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Unmasking the dynamic interplay between efflux transporters and metabolic enzymes

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Abstract

Drug efflux by intestinal P-glycoprotein (P-gp) is known to decrease the bioavailability of many CYP3A4 substrates. We have demonstrated that the interplay between P-gp and CYP3A4 at the apical intestinal membrane can increase the opportunity for drug metabolism by determining bidirectional extraction ratios across CYP3A4-transfected Caco-2 cells for two dual P-gp/CYP3A4 substrates, K77 (an experimental cysteine protease inhibitor) and sirolimus, as well as two negative control, CYP3A4 only substrates, midazolam and felodipine. Studies were carried out under control conditions, with a P-gp inhibitor (GG918) and with a dual inhibitor (cyclosporine). Measurement of intracellular concentration changes is an important component in calculating the extraction ratios. We hypothesize that the inverse orientation of P-gp and CYP3A4 in the liver will result in an opposite interactive effect in that organ. In vivo rat intestinal perfusion studies with K77 and rat liver perfusion studies with tacrolimus under control conditions and with inhibitors of CYP3A4 (troleandomycin), P-gp (GG918) and both CYP3A4/P-gp (cyclosporine) lend support to our hypotheses. These results serve as a template for predicting enzyme-transporter (both absorptive and efflux) interactions in the intestine and the liver.

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1. Introduction

Until recently attempts to define drug metabolism processes have been limited largely to understanding the importance of various metabolic isoenzymes in the liver. Furthermore, when a drug exhibited poor oral bioavailability, it was generally assumed that this was due to either physico-chemical processes, such as poor solubility in GI fluids or lack of permeability through the intestinal membranes, or alternatively due to marked first-pass metabolism in the liver. Our laboratory was among the first to hypothesize that for many drugs poor oral bioavailability could be due to the coordinated action of intestinal enzymes and efflux transporters (Benet et al., 1996; Wacher et al., 1996). Based on a series of cellular, animal and human studies, we hypothesize that intestinal metabolic enzymes and efflux transporters, working coordinatedly as a protective mechanism, could be the cause for the poor bioavailability of certain drugs.

Cytochrome P450 (CYP) 3A4 is the most prominent oxidative cytochrome P450 enzyme present in the human intestine (Watkins et al., 1987; Zhang et al., 1999), where it is localized to the columnar epithelial cells lining the intestinal lumen (Kolars et al., 1994). Despite the lower CYP3A content in the

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intestine relative to the liver, first-pass metabolism in the intestine by CYP3A has been conclusively shown to be significant, from studies performed in anhepatic patients (Paine et al., 1996) Drug absorption can also be decreased by efflux transporters in the intestine. P-glycoprotein (P-gp) is a plasma membrane-bound drug efflux protein found primarily in drug-eliminating organs. In the small intestine, P-gp has been localized in the apical membrane of the intestinal epithelial cells (Thiebaut et al., 1987), consistant with its role in effluxing compounds back into the intestinal lumen. Wacher et al. (1998) noted that most substrates of CYP3A4 are also substrates of P-gp, demonstrating the mutually broad selectivity of these proteins. The considerable overlap in the substrate selectivity, tissue localization and co-inducibility of CYP3A4 and P-gp has lead us to hypothesize that these two proteins work together in a coordinated manner to serve as an absorption barrier against drugs and other xenobiotics (Benet et al., 1996; Wacher et al., 1998; Zhang and Benet, 2001). In this manuscript we review our recent cellular studies to investigate the interplay between CYP3A4 and P-gp in the intestine and describe further work applying these principles to understanding the interactive nature of enzymes and transporters in the liver.

2. Investigating the interactive nature of CYP3A and P-glycoprotein in the intestine using cellular systems

The Caco-2 (colon carcinoma) cell line is frequently used as a model for human intestinal drug absorption (Artursson and Borchardt, 1997). Although Caco-2 cells express the P-gp efflux transporter (Gutmann et al., 1999), they are deficient in CYP3A. We recently characterized the expression of CYP3A4 and efflux transporters, P-gp as well as MRP1 and MRP2, in CYP3A4-transfected Caco-2 cells after induction with sodium butyrate and the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Cummins et al., 2001). We demonstrated that CYP3A4 protein levels were significantly increased when cells were incubated with the inducers alone or in combination, as measured by Western blot. The increase obtained with combined sodium butyrate and TPA was 40-fold over the control levels and was greater than that obtained with either inducer alone (sodium butyrate, 10-fold and TPA, 5-fold; Cummins et al., 2001). The functional activity of these cells was evaluated by measuring the extraction ratio (ER) of midazolam during transit through the polarized cell system. The ER in an in vitro system refers to the fraction of the parent drug that is metabolized as it crosses the monolayer. The extraction ratio was calculated by Eq. (1), which differs from a previous equation proposed by Fisher et al. (1999) by incorporating within the denominator the intracellular levels of unchanged drug, since if the drug is inside the cell, it could interact with the enzyme.

$$\mathrm{ER} = \frac{\sum \mathrm{metabolites}_{(\text{apical, basolateral, intracellular})}}{\sum \mathrm{parent}_{(\text{basolateral, intracellular})} + \sum \mathrm{metabolites}_{(\text{apical, basolateral, intracellular})}} \quad (1)$$

The extraction ratio of 3 μM midazolam in the apical compartment, incubated for 30 min at 37 °C, was $0.9 \pm 0.1\%$ in non-induced cells. In contrast, the extraction ratio increased 36-fold to a value of $32 \pm 1\%$ in cells induced with both sodium butyrate and TPA (Cummins et al., 2001). This functional increase closely correlates with the 40-fold increase in protein level of CYP3A4 as determined by densitometry.

The role of P-gp in modulating the extent of intestinal drug metabolism was examined in vitro (Cummins et al., 2002a) using the CYP3A4-transfected Caco-2 cells described above (Cummins et al., 2001) grown as monolayers. The transport and metabolism of two P-gp and CYP3A4 cosubstrates (K77 and sirolimus) and two CYP3A4 only substrates (midazolam and felodipine) were examined when dosed on the apical side of cells (mimicking human intestinal absorption), alone or in combination with the P-gp inhibitor GG918 (200 nM) or the dual P-gp and CYP3A inhibitor cyclosporine (10 μM). The results of these studies are shown in Table 1 as we previously reported (Benet and Cummins, 2001). We had hypothesized that for compounds that were substrates for P-gp and CYP3A4, effective inhibition of intestinal P-gp would not only increase absorption by blocking efflux transport but also decrease total metabolism, resulting in a significantly enhanced intestinal bioavailability.

The compounds tested were from a variety of drug classes and included K77 (Jacobsen et al., 2000), an

Table 1
Extraction ratios and characteristics of CYP3A4 and P-glycoprotein substrates tested across CYP3A4-Caco-2 cells

Drug	Substrate for		Efflux ratio	Extraction ratio percent (S.D.)		
	3A4[a]	P-gp[b]	B–A/A–B	Drug alone	Drug + cyclosporine	Drug + GG918
K77[c] (10 μM)	Yes	Yes	9	33 (3)	5.7 (0.3)	14 (1)
Sirolimus (1 μM)	Yes	Yes	2.5	60 (5)	15 (1)	45 (1)
Midazolam (3 μM)	Yes	No	1	25 (2)	10 (1)	23 (2)
Felodipine (10 μM)	Yes	No	1	26 (1)	14 (1)	24 (2)

[a] 3A4, CYP3A4.
[b] P-gp, P-glycoprotein.
[c] K77, K11777: *N*-methyl-piperazine-Phe-homoPhe-vinylsulfone-phenyl.

investigational cysteine protease inhibitor currently being developed to treat Chagas' disease; sirolimus, an immunosuppressive agent; midazolam, an anesthetic drug; and felodipine, a Ca^{2+}-channel blocker. All four of these drugs were known substrates of CYP3A4, each having a K_m for metabolite formation close to the dose administered as listed in Table 1. K77 was found to be the best P-gp substrate tested, exhibiting a nine-fold greater basolateral to apical flux compared with it's A–B flux. Sirolimus transport exhibited only a 2.4-fold efflux ratio across the cells and was considered a weaker P-gp substrate. Midazolam and felodipine are not P-gp substrates, as shown by their efflux ratios of 1, and acted as negative controls for these studies. Negative controls were important to ensure that the relatively specific P-gp inhibitor GG918 was not affecting the cells in any way other than to inhibit P-gp.

All compounds tested were significantly metabolized while passing through the cells as indicated in the extraction ratios ranging from 25 to 60% (Table 1, drug alone). As would be expected, incubation with cyclosporine decreased extraction ratios for all compounds, since cyclosporine is a known CYP3A4 inhibitor. The percent decrease in extraction ratio that could be attributed to the direct inhibition of CYP3A4 metabolism by cyclosporine was between 46 and 60% (as determined from the results for midazolam and felodipine, which are only substrates for CYP3A4). The percent decreases in ERs with cyclosporine for K77 and sirolimus (74–83%) were greater than this suggesting an additional factor (likely P-gp) was involved. Incubation of felodipine and midazolam with GG918 (a P-gp inhibitor that does not inhibit CYP3A4) did not change the transport profiles or the extraction ratios for either of these compounds. This was expected as these two drugs were negative controls for P-gp function and the effects of GG918 should be negligible. K77 and sirolimus transport profiles and ERs, however, were significantly affected by GG918. Consistent with complete inhibition of P-gp, efflux transport of both K77 and sirolimus was abolished. By inhibiting P-glycoprotein, the ER for K77 went from 33 to 14% (a 58% decrease), indicating that when the transporter was inactivated, there was decreased exposure of K77 to CYP3A4. The ER for sirolimus was decreased 25% in the presence of GG918 (from 60 to 45%) consistant with its moderate interaction with P-gp, when compared with K77. Therefore, for compounds that are substrates of CYP3A4 and P-gp, selective inhibition of the transporter yielded significant effects on the extent of metabolism by CYP3A4. These data support a role for P-gp in increasing the exposure of drugs to CYP3A4 in the intestine by allowing repeated cycling of drug via diffusion and active efflux.

3. The effect of transporters on intracellular drug concentrations and metabolism

In the development of Eq. (1), we recognized that intracellular drug concentrations may change when drug transporters are modulated. Therefore, it was necessary to include the intracellular parent drug levels in the denominator of Eq. (1), since this is the amount of drug available to the enzyme. The corresponding intracellular levels for the apical to basal transport studies previously shown in Table 1 are listed in Table 2.

A significant increase in intracellular amounts of K77 was found when P-glycoprotein was inhibited by GG918 or when both CYP3A and P-glycoprotein were

Table 2
Intracellular amounts of CYP3A4 and P-glycoprotein substrates in the presence of P-glycoprotein and CYP3A4 inhibitors

Drug	Intracellular amount (pmol)		
	Drug alone	Drug + cyclosporine	Drug + GG918
K77 (10 μM)	356 ± 26	1830 ± 37*	1600 ± 64*
Sirolimus (1 μM)	56 ± 10	212 ± 19*	73 ± 3
Midazolam (3 μM)	282 ± 50	354 ± 15	349 ± 30
Felodipine (10 μM)	3750 ± 125	4030 ± 92	3710 ± 218

* Significantly different from drug alone ($P < 0.05$).

inhibited by cyclosporine. In contrast, there was less of an increase for sirolimus with GG918 but a significant increase in the presence of the dual inhibitor cyclosporine. Although there was a tendency for increased intracellular amounts of the CYP3A4 substrates, midazolam and felodipine, in the presence of cyclosporine, these changes were not significant. It is obvious from the values listed in Table 2 that omission of intracellular drug levels for the dual CYP3A4 P-gp substrates would result in significant differences in ER.

Cummins et al. (2002a) reported that the bidirectional extraction ratio for K77 was 33 ± 2% in the A–B direction versus 6 ± 4% in the B–A direction, a significant difference. However, in the presence of the P-gp inhibitor GG918, the A–B extraction ratio decreased to 14 ± 1% while the B–A extraction ratio increased to 12 ± 1%, not significantly different. This shows the ability of the enzyme, independent of transporter effects, to yield equivalent extraction ratios regardless of the direction of drug absorption. This was shown for felodipine, where no differences were observed under control conditions with the A–B extraction ratio being 26 ± 1% and the B–A extraction ratio being 24 ± 1%. Similarly no changes were noted when GG918 was added (the extraction ratio in the A–B direction was 24 ± 2% and in the B–A direction 27 ± 1%). This comparability of metabolic extraction, independent of direction, when there is no active transport does not result if intracellular drug concentrations are omitted from Eq. (1).

4. The inverse special relationship between CYP3A4 and P-glycoprotein in the intestine and liver

We recently recognized that the cellular system of Cummins et al. (2001, 2002a) could mimic the topographical relationship between the enzyme CYP3A4 and the efflux transporter P-glycoprotein in both the intestine and the liver (Cummins et al., 2002b). Fig. 1 depicts this relationship. Note that in the intestine during the absorption process the drug encounters the efflux transporter, P-glycoprotein, before coming in contact with the enzyme CYP3A4. Thus, the cellular system described above (Cummins et al., 2001, 2002a) mimics the intestine when A–B transport is investigated. However, when transport is in the reverse direction, B–A, the enzyme topographically precedes the efflux transporter. This, in fact, mimics the relationship between these two proteins in the liver as depicted in Fig. 1.

4.1. Sex differences in the clearance of CYP3A4 substrates may be due to P-gp

Sex differences in the clearance of CYP3A4 substrates have frequently been observed, with women exhibiting higher clearances than men (Cummins et al., 2002b; Harris et al., 1995; Meibohm et al., 2002). This difference has not been well correlated with any sex-related differences in CYP3A4 liver enzyme levels. However, a 2.4-fold greater level of hepatic P-gp has been found in men versus women (Schuetz et al., 1995). Based upon the models we described here, we hypothesized that men, who have higher levels of P-gp, will have lower intracellular hepatic drug levels resulting in lower metabolic clearances relative to women. That is, the extraction ratio of a molecule that is a substrate for both CYP3A and P-glycoprotein will be lower for the system exhibiting higher levels of P-glycoprotein, as reflected previously in the comparison of the B–A extraction ratio of K77 under control situations versus when P-gp is inhibited with GG918. As we reviewed (Cummins et al., 2002b), in vivo animal studies in which P-gp

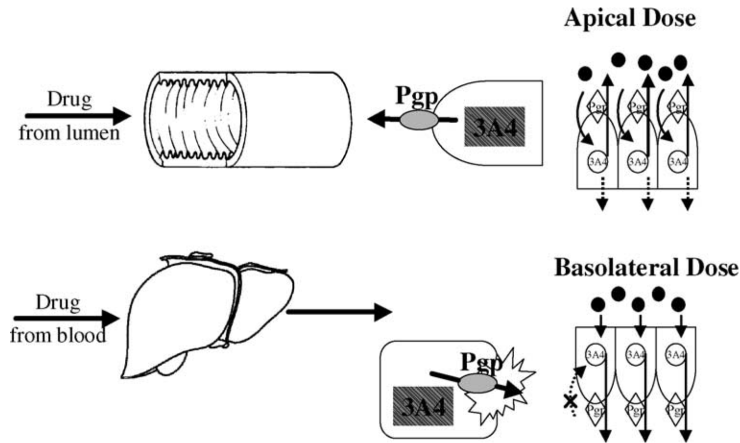

Fig. 1. The special relationship between the enzyme and transporter in the intestine and the liver as mimicked by the cellular system of Cummins et al. (2001, 2002a,b).

levels were altered and clinical data from the literature examining sex differences in drug clearances lend support to our proposed hypothesis (Cummins et al., 2002b).

4.2. *Prediction of the effects of inhibitors of CYP3A and P-gp on intestinal and hepatic metabolism*

Considering the interactive nature of CYP3A4 and P-gp on substrates of these two proteins in the intestine and the liver, it is possible to predict the metabolic changes that will occur in vivo when either P-gp or CYP3A4 or both P-gp and CY3A4 are inhibited. These predicted metabolic changes are shown in Table 3. In the intesitne, inhibition of the efflux transporter, P-gp, with no effect on the enzyme will decrease metabolism. Of course, inhibition of the enzyme will also decrease metabolism. Thus, when both the enzyme and the efflux transporter are inhibited, a significant decrease in metabolism will be observed as presented in Table 1 for the extraction ratios of K77 and sirolimus when cyclosporine, a potent inhibitor of both the enzyme and the transporter, is present. In contrast, the opposite effect is observed in the liver with respect to inhibition of P-gp where increased metabolism would be found. However, inhibition of the enzyme in the liver decreases metabolism so that a potential confounding result occurs when both the enzyme and the transporter are inhibited since the two processes result in counteractive effects.

Table 3
Predicted direction of metabolic change in the intestine and liver for dual CYP3A4 and P-gp substrates when coincubated with inhibitors

	Intestine	Liver
Inhibit P-gp	⇩	⇧
Inhibit 3A	⇩	⇩
Inhibit P-gp + 3A	⇩⇩	⇔⇧⇩

4.2.1. *Preliminary studies in rats to evaluate the effects of CYP3A and P-gp inhibition on metabolism*

4.2.1.1. Intestine. Studies using the rat single pass intestinal perfusion model (with mesenteric vein cannulation) were performed to determine whether a similar drug metabolism-efflux alliance existed in vivo (Cummins et al., 2003). The extents of metabolism of two test compounds, K77, a dual CYP3A and P-gp substrate, and midazolam, a substrate of CYP3A and not P-gp, were compared for each perfused alone and in the presence of the P-gp inhibitor GG918.

The appearance of K77 in the mesenteric blood was increased 2.5-fold in the presence of GG918, indicating that P-gp was inhibited. Midazolam permeability was unchanged by GG918, as expected since it is not a P-gp substrate. Estimates of the extent of metabolism were calculated by determining both the fraction metabolized from the disappearance of drug

from the lumen and the extraction ratio from the formation of known CYP3A metabolites. For K77, both the fraction metabolized (95 ± 3% versus 85 ± 4% with GG918) and the extraction ratio (49 ± 12% and 37 ± 3%) were decreased when P-gp was inhibited, whereas both measures of the extent of metabolism for midazolam were unchanged with P-gp inhibition (Cummins et al., 2003).

The data obtained from the single pass intestinal perfusion system were the first to show the specific interaction of P-gp with CYP3A in this isolated organ. These in vivo data support the proposed interplay between P-gp and CYP3A as P-gp, when active, appears to enhance the extent of metabolism of the dual CYP3A and P-gp substrate.

4.2.1.2. Liver. The isolated perfused liver is a useful intact organ system for examining the hepatobiliary disposition of drugs. In contrast to in vitro models like microsomes, hepatocytes and liver slices, the perfused liver preserves hepatic architecture, cell polarity and bile flow. Yet, the system avoids the influences from in vivo non-hepatic routes and hormonal effects.

Rat livers are perfused ex situ in a perfusion chamber equipped with a peristaltic pump, oxygenator, temperature and pH control. The perfusate, Krebs–Henseleit bicarbonate buffer, is run in a recirculatory mode and continuously oxygenated using the "Hamilton lung" (Hamilton et al., 1974).

The role of P-gp and CYP3A in modulating hepatobiliary drug disposition was examined ex situ using the isolated perfused rat liver (IPRL) system described above. The transport and metabolism of tacrolimus (1.2 μM), an immunosuppressive that is a substrate for both P-gp and CYP3A, was examined alone and in the presence of transporter and enzyme inhibitors. Troleandomycin (20 μM), a specific CYP3A inhibitor, GG918 (1 μM), a specific P-gp inhibitor, and cyclosporine (10 μM), a dual CYP3A and P-gp inhibitor were added 5 min before the addition of tacrolimus. Tacrolimus and its major desmethyl metabolites were measured by LC/MS/MS.

As these preliminary results had not been formally presented when this manuscript was accepted (now published as Wu and Benet, 2003), we only list here the relative effects on AUC of the various inhibitors, setting the control perfusion of tacrolimus at 1.0. In the presence of troleandomycin relative AUC increased to 1.83 as would be expected from Table 3. When liver metabolism is inhibited, less drug will be metabolized and AUC will increase. In the presence of GG918 the relative AUC for tacrolimus decreased to 0.59, again as predicted in Table 3. That is, when the efflux transporter is inhibited more drug is available to be metabolized by the enzyme and AUC will decrease. In the presence of cyclosporine the relative AUC was 1.30, intermediate between the effects of the CYP3A inhibitor and the P-gp inhibitor, again as predicted from Table 3.

5. Conclusion

We began our investigation of the interactive nature of metabolic enzymes and efflux transporters with respect to the biochemical mechanisms controlling oral drug absorption. This increased knowledge of the intestinal barriers to drug delivery has lead to a paradigm shift in the way we consider drug interactions and absorption problems. New drug candidates are routinely screened for their potential to interact with P-gp and these results can influence the future development of the compounds. The availability of in vitro systems mimicking the small intestine, as described here, enhances our ability to perform inhibition and drug interaction studies and could, in the future, provide a quantitative model for predicting human intestinal metabolism.

The cellular system described here lead us also to recognize the potential for translating basal to apical transport experiments as a model for the interactive nature of P-gp and CYP3A in the liver. Although this is one of the first drug efflux-metabolism alliances that has been discovered, there are undoubtedly other transporter-metabolism pairs exhibiting similar phenomena (such as MRP2 and UGTs), that are currently being explored. We have uncovered the dynamic interplay between P-gp and CYP3A in the intestine and the liver, demonstrating that P-gp efflux transport can enhance or impede metabolism by CYP3A4. Our findings have revealed that metabolism can be altered by changes that occur only in drug transport. Incorporating efflux, as well as uptake, processes affecting drug distribution should lead to better predictions of drug clearances from in vitro systems. Examining the effects of uptake transporters, efflux transporters and

metabolizing enzymes on metabolic processes is an exciting area for future research.

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