THE ROLE OF THE KIDNEY IN HUMAN TOXICOLOGY

P-glycoprotein-mediated drug interactions and intrarenal drug metabolism

by

Cindy Lisa Patricia Woodland

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmacology
University of Toronto

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P-glycoprotein-mediated drug interactions and intrarenal drug metabolism

CINDY LISA PATRICIA WOODLAND

Doctor of Philosophy, 2000
Department of Pharmacology, University of Toronto

ABSTRACT

The prime role of the kidney in drug elimination makes it a target for drug-induced toxicity. Using a renal tubular cell culture model that allows separation of the apical and basolateral compartments, I studied the renal tubular handling of substrates of the P-glycoprotein drug transporter. This thesis will highlight the importance of renal tubular drug interactions to systemic toxicity. The cardiac glycoside digoxin was used as an example drug because its low therapeutic index necessitates an understanding of its renal elimination in the presence of interacting drugs. The in vitro model allowed me to characterize known digoxin-drug interactions with propafenone and verapamil and to identify potential digoxin-drug interactions with mifepristone and itraconazole. Metabolites of propafenone and verapamil were found to contribute to the interactions with digoxin. Moreover, the renal tubular cells were capable of biotransforming propafenone and verapamil to their major metabolites. The importance of renal drug biotransformation to human toxicity is illustrated with the chemotherapeutic agent ifosfamide. Nephrotoxicity is often a limiting factor in the use of ifosfamide in children. Despite the co-administration of the uroprotective agent mesna (sodium 2-mercaptoethane sulfonate), ifosfamide chemotherapy is associated with nephropathy characterized by glomerular and tubular toxicity consistent with the Fanconi syndrome. This is in
distinction to cyclophosphamide, an analogue which differs solely by the position of a chloroethyl group, that is not associated with nephrotoxicity. The major nephrotoxic metabolites of ifosfamide have short half-lives and it is difficult to model renal damage caused by them after production by the liver. I hypothesized that ifosfamide is metabolized by CYP enzymes located in the renal tubular cell to the toxic metabolite chloroacetaldehyde. My results show that porcine and human kidney microsomes are capable of biotransforming ifosfamide to dechloroethylifosfamide metabolites, indicating that local production of chloroacetaldehyde in the renal tubular cell is a possible mechanism for nephrotoxicity.
ACKNOWLEDGEMENTS

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I wish to thank Dr. Irving Wainer, Camille Granvil, and Isabel Anacleto for the analysis of verapamil metabolites and the initial ifosfamide samples. Dr. Balázs Sarkadi was helpful in testing MDCK cells for MRP expression.

Permission to include my published papers in Appendix B was granted by the respective publishers.

Finally, I am grateful to the University of Toronto and the Canadian Cystic Fibrosis Foundation for their generous scholarships.
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<tr>
<td>α-MEM</td>
<td>alpha minimum essential media (Eagle's)</td>
</tr>
<tr>
<td>ABC superfamily</td>
<td>ATP binding cassette proteins</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>area under the plasma concentration-time curve</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>chiral</td>
<td>an object that cannot be superimposed on its mirror image</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>DCEI</td>
<td>dechloroethylifosfamide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>enantiomers</td>
<td>a pair of isomers that are not superimposable mirror images</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography with mass spectrometry</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
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<td>G6PD</td>
<td>glucose 6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>isoenzyme</td>
<td>a different enzyme with a different amino acid sequence, which acts on the</td>
</tr>
<tr>
<td></td>
<td>same substrate(s) to produce the same product(s)</td>
</tr>
<tr>
<td>isomer</td>
<td>different compound with the same molecular formula</td>
</tr>
<tr>
<td>Ki</td>
<td>a measure of the affinity of the inhibitor for the enzyme</td>
</tr>
<tr>
<td>Km</td>
<td>a measure of the affinity of the substrate for the enzyme</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>porcine renal tubular cell line</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>MDCK</td>
<td>canine renal tubular cell line</td>
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<tr>
<td>MDR</td>
<td>multidrug resistance</td>
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<tr>
<td>mechlorethamine</td>
<td>nitrogen mustard</td>
</tr>
<tr>
<td>melphalan</td>
<td>L-phenylalanine mustard</td>
</tr>
<tr>
<td>MESNA</td>
<td>sodium-2-mercaptoethane sulfonate</td>
</tr>
<tr>
<td>molar absorptivity</td>
<td>a reproducible value used in ultraviolet spectroscopy that takes into account concentration and cell length</td>
</tr>
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<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance-associated protein</td>
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<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
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<td>nitrogen mustard</td>
<td>mechlorethamine</td>
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<td>N-methylnicotinamide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAH</td>
<td>para-amino hippurate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PBS-G</td>
<td>phosphate-buffered saline with 5 mM glucose and 0.02% albumin</td>
</tr>
<tr>
<td>polymorphism</td>
<td>the situation in which two or more alleles at a given locus both occur with an appreciable frequency</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel</td>
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<td>electrophoresis</td>
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<td>stereoisomers</td>
<td>compounds with the same molecular formula and the same connectivity</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>---------------------------</td>
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<tr>
<td>structural isomers</td>
<td>compounds with the same molecular formula that have their atoms attached in different orders</td>
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<tr>
<td>sulfhydryl group</td>
<td>thiol group (-SH)</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
</tr>
<tr>
<td>therapeutic index</td>
<td>ratio of therapeutic-to-toxic drug concentrations</td>
</tr>
<tr>
<td>thiol</td>
<td>the sulfur analogue of an alcohol; also called an alkanethiol, or a mercaptan</td>
</tr>
<tr>
<td>thiol group</td>
<td>sulfhydryl group (-SH)</td>
</tr>
<tr>
<td>TNT</td>
<td>Tris-NaCl-Tween</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>the maximum velocity of a reaction</td>
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CHAPTER 1:
BACKGROUND TO RENAL DRUG HANDLING

1.1. Introduction

The kidney and liver, while important organs for drug elimination and hence decreasing toxicity, are themselves common targets for toxicity. Drugs may be directly toxic to the kidney as in the case of aminoglycosides. Alternatively, the presence of drugs in the kidney may indirectly cause toxicity to the kidney (as will be shown in chapter three with ifosfamide) or to other organs (as will be shown in chapter two with digoxin-drug interactions). Although the uptake and biotransformation of drugs by the liver has been well investigated, less is known about how drugs are handled by the kidney, especially with respect to renal drug biotransformation. Moreover, the role of drug metabolites in drug-induced renal toxicity is largely unexplored.

A number of anatomical and physiological characteristics predispose the kidney to the accumulation of potential toxins (Weinberg, 1993). The kidney plays a major role in drug kinetics. First, the kidney receives approximately 25% of the cardiac output and thus potential nephrotoxicants are delivered to the kidney at a high rate. Second, the large surface area of the brush border of renal tubular epithelium allows for binding and transport of drugs. Cellular accumulation of potential nephrotoxins is favoured by the increase in intraluminal drug concentrations as glomerular filtrate is reabsorbed. Moreover, the kidney has specific transport systems resulting in site-specific toxicity. For example, aminoglycosides only accumulate substantially in the kidney and inner ear (Zager, 1997).

Many drugs are excreted by the kidney, and very often drugs that are eliminated by hepatic metabolism form metabolites that are renally excreted. In the last few decades much has been learned about renally-located drug transporters and their involvement in drug interactions and toxicity. While most drugs are metabolized, little is known about the effects of hepatically-produced drug metabolites on drug-drug
interactions and toxicity in the kidney. Similarly, scant information exists on the effects of intrarenal drug biotransformation. This thesis will focus on two modes of toxicity involving extra- and intra-renally-produced drug metabolites. In chapter two, I will explore renal tubular drug interactions involving parent compounds and/or their metabolites, which may cause systemic toxicities. In chapter three, renal toxicities resulting from local production of nephrotoxic species will be examined. In essence, the goal of this thesis is to characterize mechanisms of drug-induced systemic and renal toxicities.

Drug interactions resulting from the competition of parent drug compounds for renal elimination by the organic anion and organic cation transporters are well-studied and therefore will not be extensively discussed in this thesis. Interactions occurring at the site of the P-glycoprotein drug transporter have been recently recognized and therefore will be discussed in reference to their role in toxicity. To our knowledge, this is the first report of the involvement of drug metabolites in renal tubular drug-drug interactions. We also demonstrate the ability of the kidney to metabolize drugs, a largely neglected phenomenon of drug biotransformation. Drug interactions involving drug metabolites can result from both the extrarenal (mainly hepatic) and intrarenal production of drug metabolites. Using the example of the cancer chemotherapeutic agent ifosfamide, the important contribution of local (renal) drug biotransformation to nephrotoxicity will be illustrated.

1.2 Renal Drug Handling

The kidney is made up of anatomically functional units called nephrons. When a drug enters a nephron, it may be filtered by the glomerulus and/or secreted by the renal tubules from the blood into the urine and/or reabsorbed from the urine back into the blood. During the process of glomerular filtration, approximately 20% of the plasma water and its drugs are passively filtered. The glomerular filtration rate (GFR) in human adults is approximately 120 ml/min. Diffusion of drugs across the
glomerulus and renal tubular cells is a passive process that goes with the concentration gradient and therefore does not require energy expenditure. On the other hand, active renal tubular drug secretion is an energy-dependent and saturable transport process mediated by specific drug transporters that allow drugs to move from the blood to the urine against a concentration gradient. A number of drug transporters have been identified in renal tubular cells. Renal tubular drug reabsorption is primarily a passive process, although active reabsorption also occurs (Blowey et al., 1995).

1.3 Drug Interactions

Mechanistically, drug interactions may be classified as pharmacodynamic or pharmacokinetic. Pharmacodynamic interactions result when one drug changes the effect(s) of one or more other drugs. An example of this was the initial use of chlorpromazine to potentiate central nervous system depressants (Goodman and Gilman, 1990).

Pharmacokinetic interactions occur when the disposition of a drug is altered by another drug. These interactions can occur in a number of ways at the levels of absorption, distribution, biotransformation, and excretion. Gastrointestinal absorption can be altered by drug-induced changes in physicochemical properties (e.g., pH), gastrointestinal motility, bacterial flora, and mucosal function. The distribution of a drug will change if there are alterations in blood flow, tissue uptake or binding, serum protein binding, or active transport to the site of action. The induction or inhibition of metabolic enzymes (as described later) will influence the rate of drug biotransformation. Finally, drug interactions may result in an increase or decrease in the rate of drug excretion.

Drug excretion by the kidney (as opposed to elimination in the feces, bile, sweat, tears, or lungs) is particularly vulnerable to drug interactions. For example, glomerular filtration is increased when a drug is displaced from albumin by another drug. The renal tubular reabsorption of a drug may be decreased by diuretics, or by drugs that
alter the pH of the urine. As well, tubular secretion may be altered if two or more drugs interact with a shared drug transporter. Throughout this thesis, the term drug interactions will refer to drug-drug interactions.

Drug interactions are a common source of drug-related toxicities. In recent years, the identification of several drug transporters has helped to elucidate the mechanism of some of these interactions. The kidney is one of the prime targets for drug-induced toxicity because of the central role it plays in drug excretion. The kidney generally receives about 20% to 25% of the cardiac output and therefore is greatly exposed to drugs carried by the blood. Moreover, since the kidney is an excretory organ and has urinary concentrating abilities, drugs often accumulate in the kidney to much higher levels than those witnessed elsewhere in the body. As well, the proportion of drug metabolites relative to the parent compound can be distinctly different in the kidney and extrarenal tissues if the parent drug and its metabolites are not eliminated by the same route.

1.4 Renal Tubular Drug Interactions

This thesis will focus on drug interactions at the level of the renal tubules. Renal tubular drug interactions can result from two or more parent drug compounds and/or their metabolites interacting to produce local (i.e., renal) toxicities. However, renal tubular drug interactions can also cause extrarenal drug toxicities, often without damaging the kidney.

Perhaps the best understood renal tubular drug interactions are those involving the organic anion and organic cation transporters. Also known as the organic acid and organic base transporters, respectively, these systems that are present in proximal tubular cells, are characterized by the charge of the substrates that they transport. Most often, drug interactions at these sites are competitive in nature (although they can be noncompetitive or uncompetitive). The interaction of penicillin with probenecid (a uricosuric agent) is a classic example of a drug interaction involving the organic anion
transporter. Both drugs are transported by a carrier that has higher affinity for probenecid. Consequently, at equal initial drug concentrations, probenecid inhibits the renal tubular transport of penicillin, and the half-life and serum concentrations of penicillin increase (Beyer et al., 1951; Shinn and Shrewsbury, 1985). In fact, when penicillin was expensive and difficult to obtain, probenecid was used for the purpose of preventing penicillin excretion (Blowey et al., 1995).

The interest of our laboratory in renal tubular drug interactions arose from reports of digoxin toxicity in a number of children treated with this cardiac glycoside. Upon reviewing the literature, it became apparent that a number of drugs interact with digoxin to cause increased serum digoxin concentrations (summarized in Rodin and Johnson, 1988). Furthermore, the clearance of digoxin is often decreased in the presence of interacting drugs (Pedersen et al., 1981; Herfindal et al., 1988; Hedman, 1990; Koren et al., 1983; Belz et al., 1983; Calvo et al., 1989). Since digoxin is eliminated mainly by the kidney, drug interactions at the level of the kidney were proposed as a possible mechanism for digoxin toxicity. Given the large number of patients taking digoxin and the low therapeutic index of digoxin, an understanding of digoxin renal elimination was felt to be of great importance to clinical practice.

To investigate the mechanism of digoxin renal tubular secretion, the involvement of known drug transporters was first tested. Previous studies in our laboratory and others failed to show inhibition of digoxin transport by prototypic organic acids and bases. These studies suggested that digoxin does not utilize the classic organic anion or cation transport systems for its renal tubular transport, as might be expected by its uncharged nature (Koren et al., 1988; Ito et al., 1992). Furthermore, its interactions were not associated with its pharmacological receptor, membrane Na\(^+\)-K\(^+\)-ATPase (Ito et al., 1992). This led us to investigate other, less well-studied drug transporters. The renal tubular secretion of digoxin is now believed to be mediated by the P-glycoprotein drug transporter that is associated with multidrug resistance (Tanigawara et al., 1992; de Lannoy et al., 1992b; Ito et al., 1993b*; Schinkel et al., 1996).
1.5 Multidrug Resistance

The term multidrug resistance was initially used to describe the phenomenon by which cells in culture, selected for resistance to a single natural-product cytotoxic agent, simultaneously develop broad cross-resistance to a variety of structurally and functionally unrelated compounds (Endicott and Ling, 1989). This definition has been expanded over the years to describe the resistance to a variety of unrelated drugs including those to which the patient has never been exposed. In the clinical setting, multidrug resistance is one of the major causes for failure of anticancer chemotherapy (Giaccone and Pinedo, 1996; Labroille et al., 1998; Ramachandran and Melnick, 1999). The seriousness of this phenomenon, coupled with intrigue as to how cells become resistant to drugs to which they have never even been exposed, led to an explosion of research in this field.

Since the commencement of this thesis work, information has been accumulated in the area of multidrug resistance. There are many mechanisms responsible for drug resistance such as: enzymatic inactivation or degradation of drugs, alterations of the drug target; prevention of drug entry; and active extrusion of drugs (Bolhuis et al., 1997).

Focusing on drug extrusion mechanisms, intensive study in this area has led to the identification of several transporters associated with multidrug resistance. The two most well-studied of these transporters are P-glycoprotein and the multidrug resistance-associated protein (MRP). This thesis will focus on transport mediated by P-glycoprotein with a brief discussion of MRP which has many substrates in common with P-glycoprotein.

1.5a P-Glycoprotein

In 1976 Juliano and Ling reported an association between multidrug resistance in Chinese hamster ovary (CHO) cells and the overexpression of a 170 kDa (140 kDa in the unglycosylated form) integral membrane protein called P-glycoprotein. The "P" was
named for “permeability” since drug permeability appeared to be somehow altered in the cells (Juliano and Ling 1976). Just over two decades later a wealth of information about P-glycoprotein has been gathered, yet its physiological role is still unclear.

P-glycoprotein is an ATP-dependent drug efflux pump made up of 1280 amino acids, that is expressed in the plasma membranes of not only tumour cells, but also a variety of normal cell types including: kidney, liver, pancreas, small intestine, large intestine, brain, adrenal cortex, and placenta (Thiebaut et al., 1987; 1989; Fojo et al., 1987; Arceci et al., 1988; Cordon-Cardo et al., 1989). P-glycoprotein is thought to contribute to resistance in about 50% of tumours treated by chemotherapy (Serra et al., 1995; Nussler et al., 1996; Chou et al., 1997; Lehnert 1998; Schinkel, 1998).

The expression of P-glycoprotein is associated with decreased intracellular drug accumulation. Its substrates show remarkable structural and therapeutic diversity. Among the drugs identified as P-glycoprotein substrates are chemotherapeutic agents such as vinca alkaloids, anthracyclines, epipodophyllotoxins, actinomycin D, taxanes, immunosuppressive agents such as cyclosporin A and FK506, and cardiovascular agents such as quinidine and digoxin. Loperamide, domperidone, ondansetron, and ivermectin also are substrates (Schinkel et al., 1994). Most substrates are hydrophobic, amphiphilic compounds; however, some exceptions exist. For instance, Schinkel et al. (1996) demonstrated that while P-glycoprotein mainly transports weakly basic and uncharged compounds, it also transports phenytoin, a weakly acidic drug. An even greater number of inhibitors or modulators of P-glycoprotein have been identified including: verapamil, terfenadine, midazolam, and ketoconazole (Kim, 1998).

One of the first questions raised about this polypeptide was whether it acts by a direct or indirect mechanism. All of the known ATP-dependent drug extrusion systems that hydrolyze ATP to energize drug extrusion, belong to a superfamily called the ATP-binding cassette proteins, commonly called the ABC transporters or traffic ATPases. Both P-glycoprotein and MRP are members of this family as are several uptake and efflux systems from mammals, bacteria, and lower eukaryotes.
P-glycoprotein is made up of two hydrophobic domains each containing six putative transmembrane alpha helical segments (in three close pairs) and two hydrophilic cytoplasmic components containing the highly conserved ATP binding cassette. This predicted secondary structure is thought to be the result of an internal gene duplication event (Chen et al., 1986). Both nucleotide binding domains are required for drug transport by P-glycoprotein (Loo and Clarke, 1994; Urbatsch et al., 1995). Furthermore, photoaffinity labelling studies with vinblastine and azidopine, and tryptic digestions and peptide analyses indicate that both halves of P-glycoprotein are involved in drug binding (Bruggemann et al., 1992). The similarity of the ATP-binding domains, but not the transmembrane domains, to other ABC transporters, suggests that the initial site of substrate binding is in or near the transmembrane domains.

The wide spectrum of structurally and functionally dissimilar drugs transported by P-glycoprotein raises the question of how P-glycoprotein recognizes its substrates. It was initially believed that a basic nitrogen atom and two planar aromatic domains were required (Pearce et al., 1989; Zamora et al., 1988). Another commonality between substrates is their relative hydrophobicity and amphiphilic nature (Gottesman and Pastan, 1993; Schinkel et al., 1996). However, it has now been shown that the basic nitrogen atom is not necessary and that some hydrophilic compounds have weak substrate activity (Seelig, 1998).

After screening over 100 chemically diverse compounds, Seelig (1998) concluded that the binding of a substrate to P-glycoprotein is approximately proportional to the number and the strength of hydrogen bonds formed between the two molecules, provided that substrates have similar membrane partition coefficients. The compounds that were bound by P-glycoprotein had a relatively high number of electron donor groups. All P-glycoprotein substrates were found to contain at least one type I or type II unit where type I units contain two electron donor groups with a spatial separation of 2.5 ± 0.3 Å and type II units consist of either two electron donor groups with a spatial separation of 4.6 ± 0.6 Å or three electron donor groups with a spatial separation of
4.6 ± 0.6 Å between the two outer groups. Inducers of P-glycoprotein had at least one type II unit while inhibitors seemed to need a carboxylic acid, a sulfoxy, or a mesomeric nitro group (Seelig, 1998).

While the exact mechanism by which P-glycoprotein transports its substrates has not been elucidated, a few theories have been proposed. The aqueous pore model assumes that drugs are acquired from the cytoplasm of the cell whereas the vacuum cleaner and flippase models assume that drugs are removed from the cytoplasmic membrane (Higgins and Gottesman, 1992; Gottesman and Pastan, 1993).

The aqueous pore theory of P-glycoprotein transport involves the traditional model of binding of the drug from the aqueous phase, translocation of the drug across the lipid bilayer, and release of the drug at the opposite side of the membrane. The other two models assume that P-glycoprotein binds to drugs that have diffused into the centre of the lipid bilayer. Since most P-glycoprotein substrates are hydrophobic and amphiphilic and often carry a net positive charge, they are likely to easily diffuse into the phospholipid membrane. The hydrophobic vacuum cleaner model proposes that drugs are transported from either the inner or outer leaflet of the lipid bilayer into the external medium (Gottesman and Pastan, 1993). With the flippase model, drugs are transported from the inner to the outer leaflet and then diffuse into the external medium (Higgins and Gottesman, 1992; Higgins, 1994). Based on related MDR transporters, binding of the substrate to the inner leaflet of the membrane appears to be essential for transport.

The P-glycoproteins are encoded by multidrug resistance (mdr) genes. Humans have two MDR genes. MDR1 encodes a drug transporter that can confer multidrug resistance while MDR2 (also sometimes called MDR3) is responsible for phospholipid translocation. In rodents such as the rat, mouse, and hamster, there are two genes that encode the drug transporter, namely, mdr1a and mdr1b. Mdr2 in rodents is a phospholipid translocator (i.e., the same as MDR2 in humans). For the purposes of this
discussion, the term P-glycoprotein will refer to the type-one drug-transporting P-glycoproteins.

Interestingly, mice have different expression levels of mdr1a and mdr1b P-glycoproteins in different tissues although there is overlap (Devault and Gros, 1990; Gros et al., 1991). Based on RNA assays, mdr1a is predominant in intestine, liver, brain, and testis, while mdr1b is predominant in adrenal gland, placenta, ovarium, and uterus. At least some RNA from both mdr1a and mdr1b was detectable in all major tissues. Mouse kidney contains high levels of both mdr1a and mdr1b. Significant and similar levels of mdr1a and mdr1b are also found in heart, lung, thymus, and spleen (Croop et al., 1989; Teeter et al., 1990; Arceci et al., 1988).

Schinkel et al. (1994) looked at the role of P-glycoprotein by studying mdr1a gene knock-out mice. They disrupted the mdr1a gene in mice and found that the knock-out did not affect (+/-) or (-/-) mice in terms of viability, fertility, physiology, anatomy or histology. Furthermore, serum enzymes, proteins, electrolytes, and hematological parameters did not differ. On the other hand, when mice were administered 1 mg/kg of the chemotherapeutic agent vinblastine, a P-glycoprotein substrate, and drug concentrations were determined four hours following intravenous administration, the concentrations of vinblastine in heart, muscle, and brain of (-/-) mice were approximately three-, seven-, and 20-fold higher, respectively, than those in (+/++) mice. Plasma vinblastine concentrations were two-fold higher in (-/-) mice, while the concentrations in kidney, small intestine, liver, lung, colon, and testis ranged between two- and three-fold higher. In the other tissues examined, the mice with disrupted mdr1a genes showed up to two-fold increases in vinblastine concentrations. Furthermore, the (-/-) mice showed a much slower elimination of vinblastine than the controls. Therefore, the pharmacokinetics of vinblastine were markedly altered in the (-/-) mice, especially in brain. Gros et al. (1991) reported that vinblastine is transported by mdr1b P-glycoprotein with about half the efficiency of mdr1a P-glycoprotein. The expression of mdr1b was increased in the livers and kidneys of mice with the mdr1a
disruption (+/-, -/-); however, in most tissues the levels of *mdrb* were not altered. It is possible, therefore, that *mdrb* upregulation is a compensation for the disruption of *mdra*.

Based on the findings in the *mdra* (-/-) mice, P-glycoprotein appears to have a protective role by limiting the amount of drug accumulation in important organs such as brain, heart, and muscle and speeding up the removal of drug from these tissues when plasma drug levels decrease. In addition, P-glycoprotein increases the rate at which drug is removed from plasma. Studies with mice in which both the *mdra* and *mdrb* genes have been knocked out confirm these results (Mayer et al., 1997).

Human MDR1 P-glycoprotein is highly expressed in the brush border of proximal renal tubule cells, at the biliary surface of hepatocytes, at the apical surface of mucosal cells in the small and large intestine, in capillary endothelial cells in the brain and testis, in adrenal cortex, and in placental trophoblasts (Thiebaut et al., 1987, Cordon-Cardo et al., 1989, Thiebaut et al., 1989). This interesting tissue localization suggests that MDR1 P-glycoprotein may function to protect organisms from toxic compounds that have been ingested or created through biotransformation. That is, P-glycoprotein may prevent cellular accumulation of toxins, especially in brain, by actively excreting them into blood, urine, bile, or intestinal fluid (Ito et al., 1993b*; Schinkel, 1994). Seelig (1998) suggests that the physiological role of P-glycoprotein may be to remove genetic "waste" since compounds that would interfere with the mechanism of correct genetic translation would make the cell inefficient. It is unlikely that organisms evolved to actively secrete the variety of P-glycoprotein substrates, rather, P-glycoprotein possibly functions to prevent the reabsorption of exogenous toxins once ingested. This would explain why P-glycoprotein is located throughout the body in areas where absorptive processes are substantial such as the digestive tract, blood-brain barrier, and excretory organs. In the context of renal transport, our laboratory hypothesizes that this is just one of many transporters in the urinary lumen that is equipped to pump naturally occurring toxins.
that may be reabsorbed along with water out of the cell (Ito et al., 1993b*). This "urine-blood barrier" is therefore important to maintain homeostasis in cells.

### 1.5b Multidrug Resistance-Associated Protein

Another drug transporter that causes multidrug resistance is the multidrug resistance-associated protein, called MRP. Cole et al. (1992) discovered this transporter by examining complementary DNA (cDNA) clones of messenger RNAs that were overexpressed in the H69AR multidrug-resistant variant of the H69 small cell lung cancer cell line. In this first report, Cole found expression of MRP in lung, testis, and peripheral blood mononuclear cells using Northern blot analyses of total RNA from human tissues. On the other hand, MRP transcripts could not be detected in placenta, brain, kidney, salivary gland, uterus, liver, or spleen.

Similar to P-glycoprotein, MRP functions as an ATP-dependent drug efflux pump (Zaman et al., 1994). However, while both P-glycoprotein and MRP belong to the ABC superfamily, they are only distantly related with an amino acid sequence identity of 15%. MRP is a 180-195 kDa membrane protein with 12 potential glycosylation sites and a predicted sequence of 1531 amino acids. The gene that codes for MRP is localized to band p13.1 on chromosome 16 (Cole et al., 1992). Much debate surrounds the predicted topology of MRP which is still inconclusive.

MRP is primarily located in plasma membranes (Zaman et al., 1994; Flens et al., 1994) although reports of intracellular localization may reflect variations in the intracellular trafficking of MRP in different cell types (Kavallaris, 1997). There is great overlap in the substrates of P-glycoprotein and MRP. While both transport cationic drugs such as the anthracyclines and vinca alkaloids, MRP also transports many anionic compounds such as glutathione conjugates (Zaman et al., 1994). Leukotriene C4 appears to be an endogenous substrate of MRP. Cells (SW-1573) that overexpress MRP are resistant to doxorubicin, daunorubicin, vincristine, VP-16 (etoposide), colchicine, and rhodamine 123, but not to taxol. The intracellular accumulation of drug (daunorubicin,
vincristine, and VP-16) is decreased and the efflux of drug (daunorubicin) is increased in the transfectant. Digitoxin, which can permeabilize membranes, abolishes the reduction in daunorubicin accumulation (Zaman et al., 1994).

Recently, five other MRP proteins have been discovered. Thus, the first MRP to be identified is often referred to as MRP1. Organic anions are substrates for all identified MRPs. In humans, MRP2 is identical to the multispecific organic anion transporter called canalicular multispecific organic anion transporter (cMOAT). It has a molecular mass of 190 kDa and was originally cloned from liver. MRP1 is localized in the basolateral membrane. MRP2 is localized in plasma membranes and is expressed in the apical membranes of human kidney proximal tubular cells (Schaub et al., 1997).

I.5c Other Renal Drug Transporters

Advances in molecular biology have facilitated the rapid identification of many drug transporters in the past few years. Undoubtedly, more drug transporters will be discovered in the years to come. Greater understanding of the role of drug transporters in the kidney, sheds light on the complexity of renal drug handling. Chapter two will discuss other drug transporters in more detail.

I.6 Drug Biotransformation

A variety of enzyme systems in the body metabolize both endogenous and exogenous compounds. During drug biotransformation, drugs are made more water soluble to facilitate excretion from the body. Phase I reactions involve oxidation, reduction, or hydrolysis, while phase II reactions are synthetic or conjugation reactions. For the most part, these reactions are detoxifying; however a number of metabolic reactions produce metabolites that are more active or more toxic than the parent compound.
1.6a Phase I Reactions

Oxidative reactions catalyzed by a family of heme-containing enzymes called cytochrome P450 (CYP) enzymes play an important role in drug biotransformation as well as the manufacture of cholesterol, steroids, and other important lipids. In this thesis, the discussion of phase I renal drug biotransformation is mainly limited to the CYP enzyme system. Aldehyde dehydrogenases are phase I enzymes that use NAD+ as a cofactor to oxidize aldehydes to carboxylic acids (Lohr et al., 1998). The importance of the phase I aldehyde dehydrogenase enzymes to the biotransformation of ifosfamide is discussed in chapter three.

CYP enzymes are also known as "mixed function oxidases" or "monooxygenases" since they incorporate one atom of molecular oxygen into water and one atom into the substrate. The term "cytochrome P450" abbreviated as CYP in most species was derived from the maximal absorption at or near 450 nm when carbon monoxide binds to the reduced form of heme iron (Omura and Sato, 1964). These enzymes are primarily localized in a subcellular fraction containing the smooth endoplasmic reticulum called the microsomal fraction (Anders, 1980).

The microsomal oxidase system consists of a hemoprotein, a membrane-bound flavoprotein called NADPH-cytochrome P450 reductase containing both FMN and FAD, and a lipid component. The CYP is the binding site for oxygen and the substrate. The substrate binds to the oxidized (ferric) form of CYP. This CYP-substrate complex accepts an electron from NADPH via NADPH-CYP reductase (which is identical to NADPH-cytochrome c reductase) and the resulting reduced complex binds oxygen or carbon monoxide. The reductase is required to reduce the complex since only the reduced (ferrous) form of CYP enzymes will bind oxygen. (Reduced cytochrome P-450 may also serve as an electron donor in some reductive reactions, without binding oxygen.) A second electron is then donated by NADPH via NADPH-CYP-reductase (or in some cases, by NADH via NADH-cytochrome b5 reductase) which reduces the oxygen bound to the hemoprotein. With the loss of a water molecule an "active
oxygen” species is produced that oxidizes the substrate. Therefore, the overall reaction can be written as:

$$\text{RH (drug)} + 2\text{NADPH} + \text{O}_2 \rightarrow \text{ROH} + 2\text{NADP}^+ + \text{H}_2\text{O}$$

The substrate specificities of the CYP enzymes vary greatly (Murray et al., 1988). The CYP enzymes are referred to as isoforms rather than isoenzymes because different CYPs can bind different substrates or can produce different products from the same substrate. (An isoenzyme is a different enzyme with a different amino acid sequence which acts on the same substrate(s) to produce the same product(s).) The CYP enzymes are classified such that those with greater than 40% amino acid homology belong to the same family, while those with greater than 55% homology belong to the same subfamily. An Arabic numeral represents the family. This is followed by a letter that represents the subfamily and a second Arabic numeral identifies the individual gene (e.g., CYP3A4).

Although there are many families of CYP enzymes, the biotransformation of almost all drugs is primarily handled by the CYP1, CYP2, and CYP3 families (Slaughter and Edwards, 1995; Wrighton and Stevens, 1992). For instance, more than half of all drugs are metabolized by CYP3A (Smith, 1991). The individual CYP enzymes involved in biotransforming the drugs presented in this thesis will be discussed later in the context of their role in drug interactions and intrarenal drug biotransformation.

The CYP enzyme that is responsible for biotransforming a particular xenobiotic may be identified using four different in vitro approaches called reaction phenotyping. A combination of these techniques is optimal for identifying the CYP enzyme(s). First, a correlation analysis can be used to correlate the level of activity of individual CYP enzymes with the rate of biotransformation of a xenobiotic.

Second, the effects of known inhibitors of CYP enzymes on the biotransformation of a xenobiotic can be studied. Mechanism-based inhibitors are chemical inhibitors that require biotransformation to a metabolite that inactivates or noncompetitively inhibits CYP. It should be noted that many inhibitors can inhibit more than one CYP enzyme.
Antibodies against CYP enzymes can also be used to inhibit xenobiotic biotransformation. Monoclonal antibodies are highly specific, but often lack inhibitory capacity because only a single antigen site on the enzyme is targeted and this sometimes only produces minor alterations in catalytic activity (Schmider et al., 1996). Polyclonal antibodies, on the other hand, are often only selective for CYP subfamilies and are much stronger inhibitors.

Finally, purified or cDNA-expressed CYP enzymes can be used to study which enzyme biotransforms a xenobiotic. Caution must be used in extrapolating these results to the in vivo situation because even if a particular CYP enzyme can biotransform a xenobiotic, it may not be present in sufficient amounts to contribute to the reaction in vivo. The same reaction may be catalyzed by several CYP enzymes. The biotransformation of a drug in vivo is a function of the affinity of the drug for the enzyme (Km) and the relative abundance of that enzyme in the cell. The intrinsic clearance of a drug, defined as Km/Vmax, is used to determine the relative contribution of each enzyme.

While almost all tissues except striated muscle and erythrocytes contain CYP enzymes, enzyme distribution varies greatly (Crommentuyn et al., 1998). Furthermore, many drugs and exogenous (especially environmental) compounds can induce or inhibit CYP activity. There are tissue, sex, and species differences in enzyme expression. A good example of the importance of species differences in biotransformation is the observation that ifosfamide is bioactivated in rats by CYP2B1 and CYP2C6/11, while in humans it is bioactivated by CYP3A (Walker et al., 1994). Species specificity also plays a role in CYP3A induction. For instance, rifampicin is a better inducer of CYP3A than pregnenolone 16α-carbonitrile (PCN) in humans while the opposite is true in rats (Watkins, 1998).

The induction and inhibition of drug metabolizing enzymes can have drastic effects on drug therapy. Enzyme induction can cause therapeutic failure by allowing a drug to be deactivated before it achieves its therapeutic goal. Alternatively, if drug
metabolites are toxic, enzyme induction can lead to greater toxicity. Enzyme inhibition can prevent prodrugs such as ifosfamide and cyclophosphamide (see chapter three) from being activated to their cytotoxic (that is, therapeutic) metabolites, or it can prevent toxic parent compounds from being detoxified.

Genetic polymorphisms in enzyme expression and function also exist. They can result in different phenotypes such that patients may be classified as either poor or extensive metabolizers of certain drugs. A classic example of an enzyme with a genetic polymorphism is CYP2D6. This enzyme biotransforms many classes of drugs including cardiovascular agents, psychoactive agents, and morphine derivatives. Poor metabolizers either lack this enzyme or have lessened enzyme activity and are unable to biotransform substrates for this enzyme. Hence, they have higher plasma concentrations of parent drug. The importance of the CYP2D6 polymorphism will be highlighted in the later discussion of propafenone biotransformation.

Drug interactions resulting from the inhibition of one or more drug biotransforming enzymes are common in the clinical setting. As with drug interactions occurring at drug transporters, inhibition can be competitive or noncompetitive. Competitive inhibition depends on the concentration of the inhibitor, the $K_i$, the concentration of the substrate, and the $K_m$. Inhibition of CYP enzymes can be reversible or irreversible and may require metabolic activation. Competitive inhibitors may or may not be substrates. Noncompetitive inhibitors may work by mechanism-based inhibition or suicide inactivation of CYP. That is, when CYP activates a compound to an inhibitory product, the CYP may be sequestered in a functionally inactive state or may be inactivated by heme or apoprotein modification (Crommentuyn et al., 1998).

I.6b Phase II Reactions

Phase II reactions include glucuronidation, sulfation, methylation, acetylation, amino acid conjugation, and glutathione conjugation. These reactions are generally
detoxifying reactions although a number of toxifying reactions have been observed (Parkinson, 1996). For the purposes of this thesis, only glutathione conjugation will be discussed since it plays an important role in the detoxification of ifosfamide (see chapter three).

Glutathione is a tripeptide made of glycine, cysteine, and glutamic acid. The nucleophilic attack of glutathione on electrophilic carbon atoms leads to the formation of glutathione conjugates called thioethers (Keen and Jakoby, 1978). Glutathione conjugates are often converted to mercapturic acids (S-substituted N-acetylcysteine derivatives) in the urine. The formation of these conjugates is catalyzed by glutathione transferase.

Glutathione plays a crucial role in cellular antioxidant protection and detoxification; therefore, drugs that deplete glutathione leave the cell vulnerable to toxicity. An example of this cytotoxicity is the renal- and hepatotoxicity caused by chloroacetaldehyde as described in chapter three.

1.7 Renal Drug Biotransformation

In general, the levels of drug metabolizing enzymes in the kidney are lower than those in the liver, yet, equal or greater than those in other tissues.

1.7a CYP Enzymes

The amount of total CYP enzymes in kidney is generally much less than liver in most species (Anders, 1980). Renal CYP activity is often cited as being only 10% to 20% of that of liver (Rush et al., 1986). The activity of renal NADPH-CYP reductase varies greatly across species (Anders, 1980). However, the low metabolic capacity of the kidney seems to mainly be a result of the low content of CYP enzymes (Anders, 1980).

In a study of the renal content of the mixed-function oxidase system in rabbits, the highest level of cytochrome P450 was found in the cortex of the kidney with none detectable in either the inner or outer medulla. Renal monooxygenase activities were
also highest in the cortex of the kidney (Zenzer et al., 1978). Interestingly, the hepatic sex differences in drug biotransformation in rats are not seen in kidney (Litterst et al., 1977). The activity of CYP-mediated biphenyl-4-hydroxylation was increased in kidney and decreased in liver following starvation (Litterst et al., 1975). As well, the effects of a variety of inducers and inhibitors on CYP activity differ between kidney and liver (Anders, 1980; Ronis et al., 1998; Amet et al., 1998; Villard et al., 1998; Cummings et al., 1999; Paolini et al., 1999).

I.7b Aldehyde Dehydrogenases

In both male and female rats, renal aldehyde dehydrogenase activities were approximately 20% of those found in the liver (Büttner, 1965) although more recent studies suggest that the overall activity of aldehyde dehydrogenase in rat kidney can be up to 80% of that in rat liver (Deitrich, 1966; Vasilou and Marselos, 1989; Dipple and Crabb, 1993). Using indole-3-acetaldehyde as the substrate, Dietrich (1966) found that whole renal homogenate activity was two-fold higher in female versus male rats.

I.7c Glutathione Transferases

Both glutathione and glutathione transferase concentrations are lower in kidney than in liver (Mohandras et al., 1984; Pacifici et al., 1988). The importance of differences in hepatic versus renal concentrations of glutathione are discussed in chapter three. Glutathione concentrations are 1-2 μmol/g of tissue in the kidney. Glutathione is present in the cytosol, mitochondria, and nuclei with higher concentrations in the renal cortex than in the medulla (Mohandras et al., 1984). Almost all of the glutathione that is filtered is reabsorbed from the lumen of the proximal tubule (Lohr et al., 1998). Microperfusion studies with single proximal kidney tubules from rats show that glutathione derivatives are very quickly degraded (half-life = 3.5 seconds; Wendel et al., 1978). Glutathione degradation in the proximal tubule occurs at both the apical (Hahn et al., 1978) and basolateral (Abbott, 1984) membranes.
A number of glutathione transferases have been identified in the kidney. These enzymes differ in many ways such as isoelectric point, substrate specificity and hormonal influences from hepatic glutathione transferases (Kaplowitz et al., 1976; Hales et al., 1978). Using benzo(a)pyrene-4,5-oxide, Pacifici et al. (1988) reported activities of glutathione transferase in human liver and kidney of $1.8 \pm 0.5$ and $1.41 \pm 0.33$ nmol/min/mg of protein, respectively. Despite lower activities of glutathione transferases in the kidney compared with the liver (Clifton and Kaplowitz, 1978; van Cantfort et al., 1979), glutathione transferases play an important role in the renal conversion of glutathione conjugates to mercapturic acids. The formation of mercapturic acids involves the sequential cleavage of glutamic acid and glycine from the glutathione moiety. These steps are catalyzed by gamma-glutamyltranspeptidase (which has highest activity in the kidney (Hughey et al., 1978)) and aminopeptidase M. The resulting cysteine conjugate is then N-acetylated (Parkinson, 1996). The activity of N-acetyltransferase is also highest in the kidney (Hughey et al., 1978; Green and Elce, 1975).

Obviously, there are important sex, species, tissue, and regional differences in the expression of drug metabolizing enzymes. The expression of these enzymes (especially the individual CYP isoforms) must be carefully considered in discussions of drug biotransformation by the kidney.

1.8 CYP3A and P-Glycoprotein

While individual CYP enzymes will be discussed at greater length later in this thesis, a curious relationship exists between CYP3A and P-glycoprotein and merits discussion at this point. The genes for these two proteins are both located on chromosome 7 and the proteins share a remarkable number of substrates and modulators such as cyclosporine, nifedipine, etoposide, vinblastine and ketoconazole (Wacher et al., 1995).
The percentage of CYP3A in the small intestine is even higher than that in the liver (70% versus 20% of total CYP, respectively; Watkins et al., 1987). This high expression of CYP3A in intestinal tissue explains why most drugs that are metabolized by CYP3A have low and variable bioavailabilities (Watkins, 1990; Kolars et al., 1992). However, P-glycoprotein is also expressed in high amounts on the apical membranes of enterocytes. Hepatic and intestinal expression of CYP3A each vary by at least ten-fold in patients. There is also a lack of correlation between hepatic and intestinal CYP3A expression (Lown et al., 1994). Like CYP3A, there is great interindividual variation in the intestinal expression of P-glycoprotein. Healthy volunteers show P-glycoprotein variations of up to four-fold, while medically stable, hospitalized patients have as much as ten-fold variations in P-glycoprotein expression (Lown et al., 1995).

Salphati and Benet (1998) tested the hypothesis that CYP3A and P-glycoprotein could be coordinately regulated by looking at the effects of the known CYP3A inducers triacetyloleandomycin, rifampicin, dexamethasone, and pregnenolone 16α-carbonitrile on P-glycoprotein expression in rat liver. Rifampicin caused a 50% increase in P-glycoprotein levels in male rat liver, while dexamethasone caused a five-fold increase. However, triacetyloleandomycin and pregnenolone 16α-carbonitrile did not affect P-glycoprotein expression and mRNA levels. The authors concluded that the induction of CYP3A and P-glycoprotein are regulated independently and that P-glycoprotein expression and regulation are gender specific.

Despite the independence of regulation of CYP3A and P-glycoprotein, a functional link appears to be important to the handling of xenobiotics. P-glycoprotein attempts to prevent the absorption of drugs that are substrates for it, while any drug which does get absorbed can then be biotransformed by CYP3A; again, limiting systemic bioavailability. The coordinate role of these two systems is currently being studied. It is possible that drug interactions that were previously attributed to inhibition of CYP3A may be occurring at P-glycoprotein, and vice versa, or both.
The kidney possesses various drug transport systems and drug metabolizing enzymes that facilitate the elimination of xenobiotics from the body. Naturally, drug interactions can occur at these sites. The biotransformation of drugs may lead to the production of metabolites that cause local toxicity or that contribute to drug interactions at drug transporters. Chapter two will provide examples of digoxin-drug interactions involving the P-glycoprotein drug transporter that lead to systemic toxicity. Drug metabolites produced by hepatic or extrahepatic enzymes may play a role in these interactions. Chapter three will describe how intrarenal biotransformation of ifosfamide may be associated with nephrotoxicity.
CHAPTER 2: RENAL TUBULAR DRUG INTERACTIONS AT P-GLYCOPROTEIN

II.1 Introduction

To study renal tubular drug interactions that might lead to local or systemic toxicity, I characterized an in vitro model of drug transport. This chapter will describe the utility of this renal cell culture model for studying known drug interactions and for predicting drug interactions. With the help of the model, the mechanism of the interaction between the antiarrhythmic drug propafenone and the cardiac glycoside digoxin was elucidated. The contribution of drug metabolites to this interaction and also to the digoxin-verapamil interaction was explored. Using the in vitro system, I was also able to propose a drug interaction between digoxin and the controversial abortive agent mifepristone before mifepristone was even released to North American markets. Therefore, this model may be useful as a screening tool for predicting drug interactions, especially in drug development.

I was particularly interested in studying drug interactions mediated by the P-glycoprotein drug transporter. As will be described in this chapter, with the careful choice of cell lines, almost any two (or more) drugs can be tested in this system for their interaction potential at P-glycoprotein. Digoxin has been chosen as an exemplary drug in the cell culture studies of P-glycoprotein-mediated drug interactions because of the clinical importance of these interactions. The utility of the in vitro model for screening and identifying compounds that can reverse multidrug resistance will also be discussed.

II.1a Methods to Study Renal Drug Transport

A number of methods have been used to study the renal transport of drugs including the monitoring of in vivo and in vitro clearances, the Sperber technique (infusion of a compound into the renal portal circulation), stop-flow experiments, renal slices, renal tubule suspensions, in vivo micropuncture and microperfusion, in vitro
microperfusion, membrane vesicles, and cell culture (primary culture or immortal cell lines). Each method has its own advantages and disadvantages.

Clearance studies approach the kidney as a whole, but cannot detail events at a cellular level. As a result, clearance studies do not allow one to study filtration separately from secretion and reabsorption because the end-result (that is, filtration + secretion - reabsorption) is measured. In order to get a clearer picture of renal drug handling, sub-organ preparations are useful. I chose to work with a cell culture system that enables renal tubular events to be scrutinized at a cellular level.

Improved methodologies for growing renal cells in homogeneous cultures have led to an explosion of in vitro cell culture work. In the early 1970's, renal and transport physiologists became interested in the use of permanent cell lines because they retain several kidney-specific characteristics. Renal tubular cells were found to form polarized epithelia under standard cell culture conditions (Biber et al., 1983).

An important advantage of renal cell culture is the exclusion of higher order regulatory systems with the ability to manipulate experimental conditions while maintaining adequate renal cell functions for the study of xenobiotic transport. Use of cell culture systems to investigate drug transport requires adherence to some important conditions. First, the epithelial cells should retain their correct distribution of membrane enzyme and transport systems as well as their polar architecture. Second, vectorial solute and water transport manifested by dome formation (when grown on impermeable surfaces) and generation of transepithelial electrical properties must be maintained. Third, apical and basolateral xenobiotic uptake must be the same as the in vivo et situ situation. Finally, the characteristics of the segment of nephron cultured should correspond to the appropriate expression of hormone, metabolic, and transport properties.
II.1a1 Characteristics of the MDCK cell line

We primarily used Madin-Darby canine kidney (MDCK) cells in our studies of P-glycoprotein-mediated drug transport. This cell line was established from the kidney of a female Cocker Spaniel. Established cell lines often express abnormal karyotypes, biochemical characteristics, and cellular functions (Aleo and Kostyniak, 1996). While one must bear in mind that cells in culture are not necessarily representative of the in vivo situation, in general, MDCK cells appear to have similar drug transport properties to the human kidney. Most important to our studies, P-glycoprotein expression has been confirmed in MDCK cells by our group and others (Horio et al., 1989; Ito et al., 1999). Both functional and immunohistochemical studies indicate that P-glycoprotein is located in the apical membranes of these renal tubular cells.

MDCK cells are epithelial cells with polar orientation and functional tight junctions. Based on hormonal responsiveness, they appear to have a distal tubular cell origin (Meier and Insel, 1985) and can reabsorb (i.e., transport from the apical-to-basolateral side) water and salts (Taub, 1996). Functional evidence for a collecting duct origin of MDCK cells has also been established (Ishizuka et al., 1978; Pfaller et al., 1989; Oberleithner et al., 1990a,b,c).

When MDCK cells are grown on solid supports, domes are formed. The cells are usually ciliated and bear a number of short microvilli (Valentich, 1981). Using semipermeable substrata, cell height and the formation of lateral cellular processes increase (Zuk et al., 1989).

One of the problems with established cell lines is that they are not cloned from a single parental cell. Thus, they tend to express properties of multiple cell types in culture. Two functionally distinct MDCK cell strains exist. Strain I has high transmonolayer resistance and strain II has low resistance (Barker and Simmons, 1981; Richardson et al., 1981). Cells of strain I are more columnar in appearance and display well-developed lateral cellular interdigitations, whereas the low resistance cells are fairly flat (Simons and Fuller, 1985; von Bonsdorff et al., 1985). No evidence for
differences in the morphology of junctional complexes exists; however, both MDCK strains differ considerably in their glycosphingolipid patterns (Hansson et al., 1986; Nichols et al., 1986). In addition, there is a difference between the two strains in the lipid composition of the apical and basolateral membrane domains (Nichols et al., 1988; Simons and van Meer, 1988; van Meer and Simons, 1988).

The cells used in our experiments were purchased from a commercial source and were not labelled according to strain type. The glycosphingolipid pattern of our cells suggests that they are derived from strain II (Prateek Lala, University of Toronto, personal communication). Drug transport studies with digoxin and vinblastine do not show significant differences between the two strains (Hockmann, 1999; Vicki Cook, The Hospital for Sick Children, Toronto, personal communication).

II.1b The In Vitro Model

The cell culture model that I utilized is unique in that it involves growing renal tubular cells on the permeable, inorganic membranes of commercially available tissue culture inserts. In culture, the cells retain their polar orientation such that the side of the cells that attaches to the filters represents the basal or blood side of the renal tubular cells, while the opposite end differentiates into an apical or urine side. MDCK cells form confluent monolayers on the inorganic membranes, facilitating the study of drug transport by reducing paracellular drug flux. (The permeable membrane is transparent enabling the confirmation of cell monolayer confluency prior to experimental use.)

The tissue culture insert resembles a small drinking glass in shape. It consists of a flat porous membrane attached at the bottom of a plastic cylindrical ring such that the membrane forms the bottom and the ring forms the sides (figure 1). The ring part has three short "feet" attached to the bottom of it. Therefore, if the inserts are placed in 6-well tissue culture plates, the porous-bottom of the dish is elevated such that both sides of the cell monolayer can be bathed in media (figure 2). This cell culture system
Figure 1. The tissue culture insert.
Figure 2. The tissue culture insert sitting in the well of a tissue culture plate. The inset shows the orientation of the renal tubular cells.
facilitates the study of drug transport across cell monolayers and is unique because it enables access to the apical and basal sides of the cells independently. That is, one can add a drug to the media bathing one or both sides of the cell monolayer and monitor its movement by sampling the media over a period of time.

An advantage of this model compared to tissue slices or membrane vesicles is that it is a "whole cell" model in which the apical and basolateral sides of the cell can be approached in isolation. The physical separation of the apical and basal solutions results in the electrical isolation of the solutions. This experimental system thus allows the initial manipulation of both the basal and apical xenobiotic drug concentrations. A further benefit of this cell culture system is that the tissue culture insert can be transferred to other well plates (for example, from one containing preincubation media to one containing radiolabelled media) with ease because the insert can be grasped with sterile forceps without disturbing the epithelium growing on the membrane.

II.1c Digoxin

Digoxin enhances the contractility of myocardial tissue (positive inotropy) and decreases the rhythm of the heart (negative chronotropy) (Soldin, 1986). Structurally, digoxin has an aglycone segment consisting of a steroid nucleus and an α,β-unsaturated five-membered lactone ring at the C17 position of the steroid nucleus (figure 3). Three sugars attach to the C3 hydroxyl, which, along with the C14, is in the β-form (Soldin, 1986).

Digoxin is a moderately lipophilic drug that can cross the plasma membrane by passive diffusion. It has a very large volume of distribution (5-15 L/kg) (Koren, 1987) consistent with significant digoxin tissue binding. Consequently, factors that alter the binding of digoxin to tissues may influence the volume of distribution. The elimination half-life of digoxin varies from 26-45 hours (generally about 34-36 hours) in healthy individuals but is longer if there is reduced renal function (Herfindal et al., 1988). At steady-state, the highest concentrations of digoxin can be found in the liver, kidney,
Figure 3. General structure of digitalis compounds (above). Structure of digoxin (below).
cardiac and skeletal muscles, and skin (Hastreiter, 1985). Digoxin disposition follows a multi-compartment, first-order elimination model with skeletal muscle forming the largest storage depot for digoxin (Soldin, 1986).

Digoxin is biotransformed by the gastrointestinal tract and liver to varying degrees; the metabolites are less active than the parent compound (Koren et al., 1987). Using both membrane vesicles and the multiple indicator dilution technique, Koren et al. (1987) reported metabolism by the kidney, although the metabolites were not identified. For the majority of patients, the biotransformation of digoxin is less clinically important than renal elimination; however, digoxin metabolism may be clinically important to those with renal failure. In some patients, digoxin is postulated to undergo extensive biotransformation to polar end-metabolites (some of which are active) by hydrolysis, oxidation, epimerization, and conjugation. The biotransformation of digoxin may also be influenced by inter-individual genetic variations in metabolism (Gault, 1984). In general, digoxin is mainly excreted unchanged in humans (Rodin and Johnson, 1988). When human liver microsomes or hepatocytes were incubated with digoxin, no metabolites were detected (Lacarelle et al., 1991). By contrast, in rats, biotransformation of digoxin accounts for more than 60% of the dose, while renal excretion of digoxin is less than 30% of the dose (Harrison and Gibaldi, 1976).

The metabolic pathways of digoxin are illustrated in figure 4. The sugar moieties of digoxin are subject to stepwise removal by hydrolysis, either in the stomach or in the liver, resulting in the production of 3β-digoxigenin. This species is then rapidly oxidized into 3-keto-digoxigenin which epimerizes to 3α(epi)-digoxigenin (Gault et al., 1984). The latter compound can be conjugated to the polar end-metabolites 3-epi-glucuronide and 3-epi-sulfate (Gault et al., 1984). The relative amount of substrates presented to the conjugation enzymes may influence the predominant substrate conjugated (Gault et al., 1984).

In rats, CYP3A enzymes are involved in the cleavage of digoxin and digoxigenin bis-digitoxoside (Salphati and Benet, 1999). The enzyme that cleaves digoxigenin mono-
Figure 4. Pathways of digoxin biotransformation.
digitoxoside to digoxigenin has not been identified. Digoxigenin bis-digitoxoside is the main metabolite of digoxin, likely because it is conjugated and excreted before it can be further biotransformed (Salphati and Benet, 1999). Both the mono- and bis-digitoxosides appear to have as much cardioactivity as digoxin; however, digoxigenin is considerably less cardioactive than digoxin (Soldin, 1986). In general, there is a stepwise loss in cardioactivity as sugar residues are removed in a stepwise fashion (Bach and Reiter, 1964; Brown et al., 1962; Lage and Spratt, 1966).

The lactone ring portion of digoxin can be reduced to produce dihydrometabolites such as dihydrodigoxin (Lindenbaum et al., 1981), or opened by a lactonase to produce a highly polar metabolite (Benthe, 1984). Dihydrometabolites have little cardioactivity (in fact, almost all pharmacologic activity is lost in this conversion) (Bach and Reiter, 1964; Brown et al., 1962; Lage and Spratt, 1966). Quantitatively dihydrometabolites may be important in some patients, yet one study showed them to be less than 2% of the byproducts in the urine following oral digoxin administration (Gault et al., 1984). It has been postulated that the bacterial flora in the intestine are responsible for the dihydro derivatives. If this is true, the production of these derivatives is governed by the route of administration of digoxin (Soldin, 1986). Enterohepatic recycling may also play a role in the pharmacokinetics of digoxin because the small intestine is a major site of digoxin absorption (Soldin, 1986).

In addition to being excreted renally following hepatic conjugation, digoxigenin could be eliminated by biliary excretion. In the case of renal failure, the fecal excretion of $[^3\text{H}]$-digoxin and its metabolites has been shown to increase. The nonrenal elimination (of which biliary secretion is the major component) of digoxin may account for as high as 30% of the plasma clearance in some patients. This includes metabolism by the liver, biliary secretion, and any possible intestinal secretion (seen in animal studies) (Hedman, 1990).
II.1d Renal Handling of Digoxin

In humans, almost all of the body load of digoxin is excreted unchanged by the kidney. For decades it was assumed that digoxin was eliminated through the kidney solely by glomerular filtration without subsequent secretion or reabsorption across the renal tubular cells. This belief stemmed from observations that the clearance of digoxin and its elimination half-life correlate with the glomerular filtration rate as estimated by serum creatinine, and creatinine or inulin clearances (Doherty et al., 1969; Koren et al., 1985). For instance, the plasma clearance of digoxin is decreased in proportion to the decrease in creatinine clearance in patients with renal failure. Yet in humans, creatinine is not only filtered but also secreted (Schwartz et al., 1987; Darling and Morris, 1991). Therefore, in order for the values of digoxin clearance to parallel those of creatinine clearance, digoxin must also be secreted to some extent.

In 1974, Steiness drew attention to the fact that, after correction for the plasma protein binding of digoxin, the renal clearance of the cardiac glycoside substantially exceeds that of creatinine, indicating net tubular secretion (Steiness, 1974). Digoxin displays about 25% binding to plasma albumin. With protein binding factored into the equation, the clearance of digoxin (of which only 75% is free and available for filtration) is greater than that of creatinine or inulin. Studies in humans and dogs have shown that digoxin clearance is more than double that of inulin, creatinine, and L-glucose when the protein binding of digoxin is taken into account (Steiness, 1974; de Lannoy et al., 1992a). Digoxin clearance amounting to more than that of the 'gold standard' for glomerular filtration, namely inulin, suggests that digoxin is secreted into the tubular lumen. In fact, after adjusting for protein binding, the renal tubular secretion is found to account for as much as 50% of the renal digoxin elimination (Steiness, 1974).

The in vitro studies described in this thesis, and those already published (Koren, 1987; Koren et al., 1987; Ito et al., 1992; 1993a,b,c; 1999*; Hori et al., 1993; Okamura et al., 1993), further support the presumption that digoxin is secreted by the renal tubular cells, as do in vivo studies using the single pass multiple indicator dilution technique
(Koren et al., 1987). Most importantly, the significance of the renal tubular secretion of digoxin has been evidenced in the clinic in the form of serious drug interactions. Many digoxin-drug interactions have been shown to affect the renal clearance of digoxin without affecting the GFR (Hansten, 1985); again, suggesting that digoxin renal elimination involves additional mechanisms other than filtration. However, the mechanism by which digoxin is secreted from renal tubular cells was unclear at the onset of this work.

During the seventies and eighties, several drugs that are commonly coadministered with digoxin were shown to increase the serum concentrations of this cardiac glycoside and to cause digitalis toxicity in some patients. Quinidine, an antiarrhythmic agent, was shown to cause toxic accumulation of digoxin (two- to three-fold higher digoxin concentrations) and to decrease digoxin renal clearance without affecting the glomerular filtration rate (Leahey et al., 1978; Hager et al., 1979). The total body clearance of digoxin, defined as the volume of body fluid from which the drug has been completely removed in a unit of time, fell in the presence of quinidine.

Following the description of the effects of quinidine, reports of drug interactions of digoxin with verapamil, amiodarone, and propafenone began to emerge (Moyser et al., 1981; Pedersen et al., 1981; Belz et al., 1983). The common denominator of these drug interactions is that none of these antiarrhythmics appears to change the oral bioavailability of digoxin. Moreover, because the hepatic elimination of digoxin is minimal, they must affect the renal excretion of digoxin. Yet, quinidine, verapamil, amiodarone and propafenone have not been shown to decrease the glomerular filtration rate, suggesting that these drugs primarily affect the net renal tubular secretion of the cardiac glycoside. Indeed, clearance studies have shown that quinidine, verapamil, amiodarone and propafenone decrease the renal clearance of digoxin without decreasing the GFR (Pedersen et al., 1981; Herfindal et al., 1988; Hedman, 1990; Koren et al., 1983; Belz et al., 1983; Calvo et al., 1989). The observation that these interacting drugs can cause more than a 100% increase in serum concentrations of digoxin in many
patients, indicates a predominance of the renal tubular secretion of digoxin over its glomerular filtration (Ito et al., 1993b*). By pooling various published in vivo clearance studies, Ito et al. (1993b*) found digoxin secretion to amount to as much as 140% of the GFR. The authors also noted that this value was probably an underestimation since the drugs used to inhibit digoxin renal transport were likely at submaximal inhibitory doses (to avoid toxicity).

The fact that digoxin is secreted by the renal tubules does not exclude the possibility that it is also reabsorbed. In one rat micropuncture study, about one-third of the digoxin filtered was reabsorbed into the proximal tubule (Roman and Kauker, 1976). Furthermore, both quinidine and spironolactone cause the renal clearance of digoxin to fall below that of inulin, indicative of reabsorption. Stop flow techniques (Doherty et al., 1969) have also shown that digoxin is reabsorbed.

In an analysis of published data, Ito et al. (1993b*) showed reabsorption of digoxin to amount to as high as 40% of the GFR. Again, because inhibition may not have been maximal in these studies, this figure represents a cautious estimate. The authors went further to suggest two possible mechanisms for digoxin reabsorption. The first proposes passive reabsorption of digoxin resulting from the concentration gradient created by the known reabsorption of water. The second acknowledges a potential active transport system causing digoxin efflux into the peritubular space. However, the latter possibility has not been found and therefore strongly suggests that digoxin is only reabsorbed passively. The correlation between the rate of urine flow and the reabsorption of digoxin also suggests passive reabsorption (Koren, 1987, Koren et al., 1987; Steiness et al., 1982).

II.1e P-Glycoprotein-Mediated Digoxin Transport

Several lines of evidence have shown that digoxin utilizes P-glycoprotein for its renal tubular transport. First, drugs that are known substrates of P-glycoprotein (e.g., vinblastine, vincristine) inhibit digoxin transport across the renal tubular cell (Ito et al.,
Likewise, drugs that are known to inhibit P-glycoprotein transport (e.g., verapamil, cyclosporine) also inhibit digoxin transport across the renal tubular cell (Ito et al., 1993a,b,c). In the third place, transfection of renal tubular cells with the MDR1 gene increases the renal tubular secretion of digoxin (Okamura et al., 1993). Finally, mice in which the mdr1a gene has been "knocked-out" accumulate more digoxin than their wild-type counterparts (Schinkel et al., 1995).

The importance of P-glycoprotein to the total body handling of digoxin and other P-glycoprotein substrates has only recently been appreciated. Clearly, drugs that affect this secretory mechanism may interfere with the renal tubular transport of digoxin and result in digitalis toxicity. Therefore, an understanding of P-glycoprotein and its role in the transport of digoxin is crucial to the prediction and identification of digoxin-drug interactions. I used the renal tissue culture model described above to examine a number of clinically-significant and previously unexplained digoxin-drug interactions to see if they involved P-glycoprotein.

II.1f Digoxin-Drug Interactions

The cardiac glycoside digoxin has been in clinical use for over two hundred years; yet, therapeutic drug monitoring of this drug is still a serious challenge to clinicians, both at the bedside and in the laboratory. The 1998 Compendium of Pharmaceuticals and Specialties (CPS) cites diltiazem, nicardipine, nifedipine, verapamil, amiodarone, and quinidine as precipitant drugs for drug interactions with digitalis glycosides. During steady state, the mean serum concentration of a drug is directly related to its dose and oral bioavailability and negatively related to its systemic clearance. Although the relative bioavailability of digoxin is quite high (>70%), antibiotics that alter gut flora may eradicate bacteria which metabolize digoxin, thus increasing steady state digoxin concentrations. Once absorbed, however, major changes in digoxin steady state concentrations are caused mainly by alterations in its renal elimination. Drugs or conditions that are known to decrease glomerular filtration should prompt close
monitoring of digoxin serum concentrations and vigilance for clinical signs of toxicity (e.g., aminoglycosides, amphotericin B).

Of all adult medical patients admitted to hospital, up to 15% are on digitalis therapy and 30% of these patients have signs of digoxin toxicity (Clarke and Ramoska, 1988). Many patients taking digoxin also need other medications. During the last two decades, a relatively large group of drugs has been shown to cause digitalis toxicity by inhibiting the renal tubular secretion of digoxin without affecting the GFR, thus proving the major role of this secretory mechanism in the elimination of this potentially toxic drug.

It is not surprising that the first few drugs that were shown to interact with digoxin by inhibiting its tubular secretion were antiarrhythmic agents because these drugs are commonly coadministered with digoxin. Since digoxin is now prescribed to millions of patients worldwide, it is likely that scores of medications will be coadministered with digoxin to different patients for different indications. Therefore, it is not unreasonable to expect reports of other digoxin-drug interactions to emerge in the future.

Generally, drug interactions between drugs that are not commonly co-prescribed take longer to be recognized in the literature. However, using the cell culture model, potential drug interactions can be quickly identified. For instance, we were able to show that the azole antifungal agent itraconazole is a very effective inhibitor of digoxin renal tubular secretion (Woodland et al., 1998a). Yet, because the combination of these two drugs is likely quite uncommon, the emergence of epidemiological data indicating such an interaction can be expected to be unpredictable. Recently, several case reports and small studies of the itraconazole-digoxin interactions leading to digitalis toxicity have demonstrated the relevance of our in vitro prediction (Woodland et al., 1998a).

The low therapeutic index and widespread clinical use of digoxin raises concerns that digoxin-drug interactions could lead to increases in digoxin serum concentrations and result in toxicity. Hence, a systematic approach to screen such compounds is warranted to prevent digitalis toxicity which can cause serious and even fatal
complications. The in vitro model described in this chapter may aid in the identification and characterization of such digoxin-drug interactions.

II.1g The Digoxin-Propafenone Interaction

Propafenone is a class Ic antiarrhythmic agent that blocks sodium channels and β-adrenergic receptors (Funck-Brentano et al., 1990). When propafenone is given concurrently with digoxin, steady state serum digoxin concentrations increase and digoxin clearance decreases in both adults and children (Belz et al., 1983; Calvo et al., 1989; Salerno et al., 1984; Bigot et al., 1991; Zalzstein et al., 1990). The low therapeutic index of digoxin necessitates dose adjustment and careful monitoring for digoxin toxicity. However, while the interaction between digoxin and propafenone is well known clinically, the mechanism by which propafenone interferes with digoxin elimination is unclear. This interaction is especially intriguing because the kidney, a major organ for digoxin elimination, plays virtually no role in the elimination of propafenone.

Propafenone is extensively metabolized by the liver and demonstrates negligible renal elimination with no net renal tubular secretion (Seipel and Breithardt, 1980; Hollmann et al., 1983). As shown in figure 5, the two major metabolites of propafenone are 5-hydroxypropafenone (5-OHP) and N-depropylpropafenone (NDPP). Both metabolites also have sodium channel-blocking activity; however, blockade of β-adrenergic receptors is much weaker than with the parent compound. The hydroxy metabolite is considered to be therapeutically active, while NDPP is probably less active partly because of its lower serum concentrations (Funck-Brentano et al., 1990). The biotransformation of propafenone to 5-OHP is mediated by CYP2D6 (Siddoway et al., 1987). NDPP is reportedly produced by CYP3A4 and CYP1A2 (Botsch et al., 1993). The fact that propafenone is metabolized by CYP enzymes is often cited as the reason for its interaction with quinidine and theophylline (Broly et al., 1990; Kobayashi et al., 1998).
Figure 5. Structures of propafenone and its major metabolites.
Due to a genetic polymorphism, approximately 1-7% of the population lacks activity of the CYP2D6 enzyme (Bertilsson, 1995). The prevalence of this enzyme deficiency is greatest in Caucasians (7%) and least in Orientals (1%). Recently, existence of the super-extensive metabolizer phenotype of CYP2D6-mediated biotransformation resulting from functional gene duplication was identified in 7% of a white healthy Spaniard population (Agúndez et al., 1995). Although there are substantial interindividual variations in serum concentrations of propafenone and its metabolites due to the wide range of metabolic capacities in the human population, serum concentrations of 5-OHP (0.5-1.5 μM) may reach comparable levels to those of the parent compound (1-6 μM), especially in extensive metabolizers (Siddoway et al., 1987; Haefeli et al., 1990).

If the parent propafenone compound is primarily responsible for the digoxin-propafenone interaction, poor metabolizers of propafenone should have a greater likelihood of these interactions. However, no clinical evidence exists to suggest that these interactions are more pronounced in poor metabolizers than in extensive metabolizers, implying that propafenone metabolites may play a role in digoxin-propafenone interactions. The participation of propafenone metabolites in digoxin-propafenone interactions has not been investigated; nor is it known how these metabolites are handled by the kidney. In fact, the roles of drug metabolites in renal drug-drug interactions have never been thoroughly examined.

I used the renal cell culture model described above to examine the effects of propafenone and its metabolites on the renal tubular secretion of digoxin. During the course of these experiments I found that propafenone is biotransformed by MDCK cells to NDPP. The expression of CYP enzymes is not characterized in MDCK renal tubular cells. In humans, one of the enzymes responsible for biotransformation of propafenone to NDPP, CYP3A4, is ubiquitously expressed in renal tissue and appears to have a bimodal distribution (Haehner et al., 1996). CYP1A2 is generally considered to be liver-specific; however, expression in kidney has been noted following induction (Mahajan and Rifkind, 1999). Therefore, I examined the biotransformation of propafenone by
MDCK cells and microsomes in the presence of inhibitors of CYP3A4 and CYP1A2. However, it was not the goal of this thesis to characterize the drug metabolizing enzymes responsible for propafenone biotransformation by MDCK cells or microsomes. Such a characterization would require the rigorous steps of reaction phenotyping described in chapter one.

Propafenone has a chiral carbon allowing it to exist in mirror image forms called enantiomers. According to the Cahn-Ingold-Prelog convention, the enantiomers are classified as R-enantiomers if the substituent atoms or groups of atoms around the chiral compound have a clockwise sequence of highest atomic number to lowest atomic number. When the sequence of highest to lowest atomic number of substituent atoms or groups of atoms is in the counterclockwise direction, the molecule is in the S-configuration (Roberts and Caserio, 1965). Propafenone is generally administered as a racemic mixture (that is, it contains equal numbers of each enantiomer). The R- and S-enantiomers of propafenone do not differ in their abilities to decrease the fast, inward sodium current although the S-isomer is far more potent as a β-antagonist. In addition, the area under the concentration-time curve (AUC) for S-propafenone is almost two-fold higher than that for the R-enantiomer indicating that R-propafenone has higher clearance (Kroemer et al., 1989). The relative contribution of the enantiomers of propafenone to digoxin-propafenone interactions was unknown at the onset of this work and was therefore studied using our in vitro system.

II.1h The Digoxin-Verapamil Interaction

Similar to propafenone, verapamil, an antiarrhythmic drug that acts by blocking the slow calcium channels, increases serum digoxin concentrations (Klein et al., 1980). Verapamil impairs both non-renal and renal digoxin clearance without decreasing the GFR (Pedersen et al., 1981; Herfindal et al., 1988). Verapamil also decreases the apparent central volume of distribution of digoxin. In a 1981 clinical study of the digoxin-verapamil interaction, Pedersen et al. proposed that the reduction of digoxin renal
clearance in the presence of verapamil may be due to an inhibition of renal tubular digoxin secretion (Pedersen et al., 1981). Furthermore, they found this interaction was independent of the plasma concentration of digoxin.

Using an *in vitro* tissue culture model, we were able to show that verapamil inhibits the renal tubular secretion of digoxin, at least partially explaining the increased serum concentrations of digoxin witnessed in patients taking digoxin and verapamil concurrently (Ito et al., 1993c*). The cellular uptake of digoxin was increased in the presence of verapamil indicating that verapamil does not prevent digoxin from entering renal tubular cells, rather, it keeps digoxin from exiting the cell (Ito et al., 1993c*).

Verapamil is administered clinically as a racemic mixture. The S-form of verapamil is responsible for its cardiac effects while R-verapamil is predominant in serum. This prompted us to investigate whether the two stereoisomers had different effects on digoxin transport. Our studies found that the inhibition of digoxin renal tubular secretion by verapamil is nonstereoselective (Ito et al., 1993c*). Since the renal tubular secretion of digoxin involves P-glycoprotein, and verapamil is a modulator of P-glycoprotein, our results were consistent with a drug interaction occurring at P-glycoprotein located in the apical membranes of renal tubular cells.

Multidrug resistance is a common problem in cancer chemotherapy and frequently correlates with P-glycoprotein expression. Various attempts have been made to reverse resistance to cancer chemotherapy using P-glycoprotein substrates (Raderer and Scheithauer, 1993). Before P-glycoprotein was recognized as being associated with multidrug resistance, Tsuruo et al. showed that verapamil could enhance the cytotoxicity of the chemotherapeutic agents vincristine and vinblastine both *in vitro*, using P388 leukemic cells and in mice with tumours resistant to vincristine (Tsuruo et al., 1981). These results initiated a series of investigations on the applicability of verapamil as a multidrug resistance reversal agent. Unfortunately, the success of these trials was limited by cardiac side effects (Fisher and Sikic, 1995).
Although serum concentrations of R-VER are higher than S-VER, the R-enantiomer has less calcium channel blocking activity. For this reason, R-VER has been administered to reverse multidrug resistance (Overmoyer et al., 1993; Motzer et al., 1995). While cardiotoxicity is lessened with the enantiomer (by approximately three-fold), the side effect profile is still not optimal.

VER is extensively and variably metabolized by human liver (Eichelbaum et al., 1978). NorVER is a major metabolite that is formed in human liver by CYP3A4 and CYP1A2 (Kroemer et al., 1993). The plasma concentrations of this N-demethylated metabolite approximate those of verapamil following single or multiple oral doses of the parent drug (Kates et al., 1981). As shown in figure 6, D-620 and PR-22 are N-demethyl-N-dealkyl and N-demethyl-O-demethyl verapamil metabolites, respectively.

The contribution of verapamil metabolites to the digoxin-VER interactions is unknown. While the major metabolite norVER achieves plasma concentrations that are comparable to verapamil (up to 1 μM; Shand et al., 1981; Freedman et al., 1981), it only has about 20% of the coronary vasodilator activity (in dogs) of the parent compound (Neugebauer, 1978). The D-620 and PR-22 metabolites are less well-studied; however, they are generally considered to be pharmacologically inactive (I. Wainer, McGill University, personal communication). The purpose of the present studies was to investigate the effects of the verapamil metabolites norVER, D-620, and PR-22 on the renal tubular secretion of digoxin to determine if they might contribute to the digoxin-VER interaction. The biotransformation of verapamil by MDCK cells was also investigated.

Since these verapamil metabolites lack the cardiovascular activity of the parent compound, their potential usefulness as multidrug resistance reversal agents was explored. The chemotherapeutic agent vinblastine is a well studied P-glycoprotein substrate that is secreted by renal tubular cells. Therefore, the effects of verapamil and its metabolites on the renal tubular secretion of vinblastine were investigated.
Figure 6. Structures of verapamil, norverapamil, D-620, and PR-22.
II.1i The Digoxin-Mifepristone Interaction

Mifepristone (more commonly known as RU 486 derived from the Roussel Uclaf company code number RU38486) is an antiprogestatin and antiglucocorticoid agent that was first released in France in 1990. Numerous clinical indications for mifepristone have been proposed including its use in pregnancy termination, contraception, induction of labour, cervical dilatation, breast cancer, meningiomas, gynecologic disorders, and Cushing's syndrome (Spitz and Bardin, 1993). The controversy surrounding the clinical use of mifepristone as an abortive agent has slowed its release and widespread use in other countries. As a result, comparatively less data on mifepristone-drug interactions might be expected than for other drugs available on the market for a similar time period.

Numerous steroid hormones are inhibitors and/or substrates of P-glycoprotein (Ueda et al., 1992). Substrates of P-glycoprotein tend to have a hydroxyl group at position 11 of the steroid molecule, and transport is greatly enhanced by an additional hydroxyl group at position 17 (Gruol et al., 1994). On the other hand, progesterone, which lacks a hydroxyl group at position 11, is an inhibitor, but not a substrate of P-glycoprotein (Yang et al., 1989; Qian and Beck, 1990; Yang et al., 1990). Mifepristone (figure 7), which binds to progesterone receptors, possesses structural characteristics (a hydrophobic ring and associated tertiary amine group) similar to those for other steroid group P-glycoprotein substrates (Ford and Hait, 1990). Thus, the purpose of this study was to investigate the effects of mifepristone on the renal tubular secretion of the P-glycoprotein substrates digoxin and vinblastine by MDCK renal tubular cells.

II.1j Vinblastine

Vinblastine (figure 8) is an antineoplastic drug that is a selective mitotic inhibitor of certain malignant cells. Although it is effective by itself, vinblastine is commonly coadministered with other antineoplastic drugs. Vinblastine is a well-studied substrate of P-glycoprotein (Tamai and Safa, 1991). Drug interactions with vinblastine are much
Figure 7. Structure of mifepristone.
Figure 8. Structure of vinblastine.
less commonly reported than those with digoxin. This is likely because drug interactions are often expected from chemotherapeutic agents in general given their toxicity, their coadministration with other drugs, and their low therapeutic indices.

To further study the role of P-glycoprotein in drug interactions and to investigate the potential of agents to act as reversal agents, we used vinblastine as a model substrate in our cell culture system.
II.2 Hypotheses

1. Propafenone is an inhibitor of P-glycoprotein-mediated renal tubular digoxin secretion.

2. The propafenone metabolites 5-OHP and NDPP contribute to the digoxin-propafenone interaction.

3. Renal tubular cells and microsomes biotransform propafenone to NDPP.

4. The enantiomers of propafenone contribute equally to the digoxin-propafenone renal tubular interaction.

5. The verapamil metabolites norverapamil, D-620, and PR-22 inhibit the renal tubular secretion of digoxin and vinblastine.

6. MDCK cells biotransform verapamil to norverapamil.

7. The inhibition of digoxin and vinblastine renal tubular secretion by norverapamil is not stereoselective.

8. Mifepristone inhibits the renal tubular secretion of digoxin and vinblastine.
I.3 Objectives

1. To determine whether propafenone interferes with the renal tubular secretion and uptake of digoxin or vinblastine in cell lines expressing P-glycoprotein.

2. To determine whether propafenone metabolites interfere with the renal tubular secretion and uptake of digoxin or vinblastine in cell lines expressing P-glycoprotein.

3. To determine whether propafenone is biotransformed by MDCK cells and microsomes.

4. To compare the effects of R-propafenone, S-propafenone, racemic propafenone, R-5-OHP, S-5-OHP, and racemic 5-OHP on digoxin and vinblastine renal tubular secretion.

5. To investigate the effects of the verapamil metabolites norverapamil, PR-22, and D-620 on the renal tubular transport and uptake of digoxin and vinblastine.

6. To determine whether verapamil is biotransformed by MDCK cells.

7. To compare the effects of the enantiomers of norverapamil on the renal tubular secretion of digoxin and vinblastine to those of the parent compound.

8. To examine the effects of mifepristone on the renal tubular secretion and uptake of digoxin and vinblastine.
II.4 Materials and Methods

II.4a Materials

II.4a1 Cell lines

MDCK cells (CCL-34) were obtained from the American Type Culture Collection (Rockville, MD).

II.4a2 Cell culture

Cells were grown on 75 cm² (T-75) Falcon tissue culture flasks (#7064). The cells were cultured in α-MEM (Eagle's minimum essential medium) media (not containing antibiotics) purchased in liquid form from the Ontario Cancer Institute. Fetal bovine serum (FBS) was obtained from Gibco BRL and was added to the α-MEM media in the laboratory.

Phosphate-buffered saline (PBS, at pH 7.4 unless otherwise noted) was prepared in the laboratory and filter sterilized. Cells were trypsinized with Bacto trypsin obtained from Difco Laboratories (Detroit, MI) and rehydrated in the laboratory.

For drug transport studies, cells were grown on 25 mm Nunc tissue culture inserts (Gibco BRL, Oakville, ON). The tissue culture inserts were placed in 6-well Falcon tissue culture plates (#3046).

II.4a3 Radiolabelled compounds

[³H(G)]-Digoxin (16.0 Ci/mmol) or [12α-³H(N)]-digoxin (16.1 Ci/mmol) and [¹⁴C]-mannitol (55.1 mCi/mmol) were purchased from Du Pont Canada Incorporated (Markam, ON). [³H(G)]-Vinblastine (11.2 Ci/mmol) was purchased from Amersham Canada Limited (Oakville, ON).
II.4a4 Non-radiolabelled compounds

Racemic propafenone, vinblastine, vincristine, ketoconazole, digoxin, dexamethasone, troleandomycin, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). NDPP and the enantiomers of propafenone and 5-OHP were generously donated by Knoll Pharmaceuticals Ltd. (Markam, ON). R- and S-verapamil were a gift from Dr. A. Rasymas (Faculty of Pharmacy, Ohio State University, Ohio). Norverapamil, PR-22, and D-620 were obtained from Irving Wainer (Department of Oncology, Jewish General Hospital, Montreal, Quebec) as a result of the kind donations of Searle Pharmaceuticals (Northumberland, England). Mifepristone was kindly donated by Roussel-Uclaf (Romainville, France). Bio-Rad protein reagent was purchased from Bio-Rad Laboratories (Richmond, CA).

II.4a5 HPLC reagents

Methanol, sulfuric acid, triethylamine, and boric acid were bought from Fisher Scientific (Nepean, Ontario). Acetonitrile was purchased from Caledon Laboratories Ltd. (Georgetown, Ontario). All reagents were of HPLC grade.
**II.4b Methods**

**II.4b1 Cell culture**

Renal tubular cells were grown in tissue culture flasks containing α-MEM + 10% fetal bovine serum (α-MEM/FBS) and were incubated at 37°C in an atmosphere of 5% CO2/95% air. These cells were seeded onto 25 mm Nunc tissue culture inserts at a superconfluent density of about 5 x 10^5 cells/cm^2. The inserts were placed in 6-well tissue culture plates and the confluent cell monolayers were bathed on each side with 2 ml of α-MEM + 10% fetal bovine serum (α-MEM/FBS) and incubated at 37°C in an atmosphere of 5% CO2/95% air. Media were changed every 2-3 days. Experiments were conducted 10 days after seeding.

**II.4b2 Transport experiments with propafenone**

MDCK cells between passages 61 and 142 were grown on tissue culture inserts as described in general methods.

**II.4b2a Short-term digoxin/vinblastine flux experiments**

While long-term cell culture experiments were conducted using α-MEM/FBS to ensure cell viability, in short-term experiments, cells were incubated with a less expensive mixture of PBS-G containing albumin to standardize and mimic physiological conditions. No differences were found in short-term digoxin or vinblastine fluxes between cells incubated with α-MEM/FBS or PBS-G (data not shown).

Cells in the filter preparation were gently rinsed with PBS containing 5 mM glucose and 0.02% albumin (PBS-G). These cell monolayers were preincubated at 37°C for 30 minutes with 1 ml of PBS-G in the apical compartment and 1.5 ml of PBS-G in the basal compartment.

The tissue culture inserts containing 1 ml of PBS-G in the apical compartment were placed into 6-well tissue culture plates containing 1.2 ml PBS-G solutions of 2.9 μM
[14C]-mannitol and either 0.1 µM [3H]-digoxin or 0.025 µM [3H]-vinblastine to which were added various concentrations of one of the following compounds: R-propafenone, S-propafenone, racemic propafenone, R-5-OHP, S-5-OHP, racemic 5-OHP, or racemic NDPP. Appropriate volumes of solvent were added to the wells to correct for volume differences.

The apical media were sampled (25 µl) at 10, 20, and 30 minutes. The basal media were also sampled (25 µl) at 30 minutes. The radioactivity of the samples was determined using a scintillation counter (Beckman LS5000CE).

II.4b2b Cellular uptake of digoxin and vinblastine

The cellular accumulation of radiolabelled digoxin and vinblastine in the presence and absence of either propafenone, 5-OHP, or NDPP were examined following 40 minutes of incubation. The culture inserts were rinsed on both sides with ice cold PBS-G (1 ml apical, 1.5 ml basal). The filters were cut out of the tissue culture inserts using a cutting device provided with the inserts. The membranes were placed in tissue culture plates containing 0.6 ml of 0.1% Triton X-100. Following solubilization of the cells, 0.3 ml of cell solution were sampled for determination of radioactivity. Disintegration per minute (dpm) counts were corrected for contamination by the incubation media. On average, the contamination accounted for less than 10% of the uptake.

II.4b2c Long-term digoxin flux experiments

Cell preparations on tissue culture inserts were placed in a 6-well tissue culture plate containing 2 ml of α-MEM/FBS with 0.01 µM [3H]-digoxin ± 5 µM propafenone or 0.1 µM [3H]-digoxin ± 20 µM propafenone in each well. The same media (2 ml) were slowly added to the apical compartments. The cells were incubated at 37°C under an atmosphere of 5% CO2/95% air. Apical and basal media were sampled (25 µl) at various time periods of incubation over a 24-hour period.
II.4b2d Transport of propafenone and 5-OHP

Alpha-MEM/FBS (1.5 ml) containing either 5 μM propafenone, 5 μM 5-OHP, or solvent alone were added to either side or both sides of the cell monolayer. Cells were incubated at 37°C and 5% CO₂/95% air. After a period of either 30 minutes or 48 hours, 1 ml of apical and 1 ml of basolateral solution were sampled. These samples were analyzed by the HPLC method described below. The integrity of the cell monolayers was determined following the 48-hour period of incubation by measuring the basolateral-to-apical flux of 5 μM [14C]-mannitol using the procedures described for short-term experiments.

II.4b2e Sample analysis

Propafenone and its metabolites were quantitated by high performance liquid chromatography (HPLC) in the Therapeutic Drug Monitoring lab at the Hospital for Sick Children as described by Verjee and Giesbrecht (1992). Samples (100 μl) were extracted at alkaline pH with ethyl acetate and the extract applied to a C18 Bond Elut Cartridge (1 ml size); interfering polar compounds were washed off with methanol. The drug and metabolites were eluted with 95% methanol/5% 0.1 N HCl.

After drying under nitrogen and reconstitution with 100 μl 0.1 N HCl, 75 μl were injected into a Whatman Partisil 5 ODS RAC HPLC column with in-line filter. The column was maintained at room temperature, eluted with a mobile phase of 42% acetonitrile in 10 mM phosphate buffer, pH 2.5. Detection was made at 214 nm, with a UV spectrophotometer. At a flow rate of 2.3 ml/minute chromatography was complete in 10-11 minutes.

II.4b3 Propafenone biotransformation experiments

MDCK cells between passages 87 and 106 were grown on tissue culture inserts as described in general methods.
On the day of experimentation, the tissue culture inserts were transferred to clean 6-well tissue culture plates and bathed on each side with 2 ml of α-MEM/FBS and various concentrations of propafenone with or without dexamethasone, troleandomycin, or α-naphthoflavone as outlined below. All incubations were carried out at 37°C in an atmosphere of 5% CO2/95% air. Following various periods of incubation, media were removed from each side of the cell monolayers. Samples were stored at -20°C until analysis.

II.4b3a  Time course of NDPP production

Cell monolayers were incubated with equal concentrations of propafenone (5 μM) in the apical and basal compartments. Apical and basal solutions were sampled at 1, 2, 3, 4, 5, 6, 8, 12, 24, 48, and 72 hours of incubation.

II.4b3b  Concentration-dependent propafenone biotransformation

Cell monolayers were incubated for 48 hours with apical solutions of α-MEM/FBS alone and basal solutions containing 1 μM, 2 μM, 5 μM, 10 μM, 20 μM or 40 μM propafenone. Apical and basal media were collected at 48-hours for analysis. This experiment was repeated four times.

II.4b3c  Incubation with dexamethasone or troleandomycin

Cell monolayers were incubated on the apical and basal sides for 72 hours with α-MEM/FBS alone or containing one of the following: 1 μM dexamethasone, 5 μM dexamethasone, or 50 μM troleandomycin. Media were aspirated off of the monolayers and the inserts were transferred to clean 6-well culture plates. Six filters that were previously exposed to α-MEM/FBS alone were incubated with either α-MEM/FBS + 5 μM propafenone (3 filters) or α-MEM/FBS + 5 μM 5-OHP (3 filters). Six filters previously exposed to dexamethasone (3 filters at 1 μM, 3 filters at 5 μM) were incubated with their previous solutions + 5 μM propafenone. Three filters previously
exposed to troleandomycin were incubated with troleandomycin + 5 μM propafenone. Apical and basal media were sampled at 24 hours of incubation.

**II.4b3d Incubation with α-naphthoflavone**

Cell monolayers were incubated with apical and basal solutions containing 5 μM propafenone with (three filters) or without (three filters) 50 μM α-naphthoflavone for 48 hours.

To ensure that metabolic but not transport mechanisms were affected by α-naphthoflavone, the biotransformation of propafenone was studied in microsomes prepared from MDCK cells.

**II.4b3e Preparation of microsomes from MDCK cells**

MDCK cells were scraped from 78 T-75 flasks and resuspended in 1.15% KCl solution. The cells were centrifuged at 300 x g for 15 minutes. The pellets were then resuspended in 1.15% KCl and homogenized with five strokes of a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 600 x g for 10 minutes. The supernatant was removed into a clean tube and spun at 9000 x g for 20 minutes. The supernatant was removed into a clean tube and centrifuged for 65 minutes at 105 000 x g. The pellets were resuspended in 1.15% KCl and recentrifuged at 105 000 x g for 60 minutes. The final pellet was resuspended in 1 ml of 1.15% KCl to give a total volume of 1.5 ml and frozen at -20°C.

**II.4b3f Incubation conditions**

On the day of experimentation, MDCK microsomes were thawed and added to 1 ml of 100 mM sodium phosphate/154 mM KCl/2 mM EDTA buffer. The microsomal mixture was then rehomogenized for protein determination. To each of two 1 ml Eppendorf tubes 70 μl of microsomal protein (2.4 mg/ml), 1.5 mg of NADPH, and 20 μl of 1 mM propafenone solution (to give a final concentration of 200 μM) were added. To
One tube, 10 μl of a solution containing α-naphthoflavone were added; while 10 μl of ethanol (the solvent for the inhibitor) were added to the other tube. Both tubes were vortexed and incubated at 37°C for three hours in a shaking bench-top incubator. Following incubation, 10 μl of 30% perchloric acid were added to each tube. The samples were filtered using Amicon filters and centrifuged at 600 x g for 30 minutes. The filtrates were transferred to clean Eppendorf tubes and spun at 10 000 x g for 5 minutes. The supernatants were placed in 10 ml glass extraction tubes.

II.4b3g Sample analysis

Propafenone and its metabolites were analyzed by HPLC in our laboratory. Samples (0.5 ml for cell monolayer experiments and 84 μl for microsomal experiments), internal standard [50 μl of 2 μg/ml of 2'- (2-hydroxy-3-ethylamino-propoxy)-3-phenylpropiophenone hydrochloride], and 0.3 ml of 100 mM borate buffer (pH 9.0) were vortexed in 10 ml glass extraction tubes and left to stand for 5 minutes. Diethyl ether (5 ml) was added to each tube and the tubes were mixed for 10 minutes and then centrifuged at 300 x g for 10 minutes. The organic layer was transferred to clean culture tubes and evaporated under nitrogen to approximately 1 ml. Sulphuric acid (0.2 ml of 0.05 N) was added, the samples were vortexed and left to stand for 10 minutes. Samples were centrifuged for another 10 minutes at 300 x g. The aqueous layer was injected into an HPLC. Standards containing propafenone, 5-OHP, NDPP and internal standard were extracted by the same method. For the experiments with cell monolayers, standards were prepared in α-MEM. For the microsomal experiments, standards were extracted from microsomal incubation media containing appropriate concentrations of albumin to represent protein.

The analytical column was purchased from Beckman Instruments (Ultrasphere 5 μm ODS 25 cm x 4.5 mm). The UV detector was set for a wavelength of 210 nm and 0.01 AUFS. The mobile phase consisted of 68% 0.02 M triethylamine in 0.03 N sulphuric
acid, 28.45% acetonitrile, and 3.55% methanol. All reagents were of HPLC grade. The flow rate was set to either 1.0 or 1.5 ml/min.

II.4b4 Transport experiments with verapamil

MDCK cells were used between passages 49 and 78.

II.4b4a Digoxin and vinblastine fluxes

Cells in the filter preparation were gently rinsed with PBS-G. These cell monolayers were preincubated at 37°C for 30 minutes with 1 ml of PBS-G in the apical compartment and 1.5 ml of PBS-G in the basal compartment.

The tissue culture inserts containing 1 ml of PBS-G in the apical compartment were placed into 6-well tissue culture plates containing 1.2 ml PBS-G solutions of 2.9 μM [14C]-mannitol and either 0.1 μM [3H]-digoxin or 0.025 μM [3H]-vinblastine to which were added various concentrations of one of the following compounds: verapamil, norverapamil, R-norverapamil, S-norverapamil, PR-22, and D-620. Appropriate volumes of solvent were added to the wells to correct for volume differences.

The apical media were sampled (25 μl) at 10, 20, and 30 minutes. The basal media were also sampled (25 μl) at 30 minutes. The radioactivity of the samples was determined using a scintillation counter.

II.4b4b Cellular uptake of digoxin and vinblastine

The cellular accumulations of radiolabelled digoxin and vinblastine in the presence and absence of verapamil or verapamil metabolites were examined following 40 minutes of incubation. The culture inserts were rinsed on both sides with ice cold PBS-G (1 ml apical, 1.5 ml basal). The filters were cut out of the tissue culture inserts and the membranes were placed in tissue culture plates containing 0.6 ml of 0.1% Triton X-100. Following solubilization of the cells, 0.3 ml of cell solution were sampled for determination of radioactivity. Disintegration per minute (dpm) counts were corrected
for contamination by the incubation media. On average, the contamination accounted for less than 1% of the uptake.

**II.4b4c Verapamil biotransformation**

On the day of experimentation, the tissue culture inserts were transferred to clean 6-well tissue culture plates and bathed on each side with 2 ml of α-MEM/FBS and verapamil. The cells were incubated at 37°C in an atmosphere of 5% CO₂/95% air. Following various periods of incubation, media were removed from each side of the cell monolayers. Samples were stored at -20°C and then shipped on ice to the Montreal General Hospital for HPLC analysis. Samples were assayed for the presence of verapamil, norverapamil, PR-22, PR-25, D-617, and D-620. Our main interest was in the production of norverapamil, PR-22, and D-620.

**II.4b5 Transport experiments with mifepristone**

MDCK cells were used between passages 61 and 77.

**II.4b5a Digoxin and vinblastine transport**

On the day of the experiments, cells in the filter preparation were gently rinsed with PBS-G and were preincubated at 37°C for 30 minutes with PBS-G. In all experiments, tissue culture inserts were transferred to 6-well tissue culture plates and incubated on the basolateral side with PBS-G solutions of 2.9 μM [14C]-mannitol and either 0.1 μM [3H]-digoxin or 0.025 μM [3H]-vinblastine with or without mifepristone and on the apical side with PBS-G solutions with or without mifepristone. Apical media were sampled at various periods of incubation. The radioactivity of the samples was determined.

In time course experiments, 50 μM mifepristone was added to either the apical or basal compartment. In concentration-dependency experiments, 0-50 μM mifepristone was added to the basal compartment only. In digoxin saturation experiments, 0-100 μM
unlabelled digoxin and 0, 2, 5, or 10 µM mifepristone were added to the basal compartment only.

**II.4b5b Cellular uptake of vinblastine**

The cellular accumulation of radiolabelled vinblastine in the presence and absence of mifepristone was examined following three hours of incubation with cell monolayers from vinblastine time course experiments. The culture inserts were rinsed on both sides with ice cold PBS-G. The filters were cut out of the tissue culture inserts and the membranes were placed in tissue culture plates containing 0.6 ml of 0.1% Triton X-100. Following solubilization of the cells, 0.3 ml of cell solution were sampled for determination of radioactivity. Disintegration per minute (dpm) counts were corrected for contamination by the incubation media. On average, the contamination accounted for less than 1% of the uptake.

Time course experiments were conducted with four cell monolayers per condition. Concentration-dependency experiments had one cell monolayer per concentration and were repeated three times.

**II.4b6 Data analysis**

For all radiolabelled digoxin and vinblastine flux experiments, an *a priori* decision was made to exclude the results obtained from any filters that had apical \([^{14}C]\)-mannitol (a marker of extracellular flux) concentrations greater than 5% per hour of their respective initial basal concentrations.

Data (in at least triplicates) from different experiments were compared by the two-tailed Student's t-test for unpaired data or by analysis of variance for repeated measures (StatViewTMII for Apple Macintosh). P values less than 0.05 were considered statistically significant. Results are expressed as means ± standard deviations. The nonlinear kinetic fitting program Kcat (BioMetallics Incorporated) was used to determine \(K_m\) and \(V_{max}\) values.
Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as the standard.
II.5 Results

No results had to be excluded on the basis of the a priori definition of inappropriate cell-filter preparations detected by high mannitol fluxes (see Methods: data analysis). That digoxin and vinblastine and undergo net renal tubular secretion by MDCK cells was demonstrated by the net accumulation of these drugs in the apical compartment over a period of time. When digoxin was introduced in similar concentrations at both the apical and basolateral surfaces of the MDCK tubular cell monolayers for 24 hours, the concentration gradient produced was 20:1, respectively, indicating accumulation of digoxin against a concentration gradient as a result of its active transport. The molecular species of digoxin remains unmodified by renal tubular cells at 24 hours of incubation as determined by thin layer chromatography studies suggesting that significant biotransformation of the radiolabelled compounds (digoxin and vinblastine) is highly unlikely during the time course of the following drug transport experiments (Ito et al., 1993a). Figure 9 demonstrates the transport of digoxin against a concentration gradient.

II.5a Transport Experiments with Propafenone

II.5a1 Short-term digoxin flux experiments

Racemic mixtures of propafenone, 5-OHP, and NDPP significantly inhibited the total basolateral-to-apical flux of 0.1 μM [3H]-digoxin across MDCK cell monolayers over a 30-minute time period (figure 10). The hydroxylated metabolite (5-OHP) inhibited digoxin renal tubular secretion to a somewhat lesser extent than the parent compound. NDPP was a much less potent inhibitor as illustrated in figure 10 by the lack of significant inhibition at 20 μM. The time courses of digoxin secretion were nearly linear in the presence and absence of propafenone (figure 11), 5-OHP, and NDPP.

The effects of the enantiomers of propafenone (figure 12) and 5-OHP on 0.1 μM [3H]-digoxin basolateral-to-apical flux over a period of 30 minutes were not
Figure 9. Apical digoxin accumulation against a concentration gradient. Both sides of the cell monolayers were incubated with α-MEM media containing approximately 10 nM radiolabelled digoxin. Apical (squares) and basal (circles) media were sampled over a period of 24 hours. Results are expressed as the means of three cell monolayers seeded at the same cell density ± S.D.
Figure 10. Inhibition of basolateral-to-apical digoxin flux across MDCK cell monolayers by propafenone and its metabolites. Cell monolayers were incubated on the basal side with PBS-G + 0.1 μM radiolabelled digoxin and 20 μM (solid bars) and 40 μM (hatched bars) propafenone, or 5-OHP, or NDPP, and on the apical side solely with PBS-G. Apical solutions were sampled at 30 minutes and expressed as a percentage of control. Results are expressed as the means of three cell monolayers seeded at the same cell density ± S.D. (** P < 0.01)
Figure 11. Linearity of digoxin time courses in the presence and absence of propafenone. Confluent MDCK cell monolayers were incubated on the basal side with PBS-G + 0.1 μM radiolabelled digoxin and 40 μM propafenone, 5-OHP, or NDPP, and on the apical side only with PBS-G. The time courses of inhibition were nearly linear in the absence (squares) and presence of 40 μM propafenone (triangles), 5-OHP, NDPP. For clarity, only data for 40 μM propafenone are shown. Results are expressed as the mean values of three cell monolayers seeded at the same density ± S.D.
Figure 12. Effects of the enantiomers and the racemic mixture of propafenone on basolateral-to-apical digoxin transport across MDCK cell monolayers. Cell monolayers were incubated on the basal side with PBS-G + 0.1 μM radiolabelled digoxin and various concentrations of R-propafenone (squares), S-propafenone (triangles), or racemic propafenone (circles), and on the apical side solely with PBS-G. Apical solutions were sampled at 10, 20, 30 minutes and the 30 minute results were expressed as the average percentage of control (i.e., digoxin flux in the absence of inhibitor). Results are expressed as means of three cell monolayers seeded at the same cell density ± S.D.
significantly different from each other, suggesting that the effects were nonstereospecific (P values were 0.27 and 0.37 for propafenone and 5-OHP, respectively). Because the inhibitory effects of the enantiomers of propafenone and 5-OHP were comparable to those of the racemic mixtures, the racemates were used in all subsequent transport and metabolism experiments.

II.5a2 Short-term vinblastine flux experiments

As illustrated in figure 13, propafenone, 5-OHP, and NDPP also inhibited the basolateral-to-apical flux of 0.025 μM [3H]-vinblastine across confluent MDCK cell monolayers. Concentrations of 40 μM NDPP did not produce statistically significant differences in vinblastine inhibition although significance was reached with 100 μM NDPP (61 ± 6 % of control at 30 minutes, P = 0.02). There were no significant differences in vinblastine basolateral-to-apical flux in the presence of 20 μM propafenone or 5-OHP between the R- and S-enantiomers. The time courses of vinblastine secretion were nearly linear in the presence and absence of propafenone (figure 14), 5-OHP, and NDPP.

II.5a3 Cellular uptake of digoxin and vinblastine

While increasing the concentration of propafenone decreased the net secretory flux of 50 μM [3H]-digoxin across confluent MDCK cell monolayers, the cellular uptake of digoxin did not decrease (figure 15). Similarly, the cellular uptakes of 0.1 μM [3H]-digoxin and 0.025 μM [3H]-vinblastine were not decreased relative to controls in the presence of propafenone, 5-OHP, and NDPP (table 1).

II.5a4 Long-term digoxin flux experiments

Figures 16 and 17 show the inhibition of digoxin secretion (0.01 μM and 0.1 μM) by 5 μM and 20 μM propafenone, respectively, over a 24-hour period. Similar to the short-term time course studies, propafenone inhibited the basolateral-to-apical transport of
Figure 13. Inhibition of basolateral-to-apical vinblastine flux across MDCK cell monolayers by propafenone and its metabolites. Cell monolayers were incubated on the basal side with PBS-G + 0.025 μM radiolabelled vinblastine and 20 μM (solid bars) and 40 μM (hatched bars) propafenone, or 5-OHP, or NDPP, and on the apical side solely with PBS-G. Apical solutions were sampled at 30 minutes and expressed as a percentage of control. Results are expressed as the means of three cell monolayers seeded at the same cell density ± standard deviation. (** P < 0.01)
Figure 14. Linearity of vinblastine time courses in the presence and absence of propafenone. Confluent MDCK cell monolayers were incubated on the basal side with PBS-G + 0.025 μM radiolabelled vinblastine and 40 μM propafenone, 5-OHP, or NDPP, and on the apical side only with PBS-G. The time courses of inhibition were nearly linear in the absence (squares) and presence of 40 μM propafenone (triangles), 5-OHP, NDPP. For clarity, only data for 40 μM propafenone are shown. Results are expressed as the mean values of three cell monolayers seeded at the same density ± S.D.
Figure 15. Cellular uptake and transport of 50 μM digoxin in the presence and absence of propafenone. Cell monolayers were incubated on the basal side with PBS-G + 50 μM radiolabelled digoxin and various concentrations of propafenone, and on the apical side solely with PBS-G. Apical solutions were sampled at 10, 20, and 30 minutes. Data for the 30 minute sampling are shown. Digoxin concentrations in the apical solutions were expressed as an average percentage of control (solid circles). Cellular accumulation was measured at 40 minutes (bar graph). Results are expressed as the means of three cell monolayers seeded at the same cell density ± S.D.
Table 1. Cellular uptake at 40 minutes of incubation of 0.1 μM digoxin and 0.025 μM vinblastine in the absence and presence of inhibitors. Results represent the mean cellular uptakes of three cell monolayers (seeded at the same cell density) expressed as percentages of control ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Propafenone (40 μM)</th>
<th>5-OHP (40 μM)</th>
<th>NDPP (100 μM)</th>
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<tbody>
<tr>
<td>0.1 μM [³H]-digoxin</td>
<td>133.4 ± 27.1</td>
<td>116.3 ± 10.7</td>
<td>127.7 ± 6.3</td>
</tr>
<tr>
<td>25 nM [³H]-vinblastine</td>
<td>112.6 ± 11.1</td>
<td>100.5 ± 7.3</td>
<td>123.6 ± 26.9</td>
</tr>
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</table>
Figure 16. Long-term inhibition of digoxin transport by 5 μM propafenone. Both sides of the cell monolayers were incubated with α-MEM media containing approximately 10 nM radiolabelled digoxin with (open symbols) or without (solid symbols) 5 μM propafenone. Apical (squares) and basal (circles) media were sampled over a period of 24 hours. Note that digoxin is concentrated in the apical compartment against a concentration gradient, and that propafenone inhibits this accumulation. Results are expressed as the means of three cell monolayers seeded at the same cell density ± S.D.
Figure 17. 24-hour time course of digoxin concentration changes across MDCK cell monolayers. Both sides of the cell monolayers were incubated with α-MEM media containing approximately 0.1 μM radiolabelled digoxin with (open symbols) or without (solid symbols) 20 μM propafenone. Apical (squares) and basal (circles) media were sampled over a period of 24 hours. Note that digoxin is concentrated in the apical compartment against a concentration gradient, and that propafenone inhibits this accumulation. Results are expressed as the means of three cell monolayers seeded at the same cell density ± S.D.
digoxin against a concentration gradient. The cells that were previously exposed to 5 μM propafenone (and then rinsed for one hour) were able to transport 10 nM digoxin to the same extent as those cells never exposed to propafenone, indicating that the inhibition of digoxin secretion is reversible, and that the cells were still functional after the exposure (figure 18).

II.5b Propafenone Biotransformation Experiments

To identify whether or not the inhibition of digoxin basolateral-to-apical transport by propafenone was due to propafenone itself, or rather, due to its metabolite(s) produced by the cells, the ability of MDCK cells to metabolize propafenone was examined. Following a 30-minute exposure to propafenone, 5-OHP and NDPP were undetectable. On the other hand, following a 48-hour incubation, NDPP (but not 5-OHP) was detected in approximately equal quantities from media bathing the apical and basolateral sides of cells as well as propafenone itself (figure 19). When cells were incubated with 5-OHP initially placed on both sides of the monolayer, the apical concentrations of 5-OHP were almost double that of the basal compartment (figure 20). The integrity of the cell monolayers following a 48-hour exposure to these propafenone compounds was confirmed by the 5 μM [14C]-mannitol basolateral-to-apical flux experiments.

Upon finding that MDCK cells could biotransform propafenone to NDPP, I decided to further characterize the production of NDPP. In the time course of NDPP production study, NDPP was detected by 2 hours of incubation as illustrated in figure 21. Figure 22 shows the results of a typical experiment examining the effects of incubating MDCK cell monolayers with various concentrations of propafenone for 48 hours. The average $K_m$ and $V_{max}$, respectively, for four sets of data were 26.8 ± 4.8 μM and 140.8 ± 0.03 mmol/hour/filter (means ± S.D.).

In an attempt to induce CYP3A in the MDCK cells, MDCK cells were exposed to 1 μM or 5 μM dexamethasone in α-MEM/FBS for 72 hours. NDPP formation was
Figure 18. Propafenone does not have irreversible effects on digoxin transport. Both sides of the cell monolayers were incubated with α-MEM media containing approximately 10 nM radiolabelled digoxin with (open symbols) or without (solid symbols) 5 μM propafenone. Apical and basal media were sampled over a period of 24 hours. Following the 24-hour sampling, all cell monolayers were placed in α-MEM media and rinsed for one hour. All cell monolayers were then placed in the original α-MEM media solution containing approximately 10 nM radiolabelled digoxin. The cells that were previously exposed to propafenone were able to transport digoxin similarly to those cells that were never exposed to propafenone. Results are expressed as the means of three cell monolayers seeded at the same cell density ± S.D.
Figure 19. Biotransformation of propafenone by MDCK cells. Cell monolayers were incubated with α-MEM media containing 5 µM propafenone in the apical and basal compartments. Apical (solid) and basal (hatched) media were sampled at 48 hours. NDPP was produced by the cells incubated with propafenone. Results are expressed as the means of three cell monolayers seeded at the same cell density ± S.D. (n.d. = not detected)
Figure 20. Transport of 5-OHP by MDCK cells. Cell monolayers were incubated with α-MEM media containing 5 μM 5-OHP placed in the apical and basal compartments. Apical (solid) and basal (hatched) media were sampled at 48 hours. Results are expressed as the means of three cell monolayers seeded at the same cell density ± S.D. (n.d. = not detected)
Figure 21. Time course of NDPP production. Confluent MDCK cell monolayers were incubated on both sides with α-MEM/FBS + 5 μM propafenone. Apical and basal media were sampled at various periods of incubation. Results are expressed as the sum of the apical and basal samplings.
Figure 22. Concentration-dependence of NDPP production. Confluent MDCK cell monolayers were incubated on the apical side with α-MEM/FBS and on the basal side with α-MEM/FBS + various concentrations of propafenone (1-40 μM). Apical and basal media were sampled at 48 hours of incubation. Results from a typical experiment are expressed as the sum of apical and basal concentrations.
significantly reduced relative to controls in the cells exposed to 1 μM or 5 μM dexamethasone (P < 0.0001 for both). However, it should be noted that whole cells were used to study the biotransformation of propafenone and that dexamethasone may have interfered with the ability of propafenone to enter the cells. As well, dexamethasone is a substrate for P-glycoprotein (Ueda et al., 1992) and may have been effluxed from the cell (see discussion).

When cells were exposed to propafenone in the presence of the selective CYP3A inhibitor troleandomycin, no significant decrease in NDPP formation occurred (figure 23, P = 0.18). On the other hand, the CYP1A2 selective inhibitor, α-naphthoflavone (50 μM), significantly inhibited the production of NDPP as seen in figure 24 (P < 0.0001). To ensure that the inhibition of NDPP production by α-naphthoflavone in MDCK cells was not caused by an inhibition of propafenone entering the cells, the effects of α-naphthoflavone on propafenone biotransformation were studied in microsomes prepared from MDCK cells. In the microsomal system, α-naphthoflavone appeared to inhibit the formation of NDPP (n = 1); however, this experiment should be repeated to confirm these findings.

II.5c Transport Experiments with Verapamil

In previous experiments, we had demonstrated the ability of verapamil to inhibit the renal tubular secretion of digoxin across confluent cell monolayers of LLC-PK1 cells while increasing the cellular accumulation of digoxin (Ito et al., 1993c*). In the present studies with MDCK cells, 10 μM norverapamil inhibited the renal tubular secretion of 0.1 μM digoxin similarly to verapamil (41.4 ± 2.0% and 46.7 ± 3.6% of control at 30 minutes for norverapamil and verapamil, respectively, figure 25). There were no statistically significant differences in the inhibition of digoxin secretory flux between 10 μM R-norverapamil, S-norverapamil, and RS-norverapamil.

Despite inhibitory effects of verapamil and norverapamil on secretory flux, the cellular accumulation of digoxin was not decreased. In fact, cellular digoxin uptakes at
Figure 23. The effects of dexamethasone and troleandomycin on NDPP production. Confluent MDCK cell monolayers (n = 3) were incubated on both sides with α-MEM/FBS + 5 μM propafenone + dexamethasone or troleandomycin following preincubation without propafenone in the same drug solutions. Apical and basal media were sampled at 24 hours of incubation. Results are expressed as the sum of apical and basal concentrations ± S.D. (**P < 0.0001)
Figure 24. The effects of α-naphthoflavone on NDPP production. Confluent MDCK cell monolayers were incubated on both sides with α-MEM/FBS + 5 μM propafenone with (n = 3) or without (n = 3) 50 μM α-naphthoflavone for 48 hours. Results are expressed as the sum of apical and basal concentrations ± S.D. (*P < 0.0001)
Figure 25. Time course of 0.1 μM digoxin secretory flux in the presence of verapamil and norverapamil. Confluent MDCK cell monolayers were incubated with basal PBS-G solutions containing 0.1 μM radiolabelled digoxin (squares) and either 10 μM norverapamil (triangles) or 10 μM verapamil (circles). PBS-G alone was initially placed in the apical compartment. The apical solutions were sampled at 10, 20, and 30 minutes. Data points represent the means of three filters ± S.D.
40 minutes of incubation were increased relative to controls (118.9 ± 7.4% (P = 0.05) and 137.8 ± 8.9% (P = 0.008) of control for 10 µM verapamil and norverapamil, respectively).

Likewise, 10 µM norverapamil significantly inhibited the renal tubular secretion of 0.025 µM vinblastine similarly to the parent compound, verapamil, at the same concentration (34.5 ± 4.1% versus 39.9 ± 4.4% of control at 30 minutes). This inhibition was nonstereoselective and dose-dependent (figure 26). The IC50 values for R- and S-norverapamil were 3.1 ± 0.3 µM and 3.5 ± 0.3 µM, respectively.

The N-demethyl-O-demethyl metabolite of verapamil called PR-22 (40 µM) inhibited the renal tubular secretion of 0.025 µM vinblastine by 61.8 ± 1.4% at 30 minutes of incubation (figure 27). Concentrations of 10 µM PR-22 inhibited approximately half as much. However, 40 µM D-620, the N-demethyl-N-dealkyl form of verapamil was unable to affect the secretory flux of vinblastine. Similarly, 10 µM PR-22 inhibited the secretory flux of 0.1 µM digoxin by 43.0 ± 3.5%, whereas 10 µM D-620 had no significant effect.

While there was a trend toward higher vinblastine cellular uptake in the presence of PR-22, it did not reach statistical significance (P = 0.09). Cellular digoxin accumulation was increased in the presence of 10 µM PR-22 (P = 0.03), but was not different from the control in the presence of 40 µM D-620.

II.5d Verapamil Biotransformation

Many of the samples from the verapamil biotransformation experiment were lost in Montreal; therefore, only two samples (representing basal media) were analyzed. Microgram quantities of the verapamil metabolites norverapamil, D-617, and D-620 were detected when MDCK cells were incubated with verapamil for 48 hours (injection volumes of 250 µl and 500 µl).
Figure 26. Dose-dependent, nonstereoselective inhibition of 0.025 μM vinblastine secretory flux. Confluent MDCK cell monolayers were incubated with basal PBS-G solutions containing 0.025 μM radiolabelled vinblastine and various concentrations of R-norverapamil (squares) or S-norverapamil (circles). PBS-G alone was initially placed in the apical compartment. The apical solutions were sampled at 10, 20, and 30 minutes (30 minute data shown) and expressed as a percentage of control (i.e., apical vinblastine concentration in the absence of norverapamil). Data points represent the means of three to five filters ± S.D.
Figure 27. Effects of PR-22 and D-620 on vinblastine secretory flux. PR-22 inhibited the basolateral-to-apical secretion of 0.025 μM radiolabelled vinblastine across MDCK cell monolayers, whereas D-620 did not. Results are expressed as the means of three filters ± S.D.
II.5e Transport Experiments with Mifepristone

Mifepristone significantly inhibited the secretory flux of digoxin (figure 28). The degree of inhibition was not statistically different when mifepristone was added to the basal compartment versus the apical compartment. Furthermore, the cellular uptake of digoxin was not decreased in the presence of mifepristone, suggesting that mifepristone did not prevent digoxin from entering the renal tubular cells.

Mifepristone also significantly inhibited the basolateral-to-apical (secretory) flux of vinblastine as shown in figure 29. Vinblastine flux was inhibited similarly when mifepristone was added to either the apical or basal compartments. Figure 30 illustrates that the inhibition of vinblastine basolateral-to-apical flux by mifepristone was dose-dependent. The cellular uptake of vinblastine was significantly higher in the presence of mifepristone added to the basal compartment only \((p < 0.0001)\) or the apical compartment only \((p < 0.0002)\) as shown in figure 31. Cellular uptakes of vinblastine were not statistically different between basal and apical applications.
Figure 28. Time course of mifepristone inhibition of digoxin secretion. Confluent MDCK cell monolayers were incubated with PBS-G containing 0.1 μM radiolabelled digoxin placed in the basolateral compartment and PBS-G alone in the apical compartment. Mifepristone (50 μM) was added to the apical (circles) or basal (triangles) or neither (squares) compartment. The basolateral-to-apical flux of digoxin was determined by sampling the apical media over a period of 3 hours. Results are expressed as the means of four filters ± S.D.
Figure 29. Time course of mifepristone inhibition of vinblastine secretion. Confluent MDCK cell monolayers were incubated with PBS-G containing 0.025 μM radiolabelled vinblastine placed in the basal compartment and PBS-G alone in the apical compartment. Mifepristone (50 μM) was added to the apical (circles) or basal (triangles) or neither (squares) compartment. The basolateral-to-apical flux of vinblastine was determined by sampling the apical media over a period of 3 hours. Results are expressed as the means of four filters ± S.D.
Figure 30. Dose-dependent inhibition of vinblastine secretory flux by mifepristone. Confluent MDCK cell monolayers were incubated with 0.025 μM radiolabelled vinblastine and various concentrations of mifepristone placed in the basal compartment only. Apical PBS-G solution was sampled at 30 minutes. Results are expressed as a percentage of the control condition (i.e., no mifepristone added) and represent the mean of three experiments ± S.D.
Figure 31. Cellular uptake of vinblastine in the presence of mifepristone. The cellular uptake of 0.025 μM radiolabelled vinblastine in the presence and absence of 50 μM mifepristone (added to either the apical or basal compartment) was determined following a 3-hour incubation period. Results are expressed as the means of four filters ± S.D.
Saturation curves of basolateral-to-apical digoxin fluxes in the presence of 0, 2, 5, and 10μM mifepristone did not permit a simple determination of the competitive/uncompetitive nature of inhibition. In the presence of mifepristone, digoxin appeared to be toxic to the cells before reaching saturation.
II.6 Discussion

P-glycoprotein is expressed in a variety of tissues throughout the body and appears to play an important role in protecting organisms from toxic drugs and chemicals (Ito et al., 1992a). P-glycoprotein expression is particularly high in excretory tissues such as the kidney (Fojo et al., 1987). At the onset of this research, digoxin was presumed to be eliminated mainly by the kidney by a process that involves P-glycoprotein based on studies using P-glycoprotein substrates and inhibitors, and cells that overexpress genes coding for P-glycoprotein (Tanigawara et al., 1992; de Lannoy et al., 1992b; Ito et al., 1993b). Since digoxin-drug interactions can be life-threatening, we decided to use digoxin as an example drug in our studies of the role of P-glycoprotein in renal tubular drug interactions.

An *in vitro* cell culture system was chosen to examine the transport of drugs across renal tubular cells. An important advantage of using a cell culture system versus other preparations such as membrane slices and membrane vesicles is that it allows the whole cell to be studied. The polarized orientation of epithelial cells in culture, as *in vivo*, facilitates the study of drug transport across renal tubular cells. The unique use of tissue culture inserts in the *in vitro* system that we utilized provides a further advantage of allowing independent access to both the apical and basolateral sides of the cell monolayer.

MDCK cells, which have properties of distal tubular cells, were chosen for the renal drug transport studies because they express P-glycoprotein (Horio et al., 1989; 1990). Consistent with other cell types, the localization of P-glycoprotein within these cells is primarily on the apical membranes (Thiebaut et al., 1987). The expression of P-glycoprotein in the cells used for the drug transport studies described in this thesis was verified by Western blotting techniques using monoclonal (C219) and polyclonal (4077) antibodies to P-glycoprotein (Ito et al., 1999; Appendix B) and by functional tests of calcein exclusion (Ito et al., 1999, see below).
MDCK cells have tight junctions that result in relatively high transport-to-diffusion ratios of the P-glycoprotein substrates digoxin and vinblastine across the MDCK cell monolayers. The cell-cell junctions in MDCK cells are much tighter than those found in the proximal tubular cell line LLC-PK1 with which I have also examined drug transport. Hence, the paracellular flux of digoxin and vinblastine is lower in MDCK cells than in LLC-PK1 cells. Moreover, based on functional assays and Western blotting techniques, P-glycoprotein expression is higher in MDCK cells than in LLC-PK1 cells (Ito et al., 1999*; Appendix B). Taken together, these characteristics justify the use of MDCK cells to test our hypotheses.

The pace of biological research has been greatly enhanced by recent advances in molecular biology. The ability to identify and clone genes has enabled rapid progress in the characterization of drug transporters and drug metabolizing enzymes (especially the CYPs). For instance, the transfection of genes into cells to allow overexpression of CYP enzymes and drug transporters such as MDR1 has facilitated the identification of substrates for these proteins. The availability of antibodies to proteins encoded by multidrug resistance genes and to CYP enzymes has also increased the rate at which the localization and function of these proteins are characterized.

The creation of mice lacking the mdr1a/1b genes has enabled the study of drug transport in the absence of P-glycoprotein. During the course of my research, Schinkel et al. reported that tissue levels of digoxin are as much as 35-fold higher in the brains of mice lacking the mdr1a gene compared with those mice that express P-glycoprotein (Schinkel et al., 1995). Plasma and kidney concentrations of digoxin were approximately doubled. As mentioned in chapter one, while these findings were consistent with the theory of P-glycoprotein-mediated digoxin transport, since mice have two mdr genes, the mdr1b gene could potentially compensate for the lack of mdr1a, especially since mdr1b P-glycoprotein is upregulated in the liver and kidneys of mdr1a (-/-) mice (Schinkel et al., 1994). However, the increase in digoxin concentrations was found to be similar between mice lacking mdr1a and the newly bred mice lacking both mdr1a and
The toxicological effects of this increase are different in mice than in humans because the mouse form of Na⁺-K⁺-ATPase (the pharmacological target of digoxin) is more resistant to digoxin than the human form (Schinkel et al., 1995). It should also be remembered that mice handle digoxin differently than humans with fecal excretion predominating in mice (Mayer et al., 1996).

P-glycoprotein is notable for its broad substrate specificity. Interestingly, digoxin is now one of the most commonly used substrates for studying P-glycoprotein. Of course, other transporters may also transport digoxin. In fact, studies of hepatobiliary digoxin excretion in mice with both the mdr1a and mdr1b genes knocked out indicate the involvement of another transporter or transporters. For most of my experiments with digoxin, I repeated the experiments using vinblastine, a prototype substrate for P-glycoprotein.

Vinblastine has also been identified as a modulator of MRP (Zaman et al., 1994). To determine whether MRP was a factor in our renal tubular cell experiments with digoxin and vinblastine, our MDCK cells were examined for expression of MRP. Immunoblots of our MDCK cells contained significant amounts of P-glycoprotein (0.22 μg/mg total protein), but not MRP (Ito et al., 1999*). However, the cross-reactivity of the rat MRPr1 antibody that is used to detect MRP with dog MRP is unknown. Therefore, to ensure the lack of MRP in our samples, MRP functional tests of calcein accumulation were conducted (Ito et al., 1999*). The hydrophobic compound calcein acetoxyethyl ester (calcein-AM, which is nonfluorescent) is cleaved to the fluorescent calcein dye by cytoplasmic esterase upon entrance to cells. The principle behind the use of calcein accumulation tests for MRP and P-glycoprotein expression is that only calcein-AM is transported by P-glycoprotein, whereas MRP transports calcein-AM, calcein, and a calcein glutathione conjugate (Holló et al., 1996). The functions of P-glycoprotein and MRP can be distinguished in the presence of inhibitors of each protein (Holló et al., 1996). The calcein accumulation assays conducted in the presence of the MRP inhibitor prostaglandin A₁ and the nonspecific inhibitor of both MRP and
glycoprotein, verapamil, confirmed the relatively high expression of P-glycoprotein and insignificant expression of MRP (Ito et al., 1999*).

In an effort to derive a kinetic model of the renal tubular secretion of digoxin, we analyzed our digoxin and vinblastine transport data using a nonlinear curve fitting program. The data were fit to three different hypothetical models of digoxin transport incorporating active drug transport in the basolateral-to-apical direction and in the apical-to-basolateral direction (Ito et al., 1999*). The best fit for the data was determined by the lowest values for Akaike's information criterion and Schwartz criterion that examine the number of parameters estimated and the goodness of fit. Akaike's information criterion is defined by \((n)\ln(S) + 2r\) where \(n\) equals the number of data points, \(S\) represents the sum of squares, and \(r\) denotes the number of parameters that were estimated. The Schwartz criterion is \((n)\ln(S) + (r)\ln(n)\).

MRP localizes in the basolateral membrane transporting substrates into the blood (Evers et al., 1996). Our data did not fit the model that incorporated an active drug efflux pump in the basolateral membrane very well. Rather, the data showed the best fit with a model incorporating diffusion across both the apical and basolateral membranes, paracellular diffusion, and active digoxin transport by a single itraconazole-inhibited drug transporter located in the apical membrane pumping drug into the apical compartment. This is consistent with our notion that MRP does not contribute to digoxin transport by MDCK cells.

Based on the inhibitory compounds used in this study (e.g., verapamil and propafenone are organic bases), if another mechanism is involved, the most reasonable candidate would be the classical organic cation transport system. This involvement seems unlikely since tetraethylammonium (TEA), a prototype organic cation, lacks effects on digoxin transport in LLC-PK1 renal tubular cells which express an active transport mechanism for TEA (Ito et al., 1993a; Inui et al., 1985). In addition, I was unable to inhibit the secretory flux of vinblastine by MDCK cells with two prototype organic cations, TEA and N-methyl nicotinamide (NMN) (data not shown). In fact, I
could not detect carrier-mediated TEA or NMN transport in MDCK cells (data not shown). Horio et al. (1990) also failed to show an effect of TEA on vinblastine transport by this cell line. Taken together, these findings suggest that it is unlikely that observation of the transport of P-glycoprotein substrates is complicated by organic cation transport in MDCK cells.

Recently, digoxin was identified as a substrate of the oatp2 transporter (Noé et al., 1997). This does not refute previous findings of digoxin transport by P-glycoprotein; however, the contribution of each transporter and possible others to the overall handling of digoxin needs to be addressed. Oatp2 expression has been demonstrated in murine kidney; however, the expression of oatp2 in MDCK cells has not been examined.

Inhibition of the unidirectional drug efflux pump P-glycoprotein is often implied when fluxes of substrates across cell monolayers expressing the protein are decreased in the basal-to-apical direction, and increased in the apical-to-basal direction (Horio et al., 1989; Tanigawara et al., 1992). In our studies, we assumed that basolateral-to-apical flux represents the P-glycoprotein-mediated component of digoxin transport. This assumption seems valid because we could also demonstrate in a different experimental condition that the net basolateral-to-apical transport of digoxin against a concentration gradient is inhibited by propafenone (figures 16 and 17). In addition, carrier-mediated transport appears to account for the majority of basolateral-to-apical digoxin/vinblastine flux based on experiments in which unlabelled digoxin/vinblastine significantly inhibited radiolabelled digoxin/vinblastine basolateral-to-apical flux in this experimental system (data not shown). Furthermore, our modelling analysis indicated that in MDCK cells, approximately 90% of the basolateral-to-apical digoxin flux for the first several hours reflects a functionally-single apically-located drug efflux pump (Ito et al., 1997*). Thus, based on published literature and our own data, P-glycoprotein appears to be a major apical efflux mechanism for digoxin and vinblastine in MDCK cells, although the roles of other transporters such as oatp2 remain
II.6a The Digoxin-Propafenone Interaction

My results show that propafenone and its two major metabolites, 5-OHP and NDPP, inhibit digoxin and vinblastine transport across MDCK cell monolayers. The inhibitory effects of these compounds are dose-dependent with an order of potency such that propafenone is greater than 5-OHP which is much greater than NDPP. If one attempts to extrapolate these in vitro data to the human scenario, clinically-significant differences in the nature of the digoxin-propafenone interaction are not likely among super-extensive, extensive, and poor metabolizers of propafenone. This is because the 5-OHP inhibits digoxin secretion to almost the same extent as the parent compound. To the best of our knowledge, however, digoxin-propafenone interactions have not been analyzed with respect to metabolizer phenotype.

The inhibitory effects of the enantiomers of propafenone, and 5-OHP on digoxin secretion are nonstereospecific. As a result, one would not expect to see clinically significant differences in the nature of these digoxin interactions based on serum concentrations of the enantiomers. This nonstereospecific nature of interaction is compatible with substrate/inhibitor polyspecificity of P-glycoprotein-mediated drug transport which was shown for drugs such as verapamil (Ito et al., 1993c*) and quinidine (Hedman et al., 1990).

The somewhat increased cellular accumulation of digoxin and vinblastine in the presence of propafenone, 5-OHP, and NDPP demonstrates that these compounds do not prevent digoxin from entering the cells, implying that the digoxin-propafenone interaction does not take place at the basolateral membrane. In fact, the results support the hypothesis that the digoxin-propafenone interaction takes place at P-glycoprotein located in the apical membranes of renal tubular cells.
Although the exact dynamics of the interactions are unclear from this study, since propafenone and NDPP are not accumulated in the apical compartment (figure 19), the two compounds may be inhibitors, but not substrates, of P-glycoprotein. The data also indicate that 5-OHP is accumulated on the apical side against a concentration gradient (figure 20). While this suggests a competitive nature of the digoxin-5-OHP interaction, the identification of 5-OHP as a substrate of the transport system awaits further study.

In the present experiments, digoxin concentrations were nearly ten-fold higher than those observed therapeutically (1-3 nM) due to the constraints induced by the specific activity of the available radiolabelled digoxin. Concentrations of propafenone and 5-OHP used in the experiments (1-40 μM) included relevant therapeutic serum concentrations (propafenone: 1-6 μM, 5-OHP: 0.5-1.5 μM; Siddoway et al., 1987), although protein binding was not taken into account. NDPP concentrations seen in patients receiving therapeutic doses of propafenone are much lower than those used in this study; <1.5 μM in vivo (Kates et al., 1985) versus 20-100 μM in this study in vitro. Taken together, our in vitro data seem valid to infer that propafenone and 5-OHP are responsible for digoxin-propafenone interactions in the kidney in vivo.

In vivo, propafenone is metabolized in the liver by CYP2D6 to 5-OHP (Siddoway et al., 1987). Appreciable amounts of CYP2D6 have not been detected in human kidney (de Waziers et al., 1990). I was unable to detect 5-OHP in my studies of the renal biotransformation of propafenone. This suggests that MDCK cells lack functional CYP2D6. Therefore, hepatically-produced 5-OHP is likely responsible for the majority of the in vivo interactions between digoxin and metabolites of propafenone.

On the other hand, I found that MDCK cells biotransform propafenone to NDPP. The relevance of the NDPP produced within the kidney cells is unknown at present. However, since only very high concentrations of NDPP (>40 μM) were able to significantly inhibit the renal tubular digoxin secretion, NDPP produced intra-renally probably does not play an important role in the digoxin-propafenone interaction in vivo.
Propafenone biotransformation was detected by analyzing the apical and basal media. Apical and basal concentrations of NDPP were approximately equal following a 48-hour incubation with propafenone, suggesting that NDPP is not actively transported by the cells. Likewise, propafenone does not appear to be transported.

Upon finding that MDCK cells have the ability to biotransform propafenone to NDPP, I briefly explored the production of NDPP. Following incubation of MDCK cells with propafenone, I was able to detect time- and concentration-dependent production of NDPP. Botsch et al. (1993) characterized the enzymes responsible for NDPP production in vitro using human liver microsomes, specific antibodies or inhibitors, and stably expressed CYP enzymes, and in vivo by examining NDPP formation in patients on chronic propafenone therapy. Their findings show that N-dealkylation of propafenone is mediated by CYP3A4 and CYP1A2. Human kidney was recently shown to express CYP3A4 and CYP3A5 (Haehner, 1996). While CYP1A2 is generally considered to be a liver-specific enzyme, induction of renal CYP1A2 by exposure to dietary Aroclor 1254 was demonstrated in female rat kidney (Beebe et al., 1995). Based on these findings, I performed very preliminary experiments to attempt to induce and inhibit CYP3A and to inhibit CYP1A2 enzymes in renal tubular cells, although characterization of propafenone biotransformation was not a focus of this research.

In the study by Botsch et al., the $K_m$ and $V_{max}$ values for NDPP production by human kidney microsomes were 125 $\mu$M and 4.5 pmol/$\mu$g/hr, respectively. These results cannot be directly compared with those in the present study since my experiments were conducted with whole cells from a dog kidney cell line. Still, the $K_m$ values in both studies were in the micromolar range (125 $\mu$M with microsomes versus 27 $\mu$M with whole cells).

In humans and many other species, dexamethasone induces CYP3A in liver and other tissues (Parkinson, 1996). In the present experiments, the production of NDPP was significantly inhibited in the cells that were exposed to dexamethasone. However, since the cells were incubated with dexamethasone and propafenone simultaneously in
the experimental conditions, dexamethasone may have interfered with the entry of propafenone into the cells. As well, dexamethasone is a substrate of P-glycoprotein (Ueda et al., 1992). Therefore, its effects on propafenone biotransformation are complicated by the processes of drug transport in the whole cell model. To avoid these problems, these experiments could be repeated in microsomes prepared from the cells. Moreover, attempts to study the induction of propafenone biotransformation will likely be most successful if an animal model (e.g. pretreatment of rats or mice with dexamethasone) is utilized.

Troleandomycin is both a substrate and mechanism-based inhibitor of CYP3A; however, it is also reported to induce CYP3A in humans (Parkinson, 1996). Cell monolayers were preincubated with troleandomycin before incubation with propafenone, which may explain the lack of inhibition of NDPP production seen with troleandomycin. That is, troleandomycin may have induced CYP3A in the MDCK cells; thus, negating the inhibitory effects of troleandomycin on propafenone biotransformation. Alternatively, troleandomycin may not have been sufficiently taken up into the cells to affect NDPP production.

The selective CYP1A2 inhibitor and CYP3A activator, α-naphthoflavone, significantly inhibited the production of NDPP. To ensure that the inhibition of propafenone biotransformation in the presence of α-naphthoflavone was not caused by prevention of propafenone from entering the renal tubular cells, I repeated this experiment with renal microsomes prepared from MDCK cells. Again, α-naphthoflavone appeared to inhibit NDPP formation, suggesting that CYP1A2 may play a role in the biotransformation of propafenone by the kidney. However, this experiment should be repeated to confirm these results.

In summary, my study demonstrates that drug metabolites may contribute significantly to renal tubular drug interactions, although in this case, the parent compound has a greater effect. My findings suggest that P-glycoprotein is involved in the digoxin-propafenone interaction, and that 5-OHP is possibly a substrate for
P-glycoprotein. The ability of renal tubular cells to metabolize propafenone to NDPP was established, although for this particular drug, renal biotransformation likely does not cause clinically-important drug interactions.

**II.6b The Digoxin-Verapamil Interaction**

In a paper describing the effects of verapamil on the renal tubular secretion of digoxin across confluent cell monolayers of LLC-PK1 proximal tubular cells, we suggested that the digoxin-verapamil interaction involves P-glycoprotein (Ito et al., 1993c*). Verapamil is transported by both P-glycoprotein (Horio et al., 1989) and the organic cation transporter (Sokol et al., 1989). However, direct contribution of the classical organic cation transport system to digoxin renal tubular secretion is unlikely (Ito et al., 1993a) as described above. Moreover, we were unable to detect transport of the classic organic cation substrates tetraethylammonium and N-methyl nicotinamide in MDCK cells (data not shown, see above).

As discussed in chapter one, multidrug resistance is a major cause of drug failure in the treatment of various cancers. There are two types of resistance in chemotherapy. With intrinsic resistance, there is a lack of response to the chemotherapeutic agent from the beginning of therapy. This type of resistance is common in some solid tumours such as hepatocarcinomas and adenocarcinomas of the colon and kidney. With the other type of resistance called acquired resistance, the sensitivity to anticancer drugs gradually lessens. Acquired resistance is more common with leukemias and lymphomas (Fardel et al., 1996).

Since verapamil was one of the first non-chemotherapeutic compounds to be identified as a modulator of P-glycoprotein, it was one of the first drugs to be tested as a reversal agent for multidrug resistance. Unfortunately, the serum concentrations of verapamil required to reverse multidrug resistance (6-10 μM to fully inhibit P-glycoprotein *in vitro*) were associated with significant cardiotoxicity, prohibiting the recommendation of this drug as a reversal agent for humans (Fisher and Sikic, 1995).
Yet, because the cardiotoxic effects of the R-enantiomer of verapamil are less pronounced than those of the racemic mixture, R-verapamil was also tried as a reversal agent. Again, cardiac toxicity prevented the use of this agent in patients experiencing multidrug resistance.

Verapamil is biotransformed to its major metabolite, norverapamil, by CYP3A (Kroemer et al., 1993). Given that propafenone metabolites contribute to the digoxin-propafenone interaction, we decided to go back and look at the effects of verapamil metabolites on the renal tubular secretion of digoxin. Norverapamil has less cardiac activity than verapamil, therefore, we hypothesized that it might be useful as a reversal agent if it has effects on P-glycoprotein-mediated drug transport. Moreover, the two nonactive verapamil metabolites PR-22 and D-620 were examined for their ability to affect the accumulation of P-glycoprotein substrates.

In MDCK cells, verapamil demonstrated a similar ability to decrease digoxin secretion as in LLC-PK1 cells. The effects of verapamil and norverapamil on the renal tubular secretion of vinblastine and digoxin across confluent monolayers of MDCK cells were not statistically different. The increased cellular accumulation of digoxin and vinblastine in the presence of verapamil and norverapamil demonstrates that these compounds do not interfere with the ability of digoxin to enter the cells, implying that the digoxin-verapamil/norverapamil interaction does not take place at the basolateral membrane. The inhibitory effects of the enantiomers of norverapamil on vinblastine and digoxin secretion were nonstereoselective. Again the nonstereospecific nature of the interaction is consistent with the findings of other P-glycoprotein modulators drug such as verapamil (Ito et al., 1993c*), quinidine (Hedman et al., 1990), and propafenone (Woodland et al., 1997*). Taken together, these results support the notion that the interactions of verapamil and its metabolites with digoxin and vinblastine take place at P-glycoprotein located in the apical membranes of renal tubular cells.

Norverapamil may be a useful P-glycoprotein-mediated multidrug resistance reversal agent with a better side effect profile than the more cardiotoxic parent
compound verapamil. In support of this, the percent survival of mice with doxorubicin-resistant tumours exposed to the P-glycoprotein substrates doxorubicin and adriamycin was increased to a similar extent when either verapamil or norverapamil was given concurrently (unpublished data G. Batist and I. Wainer, McGill University).

Our results showed that PR-22 inhibits digoxin and vinblastine transport across MDCK cell monolayers, while D-620 does not. The PR-22 metabolite is the N-demethyl-O-demethyl form of verapamil; whereas the D-620 molecule is both N-demethyl and N-dealkylated. Structurally, this means that the PR-22 molecule is more similar to norverapamil than the D-620 molecule which is almost cut in half. Since the larger inactive metabolite is still able to inhibit the renal tubular secretion of vinblastine and digoxin, the structure of a compound may provide important information about the requirements for P-glycoprotein modulation. Further study of P-glycoprotein modulators may allow for the identification and creation of compounds that will be useful as reversal agents.

Similar to what was found with propafenone, MDCK cells were able to biotransform verapamil. While in the case of propafenone biotransformation, the production of metabolite (NDPP) was unlikely to have a significant clinical effects on digoxin accumulation, the production of verapamil metabolites is likely important to drug interactions. This is because verapamil and norverapamil serum concentrations are similar and the metabolites have potent effects on the transport of P-glycoprotein substrates. In other words, renal biotransformation of verapamil may contribute to the digoxin-verapamil interaction.

The *in vitro* tissue culture model has utility as a tool to screen compounds that may reverse multidrug resistance. Verapamil, norverapamil, and PR-22, but not D-620, inhibit the renal tubular secretion of the P-glycoprotein substrates vinblastine and digoxin. Norverapamil, with approximately 20% of the cardiac activity of verapamil, and the inactive PR-22 molecule show promise as potential multidrug resistance reversal agents. It must be borne in mind that agents that are successful in reversing
multidrug resistance in vitro may not be successful in vivo. Still, a lot of time and money may be saved in the development of reversal agents if this model is used in the preliminary steps of this research area.

II.6c The Digoxin-Mifepristone Interaction

Mifepristone is prescribed to a very small population of people relative to other drugs. This means that clinically-significant drug interactions with mifepristone may take years to appear in the literature. We utilized our in vitro model to study the effects of mifepristone on the renal tubular secretion of digoxin and vinblastine in an attempt to identify whether mifepristone might cause drug interactions with P-glycoprotein substrates.

Mifepristone significantly inhibited the transport of digoxin and vinblastine across confluent MDCK cell monolayers. The cellular uptake experiments and addition of mifepristone to either the apical or basal compartments, demonstrated that this inhibition was not caused by blockage of the P-glycoprotein substrates from entering the renal tubular cell at the basolateral membrane. Rather, mifepristone appears to inhibit the exit of P-glycoprotein substrates from the cell across the apical membrane, consistent with drug interactions taking place at P-glycoprotein.

Three papers published during this study period support this hypothesis (Gruol et al., 1994; Lecureur et al., 1994; Fardel et al., 1996). In the first, mifepristone was found to inhibit the P-glycoprotein-dependent efflux of rhodamine 123 in cells overexpressing the mouse mdr1 gene. In the second, mifepristone strongly enhanced the intracellular accumulation of the P-glycoprotein substrate doxorubicin in both rat hepatoma RHCl and human leukemia K562 R7 drug-resistant cells. Mifepristone had no effect in SDVI drug-sensitive liver cells. The sensitivity of RHCl cells to doxorubicin was increased by mifepristone. Furthermore, mifepristone blocked P-glycoprotein labelling by azidopine, a photoactive ligand for P-glycoprotein. Finally, mifepristone also inhibited
P-glycoprotein activity in a human leukemic cell line and in tumour cells freshly isolated from patients (Fardel et al., 1996).

We attempted to characterize the kinetic nature of the digoxin-mifepristone interaction using data from digoxin saturation curves constructed in the presence of various concentrations of mifepristone. Digoxin concentrations of 100 µM did not appear to be saturating. However, in the presence of mifepristone, 200 µM digoxin appeared to be toxic to the cells. As a result, the 200 µM data points were eliminated. The interpretation of this data was not straightforward due to conflicting results from different linear transformation models (data not shown). A nonlinear kinetic fitting approach also failed to yield a conclusive result because of relatively large variations. The concentrations of digoxin required to reach saturation appeared to be toxic to the cell monolayers. We suspect that the inhibition of digoxin renal tubular secretion by mifepristone involves both competitive inhibition at P-glycoprotein as well as noncompetitive inhibition resulting from perturbations of the membrane. Alterations in apical membrane properties have been reported for other P-glycoprotein substrates and inhibitors (Wadkins and Houghton, 1993). Further experimentation is required to elucidate the mechanism(s) of inhibition.

An increase in mifepristone use can be expected due to its recent approval for release in North America. Although mifepristone will likely be used much less commonly than other drugs that interact with P-glycoprotein (e.g., cyclosporine, verapamil, propafenone), potential mifepristone-drug interactions must be identified. The ability of therapeutic concentrations of mifepristone to inhibit the secretory flux of digoxin and vinblastine suggests that clinically-relevant drug interactions might result if these drugs are given concomitantly. Moreover, mifepristone-drug interactions are likely if mifepristone is coadministered with P-glycoprotein substrates. The in vitro cell culture model that I have described may prove useful in identifying agents that will interact with mifepristone and hence, clinically-significant drug interactions may be avoided.
II.7 Conclusions

Digoxin-drug interactions can be life-threatening and hence need to be identified before patients are co-prescribed other drugs. The well-known interactions of propafenone and verapamil with digoxin and the potential interaction of mifepristone with digoxin all appear to involve P-glycoprotein. Although propafenone and verapamil are racemic mixtures, their drug interactions are not stereoselective. However, the metabolites of these drugs contribute to the drug interactions. To my knowledge, this is the first report of the role of drug metabolites in interactions due to inhibition of drug transport. In the case of propafenone, digoxin toxicity should not differ between poor and extensive metabolizers since the parent compound and 5-OHP both have inhibitory effects on the renal tubular secretion of digoxin.

Importantly, I also demonstrated the ability of the kidney to biotransform drugs. While renal propafenone biotransformation likely does not have a significant impact on the production of digoxin toxicity, the biotransformation of verapamil by the kidney may contribute to the digoxin-verapamil interaction. Given that a number of P-glycoprotein substrates are biotransformed by CYP3A, and that CYP3A is expressed in human kidney, renal drug biotransformation should be considered in the analysis of drug interactions. Furthermore, as demonstrated by norverapamil and PR-22, drug metabolites that are less toxic than their parent compounds may be attractive candidates for the reversal of multidrug resistance.

This chapter highlighted the utility of a cell culture model to the characterization of known drug interactions, the identification of possible drug interactions, and the prediction of compounds that might be useful in reversing multidrug resistance. In essence, it can be used as a tool to screen compounds that may interact with P-glycoprotein. Of course, the model is only acceptable for these applications if it is well characterized and its limitations are acknowledged. While the in vitro cell monolayer system described above should not be used in isolation, it provides an easy, relatively
inexpensive way to study drug interactions at the whole cell level and avoids the ethical concerns of human and animal studies.
CHAPTER 3:
PRODUCTION OF NEPHROTOXIC IFOSFAMIDE METABOLITES

III.1 Introduction

In the global search for the "cure for cancer" it has been recognized that different treatments have different rates of success for each type of cancer. The pharmacodynamics of antineoplastic drugs generally follow first-order kinetics such that they kill a constant percentage of cells, as opposed to a constant number of cells (Erlichman and Kerr, 1989). In general, antineoplastics are given at high doses for intermittent periods of time to allow tumour cells to be killed and normal cells to recover between drug treatment cycles. To achieve maximal effectiveness, the trend in cancer chemotherapy treatment has been to combine effective drug therapies utilizing drugs with different mechanisms of action and qualitatively different toxicities.

One important mechanistic class of antineoplastic drugs is the alkylating agents. This chemically diverse group of drugs includes nitrogen mustard (mechlorethamine), melphalan, chlorambucil, busulphan, BCNU (bischloroethyl nitrosourea, carmustine), CCNU (chloroethyl-cyclohexyl nitrosourea, lomustine), methyl-CCNU, cisplatin, cyclophosphamide, and ifosfamide. Alkylating agents are highly reactive compounds that can become strong electrophiles by forming carbonium ion intermediates or transition complexes with their molecular targets. They act by substituting alkyl groups for the hydrogen atoms of many organic compounds and by forming covalent bonds with nucleophiles such as phosphate, amino, sulfhydryl, hydroxyl, carboxyl, and imidazole groups, especially the seven nitrogen position of guanine in nucleic acids. By disrupting DNA synthesis and function, causing depurination and base-pair mismatching, alkylation can result in the scission and/or cross-linking of DNA strands (Dechant et al., 1991).

The nitrogen mustards, including mechlorethamine, melphalan, and cyclophosphamide have two chloroethyl groups on the same nitrogen atom (figure 32). When each chloroethyl substituent covalently binds to a carbon, nitrogen, or oxygen
Figure 32. Structure of nitrogen mustards.
When each chloroethyl substituent covalently binds to a carbon, nitrogen, or oxygen atom in a target molecule such as the purine and pyrimidine bases of nucleic acids, the mustard forms a bridge between the two target sites, causing irreparable DNA-DNA or DNA-protein cross-linking that interferes with normal DNA synthesis and results in cytotoxicity (Erlichman and Kerr, 1989; Wagner et al., 1997). Ifosfamide is thought to act similarly to cyclophosphamide, causing cytotoxicity by disrupting DNA.

As described below, this chapter will test the hypothesis that ifosfamide-induced renal toxicity may result from the production of toxic metabolites such as chloroacetaldehyde by renal tissues.

Ifosfamide and cyclophosphamide are oxazaphosphorine alkylating agents that are cell-cycle-independent, meaning that they can affect cells during any phase of the cell cycle, including the resting phase. The creation of these drugs followed observations of leukopenia, bone marrow aplasia, and lymphatic tissue dissolution in persons exposed to the sulfur mustard gases used for chemical warfare. In the early 1940s, nitrogen mustards were studied in mice for the treatment of lymphosarcoma.

Cyclophosphamide was developed in the late 1950s as a less reactive and more specific compound than nitrogen mustard. The strategy behind the design of cyclophosphamide was that it would require phosphatases or phosphamidases to be activated. Since the concentrations of these enzymes were thought to be higher in tumour cells than in normal tissues, cyclophosphamide was expected to be more selective than nitrogen mustard. Indeed, cyclophosphamide has demonstrated efficacy against a number of cancers, although its activity does not correlate with tumour phosphamidase concentrations (Loehrer, 1992). The main drawback of treatment with cyclophosphamide is that it produces dose-limiting myelosuppression.

In 1965, ifosfamide, which differs from cyclophosphamide by the position of a chloroethyl group (figure 33), was synthesized. It entered clinical trials only two years later. Unfortunately, soon after ifosfamide was introduced, associations with severe urotoxicity, including hemorrhagic cystitis and uremia, became apparent. Therefore,
Figure 33. Ifosfamide and cyclophosphamide differ by the position of a chloroethyl group.
ifosfamide was not widely used when it was first marketed. However, since the introduction of a uroprotective thiol agent called mesna (sodium 2-mercaptoethane sulfonate; see section III.1e) in 1980, ifosfamide has become an important mainstay in cancer chemotherapy. The co-administration of mesna with ifosfamide and cyclophosphamide has become common practice; hence, the problems of hemorrhagic cystitis have been virtually eliminated and nephron toxicity has been reduced.

When given concomitantly with mesna, the major dose-limiting toxicity of ifosfamide becomes myelosuppression, with leukopenia generally more severe than thrombocytopenia. Still, ifosfamide is less myelotoxic than cyclophosphamide and has demonstrated efficacy against a variety of cancers including: disseminated nonseminomatous testicular cancer, small cell lung cancer, pediatric solid tumours such as rhabdomyosarcoma, Wilms' tumor, Ewing's sarcoma and germ cell tumors, non-Hodgkin's and Hodgkin's lymphoma, ovarian cancer, non-small cell lung cancer, adult soft-tissue sarcomas, advanced breast cancer and advanced cervical cancer without bone marrow complications (Kornhuber, 1989; Dechant et al., 1991). Furthermore, ifosfamide has greater activity than cyclophosphamide against some types of tumours both in vitro and in vivo, and is effective in the treatment of some cyclophosphamide-resistant tumours (Rodriquez et al., 1978; Goldin, 1982; Brade et al., 1985; Wheeler et al., 1986; Bramwell et al., 1987; de Kraker et al., 1989). However, a lack of randomized clinical trials comparing the two drugs has left the question of which one is most efficacious unanswered for most cancers. Both drugs are currently used independently and in combination with other antineoplastic agents such as etoposide, dactinomycin, and cisplatin.

III.1a Physicochemical Properties of Ifosfamide

Ifosfamide, (3-[2-chloroethyl]-2-C[2-chloroethyl]-amino)-tetra-hydro-2H-1,3,2-oxazaphosphorine-2-oxide, has a molecular weight of 261.08, the same as that for cyclophosphamide, 2-(bis-[2-chloroethyl]-amino-tetrahydro-2H-1,3,2-oxazaphosphorine
-2-oxide. In general, ifosfamide is chemically similar to cyclophosphamide in many respects. For instance, the melting points of the two oxazaphosphorine agents are similar (48-51°C for ifosfamide and 41-45°C for cyclophosphamide) (Brade et al., 1986). However, the spacing of the chloroethyl groups in ifosfamide (independent of one another) makes ifosfamide more water soluble (up to 10%) than cyclophosphamide (up to 4%) (Schoenike and Dana, 1990).

### III.1b Ifosfamide Biotransformation

Both ifosfamide and cyclophosphamide are prodrugs that require bioactivation to exert their cytotoxic effects (Chang et al., 1993; Walker et al., 1994). Qualitatively, the two alkylating agents share similar pathways of biotransformation; however, there are significant quantitative differences.

As shown in figure 34, ifosfamide is biotransformed through two different pathways. Similar to cyclophosphamide, the activation pathway involves the oxidation of ifosfamide by CYP-mediated ring hydroxylation (as described later). The resulting metabolite, 4-hydroxyifosfamide, is very cytotoxic and exists in equilibrium with its tautomeric ring-opened form, aldoifosfamide. The latter metabolite is unstable and it may spontaneously decompose to equimolar amounts of acrolein and the ultimate DNA cross-linking species, isofosforamide mustard, or it may be oxidized by aldehyde dehydrogenase to produce the inactive carboxyifosfamide metabolite. Alternatively, 4-hydroxyifosfamide may be oxidized to 4-ketoifosfamide or sulfated to 4-thioifosfamide, both of which are inactive metabolites.

In a side-chain pathway, the chloroethyl groups of ifosfamide may be cleaved through oxidative N-dechloroethylation leading to the production of either 2-dechloroethylifosfamide (2-DCEI) or 3-dechloroethylifosfamide (3-DCEI) and equimolar amounts of chloroacetaldehyde, an unstable compound which possesses high chemical reactivity (Skinner et al., 1993). The biotransformation of ifosfamide to chloroacetaldehyde and 2- and 3-DCEI has often been referred to as the "deactivating"
Figure 34. Pathways of ifosfamide biotransformation.
pathway because these metabolites were not thought to have any cytostatic effects. However, Brüggemann et al. (1997) have recently challenged this assumption in showing that chloroacetaldehyde has direct cytotoxic effects on tumour cell lines exposed to chloroacetaldehyde.

There is great intra- and interindividual variation in ifosfamide biotransformation both in the ratio of unchanged to converted ifosfamide and in the ratio of ring to side-chain oxidation (Norport, 1976). In addition, there are interindividual variations in conjugation and alkylating reactions. Genetic differences in aldehyde dehydrogenase can explain the variations between individuals in the production of carboxyifosfamide, the major urinary metabolite of cyclophosphamide, and hence, in differences in cytostatic activity (Hadidi et al., 1988).

III.1c Ifosfamide Pharmacokinetics

The antineoplastic effect of cyclophosphamide depends primarily on the peak concentration achieved, whereas the period during which a particular concentration is maintained is more important to success with ifosfamide treatment (Schoenike and Dana, 1990). Clinically, this has led to single-course regimens for cyclophosphamide and a fractionated dosage schedule for ifosfamide.

Ifosfamide has an oral bioavailability of almost 100% (Cerny et al., 1986; Wagner and Drings, 1986) and a volume of distribution that approximates total body water (0.39-0.64 L/kg following intravenous administration) (Lind et al., 1989a). The pharmacokinetic properties of ifosfamide are dependent on the frequency of oral or intravenous dosing, but not the dose itself (Dechant et al., 1991). Much higher (about two-fold) plasma concentrations of 4-hydroxyifosfamide and chloroacetaldehyde are found when ifosfamide is given orally rather than by the intravenous route (Kurowski et al., 1991).

Carlson et al. (1997) described the plasma concentrations of ifosfamide, chloroacetaldehyde, and 4-hydroxyifosfamide in an anephric patient with Wilms'
tumour following a single dose of ifosfamide (1.6 g/m²). Their findings suggest that the pharmacokinetics of ifosfamide are not substantially altered by renal failure, implying that renal elimination is not an important part of ifosfamide elimination. Since the renal clearance of ifosfamide is generally lower than the creatinine clearance, tubular reabsorptive processes may be involved, although specific transporters have not been identified to date (Lind et al., 1989a).

In adults, the mean total clearance rate of ifosfamide following a single oral or intravenous dose is approximately 60 ml/min (the majority of which is nonrenal) and the terminal elimination phase half-life ranges between four and seven hours (Dechant et al., 1991). However, clearance and half-life values vary greatly with the method and duration of ifosfamide dosing. In a recent study, Kaijser et al. (1996) examined the pharmacokinetics of ifosfamide and its 2- and 3-DCEI metabolites in cancer patients treated with ifosfamide for ten days by continuous infusion. Average (semi-steady state) concentrations of ifosfamide in the first part of the plasma concentration time curve (t<24 hours) were two-fold higher than those in the second part of the curve. The half-lives for these two phases were 9.2 hours (range: 4.6-22.4) and 4.2 hours (2.4-8.4), respectively. While total clearance approximately doubled, renal clearance (11.5 ml/min; range: 2.7-20.0) did not change during the infusion. Giving ifosfamide in divided doses also decreases the half-life of ifosfamide without causing a corresponding increase in its renal clearance, suggestive of increased ifosfamide biotransformation (Nelson et al., 1976; D'Incalci et al., 1979; Wagner and Drings, 1986; Lind et al., 1989a; Lewis et al., 1990; Kurowski et al., 1991; Kurowski and Wagner, 1993).

A process of auto-induction of ifosfamide biotransforming enzymes has been proposed to explain the increased clearance and decreased half-life of ifosfamide reported after repeated dosing. The increase in ifosfamide clearance in the above-mentioned continuous infusion study by Kaijser et al. was much higher than that reported in studies where ifosfamide was given by bolus injections, suggesting that auto-induction is greater when ifosfamide is infused over a prolonged period of time.
rather than given by bolus injections. Moreover, in a comparison of ifosfamide dosing regimens, the AUC for the dechloroethylated metabolites was found to be lower following bolus administration than during a 72-hour continuous infusion (Boddy et al., 1995).

Interestingly, Kaijser et al. (1996) found that patients receiving the ten-day continuous infusion could be categorized into two groups depending on the shape of their plasma concentration versus time curves. In the first group, the maximum concentration of ifosfamide was seen at 24 hours with a steady decline in concentration thereafter. In the second group, the maximum ifosfamide concentration was also seen at 24 hours; yet, the decline in concentrations occurred much more rapidly. Essentially, the patients appeared to be either fast or slow inducers with half-lives of induction of 10 hours and 30 hours, respectively. The type of induction was independent of the dose of ifosfamide, age, and sex; however, patients remained in the same type characteristics in subsequent courses of ifosfamide.

In the continuous infusion study by Kaijser et al. (1996), about 8.5% of the total ifosfamide dose was excreted unchanged. The amounts of 2-DCEI and 3-DCEI excreted represented 3% and 11% of the ifosfamide dose, respectively. These numbers are similar to those obtained by Goren (1991) who found that 11-30% of the dose was excreted over 24 hours as unchanged drug, 3-10% was excreted as 2-DCEI, and 11-21% as 3-DCEI following a single dose (1.6 g/m²) of ifosfamide. Kaijser et al. (1992) found that urinary concentrations of 3-DCEI were generally about three-fold higher than those for 2-DCEI, although ratios of 3-DCEI/2-DCEI of up to 12 were observed following a ten-day continuous infusion of 2 g of ifosfamide per day. Expressed as a ratio to the amount of ifosfamide in the urine, the cumulative amount of 2- and 3-DCEI was 0.2 to 2.7 (Kaijser et al., 1992). As well, the half-lives of the dechloroethyl metabolites are considerably longer and more variable (especially for 2-DCEI) than that of the parent compound.
When nine adult patients received a dose of 3 g/m² of ifosfamide as a 3-hour infusion, urine collected over 24-hours recovered 17.9 ± 4.7% (range: 11.4-28.5%) of the dose as parent ifosfamide compound, 10.5 ± 2.7% (5.5%-15.8%) as 3-DCEI, 3.9 ± 1.3% (1.0%-5.7%) as 2-DCEI, 0.4 ± 0.6% (0-1.8%) as degradation products of the dechloroethylated metabolites, 3.4 ± 2.5% (1.3-9.6%) as carboxyifosfamide, 0.8 ± 0.5% (0.1-1.7%) as aldoifosfamide, and 0.2 ± 0.2% (0-0.5%) as isofosforamide mustard (Gilard et al., 1993). Silies et al. (1998) compared the excretion kinetics of children given daily doses of 400 mg/m² to 3 g/m² ifosfamide by either continuous or short-term (one-hour) infusion and found no obvious differences between the two methods of administration in the cumulative excretion of ifosfamide and its metabolites. Furthermore, the time courses of excretion for ifosfamide, 2-DCEI, and 3-DCEI in children receiving ifosfamide by either short-term or continuous infusions were superimposable. The major metabolites found in the urine were 3-DCEI, isofosforamide mustard, and 2-DCEI accounting for 14 ± 4% (range: 8-23%), 13 ± 4% (5-22%), and 8 ± 3% (4-15%) of the initial dose, respectively. Unchanged parent drug represented 23 ± 9% (8-44%).

The pharmacokinetics of ifosfamide in the elderly were studied in 20 patients (age 40-71) with advanced non small cell lung cancer (Lind et al., 1990). The autoinduction of ifosfamide biotransformation did not change with age. The total plasma clearance, nonrenal clearance and renal clearance of ifosfamide were also not affected. On the other hand, increasing age correlated positively with the elimination half-life of ifosfamide in these patients. This increase in elimination half-life was attributed to an increase in the volume of distribution that occurred with increasing age.

The clearance of ifosfamide is lower in adults than in children (Boddy et al., 1993) and the renal excretion of ifosfamide, 2-DCEI, and especially 3-DCEI is higher in children (Goren, 1991; Silies, 1998). In a pediatric population (age one to 16 years) the AUC of ifosfamide was positively correlated with patient age (Boddy et al., 1996b). Still,
interindividual differences in ifosfamide clearance and biotransformation appear to be greater than those due to age (Sladek, 1988; Skinner et al., 1993).

The use of nonspecific techniques in the determination of ifosfamide and its metabolites have created confusion in the interpretation of pharmacokinetic results. Moreover, the choice of pharmacokinetic model for ifosfamide (for example, monocompartmental, bicompartmental, multicompartmental, noncompartmental) is important. In a study of patients receiving 6 g/m² of ifosfamide by continuous infusion for five days, Passe et al. (1999) found significant differences in four of six pharmacokinetic parameters using two different monocompartmental models.

In summary, numerous studies have examined the pharmacokinetic properties of ifosfamide. The phenomenon of autoinduction of ifosfamide complicates the determination of ifosfamide pharmacokinetics. There are also great interindividual differences in the handling of ifosfamide. Therefore, careful attention must be paid to the method of ifosfamide administration (for example, oral versus intravenous, bolus versus continuous infusion, single versus repeated courses) when data are interpreted.

III.1d Ifosfamide Toxicity

The most common side effects experienced by patients taking ifosfamide are nausea and vomiting, alopecia, and leukopenia (with relative platelet-sparing activity) (Palackdharry, 1996). However, the coadministration of mesna has enabled higher and more frequent ifosfamide dosing accompanied by the emergence of a new spectrum of toxicities that include neurotoxicity, hematologic toxicity, nephrotoxicity, and acidosis.

III.1d.1 Neurotoxicity

Approximately 10% to 25% of patients taking intravenous ifosfamide experience a neurologic toxicity that is generally reversible upon discontinuation of the drug (Dechant et al., 1991). This neurotoxicity can range from mild somnolence and confusion to severe encephalopathy and coma. Typically, the symptoms of ifosfamide-
induced neurotoxicity include hallucinations, sleep disturbances, disorientation, confusion, anxiety, agitation, emotional lability, affect changes, short term memory dysfunction, ataxia, cerebellar dysfunction, cranial nerve disturbances, and temporary paresis (Palackdharry, 1996). Interestingly, the incidence of neurotoxicity increases to as high as 50% in patients receiving oral ifosfamide (Dechant et al., 1991).

In the ten-day continuous infusion study by Kaijser et al. (1996), one out of 22 patients experienced severe neurotoxicity. This patient had higher plasma concentrations of 2- and 3-DCEI, but the percentage of the total ifosfamide dose excreted as these metabolites was within a normal range, suggesting that the renal clearance of 2- and 3-DCEI was lower than that for patients who did not experience neurotoxicity.

In children, the signs of central nervous system toxicity from most to least frequent are: mental status changes, cerebellar dysfunction, urinary incontinence (which may reflect a combination of altered mental status, increased urinary output, and local irritation), transient weakness, cranial nerve dysfunction, and seizure activity (Schoenike and Dana, 1990). However in children, neurotoxicity is a less important side effect than nephrotoxicity (Smeitink et al., 1988; Skinner et al., 1990).

III.1d2 Nephrotoxicity

The incidence of clinically significant hemorrhagic cystitis from ifosfamide has been greatly reduced to less than 20% (<5% for gross hematuria and <20% for microscopic hematuria) by the routine coadministration of mesna. However, despite the use of mesna, a variety of renal toxicities including renal tubular acidosis, acute tubular necrosis, elevated serum creatinine levels, Fanconi syndrome (described below), and diffuse interstitial fibrosis, have been reported (Palackdharry, 1996). Renal tubulopathy remains a dose-limiting side effect in a significant number of patients.

Nephrotoxicity has been reported in both adults and children, but appears to be more prevalent and severe in children. One of the largest long-term outcome studies on
ifosfamide-induced nephrotoxicity in children (0.4 to 21.2 years of age) was conducted by Loebstein et al. (1999). They assessed glomerular and tubular function up to five years post-treatment in 174 children who received ifosfamide for a variety of cancers. Focussing on the clinically-significant markers of renal toxicity such as serum concentrations of phosphate and bicarbonate and urine concentrations of glucose and protein, renal dysfunction was noted in 41.4% of patients. Of these patients, all had proximal tubular dysfunction, while glomerular dysfunction was present in only 6.3%. When patients that had received platinums were excluded, nephrotoxicity and generalized tubulopathy were observed in 33.5% and 6.5%, respectively. These numbers are roughly in agreement with the incidence of ifosfamide-induced nephrotoxicity in other published studies. Using the meta-analysis tool, Stevens and Brandis (1991) calculated a 40% risk of subclinical tubular dysfunction, a 10% chance of significant glomerular damage, and a 5% risk of Fanconi syndrome following treatment with ifosfamide.

Fanconi syndrome is a generalized dysfunction of the renal proximal tubular cells. Clinically, it is defined as excessive urinary excretion of glucose, phosphate, bicarbonate, amino acids, and other solutes handled by this segment of the nephron. As well, patients frequently experience chronic acidosis, hypouricemia, and hypokalemia. Fanconi syndrome often results in growth failure, rickets, and progressive renal failure.

In the study by Loebstein et al. (1999), mild nephrotoxicity was observed in 23% of the children who received ifosfamide, whereas moderate toxicity was seen in 9.2%, and a further 9.2% had either generalized proximal tubulopathy or renal Fanconi syndrome. The most common abnormal findings in the children with nephrotoxicity were: hypophosphatemia (78%), hypomagnesemia (62%), proteinuria (37%), hypocarbia with acidosis (33%), glycosuria (20%) and reduced glomerular filtration rate (6.3%). The severity of nephrotoxicity correlated with young age, higher cumulative dose of ifosfamide, and the total dose of platinums. Reductions in the glomerular filtration rate appeared to be secondary to proximal tubulopathies.
Of the children classified with severe nephrotoxicity, 43.7% had chronic tubular dysfunction over the mean follow-up period of 5.3 years. (The range of follow-up was two to 12 years.) All of these children required daily oral phosphate and bicarbonate supplementation. Four of the seven children developed hypophosphatemic rickets and an additional two patients developed end-stage renal failure requiring renal transplantation following a progressive deterioration in glomerular filtration rate. Furthermore, among the 16 ifosfamide-treated children that were classified as having moderate nephrotoxicity, 25% required chronic oral supplementation of phosphate and bicarbonate, 12.5% had gradual deterioration of glomerular function, and 12.5% developed hypophosphatemic rickets. In summary, the long-term sequelae of ifosfamide-induced nephrotoxicity necessitate an understanding of the mechanisms responsible for this kidney damage.

III.1e Mesna

Mesna (sodium 2-mercaptoethane sulfonate) is a thiol agent with proven efficacy against cyclophosphamide and ifosfamide-induced urotoxicity. Nucleophiles like sulfhydryl or amino groups on proteins react with electrophiles such as various ifosfamide metabolites. The principle behind detoxification with mesna is that mesna forms thioether bonds with the identified urotoxic ifosfamide metabolites chloroacetaldehyde, acrolein, and 4-hydroxyifosfamide (Goren, 1992). Acrolein is generally believed to be the metabolite most responsible for urotoxicity. Mesna forms a stable thioether by combining with the double bond of acrolein. Moreover, mesna prevents the formation of acrolein in the bladder by binding to 4-hydroxyifosfamide. Since mesna does not cross plasma membranes because of the ionized sulfate moiety, it is localized extracellularly which prevents it from entering tumour cells. As a result, mesna does not interfere with the cytostatic activity of ifosfamide (Schoenike and Dana, 1990).
In the blood, mesna is oxidized to the dimer dimesna. Dimesna (mesna disulfide) does not prevent \textit{in vitro} cellular damage by metabolites of ifosfamide and cyclophosphamide (Mohrmann \textit{et al.}, 1994b; 1995). It passes through the liver unchanged. The high water solubility and poor extravascular distribution of dimesna and mesna facilitate their rapid clearance from the plasma by the kidney. The rate of renal excretion of mesna-dimesna correlates with the creatinine clearance rate, indicating that glomerular filtration is the primary means of elimination. Dimesna is filtered by the kidney, actively reabsorbed into the proximal tubule (where glutathione reductase in the renal tubular epithelium converts approximately one-third of the dimesna back to mesna at the expense of glutathione), and then secreted along with the re-formed mesna into the urine (Dechant \textit{et al.}, 1991). The free sulfhydryl groups of mesna can then combine with urotoxic metabolites to form nontoxic compounds. The conversion of dimesna to mesna by the kidney, resulting in much higher urinary than plasma concentrations of mesna, accounts for the urinary tract selectivity of ifosfamide detoxification by mesna. However, while both the apical (urine) and basal (blood) sides of renal tubular cells are exposed to dimesna, only the apical side of the cells is theoretically exposed to mesna since mesna is oxidized in the blood. Although recently, the liver was also shown to have the capability to reduce dimesna back to mesna (Goren \textit{et al.}, 1998).

Mesna has an oral bioavailability of between 50% and 75% and demonstrates about 10% serum protein binding (Dechant \textit{et al.}, 1991; Shaw \textit{et al.}, 1986). The half-life of mesna is 0.4 hours and that of dimesna is 1.2 hours (Dechant \textit{et al.}, 1991). However, urinary excretion in humans is not complete by 24 hours post-injection (James \textit{et al.}, 1987). Since the half-life of these thiols is much shorter than that of ifosfamide, the administration of mesna should continue after ifosfamide therapy has been stopped to prevent urotoxicity. Generally, mesna is administered intravenously at 20% to 60% of the ifosfamide dose at 0, 4, and 8 hours following the ifosfamide dose (Goren, 1992; Siu and Moore, 1998). Oral administration is also possible, but doses of mesna equivalent
to 40% of the ifosfamide dose are recommended and they should be administered one hour prior to their required uroprotective activity (Goren, 1992). Mesna urinary concentrations of at least 1.7μM or 100 μg/ml are thought to be required for uroprotection (James et al., 1987; Burkert et al., 1984). The urinary mesna concentrations in children and adolescents receiving intermittent doses of mesna of three times 400 mg/m² body surface area are approximately 0.3 to 3.0 mM (Goren et al., 1989). While mesna accumulates in the bladder, the renal tubules will be unprotected if mesna is not administered frequently enough (Goren et al., 1989). In children, the ability of the kidney to biotransform dimesna to mesna is thought to be saturated at concentrations greater than 10 mM. Children with preexisting renal damage do not appear to have a reduced capacity to biotransform dimesna to mesna.

III.1f Etiology of Ifosfamide-Induced Nephrotoxicity

During the last decade, several investigational efforts have attempted to clarify the etiology of ifosfamide nephrotoxicity. Interestingly, cyclophosphamide which differs from ifosfamide by the position of one chloroethyl group, does not cause significant nephrotoxicity. Therefore, careful examination of the differences between ifosfamide and cyclophosphamide may help to elucidate the mechanism of ifosfamide-induced nephrotoxicity.

Perhaps the greatest pharmacokinetic difference between ifosfamide and cyclophosphamide is found in drug biotransformation. The 4-hydroxylation of ifosfamide is much slower than that of cyclophosphamide, likely due to the steric hinderance of the bulky chloroethyl group on the adjacent endocyclic nitrogen (Dechant et al., 1991). As a result, the proportion of 4-hydroxy metabolites in human plasma from ifosfamide and cyclophosphamide is about one-to-three, respectively (Wagner et al., 1981). Therefore, in order to achieve equipotent therapeutic efficacy (i.e., equal concentrations of the alkylating isofosforamide (from ifosfamide) or phosphoramid...
(from cyclophosphamide) mustard species, three-fold higher doses of ifosfamide are generally administered.

Conversely, the magnitude of side-chain oxidation (that is, N-dechloroethylation) is higher with ifosfamide than with cyclophosphamide. While for cyclophosphamide dechloroethylation generally accounts for less than 10% of the initial dose, side chain oxidation is an important part of ifosfamide biotransformation usually accounting for 11-37% of the initial dose (Goren, 1991; Boos et al., 1991; Kaijser et al., 1992) although Norpoth (1976) reported a patient with dechloroethylifosfamide metabolites totalling 48% of the ifosfamide dose.

The different enzyme affinities for ifosfamide and cyclophosphamide coupled with the higher dosing of ifosfamide relative to cyclophosphamide, lead to substantially greater production of side-chain products (i.e., chloroacetaldehyde, 2-DCEI, and 3-DCEI) with ifosfamide than with cyclophosphamide. In fact, up to 100-fold higher concentrations of chloroacetaldehyde are reportedly produced from ifosfamide compared with cyclophosphamide (Sarosy, 1989). Since cyclophosphamide, and 2- and 3-DCEI (the sum of which are produced in equimolar amounts with chloroacetaldehyde) are not associated with nephrotoxic effects, chloroacetaldehyde has been proposed as the major nephrotoxic species in ifosfamide-induced nephrotoxicity (Skinner et al., 1993).

In support of this, recent studies have shown that some of the metabolites of ifosfamide are more nephrotoxic than the parent compound (Mohrmann et al., 1992; 1993; 1994a; 1995; Zamlauski-Tucker et al., 1994). The unstable chloroacetaldehyde metabolite was found to be especially toxic in both cell culture and perfused rat kidney experiments at concentrations found in the urine of patients receiving chemotherapy. Moreover, the chloroacetaldehyde metabolite is widely believed to be responsible for ifosfamide-induced neurotoxicity. The incidence of neurotoxicity significantly increases if ifosfamide is administered orally rather than intravenously. There is also a corresponding increase in chloroacetaldehyde concentrations when ifosfamide is given
by the oral route compared with that of the intravenous route. Some patients experiencing ifosfamide-induced neurotoxicity have also been shown to have significantly higher plasma and urinary concentrations of chloroacetaldehyde, 2-DCEI and 3-DCEI (Goren et al., 1986; Boddy et al., 1996a).

The detection of chloroacetaldehyde is complicated by its lability. As a result, the more stable 2- and 3-dechloroethyl metabolites are often measured as a reflection of chloroacetaldehyde concentrations since chloroacetaldehyde is produced in a one-to-one ratio with either 2-DCEI or 3-DCEI. In a recent study, Boddy and colleagues (1996a) studied the relationship between both acute and chronic ifosfamide nephrotoxicity and its kinetic disposition in children. Acute measures of renal toxicity did not correlate with the area under the plasma concentration-time curve (AUC) of ifosfamide and its 3-dechloroethyl metabolite. Only 15 patients were used to establish this correlation and dechloroethylation was assumed to be represented by 3-DCEI. In eight patients receiving the drug repeatedly, higher nephrotoxicity scores at one and six months correlated with decreased AUCs of 3-DCEI, which the authors suggest argues against the dechloroethyl metabolites being major nephrotoxic compounds. By this argument, chloroacetaldehyde must not play a significant role in ifosfamide-induced nephrotoxicity if 2-DCEI production is also reduced. However, an important caveat is that the metabolites were measured in plasma and therefore their origin of production is unknown.

In humans, hepatic CYP3A enzymes contribute to both the 4-hydroxylation and dechloroethylation reactions (Walker et al., 1994). Due to the instability of the nephrotoxic chloroacetaldehyde metabolite, it is unlikely that hepatic formation of this species causes significant renal damage. Given that we have previously been able to demonstrate intrarenal drug metabolism (Woodland et al., 1997*), and that many CYP enzymes, including CYP3A, are expressed in human kidney (Schuetz et al., 1992; Haehner et al., 1996), I hypothesized that ifosfamide-induced nephrotoxicity may be caused by renal tubular cell production of nephrotoxic species. The aim of this study
was to verify whether kidney microsomes are capable of biotransforming ifosfamide to 2-DCEI or 3-DCEI and therefore, also chloroacetaldehyde which would support my hypothesis that ifosfamide undergoes renal biotransformation to its nephrotoxic species.
Renal microsomes biotransform ifosfamide to 2-DCEI and 3-DCEI, as a mechanism of ifosfamide-induced nephrotoxicity.
III.3 Objective

To examine the ability of the kidney to biotransform ifosfamide to 2-DCEI and 3-DCEI and hence, chloroacetaldehyde.
III.4 Materials and Methods

III.4a Materials

IFEX was purchased from Bristol-Myers Squibb Canada Limited (Montreal, Canada). Ifosfamide, metabolites, and internal standard were kindly donated by ASTA Medica (Frankfurt, Germany). Bio-Rad protein reagent was purchased from Bio-Rad Laboratories (Richmond, California). All other chemicals were obtained from Sigma-Aldrich Canada Limited (Oakville, Canada). All reagents were of HPLC grade. Control and dexamethasone-induced rat liver microsomes were kindly donated by Dr. Steven Leeder. Human kidney microsomes were purchased from the International Institute for the Advancement of Medicine (Exton, Pennsylvania).

III.4b Preparation of Kidney Microsomes

Adult porcine kidney cortex obtained from a slaughter house was minced with a razor blade and homogenized in 0.15 M KCl and 0.25 M sucrose buffer (KCl/sucrose) with a 55 ml Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 12 000 x g at 4°C for 20 minutes. The supernatant was then centrifuged at 105 000 x g at 4°C for 80 minutes. Pellets were washed and resuspended in KCl/sucrose buffer, homogenized with a 7 ml Potter-Elvehjem glass homogenizer, and centrifuged at 105 000 x g for 60 minutes. Pellets were again resuspended and homogenized in KCl/sucrose buffer. Renal microsomes were stored in 1 ml aliquots at -80°C.

Microsomes prepared from whole kidney were purchased from a tissue bank. The kidney was taken from an obese 19 year-old Caucasian male with a history of heart murmur who died from an intracranial hemorrhage. The medications that the patient received in hospital were: albumin, dopamine, heparin, lasix, mannitol, and vasopressin.
III.4c Ifosfamide Biotransformation and Sample Preparation

On the day of experimentation, the protein content of thawed microsomes was determined using a Bio-Rad protein assay with bovine serum albumin standards. The first attempt at determining the metabolic potential of pig kidney involved adding 250 μl of renal porcine microsomes with a protein concentration of 21 mg/ml to each of four plastic tubes along with incubation medium made up of 0.06 M potassium phosphate buffer containing 0.68% KCl, 3 mM MgCl₂, 4 mM glucose-6-phosphate, 0.4 mM NADP, and 0.40 units/ml of glucose-6-phosphate dehydrogenase (G6PD) to give a final volume of 5 ml at pH 7.4. One blank tube without microsomes was also prepared. Following a five minute incubation at 37°C, 1 ml of 5 mM ifosfamide solution (prepared in deionized, distilled water) was added to each of the four tubes to give a final concentration of 0.8 mM. The mixtures were left to incubate for one hour at 37°C. Then, 5 ml of acetonitrile were added to each tube, the tubes were mixed by hand, and the proteins were precipitated by centrifuging for five minutes at 2000 x g. The supernatant was stored at -80°C. Two 1 ml samples taken from each tube were shipped on dry ice to the Montreal General Hospital for determination of ifosfamide, 2-DCEI, and 3-DCEI by gas chromatography with mass spectrometric detection (GC-MS). The samples (100 μl) were spiked with the internal standard trofosfamide, extracted with 3 ml of chloroform, vortexed for one minute, and centrifuged at 1000 x g for 10 minutes. The organic phase was evaporated to dryness. The residue was dissolved in 100 μl toluene:methanol (50:50) and 0.2-0.5 μl were injected into the GC-MS system.

In future experiments, to maximize drug biotransformation, microsomes (250 μl of 25 mg/ml) and 1 mM ifosfamide solution were incubated for up to three hours at 37°C in 100 mM potassium phosphate buffer (pH 7.4) containing 40 mM MgCl₂, 5 mM glucose-6-phosphate, 2 mM NADP, and 40 units of G6PD in a final volume of 1 ml. Boiled pig kidney microsomes were incubated with a 1 mM ifosfamide solution under identical conditions to serve as a negative control. Control and dexamethasone-induced rat liver microsomes were used as positive controls. Microsomes incubated with buffer
but no drug were used as a blank. The effects of time, an energy-generating system, and co-incubation with 5 μM ketoconazole were also investigated.

The reactions were stopped by the addition of 5 ml of ice-cold chloroform. Then 12 μl of the internal standard trofosfamide (final concentration 27 μg/ml) were added. The mixtures were vortexed at centrifuged at 2000 x g for 10 minutes. For each tube, the bottom (chloroform) layer was transferred to a glass capillary tube and evaporated to dryness. The extraction tube was rinsed twice with 3 ml of chloroform and evaporated to dryness following centrifugation as described above. The capillary tube was rinsed with 1 ml then 0.5 ml of methanol and the organic phase was evaporated to dryness each time. The final residue was dissolved in 50 μl of methanol and these samples were taken on ice to the University of Toronto Mass Spectrometry Laboratory (Dr. Jack Wang) for the identification of ifosfamide, 2-DCEI and 3-DCEI by GC-MS where the injection volume was 1 μl.

Since the detection of ifosfamide and its dechloroethyl metabolites proved expensive and difficult to quantify by GC-MS, I decided to monitor ifosfamide biotransformation using high performance liquid chromatography (HPLC) in our laboratory. For HPLC analyses, samples were prepared in the same manner as for GC-MS; however, the final residue was resuspended in 100 μl of 10% acetonitrile and the injection volume was changed to 50 μl. The extraction procedure and injection volume were varied in an attempt to optimize the quantity of metabolites detected. Performing an extra chloroform rinse and eliminating the methanol rinse step seemed to give the best results.

A series of analyses were performed using an isocratic mobile phase that only allowed for the detection of 2- and 3-DCEI. Subsequent analyses were performed on a dual pump HPLC that allowed for a mobile phase gradient. In these experiments, internal standard was also added and ifosfamide, 2-DCEI, 3-DCEI, and trofosfamide were monitored.
III.4d  Gas Chromatography-Mass Spectrometry

The GC-MS analyses were performed with a Varian 3400 GC equipped with a Finnigan A 200S GC autosampler operating in the splitless mode. The chromatographic separation was performed with a capillary column (8 mm x 0.25 mm internal diameter, 0.25 µm film thickness) coated with heptakis (2,6-di-O-methyl-3-O-pentyl)-β-cyclodextrin. The column oven temperature was linearly programmed from 140°C to 180°C at 1°C per minute. The mass spectrometer was a Finnigan MAT Model Incos 50 (Finnigan National Corporation, San Jose, CA) operated in the electron-impact ionization mode at an ion source temperature of 180°C and an ionization energy of 70 eV using multiple ion monitoring. The designated ions of interest were m/z 211 for R-ifosfamide and S-ifosfamide, m/z 149 for 2-DCEI and 3-DCEI, and m/z 273 for the internal standard.

III.4e  High-Performance Liquid Chromatography

In the first set of experiments utilizing HPLC the 2- and 3-dechloroethyl metabolites were quantified using an isocratic mobile phase. Analyses were performed using a Shimadzu C-R4A Chromatopac with a model LC-6A pump, a model SIL-6B auto injector, and a model SPD-6AV UV detector set to 195 nm (Shimadzu, Kyoto, Japan). Analytes were separated on a Phenomenex prodigy ODS 5 micron column (15 cm x 4.6 mm internal diameter). Samples were run for 10 minutes with a 10% acetonitrile mobile phase at a flow rate of 1 ml/min. With this method, metabolites, but not the parent ifosfamide could be detected.

In order to also be able to monitor the concentrations of the parent ifosfamide compound, I utilized another HPLC with a dual pump to allow a mobile phase gradient. The same column was used on a Waters HPLC system (Waters, Milford, Massachusetts) with pump model 510, autoinjector model 712WISP, and a programmable multi-wavelength (diode array) detector model 490 set to 195 nm. Mobile phase A was 10 mM sodium dihydrogen phosphate at pH 4.5. Mobile phase B
was 60% acetonitrile in HPLC grade water. Samples were run at a flow rate of 1 ml/min with the following gradient:

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Mobile Phase A (%)</th>
<th>Mobile Phase B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>6.0</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>12.0</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>15.0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>18.0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>18.5</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
III.5 Results

The GC-MS method was linear for ifosfamide concentrations ranging from 0.48 to 383 μM of each enantiomer of ifosfamide and from 0.32 to 505 μM for each enantiomer of 2-DCEI and 3-DCEI. The recoveries for the enantiomers of ifosfamide and its dechloroethylated metabolites ranged from 80 to 106%. Intra-day and inter-day coefficients of variation were less than 8%. In the eight samples from four incubation mixtures in the first set of ifosfamide biotransformation experiments, 2-DCEI and 3-DCEI were detected but not quantifiable. The mass spectrometry analysis ensured that the peaks observed corresponded to the appropriate dechloroethyl metabolites.

Since the amounts of metabolites detected were too low to quantify in the preliminary experiment, the assay was modified to contain a higher concentration of renal microsomes and the incubation period was extended from one hour to three hours. The concentrations of various energy-generating system components were also increased. To avoid freezing and thawing steps, the samples from this second set of experiments were analyzed in Toronto to allow injection of the samples following their preparation. Again, mass spectrometry confirmed that the peaks produced corresponded to the dechloroethyl metabolites of ifosfamide. However, technical difficulties with this method limited quantification, and the cost of analyzing these samples led us to pursue other methods of detection.

Therefore, for practical reasons, the 2- and 3-dechloroethyl metabolites of ifosfamide were detected using HPLC. The limit of sensitivity was 0.5 μM for 50 μl injections. Liver microsomes were used as a positive control to ensure that the incubation and assay conditions were appropriate for the production and detection of 2-DCEI and 3-DCEI. Concentrations of 2-DCEI and 3-DCEI were almost two-fold higher with the dexamethasone-treated rat liver microsomes than with the untreated liver microsomes (data not shown). Following a 3 hour incubation of pig kidney microsomes with 1 mM ifosfamide in an energy-generating phosphate buffer, concentrations of approximately 5 μM 2-DCEI and 3 μM 3-DCEI were obtained. In
addition, incubation of the pig kidney microsomes with 5 µM ketoconazole reduced the production of metabolites (data not shown), suggesting the involvement of CYP3A.

The production of metabolites was energy-, concentration-, and time-dependent (figure 35, table 2). Production of metabolites appeared to be in a plateau phase by three hours of incubation. No metabolites were detected with the blank control (i.e., no ifosfamide) or the boiled microsomes control. However, interfering amounts of 2-DCEI and 3-DCEI metabolites were occasionally identified in solutions made from ifosfamide. Therefore, the stock solutions added to the incubation mixtures were carefully analyzed for metabolite contamination. No data were used from stock solutions that had these metabolites.

The concentrations of 2-DCEI and 3-DCEI produced from porcine kidney microsomes were higher than those produced from human kidney microsomes when expressed per mg of protein (figure 36); however, human kidney microsomes were prepared from the whole kidney whereas porcine microsomes were prepared from renal cortex.
Figure 35. Production of 2-DCEI and 3-DCEI by porcine renal microsomes. A representative experiment of the production of 2-DCEI (dark bars) and 3-DCEI (light bars) by microsomes prepared from porcine renal cortex shows that metabolite production was dependent on time and concentration.
Figure 36. Human kidney biotransforms ifosfamide to 2-DCEI and 3-DCEI. A representative experiment shows the production of 2-DCEI (dark bars) and 3-DCEI (light bars) by porcine renal microsomes (prepared from renal cortex) and human renal microsomes (prepared from whole kidney) when incubated with 1 mM ifosfamide for 3 hours.
Table 2. Time course of 2-DCEI and 3-DCEI production.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>2-DCEI (µM)</th>
<th>3-DCEI (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>not detectable</td>
<td>not detectable</td>
</tr>
<tr>
<td>30</td>
<td>0.58</td>
<td>0.82</td>
</tr>
<tr>
<td>60</td>
<td>3.13</td>
<td>4.42</td>
</tr>
<tr>
<td>180</td>
<td>5.47</td>
<td>2.13</td>
</tr>
</tbody>
</table>
III.6 Discussion

In examining the role that the kidney plays in drug-induced toxicity, the focus of this study was to determine whether the kidney can biotransform ifosfamide to nephrotoxic metabolites. Ifosfamide is a useful chemotherapeutic agent in the treatment of a number of adult and pediatric cancers. However, despite the coadministration of the uroprotective thiol agent mesna, ifosfamide-induced nephrotoxicity remains a dose-limiting toxicity. Interestingly, nephrotoxicity appears to be a more common and serious complication of ifosfamide therapy in children than in adults. This is of clinical concern because ifosfamide-induced Fanconi syndrome can cause growth retardation and lead to the development of rickets.

Greater understanding of the mechanisms producing this toxicity may aid in the development of measures to reduce the often severe and sometimes permanent renal damage. By examining the differences between ifosfamide and its non-nephrotoxic structural analogue cyclophosphamide, I hypothesized that the production of large quantities of chloroacetaldehyde from ifosfamide was at least partly responsible for ifosfamide-induced nephrotoxicity.

The biotransformation of ifosfamide is well documented as it is a prodrug that requires bioactivation in order to exert its antineoplastic effects. However, to date, studies measuring plasma or urinary ifosfamide metabolites have not identified the origin of their production. While hepatic ifosfamide biotransformation has been demonstrated, the contribution of extrahepatic organs to metabolite production has been largely neglected. In rats, the lung can metabolize ifosfamide, however, to a much smaller extent than the liver (Hill et al., 1973). On the other hand, the percentage of 3-DCEI produced from ifosfamide is higher in intestinal rat microsomes than in hepatic microsomes (Lu and Chan, 1998).

The lability of chloroacetaldehyde supports a site of production proximal to its site of toxicity; therefore, I hypothesized that the kidney biotransforms ifosfamide to chloroacetaldehyde, causing the toxicity. Support for the role of the kidney in the
biotransformation of ifosfamide and cyclophosphamid, comes from the observation that concentrations of the carboxy metabolite are often undetectable in plasma, yet account for up to 25% of the dose in urine (Boddy, 1996b). In this example, the enzyme aldehyde dehydrogenase which is present in the kidney metabolizes aldoifosfamide to the nonactive metabolite carboxyifosfamide.

III.6a Renal Microsomal N-dechloroethylatation of Ifosfamide

I chose to use renal microsomes as a model of ifosfamide biotransformation. Microsomal preparations are easy to work with and concentrate drug metabolizing enzymes. In clinical studies, the quantification of chloroacetaldehyde in the urine does not reflect its total production because of its instability and nonrenal clearance. As a result, the measurement of the more stable 2- and 3-DCEI metabolites (the sum of which are each produced in equimolar amounts with chloroacetaldehyde) has become standard practice. Hence, I chose to indirectly measure chloroacetaldehyde production by monitoring the amounts of 2-DCEI and 3-DCEI. My goal was to see if the kidney had the ability to biotransform ifosfamide to 2-DCEI or 3-DCEI.

In support of my hypothesis, the microsomal studies showed that porcine and human kidney microsomes are capable of metabolizing ifosfamide to 2-DCEI and 3-DCEI. Metabolite production was time-, energy-, concentration- and microsome-dependent, indicating the involvement of an enzyme system. This is consistent with the ability of the kidney to metabolize other drugs (Woodland et al., 1997) and the renal presence of CYP3A, an enzyme responsible for biotransforming ifosfamide to 2-DCEI and 3-DCEI in the liver (Schuetz et al., 1992; Haehner et al., 1996). The importance of this study was to illustrate that local production of drug metabolites, in this case by the kidney, can contribute to toxicity.

The detection of 2-DCEI and 3-DCEI proved very difficult. While the production of 2-DCEI and 3-DCEI was verified by GC/MS, I did not have direct access to this machinery, so it was only used for preliminary experiments to confirm that peaks
corresponded to the appropriate drug metabolites. Phosphorus-31 nuclear magnetic resonance has become a useful technique for measuring ifosfamide metabolites since the parent compound and most of the metabolites contain a phosphorus atom. The advantage of using this technique is that it avoids the problems associated with pH, extraction, recovery, and chemical derivatization while measuring many metabolites simultaneously. However, $^{31}$P nuclear magnetic resonance spectroscopy has low intrinsic sensitivity compared with chromatographic methods and the signals for 3-DCEI and ifosfamide overlap without sample refinement. The time and cost of establishing this assay led to its abandonment after preliminary experiments.

As a result, I used an HPLC method to detect the dechloroethyl metabolites. Unfortunately, the detection limits of HPLC are high because the 2- and 3-DCEI metabolites have a very large molar absorptivity (molar extinction coefficient = 1000). Therefore, the measurement by HPLC was difficult because the absorbance maximum (190 nm) was at the low extreme of the detector due to poor ultraviolet absorbance qualities of ifosfamide, 2-DCEI, and 3-DCEI. At this wavelength, Goren (1991) found the absorbances of the chromatographic peaks for aqueous solutions of 2- and 3-DCEI to be 89% and 38%, respectively, of that of ifosfamide at concentrations between 100 μM and 1 mM.

My HPLC results occasionally showed higher concentrations of 2-DCEI than 3-DCEI, indicating greater side chain oxidation than ring oxidation. This differs from the literature in which plasma and urinary concentrations of 3-DCEI are approximately three-fold higher than those for 2-DCEI (Kaijser et al., 1992) reflecting greater ring oxidation, but concurs with the results of Springate et al. (1997). These differences may result from the use of an in vitro versus in vivo methodology. Interestingly, in both in vitro (Wagner, 1994a) and human studies (Hempel et al., 1997), side chain oxidation appears to prevail since higher concentrations of ifosfamide than cyclophosphamide were produced from trofosfamide. As shown in figure 37, when trofosfamide is dechloroethylated at the exocyclic position, ifosfamide is produced.
Figure 37. Structures of ifosfamide, cyclophosphamide, and trofosfamide.
Many CYP enzymes are reportedly involved in the biotransformation of oxazaphosphorine agents. In rats, the enzymes responsible for ifosfamide activation are CYP3A, CYP2B1/2, and CYP2C6/11 (Weber and Waxman, 1993), while in humans, CYP3A4 is the predominant activating enzyme (Walker et al., 1994). Cyclophosphamide and ifosfamide go through the same pathways of biotransformation; however, the contribution of CYP isoenzymes differs between the two drugs.

Murray et al. (1994) conducted experiments of testosterone 6β-hydroxylation with human liver microsomes. Cyclophosphamide (Ki = 510 ± 20 μM) and ifosfamide (Ki = 490 ± 40 μM) were competitive inhibitors of CYP3A-mediated testosterone 6β-hydroxylation. (CYP3A had approximately five-fold lesser affinity for cyclophosphamide and ifosfamide than for testosterone.) On the other hand, neither cyclophosphamide nor ifosfamide inhibited CYP1A2-dependent 7-ethoxyresorufin O-deethylolation, CYP2C-dependent tolbutamide methyl hydroxylation, or CYP2E1-mediated N-nitrosodimethylamine N-demethylation.

Chang et al. (1993) examined the 4-hydroxylase activities of cyclophosphamide and ifosfamide in human liver microsomes in the presence and absence of inhibitors and antibodies to various CYP enzymes. They also looked at the bioactivation of these drugs by cDNA expression systems for human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2E1, and CYP3A4. Both cyclophosphamide and ifosfamide were activated by CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP3A4, but not CYP1A1, CYP1A2, CYP2D6 or CYP2E1. In the human liver microsomes, CYP3A and CYP2B preferentially bioactivate ifosfamide and cyclophosphamide, respectively. Furthermore, Weber and Waxman (1993) found that pretreatment of rats with dexamethasone increased (by about 6-fold) hepatic microsomal ifosfamide activation, but not cyclophosphamide activation, suggesting that one or more dexamethasone-inducible P450s may contribute to liver microsomal activation of ifosfamide but not cyclophosphamide. While the study by Chang et al. (1993) demonstrated the
involvement of CYP2C9 in cyclophosphamide bioactivation, it was later shown to be the major enzyme involved in cyclophosphamide 4-hydroxylation by Ren et al. (1997).

The dechloroethylation pathway of ifosfamide biotransformation also involves CYP3A (Walker et al., 1994). N-dechloroethylation was inhibited by about 80% by anti-human CYP3A4 antiserum. Inhibition of dechloroethylation was also found with the CYP3A4 inhibitors naringenin (IC$_{50} = 70$ μM) and triacetyloleandomycin (IC$_{50} = 10$ μM). Nifedipine oxidase activities in 12 samples of human liver microsomes correlated with N-dechloroethylation (p < 0.001). Therefore, it was first thought that inhibition of CYP3A would have little effect on the ratio of activated products to side chain products, since both are produced by CYP3A. However, by studying the enantiomeric separation of the dechloroethylated metabolites, Wainer et al. (1996) demonstrated the involvement of at least one other enzyme in ifosfamide side chain biotransformation. CYP2B6 was recently shown to biotransform ifosfamide to its dechloroethyl metabolites (Granvil et al., 1999). This enzyme has remarkably less activity in the bioactivation pathway of ifosfamide than of cyclophosphamide. In summary, differences in bioactivation and dechloroethylation between cyclophosphamide and ifosfamide may be explained by differences in the profiles of the involved enzymes.

Most of the metabolic data on CYP3A concerns its hepatic activity. All human livers express CYP3A4, whereas until quite recently the hepatic expression of CYP3A5 was thought to only be about 20% (Schuetz et al., 1992). However, in 1998, Kostrubsky et al. reported that by loading higher amounts of protein onto their immunoblots the detection rate for CYP3A5 increased to 94% indicating that CYP3A5 is expressed in most human livers. Rifampicin and phenobarbital induced CYP3A5 in primary cultures of human hepatocytes, although the induction of CYP3A5 was less than that for CYP3A4. On the other hand, the expression of CYP3A3/4 in human kidney was shown to be polymorphic with only one in seven (14%) adult human kidneys tested found to express this protein (Schuetz et al., 1992). In a later study by Haehner et al. (1996), polymerase chain reactions with specific oligonucleotides as primers were used to
determine the expression of CYP3A4 and CYP3A5 in 27 human kidney microsomal samples. CYP3A4 mRNA was detected in eleven of 27 (40%) human kidneys, while the corresponding CYP3A4 protein was found in seven of ten kidney samples containing CYP3A4 mRNA. Interestingly, CYP3A5 was consistently detected in adult human kidney at the mRNA and protein levels (Schuetz et al., 1992; Haehner et al., 1996). Interindividual differences in the expression of drug metabolizing enzymes may explain why not all children are adversely affected by ifosfamide therapy.

Tissue distribution of CYP2B6, the other enzyme responsible for dechloroethylation of ifosfamide, is not well characterized. Mimura et al. (1993) reported infrequent and low expression of CYP2B6 in human liver. However, Gervot et al. (1998) were able to detect CYP2B6 by immunoblotting in 45 of 48 human livers tested. CYP2B6 was also detected at the protein and mRNA levels in human kidney. Phenobarbital (2 mM) and cyclophosphamide (1 mM) induced CYP2B6 protein and mRNA. However, CYP2B6 expression or inducibility could not be demonstrated in human cell lines.

My finding that incubation of renal microsomes with the relatively specific CYP3A inhibitor ketoconazole reduces metabolite production is supportive of a biotransformation pathway involving CYP3A. In in vitro studies, it must be remembered that the concentrations of substrate (that is, ifosfamide) are much higher than the in vivo situation where the maximum concentrations of the enantiomers of ifosfamide are 200 μM and by three hours following the end of the infusion have dropped to below 100 μM. Therefore, the biotransformation of ifosfamide in vitro by a high capacity enzyme such as CYP3A may not reflect the true metabolic profile of the in vivo situation (Wainer et al., 1996).

Importantly, I demonstrated the ability of kidney microsomes to metabolize ifosfamide. While these preliminary results support the hypothesis that chloroacetaldehyde produced within the kidney may contribute to nephrotoxicity, thus far, chloroacetaldehyde has been identified in an indirect manner. Interpatient
variability in the formation, and possibly the elimination, of chloroacetaldehyde may account for susceptibility to nephrotoxicity among children treated with ifosfamide.

III.6c Renal Drug Biotransformation

In support of the renal drug biotransformation hypothesis, Kharasch et al. (1993) implicate intrarenal fluoride production as a possible mechanism for nephrotoxicity due to methoxyflurane. The fluorinated ether anesthetics enflurane, isoflurane, methoxyflurane, and sevoflurane, undergo oxidative defluorination resulting in the liberation of a free fluoride ion. Nephrotoxicity due to methoxyflurane was historically believed to be a result of plasma fluoride concentrations in excess of 50 μM. Methoxyflurane-induced renal toxicity was shown to be dose-dependent in rats (Mazze et al., 1972). However, the related anesthesia drug sevoflurane, is also metabolized to fluoride, with plasma concentrations above 50 μM not causing nephrotoxicity. These observations led investigators to suspect that the area under the fluoride concentration-time curve might be a better predictor of nephrotoxicity than peak plasma fluoride concentrations. Whereas the AUC of sevoflurane is smaller than that of methoxyflurane, sustained plasma fluoride concentrations above 50 μM have been documented following prolonged enflurane and isoflurane anesthesia without associated renal toxicity. Taken together, these findings suggest that something other than increased plasma fluoride concentrations is responsible for anesthetic-induced nephrotoxicity.

In animals, renal defluorination of methoxyflurane occurs at a rate almost half as fast as with liver; whereas enflurane is only slightly metabolized (Blitt et al., 1981). In liver, CYP2E1 is primarily responsible for the biotransformation of enflurane, sevoflurane, and methoxyflurane. According to chemical inhibition studies, methoxyflurane is also metabolized by CYP1A2, CYP2C, and CYP2D6 (Kharasch and Thummel, 1993). In rats, kidney has about 10% of the CYP2E1 found in liver (de
Waziers et al., 1990). However, no significant amounts of CYP2E1 have been found in human kidney.

With these considerations in mind, the authors examined the relative rates of methoxyflurane and sevoflurane defluorination by human kidney microsomes. Incubation of kidney microsomes with methoxyflurane and sevoflurane led to fluoride production. However, the production of fluoride from methoxyflurane was substantially greater and more variable between kidneys than from sevoflurane. Production of fluoride by the kidney was 1.9% and 1.2% of that of liver for methoxyflurane and sevoflurane, respectively.

As mentioned previously, CYP2B6, CYP2E1 and CYP3A4/5 activity are characterized in human kidney. The presence of CYP2A6 activity in human kidney is suggested by enzyme activity studies (Pacifici et al., 1989; Yamano et al., 1990). Chemical inhibitors of CYP2E1, CYP2A6 and CYP3A were able to decrease the metabolism of both methoxyflurane and sevoflurane. The order of defluorination by CYP enzymes in cDNA expression systems was CYP2E1 greater than CYP2A6 greater than CYP3A4 with three- to ten-times faster CYP production of fluoride from methoxyflurane than from sevoflurane. While expressed CYP2B6 was able to catalyze methoxyflurane defluorination, its selective inhibitor had no effect on renal methoxyflurane biotransformation, suggesting that the kidney does not have appreciable amounts of CYP2B6.

The greater renal production of fluoride from methoxyflurane than from sevoflurane may explain why methoxyflurane is nephrotoxic and sevoflurane is not. However, whether fluoride production by the kidney is the cause of methoxyflurane nephrotoxicity is still under debate. Nevertheless, the study by Kharasch et al. (1995) clearly demonstrates the ability of the kidney to biotransform drugs, presenting another example of the role of the kidney in drug metabolism.
The oxidation of ifosfamide produces aldehydes such as chloroacetaldehyde, acrolein, and aldoifosfamide. Quantitatively, chloroacetaldehyde is a more important metabolite of ifosfamide biotransformation than of cyclophosphamide biotransformation. Given that the production of large amounts of chloroacetaldehyde from ifosfamide appears to be the greatest difference between the two oxazaphosphorine agents, chloroacetaldehyde is thought to explain why ifosfamide is nephrotoxic and cyclophosphamide generally is not. A further consideration is that ifosfamide is often dosed on a fractionated schedule resulting in much higher production of chloroacetaldehyde than the single dose regimen which is more commonly used with cyclophosphamide. The AUCs for DCEI metabolites (reflecting chloroacetaldehyde concentrations) are lower following a bolus injection versus a 72-hour continuous infusion (Boddy et al., 1995). Moreover, chloroacetaldehyde has been shown to produce nephrotoxicity in cell culture and animal renal perfusion models (Mohrmann et al., 1992; 1994a; 1995; Zamlauski-Tucker et al., 1994).

In my experiments, the ability of renal microsomes to biotransform ifosfamide to 2-DCEI and 3-DCEI indirectly implies that chloroacetaldehyde is also produced since ifosfamide is oxidized to equimolar amounts of 2-DCEI or 3-DCEI and chloroacetaldehyde. These data concur with a recent study by Springate et al. (1997) who demonstrated the renal production of 2- and 3-DCEI metabolites using an isolated perfused rat kidney preparation. However, these authors did not address the possibility of contamination of ifosfamide stock solutions with metabolites. In the present study, the 2- and 3-DCEI metabolites were detected in several ifosfamide stock powders; therefore, incubation solutions should be assayed for metabolites before assumptions of microsomal metabolism are made.

As well, even if the kidney demonstrates the ability to produce 2-DCEI and 3-DCEI, reflecting chloroacetaldehyde production, the amounts of metabolite produced must be sufficient to cause toxicity if the chloroacetaldehyde hypothesis is true.
Concentrations of chloroacetaldehyde of 80-100 μM and 200-220 μM in serum and urine, respectively, have been achieved in patients receiving ifosfamide (Goren et al., 1986). In cultured human proximal renal tubular (Landa Leiden) cells, incubation with 25 μM chloroacetaldehyde, a concentration that reflects typical chloroacetaldehyde plasma concentrations in ifosfamide patients, almost completely killed all of the cells within four hours (Brüggemann et al., 1997).

Given that chloroacetaldehyde is produced in equimolar concentrations with 2-DCEI or 3-DCEI, the maximal concentrations of chloroacetaldehyde generated in my experiments with kidney microsomes can be estimated. Based on the production of 2-DCEI and 3-DCEI, micromolar concentrations of chloroacetaldehyde can be generated by the kidney. However, in my experiments, the renal microsomes were incubated with higher concentrations of ifosfamide than those seen clinically. In the study by Springate et al. (1997) rat kidneys were exposed to only 77 μmol of ifosfamide in perfusate. Following 30 minutes of perfusion, urinary concentrations of 2-DCEI and 3-DCEI were 1.02 ± 0.33 μM and 0.54 ± 0.05 μM, representing 0.005% and 0.004% of the infused ifosfamide, respectively. Further study is needed to determine the concentrations that would be produced by the kidney in patients receiving standard ifosfamide therapy.

Springate and Van Liew (1995) used an animal model to examine the nephrotoxicity of ifosfamide. They administered either 40 mg/kg or 80 mg/kg of ifosfamide intraperitoneally to rats daily for three consecutive days every three weeks for a total of four treatment courses. These doses are in the range of those given to humans with cancer. Various measures of renal function were studied two to four days prior to ifosfamide administration and on day one following the week four and week ten ifosfamide treatments. One of seven rats in the 40 mg/kg group and two of eight rats in the 80 mg/kg group died compared with no rats in the control group. In addition to significantly lower body weights, the rats in the 40 mg/kg group developed isolated phosphaturia after the fourth and final treatment courses, while the rats in the 80 mg/kg group had low-grade glucosuria, phosphaturia and proteinuria throughout
the study. The rats showed no significant differences from controls with respect to plasma concentrations of sodium, glucose, potassium, total protein, inorganic phosphorus, creatinine; urine flow rate; creatinine clearance; urinary sodium and potassium excretion; nor kidney glutathione and malondialdehyde content (reflecting lipid peroxidation). Therefore, ifosfamide produced dose-dependent abnormalities in proximal tubule function. Interestingly, oxidant stress did not appear to play an important role in drug-induced toxicity based on the lack of changes in glutathione and malondialdehyde levels.

A number of in vitro studies have demonstrated the toxic effects of chloroacetaldehyde on renal tubular cells. Mohrmann et al. (1992; 1993; 1994a; 1994b; 1995) tried to produce elements of the Fanconi syndrome experienced by some patients taking ifosfamide in cultured renal tubular cells. In an attempt to identify the species responsible for tubular damage, they first investigated the effects of ifosfamide and its metabolites 4-hydroxyifosfamide, chloroacetaldehyde, and acrolein, on transport and enzyme systems in LLC-PK1 renal tubular cell monolayers (Mohrmann et al. 1992; 1993). The transport systems investigated included the sodium-dependent and sodium-independent fluxes of l-alanine, D-glucose and phosphate, representing functions commonly affected by ifosfamide in Fanconi syndrome. The activities of alkaline phosphatase, ouabain-sensitive Na⁺-K⁺-ATPase, leucine aminopeptidase, KCN-resistant NADH oxidoreductase, and succinate-cytochrome c oxidoreductase (succinate dehydrogenase) were also determined.

In these in vitro studies, incubation of renal tubular cells with ifosfamide (100 μM for 24 hours (that is, a clinically-relevant concentration) or up to 400 μM for 2 hours) did not induce any changes in the transport of l-alanine or D-glucose, whereas ifosfamide induced a moderate stimulation of phosphate transport. Using 4-hydroperoxyifosfamide at the same concentrations (100 μM for 24 hours or up to 400 μM for 2 hours) which are higher than 4-hydroxyifosfamide concentrations produced in patients on ifosfamide therapy (30 μM in urine; Wagner et al., 1981), the 4-hydroxy metabolite
derived from 4-hydroperoxyifosfamide inhibited the sodium-dependent fluxes of both l-alanine (only in the 24-hour incubation) and D-glucose. On phosphate transport, 4-hydroperoxyifosfamide had a biphasic response (stimulatory at low concentrations (100-200 µM for 2 hours), and inhibitory at high concentrations). Following a two-hour exposure to 400 µM 4-hydroperoxyifosfamide, the activity of succinate-cytochrome c oxidoreductase was significantly reduced; whereas a 24-hour exposure to 100 µM concentrations had no effect. Of course, 4-hydroxyifosfamide is in spontaneous equilibrium with aldoifosfamide which forms ifosforamide mustard and acrolein so whether it is the 4-hydroxyifosfamide itself which is toxic is unclear. When applied directly, acrolein was the most toxic metabolite tested causing damage to the cell monolayers and reducing all activities tested.

Chloroacetaldehyde had a biphasic effect on sodium-dependent l-alanine transport. Concentrations up to 200 µM (such as the blood and urine concentrations seen in patients receiving ifosfamide) were stimulatory but beyond this chloroacetaldehyde had an inhibitory effect. Sodium-dependent glucose transport was also biphasic in the presence of chloroacetaldehyde; although the inhibition of sodium-dependent glucose transport was accompanied by an increase in sodium-independent uptake of glucose. Chloroacetaldehyde had a similar biphasic effect on phosphate transport with concentrations of up to 75 µM increasing sodium-dependent phosphate transport and concentrations above 125 µM inhibiting it. The authors studied the effects of chloroacetaldehyde on sodium-dependent phosphate transport in the presence of cycloheximide and determined that protein synthesis is not required for the stimulatory effects. However, the increase in phosphate transport was accompanied by an increase in Na⁺-K⁺-ATPase activity. Chloroacetaldehyde also reduced the activity of succinate-cytochrome c oxidoreductase, implying a defect in ATP generation.

In a subsequent study, Mohrmann et al. (1994a) used the same in vitro model to examine the effects of a one-hour exposure to up to 300 µM of ifosfamide, cyclophosphamide, 4-hydroxyifosfamide, 4-hydroxycyclophosphamide, acrolein, and
chloroacetaldehyde on protein content, and the incorporation of uridine and thymidine reflecting RNA and DNA synthesis, respectively. Again, ifosfamide and cyclophosphamide were less toxic than their metabolites. The only parameter affected by the parent compounds was thymidine incorporation, with maximal inhibition of 30% with 300 μM. Acrolein and the 4-hydroxy metabolites also decreased thymidine incorporation. At high concentrations uridine incorporation was decreased by 4-hydroxycyclophosphamide but not by 4-hydroxyifosfamide. Similarly, 4-hydroxyifosfamide had a smaller effect on protein content than 4-hydroxycyclophosphamide. Acrolein reduced uridine incorporation and protein content to the greatest extent.

Chloroacetaldehyde significantly decreased total protein at concentrations of 50 μM. Thymidine incorporation was only reduced by 10-25% at low and intermediate concentrations of chloroacetaldehyde; however, concentrations of 300 μM reduced the incorporation of thymidine by 50%. On the other hand, the incorporation of uridine was strongly increased by all concentrations of chloroacetaldehyde tested except 300 μM, suggesting that a repair mechanism may be stimulated.

Next, Mohrmann et al. (1996b) examined the effects of preincubating the LLC-PK1 cells with mesna or dimesna for one hour prior to the addition of ifosfamide or cyclophosphamide or their metabolites. Thymidine or uridine incorporation and total protein were measured following exposures to the potentially toxic agents of one- to 24-hours. Mesna, but not dimesna, was able to completely prevent the alterations in thymidine and uridine incorporation caused by acrolein and 4-hydroxy-cyclophosphamide. On the other hand, even large amounts of mesna (5 mM) could not completely protect the cells from toxicity due to 4-hydroxyifosfamide or chloroacetaldehyde. These findings are consistent with the observations of nephrotoxicity in humans in the presence of high concentrations of mesna. It is unlikely that higher concentrations of mesna would afford greater protection given the constant rate of inhibition of thymidine incorporation when the authors tested a wide range of concentrations.
Chloroacetaldehyde was also shown to induce renal toxicity when Mohrmann et al. (1995) studied the effects of ifosfamide, 4-hydroperoxyifosfamide, chloroacetaldehyde, and acrolein on the Na⁺/H⁺ exchanger localized in the basolateral membrane of LLC-PK1 cells in the presence and absence of mesna or dimesna. While a two-hour incubation of LLC-PK1 cells with 100-400 µM ifosfamide had no effect on Na⁺/H⁺ exchange, a 24-hour incubation with 400 µM ifosfamide cause about a 25% inhibition. A similar incubation with 4-hydroperoxyifosfamide caused complete inhibition of Na⁺/H⁺ exchange. Likewise, complete inhibition was achieved with a two-hour incubation with 200-300 µM chloroacetaldehyde or a four-hour incubation with 100 µM. Again, acrolein demonstrated the highest toxicity.

Dimesna was unable to reverse the toxic effects of ifosfamide metabolites. On the other hand, mesna completely reversed inhibition of Na⁺/H⁺ exchange for all compounds tested. Unlike the in vivo situation, LLC-PK1 cells incubated with dimesna are unable to produce mesna, explaining the lack of effect of dimesna in the experimental system (Mohrmann et al., 1994b).

The small effect of the parent ifosfamide compound on transport and enzyme activities, even when incubated with cells for 24 hours, implies that the cells do not metabolize ifosfamide significantly to produce toxic metabolites. Cells in culture are known to lose their metabolic capabilities. However, both ifosfamide and cyclophosphamide decreased thymidine incorporation suggesting that there is some metabolic activity (Mohrmann et al., 1994a).

Chloroacetaldehyde has not only been shown to be highly nephrotoxic in cell monolayers, but also in perfused rat kidney models (Zamlauski-Tucker et al., 1994; Springate, 1997). Chloroacetaldehyde (210 µM) was found to impair function in the isolated perfused rat kidney (Zamlauski-Tucker et al., 1994). The fractional reabsorption of sodium, glucose, inorganic phosphate, and inorganic sulfate were all decreased. On the other hand, neither ifosfamide (470 µM) nor acrolein (470 µM) caused functional changes to the perfused kidney.
In another study, Springate (1997) perfused rat kidneys with 0, 65, 192, or 512 μmol of chloroacetaldehyde for 30 minutes. The initial blood concentrations of chloroacetaldehyde delivered to the kidney were calculated to be between 100 μM and 900 μM. The lowest dose of chloroacetaldehyde (64 μM) caused a significant rise in urinary glucose excretion without altering the urinary flow rate or sodium or protein excretion. However, higher doses produced progressively increasing diuresis, natriuresis, glycosuria and proteinuria. In the presence of chloroacetaldehyde, there was a dose-dependent decrease in glutathione and an increase in malondialdehyde content. Pre-equilibration for 30 minutes with 600 mg/kg of mesna was able to reduce the concentrations of malondialdehyde, but could not reverse the glutathione depletion. Perfusion of rat kidneys with mesna alone also caused a significant reduction in glutathione levels, as expected by the requirement of glutathione to convert dimesna to mesna. Administration of mesna was unable to prevent chloroacetaldehyde-induced diuresis, natriuresis, or reduced PAH clearance. Likewise, mesna significantly reduced, but was unable to normalize, the urinary excretion of glucose and protein in rats given 192 μmol of chloroacetaldehyde.

III.6e Possible Mechanisms for Chloroacetaldehyde-Induced Toxicity

Chloroacetaldehyde is an alkylating agent and potent mutagen (Malaveille et al., 1975), which forms etheno compounds with DNA adenosine and cytosine (Leonard, 1984), induces interstrand DNA cross-links (Sprengler and Singer, 1988), and inhibits the synthesis of DNA (Kandala et al., 1990). The chloroacetaldehyde metabolite is widely believed to be responsible for ifosfamide-induced neurotoxicity. Chloroacetaldehyde plasma concentrations are higher, as is the incidence of neurotoxicity, when ifosfamide is given orally rather than intravenously; although there is no relationship between the plasma concentrations of chloroacetaldehyde and the development or severity of neurotoxicity (Kurowski et al., 1991; Kaijser et al., 1994).
Sood and O'Brien (1993) examined the effects of chloroacetaldehyde on rat hepatocyte toxicity. Using trypan blue exclusion, they showed that hepatocytes incubated with 300 μM chloroacetaldehyde had a 50% loss of viability within two hours. This cytotoxicity was concentration- and time-dependent. Cytotoxicity was preceded by GSH depletion, inhibition of respiration, ATP depletion and lipid peroxidation. Moreover, the glutathione depletion and mitochondrial toxicity were not caused by the lipid peroxidation.

The removal of any unmetabolized chloroacetaldehyde following ten minutes of incubation and resuspension of the cells in fresh buffer did not affect toxicity. The toxicity was therefore irreversible. The addition of antioxidants or iron chelators following a 40-minute incubation with chloroacetaldehyde delayed cytotoxicity, while the thiol reductant dithiothreitol that breaks disulfide bonds completely prevented it. Dithiothreitol, but neither antioxidants nor iron chelators, restored protein thiols and hepatocyte respiration and prevented further depletion of ATP and cytotoxicity, indicating that chloroacetaldehyde forms reversible protein thiol adducts, allowing the proteins to regain their functions.

On the other hand, addition of dithiothreitol could not restore glutathione levels in the hepatocytes, illustrating that chloroacetaldehyde forms irreversible bonds with glutathione. Exposure to 300 μM and 500 μM chloroacetaldehyde depleted hepatocyte glutathione levels by 90% within three minutes. Lower chloroacetaldehyde concentrations only partially depleted glutathione levels when hepatocytes were exposed for one hour. Glutathione-depleted cells were more sensitive to the toxic effects of chloroacetaldehyde than undepleted cells.

Recently, methylene blue has been identified as a potential antidote for ifosfamide-induced neurotoxicity. Methylene blue has been used successfully to treat ifosfamide encephalopathy in humans (Küpf er et al., 1994; Zulian et al., 1995). Thus, an understanding of why methylene blue is protective against ifosfamide-induced
neurotoxicity may help in understanding how ifosfamide produces neurotoxicity. This may, in turn, help in the understanding of ifosfamide-induced nephrotoxicity.

One proposed explanation for ifosfamide-induced neurotoxicity involves the production of chloroethylamine. Chloroethylamine has been found in the plasma and urine of patients given ifosfamide (Highley et al., 1995). Chloroethylamine may be a product of chemical hydrolysis of ifosfamide or ifosforamide mustard. It is more prominent following intravenous rather than oral dosing. According to Highley et al. (1995), chloroethylamine is a major degradation product of ifosfamide that is found in the parent compound powder; yet Gilard et al. (1997) argue that the formation of chloroethylamine was a result of the derivatization procedure used to prepare samples for measurement. Chloroethylamine has alkylating activity, but only about one-fifth as much as the comparable metabolite from cyclophosphamide, normitrogen mustard.

In vivo, chloroethylamine combines with cysteine to produce a structural analogue of lysine called thialysine. Thialysine will follow the metabolic pathway of lysine forming a cyclic metabolite called thialysine ketamine which is a potent inhibitor of electron transfer proteins called flavoproteins. The endogenous ketamines present in several mammalian tissues are inactivated by ketamine reductases (Nardini et al., 1988a, b). However, ketamines may bind to specific sites in the brain resulting in CNS activity (Fontana et al., 1990).

Chloroethylamine is oxidized to chloroacetaldehyde. Chloroacetaldehyde should be quickly converted to chloroacetate in vivo (Norpoth, 1976). Chloroacetaldehyde may also be transformed into cysteinyld derivatives and thioacetals (Norpoth, 1976). Aldehyde dehydrogenase oxidizes chloroacetaldehyde to chloroacetic acid. Genetic variations in aldehyde dehydrogenase may result in higher concentrations of chloroacetaldehyde in patients with aldehyde dehydrogenase deficiencies. Likewise, NAD (nicotinamide adenine dinucleotide) is a cofactor for aldehyde dehydrogenase. Therefore, a reduction in NAD due to inhibition of NADH oxidation by thialysine ketamine will result in less oxidation of chloroacetaldehyde to chloroacetic acid.
Chloroacetic acid combines with cysteine to form carboxymethylcysteine which degrades to thiodiglycolic acid (Hofmann et al., 1991). This requires about 1.5 to 2.0 g of cysteine per day (Küpfer et al., 1996). Consequently, plasma concentrations of cysteine, homocysteine, and glutathione fall significantly (to about 20% of their initial values by 5 days of treatment) (Lauterburg et al., 1994). Chloroacetic acid may therefore disrupt the Krebs cycle if there is a lack of cysteine or glutathione for conjugation (Peters, 1952).

The exact mechanism(s) by which methylene blue counteracts the neurotoxic effects of ifosfamide is(are) unclear. A number of possible mechanisms have been proposed (Küpfer et al., 1996). The authors attribute ifosfamide neurotoxicity to flavoprotein inhibition by thialysine ketamine, an intracellular redox imbalance caused by inhibition of NADH oxidation by thialysine ketamine in the respiratory chain combined with an overproduction of NADH from aldehyde metabolism, and the effects of thialysine ketamine on the CNS.

Methylene blue is an electron accepting drug that is useful in treating flavoprotein deficiency and that can oxidize the excessive quantity of NADH that is formed during ifosfamide biotransformation. It is also an inhibitor of multiple amine oxidase activities and therefore, if administered prophylactically, can prevent the conversion of chloroethylamine to chloroacetaldehyde. In addition, methylene blue inhibits the formation of superoxides.

In summary, ifosfamide metabolites are toxic to mitochondria. Theoretically, if ifosfamide-induced neurotoxicity and nephrotoxicity are caused by the same mechanism, that is chloroacetaldehyde toxicity, methylene blue should alleviate nephrotoxicity as well as neurotoxicity.

III.6f Chirality of Ifosfamide and its N-dechloroethyl Metabolites

Ifosfamide and its 2- and 3-DCEI metabolites are chiral compounds. They have stereoisomers whose mirror images are not superimposable; therefore, they are classified as enantiomers. Enantiomers can differ in both pharmacokinetic and
pharmacodynamic parameters. Thus, a further consideration of ifosfamide biotransformation involves careful examination of the stereoisomers of ifosfamide and its metabolites.

Ifosfamide and its 2- and 3-DCEI metabolites contain an asymmetrically-substituted phosphorus atom which allows them to exist as (+) R- or (-) S-enantiomers. Using a rat mammary carcinoma model, Wainer et al. (1994b) concluded that the R-enantiomer of ifosfamide is metabolized to a greater extent by the activation pathway than the S-enantiomer. Conversely, the S-enantiomer is metabolized to a greater extent than the R-enantiomer by the dechloroethylation pathway (Wainer et al., 1994b). Reports in mice suggest that ketoifosfamide is produced mainly from S-ifosfamide (Blaschke and Widey, 1990). According to the Cahn-Ingold-Prelog nomenclature system, S-2-DCEI and R-3-DCEI are derived from S-ifosfamide, while R-ifosfamide is the source of R-2-DCEI and S-3-DCEI (figure 38). In mice, Blaschke and Widey (1990) found a two-fold higher production of the R-enantiomer of 2-DCEI than the S-enantiomer, while three-fold higher concentrations of the S-enantiomer of the 3-DCEI metabolites were detected.

However, the stereoselectivity of ifosfamide biotransformation varies greatly between species. Masurel et al. (1990) compared the effects of R-ifosfamide, S-ifosfamide, and the racemic mixture in vivo in mice and in vitro with microsomes prepared from mice. The authors looked at the efficacy, toxicity, and the production of the activation pathway metabolites aldoifosfamide and isofosforamide mustard. The \( K_m \) and \( V_{max} \) values for the two metabolites were similar when either of the enantiomers or the racemic mixture were used as the substrate for hepatic microsomes. Likewise, efficacy and toxicity values were similar between the three compounds in the in vivo studies. Although bladder toxicity, defined as blood in the urine, was only observed in the mice receiving the S-enantiomer.
Figure 38. Enantioselective ifosfamide biotransformation.
In humans, ifosfamide is generally administered as a racemic mixture, but it demonstrates stereoselective nonrenal clearance (Granvil et al., 1996). The renal clearance of ifosfamide does not appear to be stereoselective and represents only 24% to 30% of the total clearance for S-ifosfamide and R-ifosfamide, respectively. The authors reported 1.27-fold higher AUCs for R-ifosfamide versus S-ifosfamide following administration of the racemic mixture with the R-enantiomer representing 56% of the total ifosfamide AUC. The lower total clearance of R-ifosfamide versus S-ifosfamide was reflected in the significantly longer half-life (approximately eight hours versus six hours). The volume of distribution at steady state was lower for the R-enantiomer of ifosfamide (25.7 L/m²) than for the S-enantiomer (27.4 L/m²). Of note in this study was the large interpatient variation in dechloroethylation, suggesting that CYP expression and activities may vary greatly between patients.

Granvil et al. (1993) examined the plasma and urine concentrations of the enantiomers of ifosfamide and its dechloroethyl metabolites from both women with cervical cancer and laboratory rats. They found that in human and rat plasma, the major metabolite of side-chain metabolism was R-3-DCEI. Following R-3-DCEI, the concentrations of metabolites in human plasma were S-3-DCEI > S-2-DCEI > R-2-DCEI. In patients, the ratio of plasma concentrations of R-ifosfamide to S-ifosfamide increased from 1.03:1 at the end of a 170 minute infusion (3 g/m²) to 2.35:1 at 16-hours. Similarly, in a different study, the urine of two patients given racemic ifosfamide contained high concentrations of R-ifosfamide and S-2-DCEI with the production of dechloroethyl metabolites being 2.7 to 6.7-fold higher from the S-enantiomer of ifosfamide (Misiura et al., 1983). In a pediatric patient population, the clearance of S-ifosfamide was also significantly higher than that of R-ifosfamide (Wainer et al., 1988). However in rat plasma, concentrations of R- and S-ifosfamide remained roughly equal throughout the sampling period (Granvil et al., 1993).

Wainer et al. (1994b) used an in vivo rat mammary carcinoma model to examine the efficacy and toxicity of the stereoisomers of ifosfamide. The R- and S-enantiomers had
equal efficacy against the MatB mammary carcinoma; however R-ifosfamide was more myelotoxic and lethal. Their findings suggested that metabolism of ifosfamide by the rat is closer to that of humans than that of the mouse. In both humans and rats, the N-dechloroethylation pathway is enantioselective for S-ifosfamide. In the rat, R-ifosfamide is cleared more quickly than S-ifosfamide. This seems logical since bioactivation proceeds more rapidly and to a greater degree than N-dechloroethylation.

In an effort to better understand ifosfamide-induced neurotoxicity, Wainer et al. (1994a) examined the urinary excretion of ifosfamide and its metabolites with respect to enantiomers in seven female cancer patients. They found that the urinary excretions of R-2-DCEI and R-3-DCEI are linked as are the urinary excretions of S-2-DCEI and S-3-DCEI. Hence, by observing the pharmacokinetics of the enantiomers of ifosfamide, 2-DCEI, and 3-DCEI, it becomes obvious that in addition to CYP3A, at least one other enzyme is involved in ifosfamide dechloroethylation.

One out of the seven patients in the study by Wainer et al. (1994a) had severe neurotoxicity. This patient had higher urinary concentrations of ifosfamide and its dechloroethyl metabolites than the other patients. When the compounds were analyzed stereoselectively, it was revealed that the patient with neurotoxicity had significantly higher urinary concentrations of R-3-DCEI than the other patients. The excretion of metabolites produced from S-ifosfamide was much higher than those produced from R-ifosfamide. These findings suggest that patients who over-express the isoenzyme responsible for producing R-2-DCEI and R-3-DCEI are at greater risk of neurotoxicity from ifosfamide. However, cyclophosphamide can only produce 3-DCEI. Therefore, the lack of toxicity from cyclophosphamide suggests that if DCEI metabolites are responsible for neuro- or nephrotoxicity, it should be the 2-DCEI metabolites.

In a later study, in 11 patients treated with ifosfamide for recurrent pelvic carcinoma, the cumulative excretion of S-ifosfamide was significantly less than that of R-ifosfamide (Wainer et al., 1996). The urinary 2- and 3-DCEI metabolites accounted for 10.3% and 20.9% of the ifosfamide dose, respectively. The excretion of R-3-DCEI was
higher than that of S-3-DCEI and the excretion of S-2-DCEI was higher than that of R-2-DCEI. Two patterns of urinary excretion were observed confirming that the production of R-2-DCEI and R-3-DCEI metabolites is related as is the formation of the S-2-DCEI and S-3-DCEI metabolites.

In a study of the urinary excretion of the enantiomers of ifosfamide and its dechloroethyl metabolites, six out of 14 children had higher amounts of S-3-DCEI, while the remaining eight patients had higher levels of R-3-DCEI. The children who excreted more S-3-DCEI also excreted greater amounts of unmetabolized R-ifosfamide. The ratio of recovered R/S for 3-DCEI ranged from 67%/33% to 36%/64%. The major 2-DCEI metabolite was S-2-DCEI.

Using CYP expression systems, Granvil et al. (1999) reported the role of CYP2B6 in the dechloroethylation of ifosfamide. This enzyme is responsible for the biotransformation of ifosfamide to S-2-DCEI and S-3-DCEI, while CYP3A4 biotransforms ifosfamide to R-2-DCEI and R-3DCEI. Following Wainer's hypothesis that production of R-2DCEI and R-3DCEI is responsible for neurotoxicity, and hence, perhaps also nephrotoxicity, CYP3A expression may be a more important determinant of toxicity. However, since the kidney does not express significant amounts of CYP2B6 (Gervot et al., 1998), more ifosfamide may be converted to the R-DCEI enantiomers by CYP3A4 in kidney than in other organs such as the liver. This may explain why ifosfamide is associated with nephrotoxicity but not hepatotoxicity. Since ifosfamide crosses the blood-brain barrier, the presence of CYP2B6 in brain tissue may contribute to ifosfamide-induced neurotoxicity (Gervot et al., 1998).

Interestingly, Ducharme et al. (1997) report a case of altered N-dechloroethylation in an eight-year old girl receiving phenytoin. Following three months of phenytoin therapy, the patient received three days of etoposide (100 mg/m^2 for one hour), followed by ifosfamide (2.8 g/m^2 for one hour). She developed confusion, then obtundation, and finally, a generalized tonic-clonic seizure. Concentrations of dechloroethyl metabolites were S-3-DCEI equal to S-2-DCEI which were greater than
R-3-DCEI which were much greater than R-2-DCEI. Compared to a control population of 14 pediatric patients who had received similar doses of ifosfamide with concomitant etoposide for five consecutive days, this patient had two-three-times lower concentrations of R- and S-ifosfamide, but four-times higher concentrations of S-3-DCEI and S-2-DCEI and two-fold higher concentrations of R-3-DCEI and R-2-DCEI. These findings suggest that phenytoin induced CYP2B6 activity to a greater extent than CYP3A4 activity. Of note, the efficacy of ifosfamide therapy appeared to improve with phenytoin coadministration.

III.6g The Effects of Ifosfamide and Mesna on Glutathione Concentrations

Closely related to the concerns of chloroacetaldehyde renal toxicity is the issue of sufficient renal glutathione levels. Conjugation of an electrophilic group of the alkylating agent to a sulfhydryl group is deactivating. Such a reaction may occur spontaneously, or with the aid of an enzyme like glutathione transferase (Chasseaud, 1979). Glutathione, gamma-glutamylcysteinylglycine, plays an important role in detoxifying alkylating agents and preventing oxidative stress. Reduced glutathione can form inactive thioethers by directly binding to electrophiles, or it can be a substrate of a family of glutathione transferases that mediate conjugation to electrophiles. Thus, the effects of cytostatic drugs on both tumour and normal cells is dependent on the sulfhydryl status of the cells, especially the intracellular concentration of glutathione.

Various ifosfamide metabolites are metabolized and detoxified by glutathione and glutathione transferase. Hence, ifosfamide therapy is associated with decreased glutathione levels. The toxicity of ifosfamide may, therefore, be at least partially dependent on intracellular glutathione concentrations. Interestingly, decreasing glutathione levels has been proposed as a means to make chemotherapy more effective (Mulders et al., 1995).

A greater decline in glutathione concentrations is expected following oral administration of ifosfamide than with intravenous therapy since more glutathione-
depleting metabolites are produced. Renal tubular cells, including LLC-PK1 cells, contain relatively high concentrations of glutathione (about 3 mmol/kg; Kosower and Kosower, 1978; Siegers et al., 1987). However, whether these concentrations are sufficient to withstand therapy with ifosfamide and mesna is unclear. The biological half-life of glutathione in the kidney is only about 30 minutes (Sekura and Meister, 1974) NADPH is a cofactor in the reaction; however, the limiting step appears to be the amount of cysteine which is available for the resynthesis of glutathione (Ormstad et al., 1980).

Lind et al. (1989b) used the fluorescence indicator monochlorobimane and the technique of flow cytometry to determine the intracellular glutathione levels in P388 lymphocytes and in lymphocytes isolated from a patient taking ifosfamide therapy for epithelial ovarian cancer. Monochlorobimane is non-fluorescent but it will fluoresce when conjugated to glutathione. When cultured P388 cells were treated for one hour (37°C) with 1 mM concentrations of either ifosfamide, isofosforamide mustard, carboxyifosfamide, or monochloroethyl ifosfamide, glutathione levels were not reduced. On the other hand, both 4-hydroperoxyifosfamide and chloroacetaldehyde significantly reduced glutathione levels in a concentration-dependent manner. Chloroacetaldehyde was a more potent inhibitor with an IC50 value of approximately 100 μM, while the IC50 value for 4-hydroperoxyifosfamide was about 1 mM, well beyond therapeutic patient levels which are less than 100 nM (Klein et al., 1983). The concentration of chloroacetaldehyde, by contrast, was similar to that seen in patients receiving ifosfamide therapy. This concentration did not appear to alter the ability of the cells to hydrolyse fluorescein diacetate or to retain the fluorescent product within the cell, indicating that the cell membrane was still intact.

By measuring the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB), Lind et al. showed that 4-hydroperoxyifosfamide, chloroacetaldehyde, and isofosforamide mustard are all potent inhibitors of rat glutathione transferase. They
also found that chloroacetaldehyde inhibited human placental glutathione transferase in a competitive manner ($K_i = \sim 1.5 \text{ mM}$).

The glutathione levels in a patient receiving an eight-hour infusion of ifosfamide were followed for up to 27 hours following commencement of therapy. Glutathione levels in peripheral lymphocytes decreased to 30% of their initial value during the infusion and then gradually returned to 60% of their initial value by about 20 hours after the start of the infusion.

Moreover, Sood and O'Brien (1993) clearly showed a decrease in glutathione concentrations when human liver hepatocytes were incubated with chloroacetaldehyde. Glutathione depletion began immediately following the addition of chloroacetaldehyde. The cytotoxic effects of chloroacetaldehyde were also more pronounced in glutathione-depleted hepatocytes.

Not only does administration of ifosfamide utilize glutathione stores, but the coadministration of mesna further depletes glutathione. The conversion of dimesna to mesna by glutathione transferase requires the use of glutathione. In a study by Lauterburg et al. (1994), treatment of 14 patients with advanced sarcoma for five days with ifosfamide (2.4-3.2 g/m² daily) and mesna resulted in a decline in total glutathione concentrations from $6.9 \pm 1.1 \mu\text{M}$ to $2.5 \pm 1.1 \mu\text{M}$ (means ± 95% confidence intervals on day 6). Total cysteine concentrations consisting of cysteine, cystine, and cysteine mixed disulfides also decreased from $245 \pm 36 \mu\text{M}$ to $50 \pm 14 \mu\text{M}$. Total homocysteine concentrations dropped from $12.3 \pm 2.1 \mu\text{M}$ to $1.4 \pm 1.1 \mu\text{M}$.

Circulating concentrations of total cysteine are markedly decreased by mesna. When healthy volunteers were given 7.3 mmol of mesna by the intravenous route, total cysteine plasma levels dropped from $276 \mu\text{M}$ (215-337 \mu\text{M}) to $102 \mu\text{M}$ (89-115 \mu\text{M}) between 30 and 120 minutes after the infusion (Stofer-Vogel et al., 1993). This could be caused by increased uptake of cysteine into cells and/or increased urinary excretion of cysteine/cystine. Normal volunteers given mesna demonstrate increased cysteine
excretion (Jones et al., 1985). Hence, even though mesna is not taken up into cells, it can have a marked effect on the sulfhydryl status of cells.

A dose-dependent depletion of renal glutathione content was observed following the intra-renal infusion of chloroacetaldehyde to rats (Springate, 1997). Renal infusion of mesna also decreased the renal glutathione content as expected, although to a lesser extent than chloroacetaldehyde. The coadministration chloroacetaldehyde (180 µmol) with almost nine-fold higher concentrations of mesna was unable to prevent glutathione depletion. Interestingly, rats given intraperitoneal doses of either 40 mg/kg or 80 mg/kg ifosfamide did not have significantly lower renal glutathione concentrations than controls (Springate and Van Liew, 1995).

III.6h Why is Ifosfamide Toxic to the Kidney but not to the Liver?

Hepatotoxicity is not a clinically-reported complication of ifosfamide therapy. However, when mice are given chloroacetaldehyde in their drinking water (0.1 g/L), the primary organ for toxicity is the liver. Moreover, cytotoxicity following the incubation of hepatocytes with chloroacetaldehyde was clearly demonstrated by Sood and O’Brien (1993).

Winckler et al. (1987) prepared S9 fractions from mouse kidney and liver and looked at their ability to produce mutagenic metabolites from cyclophosphamide. While both fractions were able to generate mutagenic compounds, the S9 from liver was more effective. Moreover, while phenobarbital and Aroclor 1254 can induce CYP-mediated biotransformation of cyclophosphamide and thus cause greater mutagenicity, teratogenicity, and cytotoxicity in vivo and in vitro in the liver, the ability of the kidney to biotransform cyclophosphamide was not affected by those inducers (Hales, 1981; Hales and Jain, 1980).

If the production of chloroacetaldehyde from ifosfamide is indeed responsible for ifosfamide-induced nephrotoxicity, the question of why patients receiving ifosfamide therapy do not also experience hepatotoxicity must be raised, since quantitatively, the
liver is a more important organ of drug biotransformation. There are a few possible explanations for the discrepancy in toxicities between the two organs.

First, aldehyde dehydrogenase is responsible for the oxidation of chloroacetaldehyde to chloroacetic acid (see chapter one). The higher amounts of acetaldehyde dehydrogenase in liver may explain why the concentrations of chloroacetaldehyde required to induce 50% cellular death in hepatocytes are 30-fold higher (0.3 mM) than those required for renal tubular cells and tumour cells (Brüggemann et al., 1997). This also points out that blood chloroacetaldehyde concentrations may not reflect intratumoural or other intracellular concentrations. Boddy et al. (1993) were able to detect the carboxyifosfamide metabolite in the urine of patients from which plasma concentrations were undetectable. This suggests that aldehyde dehydrogenase in the kidney may be functional, but that carboxyifosfamide is excreted before it enters the systemic circulation.

Glutathione levels are also higher in the liver than in the kidney (by at least two-fold) suggesting that any formed toxins are detoxified to a greater extent in the liver than in the kidney (Roberts and Francetic, 1993; Asensi et al., 1994; see chapter one). In addition, since the reduction of dimesna to mesna requires glutathione, and this process is more prominent in the kidney than in the liver, renal glutathione stores may be more depleted than hepatic stores when ifosfamide is coadministered with mesna.

III.6i Why Are Ifosfamide-Induced Neurotoxicities and Nephrotoxicities Found Indepenendly of Each Other?

If high levels of chloroacetaldehyde are the only cause of ifosfamide-induced nephrotoxicity, one would expect neurotoxicity to also present with nephrotoxicity. Yet, neurotoxicity and nephrotoxicity are often reported independently; although, in a study of standard- and high-dose schedules of ifosfamide, Antman et al. (1990) found that neurotoxicity was significantly related to renal toxicity. The neurotoxicity of chloroacetaldehyde was originally hypothesized because chloroacetaldehyde is
structurally very similar to the neurotoxin chloral hydrate. It is possible that different mechanisms are responsible for the two toxicities. Regional binding of agents like thialysine ketamine suggests that there are multiple mechanisms of toxicity and that neuro- and nephrotoxicity may not necessarily result from the same compound. For example, high concentrations of R-3-DCEI are associated with neurotoxicity but not with nephrotoxicity. The distribution of CYP enzymes may also explain differences in toxicities. For instance, CYP3A concentrations in liver do not correlate with those in intestine (Lown et al., 1994). Similarly, different concentrations and expression of CYP enzymes in brain and kidney may result in local differences in toxicity. Moreover, regional activity of mesna and differences in concentrations of mesna or glutathione in brain and kidney may result in different toxicities. In other words, the total enzymatic content of each tissue may be independent of each other, explaining variations in tissue toxicities.

III.6j Why Is Ifosfamide-Induced Nephrotoxicity More Prominent in Children than in Adults?

The incidence of ifosfamide-induced chronic renal damage appears to be much lower in adults than in children (Stuart-Harriss et al., 1983; Schepper et al., 1991; Caron et al., 1992; De Skinner et al., 1992, Loebstein et al., 1999). The greater incidence of nephrotoxicity in children than adults is also puzzling. However, children often receive higher single and cumulative ifosfamide doses (Skinner et al., 1993). As well, the greater survival rates in children with cancer allow for greater detection of chronic nephrotoxicity.

Descriptions of ifosfamide-induced nephrotoxicity in adults are most commonly found in the form of case-reports in the literature. Severe renal damage reported many years after the cessation of therapy. For instance, end-stage renal interstitial fibrosis likely attributable to therapy with ifosfamide for recurrent rhabdomyosarcoma was
reported for a 33 year-old male. Late onset chronic renal disease may not be detected in patients who are much older at the time of treatment with ifosfamide.

The largest study of ifosfamide nephrotoxicity in adults reported on 131 patients with various malignancies treated with escalating doses of ifosfamide, carboplatin, and etoposide followed by autologous stem cell transplantation as part of a phase I/II therapeutic trial. While peak creatinine concentrations were significantly higher in patients receiving high-dose therapy versus those in patients receiving low or intermediate doses, the true causality of renal toxicity due to ifosfamide is difficult to assess since platinums are also associated with nephrotoxicity. Still, a greater incidence of ifosfamide-induced nephrotoxicity has been reported recently.

As mentioned previously, many pharmacokinetic properties of ifosfamide differ between children and adults. The clearance of ifosfamide is higher in children than in adults (Boddy et al., 1993). When five children and ten adults were given similar doses of ifosfamide, the sums of the 2- and 3-DCEI metabolites excreted in the urine during the first 24 hours were 15-31% and 0.75-3.91% of the initial dose, respectively (Goren, 1991). The corresponding excretions of ifosfamide were 11-30% and 0-6.37%. While theoretically the children were exposed to 250 μM chloroacetaldehyde with this relatively low dose of ifosfamide, neither neurotoxicity nor nephrotoxicity was reported in these children.

Differences in enzyme expression and function may explain the differences in ifosfamide-induced nephrotoxicity. While most enzyme systems are thought to be almost fully developed by an age when children would begin to receive chemotherapy, the ontogeny of individual biotransforming and conjugating enzymes is not well documented. Greater production of toxic metabolites by children and adolescents may explain the apparent higher incidence of nephrotoxicity in children than in adults. Loebstein et al. (1999) clearly showed that younger-children have an increased risk of nephrotoxicity from ifosfamide therapy.
The renal transport of ifosfamide, ifosfamide metabolites, and/or mesna and dimesna may also be affected by age. Little is known about how these compounds are specifically handled by the kidney. Clearly, age appears to be a factor in the incidence and severity of ifosfamide toxicity and further study into age-related changes in the handling of ifosfamide is warranted.
III.7 Conclusion

A number of possible mechanisms for ifosfamide-induced nephrotoxicity have been proposed. The detection of 2- and 3-DCEI following incubation of kidney microsomes with ifosfamide demonstrates the ability of the kidney to biotransform drugs. Indirectly, my experiments propose that chloroacetaldehyde, which is toxic to kidney cells, is co-produced with these dechloroethyl metabolites. The identification of the causative metabolite(s) of ifosfamide-induced renal damage will require further investigation. Certainly, the considerations of species differences in enantioselectivity and biotransformation leave the human kidney as the ultimate model for studying toxicity. With the availability of new technology, the future direction of our laboratory is to identify the renal enzymes responsible for ifosfamide biotransformation and to stereoselectively study the profile of all metabolites produced from ifosfamide by the kidney and the contribution of these enzymes to their respective pathways.
SUMMARY

The studies outlined in this thesis have highlighted the utility of *in vitro* models in the understanding of clinically important drug-induced toxicities. The *in vitro* tissue culture and microsomal systems provide a relatively inexpensive way to study drug-induced toxicity that is noninvasive to patients. The use of tissue culture inserts in cell culture enables the manipulation of apical and basolateral cell bathing solutions independently. The cell culture model is useful for the characterization of known drug interactions, the prediction of potential drug interactions, and the identification of reversal agents of multidrug resistance.

Drug interactions are a common source of drug-induced toxicity. The involvement of P-glycoprotein in drug interactions has been documented for a number of drugs. My results suggest that P-glycoprotein plays a role in digoxin-drug interactions with propafenone, verapamil, and mifepristone. I also demonstrated the importance of drug metabolites to digoxin-drug interactions. Although the liver is usually the main site of metabolite production, we showed that the kidney is capable of biotransforming propafenone and verapamil. Moreover, metabolites produced by the kidney (such as norverapamil) may contribute to drug interactions. These interactions may result in toxicity distal to the kidney as in the case of digoxin-drug interactions that can result in severe, often life-threatening, cardiotoxicity.

The importance of renal drug biotransformation to nephrotoxicity is illustrated by ifosfamide. Ifosfamide itself is not nephrotoxic; however, many of its metabolites are. An important difference between cyclophosphamide (which is not nephrotoxic) and ifosfamide is the almost 100-fold higher production of chloroacetaldehyde from ifosfamide. Given the instability of chloroacetaldehyde in blood, I hypothesized that nephrotoxicity results from renal production of chloroacetaldehyde. Using renal microsomes I was able to demonstrate the ability of the kidney to biotransform ifosfamide to chloroacetaldehyde. Therefore, although the kidney is not considered to
play a large role in the overall biotransformation of drugs, in the case of ifosfamide, renal drug biotransformation may be a causative factor of drug-induced toxicity.

In summary, when studying issues of drug-induced toxicity, the kidney should not be neglected as a potential site of drug interactions and drug biotransformation. The intrarenal production of drug metabolites must also be considered as a potential contributor to drug-induced toxicity. Moreover, the toxicity resulting from renal drug handling may not be limited to the kidney. For instance, drug interactions occurring in the renal tubules may cause systemic toxicity without damaging the kidney. Therefore, the kidney plays a significant role in the drug-induced toxicity.
REFERENCES


Harrison LI, Gibaldi M. Pharmacokinetics of digoxin in the rat. Drug Metabolism and Disposition 4: 88-93, 1976.


Kharasch ED, Thummel KE. Identification of cytochrome P450 2E1 as the predominant enzyme catalyzing human liver microsomal defluorination of sevoflurane, isoflurane, and methoxyflurane. Anesthesiology 79: 795-807, 1993.


Mahajan SS, Rifkind AB. Transcriptional activation of avian CYP1A4 and CYP1A5 by 2,3,7,8-tetrachlorodibenzo-p-dioxin: Differences in gene expression and regulation compared to mammalian CYP1A1 and CYP1A2. Toxicology and Applied Pharmacology 155(1): 96-106, 1999.


Salpatic L, Benet LZ. Metabolism of digoxin and digoxigenin digitoxosides in rat liver microsomes: Involvement of cytochrome P4503A. Xenobiotica 29(2): 171-185, 1999.


APPENDIX A
LIST OF PUBLICATIONS


LIST OF ABSTRACTS


Modeling of P-glycoprotein-involved epithelial drug transport in MDCK cells

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1Division of Clinical Pharmacology and Toxicology, Department of Pediatrics and Pharmacology, Hospital for Sick Children, Research Institute, and University of Toronto; 2Sunnybrook Health Science Center, Toronto, Ontario, Canada MSC 1X8; and 3The National Institute of Haematology, Blood Transfusion, and Immunology, H-1113 Budapest, Hungary

Ito, Shinya, Cindy Woodland, Balázs Sarkadi, Guido Hockmann, Scott E. Walker, and Gideon Koren. Modeling of P-glycoprotein-involved epithelial drug transport in MDCK cells. Am. J. Physiol. 277 (Renal Physiol. 46): F84–F96, 1999.—P-glycoprotein (P-gp) on the apical membranes of epithelial cells is known as a drug efflux pump. However, unclear is its integral quantitative role in the overall epithelial drug transfer, which also involves distinct diffusion processes in parallel and sequence. We used a simple three-compartment model to obtain kinetic parameters of each drug transfer mechanism, which can quantitatively describe the transport time courses of P-gp substrates, digoxin and vinblastine, across P-gp-expressing MDCK cell monolayers grown on permeable filters. Our results show that the model, which assumes a functionally single drug efflux pump in the apical membrane with diffusion across two membranes and intercellular junctions, is the least complex model with which to quantitatively reproduce the characteristics of the data. Interestingly, the model predicts that the MDCK apical membranes are less diffusion permeable than the basolateral membrane for both drugs and that the distribution volume of vinblastine is 10-fold higher than that of digoxin. Additional experiments verified these model predictions. The modeling approach appears feasible to quantitatively describe overall kinetic picture of epithelial drug transport. Further model refinement is necessary to incorporate other modes of drug transport such as transcytosis. Also, whether P-gp solely accounts for the pump function in this model awaits more studies. P-glycoprotein; multidrug resistance; diffusion; itraconazole

Studies in these polarized epithelia have shown that the expression of MDR1-type P-glycoprotein is associated with enhanced basal-to-apical net transfer of diverse classes of compounds such as vinca alkaloids (31), cyclosporin (25), and digoxin (3, 25, 31). Interpretation of these findings has been based on intuitive face validation of the conceptual model of P-glycoprotein as an apically located active drug efflux pump (Fig. 1; Refs. 5, 25, 31), which is assumed from the known localization of P-glycoprotein in the epithelial cells (32). However, to our knowledge, transport data of P-glycoprotein substrates have never been quantitatively analyzed according to this functional model. Although a steady-state linear kinetic model was previously applied to transport of vinblastine in MDCK cells (15), uncertainty of the parameter estimates was not explicit determined. Hence, in intact epithelial cells, it is difficult to infer quantitative relations among pump-mediated drug transport, equilibrium accumulation, and passive diffusion across two distinct membrane domains. This knowledge is essential to understand biological significance of drug transporters and to gain insight into roles of other distinct drug transfer processes such as diffusion in disposition of xenobiotics in vivo.

In this report, we use mathematical modeling approach to quantitatively describe in vitro time course of net transepithelial transfer of P-glycoprotein substrates, digoxin and vinblastine, through P-glycoprotein-expressing MDCK renal tubular cells. Digoxin and vinblastine were used because their transport appears to be mediated mainly by P-glycoprotein (15, 31). If not entirely. In support of this notion, mice with a disruption of mdr1a gene encoding one of the drug-transporting P-glycoproteins showed substantial increase in digoxin and vinblastine plasma and tissue levels, indicating the major role of P-glycoprotein in eliminating these compounds in vivo (26, 27). The MDCK cell line was used because 1) P-glycoprotein-mediated drug transport has been extensively studied using this cell line; 2) its intercellular junction is very tight, rendering relatively high signal-to-noise ratio of the pump function; and 3) this distal renal tubular cell line, unlike the LLC-PK1 proximal renal tubular cell line, does not show transport signals of tetraethylammonium, a prototype substrate for the organic cation transport system (6), whose overlapping substrate specificity with P-glycoprotein (4) makes interpretation of experimental results difficult.

Our results show that transport of digoxin and vinblastine across the MDCK cell monolayers is ex-
Fig. 1. Schematic representation of conventional experimental criteria intuitively derived from a conventional model of a pump-mediated drug transport (e.g., P-glycoprotein). Transport of the drug added initially to one side only (shaded areas) is monitored over time. Columns A—F denote cells with different pump functions (triangles) in the two different initial conditions. Pump function is enhanced (A and E), unaltered (C and D), or reduced (B and F). The conventional criteria state the following. The initial (i.e., for the first 1–3 h of incubation) flux from the basal to the apical side is greater than in the opposite direction, when the protein operates (A vs. B and C vs. D). When there is no unidirectional transport system (E and F), the fluxes due to passive diffusion should be the same in both directions. Alterations of the pump function result in parallel changes of the quasi-initial flux rate from the apical to the basal side (A, C, and F). In contrast, the rate of apical-to-basal fluxes is inversely correlated with the pump function (B, D, and F).

plained quantitatively by a kinetic model, which assumes a functionally single unidirectional drug pump in the apical membrane and diffusion across both membranes. Interestingly, the model predicts that the MDCK apical membranes are less diffusion permeable than the basolateral membrane for both drugs and that the distribution volume of vinblastine is 10-fold higher than that of digoxin. Further experiments cross-validated the model by verifying these predictions.

MATERIALS AND METHODS

Materials

[3H]digoxin (16.1 Ci/mmol) and [14C]mannitol (55.1 mCi/mmol) were purchased from Du Pont Canada (Markham, Ontario, Canada). [3H]vinblastine (11.2 Ci/mmol) was purchased from Amersham Canada (Oakville, Ontario). Fetal bovine serum was obtained from GIBCO-BRL and was added to media (α-MEM); Nunc TC inserts (25-mm external diameter) were from A/S Nunc. Vincristine, vinblastine, ketoconazole, and digoxin were purchased from Sigma. Itraconazole was donated by Janssen Pharmaceuticals. An anti-rabbit, horseradish peroxidase (HRP)-conjugated goat IgG was purchased from Jackson Immunoresearch (West Grove, PA). Anti-rat, peroxidase-conjugated IgG was obtained from Boehringer. Calcein acetoxymethyl ester (calcein-AM) was obtained from Molecular Probes (Eugene, OR).

Cell culture. MDCK cells obtained from the American Type Culture Collection (Rockville, MD) were grown in α-MEM containing 10% fetal bovine serum without antibiotics under an atmosphere of 95% air–5% CO2 at 37°C. They were subcultured twice weekly using 0.02% EDTA and 0.05% trypsin. NIH 3T3 cells overexpressing P-glycoprotein were kind gifts of Dr. M. M. Gottesman.

Immunoblotting and P-Glycoprotein Quantitation

Immunoblotting using a monoclonal antibody (C219; Ref. 16) against P-glycoprotein was performed as follows. Crude plasma membrane fractions were obtained from MDCK cells (23) and electrophoresed on 7.5% SDS-PAGE gels. Transfer to nitrocellulose was blocked overnight in 4% skim milk Tris-NaCl-Tween solution. The blocked nitrocellulose strips were incubated with C219 for 3 h at room temperature. Binding of C219 monoclonal antibody was visualized employing the enhanced chemiluminescence detection procedure (ECL, Amersham).

We also used an anti-MDR1 P-glycoprotein polyclonal antibody (4077), which recognizes the NH2-terminal half of MDR1 across different species including dogs (30), from which the MDCK cell line was derived. After washing twice in a protein-free (HPMI) medium, the cultured cells were dissolved and sonicated in a disaggregation buffer (1, 24). The second antibody (anti-rabbit HRP-conjugated goat IgG) was used in 20,000× dilution. HRP-dependent luminescence was detected in a similar manner to C219.

The amounts of P-glycoprotein were quantified by excising the respective bands recognized by the antibody 4077 from the polyvinylidene difluoride (PVDF) membrane and measuring their luminescence in a liquid scintillation counter (Beckman LS 6000, Single Photon Monitor mode). The standard was the known amounts of P-glycoprotein (MDRI) in the isolated SIF cell membranes (14).

Detection of multidrug resistant associated protein (MRP) with the R1 (rat) monoclonal anti-human MRP antibody, kindly provided by Dr. R. Schaper, was carried out as previously described (1). The monoclonal antibody (anti-rat, peroxidase-conjugated IgG) was used in 1,000× dilution. HRP-dependent luminescence on the immunoblots (ECL, Amersham) was determined by autoradiography.

Calcein Accumulation Assay for P-Glycoprotein and MRP Function

Calcein-AM, a nonfluorescent hydrophobic compound, is converted to fluorescent calcein by cytoplasmic esterase after entering cells. Whereas P-glycoprotein (MDRI) appears to be a pump for calcein-AM, MRP seems to be a pump for both calcein-AM and its fluorescent free form, calcein, as well as its glutathione conjugate (13). Because of differences in their inhibitor specificity, it is possible to differentiate between the functions of the two proteins (13). Calcein accumulation was measured by incubating the cells in HPMI medium (2.5 × 10⁵ cells/ml) containing 0.25 μM calcein-AM as described elsewhere (15, 25). The multidrug resistance activity factor (MAF; Refs. 13 and 14) was calculated according to the equation: MAF = (P* − F)/P*, where P* and F designate the dye accumulation rate in the presence and absence, respectively, of an inhibitor of the respective multidrug transporter. Prestarlagandin A₁ at 10 μM was used for maximum inhibition of MRP (13), whereas 100 μM verapamil was used for maximum inhibition of both MRP and P-glycoprotein (14). MAF, an empirical index ranging from 0 to 1, is correlated with the expression levels of the transporters in a nonlinear fashion, which becomes asymptotic to unity at a high level of transporter expression (14).

Filter Preparation

MDCK cells in the incubation medium were seeded at a density of about 5 × 10⁵ cells/cm² on the inorganic permeable membranes attached to 25-mm tissue culture inserts (0.2 μm pore size, 4.15-cm² growth area; Nunc TC inserts). The inserts were placed in cluster plates, fed every 48 h, and incubated in the same conditions as described above. Experiments were conducted 10 days after seeding.

Transport Experiment

The incubation was started at 37°C immediately after the culture media of both compartments were replaced with
preconditioned incubation media; only the media bathing one side of the monolayers (either basal or apical) contained 0.1 mCi [%H]digoxin or 0.1 mCi [%H]vinblastine. [14C]mannitol, 1.45 μM, was also added to the drug-containing media as a paracellular flux marker.

Two kinds of experiments were conducted: 24-h time course experiments, and 3-h time course experiments to examine side dependency of inhibitors. For 24-h incubation experiments, basal-to-apical and apical-to-basal transport was measured in the presence or absence of 5 μM itraconazole, a potent P-glycoprotein inhibitor (10), added to both basal and apical sides. When side dependency of inhibition was examined, basal-to-apical digoxin (0.1 μM) or vinblastine (0.1 μM) fluxes were monitored for 3 h in the presence or absence of inhibitors added to either basal or apical side of the monolayers.

Twenty-five microliters of the medium was sampled at given time intervals, and the radioactivity (dpm) was measured with scintillation counting. We discarded results from cell monolayers with [14C]mannitol fluxes greater than 2 SD of the mean mannitol flux in each experimental condition.

Cell Viability Assay

To ensure that overall cellular function is not disturbed even after 24-h exposures to drug, viability of the MDCK cells over prolonged periods of exposures to various concentrations of digoxin, vinblastine, and (itraconazole was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (20). Briefly, MDCK cells were plated into 96-well plates in a-MEM with 10% fetal bovine serum and incubated at 37°C in 5% CO₂. At 24- and 48-h incubation with various concentrations of digoxin, vinblastine, or itraconazole, 20 μl of a 5 mg/ml MTT solution was added to wells and incubated at 37°C in 5% CO₂ for 4 h. Supernatants were aspirated off, then 100 μl of acidified isopropanol was added and incubated in the dark at room temperature for 30 min. Plates were agitated for 2–3 min until a uniform color was achieved, then read on an ELISA reader at 490 nm. Percentage of cell survival was calculated by dividing the average value at each drug concentration by the average control value, multiplying by 100. This percentage was designated as the viability rate.

Cellular Accumulation of Digoxin and Vinblastine

The cellular accumulation of radiolabeled vinblastine from the basal side of the monolayers in the presence and absence of vincristine added to either the apical or the basal side was examined at 10, 30, and 60 min of incubation. Also, digoxin uptake from the basal side at 60 min was examined and compared to corresponding vinblastine uptake. The procedure has been described elsewhere (35). The uptake results were expressed as a ratio of the amount of drug in the cell lysates to that of the initial incubation medium.

Itraconazole Assay

Itraconazole disposition in the MDCK cells was examined as follows. Itraconazole (5 μM) was added to both sides of the monolayers at time 0. After 48 h of incubation under the usual culture conditions, apical and basal media were collected, and itraconazole was quantified with a modified HPLC method (for the original method, see Ref. 34). The inter- and intra-assay coefficients of variation were less than 5%. The quantitation limit for itraconazole was set at 10 nM.

Modeling of the Drug Transport

We developed a two-stage method to account for differences of paracellular diffusion monitored by mannitol among the four different conditions: basal-to-apical and apical-to-basal transport of drug in the presence or absence of itraconazole. This method necessitates only one assumption: that mannitol flux is proportional, if not identical, to paracellular fluxes of digoxin and vinblastine. First, the paracellular flux coefficient was estimated (first-stage fitting), which was then incorporated into the disposition equations for the final fitting of the drug transfer data (second-stage fitting).

Estimation of paracellular diffusion (first stage). The time courses of [14C]mannitol fluxes in each condition were fit to the following equations

\[
\frac{dA}{dt} = (B - A) \cdot \rho/2
\]

\[
\frac{dB}{dt} = (A - B) \cdot \rho/2
\]

in which A and B are concentrations of mannitol in the apical and basal compartment; \( \rho \) is a paracellular diffusion constant (ml/h) of mannitol through the cell monolayer; and the denominator is a volume of the apical and basal chamber (i.e., 2 ml). The estimated four different diffusion rates (\( \rho \)) for each of the four conditions were expressed as a ratio to the diffusion constant of mannitol in the basic condition, in which drug and mannitol were added to the basal side in the absence of itraconazole; the calculated ratio was designated as a relative paracellular diffusion constant (\( \rho^* \)).

Model construction (second stage). Based on the criteria (Table 1), three main models were designed (Fig. 2). All three models are based on cellular localization and function of P-glycoprotein as a unidirectional drug efflux pump on apical membranes. Model A is based on the original model (15). Model B also assumes another drug efflux system on the basal side, as suggested from the localization of MRP, although MDCK does not show significant MRP function (see RESULTS). Model C is a two-compartment model assuming the renal tubular cells to be a volumeless single barrier (instead of a substantial compartment) between the apical and basolateral compartments; this was examined for its conceptual simplicity. As variants of the most plausible model (model A), we also tested the following models: 1) model A with separate diffusion and distribution volume parameters for itraconazole-added conditions; 2) model A with an influx unidirectional pump on the basal membrane; and 3) model A with a second intracellular compartment.

There exist endocytosis-transcytosis processes that may contribute to overall drug transfer from the basal to the apical side of epithelial cells (22). Also, drugs distributed into intracellular organelles are likely to be extruded to extracellular space via vesicular trafficking. In this study, these modes of drug transport were not taken into account, because experimental validation was difficult.

MODEL A. Model A is the physiologically defined model with a single active transport system in the apical membrane (Fig. 2A), which is based on the conceptual model of P-glycoprotein

<table>
<thead>
<tr>
<th>Table 1. Criteria for model construction</th>
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<tbody>
<tr>
<td>1. Compartitional modeling is used.</td>
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<tr>
<td>3. Model has two extracellular compartments to represent the two culture chambers separated by the cell monolayer in the experimental system.</td>
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<tr>
<td>4. Model is consistent with existing data on the apical localization of P-glycoprotein in the MDCK cells.</td>
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</table>

Models are constructed on the basis of the physical and biological characteristics of the drug transport mechanisms.
Fig. 2. Compartment models of cellular digoxin and vinblastine transport across the MDCK renal tubular cells. Model A: a 3-compartment model with one unidirectional active transport system in the apical membrane and with three diffusion processes; this is based on a conventional concept illustrated in Fig. 1. Model B: model A plus another drug efflux system in the basal membrane. Model C: a simple 2-compartment model assuming the cells as a volumeless barrier. See the text for the general disposition equations for each model; $\delta_1$, $\delta_2$, and $\delta_3$ are diffusion parameters of drug through apical membranes, basal membranes, and intercellular junctions, respectively; $\delta$ is an overall diffusion parameter for the 2-compartment model; and $k$ and $\lambda$ are the intrinsic clearance of drug via the apical pump and that of the basal drug efflux pump, respectively.

as the apical pump in the renal tubular cells. The disposition equations are described as follows

$$\frac{dA}{dt} = \frac{C \cdot (k \cdot \theta + \delta_1)}{V} + B \cdot \rho \cdot \delta_3 - A \cdot (\delta_1 + \rho \cdot \delta_3)/2$$

$$\frac{dC}{dt} = A \cdot \delta_1 + B \cdot \delta_2 - C \cdot (k \cdot \theta + \delta_1 + \delta_2)/V$$

$$\frac{dB}{dt} = \frac{C \cdot \delta_2}{V} + A \cdot \rho \cdot \delta_3 - B \cdot (\delta_2 + \rho \cdot \delta_3)/2$$

in which $A$ and $B$ are concentrations of the drug in the apical and basal compartment; $C$ denotes the amount of the drug in the cellular compartment; $\delta_1$, $\delta_2$, and $\delta_3$ are diffusion parameters of drug expressed as volume per unit time through apical membrane, basal membrane, and intercellular junctions, respectively; $\rho$ is the relative paracellular diffusion constant of each condition obtained by the first stage mannitol fitting (see above); $k$ is the intrinsic clearance of drug via the pump in the absence of itraconazole; $\theta$ is a fraction (1 $\geq \theta \geq 0$) of the remaining pump parameter ($A$) in the presence of itraconazole; and $V$ is the apparent cellular distribution volume of drug. Itraconazole was assumed to inhibit the pump function ($A$) only. The denominator of the first and third equations is 2 ml, which is the volume of the apical and basal compartments in this experimental setting.

MODEL B. Model B is different from Model A in that it has another drug efflux pump on the basal membranes (Fig. 2B). Although there has been no experimental evidence suggesting that the MDCK basal membrane expresses a carrier-mediated drug efflux system for digoxin or vinblastine, we studied this model to explore such a possibility. Behind this attempt was the notion that MRP is expressed in basolateral membranes of LLC-PK1, a proximal renal tubule cell line. The general disposition equations are as follows

$$\frac{dA}{dt} = \frac{C \cdot (k \cdot \theta + \delta_1)}{V} + B \cdot \rho \cdot \delta_3 - A \cdot (\delta_1 + \rho \cdot \delta_3)/2$$

$$\frac{dC}{dt} = A \cdot \delta_1 + B \cdot \delta_2 - C \cdot (k \cdot \theta + \lambda + \delta_1 + \delta_2)/V$$

$$\frac{dB}{dt} = \frac{C \cdot (\lambda + \delta_2)}{V} + A \cdot \rho \cdot \delta_3 - B \cdot (\delta_2 + \rho \cdot \delta_3)/2$$

which are the same as model A except for the term involving $\lambda$ (an intrinsic clearance of the active transporter in the basal membrane). Itraconazole was assumed to inhibit the apical pump function ($A$) only.

MODEL C. Model C is a two-compartment model with a single active transport system (Fig. 2C). This simple two-compartment model assuming the renal tubular cells to be a volumeless single barrier (instead of a substantial compartment) between the apical and the basolateral compartments was tested because it is a simpler approach providing an easy-to-comprehend kinetic picture. The general disposition equations are as follows

$$\frac{dA}{dt} = \frac{B \cdot (k \cdot \theta + \rho \cdot \delta)}{V} - A \cdot \rho \cdot \delta)/2$$

$$\frac{dB}{dt} = \frac{A \cdot \rho \cdot \delta - B \cdot (k \cdot \theta + \rho \cdot \delta))/2$$

where $\delta$ is a hybrid diffusion constant of the three distinct diffusion processes: apical membranes, basolateral membranes, and intercellular junctions. Itraconazole was assumed to inhibit the pump function ($A$) only.

Fitting. We used MLAB (Civilized Software, Bethesda, MD) in the least-squares fitting. This program uses the Marquardt-Levenberg least-squares method for curve fitting, which is characterized by its superior fitting efficiency even when there are more than two model compartments with relatively few data points. The fitting was performed with the average data set of four filters for each of the four different initial conditions; i.e., drug was added to either the basal or the apical side in the presence and absence of itraconazole. When the experimental results were fit to those models, several initial estimates of the parameters were used to avoid entrapment in local minima. The weighting factor employed in the fitting was the reciprocal of the variance weight values formed by the standard deviation estimates of each data point. The constraints given to the fitting were the positivity of all parameters and a range of fractional pump inhibition by itraconazole (from 0 to 1). Itraconazole was assumed to change the pump function only. In all models, first-order movement of drug was assumed.

Simulation. To examine the influences of the apical pump function changes on overall cellular drug disposition, time courses of drug transport in the best-fit model (model A) were simulated using the same modeling computer program (MLAB) with the pump parameter ranging from 100% to 0% of control.

Model diagnosis and selection of a best-fit model (Table 2). To interpret the fitting results, two major questions need to be answered: 1) How well does the model fit the data? and 2) Does this model fit the data better than an alternate model?

GOODNESS OF FIT (HOW WELL DOES THE MODEL FIT THE DATA?)

The assay is done by a combination of multiple methods. The fitting with the least-squares method provides residual sum of squares (RSS) as an indicator of the average deviation of the fitting curve from the data. Here, two different situations exist (19). First, if the error variances of the experimental data are explicitly known and given, chi-squared test can be used to statistically evaluate the overall size of the RSS; specifically, compatibility of the size of RSS with the given experimental errors is tested. Also, qualitative assessment to identify apparent systematic misfitting or outright fitting errors can be done by inspecting the graph of the fitting curve superimposed on the data points and the derived $R^2$-squared value. On the other hand, if the error variance of data is unknown (such as in this study), chi-squared test is meaningless. Instead, the qualitative assessment is done.
In addition to these overall evaluations of the fitting, randomness of the residuals (i.e., random deviation of the fitting curve from the data) should be confirmed either qualitatively by patterning the residuals in a plot against time, or quantitatively by using the runs test (19). The runs test compares the number of runs of consecutive positive and negative residuals with that of runs expected from random distribution. Systematic or correlated errors identified by these methods may be the results of model error, experimental error, or failure of the weighted least-squares algorithm.

Best-fit model (does this model fit the data better than an alternate model?). We selected a best-fit model as a minimal model, whose data fitting (i.e., residual sum of squares) is statistically indistinguishable from those of more complex models. In general, higher order, more complex models tend to fit data closer than do lower order, simpler models, giving smaller residual sum of squares. However, closer fitting per se may simply reflect experimental errors rather than physiological truth. Taking these into account, we used a combination of analysis of variance (F test) and parsimony criteria such as Akaike's information criterion (AIC) and Schwartz criterion (SC) to select a best model (Table 2).

F test was used to examine statistical significance of difference in the best weighted residual sum of squares between given two models. When the F statistic becomes significant (i.e., \( P < 0.05 \)), the model with a greater residual sum of squares was rejected in favor of the other. The least complex of the remaining models was chosen as the best model, and the selection was further confirmed using AIC and SC, which represent goodness of fit (i.e., lower weighted residual sum of squares) and simplicity of the model (i.e., fewer numbers of model parameters) (19); AIC = \( n \cdot \ln (S) + 2r \); and SC = \( n \cdot \ln (S) + r \cdot \ln (n) \). In these equations, \( n \) denotes the number of data points; \( S \) is the weighted residual sum of squares; and \( r \) is the number of parameters estimated. The model with the lowest value of AIC and SC is considered as the simplest model that still provides fitting statistically as good as that of more complex models. In addition to the above statistical criteria, overall goodness of fit such as randomness of residuals (see GOODNESS OF FIT . . . , above) and uncertainty of parameter estimates were also considered.

**Data Analysis**

The results are expressed as means ± SD unless otherwise stated. ANOVA and Student's t-test were used where appropriate. \( P \) values of less than 0.05 were considered statistically significant.

**RESULTS**

**Immunoblotting of P-Glycoprotein and MRP**

The levels of expression detected by the C219 monoclonal and the 4077 polyclonal antibodies were higher in MDCK cells than in wild-type LLC-PK\(_1\) cells (Fig. 3).

**Table 2. Selection criteria for best model**

1. Goodness of fit is assessed using F-test on the RSS; the model(s) with statistically lower RSS is selected.
2. Model requires the fewest parameters, while providing reasonable fitting; the model has the smallest statistics of the parsimony criteria of Akaike and Schwartz.
3. Errors of the parameter estimates are small.
4. Data fitting to the model shows no clear systematic deviation.

The best-fit model is the least complex model with which to describe the experimental data. RSS, residual sum of squares.

The mean expression levels of P-glycoprotein quantified by the 4077 antibody were 0.22 μg/mg total cell protein for MDCK cells (~10% of the P-glycoprotein-overexpressing NIH 3T3 MDR1 cells: 2.25 μg/mg total cell protein). Total cell protein of MDCK was 212 μg/filter (4.15 cm\(^2\)) on average. Taken together, MDCK cells were estimated to be expressing ~0.05 μg of P-glycoprotein per filter preparation, corresponding to ~0.3 pmol/filter (4.15 cm\(^2\)).

The rat monoclonal antibody against human MRP failed to detect any significant antigen in the MDCK cells (data not shown). To circumvent the possible species differences of immunoreactivity of this antibody, we examined MRP function by the calcine accumulation assay (see below).

**Calcine Accumulation Assay**

Prostaglandin A\(_2\), 10 μM, which behaves as an MRP inhibitor in this concentration range (14), caused only
10% change of calcine accumulation in MDCK cells over the control; the mean MAF value was 0.10 (n = 3). In contrast, 100 μM verapamil (a nonspecific inhibitor for both P-glycoprotein and MRP; Ref. 24) induced a more than twofold increase in the accumulation; the mean MAF value was 0.53 (n = 3). Respective values for P-glycoprotein-overexpressing NIH 3T3 MDR1 cells were 0.05 (with prostaglandin A1) and 0.85 (with verapamil). This suggests that MDCK cells possess little MRP function, while they have relatively high P-glycoprotein function.

**MTT Cell Viability Assay**

Twenty-four-hour exposures to digoxin and vinblastine at concentrations of 0.1 μM resulted in MDCK cell viability rates of 99 ± 5% and 108 ± 5%, respectively (n = 5–6); after 48-h incubation, the viability rates decreased to 88 ± 7% and 68 ± 4%, respectively. The viability values for 1 μM digoxin were 65 ± 4% (24 h) and 39 ± 2% (48 h) and for 1 μM vinblastine were 109 ± 4% (24 h) and 68 ± 4% (48 h). In contrast, itraconazole (0.1–20 μM) showed few toxic effects for 48 h with the viability rates ranging from 101% to 133%. These results indicate that monolayer integrity was maintained during the transport experiments under the conditions used in this study.

**Mannitol Paracellular Flux**

When digoxin was added to the apical side for the apical-to-basal transport experiment, paracellular diffusion monitored by mannitol was higher than those of basal-to-apical experiments (Table 3; ~2–6% of mannitol added with digoxin to the apical side appeared in the basal side at 4-h incubation, whereas only 0.8% did in the basal-to-apical flux experiments). Vinblastine showed a similar trend to a lesser extent. These results were taken into account in the model fitting of drug transport by assigning the obtained relative paracellular diffusion constants for each of the four conditions to the corresponding fitting equations (see MATERIALS AND METHODS).

**Digoxin and Vinblastine Transport**

As shown in Fig. 4, in the absence of itraconazole, the basal-to-apical fluxes of digoxin and vinblastine were ~5- to 10-fold faster than those in the apical-to-basal direction. When drug was added initially to the basal side (Fig. 4, A and C), the apical concentrations increased and reached an equal level in both sides at ~10–20 h. After that period, the apical concentration of drug increased against a concentration gradient. In contrast, when drug was added initially to the apical side (Fig. 4, B and D), the basal side concentration did not exceed one-tenth of the corresponding apical concentration during the 24-h incubation, maintaining a significant concentration gradient. Vinblastine basal-to-apical flux was ~1.5-fold faster for the first several hours than digoxin. This difference gradually dissipated, and both drugs achieved comparable concentrations in the apical compartment at 24 h.

**Second-Stage Fitting Results**

None of the fitting results of the three models was under active constraints at termination. For both drugs, models A and B provided significantly better fitting than a simpler model, model C (Table 4). There was no significant difference in the goodness of fit between model A and B (Table 4). However, model A showed the lowest values in the parsimony criteria of AIC and SC (Table 4). There was no systematic deviation of the model A fitting from the data points (see Fig. 4; and nonsignificant results of the runs test, data not shown). Therefore, model A is considered as the minimal model, whose fitting is statistically indistinguishable from those of a more complex model. Compared to digoxin, vinblastine fitting to model A showed relatively small RSS and more precise parameter estimates (CV < 40%).

Data fitting with the model A variants (see MATERIALS AND METHODS) did not converge to meaningful estimates and failed to provide better statistical results (not shown). These findings indicate that model A (the physiologically defined 3-compartment model with a functionally single active transport system in the apical membrane) appears to be the model of minimal complexity that can quantitatively reproduce the characteristics of our data set on digoxin and vinblastine transport in the MDCK cells (Fig. 4).

**Cross-validation of the Model Prediction**

As shown in Table 5, the best-fit model (model A) predicted the following: 1) the apical membrane of the MDCK cell is less diffusion permeable than the basolateral membrane for both drugs; and 2) the distribution volume is 10-fold higher for vinblastine than that for digoxin, suggesting extensive cellular accumulation of vinblastine. These model predictions were experimentally cross-validated.

**Diffusion across apical membranes is slower than across basal membranes.** This prediction is compatible with the characteristics of barrier epithelia (37). If the lipid bilayer of the apical membrane is relatively impermeable, then access to the cytoplasm by diffusion from...
the extracellular milieu should be slower through the apical membrane than through the basal membrane. Given that the drug entry port of P-glycoprotein exists in its cytoplasmic domain or in the lipid phase of the apical membrane, substrates are expected to engage in the transport more readily through the basal than through the apical membranes, because the relative diffusion impermeability of barrier epithelia is due to the characteristics of the outer leaflet of the apical membranes (21).

To cross-validate this model prediction, we examined the following compounds as inhibitors of the vinblastine and digoxin transport: digoxin, vinblastine, vincristine, verapamil, itraconazole, and ketoconazole. The reported P-glycoprotein substrates such as digoxin, verapamil, vinblastine, and vincristine inhibited digoxin and vinblastine basal-to-apical transport when they were added to the basal side; when added to the apical side, the inhibition was less remarkable (Fig. 5).

Despite the inhibition of the vinblastine basal-to-apical transport by basally added vincristine (Fig. 5A), cellular uptake of vinblastine was significantly increased (Fig. 6A). In contrast, apically added vincristine showed little effect on the vinblastine uptake as well as on the transport. These findings indicate that the inhibition of the basal-to-apical transport of vinblastine by the basally added P-glycoprotein substrates is due to blockage of vinblastine extrusion at the apical membranes. We previously showed the similar phenomenon for digoxin (17, 35). Taken together, these findings are compatible with our model prediction that apical membranes of MDCK cells are an efficient diffusion barrier relative to basal membranes for P-glycoprotein substrates.

In contrast to the P-glycoprotein substrates, itraconazole exhibited strong and almost instantaneous inhibition from the apical side (Fig. 5, C and F). When the cell monolayers were incubated with 5 μM itraconazole added to both sides at time 0, no significant accumulation of itraconazole in the apical side was observed after 48 h of incubation; the apical-to-basal concentration ratio was 0.97 ± 0.13 (n = 6). Its major metabolite, hydroxyitraconazole, was undetectable both in media and cell lysates. These findings suggest either that
Table 4. Second-stage fitting statistics for models of cellular drug transport

<table>
<thead>
<tr>
<th>Model</th>
<th>Degrees of Freedom</th>
<th>Number of Parameters</th>
<th>R²</th>
<th>Residual Sum of Squares</th>
<th>AIC</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digoxin</td>
<td>A</td>
<td>58 (6)</td>
<td>0.9951</td>
<td>309.1</td>
<td>379.0</td>
<td>381.9</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>57 (7)</td>
<td>0.9951</td>
<td>311.8</td>
<td>381.5</td>
<td>386.6</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>61 (3)</td>
<td>0.9946</td>
<td>385.8</td>
<td>387.2</td>
<td>383.6</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>A</td>
<td>58 (6)</td>
<td>0.9797</td>
<td>120.3</td>
<td>318.6</td>
<td>331.5</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>57 (7)</td>
<td>0.9972</td>
<td>119.2</td>
<td>320.0</td>
<td>335.1</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>61 (3)</td>
<td>0.9735</td>
<td>604.5</td>
<td>415.9</td>
<td>422.4</td>
</tr>
</tbody>
</table>

F-test | F statistic | p value |
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<tbody>
<tr>
<td>A vs. B</td>
<td>F₁,₅₇ &lt; 1.0</td>
<td>NS</td>
</tr>
<tr>
<td>A vs. C</td>
<td>F₁,₅₇ = 4.90</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>B vs. C</td>
<td>F₁,₅₇ = 3.39</td>
<td>&lt;0.025</td>
</tr>
</tbody>
</table>

Statistical goodness of fit is assessed by Akaike's Information Criterion (AIC) and Schwartz criterion (SC), which combine the number of estimated parameters, the number of data points, and the best weighted sum of squares. The smaller the AIC and SC values, the better statistically. NS, not significant.

Itraconazole is not actively transported, or that its pump-mediated transport is extremely futile as a result of high back-diffusion possibly attributable to its high lipophilicity. Inhibition of digoxin and vinblastine transport by ketoconazole did not differ significantly between the apical and basal applications.

Larger distribution volume of vinblastine. The model predicts that distribution volume of vinblastine is nearly 10-fold higher than that of digoxin (Table 5). Interestingly, our experimental results showed that 60-min vinblastine cellular uptake was 10 times as high as digoxin uptake (Fig. 6B). Non-specific binding to the filter itself is not a contributing factor to this finding, because vinblastine and digoxin binding to the filter after 60-min incubation reached only 2.1% and ≤0.1% of the corresponding cellular uptake levels, respectively.

Simulation Based on the Best-fit Model

To understand how the differences of the pump function are reflected to the net drug fluxes in this experimental condition, digoxin and vinblastine fluxes were simulated with different degrees of the pump-mediated intrinsic clearance (i.e., 0%, 10%, 25%, 50%, and 100% of control) (Fig. 7), using model A and parameters obtained from our data (Table 5).

The results show that the basal-to-apical fluxes are more sensitive to the pump function changes than the apical-to-basal ones. Namely, the apical-to-basal fluxes, especially for the first 2 h, do not substantially change even when the drug efflux via P-glycoprotein is inhibited. This is incompatible with the conventional criteria (Fig. 1).

DISCUSSION

This study provides quantitative evidence that digoxin and vinblastine transport in the wild-type MDCK cells expressing P-glycoprotein can be explained by a three-compartment model, which assumes a functionally single itraconazole-inhibitable unidirectional transport mechanism in the apical membrane. Moreover, the model gives an overall kinetic picture of epithelial drug transport at a cellular level, elucidating functional contributions of each membrane drug translocation process. Interestingly, it predicts low diffusion permeability of the apical membranes for vinblastine and digoxin and significant kinetic differences between the two drugs. We were able to experimentally cross-validate these model predictions. This modeling approach is feasible to gain insight into kinetics of cellular drug handling.

Although modeling approach is one of the important analytical methods, there are limitations (Table 6). A

Table 5. Digoxin and vinblastine kinetic parameters derived from the models

<table>
<thead>
<tr>
<th>Drug</th>
<th>Parameters</th>
<th>Estimates</th>
<th>Standard Deviation</th>
<th>Unit</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model A</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Digoxin</td>
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<td>0.249</td>
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<td></td>
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<td>δ₂</td>
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<td>0.0452</td>
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<td>δ₃</td>
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</tr>
<tr>
<td></td>
<td>θ</td>
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<td>0.029</td>
<td>ml/h</td>
<td>97</td>
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<td></td>
<td>V</td>
<td>0.0653</td>
<td>0.0184</td>
<td>ml</td>
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<tr>
<td>Vinblastine</td>
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<tr>
<td></td>
<td>V</td>
<td>0.705</td>
<td>0.0863</td>
<td>ml</td>
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<td>Model B</td>
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<td></td>
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<tr>
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<td>ml/h</td>
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<tr>
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<td>0.0900</td>
<td>ml</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.0448</td>
<td>ml/h</td>
<td>74</td>
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<tr>
<td></td>
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<td>0.0033</td>
<td>ml/h</td>
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</tr>
<tr>
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<td>ml/h</td>
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<td>Vinblastine</td>
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<td>0.113</td>
<td>ml/h</td>
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<tr>
<td></td>
<td>δ</td>
<td>0.0195</td>
<td>0.0029</td>
<td>ml/h</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>θ</td>
<td>0.0651</td>
<td>0.0172</td>
<td>ml/h</td>
<td>26</td>
</tr>
</tbody>
</table>

CV, coefficient of variation; k, intrinsic clearance of the pump; δ₁-δ₃, diffusion parameters of apical, basal, and intercellular junctions, respectively; θ, remaining fraction of the pump in the presence of itraconazole; V, apparent distribution volume of drug; and θ_app, intrinsic clearance of the basally located drug efflux pump (e.g., multidrug resistant associated protein, MRP).
model is an abstract of a real system, simplifying an intricate network of events into a manageable format within limits of time and space resolution of the experimental data. As a result, a model can be used for simulation of the system behavior and prediction of the consequences. However, each system parameter in the model may not correspond to a single physical or molecular entity, because different molecular mecha-
models may be viewed kinetically as a single functional unit. Hence, the unidirectional transport mechanism on the apical membranes in our models may not be represented by P-glycoprotein alone, although our model describes the experimental data reasonably well.

MRP, another recently identified membrane drug transporter (2, 5, 13, 36), is expressed on the basal membrane of LLC-PK1 renal tubular cells (both the wild-type and the human MRP gene transfected line) and the transport activity toward the basal side of the cell. MRP translocates both uncharged hydrophobic compounds and anionic substances such as glucathione conjugates of drug across the basal membranes. However, using a calcine accumulation assay, we were able to show that MRP is not a major contributor in our experimental system with MDCK cells.

Contribution of other modes of cellular drug transport was not taken into account in this study. MDCK cells have been extensively studied for their endocytosis-transepithelial-exocytosis processes (7). Indeed, tracellular drug transport via vesicular trafficking exists for organic anions and cations (22), and this mode of drug transport is likely to function for most drugs including vinblastine and digoxin, although the magnitude of contribution is unknown. Also, drugs distributed into intracellular organelles may be transported to plasma membrane domains via vesicular trafficking, and this contributes to overall transepithelial drug transfer. Therefore, the pump clearance estimate in the current model is a hybrid parameter for pump(s) and transcytosis processes. To further refine the present model, functional contribution of these processes needs to be experimentally determined and then examined against a comprehensive model. Similarly, the "volume of distribution" in our model is likely to include various intracellular drug binding and uptake processes, which were kinetically combined into a single parameter in the present study. Models incorporating above-mentioned processes will have to be examined with data of higher time and space resolution.

Although a comprehensive model remains to be tested, our model provided several interesting predictions. It has been widely accepted that the pump function of P-glycoprotein in epithelial tissues is proportional to the basal-to-apical fluxes and inversely proportional to the basal-to-apical fluxes of its substrates (Fig. 1: Refs. 5, 15, 27, 31). In contrast to this conventional notion, the rate of apical-to-basal vinblastine transport up to 4 h in our study did not show significant symmetrical increase (i.e., symmetrical to the decrease in the basal-to-apical direction) in the presence of the pump inhibitor. Despite the apparent violation of the criteria, the vinblastine data are well explained by the pump model. Although the digoxin apical-to-basal flux increased from the early period of incubation when P-glycoprotein is inhibited, the nearly 10-fold increase of concomitant paracellular diffusion (i.e., monitored by mannitol) made the interpretation difficult (Table 1). Our simulation further indicates that increase in apical-to-basal digoxin and vinblastine fluxes for the first several hours (Fig. 7) necessitates increase in diffusion across plasma membranes and/or intercellular junctions. These findings exemplify pitfalls of intuitive interpretation of transport kinetics without quantitative verification. The conventional, intuitive experimental criteria may not necessarily hold true. Interpreta-
tion of transport data based on a multicompartment model should be supported by a quantitative modeling approach. Moreover, this has a significant implication when one interprets data regarding drug penetration across epithelial tissues from the apical to basal side such as seen in blood-brain barrier (30) and intestinal cells. If modification of P-glycoprotein function is associated with changes in initial apical (luminal)-to-basal drug translocation in these tissues, then concurrent alterations of diffusion may also have to be considered.

Asymmetry of apical and basal membrane diffusion was also predicted by the model. Barrier epithelia such as urinary bladder and collecting tubule are known to have apical membranes with very low permeability (9, 37), due to lower fluidity of the exofacial leaflet of the apical membranes (21). Our model prediction in MDCK cells, which are of distal tubular and collecting duct origin, is compatible with this functional difference in permeability between the apical and the basolateral membranes of barrier epithelia. However, it is unknown whether a difference in simple diffusion through plasma membranes accounts for this model prediction or whether differential expressions of carrier-mediated systems for facilitated diffusion such as aquaporins (i.e., pore-forming water channels) are also involved. Although the molecular mechanism underlying these diffusion processes and its asymmetry is unclear, the prediction was cross-validated by our results showing that verapamil, vincristine, and vinblastine inhibit basal-to-apical digoxin transport more from the basal side of the monolayers; similarly, verapamil, vincris-

Table 6. Advantages and limitations of modeling approach in cellular drug transport research

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimation of system parameters bearing possible biological significance</td>
<td>No guarantee of link between the parameters and the molecular entities</td>
</tr>
<tr>
<td>Determination of statistical uncertainty of the estimates</td>
<td>Ignoring functionally minor systems for simplicity</td>
</tr>
<tr>
<td>Quantitative prediction of overall system behavior</td>
<td>Dependence of model complexity on resolution of measurement</td>
</tr>
</tbody>
</table>

Fig. 7. Simulation of digoxin and vinblastine transport-time profiles. Based on model A and the estimated parameters (Table 5), digoxin (A and B) and vinblastine (C and D) transport was simulated for different levels of the pump function (solid lines, 100%; dashed lines, 50%; 25%, 10%, and 0%); y-axis represents drug concentrations expressed as % of the initial concentrations. A and C: changes of digoxin (A) and vinblastine (C) concentration in apical side for 2 h, when drugs are added to basal side at time 0. Insets of A and C: concentration changes in the basal and the apical sides over 50 h, when added to basal side at time 0. B and D: concentration changes of digoxin (B) and vinblastine (D) in basal side for 2 h, when added to apical side at time 0. Insets of B and D: concentration changes in basal side over 50 h, when added to apical side at time 0. Note that insets of B and D do not show the changes of concentration in apical side to provide a detailed view on basal side concentration changes. Note also that basal concentration changes for the initial 2 h in the condition shown in B and D are relatively insensitive to pump function changes.
tine, and digoxin inhibit basal-to-apical vinblastine transport more from the basal side (Fig. 5). These findings can be explained by the difference in diffusion between the apical and basal membranes of MDCK cells. If the substrate entering the MDCK cells across the apical membrane is pumped out by P-glycoprotein before reaching cytoplasm, then it must be assumed that P-glycoprotein has two or more independent drug translocation processes: one for substrates presented to the pump through the outer leaflet of the apical membrane from extracellular side, and another for substrates accessing to the pump intracellularly. Otherwise, it is inexplicable that apically added excess amounts of these drugs conspicuously lack the inhibitory effects on the pump function (Fig. 5).

In contrast to the P-glycoprotein substrates, itraconazole almost instantly inhibits P-glycoprotein more from the apical than from the basal side. Ketoconazole, another azole antifungal and a P-glycoprotein inhibitor (29), shows no such differential inhibitory effects (Fig. 5). Octanol/water partition coefficients of these compounds are as follows in a rank order of lipophilicity: vinblastine, >1.6 × 10^4; ketoconazole, >2,000; vincristine, >150; and digoxin, ~50. Although the coefficient of itraconazole has not been reported, it is more lipophilic than ketoconazole. Clearly, hydrophobicity per se does not completely explain the side-dependent inhibitory effects of these substances on P-glycoprotein. Itraconazole and ketoconazole may interact with P-glycoprotein in a manner different from other typical P-glycoprotein substrates, probably by also interacting with the extracellular domain of P-glycoprotein. In fact, we were unable to detect a signal of itraconazole active transport; i.e., accumulation of itraconazole on the apical side against a concentration gradient during the 48-h incubation.

Certain assumptions made in this modeling analysis warrant discussion. First, metabolism of digoxin and vinblastine was assumed to be negligible. This assumption seems valid, because digoxin is not extensively metabolized in vivo. Vinblastine undergoes metabolism through cytochrome P-450 3A (CYP3A) enzymes, while other groups of CYP isoforms do not seem to mediate its metabolism (38). Although kidney expresses CYP3A, its expression level is less than 1% of the hepatic level (11), and its significant contribution to overall disposition of vinblastine is unlikely. Taken together, metabolism of these probe compounds seems small enough to be ignored.

Second, itraconazole was assumed to inhibit the pump function only. We do not know whether itraconazole directly affects the pump or perturbs membrane fluidity, thereby indirectly inhibiting the pump. This may further change passive diffusion of probe compounds across the membranes. Third, the intracellular distribution volume of drug was assumed not to be affected by itraconazole, although intracellular binding of drug may be altered by the inhibitors (33). We considered these possibilities, and assigned separate diffusion and volume parameters to the model for the data obtained in the presence of itraconazole. However, the volume and diffusion parameters did not differ in the presence or absence of itraconazole by more than 10%, and this separate parameter assignment did not improve the overall fitting (data not shown). This suggests that these potential effects are insignificant, although not completely ruled out. Therefore, we opted for simplicity and fitting efficiency to assume no change in the diffusion and distribution volume of digoxin and vinblastine by itraconazole.

Third, we assumed the first-order transport kinetics for P-glycoprotein. In this study, digoxin and vinblastine concentrations of greater than 0.1 μM were not used in the 24-h time course experiments, because of the possible damaging effects on the cells after prolonged exposures (see MTT assay results). Because $K_m$ values of P-glycoprotein-mediated transport of digoxin and vinblastine are probably in the range of 1–10 μM (15, 16), our assumption of first-order kinetics appears valid. Given that the modeling approach is feasible, in situ determination of $K_m$ and $V_{max}$ may be possible, if the method is further refined.

In summary, using the model for P-glycoprotein-mediated epithelial drug transport, we were able to estimate kinetic parameters of digoxin and vinblastine transport across P-glycoprotein-expressing MDCK cells. Our simple model provides a kinetic framework with which to understand epithelial drug transport at a cellular level, predicting substantial difference in diffusion between the apical and the basal membranes. The modeling approach, however, is presently limited by resolution of experimental measurement. Detailed data on intracellular drug distribution and transcellular movement are needed, which will lead to designing more comprehensive models.

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REFERENCES


Hypothetical framework for enhanced renal tubular secretion of drugs in cystic fibrosis

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Abstract — Several clinical studies demonstrate reduced serum concentrations of renally excreted drugs in patients with cystic fibrosis (CF). To explain this phenomenon, we propose a model supporting increased proximal tubular secretion of certain drugs in individuals with CF. We hypothesize that the chloride channel located on the apical surface of renal proximal tubular cells and controlled by the cystic fibrosis transmembrane conductance regulator (CFTR) operates suboptimally in CF patients, and that the abnormal CFTR decreases Cl⁻ reabsorption, resulting in an increased concentration of Cl⁻ in the tubular lumen. We postulate that, in an effort to maintain homeostasis, luminal Cl⁻ moves intracellularly in exchange for organic anions. The result of stimulating this anion exchanger is an increased rate of organic anion secretion by the renal tubule. Hence, due to enhanced tubular secretion, individuals with CF demonstrate increased tubular clearance of organic anion drugs, resulting in lower steady state serum concentrations.

Introduction

CF, the most common lethal genetic disease in caucasians, affects approximately 4 in 10 000 (1). CF is characterized by a defect in chloride (Cl⁻) transport, and often results in the production of viscous mucus. These thick secretions cause damage and dysfunction to several organs, most notably the exocrine pancreas and the lungs. Infections of the pulmonary airways constitute a major cause of morbidity and mortality in individuals with CF (2). Consequently, antibiotics are used frequently by these patients. In order to ensure optimal therapy, detailed information regarding the pharmacokinetics and pharmacodynamics of commonly used antibiotics should be obtained.

The primary abnormality in individuals with CF appears to be an alteration in the CF gene product called CFTR (cystic fibrosis transmembrane conductance regulator). Regulated by cyclic adenosine 3'-5'-phosphate (cAMP)-dependent phosphorylation, CFTR is a small conductance, voltage- and time-independent Cl⁻ channel (3). The CFTR protein belongs to the adenosine 5'-triphosphate (ATP)-binding protein family of unidirectional solute pumps. CFTR is expressed on the apical membranes of epithelial cells and is abundant in sweat ducts, small pancreatic ducts, intestinal crypts, and renal tubules (4).
The majority of CF patients studied appear to have an abnormally controlled CFTR channel or a mal-
positioned CFTR protein subunit (5). Approximately 70% of patients with CF have a phenylalanine dele-
tion at position 508 of the CFTR gene (2). Other
CFTR mutations are associated with varying manifest-
tations of CF.

Due to the lack of significant pathology of the kidney, scant information exists concerning renal
issues in patients with CF. Pharmacokinetic studies
demonstrate reduced serum concentrations and in-
creased clearance rates of many drugs in patients with
CF including: amikacin (9), cephalaxin (8), cloxacinil-
in (12), dicloxacillin (6), epicillin (8), gentamicin (10),
methicillin (7), theophylline (16), ticarcillin (13),
tobramycin (11), fleroxacin (14), ceftazidime (15),
and ciproflox (unpublished data). The mechanism
responsible for these findings is not known.

Neither glomerular filtration (9,17,18) nor serum
protein binding (6,9,12,15,23) of drugs in general
appear to differ from normal in CF patients. In an
unpublished study examining the renal clearance
of the organic base, cispavine, the urine hydrogen ion
concentration CF patients was approximately 3.5 times
higher than in controls. In this study, renal cispavine
clearance increased as urine pH decreased (unpub-
lished data). Hence, an increase in the renal clearance
of basic drugs can be at least partially explained by
the observation that the urine of individuals with CF
is more acidic (pH = 5.30 vs 5.85) than that of non-CF
patients; therefore, basic drugs become positively
charged and cannot passively diffuse across the luminal
membrane. By this reasoning, one would expect CF
patients to show slower renal clearance rates for
anionic drugs. However, clinical observation indicates
otherwise.

Many anionic antibiotics are actively secreted
across the proximal tubule. Anionic antibiotics such
as the penicillins are taken up into the tubular cells
from the interstitial (basolateral) side by the organic
acid transporters (19) located on the basolateral
membrane of the renal tubular cell. Organic anions
accumulate in renal tubular cells following active
transport into the negative cell interior. Efflux into
the less negative tubular lumen occurs, in part, with
the aid of an apically located anion exchanger.

The lumen contains numerous ions and substances
of nutritional value, which the body requires to main-
tain a homeostatic environment. Therefore, the kidney
must selectively reabsorb some substances without
allowing metabolic end-products such as urea and
creatinine to re-enter the bloodstream. Sodium and
glucose are examples of such substances which must
be reabsorbed. Chloride follows sodium reabsorption,
maintaining electroneutrality. While Cl− may be
reabsorbed by transcellular or paracellular routes,
udies using proximal tubular cells from rat suggest
that an important part of this reabsorption is by an
active transcellular process (20).

In recent years, Cl− was shown to be a substrate
for numerous luminal anion exchangers. Para-amino-
hippurate (PAH), the prototype organic anion, has
been used in numerous studies examining anion
transport. Often referred to as the PAH exchanger, an
apically located anion exchanger was recently identi-
fied to transport both organic and inorganic anions
(21). Chloride is one such substrate. Studies with dog
tissues indicate that this exchanger is also responsible
for urate transport (22). The countertransport of
Cl− by this electroneutral anion exchanger is trans-
stimulated by PAH, Cl−, Br−, HCO3−, formate, and a
pH gradient (effective in the absence of HCO3−) (21).
Both Cl−-OH− and Cl−-CF− exchange are inhibited
by PAH (21).

Since the discovery of the organic anion exchanger,
other anion exchangers have been identified in the
surrounding region. At least two other separate anion
exchangers that mediate Cl− transport have been dis-
covered: a chloride–formate and a chloride–oxalate
exchanger have both been shown to exist in renal
microvillus membranes from rabbit (20).

A recent study of renal ticarcillin clearance in CF
patients indicated that the maximal tubular secre-
tion rate of this drug is no different from that in non-
CF patients; however, the renal secretory system in
CF patients appeared to have greater affinity for penicillin-type drugs (13). This suggests that CF
patients do not express more anion transporters than
non-CF patients.

Our hypothesis

CFTR is located in the apical membrane of proximal
and, to a lesser degree, distal renal tubular cells.
Due to a dysfunctional CFTR-regulated Cl− channel,
patients with CF may reabsorb Cl− inefficiently. To
compensate for this, and to maintain electrical neutral-
ity, Cl− is likely reabsorbed by alternate pathways.
We hypothesize that an alternate pathway for Cl−
reabsorption is the luminal anion exchanger.

Increased chloride reabsorption by an anion ex-
changer would stimulate the efflux of an intracellular
organic anion. When the organic anion that is ex-
changed is an antibiotic, an increased rate of drug
secretion occurs.

This hypothesis is indirectly supported by the
recent pharmacokinetic study with ticarcillin, where
patients with CF were shown to have total binding
capacities (Bmax) of the renal organic anion trans-
porter similar to healthy subjects as determined by ultrafiltration (13). In contrast, patients with CF had, on average, more than a two-fold higher transporter affinity (lower Kd). These data are consistent with our hypothesis in showing that there are no additional transporter sites for organic anions in patients with CF. Rather, those molecules, once transported into the cells from the interstitial (basolateral) side, are more avidly carried through the apical membrane into the urine. If there is higher exchange of organic anions at the luminal side (i.e. after the anions have crossed the basolateral membrane and got into the cell), this would translate into a higher affinity (lower Kd), with no change in Bmax.

The availability of animal models in which the CFTR protein has been manipulated allows for the future proof or disproof of our hypothesis. Beyond the explanation of changes in drug-handling in CF, this hypothesis may shed light on the secretory driving forces of organic anions in the mammalian kidney.

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References

A Model for the Prediction of Digoxin–Drug Interactions at the Renal Tubular Cell Level

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Summary: Digoxin–drug interactions are relatively common causes of digitalis toxicity. Recently, the clinical importance of the renal tubular secretion of digoxin has been proven by documenting drug interactions at this level. The authors describe a model using cultured renal tubular cell monolayers that can be used to predict drug interactions with the cardiac glycoside. This model accurately documents clinical digoxin interactions such as those with verapamil and propafenone. The common feature of these interactions is that they involve P-glycoprotein substrates (e.g., digoxin, vincristine, vinblastine) or inhibitors (e.g., quinidine, cyclosporine). In the case of the newly described interaction of digoxin with itraconazole, the model preceded the emergence of clinical cases. Key Words: Digoxin—Drug interaction—Kidney—P-glycoprotein—Model.

Many drugs are excreted by the kidney, and very often drugs that are eliminated by hepatic metabolism form metabolites that are renally excreted. Almost all of the body load of digoxin is excreted unchanged by the kidney. Because of its low therapeutic index and very wide clinical use, there are concerns that digoxin–drug interactions could lead to increases in digoxin serum concentrations and result in toxicity (1).

For decades it was assumed that digoxin was eliminated through the kidney by glomerular filtration. This belief stemmed from the fact that, in patients with renal failure, the plasma clearance of digoxin is decreased in proportion to the decrease in creatinine clearance. However, in 1974, Steiness drew attention to the fact that, after correction for the 20% plasma protein binding of digoxin, its renal clearance substantially exceeds that of creatinine, indicating net tubular secretion of the cardiac glycoside (2).

During the 1970s and 1980s, several drugs that are commonly coadministered with digoxin were shown to increase the serum concentrations of this cardiac glycoside and to cause digitalis toxicity in some patients. These included quinidine, verapamil, amiodarone, and propafenone (3). The common denominator of these drug interactions is that none of these antiarrhythmics appears to change the oral bioavailability of digoxin. Moreover, because the hepatic elimination of digoxin is minimal, they must affect the renal excretion of digoxin. Yet, quinidine, verapamil, amiodarone, and propafenone have not been shown to decrease the glomerular filtration rate, suggesting that these drugs primarily affect the net renal tubular secretion of the cardiac glycoside (4). Clearance studies have shown that quinidine, verapamil, amiodarone, and propafenone decrease the renal clearance of digoxin without decreasing the glomerular filtration rate (GFR). The observation that these interacting drugs can cause more than a 100% increase in serum concentrations of digoxin in many patients indicates predominance of the renal tubular secretion of digoxin over its glomerular filtration (4).

During the last decade, evidence has been accumu-
lated to show that digoxin does not use the classic organic anion or cation transport systems for its renal tubular transport, and its interactions are not associated with Na⁺K⁺ ATPase receptor binding (3). However, more and more evidence has pointed out that digoxin interacts with the P-glycoprotein multidrug resistance transporter encoded by the MDRI gene in humans (4–6). This transport protein, which is found in the apical membranes of the renal epithelia, has been shown to pump a large number of naturally-occurring chemotherapeutic agents out of cells, and high expression of this protein in tumors is associated with tumor resistance.

In the context of renal transport, we hypothesized that this is just one of many transporters in the urinary lumen that is equipped to pump naturally occurring toxins that may be reabsorbed along with water out of the cell (4). This “urine-blood barrier” is therefore important to maintain homeostasis in cells.

Several lines of evidence have shown that digoxin uses P-glycoprotein for its renal tubular transport:

1. Drugs that are known substrates of P-glycoprotein (e.g., vinblastine, vincristine) inhibit digoxin transport across the renal tubular cell (4).
2. Drugs that are known to inhibit P-glycoprotein transport (e.g., verapamil, cyclosporine) also inhibit digoxin transport across the renal tubular cell (4).
3. Transfection of renal tubular cells with the MDRI gene increases the renal tubular secretion of digoxin (6).
4. Mice in which the MDRIa gene has been “knocked-out” accumulate more digoxin than their wild-type counterparts (7).

Appreciating the importance of the renal tubular secretion of digoxin, it becomes clear that drugs that may affect this secretory mechanism may result in digitalis toxicity.

This paper describes an in vitro model that allows the prediction of drug-drug interactions at the renal tubular cell level. Digoxin is used as an example drug because digoxin–drug interactions are of great clinical concern because of the low therapeutic index of digoxin. Because digoxin is one of the most widely prescribed drugs in the world, and because many patients receive it along with other medications, the ability to predict clinically-important interactions before clinical toxicity occurs is highly relevant. We are also using this model to study other drugs that are secreted by renal tubular cells, some of which use systems other than P-glycoprotein for their transport.

**The Model**

The in vitro tissue culture model consists of confluent, polarized renal tubular cell monolayers grown on the permeable, inorganic membranes of commercially available tissue culture inserts. The side of the cells that attaches to these filters represents the basal or blood side of the renal tubular cells, whereas the opposite end differentiates into an apical or urine side. This orientation facilitates the study of drug transport across cell monolayers. One can add a drug to the media bathing one or both sides of the cell monolayer and monitor its movement by sampling the media over a period of time (Fig. 1). The advantage of this model as compared to studies with tissue slices or membrane vesicles is that it is a “whole cell” model and the apical and basolateral sides of the cell can be approached in isolation.

The use of different renal tubular cell lines allows for a comparison of species and regional differences. For instance, LLC-PK1 cells are derived from a male pig and have properties of proximal tubular cells; whereas MDCK cells originate from a female cocker spaniel and have properties of the distal tubule and collecting duct regions. Although one must bear in mind that cells in culture are not necessarily representative of the in vivo situation, these two cell lines appear to have similar drug transport properties to the human kidney. P-glycoprotein expression has been confirmed in both LLC-PK1 and MDCK cells by our group and others (8–10). Functional and immunohistochemical studies indicate that P-glycoprotein is located in the apical membranes of these renal tubular cells.

**MATERIALS AND METHODS**

**Cell Culture**

LLC-PK1 cells representing proximal tubular cells and MDCK cells representing distal tubular cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and were grown in α-MEM

**FIG. 1.** The in vitro model. The tissue culture insert with cells growing in a confluent monolayer on the upper side is shown sitting in the well of a tissue culture plate. The shaded area represents the sampling area for the basolateral (blood) side, and the unshaded area represents the sampling area for the apical (urine) side. The arrow indicates the direction of digoxin secretion.

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media containing 10% fetal bovine serum (FBS) without antibiotics under an atmosphere of 5% CO₂-95% air at 37°C. They were subcultured twice weekly using 0.02% EDTA and 0.05% trypsin.

Measurement of Digoxin Transport and Uptake

LLC-PK1 or MDCK cells in the incubation media were seeded at a density of 2.4 x 10⁵ cells/cm² on permeable, inorganic membranes attached to 25-mm tissue-culture inserts (0.02 μm pore size, 4.15 cm² growth area: Nunc TC inserts). The inserts were placed in six-well tissue culture plates, fed every 48 hours, and incubated in the same conditions as described earlier. Experiments were conducted 10 days after seeding the cells. The cells were preincubated with phosphate-buffered saline (PBS) for 30 minutes at 37°C. The incubation was started at 37°C immediately after the basal media were replaced with the same media (1.2 ml) containing [³H]-digoxin (0.1 μM or 50 μM) and 1.45 μM [¹⁴C]-mannitol in the presence or absence of itraconazole, ketoconazole, fluconazole, or propafenone. The concentrations of digoxin and mannitol were determined by measuring the radioisotope concentration (disintegration-per-minute) with liquid scintillation counting. We excluded results of cell monolayers with [¹⁴C]-mannitol fluxes greater than 2 SD of the mean mannitol fluxes in each experiment.

The cellular accumulation of radiolabeled digoxin in the presence and absence of propafenone was examined after 40 minutes of incubation. The culture inserts were rinsed on both sides with ice-cold phosphate-buffered saline. The filters were cut out of the tissue culture inserts using a cutting device provided with the inserts. The membranes were placed in tissue culture plates containing 0.6 ml of 0.01% Triton X-100. After solubilization of the cells, 0.3 ml cell solution was sampled for determination of radioactivity. Disintegration-per-minute (dpm) counts were corrected for contamination by the incubation media. On average, the contamination accounted for <10% of the uptake.

Materials

The [³H]-digoxin (16.1 Ci/mmol) and [¹⁴C]-mannitol (55.0 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Nonradioactive drugs and chemicals and Nunc tissue culture inserts (25mm external diameter) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.) and A/S Nunc (Roskilde, Denmark), respectively.

Data Analysis

Digoxin cumulative flux was defined as digoxin appearing in the apical compartment for a given period of incubation time and was expressed as moles per cm² of filter. When the data were expressed as a percentage of control, the cumulative fluxes at the three time points (10, 20, and 30 minutes) expressed as a percentage of control were averaged because a cumulative flux was nearly linear for 30 minutes of incubation. The results were expressed as means of at least three filters ± SD unless otherwise stated and were compared using the unpaired Student’s t-test.

RESULTS

When digoxin was introduced in similar concentrations at both the apical and basolateral surfaces of the MDCK tubular cell monolayers for 24 hours, the concentration gradient produced was 20:1, whereas for LLC-PK1 it was 3:1, indicating accumulation against a concentration gradient in both instances as a result of its active transport.

For inhibition experiments, we used 30-minute cumulative fluxes of 0.1 μM and 50 μM of digoxin from the basolateral to the apical side of the cell monolayer in the presence and absence of the interacting drugs. The fluxes of digoxin were nearly linear for the first 30 minutes, as shown in Figure 2 for LLC-PK1 cells.

Figure 3 illustrates the inhibition of 0.1 μM digoxin basolateral-to-apical flux in MDCK cells by azole antifungal drugs. Itraconazole is a more potent inhibitor of
digoxin renal tubular secretion than ketoconazole, whereas fluconazole has no effect on digoxin transport.

The cellular uptake of digoxin can also be determined quite easily in this system. Figure 4 shows that despite the decrease in the net renal tubular secretion of digoxin by MDCK cells in the presence of propafenone, the cellular accumulation of digoxin does not decrease, indicating that propafenone does not prevent digoxin from getting into the renal tubular cell.

Table 1 presents the clinical relevance of inhibitory concentrations of interacting drugs on the basolateral-to-apical fluxes of digoxin. These drugs were chosen either because of their known clinical interactions with digoxin (e.g., verapamil, propafenone) or because they are substrates or inhibitors of the P-glycoprotein system.

**DISCUSSION**

More than 200 years after the introduction of digoxin to clinical use, therapeutic drug monitoring of this cardiac glycoside is still a serious challenge to clinicians, at the bedside and in the laboratory. Patients administered digoxin are often in need of other medications. During steady state, the mean serum concentration of a drug is directly related to its dose and oral bioavailability and negatively related to its systemic clearance. Although the relative bioavailability of digoxin is quite high (>70%), antibiotics that can alter gut flora may eradicate bacteria that metabolize digoxin, thus increasing the drug's steady state concentrations. However, once absorbed, major changes in digoxin steady state concentrations are caused mainly by alterations in its renal elimination. Drugs or conditions that are known to decrease glomerular filtration should prompt close monitoring of digoxin serum concentrations and vigilance for clinical signs of toxicity (e.g., aminoglycosides, amphotericin B).

During the last two decades, a relatively large group of

![Graph](image_url)

**TABLE 1. Drugs that inhibit digoxin basolateral-to-apical flux in tubular cell monolayers**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Dose-response?</th>
<th>Clin. interaction known?</th>
<th>Clinically relevant conc?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>R-verapamil</td>
<td>y</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>S-verapamil</td>
<td>y</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>Propafenone</td>
<td>y</td>
<td>y</td>
<td>n</td>
</tr>
<tr>
<td>R-propafenone</td>
<td>y</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>S-propafenone</td>
<td>y</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>not known</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>not known</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>y</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>Vincristine</td>
<td>y</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>Mifepristone</td>
<td>y</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>Quinidine</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
</tbody>
</table>

y, yes; n, no.
drugs has been shown to cause digitalis toxicity by inhibiting the renal tubular secretion of digoxin without affecting the glomerular filtration rate, thus proving the major role of this secretory mechanism in the elimination of this potentially toxic drug.

It is not surprising that the first few drugs that were shown to interact with digoxin by inhibiting its tubular secretion were antiarrhythmic agents because these drugs are commonly coadministered with digoxin. However, as digoxin is prescribed to millions of patients worldwide, it is evident that scores of different medications will be coadministered with it to different patients for different indications. Hence, a systematic approach to screen such compounds is warranted to prevent digitalis toxicity that can cause serious and even fatal complications.

The novel method presented here has been documented to reproduce clinically important interactions with verapamil and propafenone (11,12). However, it is the case of itraconazole that proves the relevance of this technique. We have shown this antifungalazole to be a very effective inhibitor of digoxin active tubular flux (13). Yet, because the combination of the two drugs is likely quite uncommon, the emergence of epidemiologic data indicating such an interaction can be expected to be unpredictable. Recently, several case reports and small studies of the itraconazole–digoxin interaction leading to digitalis toxicity have revealed the relevance of our in vitro prediction (Table 2).

We are in the process of screening a large number of agents that are coadministered to patients on digoxin with this in vitro test. The in vitro use of tubular cell monolayers to predict clinically important drug interactions should be considered in other cases in which clinical toxicity may pose danger to patients' well being.

**REFERENCES**


**TABLE 2. Reported clinical experiences with the itraconazole–digoxin interaction**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Type</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>Single case</td>
<td>Increased serum conc. of digoxin; decreased digoxin renal clearance</td>
</tr>
<tr>
<td>14</td>
<td>Single case</td>
<td>Increased serum conc. of digoxin; digitalis toxicity</td>
</tr>
<tr>
<td>15</td>
<td>Single case</td>
<td>Increased serum conc. of digoxin</td>
</tr>
<tr>
<td>16</td>
<td>Single case</td>
<td>Increased serum conc. of digoxin</td>
</tr>
<tr>
<td>18</td>
<td>Single case</td>
<td>Increased serum conc. of digoxin</td>
</tr>
<tr>
<td>19</td>
<td>Single case</td>
<td>Increased serum conc. of digoxin; digitalis toxicity</td>
</tr>
<tr>
<td>20</td>
<td>Single case</td>
<td>Increased serum conc. of digoxin; digitalis toxicity</td>
</tr>
<tr>
<td>21</td>
<td>Study on 10 volunteers placebo–itraconazole cross-over</td>
<td>Increased serum conc. of digoxin</td>
</tr>
</tbody>
</table>

*Ther Drug Monit, Vol. 20, No. 2, 1998*
Toxic Digoxin-Drug Interactions: The Major Role of Renal P-Glycoprotein*

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ABSTRACT. The clinical use of digoxin is complicated by a large number of drug interactions leading to severe toxicity of the cardiac glycoside. The discovery that digoxin is actively secreted by the renal tubular cell via the p-glycoprotein drug efflux pump has led us to examine whether commonly interacting drugs do so by inhibiting renal tubular secretion of digoxin. We review digoxin-drug interactions which have been studied kinetically in humans, where there are sufficient data on renal clearance of digoxin and GFR.

After more than 200 y of clinical use, digoxin is still one of the most widely prescribed drugs in the world. Recent controlled studies have helped to better define its role in the pharmacotherapy of heart failure (1). Due to its very narrow therapeutic margin, toxicity associated with digoxin is common and often life-threatening (2).

Because digoxin is eliminated almost entirely unchanged by the kidney, and its clearance rate correlates well with creatinine clearance, it was assumed that the drug is filtered by the glomerulus without appreciable tubular secretion. However, over the last 2 decades it has become apparent that, after correction for 20-25% protein binding of digoxin, its renal clearance far exceeds inulin clearance, implying net renal tubular secretion (3). This new knowledge was perceived for a while to have theoretical value only, until toxic drug interactions began to become evident. In 1978 studies showed that quinidine, which is commonly co-administered with digoxin, caused toxic accumulation of the cardiac glycoside and decreased its renal clearance without affecting the glomerular filtration rate (4). Although quinidine also decreased the extrarenal clearance of the drug, the majority of the cardiac glycoside is eliminated renally, and hence this must be a major site of interaction.

Following quinidine, more and more digoxin-drug interactions causing toxicity have been identified including verapamil (5), amiodarone (6), propafenone (7) and cyclosporine (8), and in all of them a similar picture emerged: decreased renal clearance without changes in GFR. Table 1 presents these drugs, as well as other drugs shown in pharmacokinetic studies not to inhibit the renal elimination of digoxin (3-9, 28-34).

Laboratory work has revealed that digoxin does not involve its pharmacological receptor, membrane Na+, K+ ATPase (10). During the last 4 y several groups have identified the multi-drug resistance gene product (MDR1), the apical membrane P-glycoprotein as the transporter of digoxin (11,12).

P-glycoprotein (13,14), also known as multi-drug resistance (MDR) protein because of its association with cellular resistance to various cytotoxic agents, belongs to the ATP binding cassette superfamily of transport proteins (15). Epithelia, such as renal tubular cells, express one of the isoforms of the protein (ie MDR1 in humans) on their apical membranes (16,17), and its drug transport function is unidirectional across the apical membrane, extruding drugs out of the cells into the urine. Preserving their polarizing characteristics, these epithelial cells grown on permeable filters enable extensive in vitro investigations of P-gp-mediated transport (18-23).

Studies in these polarized epithelia as well as in non-polarized cells have shown that the expression of P-glycoprotein (ie its MDR1-type isoform) is associated with decreased equilibrium cellular accumulation does not involve its pharmacological receptor, membrane Na+, K+ ATPase (10). During the last 4 y several groups have identified the multi-drug resistance gene product (MDR1), the apical membrane P-glycoprotein as the transporter of digoxin (11,12).

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Studies in these polarized epithelia as well as in non-polarized cells have shown that the expression of P-glycoprotein (ie its MDR1-type isoform) is associated with decreased equilibrium cellular accumulation.

Table 1. Ability of drugs to inhibit the renal tubular secretion of digoxin.

<table>
<thead>
<tr>
<th>Interacting drugs</th>
<th>Non interacting drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinidine*</td>
<td>Quinidine</td>
</tr>
<tr>
<td>Verapamil*</td>
<td>Nifedipine</td>
</tr>
<tr>
<td>Amiodarone*</td>
<td>Dilazein</td>
</tr>
<tr>
<td>Cyclosporine*</td>
<td>Aspirin</td>
</tr>
<tr>
<td>Propafenone*</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>Spironolactone*</td>
<td>Phenobarbital</td>
</tr>
<tr>
<td>Imuranazole*</td>
<td>Cimodeline</td>
</tr>
</tbody>
</table>

*Proven clinical cases of digoxin toxicity

*Supported by a grant from MRC, Canada

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and enhanced basal-to-apical epithelial transport of diverse classes of compounds such as vinca alkaloids (18,19), cyclosporine (21-22), verapamil (18), and digoxin (18,21-24). These findings have been conventionally interpreted to be compatible with P-glycoprotein being a drug efflux pump with a broad substrate specificity.

Studies with cultured renal tubular cell monolayers have shown that digoxin employs the P-glycoprotein for its tubular transport, and that a large number of P-glycoprotein inhibitors will block digoxin tubular secretion (25).

Most drugs showing clinically important interactions with digoxin are commonly co-administered with the cardiac glycoside, and hence such interaction could be unmasked. However, because digoxin is so widely used, more rare combinations may yield toxic interactions usually difficult to predict. Recently irtraconazole, a potent inhibitor of binding to P-glycoprotein, was predicted in vitro to interact with digoxin and subsequently did so clinically (26).

To date, all drugs causing toxic interactions with digoxin at the tubular level have inhibited the P-glycoprotein in vitro in resistant tumor cells (27). Yet some drugs not interacting clinically with digoxin are also inhibitors of P-glycoprotein. For example, quinidine inhibits digoxin tubular clearance whereas its optical isomer, quinine, does not (28). This may be explained by the 25-fold higher IC50 of quinidine to P-glycoprotein as compared to quinidine (26). More work is needed defining the renal tubal IC50 of interacting drugs, and the effects of their P-glycoprotein inhibition on digoxin accumulation.

A major enigma to the mechanism of toxic digoxin-drug interactions has been solved the past few years. It is likely that more drugs will be found to have a similar pattern. With the mechanism of interaction known, an in vitro prediction model now may be developed to prevent such hazards from reaching the bedside.

REFERENCES

The Digoxin-Propafenone Interaction: Characterization of a Mechanism Using Renal Tubular Cell Monolayers

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Accepted for publication June 2, 1997

ABSTRACT

When propafenone is given with digoxin, digoxin serum concentrations increase. Although the digoxin-propafenone interaction is well known clinically, the mechanism by which propafenone interferes with digoxin elimination is unclear. To test the hypothesis that propafenone or one or both of its two major metabolites, 5-hydroxypropafenone (5-OHP) and N-depropylpropafenone (NDPP), inhibit the P-glycoprotein-mediated net renal tubular secretion of digoxin, we examined the transport of digoxin and the well-studied P-glycoprotein substrate vinblastine across confluent Madin-Darby canine kidney cell monolayers in the absence and presence of propafenone, 5-OHP and NDPP. Propafenone and its two major metabolites significantly inhibited the secretory flux of digoxin and vinblastine (propafenone > 5-OHP > NDPP). Despite decreases in net transport, cellular digoxin accumulation did not decrease, suggesting that neither propafenone nor its metabolites prohibited digoxin from entering the cells at the basolateral side. NDPP, but not 5-OHP, was detected after 48 hr of incubation of the cells with propafenone alone. When the cells were incubated with propafenone or 5-OHP, apical accumulation of 5-OHP, but neither propafenone nor NDPP, against a concentration gradient was observed. These findings are consistent with the hypothesis that the digoxin-propafenone interaction results from the inhibition of the renal tubular transport of digoxin by propafenone and its metabolites. Our data suggest that propafenone is an inhibitor of P-glycoprotein, whereas 5-OHP is a possible substrate.

Propafenone is an antiarrhythmic agent that blocks sodium channels and beta adrenergic receptors (Puncik-Brentano et al., 1990). When propafenone is given concurrently with digoxin, steady state serum digoxin concentrations increase as a result of decreased digoxin clearance (Belz et al., 1983; Calvo et al., 1989). The low therapeutic index of digoxin necessitates dose adjustment and careful monitoring for digoxin toxicity. However, although the interaction between digoxin and propafenone is well known clinically, the mechanism by which propafenone interferes with digoxin elimination is unclear. This interaction is especially intriguing because the kidney, a major organ for digoxin elimination, plays virtually no role in the elimination of propafenone.

Digoxin is eliminated mainly by the kidney, with renal tubular secretion accounting for as much as 50% of this elimination (Steiness, 1974). Recent studies have shown that digoxin transport involves an active transport mechanism that is compatible with the MDR protein P-glycoprotein (Tanigawara et al., 1992; de Lannoy and Silverman, 1992; Ito et al., 1993b; Schinkel et al., 1995). P-glycoprotein is a drug efflux pump that is expressed in the apical membranes of various cell types, including renal tubular cells (Thiebaut et al., 1987). Most of the drugs known to interact with renal tubular digoxin secretion are substrates of P-glycoprotein; examples include cyclosporine, verapamil and quinidine (Bradley et al., 1988; Ito et al., 1993a).

Propafenone is extensively metabolized by the liver and demonstrates negligible renal elimination with no net renal tubular secretion (Hollmann et al., 1983; Seipel and Breithardt, 1980). The two major metabolites of propafenone, 5-OHP and NDPP, also have sodium channel-blocking activity; however, blockade of beta adrenergic receptors is much weaker than with the parent compound. The hydroxy metabolite is considered to be therapeutically active, whereas NDPP is probably less active, in part because of its lower serum concentrations (Puncik-Brentano et al., 1990). Bio-transformation of propafenone to 5-OHP is mediated by CYP2D6 (Siddoway et al., 1987). NDPP is reportedly pro-

ABBREVIATIONS: MDR, multidrug resistance; 5-OHP, 5-hydroxypropafenone; NDPP, N-depropylpropafenone; MDCX, Madin-Darby canine kidney; α-MEM/FBS, α-minimum essential medium plus 10% fetal bovine serum; PBS-G, phosphate-buffered saline containing 5 mM glucose and 0.02% albumin; TEA, tetraethylammonium.
duced by CYP3A4 and CYP1A2 (Botsch et al., 1993). Due to a
genic polymorphism, ~1% to 7% of the population lacks
the enzyme CYP2D6 (Bertilsson, 1995). The prevalence of
this enzyme deficiency is Caucasians > African Americans >
Orientals. Recently, existence of the superextensive metabo-
lizer phenotype of CYP2D6-mediated biotransformation re-
sulting from functional gene duplication was identified in 7%
of a white population (Agündez et al., 1998). Although there
are substantial interindividual variations in serum concen-
trations of propafenone and its metabolites due to the wide
range of metabolic capacities in the population, serum con-
centrations of 5-OHP may reach levels comparable to those
of the parent compound, especially in extensive metabolizers
(Hafeli et al., 1990). NDPP usually achieves much lower
concentrations. If the parent propafenone compound is pri-
marily responsible for the digoxin-propafenone interaction,
poor metabolizers of propafenone should have a greater like-
lihood of these interactions. However, no clinical evidence
exists to suggest that these interactions are more pronounced
in poor metabolizers than in extensive metabolizers, imply-
ing that propafenone metabolites may play a role in digoxin-
propafenone interactions. The participation of propafenone
metabolites in digoxin-propafenone interactions has not been
investigated, nor is it known how these metabolites are han-
dled by the kidney. In fact, the roles of drug metabolites in
renal drug-drug interactions have never been thoroughly
examined.

Also unknown is the relative contribution of the enanti-
omers of propafenone to digoxin-propafenone interactions.
Propafenone is generally administered as a racemic mixture.
The (R)- and (S)-enantiomers do not differ in their abilities
to decrease the fast, inward sodium current, although the (S)-
isoemer is far more potent as a beta antagonist. In addition,
the area under the concentration-time curve for (S)-
propafenone is almost 2-fold higher than that for the (R)-
enantiomer, indicating that (R)-propafenone has higher
clearance (Kroemer et al., 1989).

These findings led us to hypothesize that propafenone is an
inhibitor of P-glycoprotein-mediated renal tubular digoxin
secretion and that propafenone metabolites also contribute to
this interaction. Because no enantioselective effects have
been reported for P-glycoprotein-mediated drug transport,
we also hypothesized that the enantiomers of propafenone
are handled similarly and, thus, contribute equally to the
digoxin-propafenone renal tubular interaction.

Methods

Cell culture. MDCK cells, originally derived from the kidney of a
female cocker spaniel, were obtained from the American Type Cul-
ture Collection (Rockville, MD). These cells were seeded onto 25-mm
Nunc tissue culture inserts (GIBCO BRL, Oakville, Ontario, Cana-
da) at a density of 2.5 x 10⁴ cells/cm². The inserts were placed in
six-well tissue culture plates, and the confluent cell monolayers were
bathed on each side with 2 ml of α-MEM/FBS and incubated at 37°C
in an atmosphere of 5% CO₂/95% air. Media were changed every 2 to
3 days. Experiments were conducted 10 days after seeding. Cells
were used between passages 61 and 142.

Short-term digoxin/vinblastine flux experiments. Cells in the
filter preparation were gently rinsed with PBS-G. These cell
monolayers were preincubated at 37°C for 30 min with 1 ml of PBS-G
in the apical compartment and 1.5 ml of PBS-G in the basal com-
partment. The tissue culture inserts containing 1 ml of PBS-G in the apical
compartment were placed onto six-well tissue culture plates contain-
ing 1.2-ml PBS-G solution. 2.5 μM ([¹⁴C]mannitol and either 0.1 μM
[¹⁴C]digoxin or 0.025 μM [¹⁴C]vinblastine, to which we added
various concentrations of one of the following compounds: (R)-
propafenone, (S)-propafenone, racemic propafenone, (R)-5-OHP, (S)-
5-OHP, racemic 5-OHP or racemic NDPP. Appropriate volumes of
solvent were added to the wells to correct for volume differences.

The apical media were sampled (25 μl) at 10, 20 and 30 min. The
basal media were also sampled (25 μl) at 30 min. The radioactivity
of the samples was determined using a scintillation counter (Beckman
LS5000CE).

Cellular uptake of digoxin and vinblastine. The cellular ac-
cumulation of radiolabeled digoxin and vinblastine in the presence
and absence of propafenone, 5-OHP or NDPP was examined after 40
min of incubation. The culture inserts were rinsed on both sides with
ice-cold PBS-G (1 ml apical, 1.5 ml basal). The filters were cut out of
the tissue culture inserts using a cutting device provided with the
inserts. The membranes were placed in tissue culture plates contain-
ing 0.6 ml of 0.1% Triton X-100. After solubilization of the cells, 0.3
ml of cell solution was sampled for determination of radioactivity.
Disintegration-per-minute (dpm) counts were corrected for contam-
nation by the incubation media. On average, the contamination
accounted for <10% of the uptake.

Long-term digoxin flux experiments. Six cell preparations on
tissue culture inserts were placed in a six-well tissue culture plate
containing 2 ml of α-MEM/FBS with 0.1 μM [¹⁴C]digoxin plus or
without 30 μM propafenone in each well. The same media (2 ml)
were slowly added to the apical compartments. The cells were incu-
bated at 37°C under an atmosphere of 5% CO₂/95% air. Apical and
basal media were sampled (25 μl) at various time periods of incuba-
tion over a 24-hr period.

Biotransformation of propafenone. α-MEM/FBS (1.5 ml) con-
taining 5 μM propafenone, 5 μM 5-OHP or solvent alone was added to
either side or both sides of the cell monolayer. Cells were incubated
at 37°C in 5% CO₂/95% air. After a period of 30 min or 48 hr,
1 ml of apical solution and 1 ml of basolateral solution were sampled.
These samples were analyzed by the high-performance liquid chro-
matographic method described below. The integrity of the cell mono-
layers was determined after the 48-hr period of incubation by mea-
suring the basolateral-apical flux of 5 μM [¹⁴C]mannitol using the
procedures described for short-term experiments.

High-performance liquid chromatography assay. Propafenone
and its metabolites were quantified as described by Verjee and Gies-
brecht (1992). Samples (100 μl) were extracted at alkaline pH with
ethyl acetate, and the extract was applied to a C18 Bond Elut Cartridge
(1 ml size); interfering polar compounds were washed off with metha-
nol. The drug and metabolites were eluted with 95% methanol/5% of 0.1
N HCl. After drying under nitrogen and reconstitution with 100 μl of
0.1 N HCl, 75 μl was injected into a Whatman Partisil 5 ODS RAC
HLPC column with in-line filter. The column was maintained at room
temperature and eluted with a mobile phase of 42% acetonitrile in 10
mM phosphate buffer, pH 2.5. Detection was made at 214 nm with a UV
spectrophotometer. At a flow rate of 2.3 ml/min, chromatography was
complete in 10 to 11 min.

Data analysis. For all experiments, an a priori decision was
made to exclude the results obtained from any filters that had apical
([¹⁴C]mannitol (a marker of extracellular flux) concentrations
of >5%/hr of their respective initial basal concentrations.

Data (in at least triplicates) from different experiments were com-
pared by the two-tailed Student's t test for unpaired data or by
analysis of variance for repeated measures. P values of <0.05
were considered statistically significant. Results are expressed as mean ±
S.D.

Materials. [¹⁴C]Digoxin (16.1 Ci/mmol) and [¹⁴C]mannitol (55.1
mCi/mmol) were purchased from Du Pont Canada (Markham, On-
tario, Canada). [¹⁴C]Vinblastine (11.2 Ci/mmol) was purchased from
Amer sham Canada (Oakville, Ontario, Canada). Culture medium
was brought from the Ontario Cancer Institute. FBS was obtained from GIBCO BRL and added to media in the laboratory. Racemic propafenone was purchased from Sigma Chemical (St. Louis, MO). NDPP and the enantiomers of propafenone and 5-OHP were generously donated by Knoll Pharmaceuticals (Markham, Ontario, Canada).

**Results**

No results had to be excluded on the basis of the a priori definition of inappropriate cell-filter preparations detected by high mannitol fluxes.

**Short-term digoxin flux experiments.** Racemic mixtures of propafenone, 5-OHP and NDPP significantly inhibited the total basolateral-to-apical flux of 0.1 μM [3H]digoxin across MDCK cell monolayers over a 30-min time period (fig. 1, top). The hydroxylated metabolite (5-OHP) inhibited digoxin renal tubular secretion to a somewhat lesser extent than the parent compound. NDPP was a much less potent inhibitor, as illustrated in figure 1 (top) by the lack of significant inhibition at 20 μM. The time courses of digoxin secretion were nearly linear in the presence and absence of propafenone (fig. 1, bottom), 5-OHP and NDPP.

The effects of the enantiomers of propafenone (fig. 2) and 5-OHP (data not shown) on 0.1 μM [3H]digoxin basolateral-to-apical flux over a period of 30 min were not significantly different from each other, suggesting that the effects were nonstereospecific (P = .27 and .37 for propafenone and 5-OHP, respectively). Because the inhibitory effects of the enantiomers of propafenone and 5-OHP were comparable to those of the racemic mixtures, the racemates were used in all subsequent transport and metabolism experiments.

**Short-term vinblastine flux experiments.** As illustrated in figure 3 (top), propafenone, 5-OHP and NDPP also inhibited the basolateral-to-apical flux of 0.025 μM [3H]vinblastine, a prototype P-glycoprotein substrate, across confluent MDCK cell monolayers. Concentrations of 40 μM NDPP did not produce statistically significant differences in vinblastine inhibition, although significance was reached with 100 μM NDPP (61 ± 6% of control at 30 min, P = .02). There were no significant differences in vinblastine basolateral-to-apical flux in the presence of 20 μM propafenone or 5-OHP between the (R)- and (S)-enantiomers. The time courses of vinblastine secretion were nearly linear in the presence and absence of propafenone (fig. 3, bottom), 5-OHP and NDPP.

**Cellular uptake of digoxin and vinblastine.** Although increasing the concentration of propafenone decreased the net secretory flux of 50 μM [3H]digoxin across confluent MDCK cell monolayers, the cellular uptake of digoxin did not decrease (fig. 4). Similarly, the cellular uptakes of 0.1 μM [3H]digoxin and 0.025 μM [3H]vinblastine were not decreased relative to controls in the presence of propafenone, 5-OHP and NDPP (table 1).

**Long-term digoxin flux experiments.** Figure 5 shows the inhibition of digoxin secretion by 20 μM propafenone over a 24-hr period. Similar to the short-term time course studies, propafenone inhibited the basolateral-to-apical transport of digoxin against a concentration gradient. In a separate experiment, we demonstrated the ability of cells that were previously exposed to 5 μM propafenone (and then rinsed for 1 hr) to transport 10 nM digoxin to the same extent as cells never exposed to propafenone, indicating that the inhibition of digoxin secretion is reversible and that the cells were still functional after the exposure (data not shown).

**Propafenone biotransformation.** To identify whether the inhibition of digoxin basolateral-to-apical transport by
Propafenone Concentration (µM)

Fig. 2. Effects of the enantiomers and racemic mixture of propafenone on basolateral-to-apical digoxin transport across MDCK cell monolayers. Cell monolayers were incubated on the basal side with PBS-G plus 0.1 µM (H)digoxin and various concentrations of (R)-propafenone (C), (S)-propafenone (Δ) or racemic propafenone (O) and on the apical side solely with PBS-G. Apical solution was sampled at 10, 20 and 30 min, and the 30-min results were expressed as the average percentage of control (i.e., digoxin flux in the absence of inhibitor). Results are expressed as mean values of three cell monolayers seeded at the same cell density ± S.D.

propafenone was due to propafenone itself or instead to its metabolite(s) produced by the cells, the ability of MDCK cells to metabolize propafenone was examined. After a 30-min exposure to propafenone, 5-OHP and NDPP were undetectable. On the other hand, after a 48-hr incubation, NDPP (but not 5-OHP) was detected in approximately equal quantities from media bathing the apical and basolateral sides of cells as well as propafenone itself (fig. 6, top). When cells were incubated with 5-OHP initially placed on both sides of the monolayer, the apical concentrations of 5-OHP were almost double that of the basal compartment (fig. 6, bottom). The integrity of the cell monolayers after a 48-hr exposure to these propafenone compounds was confirmed by the 5 µM [14C]mannitol basolateral-to-apical flux experiments.

Discussion

MDCK cells have properties of distal tubular cells with P-glycoprotein expression on their apical membranes (Horio et al., 1990; Tanigawara et al., 1992). We verified P-glycoprotein expression in our MDCK cell line using monoclonal (C219) and polyclonal (4077) antibodies to P-glycoprotein. In the cell culture system used in this study, these epithelial cells orient themselves in a polarized manner such that the part of the cells that adheres to the inorganic membrane of the tissue culture insert represents the basal or blood side of the renal tubular cell, whereas the opposite end represents the apical or urine side. This orientation facilitates the study of drug transport across renal tubular cells. In addition, MDCK cells have tight junction, which results in relatively high transport-to-diffusion ratios of the P-glycoprotein substrates digoxin and vinblastine across the MDCK cell mono-

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8 S. Ito, C. Woodland, and B. Sorkodi, unpublished observations.
layers. The cell-cell junctions in MDCK cells are much tighter than those found in the proximal tubular cell line LLC-PK1. Hence, the paracellular flux of digoxin and vinblastine is lower in MDCK cells than in LLC-PK1 cells. Moreover, based on functional assays and Western blotting techniques, P-glycoprotein expression is higher in MDCK cells than in LLC-PK1 cells.\footnote{S. Ito, C. Woodland, and B. Sarkadi, unpublished observations.}

P-glycoprotein is notable for its broad substrate specificity. Vinblastine is a prototype substrate for this protein, and digoxin was recently identified to also be transported by P-glycoprotein (de Launoy and Silverman, 1992; Ito et al., 1993b; Schinkel et al., 1995; Tanigawara et al., 1992). Direct contributions of other mechanisms to the renal tubular secretion of digoxin are improbable according to our modeling analysis, which suggests that digoxin renal tubular transport involves a single active transporter (Ito et al., 1997). Based on the inhibitory compounds used in this study (propafenone is a weak organic base), if another mechanism is involved, the most reasonable candidate would be the classic organic cation transport system. This involvement seems unlikely because TEA, a prototype organic cation, lacks effects on digoxin transport in LLC-PK1 renal tubular cells, which express an active transport mechanism for TEA (Imai et al., 1985; Ito et al., 1993a). In addition, we were unable to inhibit the secretory flux of digoxin or vinblastine by MDCK cells with two prototype organic cations, TEA and N-methyl-pyrroloindoline.\footnote{C. Woodland, unpublished observations.} In fact, we could not detect carrier-mediated TEA transport in MDCK cells. Horio et al. (1990) also failed to show an effect of TEA on vinblastine transport by this cell line. Therefore, P-glycoprotein appears to be a major apical efflux mechanism for digoxin and vinblastine, although the roles of other transporters remain to be explicitly elucidated.

Inhibition of the unidirectional drug efflux pump P-glycoprotein is often implied when fluxes of substrates across cell monolayers expressing the protein are decreased in the basolateral-to-apical direction and increased in the apical-basal direction (Horio et al., 1988; Tanigawara et al., 1992). In this study, we assumed that basolateral-to-apical flux represents the P-glycoprotein-mediated component of digoxin transport. This assumption seems valid because we could also demonstrate in a different experimental condition that the net basolateral-to-apical transport of digoxin against a concentration gradient is inhibited by propafenone (fig. 5). In addi-
Our results show that propafenone and its two major metabolites, 5-OHP and NDPP, inhibit digoxin and vinblastine transport across MDCK cell monolayers. The inhibitory effects of these compounds are dose-dependent with an order of potency such that propafenone > 5-OHP > NDPP. In extrapolation of these in vitro data to humans, clinically significant differences in the nature of the digoxin-propafenone interaction are not likely among superextensive, extensive and poor metabolizers of propafenone. To the best of our knowledge, however, digoxin-propafenone interactions have not been analyzed with respect to metabolizer phenotype.

The somewhat increased cellular accumulation of digoxin and vinblastine in the presence of propafenone, 5-OHP and NDPP demonstrates that these compounds do not interfere with the ability of digoxin to enter the cells, implying that the digoxin-propafenone interaction does not take place at the basolateral membrane. These results agree with the general concept that the digoxin-propafenone interaction takes place at P-glycoprotein located in the apical membranes of renal tubular cells.

Although the exact dynamics of the interactions are unclear on the basis of this study, because propafenone and NDPP are not accumulated in the apical compartment (fig. 6), the two compounds may be inhibitors, but not substrates, of P-glycoprotein. The data also indicate that 5-OHP is accumulated on the apical side against a concentration gradient. Although this suggests a competitive nature of the interaction of digoxin and 5-OHP, the identification of 5-OHP as a substrate of the transport system awaits further study.

In the present experiments, digoxin concentrations were nearly 10-fold higher than those observed therapeutically (1–3 nM) due to the constraints induced by the specific activity of the available radiolabeled digoxin. Concentrations of propafenone and 5-OHP used in the experiments (1–40 μM) included relevant therapeutic serum concentrations (propafenone, 1–6 μM; 5-OHP, 0.5–1.5 μM; Siddoway et al., 1987), although protein binding was not taken into account. NDPP concentrations seen in patients receiving therapeutic doses of propafenone are much lower than those used in this study: <1.5 μM in vivo (Kates et al., 1985) vs. 20 to 100 μM in vitro in this study. Taken together, our in vitro data seem valid to infer that propafenone and 5-OHP are responsible for digoxin-propafenone interactions in the kidney in vivo.

The inhibitory effects on digoxin secretion of the enantiomers of both propafenone and 5-OHP are nonspecific. As a result, one would not expect to see clinically significant differences in the nature of these digoxin interactions based on serum concentrations of the enantiomers. This nonspecific nature of interaction is compatible with substrate/inhibitor polyspecificity of P-glycoprotein-mediated drug transport that was shown for drugs such as verapamil (Ito et al., 1993c) and quindine (Hedman et al., 1990).

In vivo, propafenone is metabolized in the liver by CYP2D6 to 5-OHP and by CYP3A4 and CYP1A2 to NDPP (Botsch et al., 1993; Siddoway et al., 1987). The absence of production of 5-OHP suggests that MDCK cells lack functional CYP2D6. To the best of our knowledge, CYP2D6 has not been detected in human kidney. Therefore, hepatically produced 5-OHP is likely responsible for the majority of the in vivo interactions between digoxin and metabolites of propafenone.

We found that MDCK cells biotransform propafenone to NDPP. The relevance of the NDPP produced within the kid-
ney cells is unknown at present; however, because only very high concentrations of NDPP (>40 μM) were able to significantly inhibit the renal tubular digoxin secretion, NDPP produced intrarenally probably does not play an important role in the digoxin-propafenone interaction in vivo.

In summary, our study demonstrates that drug metabolites may contribute significantly to renal tubular drug interactions, although in this case, the parent compound has a greater effect. Our findings suggest that P-glycoprotein is involved in the digoxin-propafenone interaction and that 5-OHP is a possible substrate for P-glycoprotein.

References


