Variability in Human Drug Response
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# Approaches to studying the role of transporters in drug interactions in man

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## ABSTRACT

It has been suggested that some drug interactions occur due to changes in the activities of xenobiotic transport proteins, such as P-glycoprotein, a "counter transport" protein present in the epithelial cells (enterocytes) lining the small intestine. It has been difficult to study the role of P-glycoprotein in drug interactions in man in part because most substrates of P-glycoprotein are also substrates for a drug metabolizing enzyme (CYP3A4) present in hepatocytes and enterocytes. We have developed techniques to safely quantitate liver CYP3A4 activity and enterocyte CYP3A4 and P-glycoprotein concentrations in humans. The large interpatient variation in each of these three variables makes it possible to use multiple regression to estimate the contribution of each parameter to the apparent oral clearance of specific drugs. This approach has shown the role of P-glycoprotein in drug interactions involving cyclosporin A and may be readily applicable to the study of other enterocyte transport proteins. A complimentary approach would be to develop safe and selective inhibitors of transporters to "knock out" the variable. The potential value of this approach has been demonstrated with Seville orange juice, which contains inhibitors of CYP3A4 but does not appear to contain inhibitors of P-glycoprotein.

**Key words:** CYP3A4, p-glycoprotein, cyclosporin A, grapefruit juice, Seville orange juice, drug interactions.

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## INTRODUCTION

In the last decade, there has been great progress in understanding the regulation of human drug metabolizing enzymes, particularly the cytochromes P450. In some instances, this information has provided attractive explanations for known drug interactions, and successfully predicted drug interactions not yet observed in the clinic. However, it now appears likely that

some drug interactions believed to have a metabolic basis may at least in part result from changes in the activity of transport proteins. An example of this appears to be drug interactions attributed to induction or inhibition of CYP3A4.

CYP3A4 is the most abundant cytochrome P450 present in the liver [1] and small intestine [2,3]. CYP3A4 has been shown to be the major enzyme involved in the metabolism of many important drugs in several classes, including immunosuppressants, calcium channel blockers, cancer chemotherapies antihistaminics, sedatives, synthetic estrogens, and HIV protease inhibitors [4]. Indeed, it has been estimated that up to one-half of all drugs in clinical use, or under development, are capable of being metabolized by CYP3A4 [5]. Treatment with some drugs causes inhibition or induction of CYP3A4, and this has appeared to account for many drug interactions [4]. For example, ketoconazole is a potent inhibitor of CYP3A4, and this appears to account for why blood levels of CYP3A4 substrates tend to rise during therapy with ketoconazole. Conversely, rifampin is a potent inducer of CYP3A4 in both liver and intestine [3], and this provides an attractive explanation for why blood levels of CYP3A4 substrates tend to fall during treatment with rifampin. However, it is now clear that drug interactions involving CYP3A4 substrates can not always be explained by induction or inhibition of CYP3A4. For example, all but 5% of administered fexofenadine (Allegra®) is excreted from the body unmetabolized, yet ketoconazole treatment results in a 2.6 fold increase in the plasma AUC of parent drug [6].

## NEW APPROACHES

A potential additional explanation for CYP3A4 substrate/drug interactions has been provided by the discovery of p-glycoprotein (MDR1 gene product). This versatile xenobiotic transport protein is present in the brush border of the small intestine epithelial cell (enterocyte) [7], and functions to pump substrates against the absorption gradient [8]. Most substrates for CYP3A4 are also substrates for Pgp [9,10] and some CYP3A4 inhibitors (eg. ketoconazole [11]) and inducers (rifampin [12,13]) also appear to inhibit and induce Pgp. Hence, drug interactions involving CYP3A4 substrates could result from induction of inhibition of p-glycoprotein. Current pharmacokinetic approaches, such as the simultaneous administration of unlabelled drug intravenously and labelled drug orally, are useful in distinguishing liver and intestinal contributions to "first pass" effects, but are not capable of distinguishing the roles of CYP3A4 and p-glycoprotein at the level of the small intestine [14,15]. This is because metabolites generated by CYP3A4 in the enterocyte are often pumped by p-glycoprotein back into the intestinal lumen, and therefore would not be detected in blood [14].

To assess the relative contributions of CYP3A4 and p-glycoprotein in the oral availability of drugs, we have pursued a strategy that is based on the observation that there exist large interindividual differences in the activity of CYP3A4 in the liver and intestine, and in the intestinal levels of p-glycoprotein that can not be attributed to induction or inhibition by drugs [1,16-19]. In addition, there appears to be no correlation between the levels or either of these three parameters within an individual (i.e. they behave as independent variables) [18,19]. We reasoned that if **intra**individual changes in any of these three parameters caused by induction or inhibition produce corresponding changes in the clearance and dosing requirements of a CYP3A4 substrate, it logically follows that **inter**individual differences in activity of CYP3A4 should account, at least in part, for **inter**individual differences in the oral kinetics that drug.

Moreover, we reasoned that the relative strengths of the correlations between each of these three parameters and variation in the pharmacokinetics (PK) of the CYP3A4 substrate would estimate the relative importance of variable. To test this hypothesis, it would have been desirable to be able to perform liver biopsies to directly measure CYP3A4 activity. However, since the risks of liver biopsy are substantial, we developed the intravenous [14C N-methyl] erythromycin breath test (ERMBT) as an alternate means of quantitating liver CYP3A4 activity in living people [20]. This test is based on the observations that CYP3A4 catalyzes N-demethylation of erythromycin and that the carbon in the cleaved methyl group is rapidly converted to exhaled CO2 which can be easily trapped. Although there was initially some controversy [21], the ERMBT is now generally regarded as a "gold standard" probe based test for hepatic CYP3A4 activity [22,23]. In human studies we, and others, have used the ERMBT to demonstrate that variation in liver CYP3A4 activity accounts in part for interpatient differences in pharmacokinetics of several orally or intravenously administered CYP3A4 substrates [24-28], including CsA [18,28-32].We have also developed methods to quantitate CYP3A4 and p-glycoprotein in mucosal "pinch" biopsies that can be rapidly and safely obtained in people using a standard endoscopic instrument [18,33].

## THE MULTIPLE REGRESSION APPROACH

To perform a study, 20 subjects not receiving known inducers of inhibitors of CYP3A4 or p-glycoprotein receive the ERMBT and intestinal biopsies. A standard pharmacokinetic study is then performed with a drug of interest. The apparent oral clearance (CL/F) is used as the dependent variable, and the ERMBT and enterocyte concentrations of CYP3A4 and Pgp are used as independent variables in multiple regression analyses. Using this approach ([18,26] we have found that variation in enterocyte concentration of CYP3A4 (but not Pgp) correlates significantly with CL/F of felodipine ([26] supporting a substantial role of the intestinal enzyme in the disposition of this drug. However, in a similar study examining CL/F of cyclosporin A, enterocyte concentrations of Pgp (and not of CYP3A4) was a significant predictor [18]. This suggests that Pgp (and not CYP3A4) is the relevant enterocyte factor for CL/F of CsA, and the probable locus for the intestinal component of CsA drug interactions involving inducers or inhibitors. However, these observations alone do not exclude the possibility that significant CsA metabolism by CYP3A4 occurs in the intestine. As recently reviewed [15,34], Pgp may actually influence the extent of metabolism by CYP3A4 in the intestine by controlling the intraenterocyte concentrations of substrate, limiting product inhibition, or controlling the duration of absorption (i.e. total amount of metabolism occurring in the intestine would be proportional to the duration of the absorptive phase if enzyme saturation occurs). Hence, our multiple regression approach does not allow unequivocal conclusions about the role of enterocyte CYP3A4 in drug disposition.

## THE KNOCKOUT APPROACH

A novel approach for distinguishing the roles of enterocyte CYP3A4 and p-glycoprotein became apparent during our examination of the effects of grapefruit juice on enterocyte CYP3A4 and felodipine kinetics. Grapefruit juice has been shown to substantially increase the

oral availability of many CYP3A4 substrates [35-43], including felodipine [35,44,45] and cyclosporin A [46-50] We noted that after grapefruit juice treatment, variation in enterocyte concentration of CYP3A4 no longer significantly predicted CL/F of felodipine, which became chiefly a function of liver CYP3A4 activity as measured by the ERMBT([26]. Grapefruit juice had in effect eliminated or "knocked out" enterocyte CYP3A4 as a relevant variable, presumably by shifting the bulk of metabolism to the liver. We reasoned that this approach could potentially represent a useful and fairly direct strategy for assessing the role of enterocyte CYP3A4 in the disposition of drugs.

We and others [51-53] now believe that furanocoumarins (FCs) are the major active agents present in grapefruit juice accounting for the irreversible inhibition of CYP3A4 in enterocytes. We have shown that a major FC in grapefruit juice 6',7'-dihydroxybergamottin, or DHB, is a mechanism-based inactivator of CYP3A4 [51]. Mechanism-based inactivation is an attractive property for our "knock out" agent, as it is likely to be more P450-specific than reversible inhibition [54]. In addition, because it is irreversible, its duration of action will depend on the synthesis of new enzyme.

If the sole effect of grapefruit juice is to decrease enterocyte CYP3A4 activity, we might predict, based on our inability to identify a contribution of enterocyte CYP3A4 to the CL/F of cyclosporin A [18], that grapefruit juice would have no effect on the oral kinetics of cyclosporin A. However, grapefruit juice does significantly increases the oral availability of cyclosporin A [46-50]. To address the effects of DHB on cyclosporin A disposition, we used the fact that DHB is present in fruit other than grapefruit, including the Seville orange. In collaboration with David Edwards (at Wayne State University), a 2-way cross over study was performed in healthy volunteers to determine whether Seville orange juice would improve the oral availability of cyclosporin A [55]. We found that Seville orange juice (containing ~40 ·M DHB) did not at all increase mean AUC values for cyclosporin A, although whole grapefruit juice (containing ~40 ·M DHB) produced a significant increase in AUC in these same subjects [56]. To determine if Seville orange juice causes an in vivo fall in CYP3A4 protein, we performed small bowel biopsies in 2 of the subjects who participated in the trial, before and after they received Seville orange juice. An unequivocal fall in enterocyte CYP3A4 concentration was observed in each subject [56]. Hence, substantial decreases in enterocyte CYP3A4 level had no effect on the apparent oral clearance of cyclosporin A. This supports the conclusion from our multiple regression approach [18] that enterocyte CYP3A4 is unlikely to be the locus for drug interactions involving cyclosporin A. In addition, the observations suggest that the effects of grapefruit juice on CsA oral availability can not be attributed to DHB, and presumably reflect inhibitors of Pgp present in the juice.

## CONCLUSION

In summary, the multiple regression approach has appeared to provide insight into the role p-glycoprotein may play in the oral availability of cyclosporin A, and this approach should be applicable to other substrates and intestinal transporters. The "knock out" approach also appears to show promise if safe selective inhibitors of transport proteins can be developed.

Combining these approaches with traditional pharmacokinetic strategies should improve our understanding of relative contributions of liver and intestine to the oral availability of drugs.

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**Discussion: Approaches to studying the role of enzyme inhibition and induction**

**J. Urquhart:**
What role does the CYP3A4 at the brush border have in systemic metabolism?

**P.B. Watkins:**
I have assumed that the enzyme would only act on xenobiotics during the process of absorption, but there is some evidence that it may also be involved in systemic elimination. Ken Thummell in Seattle performed some studies during the phase of a liver transplant operation when the liver is removed from the body. Giving midazolam intravenously, a very good probe for CYP3A4, the mean extraction across the intestinal bed was 8% but there was one individual whose metabolic extraction was 25%. This suggests that the intestine may be a major organ of systemic extraction for some drugs in some people. Additionally, it has been known for a long time that certain drugs, particularly erythromycin, are actively transported into the gut lumen presumably by a p-glycoprotein (P-GP).

**N. Benowitz:**
You make the assumption that you can translate your data from intestinal biopsy enzyme activity to total intestinal enzyme activity. But, obviously, there are a lot of complexities in regulation, and local regulation could occur to a different degree in different parts of the small intestine. With respect to the CPY3A4 or P-GP activity, how sure are you that you can extrapolate from a single biopsy to the whole intestinal mass?

**P.B. Watkins:**
Our endoscope can only reach the first part of the small intestine. Nevertheless, we have obtained excellent correlations between the proximal and distal "phenotypes" in human intestine biopsies. Even though the level of expression falls as you proceed down the small bowel towards the colon, it appears to fall in parallel for individuals.

**D.C. Brater:**
Referring back to the differential regulation of hepatic and intestinal metabolism, if you bathe the liver in the same kind of environment that you bathe the intestinal mucosa, is there still differential regulation?

**P.B. Watkins:**
We have treated normal volunteers with known inducers of liver CYP3A4. With rifampicin we have seen induction in both liver and intestine within 24 h. However, after 48 h we have seen no evidence of induction in intestine with high therapeutic doses of phenobarbital, dexamethasone or clotrimazole. Now we plan to begin studies to treat for up to 2 weeks. These observations suggest that the actual molecular mechanisms of induction may be fundamentally different in the intestine and liver. On the other hand, the recently cloned pregnene-X receptor is expressed in the intestine as well as the liver. Therefore, at least the major receptor involved in xenobiotic induction of CYP3A4 appears to be present for xenobiotic induction in both intestine and liver.

**U. Klotz:**

You mentioned that another mechanism is the down-regulation of CYP3A4 by grapefruit juice ingredients, but you did not see a change in the mRNA of CYP3A4. Do you know a little bit more about the mechanism of this down-regulation?

**P.B. Watkins:**

We are testing at the moment in cells that express CYP3A4 whether mechanism based inactivation by furanocoumarins in the juice is followed by accelerated degradation of the protein, as has been shown with other P450s following mechanism-based inhibition.

**P. du Souich:**

The enterocyte is perfused from the mesenteric capillary through a short-cut which goes straight to the portal flow. The vasodilation of this mesenteric capillary will obviously be present during the absorption of food. But under fasting conditions, when the perfusion of the enterocyte is minimal, the access of some drugs to this enterocyte after intravenous administration will also be minimal. If you give it orally, the product needs to come from the mesenteric artery, and then reach the mesenteric capillaries in the enterocyte, which are closed when you are fasting. That is why I think the contribution of the intestine to the systemic clearance is very low and it is very difficult to interpret the exact role of the intestine in the systemic clearance of a drug. It may change significantly during the day, depending on its own perfusion.

**P.B. Watkins:**

It is the free fraction of the drug that is relevant and accessible for intestinal metabolism and the availability of this free faction is another factor, in theory, that could explain why intestinal metabolism can be low.

**D.A. Smith:**

The work that has been done on the role of P-GP in drug disposition of drugs has been very valuable. Although it is thought to be important for macrolides, at least, there is more and more data emerging that it is also just as important for small molecules. This is the case for the beta-blocker talinolol, a very small and metabolically stable compound, which is definitely secreted in the gastrointestinal tract by P-GP, and shows a marked verapamil interaction.

**P. Rolan:**

Atovaquone is a very lipophilic compound of intermediate bioavailability, that is not metabolised and reaches the bile unchanged. A population analysis showed that people on rifampin had an approximately double apparent oral clearance of this compound. At the time we did not understand this result. We thought that rifampin increases the clearance of some metabolised compounds, and this might due to an effect on transporters either in the gut or in the liver.

**P.B. Watkins:**

The other example is the follow-up compound to terfenadine in the United States, fexofenadine, which is excreted 85% unchanged from the body. Nonetheless there is an

interaction between fexofenadine and ketaconazole or erythromycin which may be explained again by modulation of P-GP activity.

**H.K. Kroemer:**
It has been published that P-GP does not have much of a role in the initial verapamil-rifampin interaction, because the amount excreted in urine does not fall significantly following rifampin administration. About the linear regression approach you mentioned, in one part of the regression you use a functional assay, the erythromycin breath test, and in the other part of the lineal regression you use a non-functional test of the expression of P-GP using Western blots. Considering the problems that exist with blotting P-GP, is there a direct relationship between the expression of P-GP and the function of transport in the intestine?

**P.B. Watkins:**
We know that we get the same answer from the immunoblot and enzymatic assays for CYP3A4. But you are right, especially when you are talking about an energy-dependent pump, it is not at all clear that the amount of protein would correlate with the actual activity, at least under all circumstances. However, in cancer cell lines the level of the P-GP protein does generally correlate with the amount of activity of the enzyme. In addition, the correlation of the P-GP protein measurements with cyclosporin oral availability also supports a close relationship between expression and function.

**A.J.J. Wood:**
Another non-metabolised substrate for P-GP is digoxin, which could probably explain the digoxin-quinidine, the digoxin-verapamil and the digoxin-ketoconazole interactions that we never really understood very well. Related to the cyclosporin studies, did you use the micro-emulsion cyclosporin?

**P.B. Watkins:**
The question refers to the fact that the older cyclosporine formulation Sandimmune®) has been replaced by a newer formulation (Neoral®) which is micro-emulsified and is absorbed much quicker and shows less inter-individual variations in kinetics. We used the Sandimmune® formulation, but in some studies with Neoral® we found that the correlations with the erythromycin breath test results were dramatically improved, presumably because of the removal of the P-GP and perhaps other variables from the regression.

**A.J.J. Wood:**
It might be because the new formulation, although it has been sold as a micro-emulsion preparation, actually contains inhibitors of CYP3A and potentially of P-GP as well, improving the bioavailability.

**S. Erill:**
The issue of the intracellular concentration of drugs was mentioned. In the case of cancer cells, P-GP plays a role in the intracellular concentration of anti-cancer drugs. Are there data on P-GP in cells other than enterocytes?

**P.B. Watkins:**
The majority of work on P-GP has been done on cancer, looking at it as a mechanism for resistance to chemotherapy. The higher levels of expression of P-GP, the lower the intracellular concentration. However, considering the presence of P-GP in the blood-brain barrier, it may be that inter-individual differences in the level of expression could account for differences between people in the CNS concentration of some drugs. The pharmacokinetic concepts are not simple ones that most of us know of, and importantly, there are not a lot of studies in progress, or even ways to study it that I know of.

**A.J.J. Wood:**
We have shown that P-GP is an important, perhaps the most important, determinant of HIV-protease inhibitors' entry into the CNS. We have recently looked at the effects of loperamide, an antidiarrhoeal agent, which is a very potent opiate. Since loperamide is a P-GP substrate it does not enter the CNS, and does not produce CNS opiate effects as other opiates do. If you inhibit P-GP with quinidine you can get respiratory depression with loperamide, but there are no respiratory effects in the absence of quinidine. So it is obvious that some of the drugs that are thought to have local effects, are actually producing them because of their ability to be pumped out of certain tissues by P-GP.

**E.M. Sellers:**
In my opinion, it is unlikely that the notions of individualisation are going to be widely accepted soon. Drug regulations and drug reimbursement policy, and socio-economic differences, along with social values and prejudice around issues of gender and race, all favor studying large groups and group differences. Practically, it is very difficult for the pharmaceutical industry to develop individualized dose approaches because of cost and labelling implications. It is very unlikely that the pharmaceutical industry is going to change, without regulatory change. I doubt whether physician education is going to have much of an impact.

**H.K. Kroemer:**
Before looking at individualisation, I think it should be made clear at some point to what extent the need for individualisation exists. The P-GP issue in cancer is a very good example. *In vitro* P-GP was a major determinant of resistance of cancer cells, yet all the clinical studies have been a complete disaster. This is because we now understand that the response of a given cancer to drug therapy is extremely complex. We should consider how far we take this individualisation, because obviously a cancer is as individual as the patient who carries this cancer.

**A.J.J. Wood:**
One of the reasons why the P-GP inhibitors did not work in cancer was that they were all potent CYP3A inhibitors as well. Part of the problem is that we need to individualise not only drugs and people, but need to have more selective probes for targeting CYP3A selectively from P-GP or vice versa. If you inhibit CYP3A, then you will get effects that are quite different from what you might have predicted from just targeting P-GP.

**P. Rolan:**

Convincing people of the importance of individualisation, and proving the clinical relevance of needing to assess it is difficult because, overall, we are so poor with most of our drugs at measuring any effect whatsoever. There is a major debate about whether the drugs of most of the major therapeutic classes do anything at all. There has been, for example, this recent issue in the popular press that all anti-depressants are placebos. There are other drugs we would expect to need to individualise because of their underlying heterogeneity, such as the treatment for Alzheimer's disease in the elderly. Since we cannot measure the effect on the population with these drugs, let alone on individuals, it is not surprising we cannot see the clinical relevance. Under these circumstances, the argument the clinical pharmacologists could put forward is best called the precautionary principle. If we have an unidentified source of variability that increases plasma concentrations four-fold, it would be very difficult to show that this is clinically relevant, because adverse event frequencies could be already low in the population, and you might not be able to detect a different principle. If you believe the drug is working through the circulation, and there are some people who have much higher concentrations, it is a reasonable starting-point to reduce the dose and then see if there are any changes. In conditions where the effect is very easy to measure, migraine for example, patients are very happy to individually titrate and use quite a lot of variability in individual response. In contrast, the pharmaceutical industry advocates one dose for everybody, because they do parallel groups studies with no individual titration therefore their recommendations are not useful and patients titrate themselves successfully anyway. We should propose the precautionary principle and the regulators should take it on board again. If we cannot see any difference in adverse drug reaction nor in efficacy it is probably because we cannot detect a difference rather than because one does not really exist. Under those circumstances it is prudent to take advance action to avoid problems, if a drug obtains a reputation for poor safety this can cause market resistance and poor sales. Since it is unlikely to have been detected in the clinical data base, I think we should be trying to propose individualisation when the size of variability is very large.