In Vitro and *In Situ* Absorption of SDZ-RAD Using a Human Intestinal Cell Line (Caco-2) and a Single Pass Perfusion Model in Rats: Comparison with Rapamycin

Andrew Crowe[1,2] and Michel Lemaire[1]

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Purpose. To compare the intestinal absorption and active efflux protein susceptibility of a new immunosuppressive agent (SDZ-RAD) with that of its analog rapamycin.
Methods. Caco-2 cell monolayers were used to examine bidirectional transport of the two compounds at low micromolar concentrations. Single pass rat intestinal perfusion was also used to examine steady state permeability.
Results. Rapamycin and SDZ-RAD showed a distinct preference for transport in the basolateral to apical direction of Caco-2 monolayers as efflux was >20 times greater than apical to basolateral transport. Efflux of SDZ-RAD was completely inhibited by verapamil while efflux of rapamycin was mostly inhibited by verapamil and partially inhibited by probenecid. Passive permeability was shown to be 20×10^{-6} cm/sec for SDZ-RAD and 10×10^{-6} cm/sec for rapamycin. *In situ* rat studies also showed the permeability of rapamycin to be half that of SDZ-RAD with permeabilities of 12.6×10^{-6} for rapamycin and 24.8×10^{-6} cm/sec for SDZ-RAD.
Conclusions. SDZ-RAD and rapamycin are strong substrates for P-gp-like mediated efflux. Rapamycin is also partially removed from cells by a second efflux system that is not responsive to SDZ-RAD. When these efflux pumps are inhibited SDZ-RAD is likely to be absorbed across the intestine at a faster rate than rapamycin.

KEY WORDS: SDZ-RAD; rapamycin; P-glycoprotein; Caco-2; probenecid; verapamil.

INTRODUCTION

Many current immunosuppressants including cyclosporin A (CsA) are known to have variable intestinal absorption and have a low therapeutic index making dosing regimes difficult (1). New formulations have helped to overcome variable absorption but the search for additional immunosuppressants continues and currently calcium independent immunosuppressants that inhibit T cell division at sites removed from that of CsA are being developed that work in synergy with CsA such as rapamycin and SDZ-RAD (2). The problems of variable absorption are still an issue to overcome, as rapamycin has also been shown to display wide intersubject variation in pharmacokinetic parameters (3). SDZ-RAD is structurally analogous to rapamycin, but differs by the inclusion of a 40-O-(2-hydroxyethyl) side group. SDZ-RAD binds to cytosolic FKBP-12 and has similar potency to rapamycin, but the added side group is expected to offer a better absorption profile for SDZ-RAD.

P-glycoprotein, an active efflux protein that was first identified in tumor cells as the means by which they became multidrug resistant has since been discovered in the functional barrier layers of human and animal organs such as the endothelial cells of the blood-brain barrier and the enterocytes of the intestinal wall (4,5). The presence of this pump in the intestine is likely to be responsible for some of the variable absorption seen with modern immunosuppressants (6,7). Apart from P-glycoprotein, multidrug resistant-associated protein (MRP) has recently been discovered (8) indicating at least two different active efflux pumps involved in multidrug resistance (9).

The human colonic cell line Caco-2 has been shown to display small intestine like qualities when fully differentiated (10). These cells also express P-glycoprotein and possibly other active efflux proteins. The intestinal characteristics and efflux protein expression in these cells made them ideal to examine the extent of rapamycin and SDZ-RAD permeability and to determine whether they are also substrates for active efflux transport. To further characterize any efflux transport observed we have used verapamil and probenecid in combination with rapamycin and SDZ-RAD since verapamil and probenecid have been shown to be inhibitors of P-gp (11) and MRP (12), respectively. Another aim of this study was to compare the steady state absorption of these two immunosuppressants *in situ* using a single pass intestinal perfusion model and further to link these results to those obtained from Caco-2 monolayers.

MATERIALS AND METHODS

Materials

[^{3}H]-Mannitol (728 MBq/μmol) and [^{3}H] Digoxin (555 MBq/μmol) were purchased from Dupont NEN (Regensdorf, Switzerland). [^{3}H]-Propranolol (1040 MBq/μmol) and [^{3}H]-Cyclosporin-A (330 MBq/μmol) were purchased from Amersham International (Buckinghamshire, England). [^{3}H]-RAD (44.67 MBq/μmol) and [^{14}C]-Rapamycin (1.57 MBq/μmol) were prepared by Novartis's isotope laboratory and shown to be higher than 99% pure by HPLC analysis. The positions of the radiolabel for rapamycin and SDZ-RAD are shown in Fig. 1.

Hanks buffered salt solution (HBSS) and phosphate buffered saline (PBS) were obtained from GibcoBRL (Paisley, Scotland). Transwell 12 mm polycarbonate filter inserts were purchased from Corning Costar Corporation (Cambridge, MA). The epithelial volt-ohm meter (EVOM) and Endohm-12 chamber were supplied by World Precision Instruments (Sarasota, FL). Verapamil was obtained from Fluka Chemicals (Buchs, Switzerland). Probenecid was purchased from ICN Biomedicals GmbH (Eschwege, Germany). Dulbecco's modified Eagles medium (DMEM), HEPES and all other chemicals were from Sigma Chemicals (St. Lous, MO).

Caco-2 Monolayers

The human colon carcinoma cell line (Caco-2) used in our laboratory originated from University Hospital, Utrecht, NL, and was cultured in a similar fashion to that done by Artursson, 1990 (13). Briefly, the cells were maintained in 25 cm^2 flasks containing DMEM supplemented with 10% fetal calf serum, 1% non-essential amino acids, penicillin (161 U/ml) and 100

[1] Drug Metabolism and Pharmacokinetics, Novartis Pharma Inc., Basel CH 4002, Switzerland.
[2] To whom correspondence should be addressed. (e-mail andrew.crowe@pharma.novartis.com)

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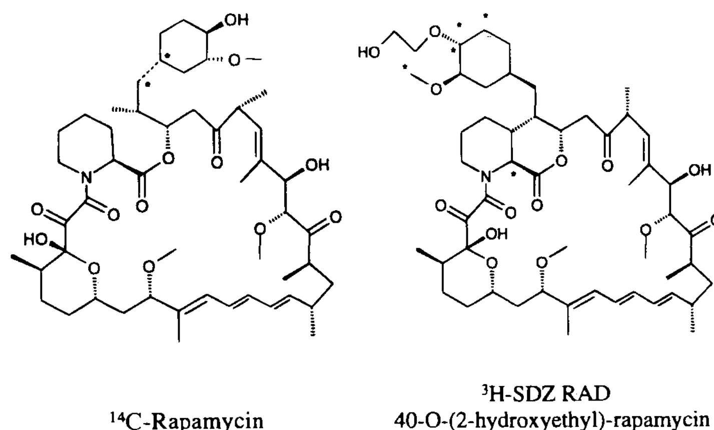

Fig. 1. Structure of Rapamycin and SDZ-RAD. Asterisk positions represent location of radiolabel.

μg/ml streptomycin. They were incubated at 37°C, 95% humidity and 5% CO_2. Before reaching confluency, at 6 days post-culturing, the cells were removed by trypsinization in 0.25% trypsin and 0.02% EDTA. Cells were resuspended in culture medium and seeded onto 1 cm^2 Transwell polycarbonate filter inserts (0.45 μm pore size) in 12 well plates at 100,000 cells/cm^2. DMEM was replaced every 2–3 days. The cells reached confluence between 5–7 days after seeding on filters, but were allowed to develop for 21–24 days before transport studies were initiated. Caco-2 cells were used between passages 75–86.

Monolayer Transport

All transport experiments were conducted at 37°C, for 2 hours in transport medium [HBSS (pH 7.4) containing 25 mM D-glucose and 25 mM HEPES buffer]. Before initiation of the study the monolayers were allowed to equilibrate at 37°C for 60 min in transport medium. Each Transwell insert was measured for trans-epithelial electrical resistance (TEER) at this time using a EVOM voltohm meter. Transport studies in the apical to basolateral direction (Ap to Bas) had 1.5 ml of transport medium placed in the basolateral chamber. Initiation of the study began when 0.5 ml of transport medium supplemented with the test compound was placed in the apical chamber. Transport studies in the basolateral to apical direction (Bas to Ap) had 0.5 ml of transport medium added first to the apical chamber followed by 1.5 ml of transport medium supplemented with test compound applied to the basolateral chamber. 200 μl was removed from the basolateral chamber (acceptor side) in Ap to Bas direction studies, while 75 μl was removed from the apical chamber in Bas to Ap studies at 5, 10, 15, 20, 30, 45, 60, 90, 105 and 120 min. Volumes taken were replaced with fresh prewarmed transport medium. At each of these times, just prior to sampling, the 12 well plates were gently swirled. After 120 min, samples were also taken from the donor side and TEER measurements were repeated to confirm that cellular integrity was maintained throughout the study. If the TEER of monolayers fell below 250 $\Omega.cm^2$ cells were considered leaky and results from these monolayers were not used. Generally TEER of our Caco-2 cells ranged from 350 to 600 $\Omega.cm^2$. Monolayers and wells were washed twice in excess PBS before the cells were trypsinized for 10 min. Cells, filters and all samples collected during the 120 min study had Lumasafe liquid scintillation cocktail (Lumac LSC, Groningen, Netherlands) added and were counted in a Beckman Tri-carb β counter to determine the amount of radiolabeled compound transported into and through the cell monolayers. Stability studies were also conducted on ^{3}H-SDZ-RAD and ^{14}C-rapamycin. They were placed in transport buffer alone or with Caco-2 cell at 37°C for over two hours and examined by HPLC. Both compounds were stable in our Caco-2 transport medium and metabolism in the Caco-2 environment did not occur to any significant degree.

In studies involving the addition of verapamil or probenecid, they were present in both apical and basolateral chambers at 0.1 mM and 1.5 mM respectively. Transport media supplemented with verapamil or probenecid were only added simultaneously with the test compounds allowing no preincubation. Otherwise these studies were identical to the normal Ap to Bas or Bas to Ap direction studies, except that the fresh media replacing that taken also contained either 0.1 mM verapamil or 1.5 mM probenecid.

CsA and digoxin are P-gp substrates and were used to confirm that a P-gp like active efflux pump operated in our Caco-2 cells, while mannitol and propranolol were each used to provide standard permeability rates for low (paracellular) and high (transcellular) passive transport respectively. Digoxin, mannitol and propranolol were each used at 1.0 μM concentrations, while CsA was used at 0.5 μM.

The standard method for determining apparent permeability through Caco-2 cells is to use Artursson's equation (Eq. 1)(14).

$$P_{app}(\text{cm/s}) = \frac{\Delta Q}{\Delta t 60 A C_o} \qquad (1)$$

where $\Delta Q/\Delta t$ is the permeability rate (μg/min), C_o is initial concentration in donor chamber (μg/ml) and A is the surface area of the membrane (cm^2). All compounds used in our study were radiolabeled, which allowed us to measure the radioactivity associated with both cells and filters. We could then correct for these factors in the determination of permeability. Therefore, a modification of Artursson's equation was created, as shown here in expanded format to first determine corrected clearance volumes (Eq. 2a), followed by the effective permeability (P_{eff})

determined from the slope of the regression line of the clearance volumes vs time (Eq. 2b).

$$\text{Cl.vol} = A_a/\{[C^*_{d0}V_d - [A_a + (C^*_{a'}V_s)] - (A_c + A_f) * n/n_{fin}]/V_d\} \quad (2a)$$

where

Cl.vol = Clearance volume (ml);
n = time (min);
A_a = Amount in acceptor compartment at time n (dpm);
C_{d_0} = Concentration in donor compartment at time 0 (dpm/ml);
V_d = Volume of donor compartment (ml);
$C_{a'}$ = Concentration in acceptor compartment at the previous time point (dpm/ml);
V_s = Sample volume of previous time point (ml);
$A_c + A_f$ = Amount of compound associated with cells and filters respectively after cells are removed from the filters at the end of the study (dpm); and
n_{fin} = final time point (min)

$$P_{eff} = J_{ss}/A \quad (2b)$$

where J_{ss} is rate of clearance which equates to Cl.vol/n (ml/s), A is the area of monolayer (cm^2) and P_{eff} = effective permeability (cm/s).

In Situ Rat Intestinal Perfusion

Adult Wistar rats (300 g) were fasted overnight before initiating the permeability study. Rats were anaesthetized for the duration of the study with intra-peritoneal injections of urethane (1.1 g/kg). The upper to middle 20 cm of rat jejunum was cannulated at both ends with silicone tubing. The initial delivery end was attached to a Harvard infusion pump, while the exit tubing was collected in liquid scintillation vials. The animal was maintained at 37°C and the cannulated segment was infused with warmed HBSS until the perfusate was clear. 1 μM ^3H- or ^{14}C- labeled test compound (4.17–7.5 kBq/ml [^3H]; 1.0–2.5 kBq/ml [^{14}C]) along with 5 μM ^{14}C- or ^3H- PEG 4000 (0.7 kBq/ml [^{14}C]; 4.2 kBq/ml [^3H]) was then warmed and pumped through the jejunal segment at 0.08 ml/min for 150 min, collecting samples every 15 min. Mucosa was scraped from the segments at the end of the study and solubilized in soluene 350 for 2–3 days. Collections were also made from the tubing before and after the study to determine the extent of non-specific binding to the tubing during the course of the study. All collections and solubilized mucosa were counted in a liquid scintillation counter and permeability values (P_{eff}) were determined using the following calculations:

$$P_{eff} = [1 - (C^*_{test}(C_{in}/C_{PEG})/C_{corr})] * Q/[2\pi rl]/60 \quad (3)$$

C_{test} = concentration of compound at a steady state point (dpm/ml);
C_{in} = final PEG-4000 inflow (dpm/ml);
C_{PEG} = concentration of PEG-4000 at the same steady state point as C_{test} (dpm/ml);
C_{corr} = corrected initial compound concentration for a particular time point (dpm/ml);
Q = flow rate (ml/min);
r = radius of intestine (cm); and
l = Section length (cm).

The protocols used for the animal studies used herein were approved by the official veterinary authority of Switzerland, and were conducted in strict accordance with Swiss animal care regulations.

Results expressed in this study are presented as the mean ± SEM. Significant differences between values were examined using Student's two-tailed unpaired t-test. Results were considered significant if $P < 0.05$.

RESULTS

Permeability characteristics for rapamycin were determined at 0.2, 1.0 and 5.0 μM. Due to solubility limitations SDZ-RAD could only be examined at 0.2 and 1.0 μM. SDZ-RAD displayed no concentration dependence at 0.2 or 1.0 μM in the Ap to Bas direction with P_{eff} values around $1.6–2.0 \times 10^{-6}$ cm/sec (Fig. 2A), which was 6–7 times higher than that obtained with our reference paracellular compound, mannitol (0.24×10^{-6} cm/sec), but was still very low when compared to the permeability coefficient from the highly absorbed transcellular compound, propranolol (31.5×10^{-6} cm/sec) (Table 1). Unlike SDZ-RAD, rapamycin showed concentration dependence in its permeability rates over the range of concentrations tested, from 0.1×10^{-6} cm/sec at 0.2 μM to 8.6×10^{-6} cm/sec at 5.0 μM (Fig. 2B). Permeability in the Bas to Ap direction was much greater than Ap to Bas transport for SDZ-RAD and rapamycin (Fig. 2). In the Bas to Ap direction, monolayers exposed to 1.0 μM SDZ-RAD displayed a higher permeability value than 0.2 μM SDZ-RAD, however, as both values were very high it may be considered that very little effective difference existed between 0.2 and 1.0 μM SDZ-RAD. Rapamycin showed progressively slower permeability rates in the Bas to Ap direction with increasing concentration, but transport in this direction was still significantly greater than in the Ap to Bas direction at the highest concentration tested (Fig. 2B).

Addition of 100 μM verapamil resulted in a 12 fold increase of Ap to Bas transport of SDZ-RAD to $\sim 23 \times 10^{-6}$ cm/sec (Fig. 2A). Concurrently, Bas to Ap transport decreased to about the same level ($18*10^{-6}$ cm/sec) regardless of the concentration of SDZ-RAD being examined, suggesting that a P-gp pump was the mechanism for active efflux for SDZ-RAD and that 100 μM verapamil was able to completely inhibit its action. Cyclosporin A at low concentrations (0.5 μM) is a known substrate for P-gp (6) and the addition of 100 μM verapamil in this study was able to increase permeability of this compound 6 fold in the Ap to Bas direction while also reducing Bas to Ap transport to similar rates (Table 1), providing further evidence that high levels of P-gp are functional in our Caco-2 monolayers. At higher concentrations, CsA becomes a competitive inhibitor of P-gp and has been shown in this laboratory to have similar effects to verapamil upon SDZ-RAD and rapamycin (results not shown).

Bidirectional transport of rapamycin was almost equivalent when 100 μM verapamil was added to 0.2 μM rapamycin. However, as the concentration of rapamycin was increased to 1.0 μM and 5.0 μM, Bas to Ap efflux increased from 10.4 to 14.7×10^{-6} and 17.7×10^{-6} cm/sec respectively (Fig. 2B). The gap between Ap to Bas and the reverse direction became

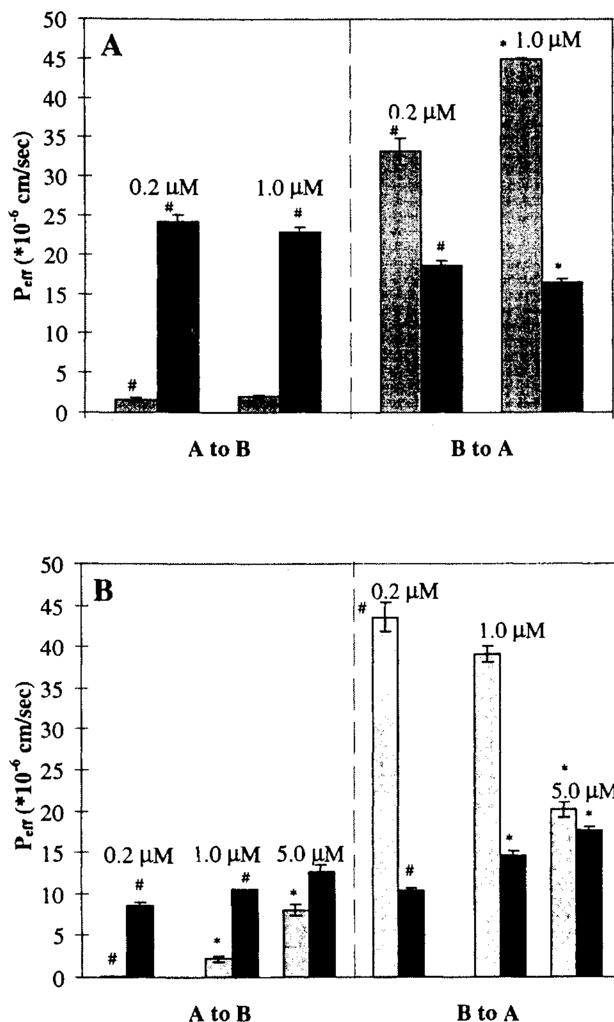

Fig. 2. Permeability coefficients (P_{eff}) for A) SDZ-RAD at 0.2 and 1.0 μM and B) Rapamycin at 0.2, 1.0 and 5.0, in both the apical to basolateral and basolateral to apical directions with (black columns) and without (light columns) the addition of 100 μM verapamil. # Significant difference between SDZ-RAD and rapamycin at the same concentration. * Significance due to increased concentration for the individual compounds.

smaller as the concentration of rapamycin increased, yet verapamil, always present at 20 times or more the concentration of rapamycin, could do little to completely equalize permeability. It became apparent that a second transporter may be providing the momentum for increased efflux.

Probenecid was added at 1.5 mM to 1.0 μM concentrations of either SDZ-RAD or rapamycin. Total transport of digoxin, another known P-gp substrate, was unaffected (Table 1), yet Bas to Ap transport was decreased for CsA, RAD and rapamycin, with the highest decreases reported for rapamycin and CsA. However, in the Ap to Bas direction only rapamycin showed significantly higher transport suggesting that a second efflux pump may be present on the Caco-2 cells and that rapamycin was more sensitive towards this pump, compared to the other P-gp substrates. Co-incubation of rapamycin with both verapamil and probenecid completely equilibrated transport through Caco-2 monolayers in both directions.

SDZ-RAD and rapamycin association with the cells was also determined after 2 hours of transport. It was found that during transport in the Ap to Bas direction there were significantly higher concentrations of compound associated with the cells compared to Bas to Ap transport (Table 2). SDZ-RAD at 1.0 μM resulted in lower cell accumulation than at 0.2 μM, while cellular accumulation of rapamycin was very high (14–19%) at all three concentrations examined (Table 2).

When using different models to examine intestinal uptake, a good correlation can be obtained from using a single pass perfusion model in conjunction with Caco-2 monolayers (15). To provide further evidence that SDZ-RAD has an inherent passive absorption profile almost double that of rapamycin, a single pass perfusion model was developed in rats. Permeabilities in this system require uptake to be at a steady state. Rapamycin reached steady state absorption in the quickest time, followed by mannitol, RAD and propranolol. All results were corrected gravimetrically and for PEG-4000 flux. Therefore the absorption of PEG-4000 in this system was assumed to be 0, and all the other compounds were related to it. The adsorption of compounds to the tubing before entering the intestinal segment had to be corrected as some compounds adsorbed to the silicone tubing used for pumping the test solution through the gut section. PEG 4000 and mannitol exhibited no binding to the tubing, but propranolol, rapamycin and SDZ-RAD had substantial binding to the silicone tubing.

Mannitol and propranolol had steady state permeabilities of 0.34×10^{-5} and 2.50×10^{-5} cm/sec respectively in this system (Table 3). The range in permeabilities for low and highly permeable compounds was more condensed in this system compared to the *in vitro* Caco-2 system, but the range was still adequate to determine whether compounds would be low, moderately or highly absorbed. SDZ-RAD had an *in situ* permeability of 2.48×10^{-5} cm/sec while that of rapamycin was only 1.26×10^{-5} cm/sec (Table 3). The relationship between *in situ* and *in vitro* permeabilities for these two compounds were plotted against those of other reference compounds (Fig. 3). Rapamycin and SDZ-RAD had *in situ-in vitro* permeabilities that correlated well with the reference compounds. The graphical representation also clearly illustrated the enhanced permeability of SDZ-RAD over rapamycin using both *in situ* and *in vitro* means.

DISCUSSION

The permeability results from both the Caco-2 monolayer system and the *in situ* single pass intestinal perfusion model show that SDZ-RAD has almost twice the rate of absorption compared to rapamycin when the effect of active efflux processes are removed.

Without removing these efflux processes, both SDZ-RAD and rapamycin were similar in their initial rates of transport through Caco-2 monolayers. Net transport was very much driven towards the apical side, with transport in this direction being 20 times faster than that in the basolateral direction, indicating the presence of an efflux pump. The massive difference between transport in the two directions also suggested that this was likely an active process. 5.0 μM solutions of SDZ-RAD were unattainable in solution due to the high lipophilicity of this compound, so we were unable to gauge whether this concentration would be enough to start saturating the efflux

Table 1. Caco-2 Monolayer Permeability Coefficients for SDZ-RAD, Rapamycin and Some Other Active Efflux and Passively Permeable Compounds After Incubation with 100 μM Verapamil and/or 1.5 mM Probenecid

Compound	Efflux inhibitor	Permeability coefficient ($\times 10^{-6}$ cm/sec)		
		Ap to Bas	Bas to Ap	Net transport A-B
SDZ-RAD	None	2.0 ± 0.2	45.0 ± 1.0	−43
	Verapamil	22.9 ± 0.8	16.5 ± 0.5	6.4
	Probenecid	1.7 ± 0.2	39.2 ± 0.7	−37.5
Rapamycin	None	2.2 ± 0.4	39.0 ± 1.0	−36.8
	Verapamil	10.5 ± 0.1	14.7 ± 0.7	− 4.2
	Probenecid	6.9 ± 0.3	31.6 ± 0.3	−24.7
	Prob + Verap	12.0 ± 0.3	12.7 ± 0.6	− 0.7
Cyclosporin A	None	0.9 ± 0.4	20.5 ± 1.2	−19.6
	Verapamil	5.7 ± 0.8	7.7 ± 1.1	− 2.0
	Probenecid	0.4 ± 0.0	15.7 ± 0.2	−15.3
Digoxin	None	0.5 ± 0.0	13.2 ± 0.3	−12.7
	Verapamil	1.3 ± 0.0	1.9 ± 0.0	− 0.6
	Probenecid	0.4 ± 0.0	12.2 ± 0.9	−11.8
Mannitol	None	0.2 ± 0.0	0.1 ± 0.0	0.1
	Verapamil	0.4 ± 0.0	0.3 ± 0.1	0.1
	Probenecid	0.2 ± 0.0	0.1 ± 0.0	0.0
Propranolol	None	31.5 ± 1.4	31.2 ± 1.3	0.3
	Verapamil	49.1 ± 2.1	36.4 ± 0.4	12.7
	Probenecid	37.0 ± 0.6	27.8 ± 0.6	9.2

Note: All compounds were given at 1.0 μM, except cyclosporin A which was at 0.5 μM. All results are the mean ± SEM of three cell monolayer studies.

pump, resulting in a decreased rate of transport towards the apical side as was shown for rapamycin. However, at low concentrations of 0.2 μM the Bas to Ap transport remained at 20 times Ap to Bas transport for SDZ-RAD, but uptake in the Ap to Bas direction slowed to almost nothing for rapamycin, resulting in the Bas to Ap transport being 427 times greater.

Table 2. Cellular Levels of ^{3}H SDZ-RAD and ^{14}C Rapamycin in Caco-2 Monolayers After 120 min of Transport in Either Ap to Bas, or Bas to Ap Directions

Conc (μM)	Direction	Efflux inhibitor[a]	Intracellular accumulation (%)	
			SDZ-RAD	Rapamycin
0.2	Ap → Bas	none	11.4 ± 0.3	14.1 ± 0.8
0.2	Ap → Bas	Verapamil	25.4 ± 1.1	21.8 ± 0.5
0.2	Bas → Ap	none	7.7 ± 0.6	7.2 ± 0.1
0.2	Bas → Ap	Verapamil	9.2 ± 1.2	6.6 ± 0.1
1.0	Ap → Bas	none	4.4 ± 0.1	13.6 ± 0.6
1.0	Ap → Bas	Verapamil	9.1 ± 0.2	14.8 ± 0.1
1.0	Ap → Bas	Probenecid	10.5 ± 0.5	16.0 ± 0.9
1.0	Bas → Ap	none	3.3 ± 0.1	3.8 ± 0.2
1.0	Bas → Ap	Verapamil	5.2 ± 0.1	5.2 ± 0.1
1.0	Bas → Ap	Probenecid	3.4 ± 0.1	5.5 ± 0.1
5.0	Ap → Bas	none	[b]	19.5 ± 0.6
5.0	Ap → Bas	Verapamil	[b]	19.0 ± 0.4
5.0	Bas → Ap	none	[b]	6.7 ± 0.2
5.0	Bas → Ap	Verapamil	[b]	6.6 ± 0.1

Note: Data shown is the mean ± SEM of three Caco-2 monolayers.
[a] Verapamil and probenecid were added to both chambers at 100 μM and 1.5 mM respectively.
[b] Not available.

Hence, it was clearly shown that Caco-2 monolayers transport rapamycin from the basolateral chamber to the apical one much more rigorously than for SDZ-RAD.

Transport of SDZ-RAD through a human intestinal cell line or by intestinal perfusion has not previously been published. However, a recent study by Dias and Yatscoff did examine rapamycin transport across Caco-2 monolayers using similar concentrations to that used in our study (16). Unfortunately, their data is dissimilar to our own. Our results showed a clear efflux mechanism in action to remove rapamycin from the basolateral (serosal) side, whereas their data did not. Our transport medium did not contain FCS, whereas theirs did, yet repeating experiments with FCS did not significantly alter our results. However, it is known that the degree of P-gp like expression can differ from one laboratory to another especially

Table 3. Permeability Coefficients for SDZ-RAD, Rapamycin and Other Compounds in a Single Pass Rat Intestinal Perfusion Model Run at 0.08 ml/min for 150 min

Compound	Effective permeability (P_{eff}) ($\times 10^{-5}$ cm/sec)
Mannitol	0.34 ± 0.05
Propranolol	2.50 ± 0.07
Testosterone	3.80 ± 0.23
Cyclosporin A	2.14 ± 0.38
SDZ-RAD	2.48 ± 0.39
Rapamycin	1.26 ± 0.10

Note: Data shown is the mean ± SEM of four rats. All compounds had reached steady state absorption between 30–75 min.

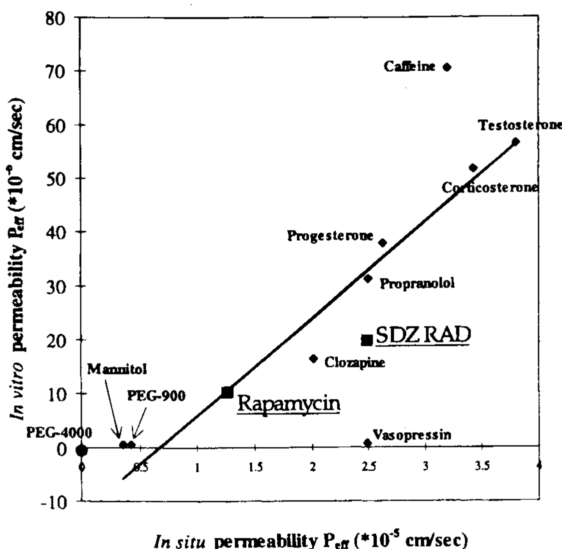

Fig. 3. *In situ/in vitro* comparison of SDZ-RAD and rapamycin permeability coefficients (P_{eff}). Mannitol, PEG-900, vasopressin, clozapine, propranolol, progesterone, corticosterone, testosterone and caffeine were used to test the *in situ* single pass intestinal perfusion system with that of the Caco-2 monolayer system, producing a linear standard curve.

if the passage numbers used are very different (17), indicating that their Caco-2 cells may have had minimal expression of this efflux protein. Data from most laboratories though show that P-gp like expression is enough to stimulate a response providing the cells have been left to mature for greater than 12 days (17–19). Our results with verapamil suggest that our Caco-2 cell line expresses this active efflux protein.

Expression of MRP has been shown to co-exist with P-gp (20) and MRP has been demonstrated in colon adenocarcinoma cell lines (21) including Caco-2 (22). Although we did not examine the expression of MRP in our cells, we explored the interaction of RAD and rapamycin with probenecid, an organic anion uptake inhibitor that has been shown to inhibit MRP (23). Rapamycin showed a 3.5 fold increase in Ap to Bas transport when co-incubated with this agent. When this is matched with the decrease in Bas to Ap transport induced by probenecid, the total efflux drops from 18 to only 5 times the Ap to Bas transport. None of the other compounds known to be transported by P-gp, such as cyclosporin A or digoxin showed this effect, and surprisingly, SDZ-RAD also did not show an increase in Ap to Bas transport considering its similar structure to rapamycin. Transport of the passively permeable control compounds, mannitol and propranolol were largely unaffected. Addition of both probenecid and verapamil together with rapamycin showed clearly that rapamycin transport could be completely equilibrated in the two directions. This suggests MRP as well as P-gp can lead to rapamycin being expelled from cells as it tries to cross Caco-2 cell membranes. It has been shown that a degree of overlap occurs in substrate specificity for MRP and P-gp (23). Rapamycin may be one of those compounds that fits into this category.

Significantly higher amounts of SDZ-RAD and rapamycin were found associated with the cells when transport was initiated in the Ap to Bas direction when compared to that from Bas to Ap studies, suggesting that these compounds may be forced to spend more time within the cytoplasm when entering from the apical side. This data is supported by work performed recently by Gan and coworkers who have shown that the residence time of cyclosporin A was increased in the Ap to Bas direction (24). It has been proposed that P-gp and intestinal enzymes, mostly CYP3A4, could act in concert, with P-gp increasing the residence time of substrates so that cytoplasmic enzymes such as CYP3A4 have more time to metabolize the compounds (24,25). It has been shown that many compounds known to be P-gp substrates are also substrates for CYP3A4 metabolism (25,26). Current results from our laboratory have shown that low doses of SDZ-RAD and rapamycin are highly metabolized at the intestinal wall *in vivo* (unpublished data), which also concurs with the P-gp/intestinal enzyme cooperation hypothesis. Very little metabolism occurred in our Caco-2 monolayers, but this is likely due to the lack of significant CYP3A4 activity in this cell line (27).

In summary, the permeability of a new rapamycin analog, SDZ-RAD was examined through the human intestinal Caco-2 cell line and an *in situ* single pass intestinal perfusion model. Both SDZ-RAD and rapamycin were found to be substrates for active efflux mechanisms, probably related to P-gp. Rapamycin was also shown to be at least partially excreted using a second distinct efflux protein. When these active efflux pathways are inhibited, SDZ-RAD was shown to have almost twice the permeability of rapamycin. In situ rat perfusion studies reiterated this view. Fagerholm and coworkers showed that there was a high correlation between the rat single pass intestinal perfusion model and *in vivo* human absorption (28), so it is likely that SDZ-RAD will be absorbed across the human intestine at a significantly faster rate than rapamycin.

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