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Use of a Caco-2 cell culture model for the characterization of intestinal absorption of antibiotics

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Abstract

The use of cell culture models, based on human cell lines derived from the intestinal epithelium, is a promising new tool for the in vitro study of oral absorption of drugs. An assay has been developed using the Caco-2 cell line with the aim of studying the in vitro permeability of antibiotics. The reproducibility of the assay conditions have been assessed by means of the transport of two different marker molecules: ^{3}H-mannitol and fluorescein, and transepithelial electrical resistance (TEER) value for cells monolayers. The results show that cells after 21 days of culture give significantly tighter monolayers than those after 15 days with higher reproducibility. Apparent permeability coefficients (Papp) have been measured for 13 antibiotics, known to be absorbed at different rates in humans. Papp values span from 0.18×10^{-6} cm/s for cephaloridine to 5.79×10^{-6} cm/s for rifampicin where the corresponding bioavailability values, known from literature, span from < 3 to 98%. A Caco-2 in vitro model appears to be suitable to investigate the transport of drugs across the intestinal epithelium. This model gives no information about the metabolic phase that follows the absorption of a drug but could provide information to investigate its pharmacokinetical behavior.

Keywords: Caco-2; Drug absorption; Intestinal transport; Antibiotics

1. Introduction

The availability of cell culture models, based on human cell lines with characteristics of the mature enterocyte, the typical cell of the small intestine, offers the possibility of studying and predicting the in vivo intestinal transport of compounds at an early stage of drug development [1,2]. One of the most characterized intestinal cell lines, Caco-2, is derived from a human colorectal carcinoma and spontaneously differentiates in culture into cells with some characteristics of the enterocyte, with the expression of the corresponding biochemical markers [3,4]. Recently, Caco-2 cells have been used to study the transport of drugs belonging to different classes such as cardiovasculars and antibiotics [5,6]. We describe here the use of the Caco-2 cell culture model for the characterization of intestinal permeability properties of a range of antimicrobial agents of natural or synthetic origin. These permeabilities have then been compared to corresponding previously published human oral bioavailability data.

2. Experimental

2.1. Drugs and markers

Rifampicin, rifapentine, rifamycin-SV, penicillin-G, teicoplanin, and novobiocin were all available from Marion Merrell Dow. Erythromycin and ampicillin were obtained from USPC Inc., Rockville, MD; trimethoprim from Roche, Switzerland; chlorotetracycline from the Istituto Superiore di Sanità, Rome, Italy; vancomycin from Eli Lilly GmbH, Germany; cycloserine, cephaloridine and fluorescein from Sigma Chemical Company, St. Louis, MO; and ^{3}H-mannitol (with spe-

Abbreviations: TEER, transepithelial electrical resistance.

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cific activity: 30 Ci/mM and radiochemical purity: 99%) from New England Nuclear, Boston, MA.

2.2. Preparation of Caco-2 differentiated monolayer

Caco-2 cells were plated at 10^4 cells/cm^2 in 75 cm^2 flasks in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum, 1% nonessential aminoacids, and 4 mM glutamine (DMEMB). All tissue culture reagents, including serum, were from Irvine Scientific, Santa Ana, CA. The medium was changed every 3 days.

At confluence the cells were washed three times with phosphate buffer saline (PBS), without Ca^{2+} and Mg^{2+} and detached from flasks by a trypsin wash (0.25% in PBS containing 0.2% EDTA). Cells were then resuspended in DMEMB and distributed (2×10^6 cells in 0.3 ml) onto polycarbonate microporous cell culture inserts, that fit into 24-well cell culture clusters (Transwell, Costar, Cambridge, MA). Then 0.5 ml of DMEMB was distributed in each basolateral chamber. The medium was changed three times a week. After 15 and 21 days of culture, the basolateral and apical medium were removed, and after washing with DMEM containing 1% nonessential aminoacids and 25 mM Hepes buffer (DMEMT), cells were used for the permeability assays.

2.3. Assay of integrity of the monolayer

2.3.1. Fluorescein passage

After washing the cells, 0.2 ml of a solution of fluorescein, 4 mg/l in DMEMT, was added to each apical chamber and 0.4 ml of DMEMT was added to each basolateral chamber. After 2 h incubation at 37°C, fluorescein content (mg/l) in the basolateral medium was determined using a Cytofluor fluorescence scanner (Cytofluor 2300, Millipore).

2.3.2. ^{3}H-Mannitol passage

^{3}H-Mannitol (30 Ci/mM) was diluted to a concentration of 1 mCi/l in DMEMT and then added to the apical side of the monolayer to a final concentration of 0.5 mCi/l. At the same time, 0.4 ml of DMEMT were dispensed into each basolateral chamber. After 2 h incubation at 37°C, the passage of the radiolabeled marker was determined measuring the radioactivity (cpm) of the basolateral medium in a liquid scintillation counter (Beckman).

2.3.3. Transepithelial electrical resistance (TEER)

The transmembrane specific resistance, expressed in ohm cm^2 (Ω cm^2), was measured using the Millicell apparatus (Millipore) before incubation with the sample. After washing the cells, DMEMT (0.2 ml in the apical chamber and 0.4 ml in the basolateral chamber) was dispensed into each filter; electrical probes (previously conditioned overnight in DMEMT) were then immersed in the apical and basolateral chambers to measure the resistance of the monolayer. TEER was measured after subtraction of the intrinsic resistance of the cell-free filter. TEER values were then transformed into the corresponding specific conductance values, expressed in milli-Siemens per cm^2 (mS/cm^2).

2.4. Antibiotic permeability assay

End-point determinations were performed for 24-well cell culture clusters. Solutions of antibiotics at different concentrations (125, 250, 500, and 1000 mg/l) were prepared in DMEMT, forming the appropriate salt in situ, if necessary, insuring complete dissolution and stability in solution at pH 7.4 for all the experiments. The solutions were preincubated at 37°C to avoid lag-time effects. A total of 0.2 ml of the antibiotic solutions was placed into the apical chamber and 0.4 ml DMEMT was placed into the basolateral chamber of the Caco-2 cells preparation. After 2 h incubation at 37°C, the experiment was stopped when $< 10\%$ of the antibiotic was permeated through the filter to assure linear kinetic conditions. The antibiotic concentration in the basolateral compartment was measured with a parallel line microbiological turbidimetric assay [7]. The test organism used for all microbiological assays was *S. aureus* ATCC 6538P incubated at 37°C in Iso-sensitest Broth (Oxoid Unipath Ltd, Basingstoke, UK) with absorbance measurements at 600 nm. A full kinetic experiment was done on D-cycloserine on 24 mm diameter Transwell plates. A total of 1.5 ml of 100 and 200 mg/l solutions of D-cycloserine in DMEMT was placed into the apical chamber and the basolateral medium was sampled (0.4 ml aliquots) at 15 min intervals during the incubation and substituted with pre-warmed medium. D-Cycloserine concentrations were measured by spectrophotometric assay at 230 nm. Apparent permeability coefficient (Papp) values were calculated from the slope of the regression of flux values versus apical concentrations. Both for the end-points determinations and for the kinetic experiment, flux (mg cm^2/s) was determined as the amount of antibiotic permeated in a given time through a known area. Under expected linear kinetics, end-point experiments provide reliable results with the advantage of avoiding perturbations of the assay system due to the sampling procedure, in particular when small basolateral volumes are involved.

3. Results

3.1. Characterization of the permeability of cell monolayer

Three different markers were used to assess the level of differentiation of the Caco-2 cell monolayers:

Table 1
TEER, conductance, fluorescein and ^{3}H-mannitol values of 15 and 21 day cultures of Caco-2 cells [a]

	15 day ($n = 41$)	21 day ($n = 48$)
TEER (Ω cm^2)	263 ± 129 (49%)	551 ± 143 (26%)
Conductance (mS/cm^2)	5.432 ± 3.775 (69%)	0.041 ± 1.052 (51%)
Fluorescein (mg/l)	0.074 ± 0.051 (69%)	0.021 ± 0.013 (62%)
^{3}H-mannitol (cpm)	3300 ± 2424 (73%)	873 ± 643 (74%)

[a] Mean (± SD) and CV% values were obtained from basolateral medium after application of 4 μg/ml of fluorescein and 0.5 mCi/l of ^{3}H-mannitol, respectively, in the apical chamber.

fluorescein and ^{3}H-mannitol permeabilities, and TEER. Culture wells were randomly chosen on the 15th and 21st days after seeding. Mean values of TEER, conductance and the passage of ^{3}H-mannitol and fluorescein are reported in Table 1. The values are all significantly different (t-test, $P < 0.01$) between the two days of sampling. The mean TEER value on the 21st day is more than twice that on the 15th day; the corresponding conductance values are reduced by the same degree. Mean passages of fluorescein and ^{3}H-mannitol are more than three times lower on the 21st day than those on the 15th day. The plots of fluorescein and ^{3}H-mannitol Papps versus TEER are reported in Fig. 1. A hyperbolic relationship of the form $y = a + b/x$ represents the best fit for the plotted values. The hyperbolic relationship shown in Fig. 1 becomes linear if conductance values are used instead of TEER. A log–log plot of ^{3}H-mannitol Papp versus fluorescein Papp (Fig. 2) for both the 15 and 21 day monolayers fell on the same straight line, adequately fitted by linear regression.

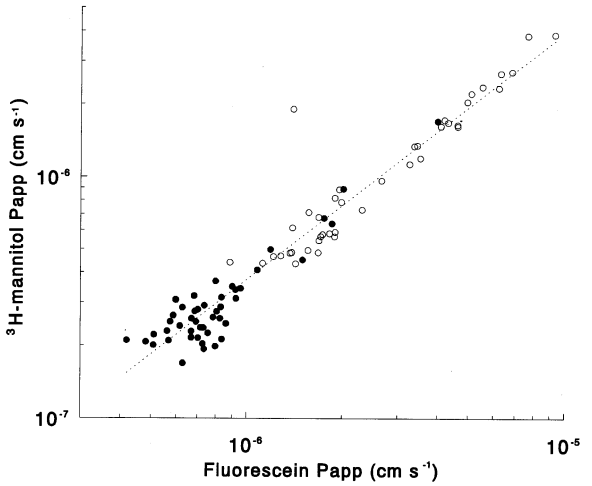

Fig. 2. Log–log plot of ^{3}H-mannitol vs. fluorescein Papp values of Caco-2 cells. The dotted line is the result of linear regression ($R^2 = 0.966$). (○) 15 day culture; (●) 21 day culture.

3.2. Permeability and absorption of antibiotics

Fig. 3 shows the results of a kinetic experiment on two apical concentrations of D-cycloserine (100 and 200 mg/l). Although this antibiotic is relatively highly permeable, measurements of the contents in the basolateral chamber indicate a linear kinetic condition of transport. Lag-time effects at the beginning of the experiment seem absent. Papp values for D-cycloserine, determined by taking into account all points or only end-point values, are very similar (3.66×10^{-6} versus 3.99×10^{-6} cm/s). Flux values of 11 antimicrobial drugs determined with the 21 day Caco-2 monolayers are reported in Fig. 4 and these were obtained from different apical concentrations for each drug. Fitted regression lines represent

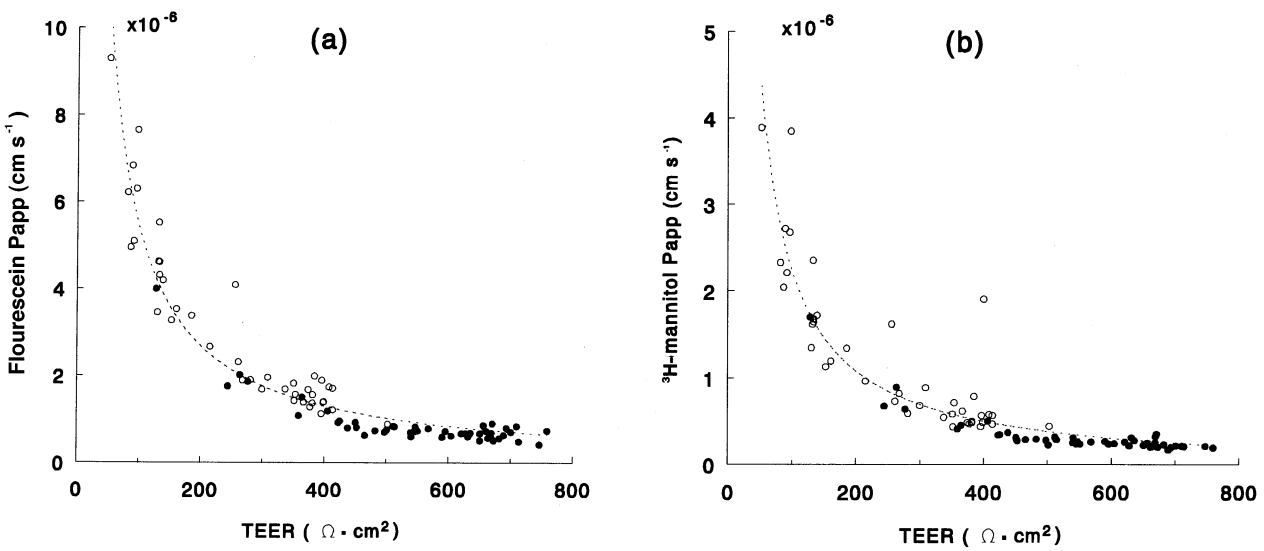

Fig. 1. Plots of ^{3}H-mannitol Papp (a) and fluorescein Papp (b), against transepithelial electrical resistance (TEER, Ω cm^2). The dotted lines are best fit predictions using a hyperbolic function ($y = a + b/x$; (b): $R^2 = 0.927$; (b): $R^2 = 0.866$). (○) 15 day culture; (●) 21 day culture.

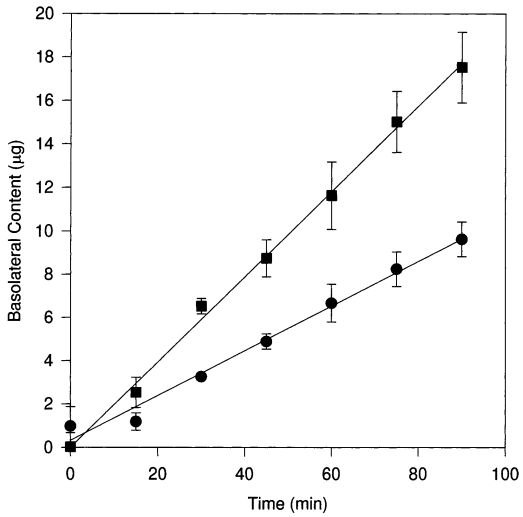

Fig. 3. Basolateral contents of D-cycloserine measured at intervals of 15 min starting from two apical concentrations of the antibiotic; passage is measured with Caco-2 cells grown on polycarbonate filters. (■) Mean (±SD) for 200 mg/l and (●) mean (±SD) for 100 mg/l, in the apical compartment.

the linear dependence of flux values from the corresponding apical concentrations; the slopes of these regressions determine Papp coefficient values. Table 2 lists Papp values for 13 tested antimicrobial drugs and human oral bioavailability taken from the literature [8–22], according to the definition reported by Gerding et al. [15]. These antibiotics are those reported in Table 2 plus teicoplanin and vancomycin. There is a good rank correlation between the two measures (Spearman $r = 0.957$). Absorption is negligible for drugs like teicoplanin with a Papp value $< 0.1 \times 10^{-6}$ cm/s and essentially complete for drugs, such as novobiocin, with a Papp value $> 4 \times 10^{-6}$ cm/s. However, although oral bioavailability tends to increase with increasing Papp, there is a large variability in the reported bioavailability values for a small change in Papp between these extremes. Thus, Papp values between 0.1 and 5×10^{-6} cm/s correspond to bioavailability values from $< 1\%$ to about 50%.

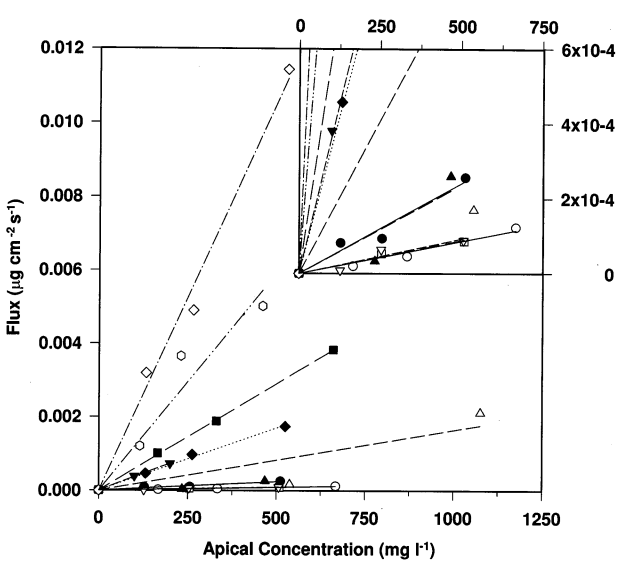

Fig. 4. Flux values of 11 antimicrobial agents measured for passage trough Caco-2 cells and plotted vs. corresponding apical concentrations with interpolated linear regressions. The square in the upper right corner provides expanded detail for the low permeating molecules. (●) Chlorotetracycline; (□) cephaloridin; (▲) erythromycin; (▽) penicillin-G; (◆) novobiocin; (◇) trimethoprim; (⬡) rifapentine; (○) rifamycin-SV; (■) rifampicin; (△) ampicillin; (▼) D-cycloserine.

4. Discussion

We have assessed the state of differentiation of Caco-2 cells by measuring paracellular permeability with three markers. Fluorescein and ^{3}H-mannitol have low lypophilicity and permeate paracellularly. TEER and its reciprocal value, conductance, are dependent upon the permeation of ions that mainly follow the paracellular route [1]; higher TEER values are expected for tighter monolayers. After 15 days of culture, our cells had TEER values of 263 ± 129 Ω cm^2, which are comparable to those of 258 ± 40 Ω cm^2 obtained by Artursson using the same cell line and culture period [1]. However, whereas he reported that the differentiation process monitored by TEER reaches a plateau after 10 days of culture, our TEER values increased further by day 21 of culture to values close to those (700–900 Ω cm^2) reported recently [23]. Although 21 day cells showed a wide range of TEER values (400–700 Ω cm^2), the corresponding range of permeability values of ^{3}H-mannitol and fluorescein is small. Measurements on day 21 versus those on day 15 show a decrease of the coefficient of variation (CV%) for fluorescein, TEER and conductance, while ^{3}H-mannitol has the highest CV% values at both times. These results suggest that after 21 days of culture Caco-2 monolayers are better suited for higher precision permeability measurements. The excellent correlation shown between the measurements of the different markers demonstrates that any one of them is suitable to measure the level of paracellular permeability of the monolayer. However, on the basis of our results, we prefer the use of fluorescein than ^{3}H-mannitol because of the lower CV%. The determination of conductance could represent a valid alternative as it is linearly correlated with the markers. TEER determinations, although not linearly correlated with markers, appear more sensitive to variations in the tightness of the monolayers at low permeability values.

We realize the potential limitation of using a biological assay instead of a direct chemical assay for the

Table 2
Human oral bioavailability together with mean (±SE) Papp values for various antibiotics

Antibiotic	Route	Human oral bioavailability (%) [a,b]	Papp (cm/s) $\times 10^{-6}$ [c]
Vancomycin	IV	<1	<0.1
Teicoplanin	IV/IM	<1	<0.1
Rifamycin-SV	IV	<1	0.17 ± 0.016
Cephaloridin	IM	<3	0.18 ± 0.017
Penicillin-G	IV/IM	20–30	0.24 ± 0.033
Chlorotetracycline	PO	30	0.48 ± 0.045
Erythromycin	PO	30–60	0.47 ± 0.181
Ampicillin	PO	30–70	1.84 ± 0.743
D-Cycloserine	PO	100	3.62 ± 0.105
Novobiocin	PO	90	3.98 ± 0.142
Rifampicin	PO	98–100	5.79 ± 0.053
Rifapentine	PO	100	11.80 ± 1.397
Trimethoprim	PO	100	21.12 ± 0.384

[a] Taken from Refs. [9–23] according to the definition reported by Gerding et al. in 1991 [15].
[b] The range of values are reported when available.
[c] Point estimations are not available for Papp values $<0.1 \times 10^{-6}$ (cm/s) due to the quantitation limit of the corresponding antibiotic assay.

determination of the quantities of the antibiotics passed through the monolayer, but radiolabeled antibiotics were not available and HPLC methods did not have sufficiently low detection and quantitation limits. However, it should be noted that most available clinical pharmacokinetic data in humans were obtained with microbiological assays and, therefore, it might be easier to correlate biological activity passage in Caco-2 with oral bioavailability of antimicrobials in man.

The establishing of a correlation between oral bioavailability in humans and cell line permeability is strongly influenced by the wide variations in bioavailability for compounds within a narrow range of permeability values. These results are similar to those published by other authors comparing Caco-2 and in vivo absorption for different drugs [24]. The tested antimicrobials could be grouped into three categories: (1) Papp values $< 0.2 \times 10^{-6}$ cm/s, very poor absorption, bioavailability $< 1\%$, (2) Papp values between 0.2×10^{-6} and 2×10^{-6} cm/s with bioavailability between 1 and 90%, and (3) Papp values $> 2 \times 10^{-6}$ cm/s, very good absorption, bioavailability over 90%. Papp appears to be a good predictor of bioavailability when antibiotics fall into either category 1 or 3, much less so when it is in category 2. Antibiotics belonging to this group evidence that absorbability is necessary but not sufficient in order to assure the use of a compound by the oral route. Bioavailability values for antibiotics in category 2 are rather variable, explained in part by the experimental conditions [15], and also whether the antibiotics were taken after food or fasting [18]. High oral bioavailability is always dependent on high permeability across the gastro-intestinal wall.

Metabolism often greatly influences the behavior of drugs before or after passage through the epithelial barrier, and low oral bioavailability could be due to first pass loss. In vitro systems have the potential to help separate the effects of these two processes. One possible example is rifamycin-SV; for rifamycin, several mechanisms have been put forward to explain its very low oral bioavailability. One is extensive first pass metabolism subsequent to good penetration [10]. Another is rapid gastric conversion to rifamycin-S, which has a very low aqueous solubility and poor permeability [12]. Our results suggest that, at least in part, the explanation lies in poor permeability with a Papp of rifamycin-SV lower than that of other rifamycins, such as rifampicin or rifapentine. We are currently investigating if this phenomenon in the Caco-2 model is dependent on the conversion of rifamycin-SV to rifamycin-S. The Caco-2 cell line is one of an increasing number of available intestinal human cell lines [2]. It shows electrical resistance values similar to colonic epithelium [1,25] much higher than those of the small intestine where most of intestinal drug absorption occurs. This epithelial cell line may reflect only a small element of the complex apparatus of the gastrointestinal tract but it can nonetheless give information on a critical phase of the antimicrobials delivery process.

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