Comparison of HT29-18-C_1 and Caco-2 Cell Lines as Models for Studying Intestinal Paracellular Drug Absorption

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Purpose. To compare the permeability characteristics of HT29-18-C_1 colonic epithelial cell line with Caco-2, an established model of intestinal drug transport.

Methods. Cell lines were grown as epithelial monolayers. Permeability was measured over a range of transepithelial electrical resistance (R_t) using a group of drug compounds.

Results. HT29-18-C_1 develop R_t slowly when grown in culture, allowing permeability to be measured over a wide range (80–600 $\Omega \cdot cm^2$). In contrast, Caco-2 monolayers rapidly develop R_t of $\approx$300 $\Omega \cdot cm^2$ and require Ca^{2+}-chelation to generate R_t equivalent to human intestine (60–120 $\Omega \cdot cm^2$). Permeability of atenolol, ranitidine, cimetidine, hydrochlorothiazide and mannitol across HT29-18-C_1 decreased 4–5 fold as R_t developed from 100–300 $\Omega \cdot cm^2$ indicating they permeate via the paracellular route. In contrast, ondansetron showed no difference in permeability with changing R_t consistent with transcellular permeation. Permeability profiles across low R_t HT29-18C_1 and pulse EGTA-treated Caco-2 monolayers were the same for all 5 paracellular drugs suggesting that transient Ca^{2+} removal does not alter selectivity of the tight junctions. Permeabilities of cimetidine, hydrochlorothiazide and atenolol across 100 $\Omega \cdot cm^2$ HT29-18-C_1 monolayers reflect more closely those reported for the human ileum *in vivo* than did mature Caco-2 monolayers.

Conclusions. HT29-18-C_1 monolayers can be used to study drug permeability at R_t values similar to human intestine without the need for Ca^{2+} chelation. As such, they offer a useful alternative to Caco-2 for modelling intestinal drug absorption.

KEY WORDS: Caco-2; HT29-18C_1 cell lines; paracellular drug absorption.

INTRODUCTION

The human adenocarcinoma cell line Caco-2 is being used increasingly as a model to predict human oral drug absorption (1–3). However, the transepithelial electrical resistance (R_t) of Caco-2 monolayers, although dependent on passage number, is reported by most workers to be significantly higher than would be expected for human small intestine (4,5) where, for most drugs, the majority of absorption occurs. Electrical resistance is closely related to tightness of the occluding junctions between epithelial cells (6) with the result that the flux of paracellularly transported compounds across Caco-2 monolayers is markedly lower than reported for mammalian intestine. For example, permeability of the paracellular marker, mannitol is reported to be $\approx$50-fold greater across rat ileum than Caco-2 monolayers (4). The structural selectivity of intestinal epithelia *in vivo* may also be different from that of "tighter" Caco-2 monolayers. Though evidence has been presented for a broad qualitative relationship between permeability across Caco-2 and tissue systems an alternative model allowing a more quantitative correlation between *in vitro* and *in vivo* systems would be useful. The HT29-18-C_1 colonic cell line is reported to develop R_t more slowly than Caco-2 (7). A previous study (8) examined permeability of various compounds in HT29-18-C_1 but only at R_t values similar to those seen in Caco-2 (around 400 $\Omega \cdot cm^2$). In this study, we have measured drug permeabilities across HT29-18-C_1 cells at different stages of monolayer development and compared these with the Caco-2 cell line using compounds transported by either paracellular or transcellular routes.

METHODS

Materials

Radiolabeled ^{3}H-atenolol, ^{3}H-cimetidine and ^{14}C-mannitol were purchased from Amersham International (UK) and unlabelled compounds from Sigma or Aldrich Chemical Company Ltd. (UK). ^{14}C-ranitidine and ^{14}C-ondansetron were prepared by Glaxo Research and Development Ltd. ^{14}C-hydrochlorothiazide was prepared by Merck Research Laboratories, PA. Tissue culture reagents were purchased from Gibco Life Technologies Ltd. (UK).

Tissue Culture

HT29-clone 18C_1 cells (passage 15–22) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 25mM $NaHCO_3$, 20mM HEPES, 10μg/ml transferrin, 4mM glutamine, 50IU/ml penicillin and 50μg/ml streptomycin in a humidified 5% CO_2/95% air atmosphere at 37°C. Caco-2 cells (passage 90–110) were maintained in DMEM containing; 10% fetal bovine serum (FBS), 40mM $NaHCO_3$, 4mM glutamine, 1% non-essential amino acids and antibiotics as for HT29-18-C_1.

For drug transport studies, cells were seeded on 12mm polycarbonate filter (0.4μM) culture inserts (Transwell, Costar, UK) at a density of $1 \times 10^5/cm^2$ and used for permeability studies from 10 to 30 days after seeding. Development of R_t was monitored with an Evometer (WPI) fitted with 'chop stick' electrodes. A value of 100 $\Omega \cdot cm^2$ equivalent to the resistance of the filter support and the fluid resistance has been subtracted from all R_t values quoted.

HPLC Analysis of Test Compounds

Purity of ^{3}H-cimetidine, ^{3}H-atenolol, ^{14}C-ranitidine, ^{14}C-hydrochlorothiazide and ^{14}C-ondansetron was determined using a high performance liquid chromatography system (Spectra

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Series AS300, USA) equipped with a radiomatic detector (Flo-One\Beta, Canberra Packard, USA). The stationary phase was Partisil ODS1 with 10μm particle size (250mm × 4.6mm i.d., Thames Chromatography, England) with a mobile phase of 90% 50mM phosphoric acid (pH 3.0) and 10% methanol except for ondansetron where 60% 50mM phosphoric acid (pH 3.0) and 40% methanol was used. The flow rate was 1.5ml/min and column temperature was maintained at 30°C. Peak area was used for quantification. Radiochemical purity of ^{3}H-atenolol, ^{14}C-ranitidine, ^{3}H-cimetidine, ^{14}C-hydrochlorothiazide and ^{14}C-ondansetron was 86%, 90%, 80%, 97% and 92% respectively. In permeability experiments comparing total radioactivity (T_r) with label specifically associated with HPLC-purified drug the T_r for atenolol, ranitidine, hydrochlorothiazide and ondansetron gave a true reflection of drug transport. However, for cimetidine, T_r significantly overestimated permeability and HPLC analysis was used routinely to quantify permeability. The inter-assay variation was less than 3% for all the drugs used.

Transport Studies

Epithelial layers on Transwell supports were washed twice in serum-free DMEM (SFDMEM) and placed in 12 well culture plates. Aliquots of SFDMEM were added to the apical (0.5 ml) and basolateral (1.5ml) compartments and monolayers were equilibrated for 30 min. 100 μl of SFDMEM was removed from the apical reservoir and replaced with 100 μl of the ^{3}H- or ^{14}C-labelled compounds together with unlabelled drug giving final concentrations of 0.1 mM (≈0.3 μCi/ml). Drug transport from apical to basolateral compartments was measured over successive (15–30 min) flux periods. After each incubation period the culture insert was moved to the next well containing fresh SFDMEM. Medium bathing the apical surface was unchanged during the course of the experiment. After each flux period a 700 μl sample of the basolateral solution was removed for analysis and 20 μl apical samples were taken at the beginning and end of each experiment. ^{14}C and ^{3}H activities were determined by liquid scintillation counting or HPLC analysis as appropriate. The background was always less than 0.5% and was not subtracted from the total. The use of short flux periods (15–30 min) maintained sink conditions and ensured a linear rate of drug transport.

For the Ca^{2+} removal step, culture inserts were incubated on apical and basolateral surfaces with SFDMEM containing 2.5mM ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) for 15 min followed by replacement of extracellular Ca^{2+}. Fluxes are expressed as apparent permeability (Papp) according to the equation:

$$\text{Papp} = \frac{dQ/dt}{A * C}\ \text{cm}\cdot\text{sec}^{-1}$$

where; dQ/dt = rate at which compound appears in basolateral compartment; A = surface area of monolayer and C = initial concentration of compound in apical compartment.

Electron Microscopy

Monolayers were fixed by submerging at room temperature for 1 hour in a primary fixative containing 2.5% v/v glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate-buffer (pH 7.2) containing 1 mM $CaCl_2$. Monolayers were rinsed in the same buffer and postfixed in cacodylate-buffered 1% OsO_4 for 1 hour and subsequently en-bloc stained in 2% uranyl acetate for 1 hour. Samples were dehydrated through increasing concentrations of alcohols prior to embedding in TAAB Spurr resin. Thin sections were mounted on formvar-coated 200-mesh copper grids and stained with uranyl acetate and lead citrate. Sections were viewed with a Phillips 400 transmission electron microscope.

Statistical Analysis

Data are presented as mean ± S.E.M. Comparisons were made using the Student's *t*-test for unpaired data or, where appropriate, the Mann-Whitney *U*-test. Values of $P < 0.05$ were considered significant.

RESULTS

Development of Electrical Resistance in HT29-18-C_1 Cells: Comparison with Caco-2

Development of electrical resistance (R_t) in HT29-18-C_1 cells over a 24 day culture period on Transwell culture inserts is shown in Fig 1. R_t increased relatively slowly during the initial phase of growth reaching approximately 100 Ω·cm^2 10–12 days after seeding. From this point R_t increased more rapidly rising to a plateau of ≈700 Ω·cm^2 after 24–28 days in culture. Ultrastructure of 10 day old HT29-18-C_1 cultures show a monolayer of polarised epithelial cells with clearly defined junctional complexes and some development of microvilli (Fig 2a). The ultrastructure of 22 day old cultures was similar (Fig 2b) though some evidence of multilayering covering 5–10% of the growth area was observed.

For comparison, R_t development and morphology in the more commonly used Caco-2 cell line was studied. The profile of R_t development in these cells was markedly different to HT29-18-C_1 (Fig 1). Caco-2 R_t developed rapidly during the early stages of culture reaching >200 Ω·cm^2 within 5 days of seeding and a maximum value of 400 ± 29 Ω·cm^2 after 14 days. This R_t was maintained for at least a further 10 days. The

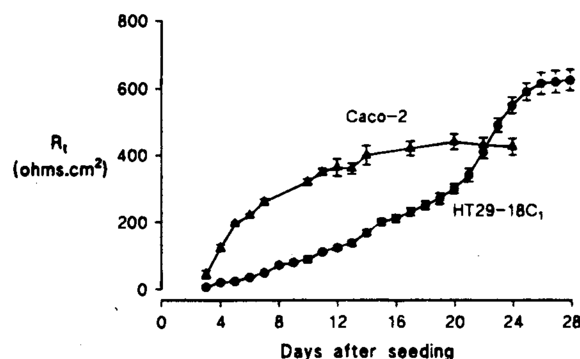

Fig. 1. Transepithelial electrical resistance (R_t) of Caco-2 and HT29-18-C_1 monolayers plotted against time grown in culture after seeding on permeable supports. Both cell lines were seeded at a density of 1 × 10^5 cells/cm^2 and the media changed daily. Data are expressed as mean ± S.E.M. for n = 24 monolayers for each cell line. A value of 100 Ω·cm^2 accounting for the fluid resistance and the resistance of the filter support alone has been subtracted in each case.

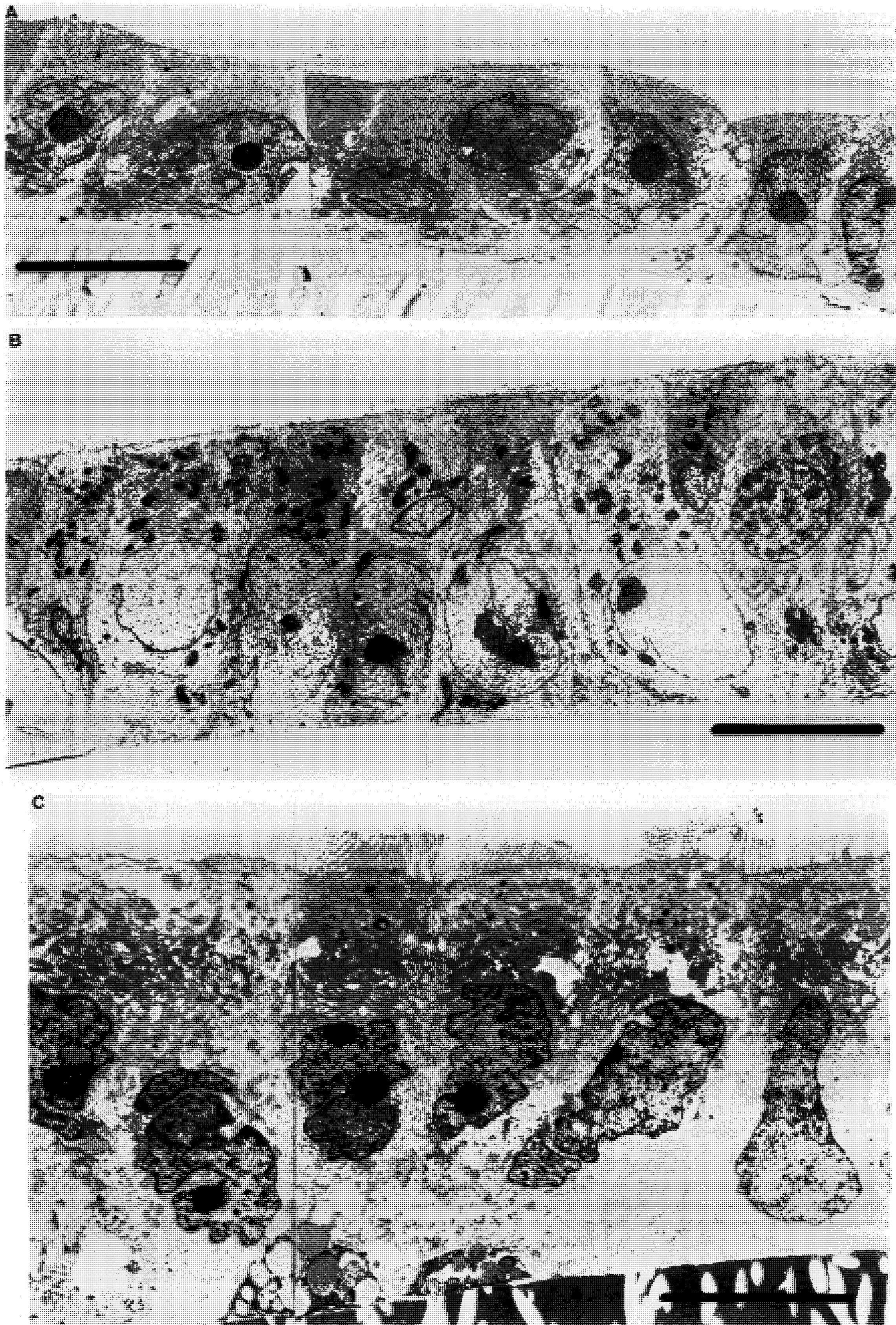

Fig. 2. Electron micrograph showing a vertical section across a HT29-18-C_1 monolayers grown for 10 (A) and 22 (B) days on Transwell filter supports. Panel C shows 20 day Caco-2 monolayers on the same support. The bar represents 10 μm.

quoted R_t values for monolayers used in the flux experiments are slightly lower due to effects of media changes during washing. Ultrastructure of Caco-2 show they form polarised monolayers similar to HT29-18-C_1 but with a more highly developed microvillus membrane (Fig 2c).

Drug Permeability Profiles at Different Stages of Development of HT29-18-C_1 Epithelial Layers

The gradual and reproducible development of R_t across HT29-18-C_1 cell layers allowed study of the permeability characteristics of a group of compounds across a range of R_t values (50–500 $\Omega \cdot cm^2$). Five compounds, atenolol (AT), cimetidine (CT), hydrochlorothiazide (HCTZ), ranitidine (RT) and mannitol (ML) are expected to be transported paracellularly while ondansetron (OT) is transported predominantly by the transcellular route (8). Figure 3 shows apical to basolateral transport of these compounds at different stages of HT29-18-C_1 development. Permeability of the five "paracellular" compounds was markedly dependent on R_t of HT29-18-C_1 cell layers. For example, Papp of atenolol declined from $4.19 \pm 0.84 \times 10^{-6}$ $cm \cdot sec^{-1}$ across 110 $\Omega \cdot cm^2$ monolayers to $1.68 \pm 0.11 \times 10^{-6}$ $cm \cdot sec^{-1}$ across 190 $\Omega \cdot cm^2$ and $1.1 \pm 0.06 \times 10^{-6}$ $cm \cdot sec^{-1}$ across 310 $\Omega \cdot cm^2$ monolayers (n = 7) with the other paracellularly transported compounds showing a similar profile (Fig. 3). In contrast, the Papp for ondansetron was significantly higher and showed no significant change ($34.0 \pm 0.5 \times 10^{-6}$ at 110 $\Omega \cdot cm^2$ vs $31.2 \pm 0.6 \times 10^{-6}$ at 310 $\Omega \cdot cm^2$, n = 5).

Comparison of Drug Transport in HT29-18-C_1 with Caco-2

The effects of R_t on permeability of these compounds was also compared in HT29-18-C_1 and Caco-2 monolayers. Low R_t was induced in Caco-2 monolayers with an EGTA-pulse technique which produces a controlled and reversible decrease in R_t. Fig 4a illustrates this approach in 20 day Caco-2 monolayers ($R_t \approx 350$ $\Omega \cdot cm^2$) exposed to a 15 min pulse of 2.5 mM EGTA. EGTA addition caused a rapid decrease in R_t to ≈ 80 $\Omega \cdot cm^2$ accompanied by a 20-fold increase in ^{14}C-mannitol permeability. Subsequent removal of EGTA and re-addition of Ca^{2+} stimulated a rapid recovery of R_t and mannitol flux back towards control values. The flux of the transcellularly transported compound ondansetron was unaffected by Ca^{2+} removal (Fig 4a). Figure 4b shows the mean flux across 20–22 day Caco-2 monolayers before, during and after treatment with 2.5mM EGTA. Only experiments in which EGTA reduced R_t from ≈ 300 to ≈ 100 $\Omega \cdot cm^2$ were included. Under these conditions, EGTA caused a ≈ 4 fold increase in the flux of CT, RT, AT and ML but only 2–3 fold for HCTZ. Following re-addition of Ca^{2+}, transport rates returned back towards pre-EGTA levels though it was noticeable that despite the virtually complete

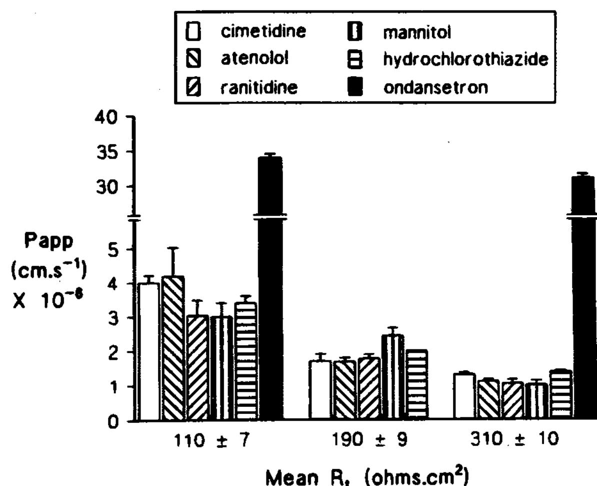

Fig. 3. Apical to basolateral flux of paracellular and transcellular drugs across HT29-18-C_1 monolayers. The permeability of the five compounds indicated were monitored in monolayers cultured for 12, 15 or 20 days showing mean R_t of 110 ± 7, 190 ± 9 and 310 ± 11 $\Omega \cdot cm^2$ respectively. The rate of flux is expressed as mean Papp ± S.E.M. for n = 7 monolayers in each group except ondansetron where n = 5.

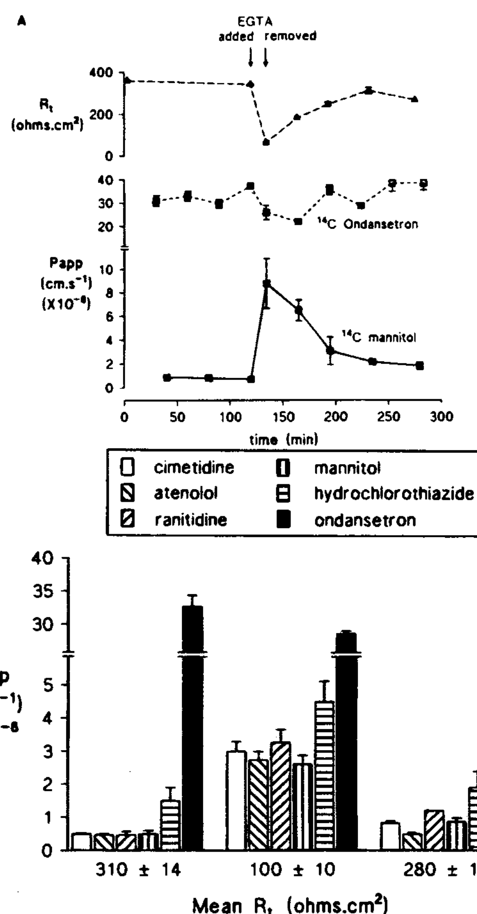

Fig. 4. A The effects of an EGTA pulse on R_t and Papp for mannitol and ondansetron in Caco-2 monolayers. Following incubation in DMEM, 2.5mM EGTA was added to apical and basolateral solutions at 120 min. After 15 min incubation in Ca^{2+}-free conditions, Ca^{2+} was replaced by washing and subsequent incubation with normal DMEM. Results shown are mean ± S.E.M. for n = 4 monolayers in each group. **B** Flux of drugs across Caco-2 monolayers prior to EGTA addition, during 15 min incubation with 2.5mM EGTA and following recovery of R_t after re-addition of Ca^{2+}. Mean R_t during these 3 periods were 310 ± 14, 100 ± 10 and 280 ± 11 $\Omega \cdot cm^2$ respectively. Papp values shown are mean ± S.E.M. for n = 8 (cimetidine, atenolol) or n = 4 (ranitidine, mannitol, hydrochlorothiazide, ondansetron) monolayers in each group.

return of R_t, fluxes of CT, RT and ML remained slightly above starting values. While the Papp of HCTZ across mature Caco-2 monolayers is significantly higher than the other 'paracellular' drugs ($P < 0.05$), this difference is not maintained on addition of EGTA. There was no significant difference in paracellular transport rate between 10 day HT29-18-C_1 and 22 day EGTA-treated Caco-2 monolayers both of which have a R_t of ≈ 100 $\Omega \cdot cm^2$ (compare figs 3 and 4b). There was a greater variability in Papp values at low R_t in both cell lines.

A more detailed relationship between R_t over the range 50–450 $\Omega \cdot cm^2$ and Papp of AT in the two cell systems is shown in Fig 5. Both HT29-18C_1 and Caco-2 exhibit a similar relationship between Papp and R_t suggesting that HT29-18-C_1 monolayers provide a valid alternative to Caco-2 for studying paracellular transport. A similar relationship was observed for CT, RT and ML (data not shown). Papp was very sensitive to changes in R_t at values <160 $\Omega \cdot cm^2$ which may explain the greater variation in Papp seen with low R_t monolayers in both cell lines (Figs 3 and 4b).

DISCUSSION

In vitro systems for predicting paracellular drug absorption should ideally be able to both model the structure selectivity of the epithelium *in vivo* and provide a reasonable quantitative prediction of drug permeability *in vivo.* As shown here and in other studies (4,9) Caco-2 cells are characterised by rapid development of R_t to levels (≈ 300 $\Omega \cdot cm^2$) considerably higher than reported for human small or large intestine *in vitro* (50 and 100 $\Omega \cdot cm^2$) (4,10). This raises the possibility that there may be significant differences in the permeability characteristics of paracellularly absorbed compounds between Caco-2 and the human intestine *in vivo.* Other studies have addressed this question and concluded that, within limits, Caco-2 does mimic the selectivity of *in vivo* systems but the overall permeability of Caco-2 monolayers is markedly lower (4). Caco-2 permeability can be increased by chelating extracellular Ca^{2+} (11) but the possibility that this manoeuvre alters the properties of tight junctions cannot be ruled out. The slower and more reproducible development of R_t during the initial stages of HT29-18-C_1 culture means that R_t can be studied in the *"in vivo"* range without the need for artificial manipulation of tight junctions. Recent studies by Wils and co-workers (8) also suggest HT29-18-C_1 as a model for intestinal drug absorption. However, in their studies, monolayers were used at high R_t (>400 $\Omega \cdot cm^2$) and the permeability characteristics of low resistance monolayers was not addressed.

Permeability across HT29-18-C_1 monolayers at different stages of development was assessed using a group of compounds expected to be transported via either paracellular or transcellular routes. The transport of the polar drugs, AT, HCTZ, CT, RT and ML showed a clear dependence on the age and R_t of HT29-18-C_1 monolayers with an approximate 4-fold decrease in permeability in going from 100 (10–12 days) to 300 $\Omega \cdot cm^2$ (20–22 days). The close relationship between permeability and R_t for these compounds suggest their route of transport is predominantly paracellular. Ondansetron was completely insensitive to changes in R_t consistent with previous observations that this compound crosses the epithelium predominantly by a transcellular route (11).

As shown in Table 1 the permeability of paracellular drugs across low R_t HT29-18-C_1 monolayers is closer to that reported for human intestine *in vivo* than are mature Caco-2 monolayers. For example, the Papp for AT in human jejunum has been measured as $10 - 37 \times 10^{-6}$ $cm \cdot sec^{-1}$ (12,13) compared to 4.19×10^{-6} across 10–12 day HT-29-18C_1 monolayers and 0.47×10^{-6} across Caco-2 monolayers. Jejunal permeability of HCTZ also shows reasonable correlation with low R_t HT29-18-C_1 monolayers though the significantly higher *in vivo* permeability of CT in the jejunum does not appear to be reflected by either model. Permeability across HT29-18C_1 and human ileum are relatively close for all three drugs. In contrast, mature Caco-2 monolayers have a much lower permeability than observed *in vivo.*

In general, the permeability characteristics of the paracellular compounds tested here are similar, with relatively small differences in Papp at a given R_t value in either cell system. HCTZ is the exception to this exhibiting a significantly higher Papp than the other "paracellular" compounds in mature (high resistance) Caco-2 monolayers. This may be due to a small transcellular component in HCTZ permeability. It would be expected that at low R_t the paracellular route would be dominant and the contribution of any small transcellular component would be minimal. In this respect, it is interesting that HCTZ permeability across low R_t HT29-18C_1 (and EGTA-treated Caco-2 monolayers) is similar to the other paracellularly absorbed compounds, reflecting the *in vivo* permeabilities. These data illustrate how high R_t monolayers may overestimate the *in vivo* permeability of drugs with a small transcellular component.

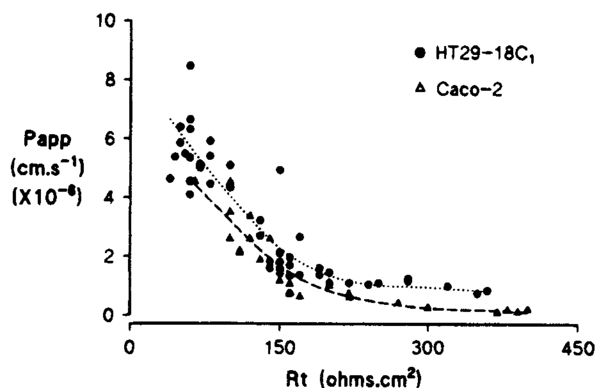

Fig. 5. Relationship between R_t and atenolol permeability across HT29-18-C_1 and Caco-2 monolayers. Caco-2 data includes flux measurements using monolayers exposed to an EGTA pulse to lower R_t.

Table I. Comparison of Papp for Various Drugs Across 300 $\Omega \cdot cm^2$ Caco-2, 100$\Omega \cdot cm^2$ HT-29-18C_1 and Human Jejunum and Ileum *in vivo*[a]

	Papp ($cm \cdot sec^{-1} \times 10^{-6}$)			
Drug	Caco-2 (300 $\Omega \cdot cm^2$)	HT29-18C_1 (100 $\Omega \cdot cm^2$)	Jejunum	Ileum
Cimetidine	0.50	4.0	77	26
Atenolol	0.47	4.2	10–37	14
Hydrochlorothiazide	1.50	3.4	18	13

[a] *In vivo* Papp values ($\times 10^{-6}$ $cm \cdot sec^{-1}$) are calculated from Sutcliffe *et al* 1988 (12) except for atenolol which also uses jejunal data from Lennernäs *et al* 1994 (11).

Lowering Caco-2 R_t to $\approx 100\ \Omega \cdot cm^2$ using an EGTA pulse technique produces drug permeability profiles similar to 10–12 day old HT29-18C_1 monolayers and suggests that EGTA treatment is a valid method for lowering R_t with no obvious deleterious effects on tight junctional integrity or selectivity. The close correlation between R_t and permeability for the two cell lines (Fig 5) also argues that simple R_t values should, within limits, allow a reliable prediction of paracellular permeability which is independent of the cell system in which it is measured or how the R_t is achieved (ie natural development or EGTA treatment). The paracellular permeability across low R_t HT29-18C_1 is closer to the human gut *in vivo* than are mature Caco-2 monolayers, for 3 of the drugs we have tested. It will clearly be important to confirm this and examine any possible correlation by studying compounds with a greater range of *in vivo* permeabilities, though this will depend on increasing the amount of reliable *in vivo* data.

Clearly, even using model systems with low R_t, significant differences between *in vitro* and human *in vivo* permeability values still exist, particularly for cimetidine in the jejunum. Such discrepancies could be due to factors *in vivo* which are not present in cell monolayers (e.g. mucus barrier, blood flow, intestinal peristalsis) which may influence the extent of absorption. Also, a simple epithelial monolayer in culture may not exactly reflect the permeability of the structurally more complex intestinal epithelium *in vivo*.

In conclusion, we have used the HT29-18-C_1 cell line to examine the relationship between transepithelial electrical resistance and paracellular permeability. An advantage of the HT29-18-C_1 system is that monolayers can be studied over a wide range of resistances during maturation, including those reported for large and small intestine *in vivo*, without the need to manipulate extracellular Ca^{2+}. HT29-18-C_1 monolayers offer a useful alternative to Caco-2 as a model for prediction of paracellular drug transport.

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