ELSEVIER

International Journal of Pharmaceutics 153 (1997) 59–66

international journal of pharmaceutics

The effect of zoledronate and pamidronate on the intestinal permeability barrier in vitro and in vivo

Jonathan R. Green [a,*], Val Clay [b], Joanne Richardson [b], Ian F. Hassan [b]

[a] *Novartis Pharma AG, K-125.9.01, Basle, CH-4002, Switzerland*
[b] *Novartis Pharmaceuticals Ltd., Horsham, RH12 4AB, England, UK*

Received 4 December 1996; received in revised form 13 January 1997; accepted 2 April 1997

Abstract

The intestinal tolerability profile of the potent bisphosphonate compound zoledronate (CGP 42'446) has been compared with that of pamidronate in 2 preclinical screening models. Despite being 2–3 orders of magnitude more potent than pamidronate as an inhibitor of bone resorption, zoledronate (1–100 mM) was 2–4 fold less potent at disrupting the permeability barrier of monolayers of an intestinal epithelial cell line (Caco-2) in vitro. In an acute in vivo rat model, luminal perfusion of ileal loops with zoledronate, pamidronate or EDTA at a concentration of 30 mM disrupted the intestinal permeability barrier within 1 h whereas 1 and 10 mM solutions had no effect. Since both EDTA and bisphosphonates are powerful calcium chelators, these changes are most probably due to calcium sequestration and a consequent loosening of tight junctions between the intestinal epithelial cells rather than to a specific pharmacological action. Thus, in comparison to pamidronate, the high potency of zoledronate as an inhibitor of bone resorption is not associated with a corresponding increase in the compound's potential to damage the intestinal mucosa. From these preclinical studies, it is predicted that zoledronate should have a higher therapeutic ratio than pamidronate (anti-resorptive potency in bone versus adverse intestinal effects) in man. © 1997 Elsevier Science B.V.

Keywords: Bisphosphonate; Zoledronate; Pamidronate; Caco-2; Intestine; Tolerability

1. Introduction

Bisphosphonates have found widespread clinical use as inhibitors of osteoclastic activity in a variety of diseases characterized by elevated bone resorption, notably tumour-induced hypercalcemia, Paget's disease, osteoporosis and more recently, bone metastases (Fleisch, 1995; Berenson et al., 1996). Bisphosphonates are powerful cation chelators (Grabenstetter and Cilley, 1971; Wiers, 1971) and form polymeric aggregates with calcium

* Corresponding author. Tel: +41 61 6964415; fax: +41 61 6963849; email: jonathan.green@chbs.mhs.ciba.com

PII S0378-5173(97)00094-X

in biological systems. As a result, these compounds have a high affinity for the mineralized bone matrix (Bisaz et al., 1978), a property which contributes to their potent pharmacological effect on this target tissue.

However, calcium complexation in the gastrointestinal tract is also a major factor contributing to the low bioavailability of these compounds, typically less than 1% for all the currently available bisphosphonates (Fleisch, 1995). In order to be therapeutically effective, these compounds have to be given either by intravenous infusion, or in much larger oral doses to compensate for their low absorption. Oral administration of bisphosphonates may be associated with a variety of adverse gastrointestinal reactions (Fleisch, 1995). Although these are usually only mild to moderate and do not normally require interruption of therapy, there have been reports of severe reactions to oral bisphosphonates (Lufkin et al., 1994; De Groen et al., 1996).

Therefore, in the preclinical profiling of new, highly potent bisphosphonates there is clearly a need to investigate intestinal tolerability to ascertain whether this unwanted adverse effect could be dissociated from the desired, potent inhibition of bone resorption. To this end, the intestinal tolerability profile of the new potent bisphosphonate zoledronate was compared to that of the reference compound pamidronate in two experimental models:

1. the human intestinal epithelial Caco-2 cell line in vitro;
2. the perfused ileal loop of the rat in vivo.

It should be emphasized that these investigations were performed as part of the preclinical profiling of research compounds in order to select development candidates. They were not intended to replace the more extensive mandatory toxicological studies.

2. Materials and methods

2.1. General materials

All tissue culture reagents were purchased from Gibco. D-[1-^{14}C]-mannitol (55 Ci/mol) and D-[^{3}H(G)]-raffinose [10 Ci/mmol) were obtained from New England Nuclear. The MTT-based in vitro cytotoxicity assay kit and reagents for the LDH assay were supplied by Sigma.

Flux experiments were performed in a 'flux medium' comprising Hank's balanced salt solution (HBSS) buffered to pH 7.4, with 25 mM HEPES and 0.1% bovine serum albumin. A 'calcium-free medium' was prepared from calcium- and magnesium-free HBSS (Gibco). Calcium chloride (100 μM) was added to the calcium-free medium to give a 'minimum calcium medium' (Nicklin et al., 1995).

2.2. Test compounds

Two bisphosphonates were investigated in this study: pamidronate [3-amino-1-hydroxypropylidene-1,1-bisphosphonic acid] and zoledronate [2-(imidazol-1-yl)-1-hydroxyethylidene-1,1-bisphosphonic acid]. They were both synthesized by the Chemistry Department, Pharmaceuticals Division, Ciba-Geigy Ltd., Basle. To improve solubility for routine use, the free acids were converted to sodium salts by addition of two equivalents of 1N NaOH. Ten-fold concentrated stock solutions of the bisphosphonates were then prepared in phosphate-buffered saline, pH 7.4 and diluted for use in 0.9% (w/v) saline or HBSS medium.

2.3. Caco-2 cells

The Caco-2 human colon adenocarcinoma cell line (Fogh et al., 1977) was obtained from Professor C. Hopkins (Imperial College, University of London). Cells of passage number 80–100 were used for these experiments.

2.4. Animals

All the in vivo experiments described in this report were performed with male rats, 180–230 g body weight, strain Tif:RAIf(SPF), supplied by the animal farm of Ciba-Geigy Ltd. (Sisseln, Switzerland).

2.5. Caco-2 cell monolayers in vitro

Caco-2 cells were seeded at a concentration of 2×10^6 cells per well onto permeable polycarbonate filters (Transwell™, Costar) and grown for 14–17 days in Dulbecco's minimal essential medium supplemented with foetal calf serum (10% v/v), non-essential amino acids (1% w/v) and penicillin/streptomycin (1% w/v). The monolayers were equilibrated in the appropriate flux medium for 15 min at 37°C. The apical medium was replaced with 2 ml of donor solution containing 20 μM [^{14}C]-mannitol at a concentration of 0.5×10^6 dpm/ml, together with various concentrations of unlabelled bisphosphonate. At various time intervals, 2.5 ml aliquots of medium were removed from the receiving chamber and replaced with fresh medium. After the final sample of receiver medium had been taken, the medium was replaced in both the donor and receiver chambers and the cells were incubated for a further 20 min at 37°C. The transepithelial electrical resistance (TER) of the monolayers was calculated from potential difference measurements as described previously (Nicklin et al., 1995). The resistance was corrected for the resistance of a blank filter without cells and multiplied by the filter area (4.71 cm^2). The filters were washed three times in phosphate-buffered saline containing 0.04% (w/v) sodium azide at 4°C and retained. Media and filters were assayed for radioactivity by liquid scintillation counting.

2.6. Cytotoxicity assays

The effect of the bisphosphonates on cell integrity was measured by the release of LDH and by a reduction in the conversion of MTT to the insoluble formazan derivative. Caco-2 cells were seeded at a concentration of 2×10^5 cells per well into 24-well plates and grown for 6 days as described above but without penicillin/streptomycin. The cells were preincubated for 30 min at 37°C in 1 ml of minimum calcium medium. The medium was replaced with 0.5 ml of the same medium containing pamidronate or zoledronate at concentrations of 1, 5, 10 and 25 mM. Controls contained medium alone or medium containing 0.05% Triton X-100. After 2 h at 37°C, 20 μl aliquots of medium were transferred to a 96-well plate to measure LDH activity. To each aliquot was added 235 μl of a solution containing 0.1M KH_2PO_4 (pH 7.2), 0.69 mM sodium pyruvate and 0.1 mg/ml reduced β-nicotinamide adenine dinucleotide (NADH) and the assay was allowed to proceed for 3 min. The reaction was stopped with 50 μl oxamic acid and the optical absorbance at 340 nm was measured in a microplate reader. The cell monolayer was then stained using the MTT assay kit as follows. To each well 0.18 ml of flux medium minus phenol red was added, followed by 20 μl of a 5 mg/ml solution of MTT in the same medium. After 1 h at 37°C, 200 μl of MTT solubilization buffer (10% Triton X-100 in acidic isopropanol) was added and the plate was shaken for 30 min to dissolve the precipitate. The optical absorbance of 100 μl aliquots was measured at 570 nm in a microplate reader.

2.7. Acute intestinal tolerability in the perfused rat ileal loop in vivo

An acute in vivo rat model, based on the leakage of a plasma marker into the ileal lumen, was developed to test the local intestinal tolerability of drugs. The non-metabolizable saccharide, raffinose (mol. wt. 504), was used as a marker compound, since it does not normally traverse the intact intestinal mucosa (Hamilton et al., 1987). Given a stable plasma level of this marker, its appearance in the luminal perfusate is an early measure of damage to the mucosal permeability barrier (e.g. loosening of the tight junctions between the epithelial cells).

Rats were anaesthetized with Inactin® (100 mg/kg i.p.), the trachea was cannulated to maintain a patent airway and cardiovascular function was monitored via a cannula in the carotid artery. Body temperature was measured with a rectal thermocouple and automatically maintained at 37°C with an infrared lamp. In order to prevent renal clearance and maintain a stable plasma level of the raffinose, the renal arteries, veins and ureters were ligated bilaterally via an abdominal incision. A 10 cm length of distal ileum was cannulated at both ends, the luminal contents

were rinsed out with 0.9% saline, and the loop was then continuously perfused with fresh saline from a reservoir at a flow rate of 10 ml/h. The perfusate was collected in 2.5 ml fractions every 15 min. All perfusion solutions were warmed to 37°C by passage through a heating coil controlled by a thermocouple mounted in the cannula at the entry point into the ileal loop.

After a 2-h stabilization period, a 0.5-ml loading bolus of raffinose (0.15 mM) was injected intravenously to saturate any binding sites, followed 10 min later by a similar bolus injection labelled with [^{3}H]-raffinose (10 μCi). Perfusion of the ileal loop and collection of the perfusate was continued, first with saline for 1 h and then for a further 2 h with a solution of the test compound (3, 10 or 30 mM) adjusted to isotonicity by the addition of solid NaCl. Arterial blood samples were collected at 1 and 3 h after injection of the radiolabel. At the end of the experiment the rat was killed with an overdose of Inactin and a urine sample was collected from the bladder. The level of radioactivity in the samples of blood, urine and ileal perfusate was determined by liquid scintillation counting.

3. Results

3.1. Effect of bisphosphonates on the integrity of Caco-2 cell monolayers

The effect of pamidronate and zoledronate on the flux of [^{14}C]-mannitol across the Caco-2 cell monolayer is shown in Fig. 1. The apical donor chamber contained calcium-free medium and the receiver chamber contained flux medium. Both bisphosphonates increased mannitol flux in a dose-dependent manner at concentrations of 5 mM and above, but the effect of pamidronate was more pronounced than that of zoledronate. As the lower concentrations of zoledronate had a relatively small effect, an additional higher concentration of 100 mM was also tested and it produced an effect similar in magnitude to that of 50 mM pamidronate. In the controls without bisphosphonate, the mean mannitol flux (expressed as a percentage $\pm$ S.D. of the donor solution) increased from 0.26 ± 0.09 at 15 min to 1.1 ± 0.27 at 120 min.

The TER was measured across the cell monolayers at the end of the experiments described above and the results are shown in Fig. 2 (solid symbols). Pamidronate at concentrations of 1 mM and above decreased the TER, whereas with zoledronate a decrease was first observed at a concentration of 5 mM. For each concentration of bisphosphonate the decrease in TER produced by zoledronate was lower than that obtained with an identical concentration of pamidronate. At the higher concentrations of bisphosphonate, the TER values reached a plateau of about -70%, indicating that the electrical resistance barrier of the cell monolayer had sustained maximal damage. The mean ($\pm$ S.D.) control TER value of cell monolayers incubated for 2 h in medium lacking bisphosphonate was 412 ± 59 ohms.cm^2.

In addition to the TER curves, Fig. 2 also shows a replot of the mannitol flux data (open symbols) from Fig. 1 to permit a direct comparison of the effects of the 2 bisphosphonates on both parameters. From these dose-response

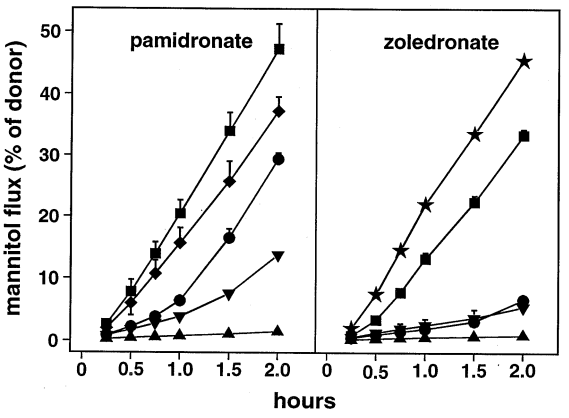

Fig. 1. The effect of pamidronate and zoledronate on the permeability barrier of Caco-2 cell monolayers, as measured by [^{14}C]-mannitol flux. Unlabelled bisphosphonate was added to the apical chamber at concentrations of 1 mM (▲), 5 mM (▼), 10 mM (●), 25 mM (◆), 50 mM (■) or 100 mM (★), together with 20 μM [^{14}C]-mannitol in calcium-free medium. The total accumulation of [^{14}C]-mannitol in the basal chamber was determined as described in Section 2. Mean values of triplicate experiments are shown; for clarity, error bars (S.D.) are only given where they are not obscured by the symbols.

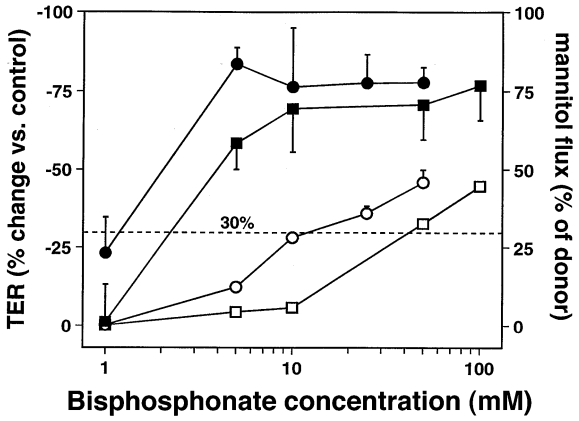

Fig. 2. Dose-response curves for the effects of pamidronate (circles) and zoledronate (squares) on the transepithelial electrical resistance (TER) and mannitol flux across Caco-2 cell monolayers. TER (solid symbols) was measured as described in Section 2 at the end of the experiments, mean percentage changes versus control ($\pm$S.D.) are given from triplicate measurements on each filter, with 3 filters per concentration. For mannitol flux (open symbols), the data for the 2 h time point from Fig. 1 have been recalculated as percent of the total counts initially present in the apical donor chamber.

curves, EC_{30} values (i.e. the concentration of bisphosphonate required to alter the given parameter by 30% – an arbitrary level) were calculated by graphical interpolation. For mannitol flux, the EC_{30} value of zoledronate is 4-fold higher than that of pamidronate (40.7 mM and 10.3 mM, respectively), whereas for the TER the EC_{30} value of zoledronate is 1.9-fold higher than that of pamidronate (2.3 mM and 1.2 mM, respectively).

Cytotoxic effects of the bisphosphonates were measured by monitoring the release of LDH or the conversion of MTT to formazan. Neither compound caused any increase in LDH levels in the medium (data not shown) so there was no evidence of gross damage to the cell membrane. By contrast, both compounds caused some reduction in the conversion of MTT (Table 1). The Caco-2 cells appeared to be slightly more sensitive to pamidronate than zoledronate, with the onset of toxicity being observed at 10 mM and 25 mM, respectively, but this limited amount of data did not permit statistical analysis.

3.2. Acute intestinal tolerability in the perfused rat ileal loop in vivo

Preliminary control experiments with the model demonstrated that stable plasma levels of [^{3}H]-raffinose were maintained for several hours with no radioactivity detectable in urine, and very little leakage of the marker into the ileal loop lumen when it was perfused with saline (data not shown). As a positive control for the model, solutions of the powerful calcium chelator ethylenediaminetetraacetic acid (EDTA) at concentrations of 1, 10 and 30 mM were perfused through the ileal loops. It was predicted that EDTA would remove calcium ions from the tight junctions between the mucosal cells and cause leakage of the plasma marker raffinose into the luminal perfusate. As shown in Fig. 3 (upper panel), perfusion of a 30-mM solution of EDTA caused a pronounced increase in marker leakage, beginning within 1 h and continuously increasing until the experiment was terminated after another hour. Perfusion with the 2 lower concentrations of EDTA (3 and 10 mM) had no detectable effect.

Fig. 3 (middle panel) shows the effect of an intraluminal perfusion of pamidronate on the leakage of the plasma label into the perfusate. Concentrations of 3 and 10 mM pamidronate had little effect whereas perfusion with 30 mM pamidronate caused a large increase in leakage of the plasma marker into the ileal lumen within 1 h.

Table 1
The effect of pamidronate and zoledronate on the viability of Caco-2 cells

Concentration (mM)	MTT activity (% of control)	
	Pamidronate	Zoledronate
0	100	100
1	96 $\pm$ 4	92 $\pm$ 6
5	92 $\pm$ 7	96 $\pm$ 4
10	85 $\pm$ 3	94 $\pm$ 6
25	72 $\pm$ 12	68 $\pm$ 14

The conversion of MTT to formazan was measured as described in Section 2 following a 2-h incubation in the presence of increasing concentrations of bisphosphonate (1–25 mM). Results are the mean and range of duplicate incubations.

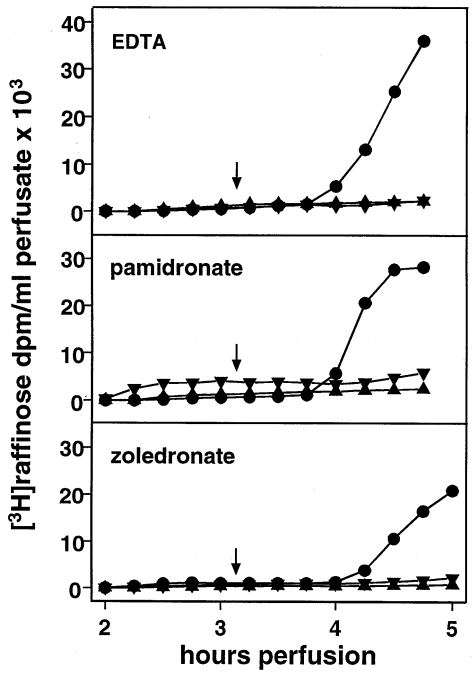

Fig. 3. Effect of intraluminal perfusion of EDTA, pamidronate or zoledronate on the mucosal permeability of rat ileal loops in vivo. Mucosal permeability was measured by the leakage of [^{3}H]-raffinose from the plasma into the luminal perfusate. Saline was perfused intraluminally at 10 ml/h for 3 h followed by a solution of the test compound (↓) at the same flow rate for approximately 2 h at concentrations of 3 mM (▲), 10 mM (▼) or 30 (●).

Similarly with zoledronate, an increase in the leakage of the marker was first observed at a bisphosphonate concentration of 30 mM, with the lower concentrations of 3 and 10 mM having no effect (Fig. 3, lower panel). Despite a slightly longer perfusion time, the maximum radioactivity (dpm/ml) measured in the luminal fluid during perfusion with 30 mM zoledronate was 25% and 40%, respectively, lower than that observed with identical concentrations of pamidronate or EDTA. Additional data would be required to test whether this difference is statistically significant.

4. Discussion

The human Caco-2 cell line has been used extensively to model the intestinal transport and cytotoxic characteristics of drugs in vitro (Artursson, 1990; Artursson and Magnusson, 1990; Hilgers et al., 1990; Wilson et al., 1990). In order to avoid the formation of insoluble bisphosphonate-calcium complexes in this model, a modified system with a minimum calcium concentration was developed by Nicklin et al. (1995).

In this minimum-calcium Caco-2 cell model, both pamidronate and zoledronate had broadly similar properties, as would be predicted from the dominant influence of the common bisphosphonate group. Nevertheless important quantitative differences were apparent: both compounds increased mannitol flux, indicating a reduction in the permeability barrier of the cell monolayer, but the EC_{30} value of zoledronate was 4 times that of pamidronate. When the TER was measured as an indicator of monolayer integrity, the EC_{30} value of zoledronate was 1.9 times that of pamidronate. Similar detrimental effects of bisphosphonates on the integrity of Caco-2 cell monolayers in vitro have been reported previously (Twiss et al., 1994; Boulenc et al., 1995; Nicklin et al., 1995). This effect of bisphosphonates on monolayer integrity is most probably due to a loosening of the tight junctions between cells as a result of calcium chelation and a consequent increase in paracellular transport. This conclusion is supported by the finding of similar changes in Caco-2 cell monolayers after treatment with the calcium chelator EGTA (Artursson and Magnusson, 1990; Boulenc et al., 1995).

In our experiments with a minimum concentration of calcium in the medium, neither pamidronate nor zoledronate (1–25 mM) had any detectable effect on the viability of the Caco-2 cell monolayer as measured by LDH release. This contrasts with the findings of Twiss et al. (1994), obtained with a conventional calcium-containing medium, who reported that pamidronate (9–33 mM) did increase LDH release. However, with the more sensitive MTT assay, cell viability was dose-dependently decreased by pamidronate whereas zoledronate appeared to be less cytotoxic.

In the acute rat model of intestinal tolerability in vivo, the threshold concentration at which luminally perfused solutions of pamidronate or zoledronate began to cause damage to the ileal

mucosa was in the range 10–30 mM. Again in this assay, the effect of zoledronate was less pronounced than that of pamidronate. As EDTA solutions produced similar effects, the most probable cause of the damage is sequestration of calcium ions from the cellular tight junctions of the mucosal epithelium, resulting in increased paracellular transport of the plasma marker. This effect of EDTA on the intestinal permeability barrier is well known (Cassidy and Tidball, 1967; Nadai et al., 1972) and can be utilized to increase the oral bioavailability of poorly absorbed drugs (Schanker and Johnson, 1961; Windsor and Cronheim, 1961), as can bisphosphonates (Shrewsbury et al., 1982; Gural et al., 1985; Van Hoogdalem et al., 1989).

From our in vitro results we conclude that both pamidronate and zoledronate (1–50 mM) have qualitatively similar effects on the integrity and viability of the Caco-2 cell monolayer. However, for a given concentration of bisphosphonate, the effect of pamidronate was approximately 2–4 fold more pronounced than that of zoledronate, despite the fact that the latter is 2–3 orders of magnitude more potent as an inhibitor of bone resorption (Green et al., 1994). Thus it appears that the observed effect on the intestinal mucosa is predominantly due to the calcium chelating properties of the bisphosphonate group rather than to a specific pharmacological effect. The in vivo results provide further evidence that the gain in potency of zoledronate as an inhibitor of osteoclastic bone resorption is not accompanied by a parallel increase in this compound's potential to damage the intestinal mucosa.

Since zoledronate is so potent, the envisaged clinical dose required to inhibit bone resorption is expected to be in the range 10–100 μg/kg, or 0.6–6.0 mg for a 60-kg patient. It can be calculated that the 6.0 mg dose dissolved in the 24 ml mean fasting volume of gastric juice in the adult stomach (Dubois et al., 1977) would produce a local concentration of 0.7 mM, which is well below the level at which zoledronate had any effect in the present studies (5–25 mM). Thus, based on these preclinical data, it is predicted that zoledronate should have a good intestinal tolerability profile and higher therapeutic ratio than pamidronate (anti-resorptive potency in bone versus adverse intestinal effects) in man.

References

Artursson, P., 1990. Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorbtive (Caco-2) cells. J. Pharm. Sci. 79, 476–482.

Artursson, P., Magnusson, C., 1990. Epithelial transport of drugs in cell culture. II: Effect of extracellular calcium concentration on the paracellular transport of drugs of different lipophilicities across monolayers of intestinal epithelial (Caco-2) cells. J. Pharm. Sci. 79, 595–600.

Berenson, J.R., Lichtenstein, A., Porter, L. et al., 1996. Efficacy of pamidronate in reducing skeletal events in patients with advanced multiple myeloma. New Engl. J. Med. 334, 488–493.

Bisaz, S., Jung, A., Fleisch, H., 1978. Uptake by bone of pyrophosphate, diphosphonates and their technetium derivatives. Clin. Sci. Mol. Med. 54, 265–272.

Boulenc, X., Roques, C., Joyeux, H., Berger, Y., Fabre, G., 1995. Bisphosphonates increase tight junction permeability in the human intestinal epithelial (Caco-2) model. Int. J. Pharm. 123, 13–24.

Cassidy, M.M., Tidball, C.S., 1967. Cellular mechanism of intestinal permeability alterations produced by chelation depletion. J. Cell Biol., 32, 685–698.

De Groen, P.C., Lubbe, D.F., Hirsch, L.J. et al., 1996. Esophagitis associated with the use of alendronate. New Engl. J. Med., 335, 1016–1021.

Dubois, A., van Eerdewegh, P., Gardner, J.D., 1977. Gastric emptying and secretion in Zollinger-Ellison Syndrome. J. Clin. Invest. 59, 255–263.

Fleisch, H., 1995. Bisphosphonates in bone disease: from the laboratory to the patient, Parthenon Publishing Group Ltd., New York and London.

Fogh, J., Wright, W.C., Loveless, J.D., 1977. Absence of HeLa cell contamination in 169 cell lines derived from human tumors. J. Natl. Cancer Inst. 58, 209–214.

Grabenstetter, R.J., Cilley, W.A., 1971. Polynuclear complex formation in solutions of calcium ion and ethane-1-hydroxy-1,1-diphosphonic acid. I. Complexometric and pH titrations. J. Phys. Chem. 75, 676–682.

Green, J.R., Müller, K., Jaeggi, K.A., 1994. Preclinical pharmacology of CGP 42'446, a new, potent, heterocyclic bisphosphonate compound. J. Bone Miner. Res. 9, 745–751.

Gural, R.P., Chungi, V.S., Shrewsbury, R.P., Dittert, L.W., 1985. Dose-dependent absorption of disodium etidronate. J. Pharm. Pharmacol. 37, 443–445.

Hamilton, I., Rothwell, J., Archer, D., Axon, A.T.R., 1987. Permeability of the rat small intestine to carbohydrate probe molecules. Clin. Sci. 73, 189–196.

Hilgers, A.R., Conradi, R.A., Burton, P.S., 1990. Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. Pharm. Res. 7, 902–910.

Lufkin, E.G., Argueta, R., Whitaker, M.D. et al., 1994. Pamidronate: an unrecognized problem in gastrointestinal tolerability. Osteoporosis Int. 4, 320–322.

Nadai, T., Kondo, R., Tatematsu, A., Sezaki, H., 1972. Drug-induced histological changes and its consequences on the permeability of the small intestinal mucosa. I. EDTA, tetracycline, and sodium laurylsulfate. Chem. Pharm. Bull. 29, 1139–1144.

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.

Schanker, L.S., Johnson, J.M., 1961. Increased intestinal absorption of foreign organic compounds in the presence of ethylenediaminetetraacetate (EDTA). Biochem. Pharmacol. 8, 421–422.

Shrewsbury, R.P., Metcalf, T.B., Weiss, D.L., Dittert, L.W., 1982. Influence of disodium etidronate on salicylic acid absorption in the rat. J. Pharm. Pharmacol. 34, 823–825.

Twiss, I.M., de Water, R., den Hartigh, J. et al., 1994. Cytotoxic effects of pamidronate on monolayers of human intestinal epithelial (Caco-2) cells and its epithelial transport. J. Pharm. Sci., 83, 699–703.

Van Hoogdalem, E.J., Wackwitz, A.T.E., de Boer, A.G., Breimer, D.D., 1989. 3-Amino-1-hydroxypropylidene-1,1-diphosphonate (APD): a novel enhancer of rectal cefoxitin absorption in rats. J. Pharm. Pharmacol. 41, 339–341.

Wiers, B.H., 1971. Polynuclear complex formation in solutions of calcium ion and ethane-1-hydroxy-1,1-diphosphonic acid. II. Light scattering, sedimentation, mobility, and dialysis measurements. J. Phys. Chem. 75, 682–687.

Wilson, G., Hassan, I.F., Dix, C.J., Williamson, I., Shah, R., Mackay, M., 1990. Transport and permeability properties of human Caco-2 cells: an in vitro model of the intestinal epithelial cell barrier. J. Control. Rel. 11, 25–40.

Windsor, E., Cronheim, G., 1961. Gastro-intestinal absorption of heparin and synthetic heparinoids. Nature 190, 263–264.