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Development of a minimum-calcium Caco-2 monolayer model: calcium and magnesium ions retard the transport of pamidronate

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Abstract

The oral absorption of some drug molecules (e.g., tetracyclines and quinolones) is limited by complexation with di- and trivalent metal cations in the gastrointestinal tract. Progress in the understanding of these absorption-limiting interactions has been restricted in the absence of a suitable in vitro model. To address this, a modification of the conventional Caco-2 transport model has been developed which has a calcium-free apical donor solution and a basolateral receiver solution containing the minimum calcium concentration required to maintain monolayer integrity (100 μM). The minimum-calcium model is proposed to be a useful universal model for studying the influence of metal cations on the transepithelial transport of drug molecules. The influence of calcium and magnesium ions on the absorption of pamidronate was evaluated by comparing its transport across the conventional and minimum-calcium Caco-2 models. In the conventional Caco-2 model, the ratio of mannitol/pamidronate flux was 5:1, whereas in the minimum-calcium model this ratio was reduced to 3:1. The elevated transepithelial transport rate for pamidronate in the minimum-calcium model cannot be explained by minor changes in the permeability of Caco-2 monolayers. It was concluded that calcium and magnesium ions retard the apical-to-basolateral flux of pamidronate across Caco-2 monolayers.

Keywords: Absorption; Caco-2; Chelation; Minimum-calcium model; Pamidronate; Transepithelial transport; Human intestine

1. Introduction

Absorption-limiting drug-metal cation interactions are well recognised. For instance, the oral bioavailability of tetracycline (Albert et al., 1979) and quinolone (reviewed by Lomestro and Baillie, 1991) antibiotics is reduced by co-administration with calcium, magnesium, aluminium or ferrous ions. In the absence of a predictive in vitro absorption model, retardation of drug absorption by chelation with metal ions is usually identified as a clinical interaction. An in vitro absorption screen would offer the following advantages over clinical

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investigations: (i) the ionic environment can be precisely controlled; (ii) convenience and rapidity; and (iii) suitability for early-phase research compounds.

1.1. Caco-2 monolayers

Enterocyte-like Caco-2 cells (Pinto et al., 1983) cultured on permeable supports represent an established drug transport model for the adult human gastrointestinal (GI) epithelium (Hidalgo et al., 1989; Artursson and Magnusson, 1990; Wilson et al., 1990). A drug compound is added to the apical (Ap) donor chamber and its rate of appearance into the basolateral (Bl) receiver chamber is monitored. A correlation between the rate of drug transport and the respective oral bioavailability in humans in vivo has been reported (Artursson and Karlsson, 1991).

Recent studies in our laboratories have shown the conventional Caco-2 model to be useful for demonstrating the influence of ferric ions on the transepithelial transport of an iron chelator (1,2-dimethyl-3-hydroxypyridin-4-one; L1) (Hamilton et al., 1994). In addition to ferric ions, other ions such as calcium can chelate with drug molecules and may influence their absorption. The major problem in studying absorption-limiting drug-calcium interactions, however, is the calcium-dependent nature of Caco-2 monolayer integrity. In the uniform presence of calcium ions (1260 μM; conventional model), Caco-2 cells form monolayers with a high transepithelial electrical resistance (TER) and a low permeability to hydrophilic transport markers (e.g., mannitol, polyethylene glycol 4000 (PEG_{4000})). Under calcium-free conditions, calcium-dependent intercellular contacts are compromised and the capacity of the paracellular pathway is increased. This loss of monolayer integrity is characterised by a decrease in TER and a high flux for hydrophilic permeability markers. Presently, the requirement of calcium ions prevents the utilisation of Caco-2 monolayers as a universal model for predicting absorption-limiting drug-metal cation interactions. This study reports a modification of the conventional Caco-2 transport model which has a calcium-free Ap donor solution and a Bl receiver solution containing the minimum calcium concentration required to maintain monolayer integrity.

1.2. Bisphosphonates

Bisphosphonates are potent inhibitors of osteoclastic bone resorption and are increasingly being used in a variety of bone disorders including Paget's disease (Cantrill and Anderson, 1990), bone metastases (Dodwell et al., 1990), tumour-induced hypercalcaemia (Coleman and Rubens, 1987) and osteoporosis (Thiebaud et al., 1994).

The oral bioavailability of this class of compounds is low. For example, Daley-Yates et al. (1991) estimated the oral bioavailability of pamidronate (3-amino-1-hydroxypropylidene-1,1-diphosphonate) to be 0.3% for patients who had breast cancer with bone metastases and were receiving an oral dose of 300 mg. The bioavailability of clodronate (dichloromethylene diphosphonate) was 1–2% in volunteers (Yakatan et al., 1982) and that of etidronate (ethane-1-hydroxy-1,1-diphosphonate) has been shown to be low and variable (Fogelman et al., 1986). The factors which limit the absorption of bisphosphonates from the GI tract have not been determined. Recent studies suggest the bisphosphonates tiludronate and pamidronate have a paracellular transepithelial transport route (Boulenc et al., 1993; Twiss et al., 1994).

The ability of pamidronate to chelate calcium and magnesium ions has been reported (Francis and Centner, 1978). This chelation potential highlights the possibility that complexation with these ions in the lumen of the GI tract may retard its absorption. In order to assess the influence of calcium and magnesium ions on the absorption of pamidronate, its transport across an equivalent permeability barrier in the presence and total absence of these ions should be compared, however, the calcium dependence of monolayer integrity makes this approach unfeasible. A minimum-calcium Caco-2 monolayer model has therefore been developed which maintains a calcium-free donor solution and has comparable permeability properties to the conventional model.

2. Materials and methods

2.1. Materials

[^{14}C]Pamidronate (8.86 Ci mol^{-1}) and pamidronate were kindly provided by Dr A. Probst (Ciba Pharmaceuticals, Basle, Switzerland). D-[^{3}H]Mannitol (30.0 Ci mol^{-1}) was purchased from New England Nuclear (Dupont, Germany). Citric acid was supplied by BDH (Poole, UK). 7-Nitro-2,1,3-benzoxadiazol-4-yl ceramide (C_6NBD ceramide) and propidium iodide were obtained from Cambridge Bioscience (Cambridge, UK) and Sigma Chemical Co. (Poole, UK), respectively. The incubation medium (IM) used for transport studies comprised Hanks' balanced salt solution (HBSS; Gibco, Paisley, UK; 076-01200 N) containing 0.1% w/v bovine serum albumin (BSA), 0.01% phenol red, 5 mM D-glucose and buffered to pH 7.2 with 14 mM Hepes. A calcium- and magnesium-free version (IM_{-Ca}) was prepared using HBSS without calcium and magnesium salts (Gibco, Paisley, UK; 042-04180). Incubation media were also prepared containing intermediate concentrations of calcium and magnesium ions by appropriate dilutions of IM ([calcium] = 1260 μM; [magnesium] = 811 μM) with IM_{-Ca} and are referred to by their calcium concentration alone.

2.2. Methods

2.2.1. Cell culture

For transport experiments, Caco-2 monolayers were cultivated for 15 days on Transwell™ polycarbonate culture inserts (4.71 cm^2; Costar, USA) as described elsewhere (Artursson, 1990). Cells between passage 95 and 105 were used for this study.

2.2.2. Titration of minimum calcium concentration

Monolayers were washed with IM_{-Ca} (1 × 2 ml (Ap + Bl) × 15 min), then IM_{-Ca} containing citrate (pH 7.4) 1 mM (1 × 1 ml (Ap + Bl) × 1 min) to chelate residual extracellular calcium ions. Finally, monolayers were washed with IM_{-Ca} (1 × 2 ml Ap × 15 min) and IM containing the appropriate concentration of calcium (1 × 2 ml Bl × 15 min). Thereafter, transport experiments were performed using IM_{-Ca} as the Ap donor solution and IM containing the appropriate calcium concentration as the Bl receiver solution.

2.2.3. Transepithelial electrical resistance (TER)

TER was measured at 37° C using an epithelial voltmeter (EVOM, World Precision Instruments, USA) and a TER medium comprising minimum essential medium supplemented with 0.1% w/v BSA (1260 μM calcium and 814 μM magnesium). TER measurements were performed 30 min after a 60 min transport experiment or a 60 min incubation under test conditions. The intrinsic resistance of the system (insert alone) was subtracted from the total resistance (monolayer + insert) to give the resistance of the monolayer. TER values were expressed as Ω cm^2.

2.2.4. Laser-scanning confocal microscopy

For laser-scanning confocal fluorescence microscopy, monolayers were washed with IM_{-Ca} (1 × 2 ml (Ap + Bl) × 15 min), then IM_{-Ca} containing citrate (pH 7.4) 1 mM (1 × 1 ml (Ap + Bl) × 1 min) to chelate residual extracellular calcium ions. Finally, monolayers were washed with IM (1260 μM calcium; conventional), IM_{-Ca} (calcium-free) (both 1 × 2 ml (Ap + Bl) × 15 min) or IM_{-Ca} (1 × 2 ml Ap × 15 min) and IM containing 100 μM calcium (1 × 2 ml Bl × 15 min) (minimum-calcium). Monolayers were incubated for 60 min with IM, IM_{-Ca} or IM_{-Ca} (Ap) and IM containing 100 μM calcium (Bl) then fixed with 4% formaldehyde and 0.25% glutaraldehyde in 0.1 M cacodylate buffer. The cells were subsequently stained for 30 min with C_6NBD ceramide (10 μg ml^{-1}; lipid stain = green) and propidium iodide (10 μg ml^{-1}; nuclear stain = red) in PBS. The samples were viewed using a BioRad 500 laser-scanning confocal microscope.

2.2.5. Pamidronate transport experiments

Caco-2 monolayers were washed with IM_{-Ca} (1 × 2 ml (Ap + Bl) × 15 min), and IM_{-Ca} containing citrate (pH 7.4) 1 mM (1 × 1 ml (Ap + Bl) × 1 min) to chelate residual extracellular calcium ions. Finally, monolayers were washed with IM (1 × 2 ml (Ap + Bl) × 15 min)(conventional

model), IM_{-Ca} (1 × 2 ml (Ap + Bl) × 15 min)(calcium-free model) or IM_{-Ca} (1 × 2 ml Ap × 15 min) and IM containing 100 μM calcium (1 × 2 ml Ap × 15 min)(minimum calcium model). The transport experiment was initiated by applying a donor solution of IM (conventional model) or IM_{-Ca} (calcium-free and minimum-calcium models) containing 0.44 μCi ml^{-1} (50 μM) [^{14}C]pamidronate and 0.50 μCi ml^{-1} D-[^{3}H]mannitol to the Ap chamber. The culture insert was immediately transferred to a receiver chamber containing 3.0 ml of IM (conventional model), IM_{-Ca} (calcium-free model) or IM containing 100 μM calcium (minimum-calcium model) receiver solutions. Ap-to-Bl transport kinetics were followed by sequentially transferring the culture insert into a fresh receiver solution at 30 min intervals. After collection of the final receiver sample, monolayers were transferred to a cold table (0–4° C), the Ap donor solutions collected and their radiochemical concentration determined by liquid scintillation counting (Beckman LS1801).

2.2.6. Data presentation

Apparent permeability coefficients (P_{app}) were calculated according to the equation given below (Artursson and Magnusson, 1990):

$$P_{app} = dQ/dt \cdot 1/(A \cdot C_o)$$

where dQ/dt is the rate of appearance in the receiver solution (mol s^{-1}), C_o represents the initial concentration of the permeant in the donor solution (mol ml^{-1}) and A is the area of the permeable support.

Data are presented as mean values and experimental errors are expressed as standard deviations about that mean. Significance testing was performed using an unpaired Student's t-test assuming equal variance.

3. Results

The influence of calcium ions on Caco-2 monolayer integrity was assessed by three methods: the transport of permeability markers ([^{14}C]PEG_{4000} and D-[^{3}H]mannitol), post-transport TER measurement and by morphological appearance using laser-scanning confocal microscopy.

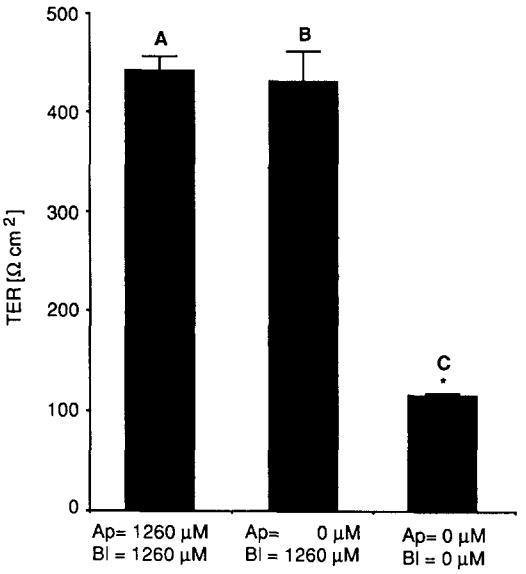

Fig. 1. Influence of the distribution of calcium ions on TER. Caco-2 monolayers were incubated in uniform calcium (A; conventional model), Bl calcium (B) or uniform calcium-free conditions (C). After 60 min these incubation media were replaced with TER medium and the TER measured at 30 min. Data are presented as mean values ± SD for six monolayers. Significant reduction from conventional model values at $^{*}p < 0.01$.

The TER across Caco-2 monolayers under uniform calcium-free conditions (112.1 ± 3.2 Ω cm^2) was 3.9-fold lower than in the presence of calcium ions (conventional; 432.4 ± 15.0 Ω cm^2). Moreover, in the absence of Ap calcium ions, but in the presence of 1260 μM Bl calcium, TER (422.5 ± 30.7 Ω cm^2) was comparable to values for the conventional model (Fig. 1). The Ap-to-Bl transport of drug molecules can therefore be studied across an intact monolayer from a calcium-containing and calcium-free donor solution. However, the use of 1260 μM calcium as the Bl receiver solution with a calcium-free Ap donor solution establishes a large Bl-to-Ap concentration gradient. Flux in proportion to this gradient would result in significant levels of calcium appearing in the Ap donor solution over the duration of the transport experiment. This would complicate the interpretation of the transport

data, therefore, Bl-to-Ap calcium flux should be minimised. The minimum concentration of calcium required in the Bl receiver solution to maintain monolayer integrity was titrated. The use of a low Bl calcium concentration would minimise the Bl-to-Ap flux and simplifies data interpretation.

All monolayer permeability indices were dependent on the Bl calcium concentration. Ap-to-Bl [^{14}C]PEG$_{4000}$ transport (Fig. 2A and B) remained constant as the Bl calcium concentration was reduced from 1260 μM ($0.32 \pm 0.02\%$ h^{-1}) to 75 μM ($0.40 \pm 0.07\%$ h^{-1}). Thereafter, further small decreases produced significantly elevated fluxes (50 μM = $0.54 \pm 0.03\%$ h^{-1}, $p < 0.05$; 25 μM = $0.91 \pm 0.27\%$ h^{-1}, $p < 0.05$). At concentrations of 10 μM and below, high rates of Ap-to-Bl [^{14}C]PEG$_{4000}$ fluxes were observed, suggesting a complete loss of monolayer integrity. Correspondingly, D-[^{3}H]mannitol transport (Fig. 2C and D) showed a similar dependency on the Bl calcium concentration. As the Bl calcium concentra-

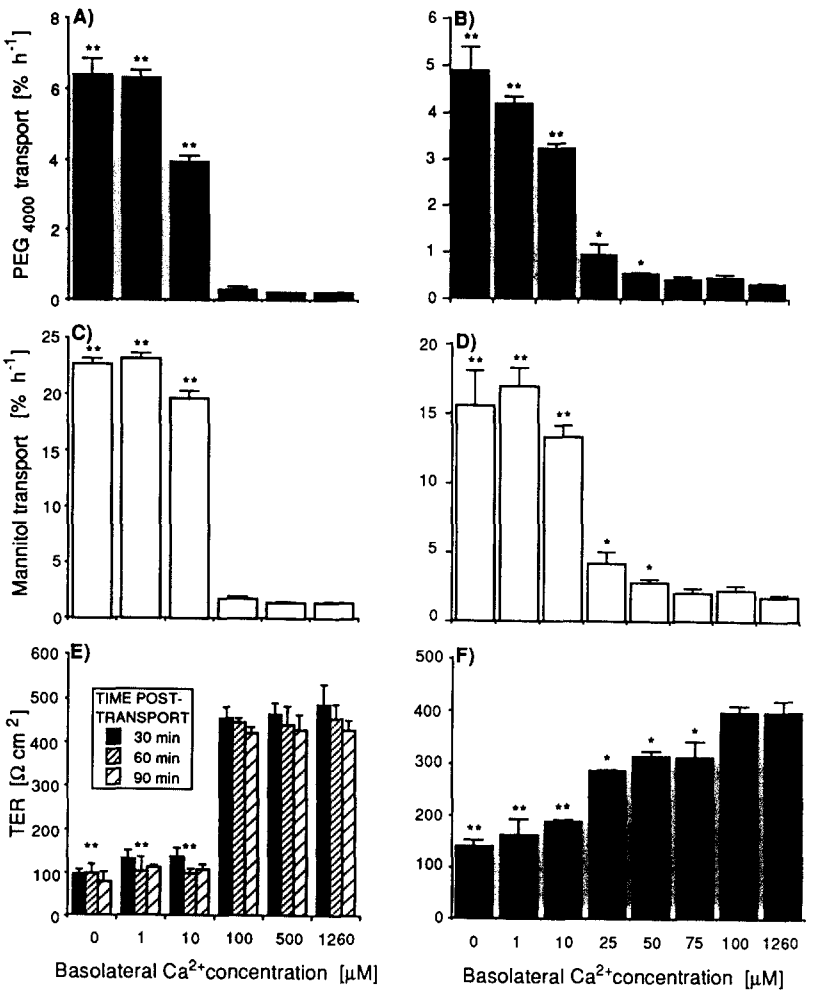

Fig. 2. Influence of Bl calcium concentration on Caco-2 monolayer permeability indices. The influence of basolateral calcium concentration on PEG4000 (A,B) and mannitol (C,D) transport and TER (E,F). Data are presented as mean values ± SD for three monolayers as a function of Bl calcium concentrations. Significant difference from conventional model values at $^{*}p < 0.05$ and $^{**}p < 0.001$.

tion decreased below 1260 μM, Ap-to-Bl D-[^{3}H]mannitol flux remained constant through to 75 μM, and thereafter its transport was increased. At concentrations of 10 μM and below, high rates of Ap-to-Bl D-[^{3}H]mannitol fluxes were observed.

Following a 60 min transport experiment, the Ap and Bl donor solutions were aspirated and replaced with TER medium containing 1260 μM calcium and 814 μM magnesium ions. Post-transport TER determinations are presented as a function of the Bl calcium concentration during the transport experiment (Fig. 2E and F). Post-transport TER was inversely related to [^{14}C]PEG$_{4000}$ and [^{3}H]mannitol transport. The 30 min post-transport TER increased as the Bl calcium concentration increased from 0 μM (139.6 $\pm$ 12.2 Ω cm^2) to 100 μM (398.9 $\pm$ 11.8 Ω cm^2). Thereafter, the 30 min post-transport TER remained constant despite further increases in Bl calcium concentration. At 100 μM Bl calcium the post-transport TER was not significantly different from control values (398.9 $\pm$ 21.7 Ω cm^2). Interestingly, there was no evidence for a recovery in post-transport TER values after 60 and 90 min (Fig. 2E). This suggests that the loss in Caco-2 monolayer integrity induced by a 60 min exposure to a low extracellular calcium concentration is not rapidly reversible upon calcium ion replacement.

Based on these observations, the following conditions were used for the minimum calcium model; IM$_{-Ca}$ as the Ap donor solution and IM containing 100 μM calcium as the Bl receiver solution. Under these conditions, the transport of permeability markers and post-transport TER were consistently indistinguishable from control values.

In the conventional model, the Caco-2 cell nuclei were well-defined, the inter-nuclear space was small and stained green, indicating the presence of lipids (Fig. 3A). This appearance is consistent with a monolayer formed from closely opposed cells with tight inter-cellular junctions. Under calcium-free conditions, cell nuclei were poorly defined and closely associated with lipids, moreover, the inter-nuclear spaces were substantially devoid of lipid staining (Fig. 3B). Under minimum-calcium conditions the monolayer appearance was indistinguishable from that of the conventional model (Fig. 3C). Laser-scanning confocal microscopy therefore confirmed that

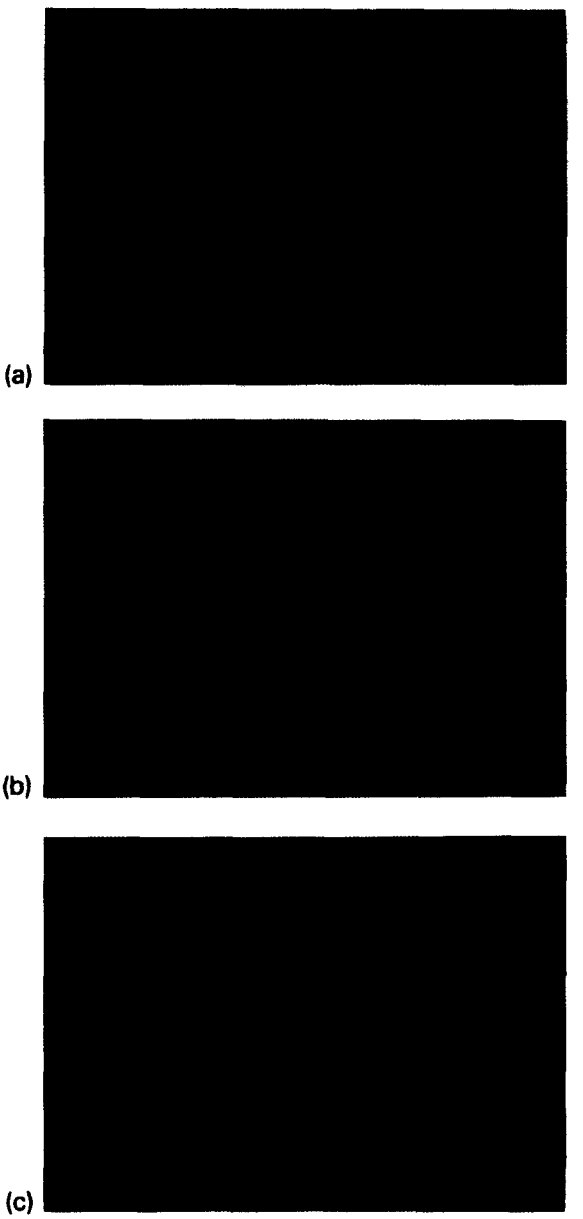

Fig. 3. Effect of calcium ions on Caco-2 monolayer appearance. Caco-2 monolayers were incubated for 60 min under uniform calcium (conventional model) (A), uniform calcium-free (B) or 100 μM Bl calcium alone (minimum-calcium model) (C). Scale bar = 25 μm.

morphological integrity was maintained under minimum-calcium conditions.

The Ap-to-Bl transport of [^{14}C]pamidronate and [^{3}H]mannitol across Caco-2 monolayers were compared in the uniform presence and total absence of calcium and in the minimum calcium model (Fig. 4). In the uniform presence of calcium ions, pamidronate transport was slow (142.6 $\pm$ 12.0 nmol h^{-1}) and linear for at least 180 min. In the total absence of calcium ions, pamidronate transport was rapid (initial rate = 12611 $\pm$ 176 nmol h^{-1}) and linear for 120 min. This represents an 88.4-fold increase in the rate of [^{14}C]pamidronate transport. Mannitol transport was concomitantly increased 15.0-fold. These data are consistent with the results of Boulenc et al. (1993) who used their data as evidence for a paracellular transepithelial transport route for tiludronate.

The ratio of mannitol to pamidronate transport across Caco-2 monolayers in the presence and absence of calcium ions was 4.892 and 1.078, respectively (Table 1). These data indicate that intact Caco-2 monolayers are approx. 5-times more permeable towards mannitol than pamidronate and that this differential flux is abolished when monolayer integrity is compromised by calcium removal. In the minimum-calcium model, the rate of pamidronate transport is 1.92-times greater than control levels, whereas mannitol flux was only slightly (1.22-fold) above control levels. The ratio of mannitol to pamidronate transport was reduced to 3.095 in the minimum calcium model (Table 1). In the absence of Ap calcium ions, therefore, pamidronate transport is increased to a much greater extent than that of mannitol. The apparent permeability coefficients for pamidronate transport in the conventional and minimum calcium models were 7.90 $\pm$ 0.68 $\times$ 10^{-8} and 2.11 $\pm$ 0.25 $\times$ 10^{-7} cm s^{-1}, respectively.

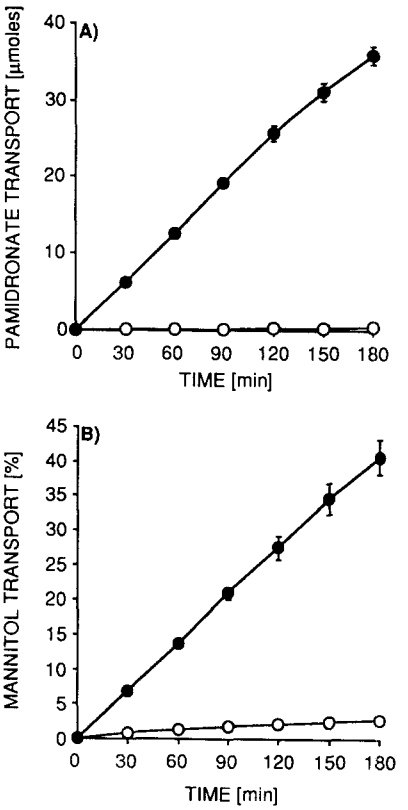

Fig. 4. Effect of calcium removal on pamidronate and mannitol transport. The kinetics of Ap-to-Bl pamidronate (A) and mannitol (B) transport in the uniform presence (○) and absence (●) of calcium ions was determined. Data are presented as mean values $\pm$ SD for three monolayers.

Table 1
Comparative effect of calcium concentration and distribution on pamidronate (CGP23339a) and mannitol transport across Caco-2 monolayers

Conditions		Mannitol transport (% h^{-1}) mean $\pm$ SD	CGP23339a transport (% h^{-1}) mean $\pm$ SD	Ratio
Ap	Bl			
+ Ca	+ Ca	0.910 $\pm$ 0.047	0.186 $\pm$ 0.048	4.892
− Ca	− Ca	13.533 $\pm$ 0.847 [b]	12.557 $\pm$ 0.442 [b]	1.078
− Ca	100 μM Ca	1.108 $\pm$ 0.033 [a]	0.358 $\pm$ 0.042 [a]	3.095

Data are presented as mean values $\pm$ SD for three monolayers.
Significant difference from conventional model values at [a] $p < 0.05$ and [b] $p < 0.001$.

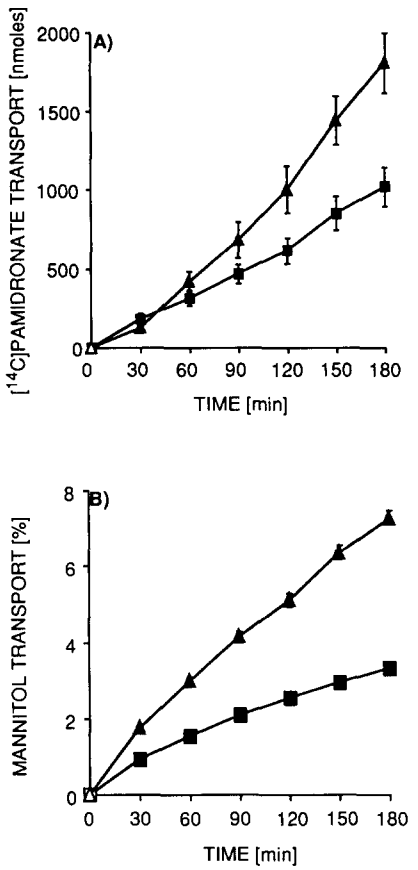

Fig. 5. Effect of Ap pamidronate on the permeability of the minimum-calcium model. The kinetics of Ap-to-Bl [^{14}C]pamidronate (A; 50 μM) and D-[^{3}H]mannitol (B) were determined in the presence (▲) and absence (■) of unlabelled pamidronate (5000 μM). Data are presented as mean values ± SD for three monolayers.

Table 2
Effect of Ap pamidronate (CGP23339a) on the permeability of Caco-2 monolayers

Conditions		CGP23339a concentration (μM)	TER (Ω cm^2) mean ± SD
Ap	Bl		
+Ca	+Ca	50	437.1 ± 28.6
−Ca	−Ca	50	94.0 ± 14.1 [b]
+Ca	100 μM Ca	50	417.8 ± 12.3
−Ca	100 μM Ca	50	415.2 ± 15.1
+Ca	100 μM Ca	5000	446.5 ± 15.4
−Ca	100 μM Ca	5000	327.4 ± 13.6 [a]

Data are presented as mean values ± SD for three monolayers.
Significant reductions from reference values at [a] $p < 0.05$ and [b] $p < 0.001$.

Finally, the proportion of [^{14}C]pamidronate transported into the Bl receiver solution in the minimum-calcium model was dependent upon its concentration in the Ap donor solution (Fig. 5A). At 50 μM and 5 mM, 0.35 ± 0.04 and 0.59 ± 0.06% h^{-1} of apically applied [^{14}C]pamidronate was transported, respectively. A 100-fold molar increase in concentration therefore increased the proportion of [^{14}C]pamidronate transport 1.7-fold. This increase in Ap-to-Bl flux was not specific since the transport of mannitol was concomitantly increased 2.2-fold (Fig. 5B). In addition, a 60 min incubation of the minimum-calcium Caco-2 model with an Ap donor solution containing 5 mM pamidronate significantly reduced TER values below reference levels (327.4 ± 13.6 vs 437 ± 28.6 Ω cm^2; $p < 0.05$). This effect was abolished by the use of an Ap donor solution containing 1260 μM calcium ions (Table 2).

4. Discussion

The GI epithelium represents a single sheet of closely opposed cells which are joined at their lateral surfaces by inter-cellular junctional complexes. These junctional complexes, comprising a tight junction, intermediate junction and desmosome, are the mainstay of an intact epithelium. The importance of calcium ions in the maintenance of epithelial tight junctions is recognised (Gumbiner, 1987; Cereijido et al., 1988). Indeed, the integrity of Caco-2 monolayers is dependent upon extracellular calcium ions. The selective calcium chelator, EGTA (2.5 mM Ap and Bl), decreases the TER across Caco-2 monolayers by approx. 75% (Artursson and Magnusson, 1990) indicating that calcium removal disrupts inter-cellular contacts. Furthermore, the present work shows that under uniform calcium-free conditions the intercellular junctions are lost and the permeability of Caco-2 monolayers is markedly increased.

Whilst Caco-2 monolayer integrity was disrupted under uniform calcium-free conditions, it was maintained after the unilateral removal of calcium ions from the Ap surface. This agrees

with the work of Noach et al. (1992) and Collares-Buzato et al. (1994) who demonstrated that apically applied calcium chelators (2.5 mM EDTA or 2 mM EGTA) did not influence monolayer integrity. Indeed, these workers demonstrated a polar sensitivity towards calcium chelators. The effectiveness of EDTA or EGTA in reducing the TER, and increasing the flux of permeability markers across Caco-2 monolayers was (Ap + Bl) > Bl ≫ Ap.

The tight junction itself seems not to be directly dependent upon calcium ions. For instance, the treatment of a tight junction-enriched liver membrane preparation with a calcium chelator at 37° C failed to dissociate tight junctions (Stevenson and Goodenough, 1984). Other junctional components, however, have been shown to be sensitive to extracellular calcium depletion including the intermediate junction (Volberg et al., 1986), the desmosomes (Watt et al., 1984) and the adhesion molecule, uvomorulin (Gumbiner and Simons, 1987). Although the integrity of epithelial tight junctions is known to be dependent on extracellular calcium ions, this dependence probably results from calcium effects on other structural elements of the junctional complex. The opening of tight junctions by the removal of extracellular calcium, therefore, results indirectly via a cascade of events which are initiated at the cell-to-cell contacts beneath the tight junctions. Since the apically positioned tight junction is the diffusion limiting component of the junctional complexes, basolateral calcium ions have free access to the uvomorulin cell-adhesion molecules. Basolateral calcium alone would therefore be expected to maintain Caco-2 monolayer integrity. In the presence of Ap calcium ions alone, the access of calcium ions to the uvomorulin cell-adhesion molecules is restricted by the tight junctions between cells. Consequently, the calcium requirement of the lateral cell-to-cell contacts would not be satisfied resulting in the loss of tight junction integrity. Since, the calcium-dependent cell-to-cell contacts occur on the lateral surfaces of cells below the tight junctions, this may explain the polarised calcium ion requirement of Caco-2 monolayers for integrity. The polarised requirement of Caco-2 cells for calcium ions is consistent with the Gumbiner model for the maintenance of tight junctions (Gumbiner, 1987).

Calcium flux across Caco-2 monolayers cultured on polycarbonate permeable supports has been characterised previously (Giuliano and Wood, 1991). It is described by a saturable carrier-mediated component superimposed onto a non-saturable diffusional flux according to the model $v = ((K_m \cdot V_{max})/(K_m + [S])) + K_d \cdot [S]$; where $K_m = 2.9 \pm 1.0$ mM, $V_{max} = 1.4 \pm 0.1$ nmol min^{-1} $(4.7\ cm^2)^{-1}$ and $(K_d = 0.13 \pm 0.2$ nmol min^{-1} $(4.7\ cm^2)^{-1}$ mM^{-1}. Assuming the diffusional component to be non-vectorial, the calcium concentration in the Ap donor solution can be estimated as a function of time. For a 1260 μM Bl-to-Ap calcium gradient, the calcium concentration in the Ap donor solution would be 14.7 μM after 180 min, assuming that the carrier-mediated pathway does not recycle calcium back to the receiver solution. This assumption is reasonable since the high affinity of pamidronate for calcium means that it will sequester calcium away from the relatively low affinity carrier-mediated transport pathway. Clearly, with an initial concentration of 50 μM pamidronate, a final calcium concentration of 14.7 μM may lead to an appreciable proportion (up to 30%) of the permeant being chelated to calcium ions. Ap-to-Bl flux of magnesium ions would further increase the divalent metal cation concentrations in the Ap donor solution. The appearance of appreciable concentrations of calcium and magnesium ions in the Ap donor solution would complicate interpretation of the transport data. In order to minimise the Bl-to-Ap flux, the minimum concentration of calcium required in the Bl receiver solution to maintain monolayer integrity was titrated. A half-maximal decrease in Caco-2 monolayer permeability indices occurred between 15 and 20 μM. At 100 μM Bl calcium concentration all permeability indices were consistently indistinguishable from control levels and this concentration was used in the minimum-calcium model. For a 100 μM Bl-to-Ap calcium gradient, the final calcium concentration in the donor solution after 180 min would be 1.2 μM. In the minimum-calcium model, pamidronate in the donor solution would therefore remain overwhelmingly in the unchelated

form. This analysis of anticipated Bl-to-Ap calcium flux as a function of Bl calcium concentration highlights the importance of developing the minimum-calcium model for these studies.

In the conventional Caco-2 transport model, the apparent permeability coefficient for pamidronate ($7.90 \pm 0.68 \times 10^{-8}$ cm s^{-1}) predicts an oral bioavailability of less than 1% in humans according to the correlation described by Artursson and Karlsson (1991). This agrees with its oral bioavailability of 0.3% in patients with breast cancer and bone metastases (Daley-Yates et al., 1991). Similarly, the estimated oral bioavailability (relative to the subcutaneous route) for pamidronate administered to rats in their powdered food diet containing 0.4% calcium was 0.2% (Reitsma et al., 1983). In the minimum-calcium model, the apparent permeability coefficient of $2.11 \pm 0.25 \times 10^{-7}$ cm s^{-1} predicts an oral bioavailability of between 30 and 50%. Moreover, this level of increased permeability was specific to pamidronate. Whilst pamidronate transport was increased 1.92-fold under minimum calcium conditions, mannitol flux was increased only 1.22-fold. Correspondingly, the ratio of mannitol to pamidronate flux in the conventional and minimum calcium models decreased from 4.892 to 3.095. These observations suggest that an interaction between calcium and/or magnesium ions with pamidronate is a limiting factor in its transepithelial transport. Preventing pamidronate chelation with calcium and magnesium ions in the lumen of the GI tract may therefore improve its absorption and result in an acceptable oral bioavailability.

Mannitol flux and TER measurements show that 5 mM pamidronate increases the permeability of the minimum-calcium Caco-2 model. That is, pamidronate non-specifically facilitates its own absorption at high doses. Pamidronate has been shown to increase the rectal absorption of cefoxitin in rats (Van Hoogdalem et al., 1989). Aqueous solutions of pamidronate at concentrations of 0.5–6% w/v (13.6–162.6 mM) increased the absorption of cefoxitin from 14 ± 12% to a maximum of 85 ± 10% at 4% w/v (108.4 mM). In the Caco-2 system, an absorption-promoting effect was observed at a concentration of 5 mM, which is lower than the minimal effective concentration in animal experiments. This probably results from the Caco-2 monolayers operating under minimum-calcium conditions. The absorption promoting effect of pamidronate in vivo was attributed to its ability to chelate calcium ions and increase the paracellular permeability. The prevention of a 5 mM pamidronate induced decrease in TER across the minimum calcium model by using IM medium (1260 μM calcium) as the donor solution supports this hypothesis.

The calcium ion concentration in the Ap and Bl solutions bathing Caco-2 cells can be manipulated without causing detrimental effects on monolayer permeability. Indeed, the transport of permeability markers (mannitol and PEG_{4000}) and TER values across the conventional and minimum-calcium models suggest they have equivalent permeability properties. These two models have been successfully employed to compare the transport of pamidronate in the presence and absence of calcium and magnesium ions. The greater rate of Ap-to-Bl pamidronate flux from a calcium- and magnesium-free donor solution suggests these ions retard its transepithelial transport. This work highlights the potential of the minimum-calcium Caco-2 transport model as a useful in vitro screen for predicting absorption-limiting interactions between drug molecules and metal cations.

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