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Effects of various absorption enhancers on transport of clodronate through Caco-2 cells

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Abstract

The major disadvantage concerning clinical use of bishosphonate drugs, like clodronate, is their poor and variable absorption after oral administration. The objective of this study was to assess the effects of four different absorption enhancers—palmitoyl carnitine chloride (PCC), *N*-trimethyl chitosan chloride (TMC), sodium caprate (C10), and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)—on the transport of clodronate using Caco-2 cell culture model. The transport experiments were performed in a normal (1.3 mM) and in a minimum-calcium concentration (apically calcium-free medium and basolaterally 100 μM calcium concentration). In the normal calcium concentration, a strong enhancement in clodronate permeation was observed with the enhancers: EGTA (2.5 mM), TMC (1.5% w/v), and PCC (0.2 mM) increased the transport of 1 mM clodronate 190-, 20-, and 10-fold, respectively, and the transport of 10 mM clodronate 130-, 70-, and 35-fold. In the minimum-calcium concentration, the effects of the absorption enhancers on the transport of clodronate were not so potent: TMC, PCC, and EGTA caused 2- to 20-fold enhancement in clodronate permeation whereas C10 (10 mM) was without any effect. According to the results, the permeation of clodronate through Caco-2 cells could be significantly promoted by the absorption enhancers, which cause widening of the tight junctions and, thus, increase the permeability of the paracellular route.

Keywords: Clodronate; Bisphosphonates; Absorption; Absorption enhancers; Caco-2 cells

1. Introduction

Clodronate, a member of bisphosphonate drugs, inhibits osteoclastic bone resorption and is, therefore, used in the therapy of bone and calcium metabolism diseases, e.g. Paget's disease, hypercalcemia of malignancy, and osteolytic bone metastases (Plosker and Goa, 1994). A major problem in the successful clinical use of bisphosphonates is their poor and variable intestinal absorption: for example, the bioavailability of clodronate is about 2% after oral administration (Yakatan et al., 1982; Pentikäinen et al., 1989; Villikka et al., 2002). The poor gastrointestinal absorption of bisphosphonates is a consequence of their very high hydrophilicity and extensive ionization, which prevents transcellular permeation across the epithelial

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cells (Lin, 1996). According to the in vitro transport studies performed using human intestinal epithelial Caco-2 cells, the paracellular route is supposed to be the main permeation route of bisphosphonates through the epithelial barrier (Boulenc et al., 1993; Twiss et al., 1994; Raiman et al., 2001). The oral absorption of bisphosphonates is dramatically diminished when the drug is taken with food (Fogelman et al., 1986; Laitinen et al., 2000). Especially, the presence of Ca^{2+} and other divalent cations in the intestinal lumen may retard the absorption since the divalent cations are able to form non-absorbable complexes with bisphosphonates (Fleisch, 1997). In addition, the negative charge and relatively large molecular size of bisphosphonates complicate their paracellular transport (Lin, 1996). Orally administrated bisphosphonates could be absorbed to a small extent from the stomach, but the main absorption site is the upper part of the small intestine (Fleisch, 1997).

In order to improve the oral absorption of bisphosphonates various methods, such as absorption enhancers and prodrug technique, have been employed (Ezra and Golomb, 2000). In the prodrug strategy, more lipophilic derivatives of clodronic (Ahlmark et al., 1999; Niemi et al., 1999) and etidronic acid (Niemi et al., 2000) have been synthesized and tested in vitro as potential bioreversible prodrug molecules of clodronate and etidronate. Moreover, dipeptidyl prodrugs (Pro-Phe-pamidronate and Pro-Phe-alendronate) which could utilize active transporters (like hPEPT1) in the absorption have been studied (Ezra et al., 2000). Among the chemical absorption enhancers, calcium chelator EDTA has been tested in promoting absorption of alendronate and clodronate in rats (Janner et al., 1991) and the effects of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Boulenc et al., 1993) and sodium lauryl sulfate (Boulenc et al., 1995a) on transport of tiludronate through the Caco-2 cells have been investigated.

In the present study, the effects of four different absorption enhancers—palmitoyl-DL-carnitine chloride (PCC), *N*-trimethyl chitosan chloride (TMC), sodium caprate (C10), and EGTA—on the transport of clodronate through Caco-2 cells were evaluated. The influence of the extracellular calcium on the absorption enhancement was studied by performing the transport experiments both in the conventional Caco-2 cell culture model and in the minimum-calcium model described by Nicklin et al. (1995).

2. Materials and methods

2.1. Materials

Clodronate and [^{14}C]clodronate (specific activity 2.7 μCi/mg) were obtained from Leiras Pharmaceutical Co. (Turku, Finland). Absorption enhancers—PCC, sodium salt of capric acid (C10) (99–100% purity), and EGTA—were all purchased from Sigma Chemical Co. (St. Louis, MA). TMC (degree of substitution ca. 60%) was synthesized using the method described previously by Kotzé et al. (1997) at the Department of Pharmaceutical Technology in the Leiden University (Leiden, The Netherlands). D-[1-^{14}C]Mannitol (specific activity 51.5 mCi/mM) was acquired from DuPont NEN® Products (Boston, MA). Medium components and other reagents for cell culture were obtained from Gibco Life Technologies (Paisley, Scotland), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) from Sigma, and rat tail type I collagen from Collaborative Biomedical Products (Bedford, MA). For the MTT test, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma, sodium dodecyl sulfate (SDS) from Merck (Darmstadt, Germany), and *N*,*N*-dimethylformamide (DMF) from Fluka Chemie AG (Buchs, Switzerland).

2.2. Cell culture

The Caco-2 cells were obtained from the University of California (San Francisco, CA). The cells were grown in a cell culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (heat-inactivated at a temperature of 56 °C for 30 min), 2 mM L-glutamine, 1% non-essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin in 75-cm^2 cell culture flasks (Nunc, Roskilde, Denmark). The cultures were kept at 37 °C in an atmosphere of 7% CO_2.

For the transport experiments, the cells between the passage numbers 45–53 were seeded at a density of 80,000 cells/cm^2 on the rat tail type I collagen-coated Transwell polycarbonate cell culture inserts (24-mm

diameter, 3.0-μm pore size) (Costar Corporation, Cambridge, MA). The medium was changed every other day and the day before use (apical volume 1.5 ml and basolateral volume 2.6 ml). The cells were used for the transport studies 21–27 days after seeding on the membranes. The integrity of each batch of the monolayers was determined by measuring the transport of [^{14}C]mannitol (0.3 μCi/ml) through the three individual monolayers.

2.3. Transport experiments

The transport experiments were performed in the normal (1.3 mM) and in the minimum-calcium concentration (a calcium-free apical donor solution and a basolateral receiver solution containing 100 μM calcium concentration) (Nicklin et al., 1995). Prior to the experiments, the cells were washed twice with Hanks' Balanced Salt Solution (HBSS) supplemented with 25 mM HEPES (pH 7.4) in the conventional Caco-2 model. In the minimum-calcium model, the cells were washed first with HBSS/25 mM HEPES (without Ca and Mg), and after that the apical sides with HBSS/25 mM HEPES (without Ca and Mg) and the basolateral sides with HBSS/25 mM HEPES containing 100 μM calcium. The transport studies were performed from the apical to the basolateral direction at 37 °C. Clodronate solution (1 or 10 mM) (0.44 μCi/ml) in HBSS/25 mM HEPES (pH 7.4) with/without absorption enhancer was added to the apical compartment, and the samples (100 μl) were withdrawn every 30 min for 3 h from the basolateral compartment. The volume removed from the basolateral compartment was always replaced with fresh prewarmed HBSS/25 mM HEPES. The concentrations of the absorption enhancers used were: 0.2 mM PCC, 1.5% w/v TMC, 10 mM C10 (only in minimum-calcium model to avoid precipitation with calcium), and 2.5 mM EGTA (both in the apical and the basolateral compartments). EGTA was added to the apical and the basolateral chambers because in previous studies (Noach et al., 1993; Collares-Buzato et al., 1994), it has been shown that application of calcium chelator (EDTA or EGTA) especially into the basolateral chamber is of major importance to provoke opening of junctional complex. To avoid precipitation of TMC with clodronate, the cells were incubated with 1.5% w/v TMC in serum-free medium for 60 min before the transport experiments were performed. In addition, the effects of PCC (0.2 mM), TMC (1.5% w/v), and EGTA (2.5 mM both apically and basolaterally) on the transport of [^{14}C]mannitol (0.3 μCi/ml) were studied in the conventional model and the effects of C10 (10 mM) in the minimum-calcium model. The samples (100 μl) were mixed with 4.5 ml of scintillation liquid (Ultima Gold, Packard, Groningen, The Netherlands), and the ^{14}C activities of the samples were measured by Wallac 1218 Rackbeta Liquid Scintillation Counter (Wallac, Turku, Finland). The apparent permeability coefficients (P_{app}) were calculated according to the equation:

$$P_{app} = \frac{dQ}{dt}\left(\frac{1}{AC_0}\right)$$

where dQ/dt is the permeability rate, A is the diffusion area of the monolayer, and C_0 is the initial donor solution concentration.

2.4. MTT test

The effects of PCC, C10, and EGTA on the Caco-2 cell viability were studied by the MTT method. The Caco-2 cells were inoculated in microwells of 96-well microtiter plates (Nunc, Roskilde, Denmark) at a density of 50,000 cells/well. After 24 h, the cells were exposed to the absorption enhancers from half-logarithmic dilution series for 3 h at 37 °C. Then, the medium was aspirated and exchanged to the serum-free medium and 25 μl MTT solution (5 mg/ml) was added. After 2 h, 100 μl SDS + DMF buffer, pH 4.7, was added to dissolve the formazan precipitate. The developed color was measured at a wavelength of 570 nm with a multiwell scanning spectrophotometer (Multiscan Plus, Labsystems, Helsinki, Finland). The results are expressed as percentage of the control value.

2.5. Statistical analysis

The results are expressed as mean ± S.D. Statistical differences were evaluated using one-way ANOVA, followed by the Fisher's LSD for multiple comparisons ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

3. Results

As a marker for integrity of the Caco-2 cell monolayers, [^{14}C]mannitol transport through the cells was followed. In the normal calcium concentration, $1.4 \pm 0.6\%$ ($P_{app} = (4.5 \pm 2.0) \times 10^{-7}$ cm/s, $n = 21$) of the initial mannitol concentration was transported through the cells in 3 h. In the minimum-calcium model, mannitol transport slightly increased being $1.9 \pm 0.6\%$ ($P_{app} = (5.6 \pm 1.6) \times 10^{-7}$ cm/s, $n = 24$).

Clodronate was poorly transported through the Caco-2 cells: the apparent permeability coefficient (P_{app}) obtained for 1 mM clodronate was $(0.5 \pm 0.2) \times 10^{-7}$ cm/s ($n = 15$) (Fig. 1a). When the concentration of clodronate was increased to 10 mM, the transport increased twofold ($P_{app} = (1.0 \pm 0.4) \times 10^{-7}$ cm/s, $n = 8$) (Fig. 1a). All the studied absorption enhancers improved the transport of clodronate through the cells. PCC (0.2 mM) increased the transport of 1 mM clodronate by a factor of about 10 ($n = 3$), the pretreatment of the cells with TMC (1.5% w/v) by a factor of 20 ($n = 3$), and EGTA (2.5 mM) by a factor of 190 ($n = 3$) (Fig. 1a). When 10 mM concentration of clodronate was used, the enhancement factors for PCC, TMC, and EGTA were 35 ($n = 3$), 70 ($n = 3$), and 130 ($n = 3$), respectively (Fig. 1a). Experiments with C10 were not performed in the normal calcium concentration because C10 precipitated with calcium.

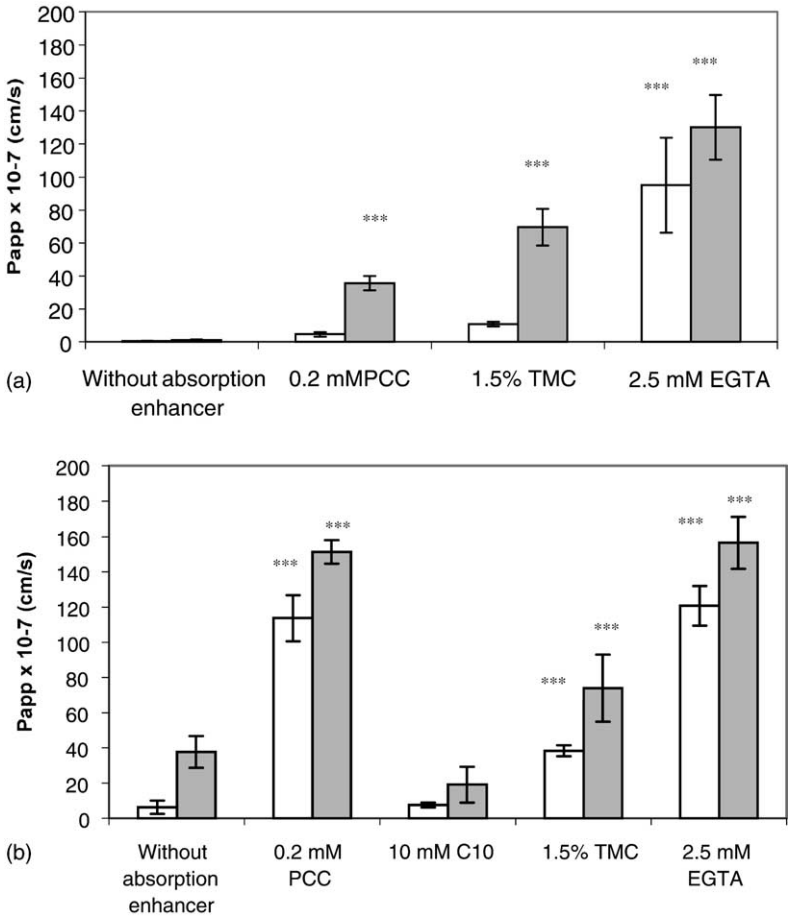

Fig. 1. The effects of the absorption enhancers on the apparent permeability coefficients (P_{app}) for 1 mM (white bars) and 10 mM (grey bars) in normal (a) and in minimum-calcium concentration (b) ($n = 3$–24).

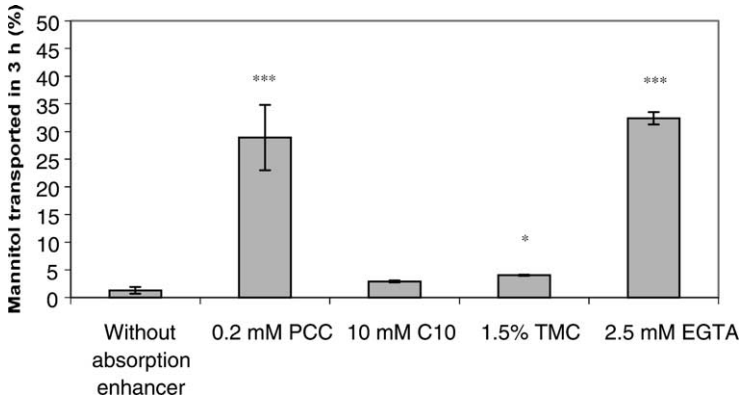

Fig. 2. The effects of the absorption enhancers on the transport of [^{14}C]mannitol through Caco-2 cells ($n = 3$–21). The experiment with C10 is performed in the minimum-calcium concentration.

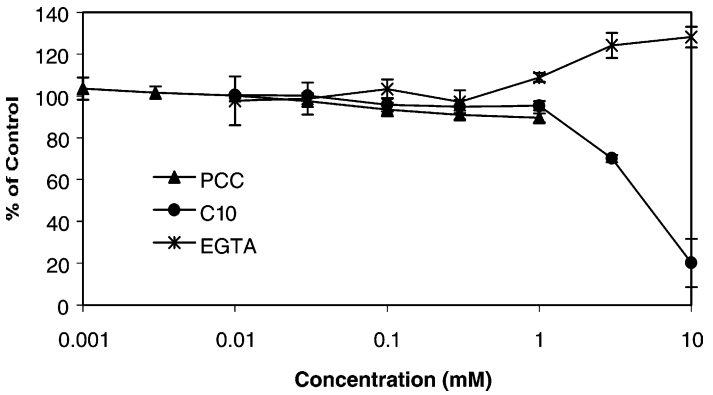

Fig. 3. The effects of PCC, C10, and EGTA on the viability of Caco-2 cells. The values are means of three independent experiments.

In the minimum-calcium model, the permeability of 1 mM clodronate increased 12-fold ($P_{app} = (6.3 \pm 3.8) \times 10^{-7}$ cm/s, $n = 8$) and that of 10 mM 38-fold ($P_{app} = (37.7 \pm 9.0) \times 10^{-7}$ cm/s, $n = 6$) as compared with the corresponding apparent permeability coefficients obtained in the presence of calcium (Fig. 1b). In the absence of calcium, EGTA and PCC were the most effective agents in enhancing clodronate's permeation (Fig. 1b). The absorption enhancers did not, however, cause such a dramatic enhancements in the clodronate permeation as compared to the permeabilities obtained in the presence of calcium. The enhancement factors in the minimum-calcium model ranged from 2 to 20, C10 being almost without any effect (Fig. 1b).

EGTA and PCC had the strongest effect also on the transport of mannitol through the Caco-2 cells: in the presence of 2.5 mM EGTA $32.4 \pm 1.1\%$ ($n = 3$) and in the presence of 0.2 mM PCC $28.9 \pm 5.9\%$ ($n = 3$) of the initial mannitol concentration was transported through the cells in 3 h (Fig. 2). The corresponding percentages for TMC and C10 were $4.0 \pm 0.1\%$ ($n = 3$) and $2.9 \pm 0.1\%$ ($n = 3$), respectively.

According to the MTT assay results, EGTA and PCC did not affect the viability of the Caco-2 cells (Fig. 3). C10, instead, decreased the viability: as a concentration of 10 mM (the same concentration which was used in the transport experiments), the viability decreased to 20% of the control value (Fig. 3).

4. Discussion

As also previously shown (Raiman et al., 2001), clodronate was very poorly transported through the

Caco-2 cells. The low permeability coefficient indicates that the paracellular pathway is the main permeation route of clodronate through Caco-2 cells. The low permeabilities of paracellularly transported drugs are a consequence of the small surface area of the intercellular spaces and the tight junctions between the epithelial cells (Artursson et al., 1996). In the minimum-calcium model, the permeability of clodronate was significantly enhanced. This indicates the strong role of calcium in the restriction of bisphosphonate absorption which is probably due to complexation of bisphosphonate into a non-absorbable form (Fleisch, 1997). The transport of clodronate was dose-dependent: higher concentration resulted in slightly increased permeability. It is earlier reported that bisphosphonates may enhance their own absorption since they are able to bind calcium and, thus, increase the permeability of the tight junctions (Boulenc et al., 1993; Twiss et al., 1994). In fact, because of the calcium binding ability of bisphosphonates, they might act as potential absorption enhancers themselves. Van Hoogdalem et al. (1989) studied the effects of 0.5–6% w/v pamidronate on rectal absorption of cefoxitin in rats. A maximal cefoxitin bioavailability of $85 \pm 10\%$ was achieved by infusion with 4% w/v of pamidronate compared with $14 \pm 12\%$ without pamidronate.

EGTA was the most effective absorption enhancer in promoting the transport of clodronate through Caco-2 cells, both in the presence and in the absence of calcium. EGTA had the strongest effect also on the transport of mannitol through the cells. As a calcium chelator, EGTA is much more potent than bisphosphonates since, for example, a 50% complexation of Ca^{2+} ions is achieved at the concentration of 0.5 mM EGTA and 10–20 mM of tiludronate (Boulenc et al., 1995b). Powerful calcium chelating agents, like EDTA and EGTA, cause Ca^{2+} depletion which, in turn, leads to considerable changes in the cells, inducing disruption of actin filaments, disruption of adherent junctions, diminished cell adhesion, and activation of protein kinases (LeCluyse and Sutton, 1997). In addition to these effects on the cells and tight junction permeability, calcium chelators may enhance bisphosphonate absorption by reducing the formation of bisphosphonate–calcium complexes (Janner et al., 1991). It is, however, unlikely that EGTA could be used in vivo, since many endogenous factors affect the calcium concentration in the gastrointestinal tract and because of the variability in the permeabilities, even obtained in controlled conditions, like in Caco-2 cell model (Artursson and Magnusson, 1990). It has also been noticed that the absorption enhancement obtained with calcium chelator EDTA is more dependent on the basolateral than the apical application of the calcium chelator (Noach et al., 1993).

A long-chain acylcarnitine PCC (0.2 mM) increased the permeability of clodronate in the presence of calcium, but in the smaller extent than EGTA. In the minimum-calcium concentration, PCC increased significantly the permeation of clodronate and almost the same permeabilities were achieved with PCC as with EGTA. PCC was also as effective as EGTA in promoting the transport of mannitol through the Caco-2 cells. It has been previously shown that 0.2 mM PCC enhances transport of hydrophilic marker molecules, lucifer yellow and ruthenium red, through Caco-2 cell monolayers and that PCC reduces rapidly and Ca^{2+} independently transepithelial electrical resistance (TEER) (Hochman et al., 1994). PCC appears not to cause apical cell membrane lysis, but PCC acts by disrupting tight junctions. However, Duizer et al. (1998) have demonstrated that, for example, leakage of lactate dehydrogenase is significantly increased at PCC concentrations of $\geq$0.2 mM in Caco-2 and $\geq$0.1 mM in IEC-18 cells.

In the case of TMC, the pretreatment of the Caco-2 cells with TMC prior to the transport experiments resulted in significantly higher clodronate permeabilities, both in the presence and the absence of calcium. Under the influence of TMC (1.5% w/v), the mannitol transport increased 2.7-fold.

Interestingly, C10 (10 mM) only very slightly increased permeability of 1 mM clodronate and did not at all affect the permeability of 10 mM clodronate. The transport of mannitol, however, was increased 1.5-fold by C10.

According to the MTT test results, EGTA and PCC did not remarkably decrease the viability of the Caco-2 cells. At the concentrations $\geq$1 mM EGTA rather increased the viability which is in agreement with the results obtained by Boulenc et al. (1995b). An increased viability could be, at least in part, due to increased transport of tetrazolium salt inside the cells. C10, however, decreased the viability of Caco-2 cells by 80% at the concentration of 10 mM which

was used in the transport experiments. Lindmark et al. (1998) have reported similar results with C10: 13 mM C10 decreased the dehydrogenase activity of Caco-2 cells to 22.3 ± 20.2%. It has also been shown that C10 at high concentration (0.5% w/v) damages plasma and nuclear membranes (Sakai et al., 1998). The effects of TMC on the viability of Caco-2 cells were not evaluated in this study due to short of the compound.

In conclusion, the effects of four different absorption enhancers on the permeation of clodronate through Caco-2 cells were studied in the presence and in the absence of calcium. A strong enhancement in clodronate permeation through the cells was observed, only C10 being without any effect. These results indicate that absorption of clodronate could be significantly increased by the absorption enhancers which affect the permeability of the tight junctions. It must, however, be emphasized that the results obtained using Caco-2 cells should not be directly extrapolated to the in vivo situation, because the opening of the tight junctions will only occur in the direct contact with the absorption enhancer and the mucosal surface which is easy to achieve in the Caco-2 cells, but most probably not in the vivo situation. Furthermore, the clinical usefulness of the absorption enhancers remains unclear and should be further clarified.

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