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The Influence of Plasma Binding on Absorption/Exsorption in the Caco-2 Model of Human Intestinal Absorption

RICHARD A. WALGREN AND THOMAS WALLE

Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC 29425, USA

Abstract

The Caco-2 cell monolayer has become an increasingly useful in-vitro model of human intestinal absorption. In this study we have determined the effect of plasma on the basolateral side on the absorption as well as exsorption of several drugs that are highly bound to plasma proteins. The drugs used included propranolol and quercetin, which both use the transcellular route of absorption, and taxol and oestradiol 17 β-D-glucuronide, which are thought to undergo efflux by P-glycoprotein and the multidrug resistance protein MRP, respectively. All experiments were carried out under sink conditions to mimic normal absorption. It was necessary to use heparin anticoagulation for generation of the plasma, as EDTA was found to make the monolayers very leaky.

The apparent permeability (P_{app}) values for absorption were $1{\cdot}54 \times 10^{-6}\,cm\,s^{-1}$ for oestradiol 17 β-D-glucuronide, $3{\cdot}33 \times 10^{-6}\,cm\,s^{-1}$ for taxol, $20{\cdot}8 \times 10^{-6}\,cm\,s^{-1}$ for quercetin, and $35{\cdot}3 \times 10^{-6}\,cm\,s^{-1}$ for propranolol. For these four compounds, plasma on the basolateral side had no influence on absorption. However, plasma on the basolateral side significantly reduced the efflux of oestradiol 17 β-D-glucuronide by 66%, taxol by 75%, propranolol by 82%, and quercetin by 94%.

Failure to consider the effect of plasma binding can result in an overestimate of basolateral to apical efflux and result in misleading net flux calculations.

The good correlation between drug transport through Caco-2 cell monolayers and that observed in-vivo has made the Caco-2 cell system useful for predicting the intestinal absorption of compounds as well as for examining mechanisms of transport (Artursson 1990; Artursson & Karlsson 1991; Meunier et al 1995; Gan & Thakker 1997; Lennernäs 1997). Caco-2 cell monolayers have been used to examine intestinal exsorption, i.e. the systemic to luminal efflux of compounds, by the active membrane transporter P-glycoprotein (Augustijns et al 1993; Hunter et al 1993; Cavet et al 1996; Walle & Walle 1998). The simplicity of the Caco-2 cell system removes many of the confounding variables of more complex systems, facilitating more readily interpretable results.

Our laboratory has recently examined the Caco-2 cell transport of two natural product drugs, taxol (Walle & Walle 1998) and quercetin (Walgren et al 1998). Both compounds demonstrated a similar apical to basolateral apparent permeability of about $5 \times 10^{-6}\,cm\,s^{-1}$, suggesting a moderate to good absorption in-vivo. As both of these compounds are highly plasma-protein bound in the circulation, approximately 95% for taxol (Kumar et al 1993) and 99% for quercetin (Boulton et al 1998), it became of interest to learn whether such high binding may affect drug transport. This may apply particularly to drug exsorption, a topic that has recently received some attention (Arimori & Nakano 1998).

This investigation examined both apical to basolateral flux (absorption) and basolateral to apical flux (exsorption) of taxol and quercetin in the presence and absence of normal human plasma on the basolateral, i.e. circulation, side. We also studied whether plasma would affect the flux of a paracellular transport marker, mannitol, a transcellular transport marker, propranolol, and a substrate for the multidrug resistance protein (MRP), oestradiol 17 β-D-glucuronide (Jedlitschky et al 1996). Propranolol is known to be highly bound to plasma proteins (McDevitt et al 1976; Walle et al 1983).

Correspondence: T. Walle, Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, 173 Ashley Avenue, PO Box 250505, Charleston, SC 29425, USA.
E-Mail: wallet@musc.edu.

Materials and Methods

D-[1-^{14}C]Mannitol (57·0 mCi mmol^{-1}) and racemic-[4′-^{3}H]propranolol hydrochloride (14·4 Ci mmol^{-1}) were purchased from Amersham Life Science (Arlington Heights, IL). [^{3}H]Taxol (16·4 Ci mmol^{-1}) was purchased from Moravek Biochemicals (Brea, CA). [6,7-^{3}H]Oestradiol 17 β-D-glucuronide (55 Ci mmol^{-1}) was purchased from DuPont-New England Nuclear (Boston, MA). [^{14}C]Quercetin (52·9 mCi mmol^{-1}) was custom-synthesized by Chemsyn Science Laboratories (Lenexa, KA) for the NCI Radiochemical Reference Standard Repository. Quercetin dihydrate, oestradiol 17 β-D-glucuronide, and taxol were purchased from Sigma Chemical Co. (St Louis, MO).

Blood was collected by venipuncture from healthy volunteers into EDTA-treated or heparinized tubes. Plasma was separated by centrifugation and stored at −20°C until used.

Plasma binding of oestradiol 17 β-D-glucuronide was determined by equilibrium dialysis (Walle et al 1983).

Caco-2 cells (American Type Culture Collection, Rockville, MD) were cultured in Eagle's Minimum Essential Medium (MEM; Cellgro, Mediatech, Herndon, VA) supplemented with 1% MEM non-essential amino acids (Mediatech), 10% foetal bovine serum (Summit, Fort Collins, CO), 100 units mL^{-1} penicillin, and 0·1 mg mL^{-1} streptomycin (Sigma), and were grown in a humidified atmosphere of 5% CO_2 at 37°C. Cells were subcultured at 80% confluence. For all transport studies, Caco-2 cells were seeded in 12-mm i.d. Transwell inserts (polycarbonate membrane, 0·4-μm pore size, Corning Costar Corp., Cambridge, MA) in 12-well plates at a density of $1·0 \times 10^5$ cells cm^{-2}. The basolateral (serosal) and apical (mucosal) compartments contained 1·5 and 0·5 mL of culture medium, respectively. Culture medium was replaced three times a week for 14 days and daily thereafter. Caco-2 cells in Transwell inserts at passage 40–66 were used for transport experiments 20 to 35 days post-seeding. Inserts with transepithelial electrical resistance (TEER) values $> 350\,\Omega\,cm^2$ in culture medium were washed twice for 30 min with warm Hanks' Balanced Salt Solution with 25 mM HEPES, pH 7·4 (HBSS).

Transepithelial fluxes were measured for [^{14}C]quercetin (50 μM), [^{3}H]taxol (10 μM), [^{3}H]propranolol (100 μM), [^{3}H]oestradiol 17 β-D-glucuronide (1·0 μM), and [^{14}C]mannitol (1·1 μM). Stock solutions of quercetin in ethanol and oestradiol 17 β-D-glucuronide and taxol in dimethylsulphoxide (DMSO) were diluted in HBSS or plasma. The resulting final concentration of ethanol, 0·5%, and DMSO, 0·1–0·5%, did not affect TEER values or the transport of mannitol. All other compounds were dissolved in transport medium.

For absorption studies, apical chambers were loaded with HBSS containing test substrates (0·5 mL), and basolateral chambers were filled with either HBSS (control) or plasma (1·5 mL). For exsorption studies, test substrates were loaded on the basolateral side in either HBSS (control) or plasma while the apical chamber was loaded with HBSS. Taxol transport studies were conducted for 120 min at 37°C. All other transport studies were conducted for 60 min. On termination, samples were collected from both sides of the cell monolayer and immediately analysed on a Beckman LS 6000SC liquid scintillation system (Fullerton, CA) after the addition of Scintisafe Econo2 liquid scintillation fluid (Fisher Scientific, Fair Lawn, NJ).

Apparent permeability coefficients (P_{app}) were calculated using the following equation:

$$P_{app} = V/AC_0 \cdot dC/dt = \text{cm s}^{-1} \qquad (1)$$

where V is the volume of the solution in the receiving compartment, A is the membrane surface area, C_0 is the initial concentration in the donor compartment, and dC/dT is the change in drug concentration in the receiver solution over time (Artursson 1990; Walgren et al 1998).

Transport data are expressed as the mean ± s.d. of five or more determinations. Analysis of variance was used to evaluate differences in flux. $P < 0·05$ was considered significant.

Results and Discussion

In the first series of experiments we examined the paracellular transport marker mannitol. With buffer on the basolateral side, mannitol demonstrated an apical to basolateral flux of 1·26 ± 0·8 pmol h^{-1} cm^{-2}, corresponding to an apparent permeability of $0·32 \pm 0·19 \times 10^{-6}$ cm s^{-1}. This is the expected result from a tight, well functioning Caco-2 epithelial cell monolayer (Walgren et al 1998). In the presence of basolateral plasma from heparin-treated blood, the transport rate was not different from the rate observed in the presence of basolateral buffer solution. The latter observation is consistent with the fact that mannitol is not plasma-protein bound. The increased mannitol flux in the presence of EDTA-treated plasma is likely due to reduced calcium levels by complexation with EDTA, thereby opening the intracellular tight junctions of the Caco-2 cells (Artursson & Magnusson 1990; Gan et al 1993).

For the transport of quercetin, the presence of plasma on the basolateral side had no influence on the apical to basolateral flux, whether EDTA or heparin was used as the anticoagulant (Table 1). Based on the results with mannitol, this suggests that the flux of quercetin is via the transcellular rather than paracellular route, emphasizing the lipophilic nature of quercetin. Efflux of quercetin was dramatically reduced when the basolateral side was loaded with plasma rather than HBSS. This is consistent with the very high plasma binding previously reported for quercetin (Boulton et al 1998). It should be noted that the P_{app} for quercetin was greater than previously reported (Walgren et al 1998). This has no obvious explanation.

The effect of basolateral plasma on the flux of two lipophilic, highly protein-bound drugs, propranolol and taxol was investigated. Propranolol is transported effectively by the transcellular pathway (Artursson 1990), whereas taxol, although showing some absorption, is effectively effluxed by P-glycoprotein (Walle & Walle 1998). For both drugs, the presence of plasma on the basolateral side, while having no influence on absorption, effectively reduced the efflux (Table 2). For these compounds, the mode of anticoagulation used to generate the plasma made no difference.

As with P-glycoprotein (Hunter et al 1993), the expression of the organic anion transporter MRP2 has been demonstrated in the Caco-2 cells (Walle et al 1999), both presumably located on the apical side of the cells (Hunter et al 1993; Jedlitschky et al 1996). Oestradiol 17 β-D-glucuronide, which is a substrate for MRP transporters (Jedlitschky et al 1996), demonstrated a $96{\cdot}0 \pm 0{\cdot}1\%$ (mean $\pm$ s.d.; $n = 7$) binding to plasma proteins. It was therefore of interest to determine the effect of plasma on the basolateral side on the flux of this substrate across the Caco-2 cell monolayer. Surprisingly, the efflux of this substrate was not significantly greater than the absorption (Table 2). However, consistent with the findings for propranolol and taxol, plasma on the basolateral side clearly inhibited efflux.

As these experiments show, the presence of plasma on the basolateral side has no influence on the net absorption of drugs, whether they are transported via the paracellular or transcellular routes or via active efflux processes. As absorption is driven by a concentration gradient, the experimental protocol of the absorption studies was, as is generally the case, designed to maintain the concentration gradient by utilizing sink conditions, i.e. the concentration on the donor side was much greater than the concentration on the receiving side throughout the experiment. Thus, under these experimental conditions, basolateral protein binding had a very limited effect on the apical to basolateral concentration gradient and hence, little effect on apical to basolateral transport. The absorption rates for substrates effluxed across the apical membrane (e.g. taxol) remained unchanged since the plasma binding of substrate on the basolateral side does not alter the concentration gradient across the apical membrane.

When plasma binding affects the concentration gradient of a substrate at the site of transport, the rate of flux is diminished, as observed for the efflux of taxol, i.e. basolateral protein binding reduced the concentration of taxol available for efflux across the apical membrane. The reduction in free drug concentration due to binding to basolateral plasma proteins also resulted in a decrease in the efflux of quercetin, propranolol, and oestradiol 17 β-D-glucuronide. Although not studied, it can be anticipated that binding to proteins on the apical side, i.e. dietary proteins, would similarly limit absorption.

The observed trapping of drug by plasma binding on the basolateral side may not be significant for compounds which are substrates for transporters that are located on the basolateral membrane and

Table 1. Effect of plasma on the basolateral side on the absorption (apical to basolateral flux) and exsorption (basolateral to apical flux) of mannitol and quercetin across the Caco-2 cell monolayer.

Transport direction	Medium on basolateral side[a]	P_{app} ($\times 10^{-6}$ cm s^{-1})	
		Mannitol 1·1 μM	Quercetin 50 μM
Absorption	Buffer	$0{\cdot}32 \pm 0{\cdot}20$	$15{\cdot}6 \pm 3{\cdot}00$
	EDTA-Plasma	$1{\cdot}62 \pm 0{\cdot}78$*	$20{\cdot}4 \pm 9{\cdot}44$
	Buffer	$0{\cdot}42 \pm 0{\cdot}20$	$20{\cdot}8 \pm 4{\cdot}00$
	Heparin-plasma	$0{\cdot}44 \pm 0{\cdot}20$	$24{\cdot}1 \pm 1{\cdot}72$
Exsorption	Buffer	$0{\cdot}51 \pm 0{\cdot}07$	$14{\cdot}2 \pm 2{\cdot}11$
	EDTA-plasma	$1{\cdot}72 \pm 0{\cdot}75$*	$3{\cdot}0 \pm 0{\cdot}72$†
	Buffer	$0{\cdot}49 \pm 0{\cdot}08$	$22{\cdot}6 \pm 2{\cdot}72$
	Heparin-plasma	$0{\cdot}52 \pm 0{\cdot}12$	$1{\cdot}3 \pm 0{\cdot}22$†

[a]Plasma was harvested from blood using either EDTA or heparin as an anticoagulant. Values are mean $\pm$ s.d., $n = 5-7$. *$P < 0{\cdot}01$, significantly higher compared with buffer. †$P < 0{\cdot}001$, significantly lower compared with buffer.

Table 2. Effect of plasma on the basolateral side on the absorption (apical to basolateral flux) and exsorption (basolateral to apical flux) of propranolol, taxol, and oestradiol 17 β-D-glucuronide across the Caco-2 cell monolayer.

Transport direction	Medium on basolateral side	P_{app} ($\times 10^6$cm s^{-1})		
		Propranolol 100 μM	Taxol 10 μM	Oestradiol 17 β-D-glucuronide 1·0 μM
Absorption	Buffer	35·3 ± 9·7	3·33 ± 1·39	1·54 ± 0·28
	Plasma	38·3 ± 20·8	4·17 ± 1·39	1·30 ± 0·21
Exsorption	Buffer	22·8 ± 8·1	29·2 ± 3·61	1·57 ± 0·70
	Plasma	4·2 ± 1·7†	7·2 ± 1·39†	0·54 ± 0·29†

Values are mean ± s.d., n = 5–7. †$P < 0{\cdot}001$, significantly lower compared with buffer.

direct basolateral to apical flux. Such a transporter may strip the drug from plasma binding sites. We were, however, unable to find such a drug to test this hypothesis.

In summary, including plasma proteins on the basolateral side of Caco-2 transport experiments represents a more physiologically relevant model. While not likely to affect apical to basolateral absorption, failure to consider the effect of plasma binding can result in an overestimate of basolateral to apical efflux and misleading net flux calculations.

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