Haen AG (Seelze, Germany). All other reagents were from various suppliers as stated and were reagent grade or better.

RAW 264 Cell Culture

Murine macrophage-like RAW 264 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) and supplemented with 10% of fetal bovine serum (Gibco, Grand Island, NY) and 100 IU/mL of penicillin and streptomycin (Gibco, Grand Island, NY) in a 7% CO_2 atmosphere. For studies on the cellular uptake and metabolism of clodronate and the influence of clodronate metabolism on the intracellular ATP concentration, the cells were seeded into 6-well plates (Nunc, Roskilde, Denmark) at a density of 5×10^6 cells/well and left to adhere for 2 h. Medium was changed just before the treatments. In the cytokine experiments the cells were dispensed into 96-well plates (Nunc, Roskilde, Denmark), 2×10^5 cells/well. After 2 h, the nonadherent cells were removed and fresh serum-free medium was added to the wells before the treatments.

The Liposome Encapsulation of Clodronate

A stock solution of clodronate at 110 mM for liposome encapsulation was prepared by dissolving the drug in deionized water, the pH was adjusted to 7.2 by sodium bicarbonate powder, and the solution was filter sterilized using a 0.22-μm syringe filter.

Negatively charged liposomes are more efficient for drug delivery to cells than neutral liposomes (15), and thus, negatively charged phospholipid distearoylphosphatidylglycerol was chosen for the liposome preparation. The liposomes contained phospholipid and cholesterol in a molar ratio of 2:1. Clodronate was encapsulated in liposomes by reverse-phase evaporation as described previously (16). The clodronate concentration was measured spectrophotometrically, the lipid content of liposomes was determined by phosphorus assay (16), and the size distribution of liposomes was analysed by Nicomp Zeta Potential/Particle Sizer (model 380 XLS, Nicomp™, Santa Barbara, CA). The concentration of liposome-encapsulated clodronate was 8.1 ± 0.8 mM (mean ± standard deviation; n = 4). The molar drug:phospholipid ratio was 0.91 ± 0.16 and a mean diameter of clodronate-liposomes was under 200 nm. Nonloaded liposomes were used as a control.

The liposome-encapsulation of ^{14}C-labeled clodronate was performed in the same way as for unlabeled clodronate, but the concentration of clodronate was measured by using a liquid scintillation counter LKB-Wallac RackBeta (Wallac Co, Turku, Finland).

The Cellular Uptake of Clodronate

The cellular uptake of clodronate was measured using ^{14}C-labeled clodronate. RAW 264 macrophage cells were treated with concentrations of 30 μM or 1000 μM liposomal or free ^{14}C-labeled drug, respectively. After 1, 3, 6, 12, and 24 h of treatment with ^{14}C-labeled clodronate, medium was collected, and the cells were washed five times with phosphate-buffered saline. The cells were then extracted with 1 mL 20% sodium dodecyl sulphate buffer (1:1 milli-Q water/DMF, pH 4.6) and scraped off from the wells. The radioactivity of the medium, washes, and cell extract were measured by liquid scintillation counting. The intracellular concentration of clodronate was determined by comparing the radioactivity of medium, washes, and cell extracts and on the basis of an estimated cellular volume of 0.408 mm^3 per million cells. Cellular volume was estimated after determining the approximate diameter of RAW 264 cells (9.2 μm) using a light microscope with an eyepiece graticule.

The Accumulation of $AppCCl_2p$

To investigate the dependence of clodronate metabolism on the extracellular drug concentration, RAW 264 macrophage cells were treated with 1–100 μM liposome-encapsulated clodronate or 30–3000 μM -free clodronate for 24 h.

The time course of metabolite accumulation was studied by exposing the cells to 30 μM liposomal or 1000 μM free clodronate for 1, 3, 6, 12, and 24 h.

After treatment with clodronate, the RAW 264 macrophage cells were scraped off from the wells, counted using a Coulter counter, centrifuged (5 min at 220 g), and washed in phosphate-buffered saline. The number of cells varied between 5.2–9.7 × 10^6 depending on the exposure time. Extracts from cells were prepared using acetonitrile as described previously (17). The molar amount of $AppCCl_2p$ was determined in cell extracts by using an ion-pairing HPLC method that is compatible with negative ion electrospray ionization mass spectrometry (ESI-MS) (17). The intracellular concentration of $AppCCl_2p$ was then estimated assuming a cellular volume of 0.408 mm^3 per million cells.

The effect of Clodronate on Intracellular ATP Concentration and Cytokine Release in RAW 264 Macrophages

To investigate the influence of clodronate metabolism on the intracellular ATP concentration, macrophages were treated with 1000 μM free or 30 μM liposomal clodronate for 1–24 h. MES-HEPES buffer and nonloaded liposomes were used as a control, respectively. Intracellular ATP concentration was measured with a luminometer Wallac Victor^2 1420 Multilabel counter (Wallac Co, Turku, Finland) using a luciferin-luciferase assay and an estimated cellular volume of 0.408 mm^3. All the assay reagents were purchased as a kit (ATP Monitoring Kit, Labsystems Oy, Helsinki, Finland) and prepared according to the manufacturer's instructions. Preliminary tests revealed that the presence of $AppCCl_2p$ did not affect the luciferase assay, and thus the method was reliable for measuring ATP concentrations despite the intracellular accumulation of $AppCCl_2p$.

The modulation of cytokine secretion by clodronate was assessed in RAW 264 cells as described previously (9). RAW 264 cells were treated with 100 μM liposome-encapsulated or 1000 μM -free clodronate for 1–24 h, and then the cells were washed free of drugs and cytokine secretion was stimulated by addition of 10 μg/mL lipopolysaccharide (LPS) (*Escherichia coli,* serotype 0127:B8, Sigma) for 24 h. After LPS treatment, the cell-free supernatants were collected and assayed for IL-6 and TNFα by time-resolved fluoroimmunoassay (18).

Statistical Analyses

Results are presented as mean ± standard deviation for three to four experiments. The Mann-Whitney U test was used to compare the effect of clodronate on intracellular ATP concentration in RAW 264 macrophages. Values of $P < 0.05$ were considered to be statistically different to control.

RESULTS

The Cellular Uptake of Clodronate

When RAW 264 macrophage cells were exposed to 30 μM liposome-encapsulated or 1000 μM -free ^{14}C-labeled clodronate for 1 h, the intracellular concentration of clodronate reached approximately 1.0 and 0.3 mM, respectively (Fig. 2). After 12 h exposure to free clodronate, the intracellular concentration reached a plateau of about 1.3 mM. In the liposomal-treated cells, the intracellular concentration of clodronate was still progressively increasing and reached about 4 mM after 24 h of incubation (Fig. 2).

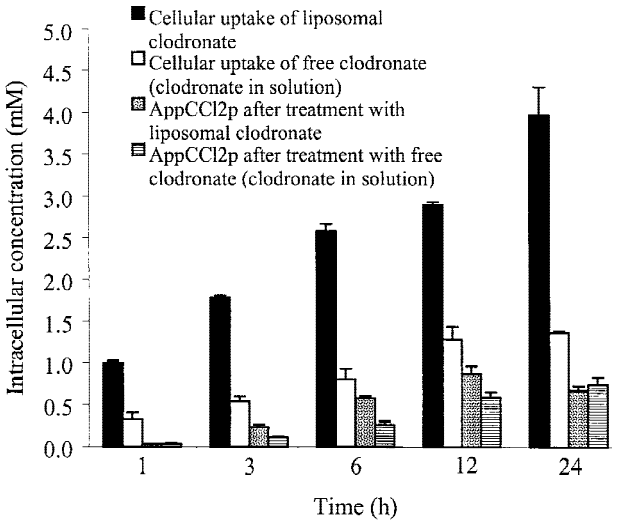

Fig. 2. The cellular uptake of clodronate and the accumulation of the metabolite of clodronate after culture for 1–24 h with 30 μM liposome-encapsulated clodronate or with 1000 μM free clodronate (mean ± standard deviation; n = 3). Five million RAW 264 cells were treated with drugs. The intracellular concentration of clodronate was determined by comparing the radioactivity of medium, washes, and cell extracts and on the basis of an estimated cellular volume of 0.408 mm^3 per million cells. The molar amount of $AppCCl_2p$ was determined in cell extracts by using HPLC-ESI-MS (17) and intracellular concentration of $AppCCl_2p$ by use of a cellular volume of 0.408 mm^3 per million cells.

The Intracellular Accumulation of $AppCCl_2p$ and the Efficiency of Metabolism

When RAW 264 cells were exposed to 1–100 μM liposome-encapsulated or 30–3000 μM liposome-free clodronate for 24 h, the highest intracellular concentration of $AppCCl_2p$ was achieved with concentrations of 30 μM liposomal clodronate or 1000 μM free clodronate (data not shown). The maximum concentration of $AppCC_2p$, which accumulated intracellularly in macrophages, was about 0.8 mM after treatment with either liposomal or free clodronate (Fig. 2). Thus, the amount of clodronate converted into $AppCCl_2p$ corresponded to 30–55% of total clodronate taken up by the cells either as liposomal or free drug. A lower proportion of the internalized liposomal clodronate was metabolized to $AppCCl_2p$ compared to free clodronate, probably because encapsulation in liposomes allows accumulation of a greater amount of clodronate, as demonstrated using ^{14}C-labeled clodronate (Fig. 2).

The metabolite was observable already after 1 h of exposure of the cells to liposomal or free clodronate. The concentration of $AppCCl_2p$ gradually increased during the first 12 h of clodronate treatment, then reached a plateau (Fig. 2). Extracts from cells that had been treated with nonloaded liposomes or vehicle for free clodronate (control) did not contain $AppCCl_2p$.

The Time Course of the Effects of Clodronate on Intracellular ATP Concentration and Cytokine Secretion

The intracellular ATP concentration in RAW 264 cells was in the range of 3–5 mM, which is similar to that reported for primary macrophages (19,20). Free or liposomal clodronate impaired intracellular ATP levels significantly only at 24 h of exposure, although liposomal clodronate seemed to slightly decrease ATP levels also at 6 h. This effect was not, however, seen at 12 h (Fig. 3). Nonloaded liposomes had similar effects on the intracellular ATP concentration (data not shown) as MES-HEPES buffer, which was used as a control.

The time course of the inhibition of TNFα and IL-6 secretion by free (1000 μM) or liposomal clodronate (100 μM) 1–24 h before the LPS challenge closely coincidenced with the appearance of $AppCCl_2p$ into the cells (Fig. 4).

Compared to free clodronate, exposure of the cells to liposome-encapsulated clodronate led to faster intracellular uptake of the drug (Fig. 2), and consequently, faster accumulation of the metabolite (Fig. 2) and faster inhibition of the cytokine secretion (Fig. 4).

DISCUSSION

Liposomes have proved to be an effective means of delivery of clodronate and other BPs to macrophages, which do not readily internalize the compounds in the free form (15,16,21). Cellular uptake of liposomal drug occurs by endocytosis and macrophages as highly endocytic cells avidly take up liposomal clodronate (Fig. 2). Endocytosis is an active mechanism of cellular uptake, which allows higher intracellular concentrations of clodronate to be achieved compared to extracellular medium. After 24 h exposure to 30 μM liposome-encapsulated clodronate, the intracellular concentration of clodronate was about 4 mM (Fig. 2). Highly hydrophilic, negatively charged compounds, such as BPs, are not membrane-permeable. However, Felix *et al.* (22) used radiolabeled BPs to study uptake by calvarial cells *in vitro* and confirmed that the BPs could enter the cytoplasm as well as

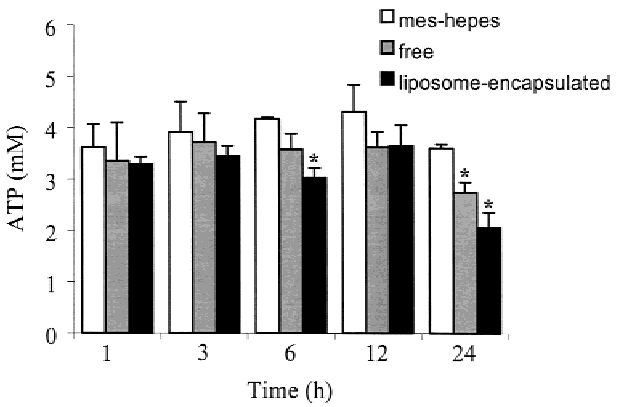

Fig. 3. The effect of clodronate on intracellular ATP concentration after culture for 1–24 h with 30 μM liposome-encapsulated or with 1000 μM free clodronate (mean ± standard deviation; n = 3). Five million RAW 264 cells were treated with drugs. The intracellular concentration of ATP was measured with a luminometer using a luciferin-luciferase assay (a cellular volume of 0.408 mm^3 per million cells). *Values significantly different from the control ($P < 0.05$ using the Mann–Whitney U test).

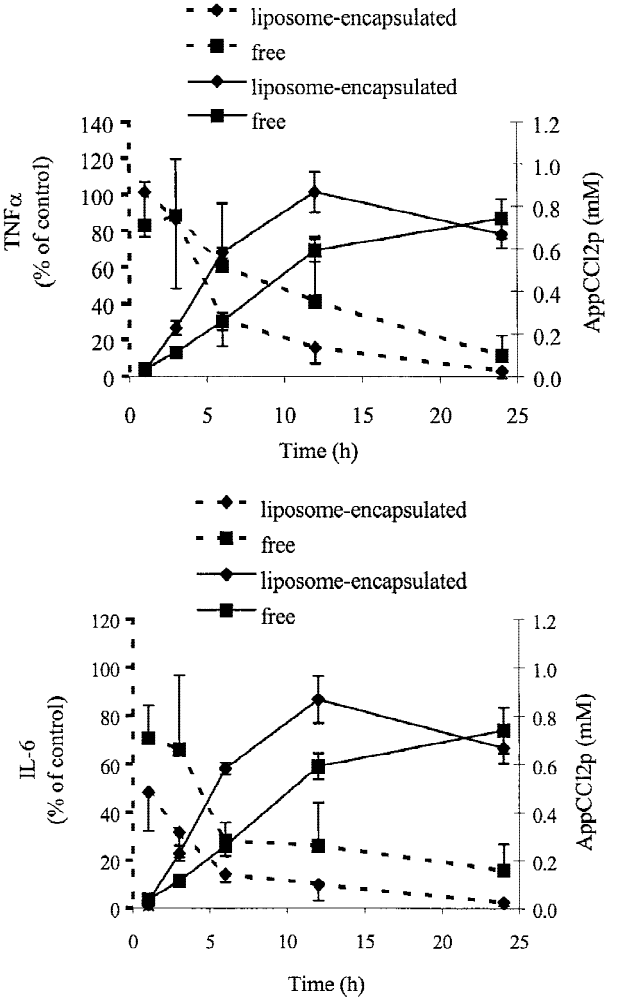

Fig. 4. The inhibition of interleukin-6 and tumor necrosis factor-alpha secretion (dotted lines, left y axis) and the accumulation of the metabolite of clodronate (solid lines, right y axis) in RAW 264 cells after culture for 1–24 h with liposome-encapsulated or free clodronate. In the cytokine experiments, the cells (2×10^5/well in 96-well plates) were pretreated with 100 μM liposome-encapsulated or 1000 μM free clodronate for 1–24 h before lipopolysaccharide (LPS) induction (mean ± standard deviation; n = 4). After pretreatments, the cells were washed free of drugs and cytokine secretion was stimulated by addition of LPS (10 μg/mL) for 24 h. The untreated LPS-stimulated cells were used as control. In the metabolism experiments, the cells (5×10^6/well in 6-well plates) were treated for 1–24 h with 30 μM liposome- encapsulated or 1000 μM free clodronate (mean ± standard deviation; n = 3). The intracellular concentration of $AppCCl_2p$ was calculated as in Figure 2.

mitochondria and other organelles. Studies with slime mould amoebae have demonstrated that the cellular uptake of free BPs occurs by fluid-phase pinocytosis (23). Pinocytosis is a passive nonspecific uptake of extracellular molecules in a pinocytic vesicle and is proportional to the molecular concentration in the extracellular solution. The study of cellular uptake of free clodronate by macrophages in the present study support the view that free clodronate is internalized by pinocytosis because the intracellular and extracellular concentrations of clodronate were approximately in equilibrium after 12 h of incubation (1.3 mM internal vs. 1 mM external) (Fig. 2).

In this study, highly endocytic RAW 264 macrophage

cells internalized free and liposome-encapsulated clodronate, which was metabolized to $AppCCl_2p$. Pelorgeas *et al.* (4) have previously examined the formation of $AppCCl_2p$ in amoebae of the slime mould Dictyostelium discoideum. In their study, $AppCCl_2p$ was detected in cell extracts by using ^{31}P NMR spectroscopy and the intracellular concentration of $AppCCl_2p$ was calculated using an estimated cellular volume of 520 μm^3. When the amoebae were exposed to 7.5 mM clodronate for 4 h, the intracellular concentration of clodronate reached 3.1 mM, and the concentration of $AppCCl_2p$ increased progressively with time, reaching a plateau of 0.20 mM after 3 h exposure. As our results show (Fig. 2), macrophages internalize and metabolize clodronate in a similar manner to amoebae. These cell types are both highly pinocytic and therefore internalize clodronate very efficiently, which is then metabolized to $AppCCl_2p$. It has also shown that clodronate accumulates mainly in the cytosol of cells (22). Aminoacyl-tRNA-synthetases, which catalyze the formation of the AppCp-type metabolites of BPs, are intracellular enzymes and therefore the metabolism of BPs is dependent on cellular uptake (23). However, the fact that the $AppCCl_2p$ concentration did not increase in the cell after 12 h of treatment, although the intracellular uptake gradually increased up to 24 h (Fig. 2) suggests that the enzyme reaction converting clodronate to $AppCCl_2p$ may also be a rate-limiting step. Another possible explanation for a plateau of metabolism may be caused by a general inhibition of cellular metabolism, which also causes an inhibition of cytokine production.

Our results indicate that 30–55% of clodronate internalized by macrophages *in vitro* is metabolized to $AppCCl_2p$, which accumulates to high intracellular concentrations during the first 12 h of exposure (Fig. 2). This strongly supports the idea that clodronate acts as a prodrug, the active form being its intracellular $AppCCl_2p$ metabolite. We and others have previously shown that clodronate has anti-inflammatory effects *in vitro* and *in vivo* (9–12) and, when delivered to RAW 264 macrophages using liposomes, $AppCCl_2p$ has similar inhibitory effects on cytokine release as clodronate itself (8). In the present study, the formation of $AppCC_2p$ occurred concomitantly with the inhibition of cytokine release (Fig. 4), further supporting the hypothesis that the metabolite of clodronate is responsible for the anti-inflammatory properties of this bisphosphonate.

The effects of $AppCCl_2p$ on cytokine secretion do not appear to be a consequence of the impairment of energy metabolism in the cells, because the intracellular ATP concentration did not drastically decrease in clodronate treated cells during the first 12 h of exposure (Fig. 3), although the intracellular concentration of $AppCCl_2p$ reached its peak level and cytokine secretion was already strongly affected by this time (Fig. 4). This suggests that the intracellular formation of $AppCCl_2p$ does not affect the ability to synthesise ATP, although the intracellular accumulation of $AppCCl_2p$ appears eventually to cause a decrease in ATP levels after 24 h, perhaps as a consequence of cell death (6).

Clodronate causes apoptotic cell death of macrophages and osteoclasts *in vitro* (6,24,25). Liposome-encapsulated $AppCCl_2p$ also causes apoptosis and necrosis of J774 macrophages *in vitro* (6). The current study provides conclusive evidence that high intracellular concentrations of $AppCCl_2p$ can be achieved after exposure of cells to clodronate, supporting the view that cell death is due to the intracellular accumulation of $AppCCl_2p$. The exact mechanism by which $AppCCl_2p$ induces apoptosis in osteoclasts and macrophages requires further study, although we recently reported that $AppCCl_2p$ inhibits the mitochondrial ATP/ADP translocase in osteoclasts and may cause apoptosis by disrupting mitochondrial membrane potential (26). The affinity of BPs for bone mineral *in vivo* leads to rapid clearance of the drugs from the circulation and localisation to bone surfaces (27). This could give rise to very high local concentrations in the osteoclast resorption lacuna after the release from bone mineral during the resorption process, as has been shown for alendronate, which reaches about 1 mM concentration in the resorption space beneath an osteoclast (28). The high concentration of BP achieved in the resorption space beneath and the high endocytic capacity of osteoclasts raise the likelihood that osteoclasts could also metabolize non-N-BPs *in vivo* and that this underlies the inhibition of osteoclastic bone resorption by this class of BPs. In support of this, we have recently confirmed that clodronate is metabolized to $AppCCl_2p$ by rabbit osteoclasts and rat peritoneal macrophages *in vivo* and that the formation of $AppCC_{22}p$ is associated with an increase in macrophage and osteoclast apoptosis (29). Taken together, these data strongly suggest that clodronate acts as a prodrug, inhibiting the cellular function of macrophages and osteoclasts in vivo as a result of intracellular accumulation of $AppCCl_2p$.

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