ELSEVIER

International Journal of Pharmaceutics 213 (2001) 135–142

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Effects of calcium and lipophilicity on transport of clodronate and its esters through Caco-2 cells

Johanna Raiman [a,*], Riku Niemi [b], Jouko Vepsäläinen [c], Kirsi Yritys [a], Tomi Järvinen [b], Jukka Mönkkönen [a]

[a] *Department of Pharmaceutics, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland*
[b] *Department of Pharmaceutical Chemistry, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland*
[c] *Department of Chemistry, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland*

Received 23 June 2000; received in revised form 31 October 2000; accepted 2 November 2000

Abstract

Clodronate, like other bisphosphonates, is poorly absorbed from the gastrointestinal tract, mainly due to its high hydrophilicity and ability to form complexes with divalent cations in the gastrointestinal tract. One strategy for improving oral absorption of these types of molecules is to develop more lipophilic derivatives. The importance of lipophilicity and calcium chelation in the absorption of clodronate was evaluated by studying the penetration of clodronate and its mono-, di-, and triphenyl esters through human intestinal Caco-2 cells. The transport rates of [^{14}C]-clodronate and its mono-, di-, and triphenyl esters were quantified by calculating their apparent permeability coefficients (P_{app}) both in normal (1.3 mM) calcium concentration and in 'minimum-calcium model'. The transport rate of 1 mM clodronate was very low (0.25×10^{-7} cm/s), while the removal of calcium from the apical side increased this transport rate 6-fold. The transport rate of clodronate was increased with increasing dose. Mono- and diphenyl esters did not significantly enhance the transport of clodronate. Triphenyl ester, however, increased the transport rate 17-fold compared with parent clodronate. Removal of calcium did not affect the transport rates of di- or triphenyl esters, which indicated that the esterification of hydroxyl groups of clodronate decreased calcium complex formation. These results indicate that clodronate is transported paracellularly through Caco-2 cells and that calcium decreases strongly its absorption. They further suggest that at least three phosphate hydroxyl groups need to be substituted until the permeation route is changed from paracellular to transcellular. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Clodronate; Bisphosphonates; Caco-2 cells

1. Introduction

Clodronate (dichloromethylene bisphosphonate) is a member of bisphosphonate drugs. Bisphosphonates are pyrophosphate analogues,

* Corresponding author. Present address: Department of Pharmacy, Pharmaceutical Technology Division, University of Helsinki, PO Box 56, FIN-00014 University of Helsinki, Finland. Tel.: +358-9-19159161; fax: +358-9-19159144.
E-mail address: johanna.raiman@helsinki.fi (J. Raiman).

0378-5173/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0378-5173(00)00655-4

which inhibit osteoclastic bone resorption and are, therefore, used for treating various disorders characterized by excessive bone loss, such as Paget's disease, hypercalcaemia of malignancy, osteoporosis, and bone metastases. When given orally, bisphosphonates are absorbed to some extent in the stomach and to a larger extent in the upper part of the small intestine (Fleisch, 1997). The oral absorption of bisphosphonates is very low, for example, the bioavailability of oral clodronate in humans is only 1–2% (Yakatan et al., 1982; Pentikäinen et al., 1989). The poor absorption of bisphosphonates is often attributed to their high hydrophilicity, which prevents absorption across the epithelial cells. In addition, poor absorption can also be due to the ability of bisphosphonates to form calcium complexes with divalent cations and in particular with calcium. Further, at the physiological pH of small intestine, bisphosphonates are expected to be highly ionized and negatively charged. Since the paracellular spaces between the cells are also negatively charged, this leads to electrical rejection, which hinders paracellular transport of bisphosphonates (Lin, 1996). There are also large individual differences in the absorption of bisphosphonates, and food reduces the absorption almost to zero (Fogelman et al., 1986).

Hydrophilic drugs, like clodronate, distribute poorly into biological membranes and such drugs are transported across the intestinal epithelium preferably by the paracellular route, though it is not totally excluded that even very hydrophilic drugs may also partly utilize the transcellular route (Artursson et al., 1996). The efficacy of the paracellular route is, however, low due to the small surface area (about 0.01%) of the paracellular route compared with the transcellular route and the tight junctions, which gate the entrance to the paracellular pathway further restricting the transport of drugs (Artursson, 1991). It has previously been shown that the bisphosphonates, tiludronate and pamidronate, are transported across Caco-2 cells by the paracellular route (Boulenc et al., 1993; Twiss et al., 1994). Otherwise the absorption mechanisms and the factors affecting the absorption of bisphosphonates are poorly characterized.

One strategy for improving oral absorption of hydrophilic molecules is to design more lipophilic prodrugs, which could utilize transcellular route for absorption. A suitable promoiety is attached to the parent drug molecule and the physicochemical properties of the drug are improved allowing better absorption through the limiting barrier. The prodrug should release the active parent drug in the gut wall or bloodstream to induce the pharmacological effect (Stella et al., 1985). Partial phenyl esters of clodronate used in this study do not release active clodronate, and thus do not fulfil the criteria for the prodrug approach (Niemi et al., 1997). These phenyl esters are, however, suitable for the use as model compounds to study the effect of increasing lipophilicity on the transport of clodronate.

In this study, Caco-2 cells, which are derived from human colon adenocarcinoma, were used as an in vitro model for assessing clodronate absorption across the intestinal epithelium. Caco-2 cells form confluent monolayers with an apical brush border, well-developed tight junctions, and several other characteristics of differentiated absorptive epithelial cells in the small intestine (Hidalgo et al., 1989). The aim of this study was to evaluate the effect of lipophilicity of clodronate esters on clodronate absorption, as well as to investigate the effects of calcium on the permeability of clodronate and its phenyl esters. The effect of calcium was studied in a 'minimum calcium model' (Nicklin et al., 1995), which has a calcium-free apical donor solution and a basolateral receiver solution containing a calcium concentration of 100 μM to retain the integrity of the monolayer.

2. Materials and methods

2.1. Materials

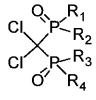
Clodronate and [^{14}C]-clodronate (specific activity of 2.7 μCi/mg) were obtained from Leiras Pharmaceutical Co. (Turku, Finland). The phenyl esters (Table 1) were prepared using the known methods (Vepsäläinen et al., 1993, 1995) at the Department of Chemistry of the University of Kuopio (Kuopio, Finland). D-[1-^{14}C]-mannitol

(specific activity of 51.5 mCi/mmol) was purchased from New England Nuclear (Boston, MA). All the medium components and reagents for cell culture were obtained from Gibco Life Technologies (Paisley, Scotland) and rat tail type I collagen from Collaborative Biomedical Products (Bedford, MA). For the MTT-assay 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MA), sodium dodecyl sulphate (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 maintained in 75 cm^2 culture flasks from Nunc (Roskilde, Denmark) in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum, 1% MEM (100 ×) non-essential amino acids, 1% L-glutamine, and 100 IU/ml of penicillin G and 100 μg/ml streptomycin sulphate, in an atmosphere of 7% CO_2 at 37°C. Before the cells reached full confluency, i.e. 3–4 days, the cells were trypsinated with 0.25% trypsin and 0.02% EDTA and removed from the surface of the flask. After that the cells were collected and counted with a hemocytometer, and about 1 000 000 cells were transferred into a new cell culture flask.

Table 1
Structures of disodium clodronate and phenyl esters of clodronic acid



Compound	R_1	R_2	R_3	R_4
Disodium clodronate	$-O^-Na^+$	-OH	-OH	$-O^-Na^+$
Monophenyl ester		$-O^-Na^+$	$-O^-Na^+$	$-O^-Na^+$
P,P-diphenyl ester			$-O^-Na^+$	$-O^-Na^+$
P,P'-diphenyl ester				
Triphenyl ester				$-O^-Na^+$

For the transport experiments the cells between the passage number of 35–50 were seeded at a density of 80 000 cells per cm^2 on the permeable polycarbonate membranes (Transwell cell culture inserts; 24-mm diameter, 3.0-μm pore size; Costar Corporation, Cambridge, MA), which had previously been coated with rat tail type I collagen. The fresh culture medium (apical volume 1.5 ml and basolateral volume 2.6 ml) was replaced every other day to the cells. The cell monolayers were used for the transport experiments 21–28 days after the cells were seeded on the membranes. The integrity of each batch of the cell monolayers was determined by measuring the transport of [^{14}C]-mannitol (0.3 μCi/ml) through three individual monolayers.

2.3. Transport experiments

The transport experiments were performed in normal (1.3 mM) and in 'minimum calcium concentration'. In the 'minimum calcium model', a calcium- and magnesium-free Hank's balanced salt solution (HBSS) was used as an apical donor solution and HBSS containing 100 μM calcium concentration as a basolateral receiver solution. Prior to the experiments, the cells were washed twice with HBSS containing Ca and Mg in normal model and in the minimum calcium model first with HBSS without Ca and Mg, and after that the apical sides were washed with HBSS without Ca and Mg and the basolateral sides with HBSS containing 100 μM calcium. The transport experiments were performed at 37°C in a water bath from the apical to basolateral direction or in the opposite direction. The experiments were initiated by adding clodronate (1, 10, or 50 mM) (0.44 μCi/ml) or phenyl ester (1 mM) solution in HBSS pH 7.4 to the apical or basolateral compartment, and the samples were withdrawn from the opposite side at various times up to 180 min. The volume removed was always replaced with fresh prewarmed HBSS. The influence of various clodronate concentrations (1, 10, 50 mM) and phenyl esters (1 mM) on the integrity of Caco-2 cell monolayers was studied by measuring the effect of the drugs on the transport of [^{14}C]-mannitol. [^{14}C]-mannitol or [^{14}C]-clodronate samples

Table 2
The chromatographic conditions and retention times for the partial phenyl esters of clodronate

Compound	% A[a]	% B[b]	Retention time (min)[c]
Monophenyl ester	88	12	4.4
P,P-Diphenyl ester	70	30	4.2
P,P′-Diphenyl ester	75	25	5.0
Triphenyl ester	55	45	5.2

[a] KH_2PO_4 (20 mM; pH 5.8), containing 60 mM butylamine.
[b] 80% Acetonitrile in water.
[c] At a flow rate of 1.0 ml/min.

(100 μl) were mixed with 4.5 ml of scintillation liquid (Ultima Gold, Packard, Groningen, the Netherlands) and the [^{14}C] activity was determined by liquid scintillation counting (LKB Wallac 1218 Rackbeta Liquid Scintillation Counter, Wallac, Turku, Finland). The concentrations of phenyl ester samples were analyzed by HPLC–UV assay. HPLC determinations were performed with a Merck LaChrom HPLC system consisting of a Model L-7250 programmable autosampler, Model L-7100 HPLC pump, Model D-7000 interface module, Model L-7400 UV-detector, and a Model D-7000 HPLC system manager (Hitachi Ltd., Tokyo, Japan). The injection loop was a 100 μl stainless steel loop and injection volumes of 20 μl were used. The UV-detector was set at a wavelenght of 205 nm. A Purospher RP-18 (125 × 4 I.D., 5 μm) (Merck, Darmstadt, Germany) was used as an analytical column. The mobile phase consisted of a mixture of (A) 20 mM phosphate buffer (KH_2PO_4 pH 5.8) containing 60 mM butylamine as an ion-pair reagent, and (B) 80% acetonitrile in water. The mobile phase was delivered at a flow-rate of 1.0 ml/min. All separations were carried out by isocratic elutions and the mobile phase compositions and retention times for the analytes are shown in Table 2.

The apparent permeability coefficients (P_{app}) were calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{1}{AC_0},$$

where dQ/dt is the permeability rate; C_0 is the initial concentration in the donor compartment and A is the surface area of the monolayer.

2.4. MTT assay

The effect of clodronate and its phenyl ester derivatives on intracellular dehydrogenase activity of the cells was studied by the MTT method. MTT assay is a colorimetric method for determining cell viability based on reduction of the yellow tetrazolium salt MTT to a purple formazan dye by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). Briefly, the Caco-2 cells were seeded on 96-well tissue culture plate (Nunc, Roskilde, Denmark) at a density of 50 000 cells per well. After 24 h, the cells were exposed to drug solutions from half-logarithmic dilution series for 3 h at 37°C in air. Then the medium was aspirated and exchanged to the serum-free medium and 25 μl of MTT-solution (5 mg/ml) was added. After 2 h, 100 μl SDS + DMF buffer pH 4.7 was added to the wells. 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. Determination of distribution coefficients

The distribution coefficients ($\log D_{oct}^{7.4}$) of [^{14}C]-clodronate and its phenylesters were determined by the distribution of the compound between octanol and 0.16 M phosphate buffer (pH 7.4). By overnight shaking, 0.16 M phosphate buffer and 1-octanol were equilibrated and the phases were separated. The drug studied was dissolved in the buffer and the pH was adjusted to 7.4 and mixed with 1-octanol. This mixture was shaken for 60 min at room temperature, and after that octanol and buffer were separated by centrifugation. The phenyl ester concentrations in the buffer phase before and after distribution were analyzed by HPLC. [^{14}C]-clodronate concentration was determined from both 1-octanol and buffer phases by liquid scintillation counting.

2.6. Statistics

All results are expressed as means ± S.D. Statistical differences were determined using one-way analysis of variance (ANOVA), followed by the Fisher's LSD for multiple comparisons (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3. Results

The integrity of the cell monolayer was determined by measuring the transport of [^{14}C]-mannitol through a monolayer. In the normal calcium concentration, $1.1 \pm 0.7\%$ (mean ± S.D., $n = 33$) of the initial mannitol concentration penetrated through the cells in 3 h. Removal of calcium did not affect the integrity of the cell monolayers; $1.1 \pm 0.3\%$ (mean ± S.D., $n = 9$) of mannitol penetrated through the cells in 'minimum calcium model'.

The transport of 1 mM clodronate through Caco-2 cells was very low; the apparent permeability coefficient for 1 mM clodronate from the apical to basolateral direction was $(0.25 \pm 0.07) \times 10^{-7}$ cm/s (mean ± S.D., $n = 6$) and from the basolateral to apical direction $(0.60 \pm 0.04) \times 10^{-7}$ cm/s (mean ± S.D., $n = 3$). Such a low permeability coefficient indicates paracellular transport for clodronate. Removal of calcium from the apical compartment increased 1 mM clodronate transport by a factor of six. Apparent permeability coefficients for 10 and 50 mM clodronate were $(1.65 \pm 1.26) \times 10^{-7}$ ($n = 6$) and $(110.5 \pm 43.5) \times 10^{-7}$ cm/s ($n = 6$), respectively. When the calcium was removed from the medium, the transport of 10 mM clodronate increased about 60-fold, but the transport of 50 mM clodronate did not change in the absence of calcium. In normal calcium concentration, 1 and 10 mM clodronate did not affect the transport of mannitol, while 50 mM clodronate increased significantly mannitol transport through the cellsabout $35.4 \pm 5.2\%$ ($n = 3$) of mannitol being transported in 3 h.

Mono- and diphenyl esters did not considerably enhance the transport of clodronate. The apparent permeability coefficients for monophenyl, *P*,*P*-diphenyl, and *P*,*P*′-diphenyl esters were 1.07, 1.01, and 0.61×10^{-7} cm/s, respectively (Fig. 1). There was no great difference between the transport rates from apical to basolateral and from

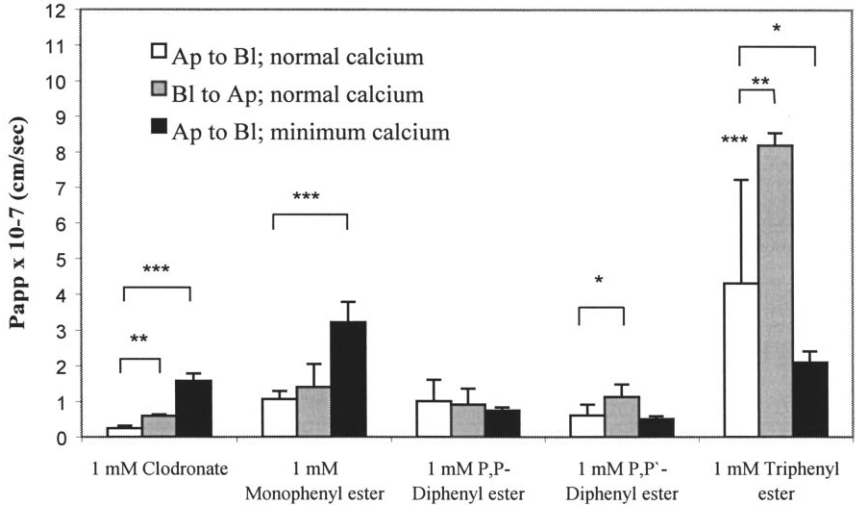


Fig. 1. Apparent permeability coefficients (P_{app}) for 1 mM clodronate, monophenyl ester, *P*,*P*-diphenyl ester, *P*,*P*′-diphenyl ester, and triphenyl ester from the apical to basolateral direction (white columns), from the basolateral to apical direction (grey columns) in normal calcium concentration and in 'minimum calcium model' from the apical to basolateral direction (black columns) ($n = 3$–8) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Table 3
Molecular weights and log $D_{oct}^{7.4}$ values for clodronate and its phenyl esters

	Molecular weight (g/mol)	log $D_{oct}^{7.4a}$
Clodronate disodium salt	289.00	-5.05 ± 0.03
Monophenyl ester trisodium salt	386.93	-3.77 ± 0.04
P,*P*-Diphenyl ester disodium salt	441.05	-3.07 ± 0.06
P,*P*′-Diphenyl ester dipiperidium salt	567.39	-2.68 ± 0.10
Triphenyl ester sodium salt	495.17	0.98 ± 0.00

[a] Each value represents the mean ± S.D. of three experiments.

basolateral to apical directions. Triphenyl ester, however, increased the transport rate 17-fold compared with parent clodronate. From the basolateral to apical direction the transport rate of triphenyl ester was almost twice as fast as the transport rate in the opposite direction, although the deviation from the apical to basolateral direction is large (Fig. 1). The removal of calcium did not affect the transport rates of di- or triphenyl esters, suggesting that these phenyl esters do not form calcium complexes as strongly as clodronate does. The mono-, di-, and triphenyl esters did not have an effect on the transport of mannitol through the cells.

Clodronate and mono-, di-, and triphenyl esters did not affect the viability of the Caco-2 cells, which suggests that these compounds are not toxic for the Caco-2 cells.

For [^{14}C]-clodronate the distribution coefficient (log $D_{oct}^{7.4}$) of -5.05 ± 0.03 (mean ± S.D., $n = 3$) (Table 3) was obtained. This is the first time, to our knowledge, when distribution coefficient for clodronate has been determined experimentally. For the phenyl esters the log $D_{oct}^{7.4}$ values ranged from -3.8 for monophenyl ester to 1.0 for triphenyl ester (Table 3).

4. Discussion

The present study demonstrates that clodronate is poorly absorbed through the Caco-2 cell monolayer. The low permeability coefficient (0.25×10^{-7} cm/s) obtained for 1 mM clodronate confirms that clodronate is transported through the cells paracellularly. Previously it has been shown that two other bisphosphonates, tiludronate and pamidronate, are also absorbed through Caco-2 cells by paracellular route (Boulenc et al., 1993; Twiss et al., 1994). Removal of calcium from the apical side increased the transport 6-fold, which indicates that calcium limits strongly the absorption of clodronate due to the ability of clodronate to form complexes with calcium, and these complexes are poorly water soluble (Ahlmark et al., 1999) and too large to be absorbed through the intercellular spaces. The transport of clodronate was increased when the dose was increased. This effect has also been noticed with other bisphosphonates (Boulenc et al., 1993; Twiss et al., 1994). In our study clodronate also increased the transport of paracellular marker molecule, mannitol, through Caco-2 cells in a concentration-dependent way. This is probably because bisphosphonates as powerful calcium chelators chelate calcium and thus cause loosening of the tight junctions between the epithelial cells (Green et al., 1997).

One strategy to improve oral absorption of hydrophilic molecules is to use more lipophilic derivatives. There has also been interest to modify physicochemical properties of clodronate by synthesizing chemically different kinds of prodrugs (Björkroth et al., 1991; Niemi et al., 1998, 1999; Ahlmark et al., 1999). In this study we have used mono-, di, and triphenyl esters as model compounds. According to our results, one or two phenyl groups in the molecule did not enhance the permeability of the molecule through Caco-2 cells significantly. This is expected, because according to their log $D_{oct}^{7.4}$ values, which range from -3.8 for monophenyl ester to -2.7 for *P*,*P*′-diphenyl ester (Table 3), these molecules are still very hydrophilic. Triphenyl ester has considerably better permeability across the Caco-2 monolayer suggesting that it probably employs transcellular

route. The log $D_{oct}^{7.4}$ value of triphenyl ester (1.0, Table 3) approaches the range needed for efficient passive transcellular transport, but is still below the optimum value of 2–3 (Taylor, 1996). Similar apparent permeability coefficients in the apical to basolateral and in the opposite direction for monophenyl and diphenyl esters indicate that these molecules are not substrates for *P*-glycoprotein efflux pump. However, the two times lower permeability of triphenyl ester from the apical to basolateral side compared with the basolateral to apical side suggests that triphenyl ester could be a substrate of *P*-glycoprotein efflux mechanism. This needs, however, further clarification.

According to the MTT assay results, concentrations of 1 mM clodronate and its partial phenyl esters are not toxic to the cells. In the clinical use, clodronate is generally well tolerated and causes only mild adverse gastrointestinal effects (Plosker and Goa, 1994). More serious gastrointestinal compliants are often attributed to the nitrogen containing bisphosphonates (Fleisch, 1997).

In summary, clodronate is transported through the paracellular route and calcium strongly limits its absorption. The present study shows that the absorption mechanism of clodronate can be changed from a paracellular to a transcellular pathway by more lipophilic esters, but at least three phosphate hydroxyl groups need to be substituted until the change of absorption pathway is realized.

Acknowledgements

This study was part of Biomacromolecule Research Program at the University of Kuopio and supported in part by the Technical Development Center, Finland (TEKES) and Leiras Pharmaceutical Co., Finland.

References

Ahlmark, M., Vepsäläinen, J., Taipale, H., Niemi, R., Järvinen, T., 1999. Bisphosphonate prodrugs: synthesis and in vitro evaluation of novel clodronic acid dianhydrides as bioreversible prodrugs of clodronate. J. Med. Chem. 42, 1473–1476.

Artursson, P., 1991. Cell cultures as models for drug absorption across the intestinal mucosa. Crit. Rev. Ther. Drug Carrier Syst. 8, 305–330.

Artursson, P., Palm, K., Luthman, K., 1996. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv. Drug Deliv. Rev. 22, 67–84.

Björkroth, J.-P., Pakkanen, T.A., Lindroos, J., Pohjala, E., Hanhijärvi, H., Laurén, L., Hannunniemi, R., Juhakoski, A., Kippo, K., Kleimola, T., 1991. Comparative molecular field analysis of some clodronic acid esters. J. Med. Chem. 34, 2338–2343.

Boulenc, X., Marti, E., Joyeux, H., Roques, C., Berger, Y., Fabre, G., 1993. Importance of the paracellular pathway for the transport of a new bisphoshonate using the human Caco-2 monolayers model. Biochem. Pharmacol. 46, 1591–1600.

Fleisch, H., 1997. Bisphosphonates in bone disease from the laboratory to the patient, third ed. The Parthenon Publishing Group, New York.

Fogelman, I., Smith, L., Mazess, R., Wilson, M.A., Bevan, J.A., 1986. Absorption of oral diphosphonate in normal subjects. Clin. Endocrinol. 24, 57–62.

Green, J.R., Clay, V., Richardson, J., Hassan, I.F., 1997. The effect of zoledronate and pamidronate on the intestinal permeability barrier in vitro and in vivo. Int. J. Pharm. 153, 59–66.

Hidalgo, I.J., Raub, T.J., Borchardt, R.T., 1989. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96, 736–749.

Lin, J.H., 1996. Bisphosphonates: a review of their pharmacokinetic properties. Bone 18, 75–85.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Nicklin, P.L., Irwin, W.J., Hassan, I.F., Mackay, M., 1995. Development of a minimum-calcium Caco-2 monolayer model: calcium and magnesium ions retard the transport of pamidronate. Int. J. Pharm. 123, 187–197.

Niemi, R., Taipale, H., Ahlmark, M., Vepsäläinen, J., Järvinen, T., 1997. Simultaneous determination of clodronate and its partial ester derivatives by ion-pair reversed-phase high-performance liquid chromatography coupled with evaporative light-scattering detection. J. Chromatogr. B 701, 97–102.

Niemi, R., Pennanen, H., Vepsäläinen, J., Taipale, H., Järvinen, T., 1998. Bisphosphonate prodrugs: synthesis and in vitro evaluation of novel partial amides of clodronic acid. Int. J. Pharm. 174, 111–115.

Niemi, R., Vepsäläinen, J., Taipale, H., Järvinen, T., 1999. Bisphosphonate prodrugs: synthesis and in vitro evaluation of novel acyloxyalkyl esters of clodronic acid. J. Med. Chem. 42, 5053–5058.

Pentikäinen, P.J., Elomaa, I., Nurmi, A.-K., Kärkkäinen, S., 1989. Pharmacokinetics of clodronate in patients with

metastatic breast cancer. Int. J. Clin. Pharmacol. Ther. Toxicol. 27, 222–228.

Plosker, G.L., Goa, K.L., 1994. Clodronate a review of its pharmacological properties and therapeutic efficacy in resorptive bone disease. Drugs 47, 945–982.

Stella, V.J., Charman, W.N.A., Naringrekar, V.H., 1985. Prodrugs do they have advantages in clinical practice. Drugs 29, 455–473.

Taylor, M.D., 1996. Improved passive oral drug delivery via prodrugs. Adv. Drug Deliv. Rev. 19, 131–148.

Twiss, I.M., De Water, R., Den Hartigh, J., Sparidans, R., Ramp-Koopmanschap, W., Brill, H., Wijdeveld, M., Vermeij, P., 1994. Cytotoxic effects of pamidronate on monolayers of human intestinal epithelial (Caco-2) cells and its epithelial transport. J. Pharm. Sci. 83, 699–703.

Vepsäläinen, J., Nupponen, H., Pohjala, E., 1993. Bisphosphonic compounds. V. Selective preparation of (dichloromethylene) bisphosphonate partial esters. Tetrahedron Lett. 34, 4551–4554.

Vepsäläinen, J., Kivikoski, J., Ahlgren, M., Nupponen, H., Pohjala, E., 1995. An improved synthetic method and the first crystal structures for (dihalomethylene)bisphosphonate partial esters. Tetrahedron 51, 6805–6818.

Yakatan, G.J., Poynor, W.J., Talbert, R.L., Floyd, B.F., Slough, C.L., Ampulski, R.S., Benedict, J.J., 1982. Clodronate kinetics and bioavailability. Clin. Pharmacol. Ther. 31, 402–410.