Title
Modulation of multidrug transporters--a potential pharmacokinetic mechanism of clinical drug-drug interactions

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Publication Date
1999

Peer reviewed|Thesis/dissertation

Modulation of Multidrug Transporters--
A Potential Pharmacokinetic Mechanism of Clinical Drug-Drug Interactions: Digoxin as a Model

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

To Yaomin and Florence

ACKNOWLEDGEMENTS

I first would like to convey my most sincere appreciation to Dr. Leslie Z. Benet for his intelligent mentorship. His never-ending enthusiasm, tireless support and inspired guidance have made my graduate studies at UCSF a truly rewarding experience. I thank him for critically reading and tremendously improving this dissertation. It is my good fortune to graduate from LZB's laboratory.

Thanks to the Department of Pharmaceutical Chemistry, especially the Department of Biopharmaceutical Sciences and the Graduate Division of University of California San Francisco for their financial support and making my student life in San Francisco possible.

I would like to thank Drs. Kathleen M. Giacomini and Robert A. Upton, my thesis committee members, for their timely review of this manuscript and helpful comments. Heartfelt thanks to the members of my oral committee— Drs. Kathleen M. Giacomini, Richard Shafer, Barry Massie and Chair, Robert A. Upton for their supportive suggestions and comments on my thesis research, and for providing a successful and enjoyable qualifying examination.

Thanks to Drs. Svein Øie and Wolfgang Sadee for contributing critical feedback during the preparation and development as well as strengthening my orals proposal. Dr. Emil T. Lin and his associates in the Drug Study Unit at UCSF have provided tremendous analytical support and have been extremely helpful in quantitation of my clinical study samples.

Many current and previous members of the LZB group have enabled our lab to be a great place to learn and make friends. I thank Drs. Laurent Salphati and Hiroyuki Sasabe for helping me greatly at the start of this thesis project and for very useful discussions of my research. Many thanks to Dr. Naonori Kohri and Mr. Robert Fiorentino for their friendship, and to Ms. Karen Baner and Milagros Hann for wonderful lab assistance. I appreciate Dr. Lynda Frassetto for help in conducting my clinical study, and Ms. Gloria Johnson for "bridging" us to LZB.

I have been very fortunate to have been located on the 8th Floor and to have learned much from the interactions with many talented people from other labs, especially the KMG group. Thanks to Dr. Juan Wang, Mr. Carlo Bello and Mr. Mark Dresser for their expertise in cell transport studies and saving me from much frustration.

My parents and sister have been extremely supportive and have always been my timely "backup" during the whole process of pursuing higher education. Their love, care, encouragement and advice have helped me through many difficult moments. My clever and beautiful daughter, Florence (Tingwei), who was born in the middle of my school years at UCSF, has brought so much fulfillment, joy and excitement to my daily life.

Finally and most importantly, my deepest gratitude goes to my wonderful husband, Yaomin. Without his love, care, encouragement and friendship, I could not have accomplished this work. His strength, insight, support and excellent baby-sitting have made my success possible and worthwhile.

Lingling Guan

December, 1999

ABSTRACT

Modulation of Multidrug Transporters— A Potential Pharmacokinetic Mechanism of Clinical Drug-Drug Interactions: Digoxin as a Model

Lingling Guan

P-glycoprotein (Pgp) and cytochrome P450 3A (CYP3A) enzymes share a large number of substrates and modulators and play major roles in drug absorption and disposition. Digoxin is a substrate of Pgp, and could be used in humans as a probe of Pgp since this drug undergoes very limited metabolism.

Digoxin metabolism was compared in rat and human liver microsomes. No extensive metabolite was formed after incubation of digoxin with human liver microsomes. Ketoconazole (KCZ) and itraconazole (ICZ) reduced digoxin metabolism by CYP3A in rat liver microsomes.

The interactions of digoxin and various drugs with multidrug transporters were studied in wild-type and human transporter cDNA transfected kidney or intestine derived cell lines. Digoxin B to A transport was greater than A to B transport in all cell lines tested, and trans-epithelial secretion of digoxin displayed temperature-dependence.

Digoxin secretion was enhanced in Pgp over-expressed than in wild-type cells, and was affected by inhibitors of Multidrug Resistance-Associate Proteins (MRPs) in MRP transporter over-expressed cells. Many drugs including KCZ and ICZ demonstrated an apparent inhibition of digoxin secretion in Pgp and MRP over-expressed cells.

KCZ increased digoxin absorption and decreased digoxin elimination, leading to enhanced digoxin exposure in rats. The increased digoxin bioavailability could be caused by inhibition of CYP3A and Pgp/MRPs, however, the significant reduction of digoxin T_{peak} and MAT could only be explained by the inhibition of intestinal Pgp/MRPs.

Kinetic parameters from our clinical study show that digoxin kinetics were affected by azoles, and the extent of digoxin-KCZ interaction, although highly variable, was greater than the digoxin-ICZ interaction. The major site of the KCZ/digoxin interaction appeared to be the small intestine and the major effect of ICZ was on digoxin elimination.

Azole kinetics were not altered by digoxin co-administration, and no extensive digoxin metabolites were detected in the subjects' plasma. The results support our hypothesis that azoles increase digoxin exposure by differential inhibition of Pgp/MRPs transporters, but not CYP3A, in tissues such as intestine, liver and kidney.

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ABBREVIATIONS

A	Apical
ABC	ATP-Binding Cassette
ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionization
AUC	Area under the concentration-time curve
AUMC	Area under the (first) moment-time curve
B	Basal
BBB	Blood-brain barrier
BBM	Brush-border membrane
BLM	Baso-lateral membrane
BSA	Bovine serum albumin
BSEP	Bile salt export pump
Caco-2 cells	Human adenocarcinoma cells
cBAT	Canalicular bile acid transporter
CFTR	Cystis fibrosis transmembrane regulator
CHF	Congestive heart failure
CHO	Chinese Hamster ovary
CL	Plasma clearance
C_{max}	Maximum plasma concentration
cMOAT	Canalicular multispecific organic anion transporter
C_{peak}	Peak plasma concentration

CYP	Cytochrome P450
CsA	Cyclosporine
Dg0	Digoxigenin
Dg1	Digoxigenin mono-digitoxoside
Dg2	Digoxigenin bis-digitoxoside
Dg3	Digoxin
DME	Dulbecco's Modified Eagle's
DMEM	Dubelco's modified Eagle's medium
DRP	Digoxin reduction product
EHBR	Esai hyperbilirubinemic, Sprague-Dawley rats deficient of cMOAT (MRP2)
EIA	Enzyme-immunoassay
F	Bioavailability
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FCZ	Fluconazole
FMN	Flavin mononucleotide
GFR	Glomerular filtration rate
GI	Gastrointestinal
GSH	Glutathione
GS-X	Glutathione conjugates
ICZ	Itraconazole
IPA	Isopropanol

IS	Internal standard
KCZ	Ketoconazole
α-KG	α-Ketoglutarate
LLC-PK1 cells	Proximal tubular cells from porcine kidney
LRP	Lung resistance-related protein
MAT	Mean absorption time
MCZ	Miconazole
MDCK cells	Distal tubular cells from Madin-Darby canine kidney
MDR	Multidrug resistance
MDR1	Human gene coding for class I P-glycoprotein
MDR2	Human gene coding for class II P-glycoprotein
mdr1a (+/+)	Wild type mouse
mdr1a (-/+)	Mouse heterozygous for a disruption of the mdr1a gene
mdr1a (-/-)	Mouse homozygous for a disruption of the mdr1a gene
mdr1a/1b (+/+)	Wild type mouse
mdr1a/1b (-/-)	Mouse homozygous for a disruption of the mdr1a and mdr1b genes
mdr1b (+/+)	Wild type mouse
mdr1b (-/+)	Mouse heterozygous for a disruption of the mdr1b gene
mdr1b (-/-)	Mouse homozygous for a disruption of the mdr1b gene
mdr1a	Rodent gene coding for mdr1a P-glycoprotein
mdr1b	Rodent gene coding for mdr1b P-glycoprotein
mdr2	Rodent gene coding for mdr2 P-glycoprotein
MEIA	micro-particle enzyme immuno-assay

MEM	Minimum essential medium
MFO	Mixed function oxidases
MRP	MDR-associated protein
MRT	Mean residence time
MTBE	Methyl-terbutyl ether
NBDs	Nucleotide-binding domains
NTCP	Na^+-dependent taurocholate protein
	or Na^+-taurocholate co-transporting polypepetide
OATP	Organic anion transport protein
	or Na^+-independent organic anion transport protein
OCT	Organic cation transporter
OK	Opossum kidney
PC	Phosphatidylcholine
PC-TP	Phosphatidylcholine-transfer protein
Pgp	P-glycoprotein
PKC	Protein kinase C
PM	Poor metabolizer
Rh 123	Rhodamine 123
RIA	Radio-immunoassay
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAR	Structure-activity relationship
SD	Standard deviation
SPGP	Sister P-glycoprotein

$t_{1/2}$	Half-life
TEER	Transepithelial electrical resistance
TFA	Trifluoracetic acid
T_{max}	Time to reach maximum plasma concentration
TMDs	Trans-membrane domains
T_{peak}	Time to reach peak plasma concentration
TR^- rats	Groningen yellow (GY), wistar rats deficient of cMOAT (MRP2)
V_{ss}	Steady-state volume of distribution

Chapter 1

TRANSPORTERS AND ENZYMES

1.1 P-glycoprotein

P-glycoprotein (Pgp), named from permeability-glycoprotein (Juliano and Ling, 1976), was originally discovered in Chinese Hamster Ovary (CHO) cells (Juliano and Ling, 1976), that display cross resistance to a broad variety of anticancer agents, such as colchicine, anthracyclines, epipodophyllotoxins and vinca alkaloids (Ling, 1975). At that time, it was thought that Pgp increases membrane permeability thereby leaking toxic therapeutic agents out of cancer cells, but later Pgp was found to be an efflux pump. Pgp is over-expressed in many types of cancer and has been extensively studied and characterized, so it is also referred to as the multidrug resistance (MDR) protein. Substrate transport mediated by Pgp is an ATP-consuming process (ATP hydrolysis). MDR1 belongs to the superfamily most commonly referred to as "ABC Transporters", also called "traffic ATPase".

1.1.1 ABC transporters

The ABC superfamily, one of the largest and most diverse of transport families, includes over 50 members, such as cystis fibrosis transmembrane regulator (CFTR) and MDR-associated protein (MRP) (Higgins, 1992). Most of these transporters have been identified in prokaryotes (Lomri *et al.*, 1996), and the majority of family members have no role in drug resistance, but are rather involved in the transport of ions and metabolites, polysaccharides, peptides or proteins (Pedersen, 1995).

ABC transporters generally consist of four domains. The transmembrane domains (TMDs), each of which consists of six putative membrane-spanning segments, appear largely responsible for the substrate specificity of the transporter. The nucleotide-binding domains (NBDs), which are located at the cytoplasmic face of the membrane, bind ATP and couple ATP hydrolysis to transport. Both NBDs are required for the normal function of ABC proteins. Certain ABC proteins exhibit structural variations that reflect their specific function (Lomri *et al.*, 1996). Other than CFTR, all ABC transporters are ATP-dependent active pumps.

1.1.2 Pgp structure

Pgp has 1280 amino acids and is a plasma membrane glycoprotein of about 170 kDa. Pgp has two homologous halves each having 6 transmembrane domains and glycosylation sites on the first extra cellular loop. Each homologous half contains an ATP binding site (Leveille-Webster and Arias, 1995). It seems evident that the linker region located inside the cell is essential for the ATPase and drug efflux activity. Recent low-resolution 2.5 nm structural data obtained by electron microscopy analysis confirmed the secondary structure that had been deduced initially (Rosenberg *et al.*, 1997).

Phosphorylation seems to play a role in regulating Pgp function. The Cl^- current was regulated by protein kinase C (PKC) mediated phosphorylation of Pgp (Higgins, 1995) in cells transfected with human MDR1 cDNA. ATP hydrolysis is required in the process of Pgp extruding drug out of the cell (Shapiro and Ling, 1995b), however, the mechanism is still not fully understood. Blocking N-linked glycosylation did not affect Pgp function in MDR cells (Beck and Cirtain, 1982). Experiments showed that N-glycosylation deficient protein conserved a normal transport activity (Schinkel *et al.*, 1993). However, glycosylation might be necessary in the trafficking and stability of the protein (Beck and Cirtain, 1982; Kramer *et al.*, 1995). The model representing Pgp structure is depicted in Figure 1-1.

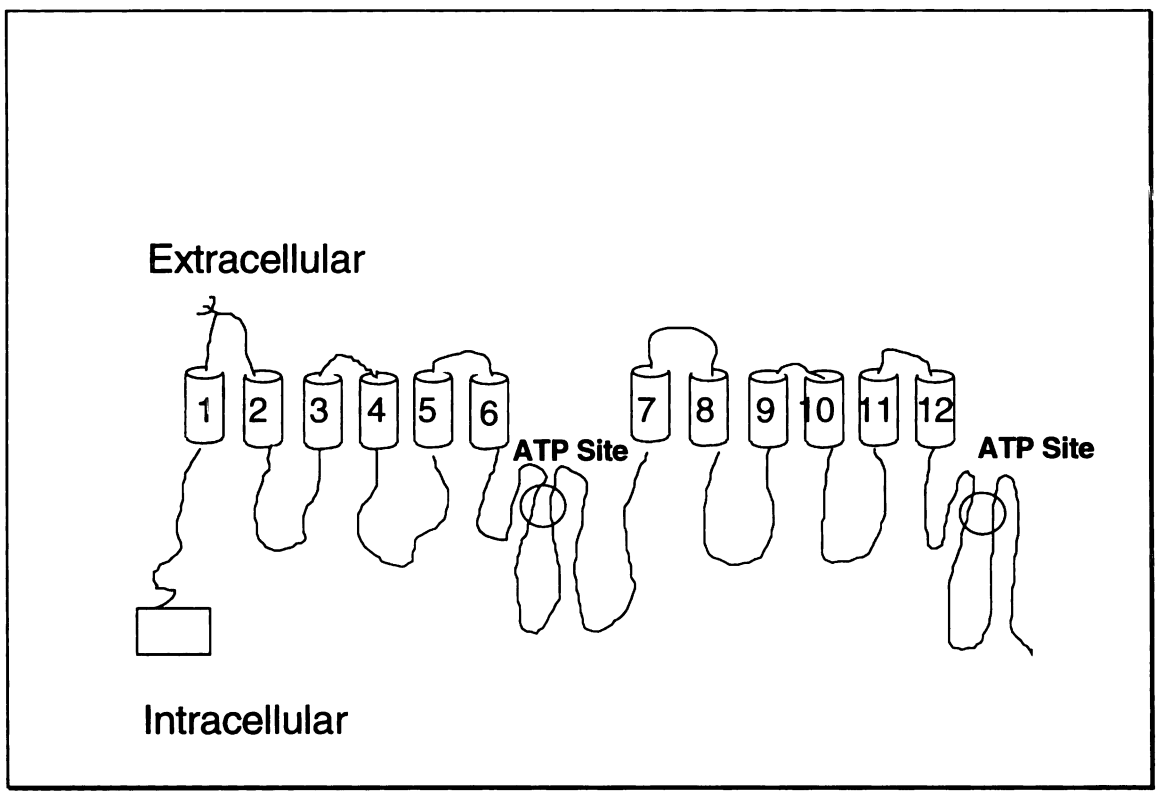


Figure 1-1. The model of Pgp structure.

In the model described above, Pgp is assumed to have only one binding site. Kinetic inhibition studies showed that verapamil seems to non-competitively inhibit daunorubicin efflux (Garrigos *et al.*, 1997). Verapamil, vinblastine and progesterone were found to interact with each other non-competitively (Martin *et al.*, 1997; Shao *et al.*, 1997). These results are consistent with findings by others (Dey *et al.*, 1997) that the photoactive analog of prazosin interacts with two distinct substrate interaction sites on the C- and N- terminal halves of the human Pgp.

1.1.3 Substrates of Pgp

The substrate specificity of Pgp is extremely broad, possibly reflecting more than one binding site since substrates exhibit diverse chemical structures, such as calcium channel blockers, anthracycline and vinca alkyloid analogues, as well as steroids and hormonal analogues (Ford and Hait, 1990). Structural studies indicate that the requirements for substrates of Pgp are a molecular weight usually in the range of 350 to 1000 (exceptions with CsA and PSC833), hydrophobicity, two planar rings and a weak cationic charge (Arias *et al.*, 1990). Studies with Pgp also suggest at least eight theoretical requirements for an ideal MDR probe (Broxterman *et al.*, 1996) (Table 1-1).

1.1.4 Transport mechanisms of Pgp

In order to explain the extremely broad substrate specificity of Pgp (Gottesman and Pastan, 1993), direct and indirect transport mechanisms have been envisaged. One indirect transport mechanism hypothesis is that Pgp regulates the plasma membrane by pH gradient or electrical potential (Higgins and Gottesman, 1992; Luz *et al.*, 1994; Roepe, 1992; Roepe *et al.*, 1993 and 1994). Since most Pgp substrates are weak bases, an increased intracellular pH or a reduction in the negative electrical potential would

therefore reduce the intracellular drug concentration by passive diffusion across the plasma membrane. However, these indirect mechanisms can not explain the change in substrate specificity resulting from a single amino acid substitution (Choi *et al.*, 1988). No correlation between efflux and intracellular pH was shown for Rhodamine 123 (Rh123) (Altenberg *et al.*, 1993 and 1994). Purified Pgp reconstituted in a liposome system with a fluoresent substrate yielded similar results (Shapiro and Ling, 1995a).

Table 1-1. Eight theoretical requirements for an ideal MDR probe.

1	General fluorescence properties for flow cytometry (high fluorescence and no reproducible quenching)
2	High cellular accumulation (or high distribution volume)
3	Rapid equilibration
4	High ratio of active to passive plasma membrane passage
5	Rate of plasma membrane transport determines the total loss rate of the probe from the cell
6	pH-independent transmembrane transport
7	Membrane potential-independent transport
8	All properties, except MDR-related efflux, unaffected by pump inhibitors (among others, intracellular probe distribution and passive membrane transport)

The evidence tends to support a direct transport mechanism. At least three different models have been proposed for MDR1-mediated substrate translocation (Müller and Jansen, 1998; van Veen and Konings, 1997) (Table 1-2).

Table 1-2. Three different models proposed for MDR1-mediated substrate translocation.

"flippase" model	Drug-transporting P-glycoproteins act by flipping drugs from the inner to the outer leaflet of the plasma membrane (Higgins and Gottesman, 1992).
"vacuum cleaner" model	P-glycoproteins act by binding hydrophobic compounds at the inner or the outer leaflet of the plasma membrane, followed by extrusion into the external medium (Gottesman and Pastan, 1993; Shalinsky *et al.*, 1993; Stein *et al.*, 1994).
"aqueous pore" model	P-glycoproteins act by directly transporting amphiphilic compounds from the cytosol to the outside of the cell (Homolya *et al.*, 1993).

1.1.5 Expression of Pgp in normal tissues

The major role of Pgp is in the development of MDR in tumor cells. However, Pgp is also expressed in normal tissues. The development of antibody (Kartner *et al.*, 1985) facilitated the detection of this transporter in tissues. There are many monoclonal antibodies that have been used to detect Pgp as listed in Table 1-3 (Ferry, 1998; Okochi *et al.*, 1997; Woodhouse and Ferry, 1995).

In humans (Cordon-Cardo *et al.*, 1990; Thiebaut *et al.*, 1987), Pgp is found primarily lining the apical surface of epithelial tissues such as the liver (principally in the bile canaliculi), kidneys (proximal tubules), intestine (mucosal cells), and pancreas (ductal and acinar cells). High levels of Pgp are present in endothelial cells in the brain, skin and testis where it essentially serves a protective mechanism for the body. Pgp is also present in the adrenal glands, cortex and medulla. Its ubiquitous presence in the adrenal glands may indicate a role in steroid secretion (Gottesman and Pastan, 1993). The

degrees of MDR1 expression in various normal tissues can be classified into three levels as given in Table 1-4 (Lum *et al.*, 1993; van der Heyden *et al.*, 1995).

Table 1-3. Pgp monoclonal antibodies.

Monoclonal antibody	**Pgp epitope recognized**	**comments**
C219	cytoplasmic	cross-reacts with MDR3 and myosin
C494	cytoplasmic	MDR1 specific, only reacts with fixed tissues
UIC2	extracellular	MDR modulator, better than verapamil
4E3	extracellular	no effect on drug transport, non-glycosylated forms
MM4.17	extracellular linear	human MDR1 specific
JSB-1	cytoplasmic	MDR1 specific
MRK16	extracellular loop	human MDR1 specific, used in flow cytometry
MRK17	external	
HYB-241	internal	
HYB-612	internal	
HYB-195	internal	
265/F4	external	
15D3	external	
17F9	external	

Table 1-4. Level of MDR1 expression in normal tissues.

High	**Moderate**	**Low**
Adrenal cortex	Adrenal medulla	Skin
Kidney (renal proximal tubule)	Trachea (apical)	Skeletal muscle
Liver (biliary lining)	Lung (major bronchi)	Heart
Placenta (trophoblasts)	Prostate (glandular)	Spleen
Colon (luminal)		Esophagus
Small bowel (apical)		Stomach
Brain (endothelial cells)		Ovary
Testis (endothelial cells)		Spinal cord
Pancreas (epithelial cells)		Bone marrow (stem
Macrophages (malignant effusions)		cells, moderate)

1.1.6 Physiological function of Pgp

Despite the identification of substrates and inhibitors and the determination of some of its structural features, the physiologic function(s) of Pgp is still unidentified. The localization of Pgp suggests that it plays a major role in detoxification processes and protection against xenobiotics, in excretion of steroid hormones, drugs and their metabolites (Arias *et al.*, 1990), but no natural substrate of Pgp has been identified. However, Pgp is also found in high concentration in capillaries of the central nervous system and uterus, suggesting a possible function of Pgp in the blood-brain barrier and in the placental transport mechanism. Schematic representations of the position of the Pgp pump in the transport mechanism in the intestinal epithelium and the blood-brain barrier are depicted in Figure 1-2 and Figure 1-3, respectively (van Asperen *et al.*, 1997).

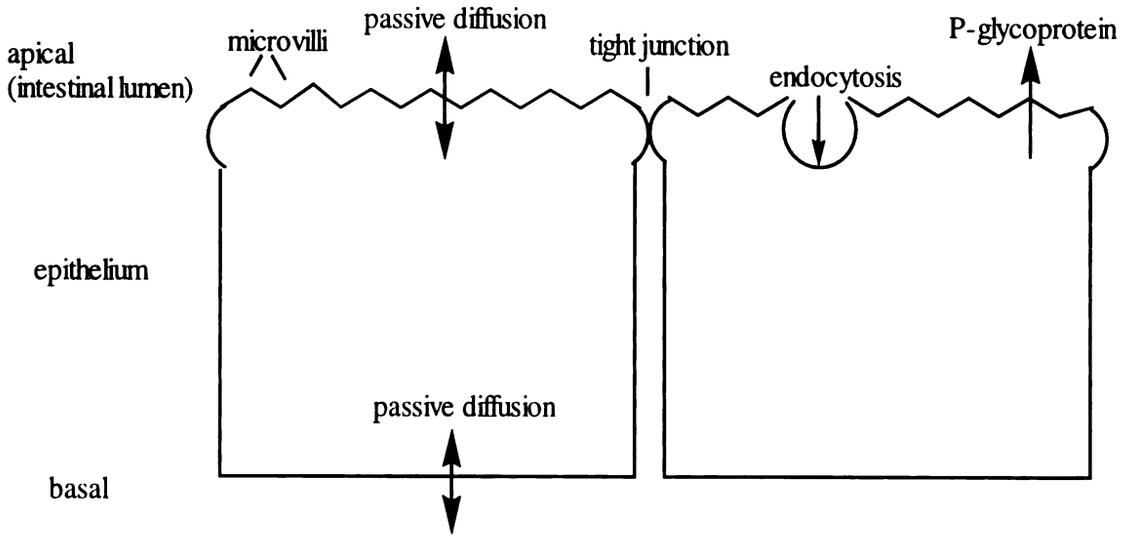


Figure 1-2. Pgp pump transport mechanism at the intestinal epithelium.

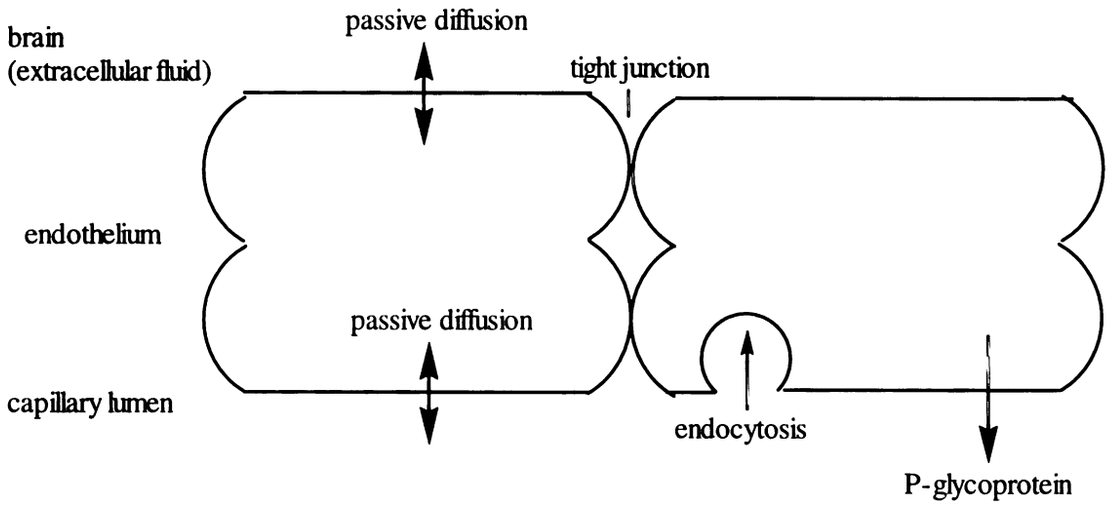


Figure 1-3. Pgp pump transport mechanism in blood-brain barrier.

It is hypothesized that small peptides are the natural substrates for the normal Pgp system and that following their transmembrane transport, the peptides are rapidly hydrolyzed to the amino acids. During normal protein turnover, many hydrophobic peptides are formed intracellularly and some are rapidly secreted (Arias *et al.*, 1990). Pgp in mammals can have at least eight possible physiological functions (Borst *et al.*, 1993; Borst and Schinkel, 1996) as listed below in Table 1-5.

Table 1-5. Eight possible physiological functions of Pgp in mammals.

1	Protection against exogenous toxins ingested with food: Expression in small intestine, colon, blood-tissue barrier sites.
2	Excretion of metabolites or toxins: Expression in liver canalicular membrane and kidney (digoxin transport).
3	Transport of steroid hormones: Expression in adrenal gland, demonstrated transport of cortisol, corticosterone, aldosterone.
4	Extrusion of (poly-)peptides (cytokines) not exported from the cell via the classical signal/cleavage pathway: Mammalian endoplasmic reticulum peptide transporters.
5	Ion transport and cell volume regulation: Activation of an endogenous Cl^- channel activity.
6	Lymphocyte cytotoxicity: Possible involvement in NK-cell-mediated cytotoxicity.
7	Transport of prenylcysteine methyl esters.
8	Intracellular vesicular transport of cholesterol.

1.1.7 MDR gene across species

Highly conserved genes encode Pgp across species (Ng *et al.*, 1989). In mice, hamsters and rats, the Pgps listed in Table 1-6, locate at similar sites, as in humans (Biedler, 1992; Borst, 1997; Croop, 1993; Salphati, 1998).

Table 1-6. Pgp genes across species.

		Chinese		Mouse	
Class	**Human**	**hamster**	**Rat**	**Scheme A**	**Scheme B**
I	MDR1	mdr1a (pgp1)	mdr1a	mdr3	mdr1a
II		mdr1b (pgp2)	mdr1b	mdr1	mdr1b
III	MDR2/3	mdr2 (pgp3)	mdr2	mdr2	mdr2

The implication of MDR1, mdr1a and mdr1b (Devault and Gros, 1990; Gros *et al.*, 1986; Ueda *et al.*, 1987) in the development of the MDR phenotype was demonstrated when sensitive cells became resistant after transfection of Pgp cDNA. Classes I and II transporters translocate drugs from the inner to the outer side of the plasma membrane, whereas class III transporters locate primarily on the canalicular side of liver cells and act as phosphatidylcholine (PC) translocaters. All of the mammalian Pgps display a high level of amino-acid identity (> 70 %). The human MDR1 transcript shows a higher identity to mouse mdr1a and mdr1b transcripts than to the human MDR2 transcript.

In rodents, mdr1a and mdr1b differ in their substrate specificity and expression; the latter is also gender dependent. The mouse mdr1b confers a preferential drug resistance to adriamycin and colchicine while mdr1a is more efficient against

actinomycin D. In mice and rats, mdr1a is the main Pgp expressed in intestine whereas mdr1b is predominant in adrenal glands and ovaries (Croop *et al.*, 1989; Silverman and Schrenk, 1997). In rats, mdr1b is highly expressed in lung whereas it is the least abundant isoform in the liver. In hamsters, mdr1b is present only in the cortex and not in the medulla of the adrenal glands (Croop *et al.*, 1989). No Pgp can be detected in the adrenal gland of female hamsters (Bradley *et al.*, 1990). Moreover, the development of MDR in mouse cell lines can result from the independent over-expression of mdr1b or mdr1a or both (Devault and Gros, 1990).

1.1.8 MDR2 gene

Transfection of sensitive cells with the mdr2 cDNA failed to confer MDR (Buschman *et al.*, 1992; Buschman and Gros, 1991; Gros *et al.*, 1988; van der Bliek *et al.*, 1988), indicating mdr2 protein is not a drug transporter. Studies (Ruetz and Gros, 1994; Smith *et al.*, 1994) showed that this protein is a phospholipid translocator. Its significant role in phospholipid secretion was confirmed by the generation of mice homozygous with the mdr2 gene disruption (Smit *et al.*, 1993). Bile salts secretion in these animals is unchanged, but bile is devoid of phospholipids and has a lower content of cholesterol.

It was hypothesized that mdr2 would behave like a flippase (Ruetz and Gros, 1994; Smit *et al.*, 1993) to make phosphatidylcholine accessible to bile salts (Oude Elferink *et al.*, 1997). But bile acids cannot be solubilized in the absence of phospholipids, so that they damage the canalicular membrane (Ruetz and Gros, 1994). A faster translocation of PC in cells expressing the human MDR2 Pgp as compared to control cells was observed (Oude Elferink *et al.*, 1997).

The mdr1a (-/-) knockout mice display normal phenotypes, organ functions, lifespan and fertility (Schinkel *et al.*, 1997), and pharmacologically mdr1a/1b (-/-) mice behaved similarly to mdr1a (-/-) mice (Schinkel *et al.*, 1997). But mice with disrupted class III Pgp show signs of a disturbed hepatic architecture and an expansion of biliary ducts. Additionally, MDR2 Pgp may be implicated in a human liver hereditary disease (Deleuze *et al.*, 1996).

1.1.9 Inducers of Pgp

Dexamethasone was found to repress the "natural" induction that occurs in primary culture (Fardel *et al.*, 1993), but the opposite effect was observed in the rat hepatoma cell line (Schuetz *et al.*, 1995). However, induction by dexamethasone seems to be cell line dependent since it induced Pgp in a mouse hepatoma cell line and in HepG2 cells, whereas no effect was observed in NIH3T3 and HeLa cells (Zhao *et al.*, 1993). The basal level of Pgp was found to be 40% lower in male rats than in females and that mdr2 mRNA levels in male rats were one-half those in females (Salphati and Benet, 1998). In male rats, rifampicin and dexamethasone caused 50% and 5-fold increases in Pgp levels, respectively. Dexamethasone reduced Pgp expression by about 60% and caused a 30% decrease in mdr2 mRNA levels in female rats. Mdr1a was not affected and mdr1b was not detected in female or male rats (Salphati and Benet, 1998).

1.2 Other transporters

1.2.1 MRP

Recently, MDR-associated protein (MRP1) has been discovered to be a 190 kDa protein and, like Pgp, belongs to the ABC protein family (Cole *et al.*, 1994). The human MRP1 gene is localized on chromosome 16 at band p13.1. MRP1 shows a similar but not

identical pattern of resistance to Pgp and is the only member of the MRP family that has been shown to associate with MDR (Kool *et al.*, 1997). In humans only one Pgp (MDR1) transports drugs, whereas the MRP family has at least six members serving this function (Kool *et al.*, 1997).

MRP1 is functionally similar to Pgp in transporting many of the same substrates, such as vinblastine, cyclosporine and verapamil (Holló *et al.*, 1996), but only has 15% amino acid identity with Pgp (Lautier *et al.*, 1996). Unlike Pgp, that prefers lipophilic or weakly basic substrates, MRPs prefer anionic compounds including the leukotriene LTC4 (substrate with the highest affinity for MRP), and products of phase II metabolism such as glutathione-conjugates (Heijn *et al.*, 1997; Shen *et al.*, 1996). MRP1 has been referred as a glutathione conjugate efflux pump (Flens *et al.*, 1994).

MRP1 is found in solid tumors as well as expressed at low levels in many tissues (Kruh *et al.*, 1995) including liver, intestinal and hematopoietic cells, and at higher levels in skeletal muscle, heart, testes, kidney and lung. However, the physiological relevance of MRP in these tissues is unknown. MRP1 expression in the liver was found to be very low (Paulusma *et al.*, 1996), while its homologous transporter MRP2 (also termed cMOAT) is found almost exclusively in the liver and in lesser amounts in the small intestine and other tissues.

The transport properties of cMOAT have been well-characterized (Ishikawa *et al.*, 1990; Oude Elferink and Jansen, 1994). In the TR^- rats (Wistar rats deficient in cMOAT), the ATP-dependent transport of anionic compounds from liver cells into the bile is disturbed. Early evidence suggests that MRP3 is similar to MRP2, and MRP5 similar to MRP1. MRP4 is rare and the role of MRP6 is unclear. Homology between family

members suggests that all MRPs function as GS-X (glutathione conjugates) transporters (Kool *et al.*, 1997).

1.2.2 LRP

The lung resistance-related protein (LRP) is another protein associated with MDR (den Boer *et al.*, 1998). The human LRP genes map on the short arm of chromosome 16 (16p 13.1-16p 11.2) proximal to MRP (16p 13.1). This 110 kDa protein shows a high amino-acid identity (60-90%) to MRP1.

LRP is expressed in cancerous tissue (Izquierdo *et al.*, 1996), especially in non-Pgp MDR tumor cells, as well as in normal tissues. Epithelial cells with secretory functions and cells exposed to xenobiotics, such as bronchial, intestinal, proximal and renal tubular cells, keratinocytes, macrophages and adrenal cortex cells show the highest LRP expression. Even though LRP expression does not directly result in MDR (Sugawara *et al.*, 1997), this protein has been speculated to be involved in drug transport since it serves as a better prognosticator for MDR cancer strains than either Pgp or MRP. LRP can only be found in the cytosol, with no evidence of either transport or sequestering of drugs (Borst *et al.*, 1997). Table 1-7 summarizes the similarities and differences between various MDR transporters (Borst, 1997; Müller, 1999) as discussed above together with cystis fibrosis transmembrane regulator (CFTR) and bile salt export pump (BPEP), also termed sister Pgp (SPGP), to be discussed in subsequent sections.

Table 1-7. The similarities and difference between various human MDR transporters.

	MDR1	MDR3	BSEP (SPGP)	CFTR	LRP	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6
AA	1280	1279	1321	1480	896	1531	1545	1527	1325	1437	1503
MW (kD)	170	170	170		110	190	200				
Chromosome	7q21	7q21	2q24/ 2q31	7q31	16p 13.1-11.2	16p 13.12-13	10q24	17q21.3	13q 31/32	3q27	16p13
Homology		74.9% (MDR1)	82-88% (rat)			15% (MDR1) 19% (CFTR)	46% (MRP1) 25% (MDR1) 24% (MDR3)	58% (MRP1)			
Tissue Distribution	Kidney, liver, intestine, brain	High in liver, kidney, intestine	Kidney, intestine		Kidney	Kidney, intestine	Liver, kidney, intestine, BBB cells	Liver?		Liver?	
Membrane Domain	Apical	Apical	Apical			Lateral	Apical	Baso-lateral	?	?	Lateral
Substrates	Hydrophobic compounds; organic cations; Estradiol-17β-D-glucuronide		Bile salts			Conjugates of GSH, sulfate, glucuronide; GSSG, GSH; organic anions	Anionic conjugates; GSH biliary secretion				

1.2.3 Transporters in various organs

Three other transport systems capable of transporting a variety of drugs are the organic cation transporters (OCTs), organic anion transport polypeptides (OATPs) and Na^+-dependent taurocholate proteins (NTCPs). Figure 1-4 compares the structures and sizes of the five transporter families (Müller and Jansen, 1998).

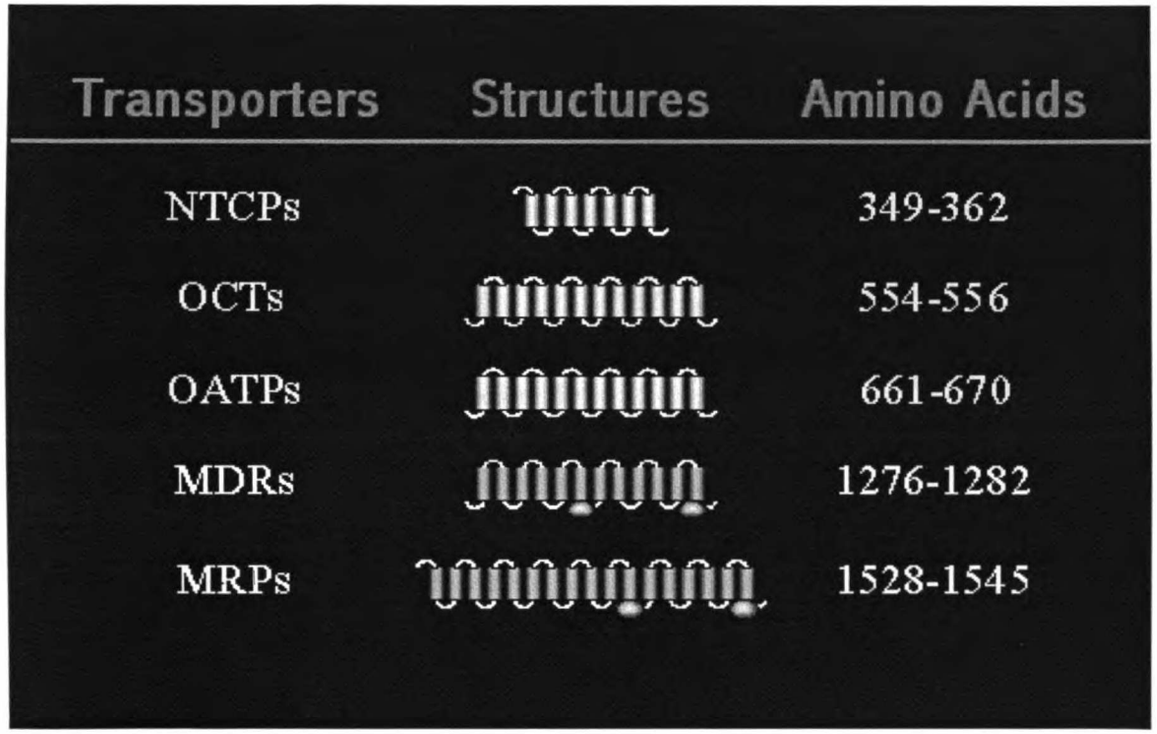


Figure 1-4. Comparison of the structure and size of five transporter families.

OCTs and OATPs have long been studied in uptake and secretion of anionic and cationic endogenous substances and xenobiotics in the kidney and liver, but less well studied in other tissues. NTCPs are well studied in the liver where they uptake bile salts into hepatocytes. Figure 1-5 shows the inter-relationship and how various transporters

function in the liver (Keppler and Arias, 1997; Müller *et al.*, 1996; Müller and Jansen, 1997; Zhang *et al.*, 1998).

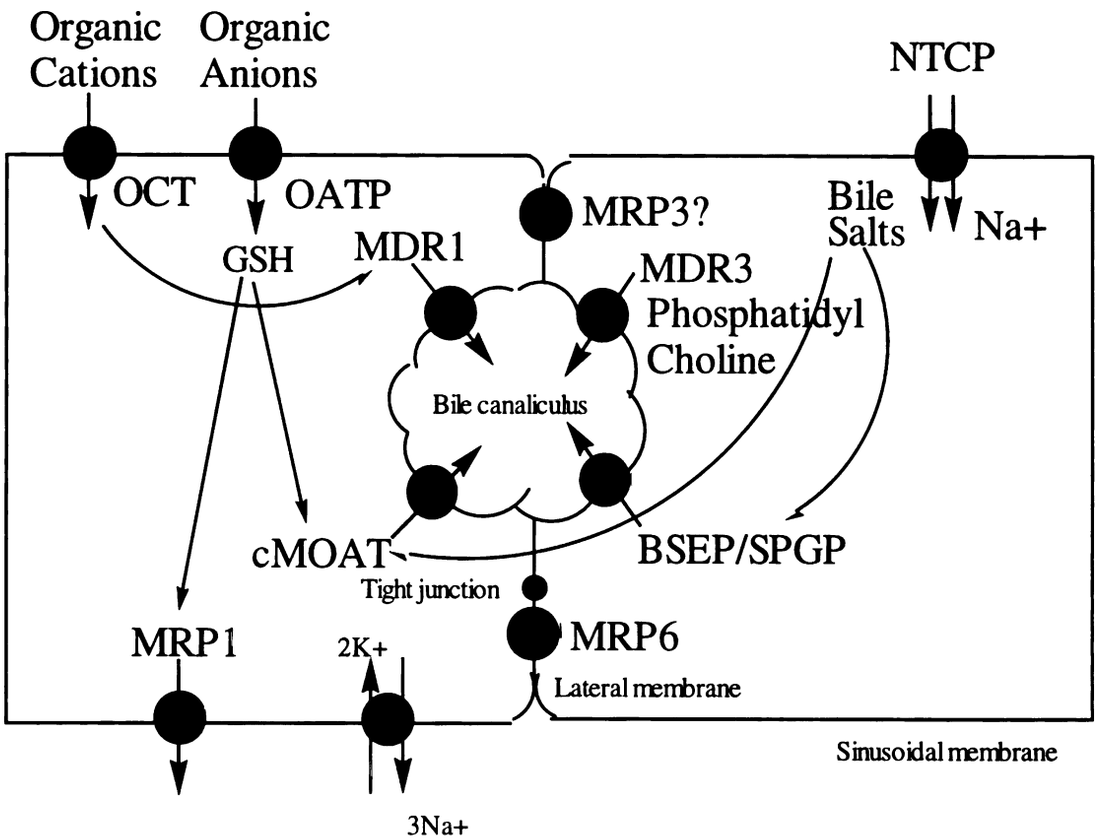


Figure 1-5. Liver transporters.

BSEP (the bile salt export pump), also termed cBAT (canalicular bile acid transporter), that appears to be identical to what has been called sister Pgp (SPGP) (Müller and Jansen, 1997), pumps mono-anionic bile salts, such as taurocholate, across the canalicular membrane (Keppler and Arias, 1997). NTCP is called Na^+-dependent taurocholate protein (Müller *et al.*, 1996) or Na^+-taurocholate co-transporting polypeptide (Müller and Jansen, 1997). Poly-specific OATP is called organic anion transport protein

(Müller *et al.*, 1996) or Na^+-independent organic anion transport protein (Müller and Jansen, 1997).

Renal secretion of anions and cations involves uptake or efflux transporters at either the basolateral (BLM) or brush border membrane (BBM). Figure 1-6 illustrates the various transporters important in kidney function (Bendayan, 1996; Pritchard and Miller, 1993; Somogyi, 1996; Zhang *et al.*, 1998).

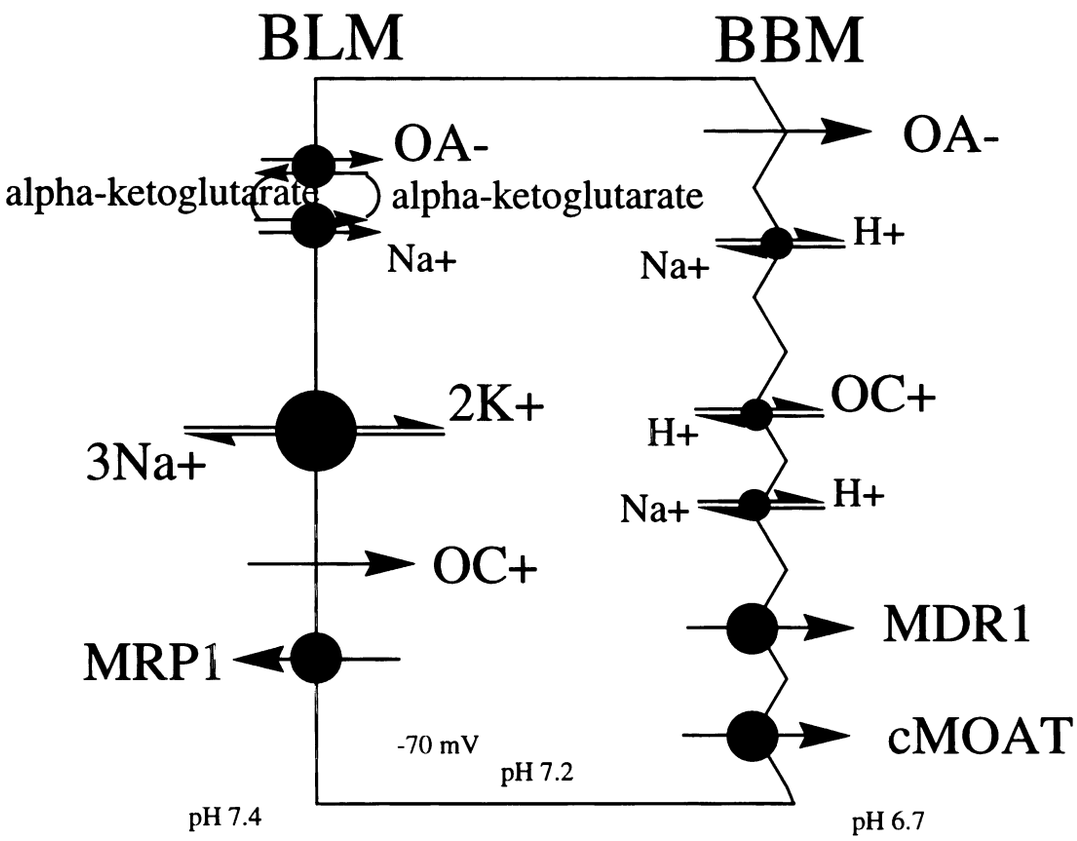


Figure 1-6. Kidney transporters.

Na^+/K^+-ATPase and Pgp are primary active transporters. Symporter or co-transporter (Na^+/glucose), counter-transport (galactose/glucose) and antiporter (Na^+/H^+

and Na^+/Ca^{++}) are secondary active, also called exchange-only, systems. One example of tertiary active transporters is the α-ketoglutarate (α-KG) system, which involves Na^+/K^+-ATPase, Na^+/α-KG cotransporter and OA^-/α-KG anion exchanger.

1.2.4 Overlaps between transporters

Substrate specificity varies among the various isoforms of these transporters, but is in general quite broad (Bendayan, 1996). The canalicular transport of organic anions and cations against a high concentration gradient from the liver cell into the bile seems to be caused, at least partly, by the class I/II Pgp and cMOAT. It is believed that Pgp is one of the putative cation carriers responsible for biliary excretion of bulky (amphiphilic) organic cations. Regarding the canalicular transport of phospholipids, at least the secretion of phosphatidylcholine seems to be mediated by the class III Pgp.

There is also some overlap between OCT and OATP transporters (Bendayan, 1996; Ott *et al.*, 1990). Cation guanidine transport in rabbit BBM vesicles was not effected by typical OCT inhibitors (Miyamoto *et al.*, 1988). "Bisubstrates" interacting with both organic anion and cation transporters are also observed (Ullrich *et al.*, 1993a and b).

1.3 Cytochrome P450s

Over the past years, knowledge at the molecular level has expanded rapidly and substrate specificity and regulatory determinants of various cytochromes P450 (CYP) enzymes have been characterized. About 30 human CYP isoforms (Thummel and Wilkinson, 1998) have been recognized, and drug metabolism studies are now increasingly being used in early drug development.

1.3.1 Drug metabolism reactions and enzymes

Metabolism can occur in many tissues, such as liver, GI tract, lungs, skin and kidneys. Drug metabolism can be classified into phases I and II reactions (Meyer, 1996). Phase I reactions are functional group conversion reactions including dehydrogenation, oxidation and monooxygenation. Phase II reactions are derivatization of functional groups, which include glucuronidation, sulfation, acetylation, GSH-conjugation and methylation. Many enzymes can be involved in drug metabolism (Guengerich, 1996). Oxygenases include cytochromes P450 and flavin-containing monooxygenases. Glutathione S-transferases, sulfotransferases and UDP-glucuronyl transferases are the common conjugating enzymes.

1.3.2 Microsomal enzymes

Microsomes are on the cell smooth endoplasmic reticulum (no ribosome). Microsomal drug oxidations are carried out by oxygenases, also called mixed function oxidases (MFOs). Microsomal enzyme system includes four components— NADPH, O_2, NADPH-cytochrome P450 reductase flavoprotein and cytochrome P450 hemoprotein.

The cytochrome P450 enzymes are involved in oxidative metabolism of endogenous and exogenous compounds. P450s have over 500 genes in all species, with about 50 expressed in each human organism. CYP enzymes are divided into families (1, 2, 3, *etc.*), and further subdivided into subfamilies (1A2, 2A6, *etc.*). Family members have >40% amino acid sequence identity, subfamily members have >55% related identity. Human P450s mainly consist of three families (Table 1-8).

Table 1-8. Three major human P450 families.

CYP1	1A1	in <1% of human liver specimens, in lungs, placenta, inducible
	1A2	not in extrahepatic tissues, only in liver, inducible
CYP2 (largest number of P450s)	2A6	liver
	2B6	in <50% liver samples, only 1% of total P450 in those are 2B6
	2C8	liver, renal
	2C9 and 2C10	differ by two amino acids in liver
	2C19	liver, 5% Caucasians and 20% Asians are poor metabolizers (PMs)
	2D6	liver, 5-10% Caucasians and 1% Asian, African are PMs
	2E1	liver and extrahepatic
CYP3	3A3	liver?, inducible?
	3A4	liver, gut, 50% of total P450s in liver is 3A4, inducible
	3A5	adult liver, in 20-25%
	3A7	fetal liver

1.3.3 P450 isoforms

Table 1-9 notes some substrates (Krishna, 1999), inhibitors and inducers of human P450 isoforms (Flockhart, 1999). Some human P450s also have polymorphisms (Flockhart, 1999) (Table 1-9).

Table 1-9. Some substrates, inhibitors, inducers, location and polymorphisms of the main human P450s.

	1A2	2C19	2C9	2D6	2E1	3A4,5,7
Substrates	acetaminophen caffeine estradiol phenacetin theophylline warfarin	diazepam (S)-mephenytoin propranolol	diclofenac phenytoin tolbutamide (S)-warfarin	codeine debrisoquine dextromethorphan encainide metoprolol propranolol	acetaminophen alcohols benzene caffeine chlorzoxazone chloroform dapsone ethanol theophylline	amiodarone cyclosporine dapsone diazepam erythromycin estradiol etoposide lidocaine midazolam nifedipine quinidine steroids (cortisol) taxol testosterone verapamil warfarin
Inhibitors	amiodarone furafylline	indomethacin ketoconazole steroids	amiodarone fluconazole lovastatin sulfaphenazole (0.5 μM) sulfinpyrazone	amiodarone (0.074 μM) doxorubicin? quinidine (0.04 μM)	diethyl- dithiocarbamate dimethyl sulfoxide disulfiram	amiodarone erythromycin fluconazole grapefruit juice itraconazole ketoconazole miconazole troleandomycin
Inducers	broccoli? Brussels sprouts? insulin 3-methyl-cholanthrene tobacco	not pentobarbital prednisone rifampin	rifampin	dexamethasone? rifampin?	ethanol isoniazid	barbiturates carbamazepine dexamethasone glucocorticoids phenobarbital phenytoin rifampin sulfinpyrazone troleandomycin
Gene location, polymorphisms	Chromosome 15	Chromosome 10 3-5% Caucasian, 15-20% Asian, PMs	Chromosome 10 1-3% Caucasian, PMs	Chromosome 22 5-10% Caucasian, 1% Asian, African PMs	Chromosome 10	Chromosome 7

1.3.4 Substrate specificity of P450 enzymes

Metabolism by CYPs is determined by three generic rules (Smith *et al.*, 1996): topography of the active site, degree of steric hindrance of the iron-oxygen complex access to the possible metabolism sites, and possible ease of electron or hydrogen abstraction from the various carbons or heteroatoms of the substrate. The SAR of three P450s is summarized below (Table 1-10) (Smith *et al.*, 1996).

Table 1-10. The SAR of three major P450s.

CYP2D6	Arylalkylamines (basic) with site of oxidation 5-7 Å from protonated nitrogen.
CYP2C9	Neutral or acidic molecules with site of oxidation 5-8 Å from H-bond donor heteroatom. Molecules tend to be amphipathic with a region of lipophilicity at the site of hydroxylation and an area of hydrophobicity around the H-bond forming region.
CYP3A4	Lipophilic, neutral or basic molecules with site of oxidation often basic nitrogen (N-dealkylation) or allylic positions.

1.3.5 Cytochrome P450 3A

By far, cytochrome P450 3As are the most abundant human CYP isoforms and are of major importance in the metabolism of drugs in humans as shown in Table 1-11 (Guengerich, 1996). CYP3As are localized in organs relevant to drug absorption (gastrointestinal tract) and disposition (kidney, and liver) and their catalytic activity is readily modulated by a variety of compounds. Estimates based on in vitro studies suggest that the metabolism of 40–50% of drugs used in humans involve CYP3A-mediated

oxidation. However, whether this reflects the importance of such metabolism in drug elimination *in vivo* is not so apparent.

Table 1-11. Percentage of drugs oxidazed by different P450 isoforms.

P450	**Location, regulation**	**% of drugs oxidized**
1A2	hepatic, inducible	4
2C9/10	hepatic	10
2C19	hepatic, polymorphic	2
2D6	hepatic, polymorphic	30
2E1	hepatic and extrahepatic	2
3A4	hepatic, small intestine, inducible	50
2A6	hepatic	2

1.3.6 Tissue localization of P450 3A

Humans have at least three functional proteins of CYP3A. CYP3A4 is universally found and is the major isoform, comprising about 30% of total CYP proteins in the liver (Shimada *et al.*, 1994). Relatively high CYP3A4 levels are also present in small intestinal epithelia, particularly in the apical region of enterocytes at the tip of the microvillus, comprising about 70% of total CYP intestinal enzymes (Kolars *et al.*, 1994; McKinnon *et al.*, 1995). The isoform amount falls along the gastrointestinal tract. In the kidney, CYP3A4 is present in only about 30% of renal tissue samples and mainly in the collecting ducts (Haehner *et al.*, 1996; Schuetz *et al.*, 1992), but the mechanism of such polymorphic expression is not clearly understood.

CYP3A3 is a very closely related isoform to CYP3A4 (>98% cDNA sequence similarity), but it is not known whether this reflects a separate gene product or an allelic

variant. Therefore, the term CYP3A4 is generally used to indicate a collective contribution of the two isoforms.

By contrast, CYP3A5 is distinct from CYP3A4 structurally. This polymorphism may be due to a point mutation resulting in the synthesis of an unstable protein (Jounaïdi *et al.*, 1996). CYP3A5 is found in the liver but only in about 10–30% of hepatic samples and only at 10–30% of CYP3A4 levels (Wrighton *et al.*, 1989 and 1990). CYP3A5 is the predominant CYP3A isoform that is universally expressed in the kidney (Haehner *et al.*, 1996; Schuetz *et al.*, 1992). It is also heterogeneously expressed throughout the gastrointestinal tract, but in lower amounts than CYP3A4 except in stomach parietal cells (Kolars *et al.*, 1994; McKinnon *et al.*, 1995). CYP3A5 expression does not appear to be up-regulated by well-established inducers of CYP3A4 (Schuetz *et al.*, 1993; Wrighton *et al.*, 1989).

CYP3A7 was originally found in fetal liver. However, it also appears to be selectively expressed in adult livers at lower levels than CYP3A4 and CYP3A5 (Schuetz *et al.*, 1994).

1.3.7 P450 3A substrate specificity

The substrate specificity of the CYP3A enzymes is very broad, with many structurally divergent chemicals metabolized by various different pathways in a regio- and stereo-selective fashion (Guengerich, 1995; Maurel, 1996).

CYP3A4 is the most thoroughly studied isoform, and it is assumed that the other isoforms have essentially similar characteristics. However, possible important differences in the isoforms' substrate specificity may exist. For instance, neither quinidine nor erythromycin appear to be metabolized by CYP3A5 although they are good CYP3A4

substrates (Aoyama *et al.*, 1989; Wrighton *et al.*, 1990). Furthermore, only one of three primary metabolites of cyclosporine, all formed by CYP3A4, was also produced by CYP3A5 (Aoyama *et al.*, 1989). CYP3A5 exhibited greater catalytic activity in the 1'-hydroxylation of midazolam, but the level of CYP3A5-mediated metabolism was less than that of CYP3A4 (Gorski *et al.*, 1994). Optimal *in vitro* conditions may not be the same for the two isoforms causing the differences in these comparative studies.

1.3.8 Variability of P450 3A

CYP3A exhibits large inter-individual variability in activity resulting from a genetic effect combined with modulation by environmental factors. Hepatic microsomal activity often differs by up to 40-fold (Guengerich, 1995; Shimada *et al.*, 1994), and large variability also has been noted with intestinal (Lown *et al.*, 1994) and renal microsomes (Haehner *et al.*, 1996). Human *in vivo* studies demonstrate considerable interindividual variability that is generally smaller (five-fold) than microsomal activity, although inhibition and induction can significantly increase the range. The reason for such a discrepancy is unclear, but care must be taken in quantitatively extrapolating *in vitro* studies to the *in vivo* situation.

Even though the term CYP3A primarily reflects CYP3A4, it usually reflects all of the isoforms because of the difficulty in distinguishing between the isoforms' catalytic activities and more often several isoforms may be present in a single organ.

1.3.9 P450 3A in drug interactions

Not all drug interactions caused by CYP3A are necessarily of clinical importance, unless CYP3A-mediated metabolism is the drug's major elimination pathway. Important clinical drug interactions generally occur only with drugs that have a narrow and/or steep

concentration-response relationship when coadministered with the most potent inhibitors or inducers. More modest interactions are generally of less concern, since their consequences are within the normal population variability.

The involvement of CYP3A in the metabolism of numerous drugs leading to potential drug-drug interactions has been reported. The order of *in vivo* inhibitory potency for azole antifungal agents is consistent with their *in vitro* K_i values, *i.e.* ketoconazole>itraconazole>fluconazole, with miconazole showing no effect (Baciewicz and Baciewicz, 1993; Gillum *et al.*, 1993). Erythromycin, which inactivates CYP3A by the formation of complexes with the heme moiety, is also a potent inhibitor, but less so than ketoconazole (Gillum *et al.*, 1993; Lindstrom *et al.*, 1993; Periti *et al.*, 1992; von Rosensteil and Adam, 1995). Several calcium-channel blockers, such as verapamil and diltiazem, produce a weak to moderate inhibitory interaction with other CYP3A drugs *in vivo* (Rosenthal and Ezra, 1995).

The increase in cyclosporine blood concentration after co-administration of various inhibitors (*e.g.*, azole antifungal agents) is consistent with *in vitro* studies. Use of ketoconazole (Butman *et al.*, 1991; First *et al.*, 1991 and 1993; Hebert *et al.*, 1992; Keogh *et al.*, 1995; Patton *et al.*, 1994; Sobh *et al.*, 1995) together with cyclosporine reduce the immunosuppressives metabolism by CYP3A. Co-administration of azole antifungal agents has been reported to increase tacrolimus (FK506) blood concentrations (Floren *et al.*, 1997; Venkataramanan *et al.*, 1995).

The azole antifungal agents and erythromycin (Ahonen *et al.*, 1995 and 1997; Backman *et al.*, 1994a and b, 1995; Mattila *et al.*, 1994; Olkkola *et al.*, 1993, 1994 and 1996; Yeates *et al.*, 1996; Zimmermann *et al.*, 1996) have also been found to markedly

enhance midazolam plasma levels. Terfenadine's elimination was predominantly determined by CYP3A (Jurima-Romet *et al.*, 1994; Ling *et al.*, 1995; Yun *et al.*, 1993) and serious side effects were associated with co-administration of ketoconazole or erythromycin (Woosley, 1996). Fexofenadine, the active metabolite of terfenadine, is not extensively metabolized but is a Pgp substrate.

1.4 Cytochrome P450 3A and P-glycoprotein

Pgp is often co-localized to cells in which CYP3A is extensively expressed, *e.g.* enterocytes and hepatocytes, so that the two proteins appear to function in concert to reduce the intracellular concentration of xenobiotics. Considerable overlap exists between compounds interacting with Pgp and CYP3A (Wacher *et al.*, 1995), probably reflective of the broad substrate specificity of the individual proteins. A considerable number, but not all of CYP3A substrates, interact with Pgp either as substrates and/or inhibitors (calcium-channel blockers, azole antifungal agents, immunosuppressants and macrolide antibiotics).

The co-administration of two Pgp and/or CYP3A substrates can result in interactions that reflect inhibition of metabolism alone or reduced Pgp efflux only or a combination of both (Kivistö *et al.*, 1995; Wacher *et al.*, 1998). Several studies in animals and *in vitro* cultured cell lines indicate that the interactions previously considered to only reflect inhibition of metabolism could also involve a Pgp mechanism (Hunter *et al.*, 1993; Leu and Huang, 1995; Terao *et al.*, 1996). Moreover, it has been shown that cellular levels of expression of the efflux pump relate to the extent of CYP3A induction by rifampin *in vitro* (Schuetz *et al.*, 1996).

Benet and his colleagues (reviewed in Wacher *et al.*, 1998) proposed that Pgp expression in the gut could influence the extent of drug metabolism, depending on the efficiency of the active counter-transport. The intestinal barrier of CYP3A and Pgp is depicted in Figure 1-7.

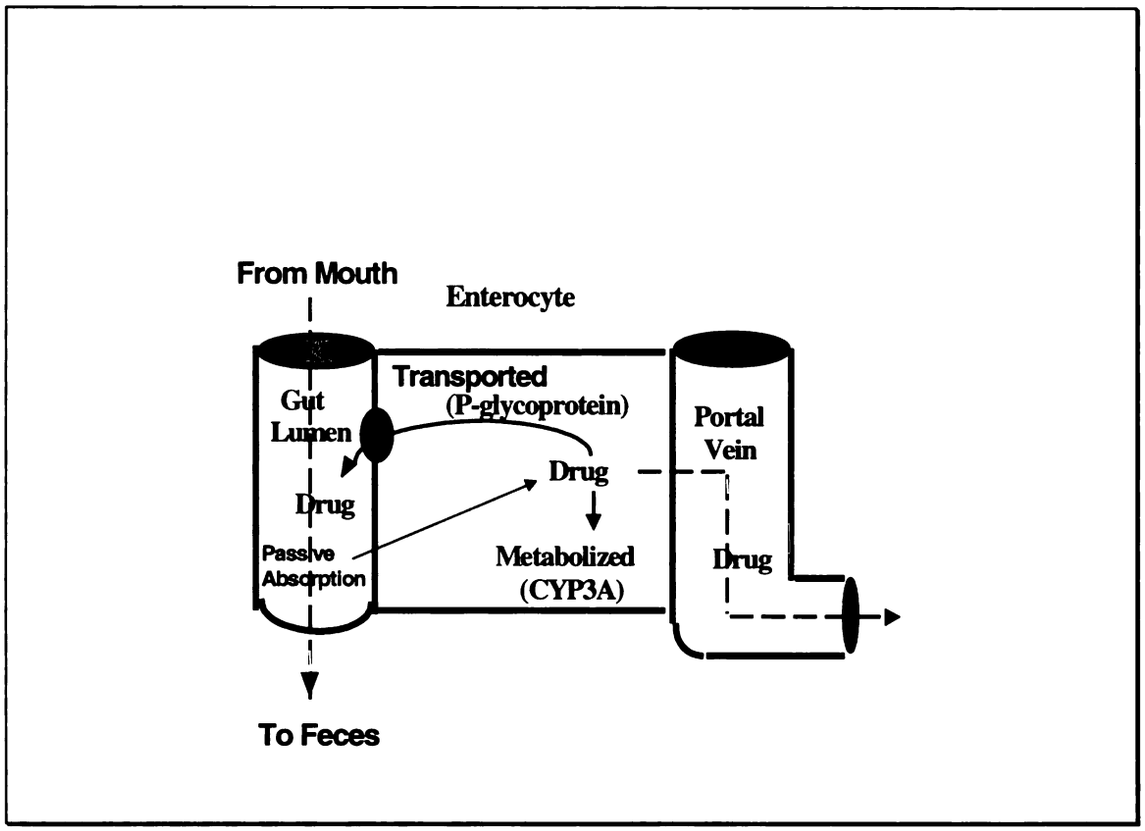


Figure 1-7. The intestinal barrier of CYP3A and Pgp.

When an orally administered drug reaches the intestine, it can cross the plasma membrane of the enterocytes by passive diffusion. Of the drug absorbed, a fraction reaches the portal vein by-passing the enzyme, another fraction is metabolized by CYP3A and an additional fraction is pumped out of the enterocyte. The expelled unchanged drug may re-enter the enterocyte and again be partially absorbed, metabolized and extruded. Blocking Pgp counter-transport leads to a lower extraction of the drug by

the enzyme, and thus to a higher fraction absorbed because a larger fraction of the drug reaches and saturates the capacity-limited enzyme. When CYP3A is inhibited, the fraction of the drug getting metabolized is reduced since Pgp is the only mechanism limiting drug absorption. Inhibition of both proteins will significantly increase the fraction of the drug able to reach the portal vein.

Based on this model, it appears that Pgp not only affects drug absorption by its own activity, but also influences the fraction of the drug metabolized by regulating the drug's access to the enzyme. It is difficult to discriminate between the effects resulting from the interaction with the transporter or with the enzyme because many of the drugs that have been used as Pgp modulators, such as cyclosporine, verapamil, amiodarone and quinidine, are also CYP3A substrates. This thesis research attempts to separate out the effects of Pgp *versus* CYP3A as discussed in Chapter 2 for the model substrate digoxin.

1.5 References

Ahonen, J., Olkkola, K. T. and Neuvonen, P. J. (1995). Effect of itraconazole and terbinafine on the pharmacokinetics and pharmacodynamics of midazolam in healthy volunteers. *British Journal of Clinical Pharmacology* **40,** 270-2.

Ahonen, J., Olkkola, K. T. and Neuvonen, P. J. (1997). Effect of route of administration of fluconazole on the interaction between fluconazole and midazolam. *European Journal of Clinical Pharmacology* **51,** 415-9.

Altenberg, G. A., Young, G., Horton, J. K., Glass, D., Belli, J. A. and Reuss, L. (1993). Changes in intra- or extracellular pH do not mediate P-glycoprotein-dependent multidrug resistance. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 9735-8.

Altenberg, G. A., Vanoye, C. G., Han, E. S., Deitmer, J. W. and Reuss, L. (1994). Relationships between rhodamine 123 transport, cell volume, and ion-channel function of P-glycoprotein. *Journal of Biological Chemistry* **269,** 7145-9.

Aoyama, T., Yamano, S., Waxman, D. J., Lapenson, D. P., Meyer, U. A., Fischer, V., Tyndale, R., Inaba, T., Kalow, W. and Gelboin, H. V. (1989). Cytochrome P-450 hPCN3, a novel cytochrome P-450 IIIA gene product that is differentially expressed in adult human liver. cDNA and deduced amino acid sequence and distinct specificities of cDNA-expressed hPCN1 and hPCN3 for the metabolism of steroid hormones and cyclosporine. *Journal of Biological Chemistry* **264,** 10388-95.

Arias, I. M., Gatmaitan, Z., Mazzanti, R., Shu, H. and Kumamoto, Y. (1990). Structure and function of P-glycoprotein in the normal liver and intestine. *Princess Takamatsu Symposia* **21,** 229-39.

Baciewicz, A. M. and Baciewicz, F. A., Jr. (1993). Ketoconazole and fluconazole drug interactions [see comments]. *Archives of Internal Medicine* **153,** 1970-6.

Backman, J. T., Aranko, K., Himberg, J. J. and Olkkola, K. T. (1994a). A pharmacokinetic interaction between roxithromycin and midazolam. *European Journal of Clinical Pharmacology* **46,** 551-5.

Backman, J. T., Olkkola, K. T., Aranko, K., Himberg, J. J. and Neuvonen, P. J. (1994b). Dose of midazolam should be reduced during diltiazem and verapamil treatments. *British Journal of Clinical Pharmacology* **37,** 221-5.

Backman, J. T., Olkkola, K. T. and Neuvonen, P. J. (1995). Azithromycin does not increase plasma concentrations of oral midazolam. *International Journal of Clinical Pharmacology and Therapeutics* **33,** 356-9.

Beck, W. T. and Cirtain, M. C. (1982). Continued expression of vinca alkaloid resistance by CCRF-CEM cells after treatment with tunicamycin or pronase. *Cancer Research* **42,** 184-9.

Bendayan, R. (1996). Renal drug transport: a review. *Pharmacotherapy* **16,** 971-85.

Biedler, J. L. (1992). Genetic aspects of multidrug resistance. *Cancer* **70,** 1799-809.

Borst, P. (1997). Multidrug resistant proteins. *Seminars in Cancer Biology* **8,** 131-4.

Borst, P. and Schinkel, A. H. (1996). What have we learnt thus far from mice with disrupted P-glycoprotein genes? *European Journal of Cancer* **32A,** 985-90.

Borst, P., Schinkel, A. H., Smit, J. J., Wagenaar, E., Van Deemter, L., Smith, A. J., Eijdems, E. W., Baas, F. and Zaman, G. J. (1993). Classical and novel forms of multidrug resistance and the physiological functions of P-glycoproteins in mammals. *Pharmacology and Therapeutics* **60,** 289-99.

Borst, P., Kool, M. and Evers, R. (1997). Do cMOAT (MRP2), other MRP homologues, and LRP play a role in MDR? *Seminars in Cancer Biology* **8,** 205-13.

Bradley, G., Georges, E. and Ling, V. (1990). Sex-dependent and independent expression of the P-glycoprotein isoforms in Chinese hamster. *Journal of Cellular Physiology* **145,** 398-408.

Broxterman, H. J., Lankelma, J. and Pinedo, H. M. (1996). How to probe clinical tumour samples for P-glycoprotein and multidrug resistance-associated protein. *European Journal of Cancer* **32A,** 1024-1033.

Buschman, E. and Gros, P. (1991). Functional analysis of chimeric genes obtained by exchanging homologous domains of the mouse mdr1 and mdr2 genes. *Molecular and Cellular Biology* **11,** 595-603.

Buschman, E., Arceci, R. J., Croop, J. M., Che, M., Arias, I. M., Housman, D. E. and Gros, P. (1992). mdr2 encodes P-glycoprotein expressed in the bile canalicular membrane as determined by isoform-specific antibodies. *Journal of Biological Chemistry* **267,** 18093-9.

Butman, S. M., Wild, J. C., Nolan, P. E., Fagan, T. C., Finley, P. R., Hicks, M. J., Mackie, M. J. and Copeland, J. G. 3d. (1991). Prospective study of the safety and financial benefit of ketoconazole as adjunctive therapy to cyclosporine after heart transplantation. *Journal of Heart and Lung Transplantation* **10,** 351-8.

Choi, K. H., Chen, C. J., Kriegler, M. and Roninson, I. B. (1988). An altered pattern of cross-resistance in multidrug-resistant human cells results from spontaneous mutations in the mdr1 (P-glycoprotein) gene. *Cell* **53,** 519-29.

Cole, S. P., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M. and Deeley, R. G. (1994). Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Research* **54,** 5902-10.

Cordon-Cardo, C., O'Brien, J. P., Boccia, J., Casals, D., Bertino, J. R. and Melamed, M. R. (1990). Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *Journal of Histochemistry and Cytochemistry* **38,** 1277-87.

Croop, J. M. (1993). P-glycoprotein structure and evolutionary homologies. *Cytotechnology* **12,** 1-32.

Croop, J. M., Raymond, M., Haber, D., Devault, A., Arceci, R. J., Gros, P. and Housman, D. E. (1989). The three mouse multidrug resistance (mdr) genes are expressed in a tissue-specific manner in normal mouse tissues. *Molecular and Cellular Biology* **9,** 1346-50.

Deleuze, J. F., Jacquemin, E., Dubuisson, C., Cresteil, D., Dumont, M., Erlinger, S., Bernard, O. and Hadchouel, M. (1996). Defect of multidrug-resistance 3 gene expression in a subtype of progressive familial intrahepatic cholestasis. *Hepatology* **23,** 904-8.

den Boer, M. L., Pieters, R., Kazemier, K. M., Rottier, M. M., Zwaan, C. M., Kaspers, G. J., Janka-Schaub, G., Henze, G., Creutzig, U., Scheper, R. J. and Veerman, A. J. (1998). Relationship between major vault protein/lung resistance protein, multidrug resistance-associated protein, P-glycoprotein expression, and drug resistance in childhood leukemia. *Blood* **91,** 2092-8.

Devault, A. and Gros, P. (1990). Two members of the mouse mdr gene family confer multidrug resistance with overlapping but distinct drug specificities. *Molecular and Cellular Biology* **10,** 1652-63.

Dey, S., Ramachandra, M., Pastan, I., Gottesman, M. M. and Ambudkar, S. V. (1997). Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 10594-9.

Fardel, O., Lecureur, V. and Guillouzo, A. (1993). Regulation by dexamethasone of P-glycoprotein expression in cultured rat hepatocytes. *FEBS Letters* **327,** 189-93.

Ferry, D. R. (1998). Testing the role of P-glycoprotein expression in clinical trials: applying pharmacological principles and best methods for detection together with good clinical trials methodology. *International Journal of Clinical Pharmacology and Therapeutics* **36,** 29-40.

First, M. R., Schroeder, T. J., Alexander, J. W., Stephens, G. W., Weiskittel, P., Myre, S. A. and Pesce, A. J. (1991). Cyclosporine dose reduction by ketoconazole administration in renal transplant recipients. *Transplantation* **51,** 365-70.

First, M. R., Schroeder, T. J., Michael, A., Hariharan, S., Weiskittel, P. and Alexander, J. W. (1993). Safety and efficacy of long-term cyclosporine-ketoconazole administration and preliminary results of a randomized trial. *Transplantation Proceedings* **25,** 591-4.

Flens, M. J., Izquierdo, M. A., Scheffer, G. L., Fritz, J. M., Meijer, C. J., Scheper, R. J. and Zaman, G. J. (1994). Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Research* **54,** 4557-63.

Flockhart, D. A. (1999). Cytochrome P450 Drug Interaction Table. *http://www.dml.georgetown.edu/depts/pharmacology/davetab.html.*

Floren, L. C., Bekersky, I., Benet, L. Z., Mekki, Q., Dressler, D., Lee, J. W., Roberts, J. P. and Hebert, M. F. (1997). Tacrolimus oral bioavailability doubles with coadministration of ketoconazole. *Clinical Pharmacology and Therapeutics* **62,** 41-9.

Ford, J. M. and Hait, W. N. (1990). Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacological Reviews* **42,** 155-99.

Garrigos, M., Mir, L. M. and Orlowski, S. (1997). Competitive and non-competitive inhibition of the multidrug-resistance-associated P-glycoprotein ATPase--further experimental evidence for a multisite model. *European Journal of Biochemistry* **244,** 664-73.

Gillum, J. G., Israel, D. S. and Polk, R. E. (1993). Pharmacokinetic drug interactions with antimicrobial agents. *Clinical Pharmacokinetics* **25,** 450-82.

Gorski, J. C., Hall, S. D., Jones, D. R., VandenBranden, M. and Wrighton, S. A. (1994). Regioselective biotransformation of midazolam by members of the human cytochrome P450 3A (CYP3A) subfamily. *Biochemical Pharmacology* **47,** 1643-53.

Gottesman, M. M. and Pastan, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual Review of Biochemistry* **62,** 385-427.

Gros, P., Ben Neriah, Y. B., Croop, J. M. and Housman, D. E. (1986). Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature* **323,** 728-31.

Gros, P., Raymond, M., Bell, J. and Housman, D. (1988). Cloning and characterization of a second member of the mouse mdr gene family. *Molecular and Cellular Biology* **8,** 2770-8.

Guengerich, F. P. (1995). Human cytochrome P450 enzymes. In *Cytochrome P450: Structure, Mechanism and Biochemistry* (ed. Ortiz de Montellano, P.), pp. 473-535. New York: Plenum.

Guengerich, F. P. (1996). In vitro techniques for studying drug metabolism. *Journal of Pharmacokinetics and Biopharmaceutics* **24,** 521-33.

Haehner, B. D., Gorski, J. C., Vandenbranden, M., Wrighton, S. A., Janardan, S. K., Watkins, P. B. and Hall, S. D. (1996). Bimodal distribution of renal cytochrome P450 3A activity in humans. *Molecular Pharmacology* **50,** 52-9.

Hebert, M. F., Roberts, J. P., Prueksaritanont, T. and Benet, L. Z. (1992). Bioavailability of cyclosporine with concomitant rifampin administration is markedly less than predicted by hepatic enzyme induction. *Clinical Pharmacology and Therapeutics* **52,** 453-7.

Heijn, M., Hooijberg, J. H., Scheffer, G. L., Szabó, G., Westerhoff, H. V. and Lankelma, J. (1997). Anthracyclines modulate multidrug resistance protein (MRP) mediated organic anion transport. *Biochimica et Biophysica Acta* **1326,** 12-22.

Higgins, C. F. (1992). ABC transporters: from microorganisms to man. *Annual Review of Cell Biology* **8,** 67-113.

Higgins, C. F. (1995). P-glycoprotein and cell volume-activated chloride channels. *Journal of Bioenergetics and Biomembranes* **27,** 63-70.

Higgins, C. F. and Gottesman, M. M. (1992). Is the multidrug transporter a flippase? *Trends in Biochemical Sciences* **17,** 18-21.

Holló, Z., Homolya, L., Hegedüs, T. and Sarkadi, B. (1996). Transport properties of the multidrug resistance-associated protein (MRP) in human tumour cells. *FEBS Letters* **383,** 99-104.

Homolya, L., Holló, Z., Germann, U. A., Pastan, I., Gottesman, M. M. and Sarkadi, B. (1993). Fluorescent cellular indicators are extruded by the multidrug resistance protein. *Journal of Biological Chemistry* **268,** 21493-6.

Hunter, J., Hirst, B. H. and Simmons, N. L. (1993). Drug absorption limited by P-glycoprotein-mediated secretory drug transport in human intestinal epithelial Caco-2 cell layers. *Pharmaceutical Research* **10,** 743-9.

Ishikawa, T., Müller, M., Klünemann, C., Schaub, T. and Keppler, D. (1990). ATP-dependent primary active transport of cysteinyl leukotrienes across liver canalicular membrane. Role of the ATP-dependent transport system for glutathione S-conjugates. *Journal of Biological Chemistry* **265,** 19279-86.

Izquierdo, M. A., Scheffer, G. L., Flens, M. J., Giaccone, G., Broxterman, H. J., Meijer, C. J., van der Valk, P. and Scheper, R. J. (1996). Broad distribution of the multidrug resistance-related vault lung resistance protein in normal human tissues and tumors. *American Journal of Pathology* **148,** 877-87.

Jounaïdi, Y., Hyrailles, V., Gervot, L. and Maurel, P. (1996). Detection of CYP3A5 allelic variant: a candidate for the polymorphic expression of the protein? *Biochemical and Biophysical Research Communications* **221,** 466-70.

Juliano, R. L. and Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta* **455,** 152-62.

Jurima-Romet, M., Crawford, K., Cyr, T. and Inaba, T. (1994). Terfenadine metabolism in human liver. In vitro inhibition by macrolide antibiotics and azole antifungals. *Drug Metabolism and Disposition* **22,** 849-57.

Kartner, N., Evernden-Porelle, D., Bradley, G. and Ling, V. (1985). Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature* **316,** 820-3.

Keogh, A., Spratt, P., McCosker, C., Macdonald, P., Mundy, J. and Kaan, A. (1995). Ketoconazole to reduce the need for cyclosporine after cardiac transplantation [see comments]. *New England Journal of Medicine* **333,** 628-33.

Keppler, D. and Arias, I. M. (1997). Hepatic canalicular membrane. Introduction: transport across the hepatocyte canalicular membrane. *FASEB Journal* **11,** 15-8.

Kivistö, K. T., Kroemer, H. K. and Eichelbaum, M. (1995). The role of human cytochrome P450 enzymes in the metabolism of anticancer agents: implications for drug interactions. *British Journal of Clinical Pharmacology* **40,** 523-30.

Kolars, J. C., Lown, K. S., Schmiedlin-Ren, P., Ghosh, M., Fang, C., Wrighton, S. A., Merion, R. M. and Watkins, P. B. (1994). CYP3A gene expression in human gut epithelium. *Pharmacogenetics* **4,** 247-59.

Kool, M., de Haas, M., Scheffer, G. L., Scheper, R. J., van Eijk, M. J., Juijn, J. A., Baas, F. and Borst, P. (1997). Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Research* **57,** 3537-47.

Kramer, R., Weber, T. K., Arceci, R., Ramchurren, N., Kastrinakis, W. V., Steele, G., Jr. and Summerhayes, I. C. (1995). Inhibition of N-linked glycosylation of P-glycoprotein by tunicamycin results in a reduced multidrug resistance phenotype. *British Journal of Cancer* **71,** 670-5.

Krishna, G. (1999). Some substrates, inducers, and inhibitors for the human cytochrome P450 isoforms. *http://griffin.vcu.edu/~gkrishna/PK/pk.html.*

Kruh, G. D., Gaughan, K. T., Godwin, A. and Chan, A. (1995). Expression pattern of MRP in human tissues and adult solid tumor cell lines. *Journal of the National Cancer Institute* **87,** 1256-8.

Lautier, D., Canitrot, Y., Deeley, R. G. and Cole, S. P. (1996). Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. *Biochemical Pharmacology* **52,** 967-77.

Leu, B. L. and Huang, J. D. (1995). Inhibition of intestinal P-glycoprotein and effects on etoposide absorption. *Cancer Chemotherapy and Pharmacology* **35,** 432-6.

Leveille-Webster, C. R. and Arias, I. M. (1995). The biology of the P-glycoproteins. *Journal of Membrane Biology* **143,** 89-102.

Lindstrom, T. D., Hanssen, B. R. and Wrighton, S. A. (1993). Cytochrome P-450 complex formation by dirithromycin and other macrolides in rat and human livers. *Antimicrobial Agents and Chemotherapy* **37,** 265-9.

Ling, V. (1975). Drug resistance and membrane alteration in mutants of mammalian cells. *Canadian Journal of Genetics and Cytology* **17,** 503-15.

Ling, K. H., Leeson, G. A., Burmaster, S. D., Hook, R. H., Reith, M. K. and Cheng, L. K. (1995). Metabolism of terfenadine associated with CYP3A(4) activity in human hepatic microsomes. *Drug Metabolism and Disposition* **23,** 631-6.

Lomri, N., Fitz, J. G. and Scharschmidt, B. F. (1996). Hepatocellular transport: role of ATP-binding cassette proteins. *Seminars in Liver Disease* **16,** 201-10.

Lown, K. S., Kolars, J. C., Thummel, K. E., Barnett, J. L., Kunze, K. L., Wrighton, S. A. and Watkins, P. B. (1994). Interpatient heterogeneity in expression of CYP3A4 and CYP3A5 in small bowel. Lack of prediction by the erythromycin breath test

[published erratum appears in Drug Metab Dispos 1995 Mar;23(3):following table of contents]. *Drug Metabolism and Disposition* **22,** 947-55.

Lum, B. L., Fisher, G. A., Brophy, N. A., Yahanda, A. M., Adler, K. M., Kaubisch, S., Halsey, J. and Sikic, B. I. (1993). Clinical trials of modulation of multidrug resistance. Pharmacokinetic and pharmacodynamic considerations. *Cancer* **72,** 3502-14.

Luz, J. G., Wei, L. Y., Basu, S. and Roepe, P. D. (1994). Transfection of mu MDR 1 inhibits Na(+)-independent Cl-/-HCO3 exchange in Chinese hamster ovary cells. *Biochemistry* **33,** 7239-49.

Martin, C., Berridge, G., Higgins, C. F. and Callaghan, R. (1997). The multi-drug resistance reversal agent SR33557 and modulation of vinca alkaloid binding to P-glycoprotein by an allosteric interaction. *British Journal of Pharmacology* **122,** 765-71.

Mattila, M. J., Vanakoski, J. and Idänpään-Heikkilä, J. J. (1994). Azithromycin does not alter the effects of oral midazolam on human performance. *European Journal of Clinical Pharmacology* **47,** 49-52.

Maurel, P. (1996). The CYP3 family. In *Cytochrome P450: Metabolic and Toxicological Aspects* (ed. Ioannides, C.), pp. 241-70. Boca Raton, FL: CRC.

McKinnon, R. A., Burgess, W. M., Hall, P. M., Roberts-Thomson, S. J., Gonzalez, F. J. and McManus, M. E. (1995). Characterisation of CYP3A gene subfamily expression in human gastrointestinal tissues. *Gut* **36,** 259-67.

Meyer, U. A. (1996). Overview of enzymes of drug metabolism. *Journal of Pharmacokinetics and Biopharmaceutics* **24,** 449-59.

Miyamoto, Y., Coone, J. L., Ganapathy, V. and Leibach, F. H. (1988). Distribution and properties of the glycylsarcosine-transport system in rabbit renal proximal tubule. Studies with isolated brush-border-membrane vesicles. *Biochemical Journal* **249,** 247-53.

Müller, M. (1999). Human ATP-Binding Cassette Transporters (39). *http://www.med.rug.nl/mdl/humanabc.htm.*

Müller, M. and Jansen, P. L. (1997). Molecular aspects of hepatobiliary transport. *American Journal of Physiology* **272,** G1285-303.

Müller, M. and Jansen, P. L. (1998). The secretory function of the liver: new aspects of hepatobiliary transport. *Journal of Hepatology* **28,** 344-54.

Müller, M., de Vries, E. G. and Jansen, P. L. (1996). Role of multidrug resistance protein (MRP) in glutathione S-conjugate transport in mammalian cells. *Journal of Hepatology* **24 Suppl 1,** 100-8.

Ng, W. F., Sarangi, F., Zastawny, R. L., Veinot-Drebot, L. and Ling, V. (1989). Identification of members of the P-glycoprotein multigene family. *Molecular and Cellular Biology* **9,** 1224-32.

Okochi, E., Iwahashi, T. and Tsuruo, T. (1997). Monoclonal antibodies specific for P-glycoprotein. *Leukemia (Basingstoke)* **11,** 1119-23.

Olkkola, K. T., Aranko, K., Luurila, H., Hiller, A., Saarnivaara, L., Himberg, J. J. and Neuvonen, P. J. (1993). A potentially hazardous interaction between erythromycin and midazolam. *Clinical Pharmacology and Therapeutics* **53,** 298-305.

Olkkola, K. T., Backman, J. T. and Neuvonen, P. J. (1994). Midazolam should be avoided in patients receiving the systemic antimycotics ketoconazole or itraconazole. *Clinical Pharmacology and Therapeutics* **55,** 481-5.

Olkkola, K. T., Ahonen, J. and Neuvonen, P. J. (1996). The effects of the systemic antimycotics, itraconazole and fluconazole, on the pharmacokinetics and pharmacodynamics of intravenous and oral midazolam. *Anesthesia and Analgesia* **82,** 511-6.

Ott, R. J., Hai, A. C. and Giacomini, K. M. (1990). Mechanisms of interactions between organic anions and the organic cation transporter in renal brush border membrane vesicles. *Biochemical Pharmacology* **40,** 659-61.

Oude Elferink, R. P. and Jansen, P. L. (1994). The role of the canalicular multispecific organic anion transporter in the disposal of endo- and xenobiotics. *Pharmacology and Therapeutics* **64,** 77-97.

Oude Elferink, R. P., Tytgat, G. N. and Groen, A. K. (1997). Hepatic canalicular membrane 1: The role of mdr2 P-glycoprotein in hepatobiliary lipid transport. *FASEB Journal* **11,** 19-28.

Patton, P. R., Brunson, M. E., Pfaff, W. W., Howard, R. J., Peterson, J. C., Ramos, E. L. and Karlix, J. L. (1994). A preliminary report of diltiazem and ketoconazole. Their cyclosporine-sparing effect and impact on transplant outcome. *Transplantation* **57,** 889-92.

Paulusma, C. C., Bosma, P. J., Zaman, G. J., Bakker, C. T., Otter, M., Scheffer, G. L., Scheper, R. J., Borst, P. and Oude Elferink, R. P. (1996). Congenital jaundice in

rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271,** 1126-8.

Pedersen, P. L. (1995). Multidrug resistance--a fascinating, clinically relevant problem in bioenergetics. *Journal of Bioenergetics and Biomembranes* **27,** 3-5.

Periti, P., Mazzei, T., Mini, E. and Novelli, A. (1992). Pharmacokinetic drug interactions of macrolides [published erratum appears in Clin Pharmacokinet 1993 Jan;24(1):70]. *Clinical Pharmacokinetics* **23,** 106-31.

Pritchard, J. B. and Miller, D. S. (1993). Mechanisms mediating renal secretion of organic anions and cations. *Physiological Reviews* **73,** 765-96.

Roepe, P. D. (1992). Analysis of the steady-state and initial rate of doxorubicin efflux from a series of multidrug-resistant cells expressing different levels of P-glycoprotein. *Biochemistry* **31,** 12555-64.

Roepe, P. D., Wei, L. Y., Cruz, J. and Carlson, D. (1993). Lower electrical membrane potential and altered pHi homeostasis in multidrug-resistant (MDR) cells: further characterization of a series of MDR cell lines expressing different levels of P-glycoprotein. *Biochemistry* **32,** 11042-56.

Roepe, P. D., Weisburg, J. H., Luz, J. G., Hoffman, M. M. and Wei, L. Y. (1994). Novel Cl(-)-dependent intracellular pH regulation in murine MDR 1 transfectants and potential implications. *Biochemistry* **33,** 11008-15.

Rosenberg, M. F., Callaghan, R., Ford, R. C. and Higgins, C. F. (1997). Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. *Journal of Biological Chemistry* **272,** 10685-94.

Rosenthal, T. and Ezra, D. (1995). Calcium antagonists. Drug interactions of clinical significance. *Drug Safety* **13,** 157-87.

Ruetz, S. and Gros, P. (1994). Phosphatidylcholine translocase: a physiological role for the mdr2 gene. *Cell* **77,** 1071-81.

Salphati, L. (1998). P-glycoprotein and Cytochrome P450 3A: Potential Interactions and Complementary Roles in Drug Absorption and Disposition, Ph.D. Thesis: Universite Paris.

Salphati, L. and Benet, L. Z. (1998). Modulation of P-glycoprotein expression by cytochrome P450 3A inducers in male and female rat livers. *Biochemical Pharmacology* **55,** 387-95.

Schinkel, A. H., Kemp, S., Dolle, M., Rudenko, G. and Wagenaar, E. (1993). N-glycosylation and deletion mutants of the human MDR1 P-glycoprotein. *Journal of Biological Chemistry* **268,** 7474-81.

Schinkel, A. H., Mayer, U., Wagenaar, E., Mol, C. A., van Deemter, L., Smit, J. J., van der Valk, M. A., Voordouw, A. C., Spits, H., van Tellingen, O., Zijlmans, J. M., Fibbe, W. E. and Borst, P. (1997). Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 4028-33.

Schuetz, E. G., Schuetz, J. D., Grogan, W. M., Naray-Fejes-Toth, A., Fejes-Toth, G., Raucy, J., Guzelian, P., Gionela, K. and Watlington, C. O. (1992). Expression of cytochrome P450 3A in amphibian, rat, and human kidney. *Archives of Biochemistry and Biophysics* **294,** 206-14.

Schuetz, E. G., Schuetz, J. D., Strom, S. C., Thompson, M. T., Fisher, R. A., Molowa, D. T., Li, D. and Guzelian, P. S. (1993). Regulation of human liver cytochromes P-450 in family 3A in primary and continuous culture of human hepatocytes. *Hepatology* **18,** 1254-62.

Schuetz, J. D., Beach, D. L. and Guzelian, P. S. (1994). Selective expression of cytochrome P450 CYP3A mRNAs in embryonic and adult human liver. *Pharmacogenetics* **4,** 11-20.

Schuetz, J. D., Silverman, J. A., Thottassery, J. V., Furuya, K. N. and Schuetz, E. G. (1995). Divergent regulation of the class II P-glycoprotein gene in primary cultures of hepatocytes versus H35 hepatoma by glucocorticoids. *Cell Growth and Differentiation* **6,** 1321-32.

Schuetz, E. G., Schinkel, A. H., Relling, M. V. and Schuetz, J. D. (1996). P-glycoprotein: a major determinant of rifampicin-inducible expression of cytochrome P4503A in mice and humans. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 4001-5.

Shalinsky, D. R., Jekunen, A. P., Alcaraz, J. E., Christen, R. D., Kim, S., Khatibi, S. and Howell, S. B. (1993). Regulation of initial vinblastine influx by P-glycoprotein. *British Journal of Cancer* **67,** 30-6.

Shao, Y. M., Ayesh, S. and Stein, W. D. (1997). Mutually co-operative interactions between modulators of P-glycoprotein. *Biochimica et Biophysica Acta* **1360,** 30-8.

Shapiro, A. B. and Ling, V. (1995a). Reconstitution of drug transport by purified P-glycoprotein. *Journal of Biological Chemistry* **270,** 16167-75.

Shapiro, A. B. and Ling, V. (1995b). Using purified p-glycoprotein to understand multidrug resistance. *Journal of Bioenergetics and Biomembranes* **27,** 7-13.

Shen, H., Paul, S., Breuninger, L. M., Ciaccio, P. J., Laing, N. M., Helt, M., Tew, K. D. and Kruh, G. D. (1996). Cellular and in vitro transport of glutathione conjugates by MRP [retracted in Biochemistry 1997 Nov 11;36(45):13972]. *Biochemistry* **35,** 5719-25.

Shimada, T., Yamazaki, H., Mimura, M., Inui, Y. and Guengerich, F. P. (1994). Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics* **270,** 414-23.

Silverman, J. A. and Schrenk, D. (1997). Hepatic canalicular membrane 4: expression of the multidrug resistance genes in the liver. *FASEB Journal* **11,** 308-13.

Smit, J. J., Schinkel, A. H., Oude Elferink, R. P., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A., Ottenhoff, R., van der Lugt, N. M., van Roon, M. A., Offerhaus, G. J. A., Berns, A. J. M. and Borst, P. (1993). Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* **75,** 451-62.

Smith, A. J., Timmermans-Hereijgers, J. L., Roelofsen, B., Wirtz, K. W., van Blitterswijk, W. J., Smit, J. J., Schinkel, A. H. and Borst, P. (1994). The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. *FEBS Letters* **354,** 263-6.

Smith, D. A., Jones, B. C. and Walker, D. K. (1996). Design of drugs involving the concepts and theories of drug metabolism and pharmacokinetics. *Medicinal Research Reviews* **16,** 243-66.

Sobh, M., el-Agroudy, A., Moustafa, F., Harras, F., el-Bedewy, M. and Ghoneim, M. (1995). Coadministration of ketoconazole to cyclosporin-treated kidney transplant recipients: a prospective randomized study. *American Journal of Nephrology* **15,** 493-9.

Somogyi, A. (1996). Renal transport of drugs: specificity and molecular mechanisms. *Clinical and Experimental Pharmacology and Physiology* **23,** 986-9.

Stein, W. D., Cardarelli, C., Pastan, I. and Gottesman, M. M. (1994). Kinetic evidence suggesting that the multidrug transporter differentially handles influx and efflux of its substrates. *Molecular Pharmacology* **45,** 763-72.

Sugawara, I., Akiyama, S., Scheper, R. J. and Itoyama, S. (1997). Lung resistance protein (LRP) expression in human normal tissues in comparison with that of MDR1 and MRP. *Cancer Letters* **112,** 23-31.

Terao, T., Hisanaga, E., Sai, Y., Tamai, I. and Tsuji, A. (1996). Active secretion of drugs from the small intestinal epithelium in rats by P-glycoprotein functioning as an absorption barrier. *Journal of Pharmacy and Pharmacology* **48,** 1083-9.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I. and Willingham, M. C. (1987). Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National Academy of Sciences of the United States of America* **84,** 7735-8.

Thummel, K. E. and Wilkinson, G. R. (1998). In vitro and in vivo drug interactions involving human CYP3A. *Annual Review of Pharmacology and Toxicology* **38,** 389-430.

Ueda, K., Cardarelli, C., Gottesman, M. M. and Pastan, I. (1987). Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proceedings of the National Academy of Sciences of the United States of America* **84,** 3004-8.

Ullrich, K. J., Rumrich, G., David, C. and Fritzsch, G. (1993a). Bisubstrates: substances that interact with renal contraluminal organic anion and organic cation transport systems. I. Amines, piperidines, piperazines, azepines, pyridines, quinolines, imidazoles, thiazoles, guanidines and hydrazines. *European Journal of Physiology* **425,** 280-99.

Ullrich, K. J., Rumrich, G., David, C. and Fritzsch, G. (1993b). Bisubstrates: substances that interact with both, renal contraluminal organic anion and organic cation transport systems. II. Zwitterionic substrates: dipeptides, cephalosporins, quinolone-carboxylate gyrase inhibitors and phosphamide thiazine carboxylates; nonionizable substrates: steroid hormones and cyclophosphamides. *European Journal of Physiology* **425,** 300-12.

van Asperen, J., Mayer, U., van Tellingen, O. and Beijnen, J. H. (1997). The functional role of P-glycoprotein in the blood-brain barrier. *Journal of Pharmaceutical Sciences* **86,** 881-4.

van der Bliek, A. M., Kooiman, P. M., Schneider, C. and Borst, P. (1988). Sequence of mdr3 cDNA encoding a human P-glycoprotein. *Gene* **71,** 401-11.

van der Heyden, S., Gheuens, E., DeBruijn, E., Van Oosterom, A. and Maes, R. (1995). P-glycoprotein: clinical significance and methods of analysis. *Critical Reviews in Clinical Laboratory Sciences* **32,** 221-64.

van Veen, H. W. and Konings, W. N. (1997). Multidrug transporters from bacteria to man: similarities in structure and function. *Seminars in Cancer Biology* **8,** 183-91.

Venkataramanan, R., Swaminathan, A., Prasad, T., Jain, A., Zuckerman, S., Warty, V., McMichael, J., Lever, J., Burckart, G. and Starzl, T. (1995). Clinical pharmacokinetics of tacrolimus. *Clinical Pharmacokinetics* **29,** 404-30.

von Rosensteil, N. A. and Adam, D. (1995). Macrolide antibacterials. Drug interactions of clinical significance. *Drug Safety* **13,** 105-22.

Wacher, V. J., Wu, C. Y. and Benet, L. Z. (1995). Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: Implications for drug delivery and activity in cancer chemotherapy. *Molecular Carcinogenesis* **13,** 129-134.

Wacher, V. J., Silverman, J. A., Zhang, Y. and Benet, L. Z. (1998). Role of P-glycoprotein and cytochrome P450 3A in limiting oral absorption of peptides and peptidomimetics. *Journal of Pharmaceutical Sciences* **87,** 1322-30.

Woodhouse, J. R. and Ferry, D. R. (1995). The genetic basis of resistance to cancer chemotherapy. *Annals of Medicine* **27,** 157-67.

Woosley, R. L. (1996). Cardiac actions of antihistamines. *Annual Review of Pharmacology and Toxicology* **36,** 233-52.

Wrighton, S. A., Brian, W. R., Sari, M. A., Iwasaki, M., Guengerich, F. P., Raucy, J. L., Molowa, D. T. and Vandenbranden, M. (1990). Studies on the expression and

metabolic capabilities of human liver cytochrome P450IIIA5 (HLp3). *Molecular Pharmacology* **38,** 207-13.

Wrighton, S. A., Ring, B. J., Watkins, P. B. and VandenBranden, M. (1989). Identification of a polymorphically expressed member of the human cytochrome P-450III family. *Molecular Pharmacology* **36,** 97-105.

Yeates, R. A., Laufen, H. and Zimmermann, T. (1996). Interaction between midazolam and clarithromycin: comparison with azithromycin. *International Journal of Clinical Pharmacology and Therapeutics* **34,** 400-5.

Yun, C. H., Okerholm, R. A. and Guengerich, F. P. (1993). Oxidation of the antihistaminic drug terfenadine in human liver microsomes. Role of cytochrome P-450 3A(4) in N-dealkylation and C-hydroxylation. *Drug Metabolism and Disposition* **21,** 403-9.

Zhang, L., Brett, C. M. and Giacomini, K. M. (1998). Role of organic cation transporters in drug absorption and elimination. *Annual Review of Pharmacology and Toxicology* **38,** 431-60.

Zhao, J. Y., Ikeguchi, M., Eckersberg, T. and Kuo, M. T. (1993). Modulation of multidrug resistance gene expression by dexamethasone in cultured hepatoma cells. *Endocrinology* **133,** 521-8.

Zimmermann, T., Yeates, R. A., Laufen, H., Scharpf, F., Leitold, M. and Wildfeuer, A. (1996). Influence of the antibiotics erythromycin and azithromycin on the pharmacokinetics and pharmacodynamics of midazolam. *Arzneimittel-Forschung* **46,** 213-7.

Chapter 2

DIGOXIN AS A MODEL

2.1 Introduction

Digoxin is the most commonly used drug for congestive heart failure (CHF) (Smith, 1984b) and atrial fibrillation. In the United States digoxin is the cardiac glycoside used most frequently, owing to the flexibility of its route of administration, its intermediate duration of action, and the readily available techniques for assaying serum digoxin levels.

Digoxin is an inotropic agent that exerts its direct cardiac effects by binding to myocardial Na^+/K^+-ATPase (Smith, 1984a). In cells, there are Na^+ and Ca^{++} pumps that pump these ions out of the cells, and a Na^+/Ca^{++} anti-porter, which exchanges three Na^+ in for one Ca^{++} ion out. Digoxin inhibits Na^+/K^+-ATPase activity thereby raising intracellular Na^+ concentrations that in turn lead to increased free intracellular Ca^{++} concentrations, which result in enhanced myocardial contractility. Digoxin shortens the duration of the action potential by shortening atrial and ventricular refractoriness and by

increasing AV nodal refractoriness. The effects of digoxin on ECG are PR interval increase, ST segment depression, QT interval decrease and T wave inversion (Katzung, 1998).

Adverse effects are most common in the heart (70-95 % of total occurrences), the GI tract (50-75 % of total occurrences), and the central nervous system (infrequent and less worrisome) (Smith *et al.*, 1984a, b and c). The most serious adverse effects are premature ventricular contractions and various degrees of atrio-ventricular block. Nausea, vomiting and diarrhea, often accompanied by abdominal discomfort and pain, are the common adverse effects associated with the GI tract. Central nervous system toxicity includes headache, fatigue, malaise, neurologic pain, confusion, and seizures. Visual symptoms and disturbed color vision are also frequent complaints in patients with digitalis toxicity.

Digoxin was isolated initially from the leaves of *Digitalis lanata*, introduced to clinical use in 1934, but its chemical structure was not finalized until 1953 (Reuning and Geraets, 1986). The digoxin chemical structure has both lipophilic (steroid) and hydrophilic (sugar) parts as shown in Figure 2-1. Digoxin lacks an easily ionizable group, and therefore solubility is not pH dependent. It is very stable if protected from strong light and low pH. The SAR indicates that the lactone and steroid moieties are essential for activity, while the sugars influence pharmacokinetic parameters including absorption, $t_{1/2}$ and metabolism (Katzung, 1998). The pharmacokinetics of digoxin is considered to be linear within the therapeutic dosage range.

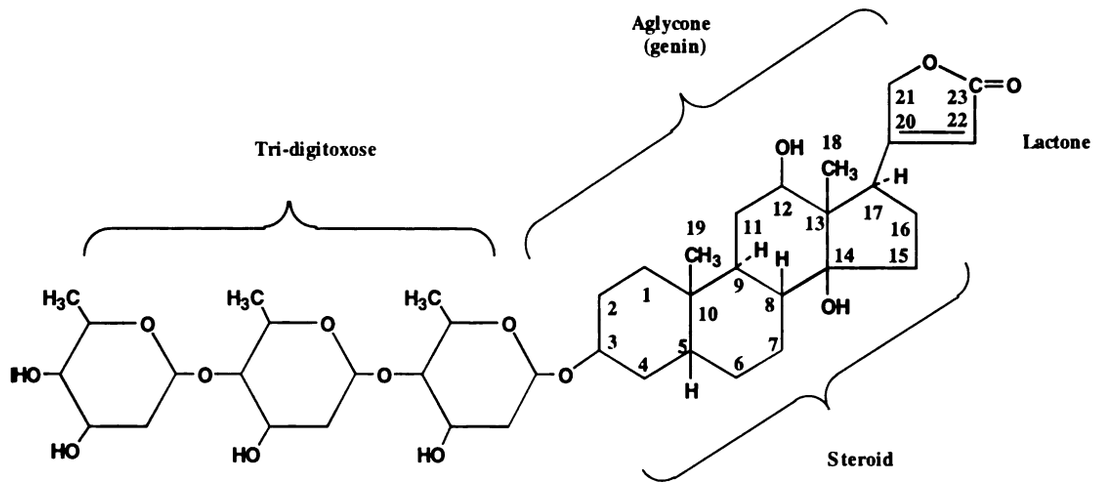


Figure 2-1. Chemical structure of digoxin.

2.2 Digoxin pharmacokinetics

2.2.1 *Absorption*

The intestinal absorption of digoxin has previously been thought to be a passive, non-saturable transport process (Beermann *et al.*, 1972; Caldwell *et al.*, 1969) that is incomplete and highly variable between individuals. No significant first-pass effect associated with liver metabolism for digoxin has been characterized. The current literature summarizes the average systemic availability to be: conventional tablets 70 %, elixir 80 % and capsules 85 % (Brown, 1984; Kholodov *et al.*, 1980). A normal breakfast does not appear to influence the extent of bioavailability although a meal may delay digoxin absorption (White *et al.*, 1971).

2.2.2 *Distribution*

Digoxin disposition following intravenous injection can be described mathematically as an tri-exponential serum concentration-time curve or according to a three-compartment pharmacokinetic model (Kramer *et al.*, 1974). Only a small fraction of digoxin in the body is present in blood, that is, it exhibits a large volume of distribution. In the dog, 50 % of total amount of digoxin radioactivity in the body was in the skeletal muscle because of large mass, followed by kidney, liver, small intestine and heart (Harrison *et al.*, 1966).

Age influences digoxin distribution (Gorodischer *et al.*, 1976; Wettrell, 1977). Obesity does not alter digoxin distribution appreciably in humans, since digoxin distributes little into body fat (Ewy *et al.*, 1971). The volume of distribution of digoxin decreases in chronic renal failure (Koup *et al.*, 1975b) due to decreased binding of digoxin to tissues.

2.2.3 Protein binding

Only the unbound drug is active, but protein binding of digoxin is a minor pharmacokinetic factor. The binding of digoxin to plasma proteins is independent of concentration over a very wide range and averages 25 % in normal humans (Hinderling, 1984). The important binding protein is albumin (Evered, 1972). The concentration ratio (cells: plasma) is approximately 0.9 (Abshagen *et al.*, 1971).

2.2.4 Metabolism

Hepatic biotransformation of digoxin is not extensive. Digoxin metabolism may occur by two pathways as described in Figure 2-2 (Reuning and Geraets, 1986). One pathway consists of sequential hydrolysis of the digitoxose sugar moieties attached to the 3-position of the steroid nucleus, which may occur in the acidic stomach environment

(Gault *et al.*, 1980 and 1981; Magnusson, 1983) to form digoxigenin bis-digitoxoside (Dg2), digoxigenin mono-digitoxoside (Dg1) and digoxigenin (Dg0) (Magnusson *et al.*, 1982). The second pathway is the reduction of the double bond in the lactone ring of digoxin by intestinal bacteria called *Eubacterium lentum* (Dobkin *et al.*, 1982), to form dihydro-digoxin.

Hydrolysis products usually account for less than 15% of total urinary excretion (Gault *et al.*, 1979; Magnusson *et al.*, 1982), but the dihydro pathway in 10% of people accounts for more than 40% of total urinary elimination (Lindenbaum *et al.*, 1981a and b). Dg2 and Dg1 have cardio-activity at least comparable to that of digoxin, Dg0 is considerably less active and, 3-keto digoxigenin and the dihydro metabolites are nearly inactive (Lage and Spratt, 1966).

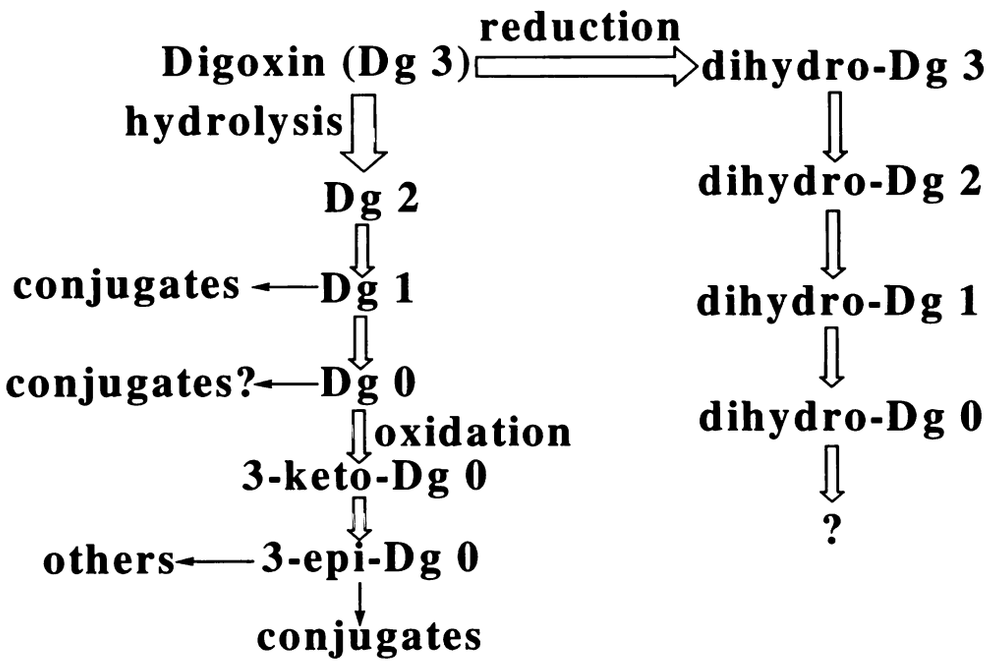


Figure 2-2. The metabolic pathways of digoxin.

Although the GI tract plays a key role in both major metabolic pathways, it is likely that liver and possibly other organs also contribute. Dg1 (Kuhlmann *et al.*, 1974) may be further metabolized by conjugation in the liver in man (Abshagen *et al.*, 1974). Further metabolism of Dg0 is mainly by oxidation to 3-keto-digoxigenin, which can subsequently be reduced to 3-α-digoxigenin (3-epi-digoxigenin) and conjugated, all of which probably occurs predominately in the liver (Schmoldt and Ahsendorf, 1980; Schmoldt and Promies, 1982; Talcott *et al.*, 1972).

2.2.5 Excretion

Irrespective of the route of administration, digoxin appears to be excreted by first order processes, with an average half-life of 39 hours in healthy individuals with normal renal function. Non-renal excretion of digoxin and/or digoxin metabolites includes hepatic metabolism, biliary excretion, possible intestinal secretion, and subsequent fecal elimination of digoxin and/or metabolites that are not absorbed. The total fecal excretion of radioactivity averaged 11 % after an *iv* dose (Doherty, 1968) and 20% after a *p.o.* dose.

Digoxin is predominately eliminated in the urine as unchanged drug. Renal clearance accounts for most of total body clearance for digoxin, and exceeds creatinine and inulin clearances (Koup *et al.*, 1975a; Steiness, 1974b). An enhanced renal excretory function of digoxin in the young appears to be due to an enhanced tubular secretory component (Gorodischer *et al.*, 1977). Since the renal clearance of digoxin has a significant secretory component (Steiness, 1974b), the efficiency of renal excretion may be influenced by interacting factors.

Digoxin is sold under the brand names Lanoxicaps or Lanoxin, and dosage forms include elixir (pediatric), capsules, injection and tablets (Steiness, 1974a). Bioavailability

varies among the dosage forms (Steiness *et al.*, 1974). Digoxin kinetic parameters can be summarized as given (Lewis, 1992; Reuning and Geraets, 1986) in Table 2-1.

Table 2-1. Pharmacokinetic parameters of digoxin.

Usual daily p.o. dose (mg)	0.25 - 0.5
T_{peak} (hr)	1.5 - 5
F (%)	70 (tablets); 85 (capsules); 80 (elixir)
f_u (%)	75
Compartment model	Multi-compartment
V_{ss} (L/kg)	6.7
CL (ml/min/kg)	2.7 (CL_r 1.86, CL_{nr} 0.82)
$t_{1/2}$ (hr)	39
Therapeutic concentration (ng/ml)	0.8 - 2
Excreted unchanged in urine (%)	72 (*iv*), 54 (*p.o.*)

Some physiological and pathological factors that have influence on digoxin pharmacokinetics are summarized in Table 2-2.

Among patients overlap exists between effective (0.8 to 2.0 ng/ml) and toxic ranges (>1.6 ng/ml) of digoxin serum concentrations (Evered and Chapman, 1971; Smith and Haber, 1970). The most appropriate time interval of blood sampling for therapeutic drug monitoring is 12-24 hours, while samples obtained prior to 8 hours during the distribution phase can be very misleading as an indicator of response (Kramer *et al.*, 1979). Age is a factor influencing the response to digoxin. Increased dosage requirement in infants and children is due to a larger V/kg in the newborn (Wettrell, 1977; Wettrell and Andersson, 1977) and a decreased sensitivity to digoxin, which may be due to

differences in the number and affinity of the receptors that bind digoxin (Kearin *et al.*, 1980).

Table 2-2. Physiological and pathological factors that influence digoxin kinetics.

Meal	C_{peak} decreased, T_{peak} increased (Greenblatt *et al.*, 1974)
Milk	may be taken with milk?
Alcohol	no interactions with alcohol
Age	decreased distribution (Lewis, 1992); decreased elimination (Lewis, 1992; Wettrell, 1977)
Obesity	no effect (Ewy *et al.*, 1971)
Pregnancy	increased distribution (Lewis, 1992)
Physical activity	increased distribution (Joreteg and Jogestrand, 1983)
CHF	Sheiner equation-- $CL = CL_{cr} + 0.8$ ml/min/kg no CHF $CL = 0.9\ CL_{cr} + 0.33$ ml/min/kg with CHF
Renal failure	decreased elimination (Lewis, 1992)-- decreased urinary excretion and increased fecal excretion decreased distribution (Lewis, 1992)-- f_{uT} increased, V (L/kg) = $3.8 + 3.1\ CL_{cr}$ (ml/min/kg)

2.3 Digoxin assay methods

Many analytic methods have been used to assay digoxin concentrations both in the clinic and in research, as presented in Table 2-3.

Table 2-3. Assay methods of digoxin.

Methods	Specificity	Sensitivity (ng/ml)	Metabolite analysis	Plasma or serum analysis	Urine analysis	Minimum volume (ml)	Comments
RIA	Poor	0.2-0.4	No	Yes	Yes	0.1	Metabolites and endogenous
Enzyme immunoassay	Poor	0.3-0.5	No	Yes	Yes	0.1~0.2	Metabolites and endogenous
Fluorescence polarization immunoassay	Poor	0.2	No	Yes	?	0.2	Metabolites and endogenous
Derivative HPLC	Excellent	≥10	Yes	No	Yes	1-10	Sensitivity inadequate for serum
HPLC-RIA	Excellent	~0.1	Some	Yes	Yes	1	Some metabolites not immuno-logically active
MEIA	Poor	0.3-4.0	No	Yes	No	0.03	Metabolites and endogenous
LC/MS/MS	Excellent	0.1~0.2	Yes	Yes	Yes	0.5~1	Specific for metabolites and endogenous; Sensitivity adequate for plasma, serum and urine samples

2.3.1 *Radio-immunoassay (RIA)*

RIA (Evered *et al.*, 1970; Smith *et al.*, 1969) is a method in which digoxin in the sample competes with a radio-labeled digoxin (or digoxin derivative) tracer for binding sites on the antibody to digoxin. The unbound digoxin (labeled and unlabeled) is then separated from the bound and quantitated by radioactivity counting. The sensitivity is 0.2-0.4 ng/ml. Dg2 and Dg1 cross-react equally to Dg3, but Dg0 is much less cross-reactive (Stoll *et al.*, 1972). The cross-reactivity is of clinical significance since the digoxin antibody will measure the relatively inactive dihydro-digoxin (Kramer *et al.*, 1976 and 1978).

2.3.2 *Enzyme-immunoassay (EIA)*

In the EIA, digoxin-enzyme (digoxin chemically bonded to an enzyme) competes with digoxin in the sample for digoxin-antibody binding sites. Then free or antibody-bound digoxin-enzyme reacts with an excess enzyme substrate to produce an UV chromophore that is measured spectrophotometrically. EIA has similar precision and sensitivity to RIA, but EIA is technically easier and requires less investment in equipment. Inactive dihydro-digoxin did not cross-react with digoxin and its active metabolites (Linday and Drayer, 1983).

2.3.3 *Fluorescence polarization immunoassay*

The fluorescence polarization immunoassay method uses a fluorescein-labeled digoxin tracer to compete with digoxin in the sample for antibody binding sites. A single-wavelength, polarized light excites the unbound and antibody-bound fluorescent label; quantitation is based on the exhibited widely different degrees of polarization of emission

fluorescence. A high cross reactivity exists for all of the hydrolyzed metabolites. Little cross-reactivity was found for dihydro-digoxin.

2.3.4 High performance liquid chromatography

Because of the lack of detectable functional groups on the digoxin molecule, chromatography cannot analyze digoxin concentrations, unless coupled with RIA (Morais *et al.*, 1981) or liquid scintillation (Eriksson *et al.*, 1981) or chemical derivatization (Nachtmann *et al.*, 1976a) using UV-absorbing (Nachtmann *et al.*, 1976b) or fluorescent derivatives. Even though the coupled methods can assay digoxin and its metabolites and, analyze these substances in urine, tissues and serum, the methods are labor-intensive and not feasible for routine clinical use.

2.3.5 Micro-particle enzyme immuno-assay (MEIA)

The MEIA method can not distinguish between digoxin and its active metabolites. When parent digoxin concentrations were below assay sensitivity in patients, Dg2, Dg1 and Dg0 tested at concentrations of 0.22, 0.088 and 0.132 ng/ml, respectively. The assay was calibrated for concentrations from 0.3 to 5 ng/ml.

2.3.6 LC/MS/MS

The LC/MS/MS method has recently been recognized to have more advantages as demonstrated in Table 2-3. It is a new technology of high sensitivity and specificity, allowing fast determination of both serum and urine concentrations of digoxin. This method has a sensitivity of 0.1-0.2 ng/ml.

2.4 P-glycoprotein and digoxin

Many studies have be done to show that Pgp plays a significant role in digoxin absorption, distribution and disposition.

2.4.1 *In vitro studies*

Several *in vitro* cell culture studies have shown that digoxin is a Pgp substrate. Digoxin B to A transport in LLC-PK1 cells was saturable, with a V_{max} = 184.5 ± 38.0 pmol/cm^2/h and K_m = 14.1 ± 1.6 μM. Metabolic inhibitors and Pgp inhibitors, such as quinidine, verapamil, vincristine and cyclosporine, reduced B to A flux and conversely increased the A to B flux of digoxin without affecting the non-specific flux significantly (Ito *et al.*, 1993).

Digoxin secretion by intestinal epithelium is likely to involve both diffusional uptake and Na^+/K^+ pump-mediated endocytosis, followed by active extrusion at the apical membrane (Cavet *et al.*, 1996). But digoxin did not compete with azidopine (which binds preferentially to Pgp) for binding in rat BBM or membranes prepared from the multidrug resistant CHO cell line (de Lannoy *et al.*, 1992). Verapamil, nifedipine and vinblastine all abolished net secretion of digoxin. Furthermore, de Lannoy and Silverman (1992) showed that accumulation of digoxin was 3 to 5-fold greater in drug sensitive parent cells than in multidrug resistant CHO cells, which were 180 times more resistant to colchicine relative to drug sensitive parent cells, suggesting transport of digoxin is mediated by Pgp.

Studies in LLC-PK1 cells transfected with the human MDR1 cDNA (Tanigawara *et al.*, 1992), in which transfected cells exhibited markedly greater B to A transport and less A to B transport than the host cells (8 fold difference), showed that digoxin was transported by Pgp and digoxin oriented transport was inhibited by vinblastine, quinidine and verapamil. Studies in LLC-PK1, L-MDR1, L-mdr1a cell lines showed that mouse

mdr1a and human MDR1 Pgp actively transport digoxin and cyclosporine A (Schinkel *et al.*, 1995).

Digoxin-drug interactions have also been studied *in vitro*. Uptake of digoxin by BBM and binding of digoxin to ALM was not affected by quinidine and verapamil (Koren *et al.*, 1986). In dog studies, uptake of digoxin by BBM and binding kinetics of digoxin to Na^+/K^+-ATPase were not affected by quinidine (Koren *et al.*, 1988). Inhibition of Pgp-mediated drug transport has been shown to be an unifying mechanism to explain the interaction between digoxin and quinidine (Fromm *et al.*, 1999).

Propafenone significantly inhibited the secretory flux of digoxin across confluent MDCK cells, but cellular digoxin accumulation did not decrease, suggesting propafenone did not prohibit digoxin from entering the cells at the basolateral side (Woodland *et al.*, 1997). In LLC-PK1 and MDR1-LLC-PK1 cells, CsA inhibited digoxin transepithelial transport mediated by human Pgp (Okamura *et al.*, 1993).

2.4.2 In situ animal studies

Early *in vivo* multiple indicator dilution results showed that two steps were involved in digoxin secretion: sequestration from the postglomerular circulation and trans-tubular secretion (Koren *et al.*, 1986). By using this technique, renal-artery infusion of quinidine did not affect the recovery of digoxin in the renal vein or urine. It was concluded that quinidine inhibits renal clearance of digoxin not by competition at the tubular cell membrane level, but rather by decreasing renal blood flow. A similar mechanism was suggested to exist for biliary clearance of digoxin (Koren *et al.*, 1988).

However, it is only recently that Pgp emerged as the possible site of the pharmacokinetic interaction between digoxin and quinidine. Su *et al.* (1996) studied

digoxin exsorption and absorption using everted sac and single-pass perfusion, and found that quinidine may affect digoxin elimination, as well as digoxin absorption/exsorption in the gastrointestinal tract. This study (Su and Huang, 1996) indicated that Pgp is involved in the drug interaction between digoxin and quinidine in the small intestine.

Using isolated perfused rat kidney, digoxin was administered *iv* into the renal artery together with inulin and Evans blue-albumin. The ratio of fractional excretion to filtration for digoxin was 2.4 ± 0.4, indicating involvement of tubular secretion. Quinidine, verapamil, vinblastine, daunorubicin and 2,4-dinitrophenol markedly inhibited digoxin secretion (Hori *et al.*, 1993). CsA also reduced renal tubular secretion of digoxin by isolated perfused rat kidney, but digoxin did not affect CsA transport by Pgp (Okamura *et al.*, 1993).

de Lannoy *et al.* (1992) studied the *in vivo* luminal and contra-luminal uptake of digoxin in dog kidney using single-pass multiple indicator dilution method. Uptake of digoxin across basolateral membrane was large and non-saturable, and the urine recovery ratio indicated net digoxin secretion. CsA and quinidine decreased digoxin urinary recovery (de Lannoy *et al.*, 1992).

2.4.3 In vivo animal studies

Knockout mice studies further demonstrated the determinant role of Pgp in digoxin distribution and disposition. Schinkel *et al.* (1997) found no physiological abnormalities in either mdr1a (-/-) or mdr1a/1b (-/-) mice. The high level of mdr1b Pgp normally present in the pregnant uterus did not protect fetuses from digoxin in the bloodstream of the mother. Pharmacologically, mdr1a/1b (-/-) behaved similarly to

mdr1a(-/-) mice, displaying increased brain penetration and reduced elimination of digoxin (Schinkel *et al.*, 1997).

For digoxin, effects of Pgp on the bioavailability and secretion into the intestine were observed when wild-type were compared to knock-out mice (Mayer *et al.*, 1996; Sparreboom *et al.*, 1997). Predominant fecal excretion of digoxin in wild-type mice shifted towards predominantly urinary excretion in mdr1a(-/-) mice. Approximately 16% of administered digoxin was excreted via the gut mucosa in wild-type mice, while only 2% in mdr1a(-/-) mice after gallbladder cannulation in both sets of animals. Biliary excretion of digoxin was not markedly decreased (24% in wild-type mice *vs.* 16% in mdr1a (-/-) mice) (Mayer *et al.*, 1996). However, mice homozygous for mdr1a/1b gene disruption (Schinkel *et al.*, 1997) suggested that another mechanism of digoxin biliary excretion exists in the liver.

Brain accumulation of digoxin (van Asperen *et al.*, 1996) was also significant in knock-out mice. The mouse mdr1a Pgp is abundant in the blood-brain barrier, and its absence in mdr1a (-/-) mice leads to highly increased levels of digoxin in the brain (Schinkel *et al.*, 1996). Injection of radiolabeled drugs in mdr1a (-/-) and wild-type mice resulted in markedly (20 to 50 fold) higher levels of radioactivity in mdr1a (-/-) mice brains for digoxin. Digoxin was also more slowly eliminated from mdr1a(-/-) mice (Schinkel *et al.*, 1995).

PSC833 can inhibit blood-brain barrier Pgp extensively, and intestinal Pgp completely. Hepatobiliary excretion of digoxin was markedly decreased in both wild-type and mdr1a/1b (-/-) mice by PSC833, indicating PSC833 not only inhibits mdr1-type Pgp, but also other drug transporters (Mayer *et al.*, 1997).

2.4.4 *Human studies*

Clinically digoxin interacts with many drugs (Rodin and Johnson, 1988). Generally, when digoxin is administered with one of these drugs, digoxin C_p, AUC, F and $t_{1/2}$ increase, and CL_{nr} and CL_r decrease. The pharmacokinetic interaction between digoxin and quinidine is characterized by an increased digoxin plasma concentration, a reduced digoxin clearance with no change in renal function (Bussey, 1982). Quinidine does not affect equilibrium binding of digoxin (Colvin *et al.*, 1990), while it reduces biliary clearance of digoxin in man (Angelin *et al.*, 1987).

Seven cases of digoxin-itraconazole interactions (Table 2-4) have been reported (Alderman and Allcroft, 1997; Alderman and Jersmann, 1993; Cone *et al.*, 1996; Kauffman and Bagnasco, 1992; McClean and Sheehan, 1994; Meyboom *et al.*, 1994; Rex, 1992; Sachs *et al.*, 1993). However, in two cases, therapy (in the same patients exhibiting an itraconazole interaction) with ketoconazole (KCZ) (Rex, 1992) or miconazole (MCZ) (Alderman and Allcroft, 1997) did not produce side effects in the patient.

Two clinical studies have been conducted to investigate the digoxin-itraconazole interactions. In one study, volunteers, receiving 0.25 mg of oral digoxin once daily for 20 days, were randomized to receive concomitantly either 200 mg itraconazole or placebo orally for the first 10 days. Over the next 10 days, itraconazole was changed to placebo and *vice versa*. On day 10 after dosing itraconazole, digoxin concentrations at 12 hr in each subject increased on average from 1.0 ± 0.1 nmol/L (placebo phase) to 1.8 ± 0.1 nmol/L (itraconazole phase) (Partanen *et al.*, 1996).

Table 2-4. Cases of digoxin-itraconazole interactions.

Cases	Digoxin dose (mg/day)	Itraconazole dose (mg/day)	Initial digoxin conc. (µg/L)	End digoxin conc. (µg/L)	Change in digoxin conc. (µg/L) (interval)
Alderman and Jersmann (1993)	0.25	200	1.6	4.2	2.6 (9 days)
McClean and Sheehan (1994)	0.25	400	1.4	5.6	4.2 (4 months, 7days)
Kauffman and Bagnasco (1992)	0.25	400	1.4	2.4	1.0 (22 days)
Sachs *et al.* (1993)	0.125	100→ 400	1.2	4.6	3.4 (34 days)
Rex (1992)[a]	0.25 (*b.i.d.*)	400	1.6	3.2	1.6 (7 days)
Alderman and Allcroft (1997)[b]	0.187	200	0.7	1.25	0.55 (14 days)

[a] No toxicity with ketoconazole 200 mg *b.i.d.*

[b] No toxicity with miconazole.

In another double-blind, randomized, two-phase crossover study, healthy volunteers received either 200 mg itraconazole or placebo orally once daily for 5 days. On day 3, each volunteer ingested a single oral dose of digoxin (0.5 mg). Itraconazole increased digoxin $AUC_{0\rightarrow72}$ by 50%, decreased Cl_r by 20%, but did not increase C_{max}, while $t_{1/2}$ increased significantly (Jalava *et al.*, 1997).

2.4.5 Hypothesis by others

Many drugs interact with digoxin at the tubular level, such as verapamil (Belz *et al.*, 1983), propafenone (Belz *et al.*, 1983; Calvo *et al.*, 1989) and itraconazole (Koren *et al.*, 1998). Many drugs that interact with digoxin at the tubular level exhibit Pgp inhibition *in vitro* in resistant tumor cells, *e.g.* quinidine (Tsuruo *et al.*, 1984), amiodarone (van der Graaf *et al.*, 1991), propafenone (Woodland *et al.*, 1997) and verapamil (Burton *et al.*, 1993; Hunter *et al.*, 1993a and b; Yusa and Tsuruo, 1989).

At concentrations that have previously been safely used *in vivo* for treatment of fungal infection, KCZ was found *in vitro* to overcome resistance to vinblastine and doxorubicin (Siegsmund *et al.*, 1994). The results that ICZ reversed adriamycin resistance at dosage compatible to the plasma levels achieved by the therapeutic dosages used for fungal infections, suggested involvement of ICZ in MDR gene and/or MRP gene associated resistance (Kurosawa *et al.*, 1996).

Both ketoconazole and itraconazole have been shown to inhibit digoxin transport across MDCK cells (Ito *et al.*, 1994 and 1995), while fluconazole has little effect (Ito and Koren, 1997). The observed digoxin-itraconazole interactions are very likely, at least in part, caused by inhibition of digoxin secretory transport in the body by itraconazole, although Pgp may not be the sole factor. It has been proposed that Pgp plays an important

role in clinically relevant digoxin-drug interactions, such as digoxin-itraconazole interactions (Koren *et al.*, 1998).

2.5 Our hypothesis, objectives and specific aims

Recently, a novel multi-specific organic anion transporting polypeptide (oatp2), homologous to other members of the oatp gene family of membrane transporters, has been isolated from rat brain (Noe *et al.*, 1997) and was found to mediate high-affinity uptake of digoxin (K_m = 0.24 μM) in functional expression studies in oocytes. On the basis of Northern blot analysis, oatp2 is highly expressed in brain, liver, and kidney but not in heart, spleen, lung, skeletal muscle, and testes. This indicates that oatp2 may play an especially important role in the brain accumulation and toxicity of digoxin and in the hepatobiliary and renal excretion of cardiac glycosides from the body.

After administration of indomethacin to an infant on digoxin, a therapeutic digoxin dose resulted in toxic serum digoxin concentrations and C_{cr} rose accordingly (Haig and Brookfield, 1992). Administration of indomethacin to patients on chronic digoxin treatment also increased serum digoxin concentrations (Jørgensen *et al.*, 1991). Indomethacin has been shown to decrease vincristine accumulation and increased susceptibility of both murine and human cell lines over-expressing MRP, but not Pgp (Kobayashi *et al.*, 1997).

It has been demonstrated that vinblastine, an anticancer drug which was previously believed to be a typical Pgp substrate, can be transported to the apical side of cell monolayers by cMOAT (Evers *et al.*, 1998). These results, along with the fact that some drugs not interacting clinically with digoxin are also inhibitors of Pgp, *e.g.*

ketoconazole (Siegsmund *et al.*, 1994), provide further support for the substrate specificity may overlap between substrates of Pgp and MRP transporters.

The hypothesis of this thesis is:

The locations of MRP1 and cMOAT (Figure 2-3), such as liver, kidney and intestine, suggest that these MRP transporters, like Pgp, may play roles in digoxin absorption, distribution and disposition, as well as various digoxin-drug interactions.

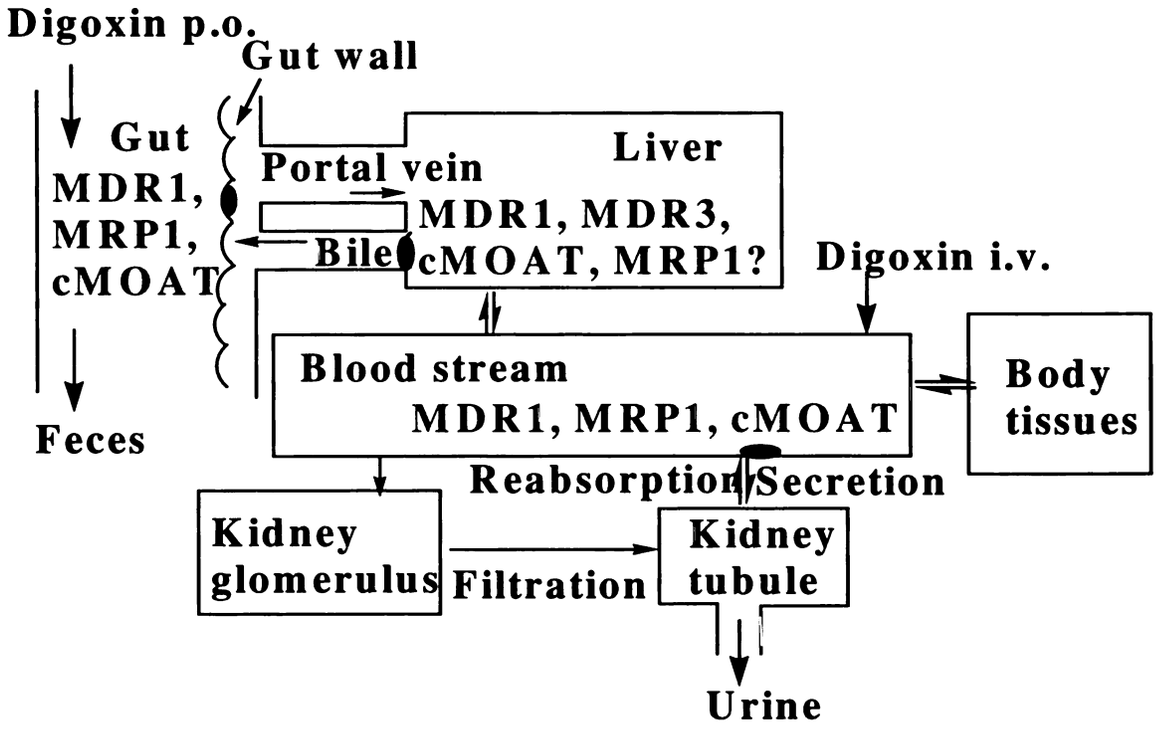


Figure 2-3. The potential roles of MDR and MRP transporters in the body.

Azole anti-fungal agents are potent inhibitors of CYP3A4, *e.g.* KCZ (Bourrié *et al.*, 1996; Gibbs *et al.*, 1999; Lampen *et al.*, 1995; Newton *et al.*, 1995; von Moltke *et al.*, 1996; Wrighton and Ring, 1994), ICZ (von Moltke *et al.*, 1996), MCZ (Maurice *et al.*, 1992; Pichard *et al.*, 1991) and FCZ (Gibbs *et al.*, 1999; Kunze *et al.*, 1996; Maurice *et*

al., 1992; von Moltke *et al.*, 1996). KCZ has a significantly greater *in vitro* inhibitory effect than ICZ and FCZ on CYP3A4 (Como and Dismukes, 1994).

Substrate specificity strongly overlaps between CYP3A and Pgp substrates and modulators (Wacher *et al.*, 1995). But in humans, Dg3 has been reported to not be extensively metabolized but mostly excreted unchanged by the kidney (Rodin and Johnson, 1988). Because the stepwise cleavage of Dg3 was not cytochrome P450 dependent in human hepatocytes and liver microsomes (Lacarelle *et al.*, 1991), digoxin might serve as a selective Pgp probe to differentiate the contributions of each protein on the absorption and disposition of a drug.

The field of drug transport in pharmacokinetics is still in its infancy. It will be very important to characterize other transporter(s), such as MRP1 and cMOAT, in transport of digoxin *in vitro*, and the potential role of these transporters in digoxin-drug interactions.

The objectives of this thesis are:

1. To characterize transporter(s) which may be responsible for digoxin disposition;
2. To determine the digoxin metabolism and pharmacokinetics in rats and humans;
3. To understand the pharmacokinetic mechanisms of clinical digoxin-drug interactions.

The specific aims of this thesis are:

1. To characterize digoxin metabolism in human and rat liver microsomes.
2. To characterize transporter(s) for net secretion and/or absorption of digoxin in cell culture systems.

3. To characterize transporter(s) of the drugs known to have clinical pharmacokinetic interactions with digoxin, and to determine the effects of these drugs on digoxin transport.
4. To determine the pharmacokinetics of digoxin alone and with ketoconazole in rats.
5. To determine the clinical pharmacokinetics of digoxin alone and co-administered with ketoconazole/itraconazole.

A clinical pharmacokinetic study of digoxin alone or with azoles is planned to separate out the transporter *versus* CYP3A effects on digoxin pharmacokinetic and drug interactions. This study will not only be able to determine the pharmacokinetic mechanisms of digoxin-itraconazole interactions *in vivo*, but also help us predict and prevent clinical digoxin-drug interactions. Using digoxin as a model, the results from this study will provide us with useful information for understanding the importance of secretory transport process in digoxin pharmacokinetics and interactions.

2.6 References

Abshagen, U., Kewitz, H. and Rietbrock, N. (1971). Distribution of digoxin, digitoxin and ouabain between plasma and erythrocytes in various species. *Naunyn-Schmiedebergs Archives of Pharmacology* **270,** 105-16.

Abshagen, U., Rennekamp, H., Küchler, R. and Rietbrock, N. (1974). Formation and disposition of bis- and mono-glycosides after administration of ^{3}H-4'-methyldigoxin to man. *European Journal of Clinical Pharmacology* **7,** 177-81.

Alderman, C. P. and Allcroft, P. D. (1997). Digoxin-itraconazole interaction: possible mechanisms [see comments]. *Annals of Pharmacotherapy* **31,** 438-40.

Alderman, C. P. and Jersmann, H. P. (1993). Digoxin-itraconazole interaction [letter]. *Medical Journal of Australia* **159,** 838-9.

Angelin, B., Arvidsson, A., Dahlqvist, R., Hedman, A. and Schenck-Gustafsson, K. (1987). Quinidine reduces biliary clearance of digoxin in man. *European Journal of Clinical Investigation* **17,** 262-5.

Beermann, B., Hellström, K. and Rosén, A. (1972). The absorption of orally administered (12 - ^{3}H)digoxin in man. *Clinical Science* **43,** 507-18.

Belz, G. G., Doering, W., Munkes, R. and Matthews, J. (1983). Interaction between digoxin and calcium antagonists and antiarrhythmic drugs. *Clinical Pharmacology and Therapeutics* **33,** 410-7.

Bourrié, M., Meunier, V., Berger, Y. and Fabre, G. (1996). Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalyzed by human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics* **277,** 321-32.

Brown, D. (1984). Lanoxicaps: a new digoxin formulation with improved bioavailability. *Iowa Medicine* **74,** 303-4.

Burton, P. S., Conradi, R. A., Hilgers, A. R. and Ho, N. F. (1993). Evidence for a polarized efflux system for peptides in the apical membrane of Caco-2 cells. *Biochemical and Biophysical Research Communications* **190,** 760-6.

Bussey, H. I. (1982). The influence of quinidine and other agents on digitalis glycosides. *American Heart Journal* **104,** 289-302.

Caldwell, J. H., Martin, J. F., Dutta, S. and Greenberger, N. J. (1969). Intestinal absorption of digoxin-3H in the rat. *American Journal of Physiology* **217,** 1747-51.

Calvo, M. V., Martin-Suarez, A., Martin Luengo, C., Avila, C., Cascon, M. and Domínguez-Gil Hurlé, A. (1989). Interaction between digoxin and propafenone. *Therapeutic Drug Monitoring* **11,** 10-5.

Cavet, M. E., West, M. and Simmons, N. L. (1996). Transport and epithelial secretion of the cardiac glycoside, digoxin, by human intestinal epithelial (Caco-2) cells. *British Journal of Pharmacology* **118,** 1389-96.

Colvin, R. A., Ashavaid, T. F., Katz, A. M. and Messineo, F. C. (1990). Amiodarone, verapamil, and quinidine do not affect equilibrium binding of digoxin. *Journal of Cardiovascular Pharmacology* **16,** 519-22.

Como, J. A. and Dismukes, W. E. (1994). Oral azole drugs as systemic antifungal therapy [see comments]. *New England Journal of Medicine* **330,** 263-72.

Cone, L. A., Himelman, R. B., Hirschberg, J. N. and Hutcheson, J. W. (1996). Itraconazole-related amaurosis and vomiting due to digoxin toxicity [letter]. *Western Journal of Medicine* **165,** 322.

de Lannoy, I. A. and Silverman, M. (1992). The MDR1 gene product, P-glycoprotein, mediates the transport of the cardiac glycoside, digoxin. *Biochemical and Biophysical Research Communications* **189,** 551-7.

de Lannoy, I. A., Koren, G., Klein, J., Charuk, J. and Silverman, M. (1992). Cyclosporin and quinidine inhibition of renal digoxin excretion: evidence for luminal secretion of digoxin. *American Journal of Physiology* **263,** F613-22.

Dobkin, J. F., Saha, J. R., Butler, V. P., Jr., Neu, H. C. and Lindenbaum, J. (1982). Inactivation of digoxin by *Eubacterium lentum*, an anaerobe of the human gut flora. *Transactions of the Association of American Physicians* **95,** 22-9.

Doherty, J. E. (1968). The clinical pharmacology of digitalis glycosides: a review. *American Journal of the Medical Sciences* **255,** 382-414.

Eriksson, B. M., Tekenbergs, L., Magnusson, J. O. and Molin, L. (1981). Determination of tritiated digoxin and metabolites in urine by liquid chromatography. *Journal of Chromatography* **223,** 401-8.

Evered, D. C. (1972). The binding of digoxin by the serum proteins. *European Journal of Pharmacology* **18,** 236-44.

Evered, D. C. and Chapman, C. (1971). Plasma digoxin concentrations and digoxin toxicity in hospital patients. *British Heart Journal* **33,** 540-5.

Evered, D. C., Chapman, C. and Hayter, C. J. (1970). Measurement of plasma digoxin concentration by radioimmunoassay. *British Medical Journal* **3,** 427-8.

Evers, R., Kool, M., van Deemter, L., Janssen, H., Calafat, J., Oomen, L. C., Paulusma, C. C., Oude Elferink, R. P., Baas, F., Schinkel, A. H. and Borst, P. (1998). Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. *Journal of Clinical Investigation* **101,** 1310-9.

Evers, R., Zaman, G. J., van Deemter, L., Jansen, H., Calafat, J., Oomen, L. C., Oude Elferink, R. P., Borst, P. and Schinkel, A. H. (1996). Basolateral localization and export activity of the human multidrug resistance-associated protein in polarized pig kidney cells. *Journal of Clinical Investigation* **97,** 1211-8.

Ewy, G. A., Groves, B. M., Ball, M. F., Nimmo, L., Jackson, B. and Marcus, F. (1971). Digoxin metabolism in obesity. *Circulation* **44,** 810-4.

Fromm, M. F., Kim, R. B., Stein, C. M., Wilkinson, G. R. and Roden, D. M. (1999). Inhibition of P-glycoprotein-mediated drug transport: A unifying mechanism to explain the interaction between digoxin and quinidine [see comments]. *Circulation* **99,** 552-7.

Gault, H., Kalra, J., Ahmed, M., Kepkay, D. and Barrowman, J. (1980). Influence of gastric pH on digoxin biotransformation. I. Intragastric hydrolysis. *Clinical Pharmacology and Therapeutics* **27,** 16-21.

Gault, H., Kalra, J., Ahmed, M., Kepkay, D., Longerich, L. and Barrowman, J. (1981). Influence of gastric pH on digoxin biotransformation. II. Extractable urinary metabolites. *Clinical Pharmacology and Therapeutics* **29,** 181-90.

Gault, H., Sugden, D., Maloney, C., Ahmed, M. and Tweeddale, M. (1979). Biotransformation and elimination of digoxin with normal and minimal renal function. *Clinical Pharmacology and Therapeutics* **25,** 499-513.

Gibbs, M. A., Thummel, K. E., Shen, D. D. and Kunze, K. L. (1999). Inhibition of cytochrome P-450 3A (CYP3A) in human intestinal and liver microsomes: comparison of Ki values and impact of CYP3A5 expression. *Drug Metabolism and Disposition* **27,** 180-7.

Gorodischer, R., Jusko, W. J. and Yaffe, S. J. (1976). Tissue and erythrocyte distribution of digoxin in infants. *Clinical Pharmacology and Therapeutics* **19,** 256-63.

Gorodischer, R., Jusko, W. J. and Yaffe, S. J. (1977). Renal clearance of digoxin in young infants. *Research Communications in Chemical Pathology and Pharmacology* **16,** 363-74.

Greenblatt, D. J., Duhme, D. W., Koch-Weser, J. and Smith, T. W. (1974). Bioavailability of digoxin tablets and elixir in the fasting and postprandial states. *Clinical Pharmacology and Therapeutics* **16,** 444-8.

Haig, G. M. and Brookfield, E. G. (1992). Increase in serum digoxin concentrations after indomethacin therapy in a full-term neonate. *Pharmacotherapy* **12,** 334-6.

Harrison, C. E., Jr., Brandenburg, R. O., Ongley, P. A., Orvis, A. L. and Owen, C. A., Jr. (1966). The distribution and excretion of tritiated substances in experimental animals following the administration of digoxin-^{3}H. *Journal of Laboratory and Clinical Medicine* **67,** 764-77.

Hinderling, P. H. (1984). Kinetics of partitioning and binding of digoxin and its analogues in the sub-compartments of blood. *Journal of Pharmaceutical Sciences* **73,** 1042-53.

Hori, R., Okamura, N., Aiba, T. and Tanigawara, Y. (1993). Role of P-glycoprotein in renal tubular secretion of digoxin in the isolated perfused rat kidney. *Journal of Pharmacology and Experimental Therapeutics* **266,** 1620-5.

Hunter, J., Hirst, B. H. and Simmons, N. L. (1993a). Drug absorption limited by P-glycoprotein-mediated secretory drug transport in human intestinal epithelial Caco-2 cell layers. *Pharmaceutical Research* **10,** 743-9.

Hunter, J., Jepson, M. A., Tsuruo, T., Simmons, N. L. and Hirst, B. H. (1993b). Functional expression of P-glycoprotein in apical membranes of human intestinal

Caco-2 cells. Kinetics of vinblastine secretion and interaction with modulators. *Journal of Biological Chemistry* **268,** 14991-7.

Ito, S. and Koren, G. (1997). Comment: possible mechanism of digoxin-itraconazole interaction [letter; comment]. *Annals of Pharmacotherapy* **31,** 1091-2.

Ito, S., Koren, G., Harper, P. A. and Silverman, M. (1993). Energy-dependent transport of digoxin across renal tubular cell monolayers (LLC-PK1). *Canadian Journal of Physiology and Pharmacology* **71,** 40-7.

Ito, S., Woodland, C. and Koren, G. (1994). Ketoconazole and itraconazole inhibit digoxin transport across renal tubular cell monolayer (MDCK). *Pediatric Research* **37,** 77A.

Ito, S., Woodland, C. and Koren, G. (1995). Characteristics of itraconazole and ketoconazole inhibition of digoxin transport across renal tubular cells. *FASEB Journal* **9,** A686.

Jalava, K. M., Partanen, J. and Neuvonen, P. J. (1997). Itraconazole decreases renal clearance of digoxin. *Therapeutic Drug Monitoring* **19,** 609-13.

Joreteg, T. and Jogestrand, T. (1983). Physical exercise and digoxin binding to skeletal muscle: relation to exercise intensity. *European Journal of Clinical Pharmacology* **25,** 585-8.

Jørgensen, H. S., Christensen, H. R. and Kampmann, J. P. (1991). Interaction between digoxin and indomethacin or ibuprofen. *British Journal of Clinical Pharmacology* **31,** 108-10.

Katzung, B. G. (1998). Basic & Clinical Pharmacology, pp. xii, 1151. Stamford, Conn.: Appleton & Lange.

Kauffman, C. A. and Bagnasco, F. A. (1992). Digoxin toxicity associated with itraconazole therapy [letter]. *Clinical Infectious Diseases* **15,** 886-7.

Kearin, M., Kelly, J. G. and O'Malley, K. (1980). Digoxin "receptors" in neonates: an explanation of less sensitivity to digoxin than in adults. *Clinical Pharmacology and Therapeutics* **28,** 346-9.

Kholodov, L. E., Grigor'eva, E. A., Glezer, M. G. and Sabel'nikova, E. A. (1980). [Bioavailability of digoxin tablets in cardiac insufficiency patients]. *Kardiologiia* **20,** 68-73.

Kobayashi, S., Okada, S., Yoshida, H. and Fujimura, S. (1997). Indomethacin enhances the cytotoxicity of VCR and ADR in human pulmonary adenocarcinoma cells. *Tohoku Journal of Experimental Medicine* **181,** 361-70.

Koren, G. (1987). Clinical pharmacokinetic significance of the renal tubular secretion of digoxin. *Clinical Pharmacokinetics* **13,** 334-43.

Koren, G., Klein, J., Giesbrecht, E., Ben Dayan, R., Soldin, S., Sellers, E., MacLeod, S. and Silverman, M. (1988). Effects of quinidine on the renal tubular and biliary transport of digoxin: in vivo and in vitro studies in the dog. *Journal of Pharmacology and Experimental Therapeutics* **247,** 1193-8.

Koren, G., Klein, J., MacLeod, S. and Silverman, M. (1986). Cellular mechanisms of digoxin transport and toxic interactions in the kidney. *Veterinary and Human Toxicology* **28,** 549-52.

Koren, G., Woodland, C. and Ito, S. (1998). Toxic digoxin-drug interactions: the major role of renal P-glycoprotein. *Veterinary and Human Toxicology* **40,** 45-6.

Koup, J. R., Greenblatt, D. J., Jusko, W. J., Smith, T. W. and Koch-Weser, J. (1975a). Pharmacokinetics of digoxin in normal subjects after intravenous bolus and infusion doses. *Journal of Pharmacokinetics and Biopharmaceutics* **3,** 181-92.

Koup, J. R., Jusko, W. J., Elwood, C. M. and Kohli, R. K. (1975b). Digoxin pharmacokinetics: role of renal failure in dosage regimen design. *Clinical Pharmacology and Therapeutics* **18,** 9-21.

Kramer, W. G., Bathala, M. S. and Reuning, R. H. (1976). Specificity of the digoxin radioimmunoassay with respect to dihydrodigoxin. *Research Communications in Chemical Pathology and Pharmacology* **14,** 83-8.

Kramer, W. G., Kinnear, N. L. and Morgan, H. K. (1978). Variability among commercially available digoxin radioimmunoassay kits in cross reactivity to dihydro-digoxin. *Clinical Chemistry* **24,** 155-7.

Kramer, W. G., Kolibash, A. J., Lewis, R. P., Bathala, M. S., Visconti, J. A. and Reuning, R. H. (1979). Pharmacokinetics of digoxin: relationship between response intensity and predicted compartmental drug levels in man. *Journal of Pharmacokinetics and Biopharmaceutics* **7,** 47-61.

Kramer, W. G., Lewis, R. P., Cobb, T. C., Forester, W. F., Jr., Visconti, J. A., Wanke, L. A., Boxenbaum, H. G. and Reuning, R. H. (1974). Pharmacokinetics of digoxin: comparison of a two- and a three-compartment model in man. *Journal of Pharmacokinetics and Biopharmaceutics* **2,** 299-312.

Kuhlmann, J., Abshagen, U. and Rietbrock, N. (1974). Pharmacokinetics and metabolism of digoxigenin-mono-digitoxoside in man. *European Journal of Clinical Pharmacology* **7,** 87-94.

Kunze, K. L., Wienkers, L. C., Thummel, K. E. and Trager, W. F. (1996). Warfarin-fluconazole. I. Inhibition of the human cytochrome P450-dependent metabolism of warfarin by fluconazole: in vitro studies. *Drug Metabolism and Disposition* **24,** 414-21.

Kurosawa, M., Okabe, M., Hara, N., Kawamura, K., Suzuki, S., Sakurada, K. and Asaka, M. (1996). Reversal effect of itraconazole on adriamycin and etoposide resistance in human leukemia cells. *Annals of Hematology* **72,** 17-21.

Lacarelle, B., Rahmani, R., de Sousa, G., Durand, A., Placidi, M. and Cano, J. P. (1991). Metabolism of digoxin, digoxigenin digitoxosides and digoxigenin in human hepatocytes and liver microsomes. *Fundamental and Clinical Pharmacology* **5,** 567-82.

Lage, G. L. and Spratt, J. L. (1966). Structure-activity correlation of the lethality and central effects of selected cardiac glycosides. *Journal of Pharmacology and Experimental Therapeutics* **152,** 501-8.

Lampen, A., Christians, U., Guengerich, F. P., Watkins, P. B., Kolars, J. C., Bader, A., Gonschior, A. K., Dralle, H., Hackbarth, I. and Sewing, K. F. (1995). Metabolism of the immunosuppressant tacrolimus in the small intestine: cytochrome P450, drug interactions, and interindividual variability. *Drug Metabolism and Disposition* **23,** 1315-24.

Lewis, R. P. (1992). Clinical use of serum digoxin concentrations. *American Journal of Cardiology* **69,** 97G-106G; discussion 106G.

Linday, L. and Drayer, D. E. (1983). Cross reactivity of the EMIT digoxin assay with digoxin metabolites, and validation of the method for measurement of urinary digoxin. *Clinical Chemistry* **29,** 175-7.

Lindenbaum, J., Rund, D. G., Butler, V. P., Jr., Tse-Eng, D. and Saha, J. R. (1981a). Inactivation of digoxin by the gut flora: reversal by antibiotic therapy. *New England Journal of Medicine* **305,** 789-94.

Lindenbaum, J., Tse-Eng, D., Butler, V. P., Jr. and Rund, D. G. (1981b). Urinary excretion of reduced metabolites of digoxin. *American Journal of Medicine* **71,** 67-74.

Magnusson, J. O. (1983). Metabolism of digoxin after oral and intra-jejunal administration. *British Journal of Clinical Pharmacology* **16,** 741-2.

Magnusson, J. O., Bergdahl, B., Bogentoft, C., Jonsson, U. E. and Tekenbergs, L. (1982). Excretion of digoxin and its metabolites in urine after a single oral dose in healthy subjects. *Biopharmaceutics and Drug Disposition* **3,** 211-8.

Maurice, M., Pichard, L., Daujat, M., Fabre, I., Joyeux, H., Domergue, J. and Maurel, P. (1992). Effects of imidazole derivatives on cytochromes P450 from human hepatocytes in primary culture. *FASEB Journal* **6,** 752-8.

Mayer, U., Wagenaar, E., Beijnen, J. H., Smit, J. W., Meijer, D. K., van Asperen, J., Borst, P. and Schinkel, A. H. (1996). Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. *British Journal of Pharmacology* **119,** 1038-44.

Mayer, U., Wagenaar, E., Dorobek, B., Beijnen, J. H., Borst, P. and Schinkel, A. H. (1997). Full blockade of intestinal P-glycoprotein and extensive inhibition of

blood-brain barrier P-glycoprotein by oral treatment of mice with PSC833. *Journal of Clinical Investigation* **100,** 2430-6.

McClean, K. L. and Sheehan, G. J. (1994). Interaction between itraconazole and digoxin [letter; comment]. *Clinical Infectious Diseases* **18,** 259-60.

Meyboom, R. H., de Jonge, K., Veentjer, H., Dekens-Konter, J. A. and de Koning, G. H. (1994). [Potentiation of digoxin by itraconazole]. *Nederlands Tijdschrift voor Geneeskunde* **138,** 2353-6.

Morais, J. A., Zlotecki, R. A., Sakmar, E., Stetson, P. L. and Wagner, J. G. (1981). Specific and sensitive assays for digoxin in plasma, urine and heart tissue. *Research Communications in Chemical Pathology and Pharmacology* **31,** 285-98.

Nachtmann, F., Spitzy, H. and Frei, R. W. (1976a). Rapid and sensitive high-resolution procedure for digitalis glycoside analysis by derivatization liquid chromatography. *Journal of Chromatography* **122,** 293-303.

Nachtmann, F., Spitzy, H. and Frei, R. W. (1976b). Ultraviolet derivatization of digitalis glycosides as 4-nitrobenzoates for liquid chromatographic trace analysis. *Analytical Chemistry* **48,** 1576-9.

Newton, D. J., Wang, R. W. and Lu, A. Y. (1995). Cytochrome P450 inhibitors. Evaluation of specificities in the in vitro metabolism of therapeutic agents by human liver microsomes. *Drug Metabolism and Disposition* **23,** 154-8.

Noe, B., Hagenbuch, B., Stieger, B. and Meier, P. J. (1997). Isolation of a multispecific organic anion and cardiac glycoside transporter from rat brain. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 10346-50.

Okamura, N., Hirai, M., Tanigawara, Y., Tanaka, K., Yasuhara, M., Ueda, K., Komano, T. and Hori, R. (1993). Digoxin-cyclosporin A interaction: modulation of the multidrug transporter P-glycoprotein in the kidney. *Journal of Pharmacology and Experimental Therapeutics* **266,** 1614-9.

Partanen, J., Jalava, K. M. and Neuvonen, P. J. (1996). Itraconazole increases serum digoxin concentration. *Pharmacology and Toxicology* **79,** 274-6.

Pichard, L., Fabre, J. M., Domergue, J., Fabre, G., Saint-Aubert, B., Mourad, G. and Maurel, P. (1991). Molecular mechanism of cyclosporine A drug interactions: inducers and inhibitors of cytochrome P450 screening in primary cultures of human hepatocytes. *Transplantation Proceedings* **23,** 978-9.

Reuning, R. H. and Geraets, D. R. (1986). Digoxin. In *Applied Pharmacokinetics : Principles of Therapeutic Drug Monitoring* (Eds. Evans, W. E. , Schentag, J. J. and Jusko, W. J.), pp. 570-623. Spokane, WA: Applied Therapeutics.

Rex, J. (1992). Itraconazole-digoxin interaction [letter]. *Annals of Internal Medicine* **116,** 525.

Rodin, S. M. and Johnson, B. F. (1988). Pharmacokinetic interactions with digoxin. *Clinical Pharmacokinetics* **15,** 227-44.

Sachs, M. K., Blanchard, L. M. and Green, P. J. (1993). Interaction of itraconazole and digoxin [see comments]. *Clinical Infectious Diseases* **16,** 400-3.

Schinkel, A. H., Mayer, U., Wagenaar, E., Mol, C. A., van Deemter, L., Smit, J. J., van der Valk, M. A., Voordouw, A. C., Spits, H., van Tellingen, O., Zijlmans, J. M., Fibbe, W. E. and Borst, P. (1997). Normal viability and altered pharmacokinetics

in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 4028-33.

Schinkel, A. H., Wagenaar, E., Mol, C. A. and van Deemter, L. (1996). P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *Journal of Clinical Investigation* **97,** 2517-24.

Schinkel, A. H., Wagenaar, E., van Deemter, L., Mol, C. A. and Borst, P. (1995). Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *Journal of Clinical Investigation* **96,** 1698-705.

Schmoldt, A. and Ahsendorf, B. (1980). Cleavage of digoxigenin digitoxosides by rat liver microsomes. *European Journal of Drug Metabolism and Pharmacokinetics* **5,** 225-32.

Schmoldt, A. and Promies, J. (1982). On the substrate specificity of the digitoxigenin mono-digitoxoside conjugating UDP-glucuronyl-transferase in rat liver. *Biochemical Pharmacology* **31,** 2285-9.

Siegsmund, M. J., Cardarelli, C., Aksentijevich, I., Sugimoto, Y., Pastan, I. and Gottesman, M. M. (1994). Ketoconazole effectively reverses multidrug resistance in highly resistant KB cells. *Journal of Urology* **151,** 485-91.

Smith, T. W. (1984a). The basic mechanism of inotropic action of digitalis glycosides. *Journal de Pharmacologie* **15 Suppl 1,** 35-51.

Smith, T. W. (1984b). Digitalis in the management of heart failure. *Hospital Practice (Office Edition)* **19,** 67-73, 77-86, 91-2.

Smith, T. W. and Haber, E. (1970). Digoxin intoxication: the relationship of clinical presentation to serum digoxin concentration. *Journal of Clinical Investigation* **49,** 2377-86.

Smith, T. W., Butler, V. P., Jr. and Haber, E. (1969). Determination of therapeutic and toxic serum digoxin concentrations by radioimmunoassay. *New England Journal of Medicine* **281,** 1212-6.

Smith, T. W., Antman, E. M., Friedman, P. L., Blatt, C. M. and Marsh, J. D. (1984a). Digitalis glycosides: mechanisms and manifestations of toxicity. Part I. *Progress in Cardiovascular Diseases* **26,** 413-58.

Smith, T. W., Antman, E. M., Friedman, P. L., Blatt, C. M. and Marsh, J. D. (1984b). Digitalis glycosides: mechanisms and manifestations of toxicity. Part II. *Progress in Cardiovascular Diseases* **26,** 495-540.

Smith, T. W., Antman, E. M., Friedman, P. L., Blatt, C. M. and Marsh, J. D. (1984c). Digitalis glycosides: mechanisms and manifestations of toxicity. Part III. *Progress in Cardiovascular Diseases* **27,** 21-56.

Sparreboom, A., van Asperen, J., Mayer, U., Schinkel, A. H., Smit, J. W., Meijer, D. K., Borst, P., Nooijen, W. J., Beijnen, J. H. and van Tellingen, O. (1997). Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 2031-5.

Steiness, E. (1974a). [Digoxin tablets]. *Ugeskrift for Laeger* **136,** 658.

Steiness, E. (1974b). Renal tubular secretion of digoxin. *Circulation* **50,** 103-7.

Steiness, E., Rasmussen, F., Svendsen, O. and Johansen, H. (1974). [Forms of digoxin administration]. *Ugeskrift for Laeger* **136,** 635-8.

Stoll, R. G., Christensen, M. S., Sakmar, E. and Wagner, J. G. (1972). The specificity of the digoxin radioimmunoassay procedure. *Research Communications in Chemical Pathology and Pharmacology* **4,** 503-10.

Su, S. F. and Huang, J. D. (1996). Inhibition of the intestinal digoxin absorption and exsorption by quinidine. *Drug Metabolism and Disposition* **24,** 142-7.

Talcott, R. E., Stohs, S. J. and el-Olemy, M. M. (1972). Metabolites and some characteristics of the metabolism of ^{3}H-digoxigenin by rat liver homogenates. *Biochemical Pharmacology* **21,** 2001-6.

Tanigawara, Y., Okamura, N., Hirai, M., Yasuhara, M., Ueda, K., Kioka, N., Komano, T. and Hori, R. (1992). Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). *Journal of Pharmacology and Experimental Therapeutics* **263,** 840-5.

Tsuruo, T., Iida, H., Kitatani, Y., Yokota, K., Tsukagoshi, S. and Sakurai, Y. (1984). Effects of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and adriamycin in drug-resistant tumor cells. *Cancer Research* **44,** 4303-7.

van Asperen, J., Schinkel, A. H., Beijnen, J. H., Nooijen, W. J., Borst, P. and van Tellingen, O. (1996). Altered pharmacokinetics of vinblastine in Mdr1a P-glycoprotein-deficient Mice. *Journal of the National Cancer Institute* **88,** 994-9.

van der Graaf, W. T., de Vries, E. G., Uges, D. R., Nanninga, A. G., Meijer, C., Vellenga, E., Mulder, P. O. and Mulder, N. H. (1991). In vitro and in vivo modulation of

multi-drug resistance with amiodarone. *International Journal of Cancer* **48,** 616-22.

von Moltke, L. L., Greenblatt, D. J., Schmider, J., Duan, S. X., Wright, C. E., Harmatz, J. S. and Shader, R. I. (1996). Midazolam hydroxylation by human liver microsomes in vitro: inhibition by fluoxetine, norfluoxetine, and by azole antifungal agents. *Journal of Clinical Pharmacology* **36,** 783-91.

Wettrell, G. (1977). Distribution and elimination of digoxin in infants. *European Journal of Clinical Pharmacology* **11,** 329-35.

Wettrell, G. and Andersson, K. E. (1977). Clinical pharmacokinetics of digoxin in infants. *Clinical Pharmacokinetics* **2,** 17-31.

White, R. J., Chamberlain, D. A., Howard, M. and Smith, T. W. (1971). Plasma concentrations of digoxin after oral administration in the fasting and postprandial state. *British Medical Journal* **1,** 380-1.

Woodland, C., Verjee, Z., Giesbrecht, E., Koren, G. and Ito, S. (1997). The digoxin-propafenone interaction: characterization of a mechanism using renal tubular cell monolayers. *Journal of Pharmacology and Experimental Therapeutics* **283,** 39-45.

Wrighton, S. A. and Ring, B. J. (1994). Inhibition of human CYP3A catalyzed 1'-hydroxy midazolam formation by ketoconazole, nifedipine, erythromycin, cimetidine, and nizatidine. *Pharmaceutical Research* **11,** 921-4.

Yusa, K. and Tsuruo, T. (1989). Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and

transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Research* **49,** 5002-6.

Chapter 3

DIGOXIN METABOLISM *IN VITRO* AND THE EFFECTS OF KETOCONAZOLE ON DIGOXIN PHARMACOKINETICS IN RATS

3.1 Abstract

Studies have shown that Digoxin (Dg3), a cardiac glycoside, is a substrate of P-glycoprotein (Pgp) and that the drug is a substrate for cytochrome P450 3A (CYP3A) in rats. The objective of this study was to characterize the digoxin metabolism in rats and humans, as well as to determine the pharmacokinetics of digoxin alone and with ketoconazole (KCZ), an anti-fungal agent known to be a potent inhibitor of Pgp and CYP3A, in rats.

The metabolism of Dg3 to digoxigenin bis-digitoxoside (Dg2) was compared in rat and human liver microsomes. No extensive Dg2 was formed after incubation of Dg3 with human liver microsomes. Chemical inhibition by ketoconazole (KCZ) and

itraconazole (ICZ) decreased the formation of Dg2 in rat liver microsomes. Antibodies specific to rat CYP3A2 also reduced the oxidative cleavage of Dg3 in rat liver microsomes. These data support the involvement of CYP3A in the cleavage of Dg3 in rats but not in humans.

The effects of oral KCZ at 80 mg/kg and 10 mg/kg on digoxin pharmacokinetics in rats were determined. In the presence of 80 mg/kg of KCZ, C_{max} of oral digoxin increased from 18.4 ng/ml to 83.8 ng/ml, T_{peak} decreased from 0.75 hr to 0.38 hr, AUCs increased 6 fold (*p.o.*) and 4.6 fold (*iv*), and the apparent F increased from 62% to 83%. Time averaged CL decreased from 1.8 L/hr/kg to 0.4 L/hr/kg, and V_{ss} decreased from 4.4 L/kg to 1.9 L/kg when controls are compared to concomitant 80 mg/kg KCZ. But at 10 mg/kg, KCZ produced a less effect on digoxin pharmacokinetics.

The excellent linear fits of the digoxin clearance *vs.* KCZ dose suggest that the pharmacokinetics of digoxin in the rat are well represented by assuming that digoxin's elimination kinetics are linear both without and with concomitant KCZ and that a competitive modulator model is appropriate *in vivo*. Although the effects of KCZ on AUC could be explained by inhibition of both CYP3A and Pgp, which can not be differentiated in this study, the decreased digoxin mean absorption time MAT, which dropped significantly from 0.97 hr to 0.52 hr then to 0.32 hr with increasing KCZ doses, can only be explained by inhibition of Pgp by KCZ in the intestine.

3.2 Introduction

Digoxin is a cardiac glycoside that has been used clinically for more than 200 years (Antman and Smith, 1985; Heller, 1990). It is currently utilized as an antiarrhythmic and is the most commonly prescribed drug in congestive heart failure

(CHF) (Kelly and Smith, 1996). Chemical structures of digoxin and its metabolites (Dg0, Dg1 and Dg2) are shown in Figure 3-1.

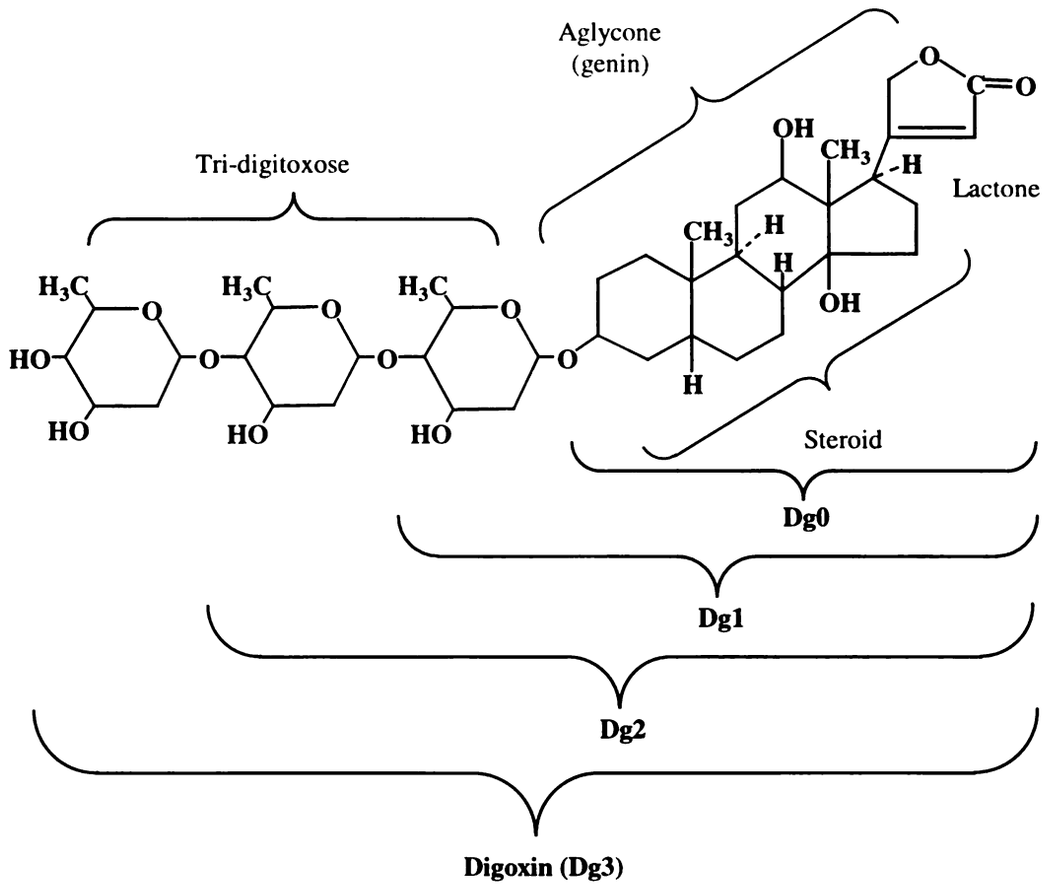


Figure 3-1. Chemical structures of digoxin and its metabolites- Dg0, Dg1 and Dg2.

Digoxin is extensively metabolized in rats (Harrison and Gibaldi, 1976), and we have recently shown that CYP3A is the primary enzyme family involved (Salphati and Benet, 1999). In rats more than 60% of a dose is metabolized while renally excreted drug accounts for about 30% (Harrison and Gibaldi, 1976). Dg3 biotransformations involve the stepwise cleavage of the digitoxoses to form Dg2, Dg1 and Dg0, which then is further

conjugated and eliminated (Harrison and Gibaldi, 1976). The sequential release of each sugar residue requires a dehydrogenation of the axial hydroxyl group from the terminal digitoxose before it can be split off (Schmoldt and Ahsendorf, 1980). The dehydrogenation is mediated by CYP (Schmoldt and Ahsendorf, 1980).

P-glycoprotein (Pgp), is a plasma membrane protein initially studied for its prominent role in the development of MDR in cancer chemotherapy (Borst *et al.*, 1993; Chin *et al.*, 1993; Gottesman and Pastan, 1993; Leveille-Webster and Arias, 1995). It is also normally expressed on the apical side in many eliminating organs (liver, kidney, intestine) (Cordon-Cardo and O'Brien, 1991) and plays a role in blood brain barrier (BBB) (Schinkel *et al.*, 1994) and placenta function. Rats possess mdr1a, mdr1b and mdr2, three MDR genes (Silverman and Thorgeirsson, 1995). Mdr1a and mdr1b encoded Pgps are drug transporters while mdr2 is a phospholipid translocator (Ruetz and Gros, 1994; Smit *et al.*, 1993). Mdr1a is the main Pgp expressed in rat intestine whereas both mdr1 and mdr2 are present in the liver (Silverman and Schrenk, 1997).

Pgp transports a broad variety of drugs such as etoposide, vinblastine, doxorubicin, cyclosporine (CsA), verapamil and quinidine (Wacher *et al.*, 1995). Studies in LLC-PK1 cells transfected with the human MDR1 cDNA (Tanigawara *et al.*, 1992) showed that Dg3 was transported by Pgp. Investigations using perfused rat kidney (Hori *et al.*, 1993) and rat everted gut sacs (Su and Huang, 1996) concluded that this drug was a rat Pgp substrate. In mdr1a and mdr1a/1b knock-out mice, it was demonstrated that elimination of digoxin was mediated, at least partly, by intestinal Pgp (Mayer *et al.*, 1996; Schinkel *et al.*, 1997).

These data provide an explanation to the numerous drug interactions reported in human between Dg3 and verapamil (Belz *et al.*, 1983), amiodarone, quinidine (Rodin and Johnson, 1988) and propafenone (Belz *et al.*, 1983; Calvo *et al.*, 1989), which are Pgp substrates/inhibitors (Tsuruo *et al.*, 1984; van der Graaf *et al.*, 1991; Woodland *et al.*, 1997; Yusa and Tsuruo, 1989).

Azole anti-fungal agents such as ketoconazole (KCZ) (Newton *et al.*, 1995) are potent inhibitors of CYP3A (Table 3-1), a major enzyme representing 30% of the total CYPs in human liver (Kolars *et al.*, 1994) and accounting for the metabolism of more than 50% of drugs eliminated by the P450 enzymes. KCZ has also been shown to inhibit Pgp in a vinblastine resistant cell line (Siegsmund *et al.*, 1994).

Table 3-1. Reversible inhibitors of human CYP3A.

Inhibitor	Apparent K_i (μM)	Mechanism	References
Ketoconazole	0.0037–8	Noncompetitive, mixed	Bourrié *et al.*, 1996; Gibbs *et al.*, 1999; Lampen *et al.*, 1995; von Moltke *et al.*, 1996; Wrighton and Ring, 1994
Itraconazole	0.27		von Moltke *et al.*, 1996
Miconazole	0.9–1.3	Competitive	Maurice *et al.*, 1992; Pichard *et al.*, 1991
Fluconazole	1.27–63	Competitive, noncompetitive	Gibbs *et al.*, 1999; Kunze *et al.*, 1996; Maurice *et al.*, 1992; von Moltke *et al.*, 1996

The fact that specificity strongly overlaps between CYP3A and Pgp substrates and modulators (Wacher *et al.*, 1995) makes it difficult to measure the contributions of each protein on the absorption and disposition of a drug (Lum *et al.*, 1993). In humans, Dg3

has been reported to not be extensively metabolized but mostly excreted unchanged by the kidney (Rodin and Johnson, 1988). Since the stepwise cleavage of Dg3 was not cytochrome P450 dependent in human hepatocytes and liver microsomes (Lacarelle *et al.*, 1991), digoxin might serve as a selective Pgp probe to differentiate these effects.

Prior to studying the effects of inhibition of the MDR transporters on digoxin concentration *versus* time profiles clinically, we compared digoxin metabolism in rat and human liver microsomes, in which inhibition was also used to confirm the possible enzyme involved in digoxin metabolism in rats. We then evaluated the effects of KCZ on digoxin pharmacokinetics by administering digoxin to rats without and with concomitant low and high doses of KCZ. Since KCZ is a inhibitor of CYP3A and Pgp, and Dg3 is a substrate of CYP3A and Pgp, digoxin absorption and disposition changed markedly after co-administration of KCZ. This allows us to estimate the apparent inhibitory potency of KCZ on Dg3 elimination and absorption in rats.

3.3 Materials and Methods

3.3.1 Chemicals

For metabolism studies

Dg3, NADPH, isocitrate dehydrogenase, sodium phosphate and corticosterone were purchased from the Sigma Chemical Co. (St. Louis, MO).

Dg0 was from Indofine Chemical Company, Inc. (Belle Mead, NJ) or Fluka Chemika or Sigma Chemical Co. (St. Louis, MO). Dg1 was from Boehringer Mannheim GmbH (Germany). Dg2 was from Crescent Chemical Co. (Hauppauge, NY) or Serva Boehringer Ingelheim or Serva Feinbiochemica GmbH (Germany).

KCZ were purchased from the U.S. Pharmacopeia (Rockville, MD) and Research Biochemical International (Natick, MA), respectively. Janssen Pharmaceutica (Belgium) kindly supplied ICZ. Antibodies to rat CYP were obtained from Gentest Corp. (Woburn, MA). HPLC grade methanol and acetonitrile were from Fisher Scientific (Pittsburgh, PA).

For pharmacokinetic studies

Digoxin (Lanoxin injection, 0.1 mg/ml) was obtained from GlaxoWellcome (Research Triangle Park, NC) and KCZ was purchased from U.S.P.C., Inc. (Rockville, MD). Ketamine and xylazine were obtained from Parke Davis (Morris Plains, NJ) and Lloyd Laboratories (Shenandoah, IO), respectively. Propylene glycol, citric acid and sodium phosphate were purchased from the Sigma Chemical Co. (St. Louis, MO).

3.3.2 Animals

Preparation of microsomes for metabolism studies

Adult male and female Sprague-Dawley rats (280-300 g) were obtained from Charles River Laboratories (Wilmington, MA) and maintained on a 12-hr light/dark cycle. Rats were fed standard laboratory chow *ad lib*. Microsomes were prepared from pooled fresh livers at 4°C by homogenization and differential centrifugation following established protocols (Guengerich, 1989). The microsomes were stored at -80°C until use in 10mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 20% (w/v) glycerol. Protein concentrations were determined using BSA as the standard and the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Surgical procedure for pharmacokinetic studies

Adult male Sprague-Dawley rats (8-10 weeks old) were obtained from Charles River Laboratories (Wilmington, MA) and maintained on a 12-hr light/dark cycle. Rats were fed standard laboratory chow *ad lib*. The surgical procedure and the implantation of the cannula in the jugular veins were as described before (Upton, 1975). Briefly, rats were anesthetized by an *i.p.* injection of ketamine (22 mg/kg) and xylazine (2.5 mg/kg). The right jugular vein was exposed, about 30 mm of silastic (0.02 in. i.d.) tubing (Dow Corning, Midland, MI) was inserted in the incised vein toward the heart. Discontinuous sutures reunited the musculature and ventral skin. The other end of the tubing emerged at the back of the neck. Blood samples were taken from this cannula and in case of *iv* administration of digoxin, a second catheter was implanted in the left jugular vein. After a 24 hr recovery period, the rats were administered digoxin (0.2 mg/kg) intravenously or by oral gavage using a blunt ended needle with or without ketoconazole.

When digoxin was given orally, ketoconazole was administered concomitantly as a suspension in the digoxin solution. When digoxin was injected intravenously, ketoconazole was given orally about 1 min prior to the digoxin injection, as a suspension in the digoxin buffer- propylene glycol 40% (v/v), alcohol 10% (v/v), sodium phosphate 0.17% (w/v) and anhydrous citric acid 0.08% (w/v), pH 6.8 to 7.2. 10 to 12 blood samples were taken through the implanted catheter from 5 min to 24 hr following drug administration. The blood was collected in microtainer tubes (Becton Dickinson, Franklin Lakes, NJ), containing EDTA. They were spun at 4000 g (Eppendorf centrifuge 5415C, Westbury, NY) for 5 min and the plasma was transferred into clean tubes and stored at 4°C until analysis.

3.3.3 Incubation conditions

Incubations of Dg3 as a substrate were conducted at 37°C with 0.75 mg protein/ml in K_2HPO_4 (0.1 M, pH 7.4), $MgCl_2$ (5 mM), isocitrate (5 mM) and isocitrate dehydrogenase (10 U/ml). Substrates and inhibitors were added in methanol (final methanol concentration = 1%). Reactions were initiated by adding NADPH (to 1 mM) after a 5 min of pre-incubation and quenched after 15 min to 2 hours by addition of 5 ml of methylene chloride containing the internal standard. The samples were vortexed, centrifuged (2000 g for 15 min) and an aliquot of the organic phase (3 ml) was evaporated under nitrogen. The residue was dissolved in 200 µl of mobile phase and 100 µl were analyzed by HPLC.

In immuno-inhibition experiments, microsomes were incubated for 30 min at room temperature with pre-immune sera or antibodies specific to rat CYP 3A. Thereafter, substrates and NADPH were added and the reactions conducted as described above.

3.3.4 Analytical methods

Assay for digoxin and its metabolites in metabolism studies

Digoxin, its metabolites and the internal standard corticosterone were resolved on a Beckman C_8 (5 µm x 4.6 mm i.d. x 150 mm) column with a Schimadzu SCL-10A system controller and a LC-10AD pump equipped with an SIL-10A auto injector. The absorbance was monitored at 220 nm with an UV-VIS spectrophotometric detector SPD-10AV and the compounds were quantified by comparison of the ratio of their peak area (measured with a Hewlett-Packard 3392A integrator) to those of the internal standards. Digoxin and metabolites were eluted isocratically over about 20 min at room temperature. The flow rate was 1 ml/min and the mobile phase was 26% acetonitrile in water. The limits of detection for Dg2 were lower than 0.03 µM.

Determination of digoxin and metabolite levels for pharmacokinetic studies

The micro-particle enzyme immuno-assay (MEIA) method was used. The method can not distinguish between digoxin and its active metabolites. The specificity of Abbott Laboratories AxSYM Digoxin II assay (Abbott Park, IL) is described as "The digoxin metabolites Dg0, Dg1 and Dg2, tested at concentrations corresponding to high plasma levels in digoxin patients (0.132, 0.088 and 0.22 ng/ml, respectively), showed digoxin concentrations which were below assay sensitivity". The assay was calibrated for concentrations from 0.3 to 5 ng/ml.

3.3.5 Data Analysis

Pharmacokinetic values for AUC, AUMC, CL, F, MRT, V_{ss}, C_{max}, T_{max} and MAT were calculated by the WinNonlin program using non-compartmental methods (Benet and Galeazzi, 1979).

Calculation of CL = Dose/AUC is valid under the assumption that all disposition processes follow first order kinetics. With co-administration of ketoconazole, digoxin plasma concentrations may exhibit a non-linear profile, which implies that digoxin plasma clearance is not constant. A time-averaged clearance term has been defined as CL_{av} = Dose/AUC in the presence of non-linear processes (Staubus and Smith, 1984). Using this method, the values calculated for MRT, V_{ss}, F and MAT will also be time averaged and may be underestimated compared to the "true" values by integrating non-linear kinetics parameters (Cheng and Jusko, 1989). Statistical comparisons between pharmacokinetic parameters with and without ketoconazole administration were made using Student's t-test. Statistical significance was accepted as $p<0.05$.

Using the time averaged values calculated assuming linearity, an attempt was

made to test the linearity assumption in vivo in the presence of the high and low doses of KCZ in the rat studies performed here. Each pharmacokinetic parameter determined here in the absence of KCZ may be considered as the no modulator parameter (P_{NM} or P_0). The time-averaged parameters calculated for digoxin following the 10 (P_{10}) and 80 (P_{80}) mg concomitant doses of KCZ may be expected to vary from (P_{NM} or P_0) if KCZ has an effect on that parameter. For example, since KCZ is a more potent inhibitor of CYP3A and only a moderate inhibitor of P-glycoprotein, clearance of digoxin in rats would be expected to decrease with increasing KCZ doses (i.e. $CL_{NM} > CL_{10} > CL_{80}$). In contrast, bioavailability would be expected to increase with increasing KCZ doses ($F_{80} > F_{10} > F_{NM}$). Using an integrated approach to the competitive drug interaction model, it may be possible to calculate the MD_{50}, the dose of the modulator (KCZ) required to decrease a pharmacokinetic parameter by 50%.

$$P_D = P_{NM} / [1 + (D/MD_{50})] \qquad \text{(Eq. 1)}$$

where D is the dose of the modulator.

Equation 1 can be linearized to Eq. 2:

$$\frac{1}{P_D} = \frac{1}{P_{NM}} + \frac{D}{P_{NM} \cdot MD_{50}} \qquad \text{(Eq. 2)}$$

Therefore, a plot of the inverse of a parameter *versus* the dose of the modulator will allow calculation of the parameter in the absence of the modulator (P_{NM}) as the inverse of the intercept and MD_{50} as the inverse of the slope divided by P_{NM}. These calculations were carried out for the time-averaged non compartmental values for CL, V_{ss} and MAT. The inhibitory model, as exemplified by Eq. 1, is only relevant for those parameters that decrease, or are inhibited, by ketoconazole. The linear fits of the

parameters from the three sets of studies (0, 10 and 80 mg KCZ) were calculated using both un-weighted linear regression and weighted linear regression. For CL and V_{ss}, the weights were taken as the inverse of the standard deviations squared for the means of the parameters in each set of studies. Since cross-over studies were not possible here, even for *iv* and *p.o.* dosing at each KCZ dose, the weights for the MAT fits were taken as the inverse of the parameter squared.

Fitting the mean parameters to Eq. 2 offers a number of useful insights. First, a good fit of the mean values to the linearized equation suggests that the assumptions employed in the calculations may be valid. That is, on average the pharmacokinetics of digoxin in the rat are well represented by assuming that the drug's kinetics are linear both without and with concomitant KCZ and that a competitive modulator model is appropriate *in vivo*, even though *in vitro* evaluation of the effects of the modulator may be best described by an alternate model. Second, the intercept of the linearized plot should yield a calculated value of P_{NM} from the regression which approximates the measured mean P_0, *i.e.*, the calculated parameter in the absence of concomitant modulator dosing.

3.4 Results

3.4.1 Metabolic profile

The sequential metabolism of Dg3 to Dg2 was investigated in incubations using authentic standard Dg2 metabolite. Preliminary experiments showed that metabolite Dg2 formation from substrate Dg3 was linear up to 1.5 mg protein/ml. Thus, subsequent incubations were performed with a protein concentration of 0.75 mg/ml. Figure 3-2 demonstrates that 6% of Dg3 was metabolized to Dg2 after 1 hour incubation of Dg3

with rat liver microsomes. In contrast, hardly any Dg2 was formed after incubation of Dg3 with human liver microsomes for 1 hour (Figure 3-3).

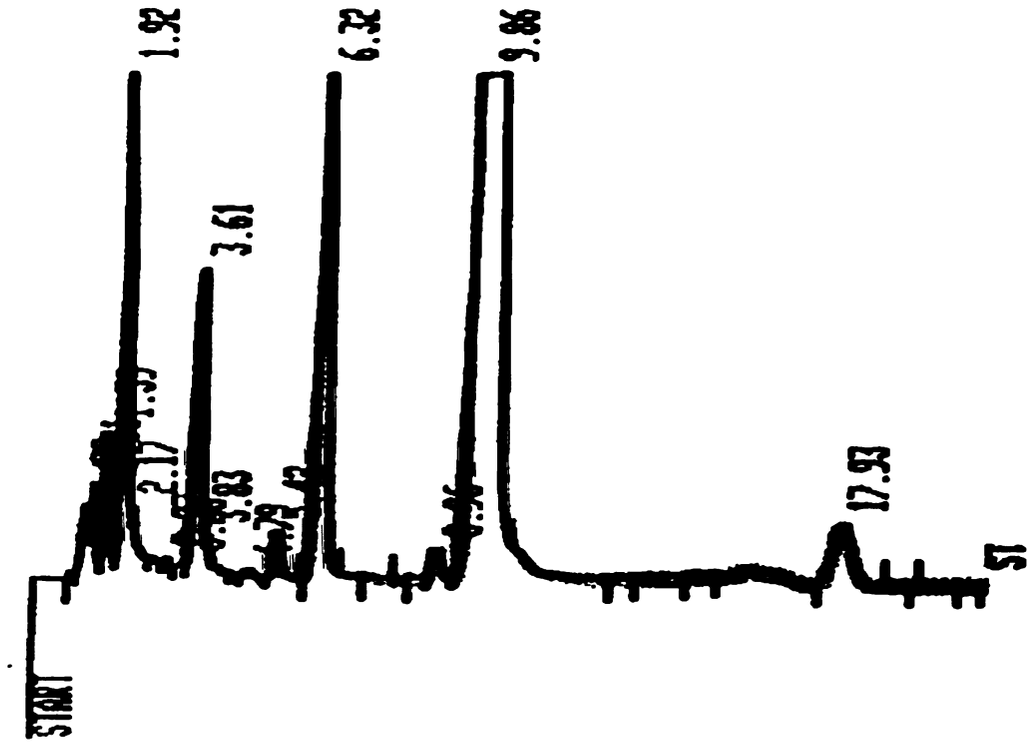


Figure 3-2. Digoxin incubated in rat liver microsomes for 1 hour (Dg3 at retention time of 9.86 min and Dg2 retention at 6.32 min).

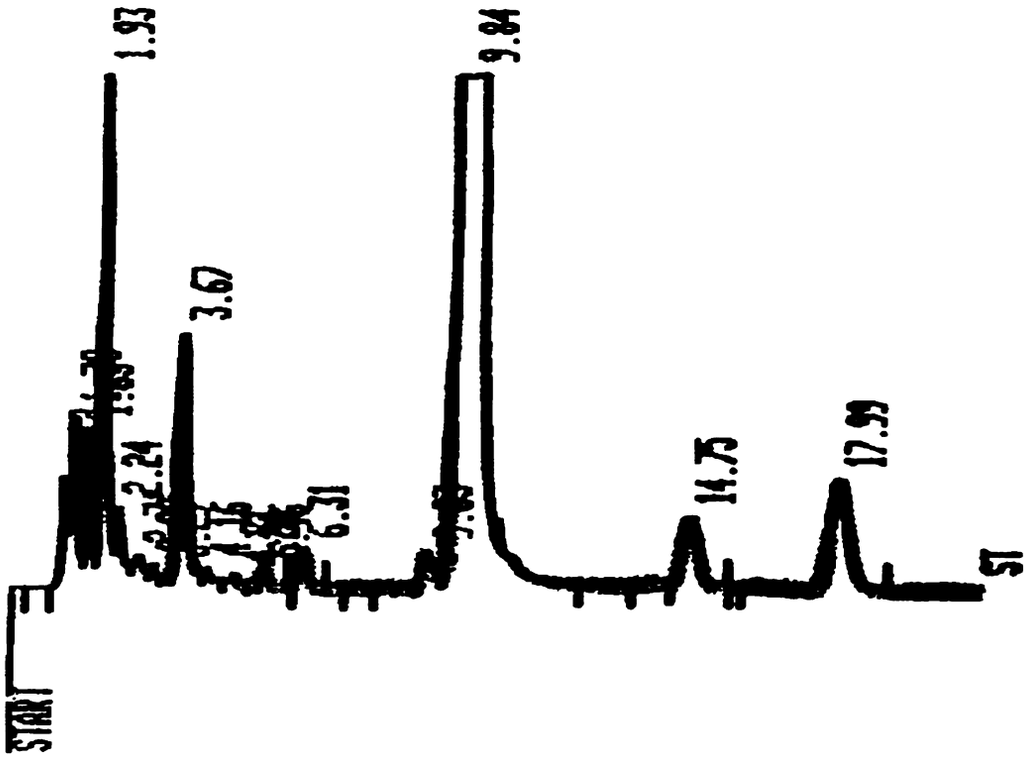


Figure 3-3. Digoxin incubated in human liver microsomes for 1 hour (Dg3 retention time at 9.84 min).

3.4.2 Effects of chemical inhibitors on metabolite formation

We measured the effects of competitive (KCZ and ICZ) inhibitors on the metabolism of Dg3 in male rat liver microsomes. KCZ and ICZ, selective inhibitors of CYP3A, caused inhibition of 50 μM of Dg3 metabolism to Dg2 in rat liver microsomes, with an IC_{50} = 54.6 ± 2.98 nM for KCZ (Figure 3-4) and an IC_{50} = 0.29 ± 0.02 μM for ICZ (Figure 3-5). The reported K_i values of ketoconazole and itraconazole on midazolam metabolism were 3.7 nM and 0.27 μM, respectively (von Moltke *et al.*, 1996).

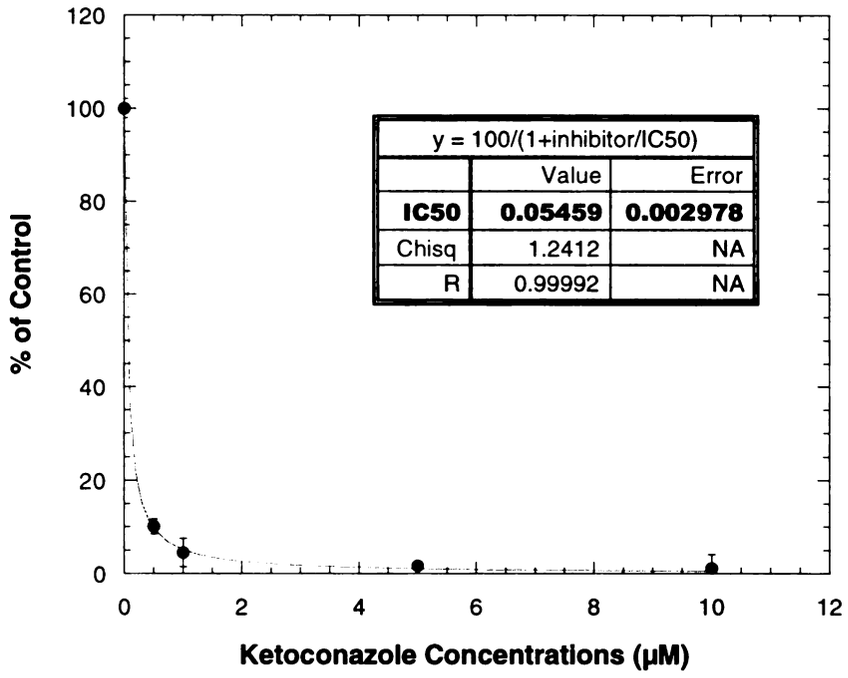


Figure 3-4. KCZ inhibited Dg3 metabolism to Dg2 in rat liver microsomes.

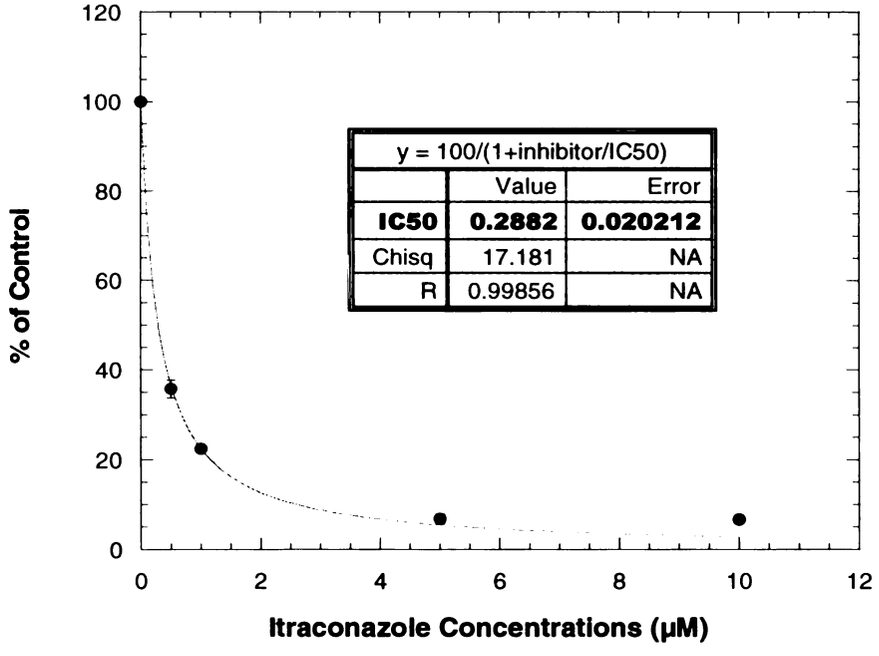


Figure 3-5. ICZ inhibited Dg3 metabolism to Dg2 in rat liver microsomes.

3.4.3 Immuno-inhibition

Rat monoclonal antibody to CYP3A2 strongly inhibited digoxin metabolism as shown in Figure 3-6, with an IC_{50} = 54.1 ± 10.0 (µl/mg protein). While pre-immune serum did not have any effect after incubation of 50 µM digoxin with rat liver microsomes (Figure 3-7). This and other inhibition studies (Salphati and Benet, 1999) further confirmed that CYP3A is involved in Dg3 metabolism to Dg2.

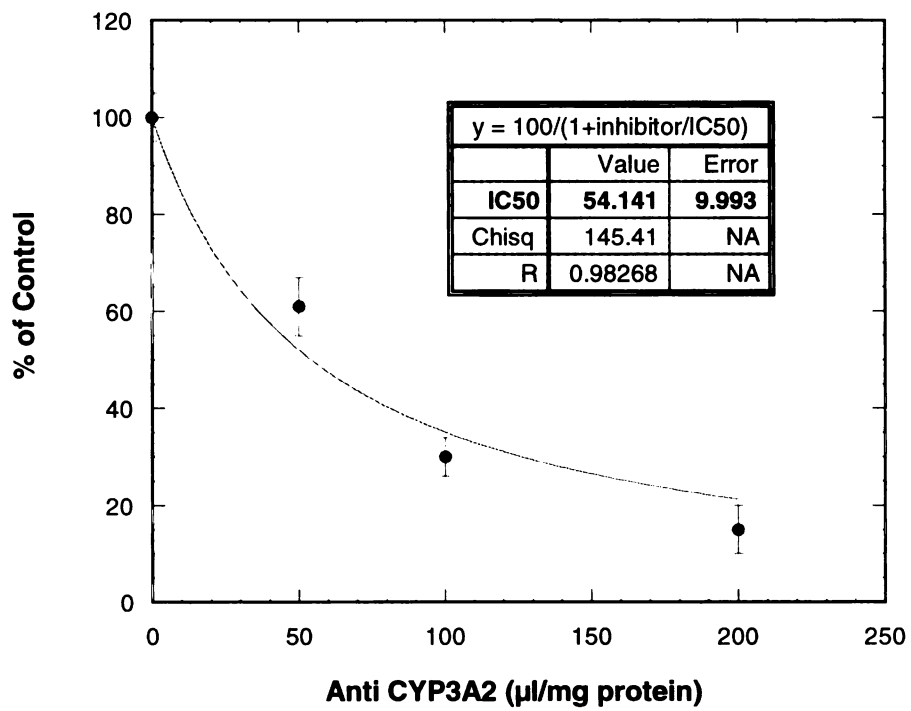


Figure 3-6. Antibody to CYP3A2 inhibited Dg3 metabolism in rat liver microsomes.

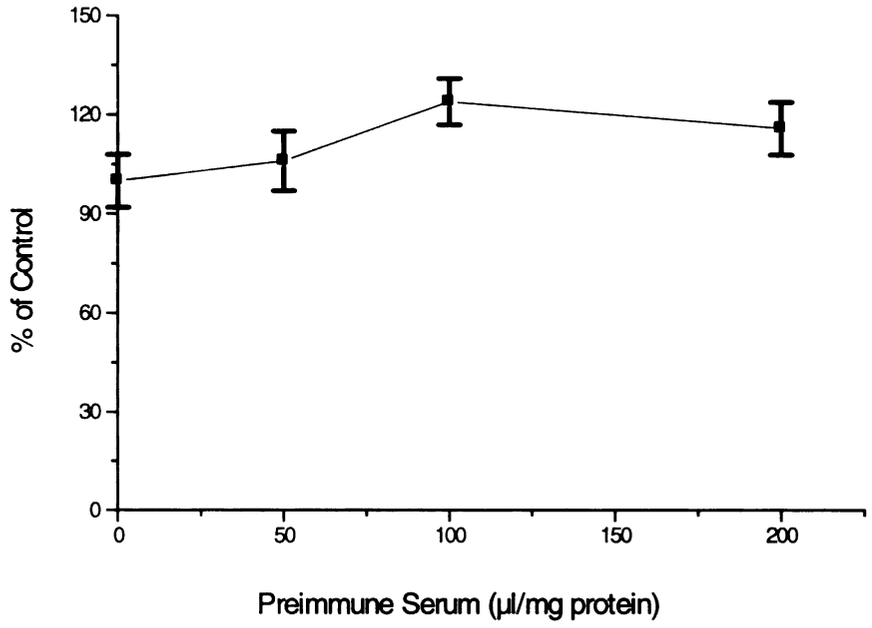


Figure 3-7. The effect of pre-immune serum on Dg3 metabolism in rat liver microsomes.

3.4.4 Pharmacokinetic parameters

The effects of ketoconazole on digoxin pharmacokinetics were also investigated in rats. Plasma concentration *vs.* time profiles after *iv* and oral administration of digoxin at 0.2 mg/kg to rats are shown in Figure 3-8. The effects of oral KCZ at 80 mg/kg, a dose that was shown to induce Pgp in rat intestine after chronic administration (Lown *et al.*, 1996), on digoxin pharmacokinetics *via iv* and *p.o.* administration routes are shown in Figures 3-9 and 3-10, respectively. For both *iv* and oral administrations, digoxin AUCs markedly increased and elimination decreased in the presence of 80 mg/kg of ketoconazole. In the case of oral digoxin, digoxin C_{max} also increased, while T_{max} decreased.

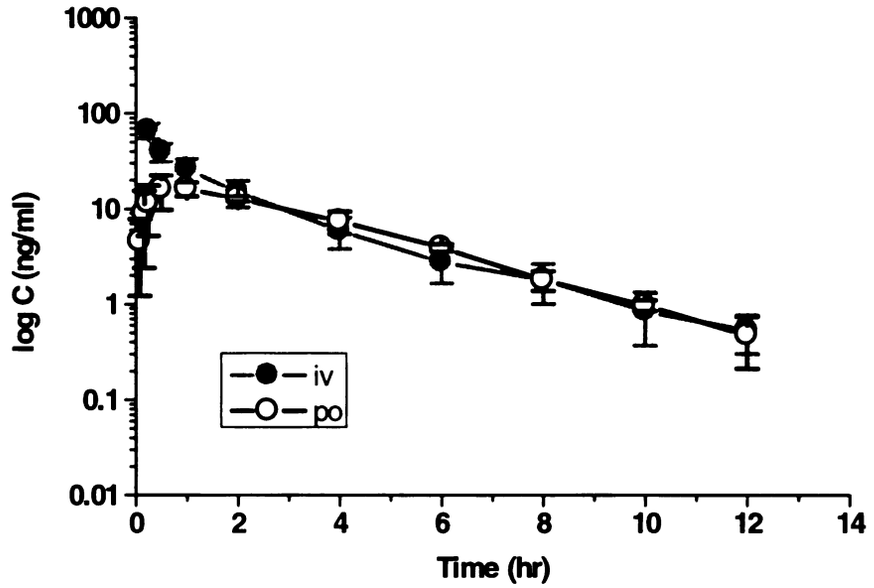


Figure 3-8. Time courses of intravenously and orally administered digoxin (0.2 mg/kg). Data represent the means of at least three rats ± SD.

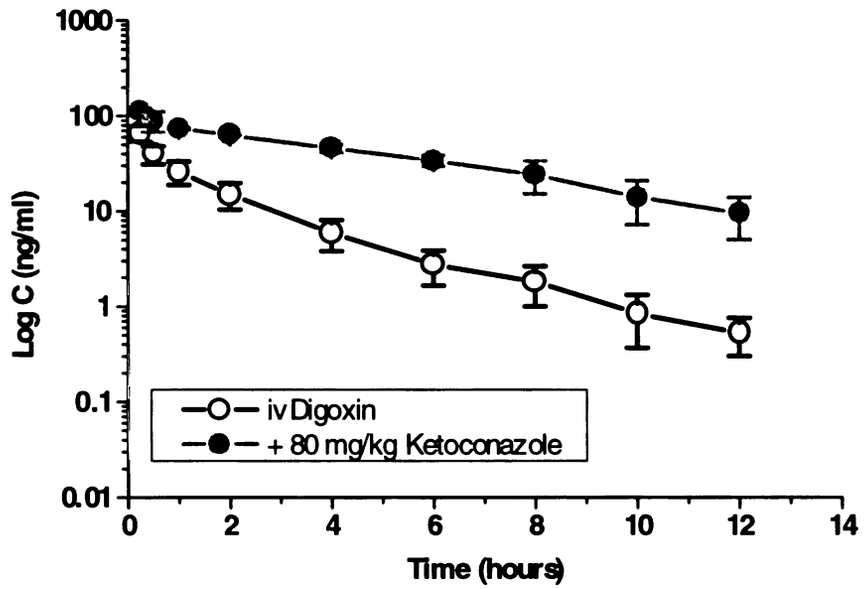


Figure 3-9. Time course of *iv* administered digoxin (0.2 mg/kg), with and without a concomitant oral dose (80 mg/kg) of ketoconazole. Data represent the mean of at least three rats ± SD.

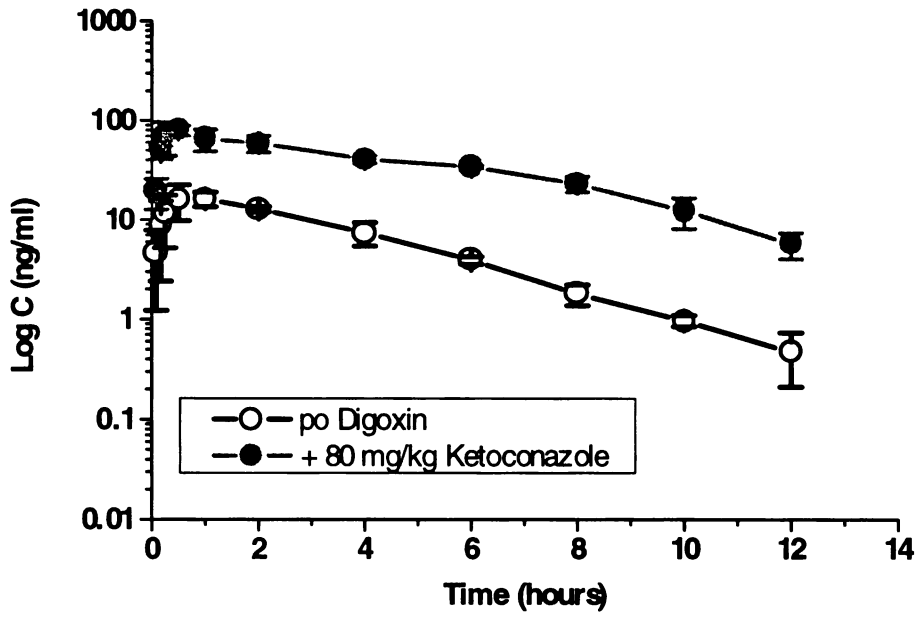


Figure 3-10. Time course of orally administered digoxin (0.2 mg/kg), with and without a concomitant oral dose (80 mg/kg) of ketoconazole. Data represent the mean of at least three rats ± SD.

We also dosed rats KCZ at a lower dose with *iv* and *p.o.* digoxin. Figures 3-11 and 3-12 show the effects of 10 mg/kg KCZ on digoxin concentration *vs.* time profiles (*via iv* and *p.o.* administrations, respectively). Compared to 80 mg/kg, 10 mg/kg of ketoconazole had a much smaller effect of digoxin (*iv* and *p.o.*) plasma concentration *vs.* time profiles.

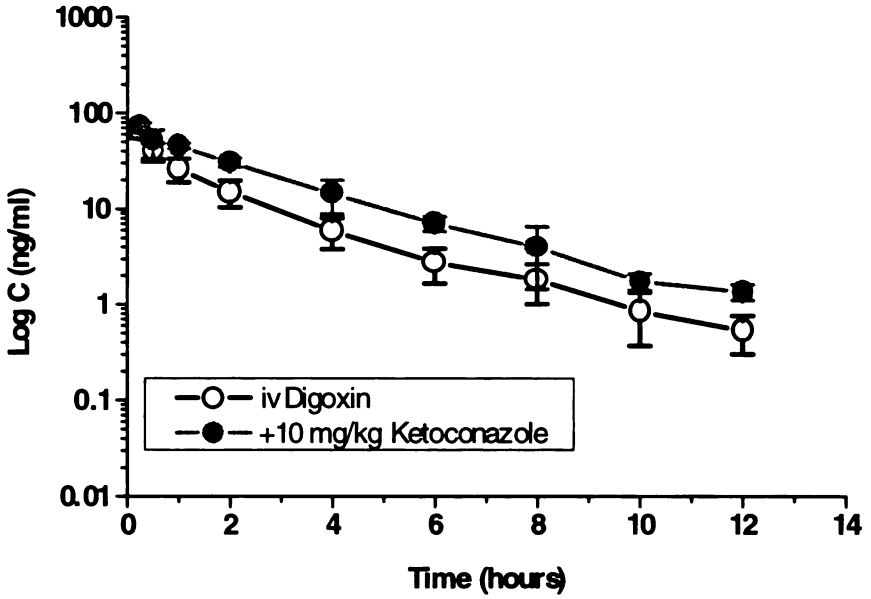


Figure 3-11. Time course of *iv* administered digoxin (0.2 mg/kg), with and without a concomitant oral dose (10 mg/kg) of ketoconazole. Data represent the mean of at least three rats ± SD.

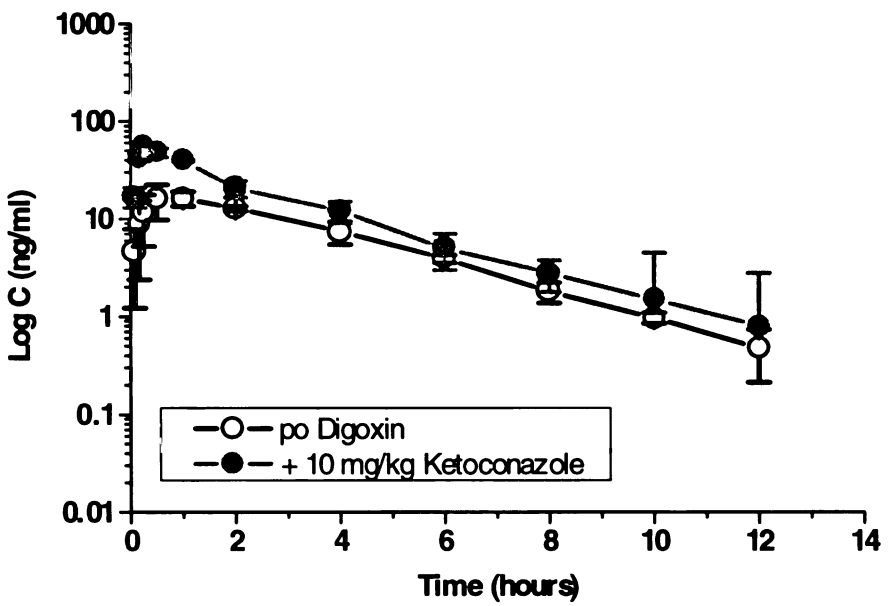


Figure 3-12. Time course of orally administered digoxin (0.2 mg/kg), with and without a concomitant oral dose (10 mg/kg) of ketoconazole. Data represent the mean of at least three rats ± SD.

Assuming digoxin disposition is linear following oral and *iv* dosing with concomitant ketoconazole, the kinetic parameters for digoxin dosed at 0.2 mg/kg in rats are listed in Table 3-2.

C_{max} of oral digoxin increased from 18.4 ng/ml to approximately 84 ng/ml after both 10 and 80 mg/kg oral administrations of ketoconazole. T_{peak} decreased from 0.75 hr to 0.5 hr and 0.38 hr with concomitant KCZ at 10 mg/kg and 80 mg/kg, respectively.

In the presence of 80 mg/kg of ketoconazole, digoxin AUCs increased 6 and 4.6 fold after oral or *iv* administration, respectively, and the apparent bioavailability increased from 62% to 83%. While 10 mg/kg ketoconazole increased *iv* and *p.o.* digoxin AUCs by 1.6 and 1.9 fold, respectively and F increased slightly from 61.9% to 70.2%. Time averaged CL decreased from 1.8 L/hr/kg to 1.1 L/hr/kg to 0.4 L/hr/kg, and V_{ss} decreased from 4.4 L/kg to 2.8 L/kg to 1.9 L/kg when controls are compared to concomitant 10 mg/kg and 80 mg/kg ketoconazole, respectively.

Digoxin MRT increased in the presence of ketoconazole in a dose dependent manner. Note that the terminal half-life does not parallel the changes in MRT. This is not unexpected since MRT represents an overall "half-life" measure for total drug in the body, *versus* $T_{1/2,z}$ which is only the terminal elimination half-life from the plasma.

MAT of oral digoxin decreased from 0.97 hr to 0.52 hr by co-administration of 10 mg/kg KCZ and was further reduced to 0.32 hr by 80 mg/kg KCZ.

Table 3-2. Pharmacokinetic parameters, obtained by non-compartmental WinNonlin methods, of digoxin at 0.2 mg/kg (*iv* and *p.o.*) with or without ketoconazole.

	C_{max} (ng/ml)	T_{peak} (hr)	AUC (ng*hr/ml)	Time Avg CL (L/hr/kg)	$T_{1/2,z}$ (hr)	MRT (hr)	F (%)	V_{ss} (L/kg)	MAT (hr)
***iv* Digoxin (n=4)**			114 ± 25	1.8 ± 0.5	2.7 ± 1.1	2.4 ± 0.6		4.4 ± 1.4	
+ 10 mg/kg KCZ (n=3)			188 ± 12	1.1 ± 0.1	2.0 ± 0.2	2.6 ± 0.4		2.8 ± 0.6	
+ 80 mg/kg KCZ (n=3)			519 ± 62	0.4 ± 0.1	2.9 ± 1.1	4.9 ± 1.2		1.9 ± 0.3	
***p.o.* Digoxin (n=4)**	18.4 ± 4.6	0.75 ± 0.29	70.6 ± 11.1		1.8 ± 0.6		61.9		0.97
+ 10 mg/kg KCZ (n=3)	84.5 ± 2.0	0.50 ± 0.10	132 ± 10		1.9 ± 0.5		70.2		0.52
+ 80 mg/kg KCZ (n=4)	83.8 ± 13.6	0.38 ± 0.14	432 ± 62		2.3 ± 0.6		83.2		0.32

Data are the mean of rats ± SD.

No SD for F and MAT due to non-crossover study.

The calculated values for CL, V_{ss} and MAT, as given in Table 3-2, were fit to Eq. 2 using linear un-weighted and weighted regressions. The un-weighted regression for CL is depicted in Figure 3-13.

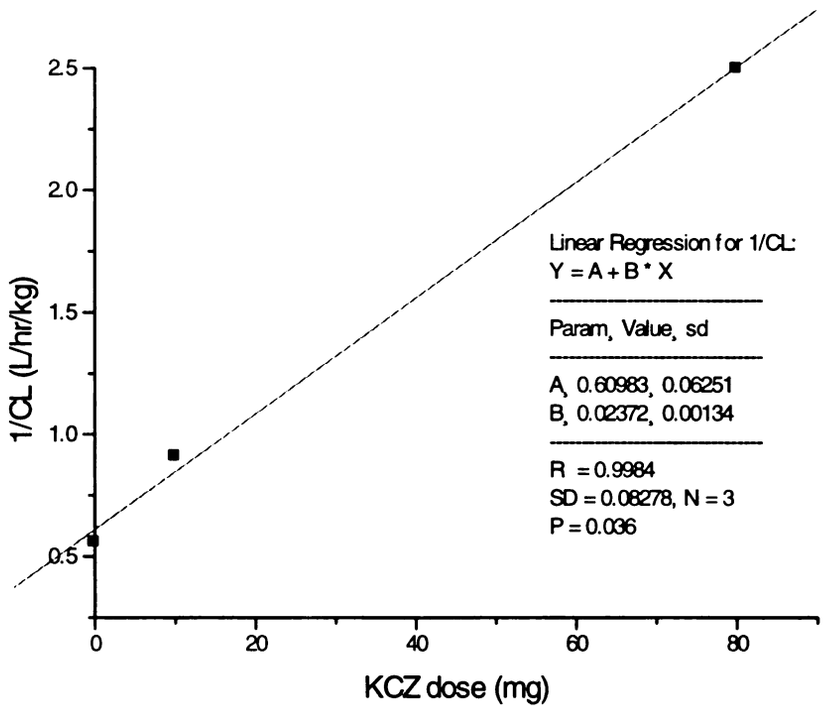


Figure 3-13. The un-weighted linear fits of the digoxin clearance *vs.* ketoconazole dose.

The y-axis intercept for this plot (CL_{NM} = 1.6) compares favorably with the CL_0 value of 1.8 from Table 3-2. The dose of ketoconazole calculated to halve CL (MD_{50}) is 26 mg. The r^2 for this fit is 0.997. The results of the data fits to Eq. 2 for CL, V_{ss} and MAT are summarized in Table 3-3.

Table 3-3. The calculated linear fits of Eq. 2 for the time-averaged non-compartmental values for CL, V_{ss} and MAT from the three sets of studies (0, 10 and 80 mg KCZ) using both un-weighted and weighted linear regressions.

Pharmacokinetic parameters		**Regression r^2**	**Un-weighted linear fit**		**[a]Weighted linear fit**	
P_D	**P_0**		**P_{NM}**	**MD_{50} (mg)**	**P_{NM}**	**MD_{50} (mg)**
CL (L/hr/kg)	1.8	0.997	1.6	26	1.5	30
V_{ss} (L/kg)	4.4	0.893	3.7	84	3.2	117
MAT (hr)	0.97	0.899	0.75	59	0.64	79

[a]For CL and V_{ss}, the weights were taken as the inverse of the standard deviations squared for the means of the parameters in each set of studies. For MAT, the weights were taken as the inverse of the parameter squared.

Weighting had little effect on the calculated P_{NM} and MD_{50} for CL, reflective of the excellent fit obtained. As would be expected for V_{ss} and MAT, where $r^2 = 0.9$, weighting gave the parameters from 80 mg/kg ketoconazole more influence resulting in lower P_{NM} and higher MD_{50} values (Table 3-3).

3.5 Discussion

In preparation for carrying out a digoxin-ketoconazole interaction study in humans, we evaluated the effects of ketoconazole on digoxin *iv* and oral pharmacokinetics in rats. Since digoxin is not a substrate for CYP3A enzymes in humans

(Lacarelle *et al.*, 1991), as confirmed by the present metabolism studies of digoxin in rat and human liver microsomes, our proposed study should allow us to evaluate the importance of Pgp in digoxin elimination in humans.

Digoxin is metabolized by stepwise cleavage of the sugar residues to Dg2, Dg1 and Dg0 (Harrison and Gibaldi, 1976). It was also observed in rat liver microsomes (Salphati and Benet, 1999) that the cleavage of Dg2 was much slower than its formation. This is consistent with the fact that *in vivo* in rat, Dg2 is the main metabolite (Klaassen, 1974; Wirth and Frölich, 1974). After being formed, Dg2 is most likely conjugated and excreted at a much faster rate.

In the previous metabolism study (Lacarelle *et al.*, 1991), tritiated substrates were separated by HPLC. Here, an HPLC analytical method was used for non-radioactive substrates to study digoxin metabolism in human and rat liver microsomes. This method is specific for Dg2, with no interference from Dg3 in the assay. However, the sensitivity of the method is low, requiring that a high concentration of digoxin, such as 50 μM to be used in the incubation. In the previous (Salphati and Benet, 1998) and present rat kinetic study, the micro-particle enzyme immuno-assay (MEIA) method was utilized. This method can not distinguish between digoxin and its active metabolites. However, according to the supplier of the assay, the cross reactivity is low: "The digoxin metabolites Dg0, Dg1 and Dg2, tested at concentrations corresponding to high plasma levels in digoxin patients (0.132, 0.088 and 0.22 ng/ml, respectively), showed digoxin concentrations which were below assay sensitivity". This method is easy to carry out and sensitive, and thus the method is widely used in clinical assays of digoxin.

The cleavage of the terminal digitoxosyl from Dg3, Dg2 and Dg1 is thought to involve two steps- a dehydrogenation of the axial hydroxyl group from the terminal digitoxose, which is CYP-mediated, followed by a non-enzymatic terminal dehydro-digitoxosyl split off (Schmoldt and Ahsendorf, 1980). The apparent Michaelis-Menten parameters reported for the first time by Salphati and Benet (1999), represent the two necessary steps in the cleavage of each digitoxose.

Salphati and Benet (1999) showed that from among the many inhibitors tested, that is competitive inhibitors- quinidine (CYP2D), sulfinpyrazone (CYP2C) and chlorzoxazone (CYP2E1) and mechanism-based inhibitors– triacetyloleandomycin (CYP3A), diethyldithiocarbamate (CYP2E1) and furafylline (CYP1A2), only triacetyloleandomycin markedly inhibited Dg3 metabolism. The minor inhibition observed with diethyldithiocarbamate was likely to be due to a slight inhibition of CYP3A (Chang *et al.*, 1994; Newton *et al.*, 1995). However, furafylline, although specific, is not a very potent inhibitor of the rat CYP1A2 (Sesardic *et al.*, 1990).

Here, chemical inhibition of CYP3A by ICZ and KCZ resulted in significant inhibition of Dg3 metabolism in rat liver microsomes. This was further confirmed using specific antibodies to rat CYP3A2, while pre-immune sera had no effect on Dg3 metabolism. The involvement of CYP3A was also consistent with the increased metabolism of Dg3 in rats following chronic administration of inducers of this enzyme (Klaassen, 1974; Wirth and Frölich, 1974).

In our study, the bioavailability of digoxin was 61.9%, which is comparable to what have been previously reported in rat (Rietbrock *et al.*, 1972) and guinea pig (Balkon and Donnelly, 1992). Evans *et al.* (1990) and Harrison and Gibaldi (1976) observed

systemic intravenous digoxin clearances ranging from 7.5 to 16 ml/min/kg after *i.v.* dosing. Our higher CL_{iv} (30 ml/min/kg) may be attributed to the differences in age and strains of the rats used, as reported in the latter study. In the previous studies (such as Harrison and Gibaldi 1976), a thin-layer chromatography method was used to separate tritium labeled Dg3 and Dg2 from other metabolites in plasma and urine. Here, the MEIA method was used and the cross-reactivity between digoxin and its metabolites may also be responsible for the differences observed.

In the present study, ketoconazole markedly decreased digoxin clearance following *iv* digoxin administration, 1.8 L/hr/kg to 1.1 L/hr/kg to 0.4 L/hr/kg. Our data do not allow us to determine which pathway was most affected, but previous studies (Braunschweig *et al.*, 1987) demonstrate that digoxin biliary clearance was reduced by 50% after administration of amiodarone. Since renal elimination accounts for only 10-15% of digoxin clearance in rats (Harrison and Gibaldi, 1976), it is likely that ketoconazole affects the biliary clearance of digoxin more than its effects on the metabolism of digoxin. These data and the results of Dg3 metabolism *in vitro* (Lacarelle *et al.*, 1991; Salphati and Benet, 1999 and here) suggest that the ketoconazole-digoxin interaction observed here may be more reflective of an effect on transport processes than metabolic processes.

As previously described in Chapter 2, digoxin is a known substrate of Pgp. Studies in LLC-PK1 cells transfected with the human MDR1 cDNA (Tanigawara *et al.*, 1992 and Chapter 4) showed that digoxin was transported by Pgp. Okamura *et al.* (1993), using a kidney epithelial cell line and the isolated perfused rat kidney, showed that apical to basal transport and renal tubular secretion of digoxin were reduced by cyclosporine. Su

and Huang (1996) studied digoxin bioavailability in everted gut sacs from rats and showed that digoxin intestinal absorption increased in the presence of quinidine. The intestinal and total clearance of digoxin were reduced when rats were co-infused with quinidine, which may also affect extra-intestinal such as biliary or renal elimination pathways involving Pgp. Similarly, investigations using perfused rat kidney (Hori *et al.*, 1993) concluded that this drug was a Pgp substrate in rats.

Studies in knockout mice showed that digoxin tissue distribution and pharmacokinetics (Schinkel *et al.*, 1995) were strongly affected by the mouse mdr1a P-gp activity. Mayer *et al.* (1996) and Schinkel *et al.* (1997) demonstrated in mdr1a and mdr1a/1b knock-out mice that elimination of digoxin was mediated, at least partly, by the Pgp located in the intestine. Digoxin bioavailability was improved in knock-out mice and the direct intestinal secretion decreased from 16% of the dose in wild-type to 2% in the knock-out mice (Mayer *et al.*, 1996). Brain accumulation of digoxin was also significant in knock-out mice (Mayer *et al.*, 1996; Schinkel *et al.*, 1996). However, mice homozygous for mdr1a/1b gene disruption (Schinkel *et al.*, 1997) showed that digoxin biliary secretion, although reduced, still occurred, suggesting that another mechanism for biliary excretion exists in the liver.

Increases in digoxin concentrations have previously been reported in rats after intravenous administration of amiodarone (Braunschweig *et al.*, 1987; Weinhouse *et al.*, 1988) and verapamil (Weinhouse *et al.*, 1985), both inhibitors of Pgp (Ford and Hait, 1990) and substrates for CYP3A (Wacher *et al.*, 1995). In all of these studies, either the clinically relevant nonspecific immunoassay methods or radiolabeled compounds coupled HPLC methods have been used. The immunoassay methods do not have the specificity to

distinguish digoxin from its active metabolites and cannot be used to determine digoxin concentrations in urine (Chapter 2). While the HPLC methods are not sensitive enough to analyze digoxin at low concentrations (Chapter 2).

In the present work, MAT dropped significantly from 0.97 hr to 0.52 hr then to 0.32 hr with increasing ketoconazole doses, reflecting the effect of ketoconazole increasing the digoxin rate of absorption. Since CYP3A is involved in digoxin elimination in rats (Salphati and Benet, 1999), it is not possible to differentiate the effects of ketoconazole on metabolism *vs.* Pgp inhibition in terms of AUC, elimination and bioavailability in rats. However, the marked effect of ketoconazole on digoxin mean absorption time can be explained only by an effect of ketoconazole on intestinal Pgp in rats.

This is consistent with our proposed intestinal barrier of CYP3A and Pgp (Salphati, 1998). KCZ would tend to reduce T_{peak} by inhibiting Pgp and increasing absorption rate, and also tend to increase T_{peak} by decreasing elimination of digoxin by inhibiting CYP3A and Pgp. Looking only at T_{peak}, it may be difficult to attribute changes in T_{peak} to changes in absorption versus changes in elimination, although the decrease in T_{peak} observed here (Table 3-2) is consistent with a greater effect of KCZ on absorption rate. However, the MAT measures are only an effect of the absorption rate.

Our results are consistent with the previous study from our laboratory (Salphati and Benet, 1998), in which only 80 mg/kg ketoconazole was dosed. The data reported here for digoxin alone and digoxin plus 80 mg/kg ketoconazole are from rats, reported by Salphati and Benet (1998) where I assisted in the studies. Calculated parameters differ slightly from the previous study where the log trapezoidal rules was used to calculate

AUC values for declining plasma concentrations. Here, we obtained the pharmacokinetic parameters by WinNonlin analysis, using the linear trapezoidal rules for all AUC measurements as recommended by the FDA.

The excellent linear fits of the digoxin clearance *vs.* ketoconazole dose (Figure 3-13) suggests that the pharmacokinetics of digoxin in the rat are well represented by assuming that the drug's elimination kinetics are linear both without and with concomitant KCZ and that a competitive modulator model is appropriate *in vivo*. Although the shapes of the terminal portions of the mean curves following *p.o.* and *iv* digoxin with 80 mg ketoconazole (Figures 3-9 and 3-10) might suggest some degree of non-linearity, the total areas are not sufficiently affected to skew the regression plot (Figure 3-13).

We did not observe Dg2 formed after incubation of Dg3 in human liver microsomes, which is consistent with Dg3 being mostly excreted unchanged in humans (Rodin and Johnson, 1988) and with previous studies reporting that no metabolism can be detected after incubation of Dg3 with human liver microsomes or hepatocytes (Lacarelle *et al.*, 1991). In addition, when stepwise cleavage of Dg3 does occur in humans, this reaction is mediated by intra-gastric hydrolysis, in which the extent varies depending on the gastric pH (Gault *et al.*, 1980 and 1981) or intestinal bacteria (Chapter 2). Our results and the previous studies (Salphati and Benet, 1998 and 1999) indicate that digoxin metabolic and kinetic profiles in rats and humans are quite different.

3.6 References

Antman, E. M. and Smith, T. W. (1985). Digitalis toxicity. *Annual Review of Medicine* **36,** 357-67.

Balkon, J. and Donnelly, B. (1992). Comparative kinetics of digoxin in serum and vitreous humor in a guinea pig model. II. Single oral dose administration. *Journal of Analytical Toxicology* **16,** 155-7.

Belz, G. G., Doering, W., Munkes, R. and Matthews, J. (1983). Interaction between digoxin and calcium antagonists and antiarrhythmic drugs. *Clinical Pharmacology and Therapeutics* **33,** 410-7.

Benet, L. Z. and Galeazzi, R. L. (1979). Non-compartmental determination of the steady-state volume of distribution. *Journal of Pharmaceutical Sciences* **68,** 1071-4.

Borst, P., Schinkel, A. H., Smit, J. J., Wagenaar, E., Van Deemter, L., Smith, A. J., Eijdems, E. W., Baas, F. and Zaman, G. J. (1993). Classical and novel forms of multidrug resistance and the physiological functions of P-glycoproteins in mammals. *Pharmacology and Therapeutics* **60,** 289-99.

Bourrié, M., Meunier, V., Berger, Y. and Fabre, G. (1996). Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalyzed by human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics* **277,** 321-32.

Braunschweig, J., Stäubli, M. and Studer, H. (1987). Interactions of amiodarone with digoxin in rats. *British Journal of Pharmacology* **92,** 553-9.

Calvo, M. V., Martin-Suarez, A., Martin Luengo, C., Avila, C., Cascon, M. and Domínguez-Gil Hurlé, A. (1989). Interaction between digoxin and propafenone. *Therapeutic Drug Monitoring* **11,** 10-5.

Chang, T. K., Gonzalez, F. J. and Waxman, D. J. (1994). Evaluation of triacetyloleandomycin, alpha-naphthoflavone and diethyldithiocarbamate as

selective chemical probes for inhibition of human cytochromes P450. *Archives of Biochemistry and Biophysics* **311,** 437-42.

Cheng, H. Y. and Jusko, W. J. (1989). Application of mean residence-time concepts to pharmacokinetic systems with non-instantaneous input and nonlinear elimination. *Pharmaceutical Research* **6,** 4-12.

Chin, K. V., Pastan, I. and Gottesman, M. M. (1993). Function and regulation of the human multidrug resistance gene. *Advances in Cancer Research* **60,** 157-80.

Cordon-Cardo, C. and O'Brien, J. P. (1991). The multidrug resistance phenotype in human cancer. *Important Advances in Oncology* **27,** 19-38.

Evans, R. L., Owens, S. M., Ruch, S., Kennedy, R. H. and Seifen, E. (1990). The effect of age on digoxin pharmacokinetics in Fischer-344 rats. *Toxicology and Applied Pharmacology* **102,** 61-7.

Ford, J. M. and Hait, W. N. (1990). Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacological Reviews* **42,** 155-99.

Gault, H., Kalra, J., Ahmed, M., Kepkay, D. and Barrowman, J. (1980). Influence of gastric pH on digoxin biotransformation. I. Intragastric hydrolysis. *Clinical Pharmacology and Therapeutics* **27,** 16-21.

Gault, H., Kalra, J., Ahmed, M., Kepkay, D., Longerich, L. and Barrowman, J. (1981). Influence of gastric pH on digoxin biotransformation. II. Extractable urinary metabolites. *Clinical Pharmacology and Therapeutics* **29,** 181-90.

Gibbs, M. A., Thummel, K. E., Shen, D. D. and Kunze, K. L. (1999). Inhibition of cytochrome P-450 3A (CYP3A) in human intestinal and liver microsomes:

comparison of Ki values and impact of CYP3A5 expression. *Drug Metabolism and Disposition* **27,** 180-7.

Gottesman, M. M. and Pastan, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual Review of Biochemistry* **62,** 385-427.

Guengerich, F. P. (1989). Analysis and characterization of enzymes. In *Principles and Methods of Toxicology* (ed. Hayes, A. W.), pp. 777-814. New York: Raven Press.

Harrison, L. I. and Gibaldi, M. (1976). Pharmacokinetics of digoxin in the rat. *Drug Metabolism and Disposition* **4,** 88-93.

Heller, M. (1990). Cardiac glycosides. New/old ideas about old drugs. *Biochemical Pharmacology* **40,** 919-25.

Hori, R., Okamura, N., Aiba, T. and Tanigawara, Y. (1993). Role of P-glycoprotein in renal tubular secretion of digoxin in the isolated perfused rat kidney. *Journal of Pharmacology and Experimental Therapeutics* **266,** 1620-5.

Kelly, R. A. and Smith, T. W. (1996). Pharmacological treatment of heart failure. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (ed. Hardman, J. G. and Limbird, L. E.), pp. 809-838. New York: McGraw-Hill.

Klaassen, C. D. (1974). Effect of microsomal enzyme inducers on the biliary excretion of cardiac glycosides. *Journal of Pharmacology and Experimental Therapeutics* **191,** 201-11.

Kolars, J. C., Lown, K. S., Schmiedlin-Ren, P., Ghosh, M., Fang, C., Wrighton, S. A., Merion, R. M. and Watkins, P. B. (1994). CYP3A gene expression in human gut epithelium. *Pharmacogenetics* **4,** 247-59.

Kunze, K. L., Wienkers, L. C., Thummel, K. E. and Trager, W. F. (1996). Warfarin-fluconazole. I. Inhibition of the human cytochrome P450-dependent metabolism of warfarin by fluconazole: in vitro studies. *Drug Metabolism and Disposition* **24,** 414-21.

Lacarelle, B., Rahmani, R., de Sousa, G., Durand, A., Placidi, M. and Cano, J. P. (1991). Metabolism of digoxin, digoxigenin digitoxosides and digoxigenin in human hepatocytes and liver microsomes. *Fundamental and Clinical Pharmacology* **5,** 567-82.

Lampen, A., Christians, U., Guengerich, F. P., Watkins, P. B., Kolars, J. C., Bader, A., Gonschior, A. K., Dralle, H., Hackbarth, I. and Sewing, K. F. (1995). Metabolism of the immunosuppressant tacrolimus in the small intestine: cytochrome P450, drug interactions, and interindividual variability. *Drug Metabolism and Disposition* **23,** 1315-24.

Leveille-Webster, C. R. and Arias, I. M. (1995). The biology of the P-glycoproteins. *Journal of Membrane Biology* **143,** 89-102.

Lown, K. S., Kolars, J. C., Ghosh, M., Schmiedlin-Ren, P. and Watkins, P. B. (1996). Induction of MDR1 expression in normal rat and human intestine in vivo. *Gastroenterology* **110,** A344.

Lum, B. L., Fisher, G. A., Brophy, N. A., Yahanda, A. M., Adler, K. M., Kaubisch, S., Halsey, J. and Sikic, B. I. (1993). Clinical trials of modulation of multidrug resistance. Pharmacokinetic and pharmacodynamic considerations. *Cancer* **72,** 3502-14.

Maurice, M., Pichard, L., Daujat, M., Fabre, I., Joyeux, H., Domergue, J. and Maurel, P. (1992). Effects of imidazole derivatives on cytochromes P450 from human hepatocytes in primary culture. *FASEB Journal* **6,** 752-8.

Mayer, U., Wagenaar, E., Beijnen, J. H., Smit, J. W., Meijer, D. K., van Asperen, J., Borst, P. and Schinkel, A. H. (1996). Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. *British Journal of Pharmacology* **119,** 1038-44.

Newton, D. J., Wang, R. W. and Lu, A. Y. (1995). Cytochrome P450 inhibitors. Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metabolism and Disposition* **23,** 154-8.

Okamura, N., Hirai, M., Tanigawara, Y., Tanaka, K., Yasuhara, M., Ueda, K., Komano, T. and Hori, R. (1993). Digoxin-cyclosporin A interaction: modulation of the multidrug transporter P-glycoprotein in the kidney. *Journal of Pharmacology and Experimental Therapeutics* **266,** 1614-9.

Pichard, L., Fabre, J. M., Domergue, J., Fabre, G., Saint-Aubert, B., Mourad, G. and Maurel, P. (1991). Molecular mechanism of cyclosporine A drug interactions: inducers and inhibitors of cytochrome P450 screening in primary cultures of human hepatocytes. *Transplantation Proceedings* **23,** 978-9.

Rietbrock, N., Abshagen, U., Bergmann, K. v. and Kewitz, H. (1972). Pharmacokinetics of digoxin and its 4'-acetyl- and methyl- derivatives in the rat. *Naunyn-Schmiedebergs Archives of Pharmacology* **274,** 171-81.

Rodin, S. M. and Johnson, B. F. (1988). Pharmacokinetic interactions with digoxin. *Clinical Pharmacokinetics* **15,** 227-44.

Ruetz, S. and Gros, P. (1994). Phosphatidylcholine translocase: a physiological role for the mdr2 gene. *Cell* **77,** 1071-81.

Salphati, L. (1998). P-glycoprotein and Cytochrome P450 3A: Potential Interactions and Complementary Roles in Drug Absorption and Disposition. Ph.D. Dissertation, p. 18-22. Paris, France: Universite Paris.

Salphati, L. and Benet, L. Z. (1998). Effects of ketoconazole on digoxin absorption and disposition in rat. *Pharmacology* **56,** 308-13.

Salphati, L. and Benet, L. Z. (1999). Metabolism of digoxin and digoxigenin digitoxosides in rat liver microsomes: involvement of cytochrome P4503A. *Xenobiotica* **29,** 171-85.

Schinkel, A. H., Smit, J. J., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Mol, C. A., van der Valk, M. A., Robanus-Maandag, E. C., te Riele, H. P., Berns, A. J. M. and Borst, P. (1994). Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77,** 491-502.

Schinkel, A. H., Wagenaar, E., van Deemter, L., Mol, C. A. and Borst, P. (1995). Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *Journal of Clinical Investigation* **96,** 1698-705.

Schinkel, A. H., Wagenaar, E., Mol, C. A. and van Deemter, L. (1996). P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *Journal of Clinical Investigation* **97,** 2517-24.

Schinkel, A. H., Mayer, U., Wagenaar, E., Mol, C. A., van Deemter, L., Smit, J. J., van der Valk, M. A., Voordouw, A. C., Spits, H., van Tellingen, O., Zijlmans, J. M., Fibbe, W. E. and Borst, P. (1997). Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 4028-33.

Schmoldt, A. and Ahsendorf, B. (1980). Cleavage of digoxigenin digitoxosides by rat liver microsomes. *European Journal of Drug Metabolism and Pharmacokinetics* **5,** 225-32.

Sesardic, D., Boobis, A. R., Murray, B. P., Murray, S., Segura, J., de la Torre, R. and Davies, D. S. (1990). Furafylline is a potent and selective inhibitor of cytochrome P450IA2 in man. *British Journal of Clinical Pharmacology* **29,** 651-63.

Siegsmund, M. J., Cardarelli, C., Aksentijevich, I., Sugimoto, Y., Pastan, I. and Gottesman, M. M. (1994). Ketoconazole effectively reverses multidrug resistance in highly resistant KB cells. *Journal of Urology* **151,** 485-91.

Silverman, J. A. and Schrenk, D. (1997). Hepatic canalicular membrane 4: expression of the multidrug resistance genes in the liver. *FASEB Journal* **11,** 308-13.

Silverman, J. A. and Thorgeirsson, S. S. (1995). Regulation and function of the multidrug resistance genes in liver. *Progress in Liver Diseases* **13,** 101-23.

Smit, J. J., Schinkel, A. H., Oude Elferink, R. P., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A., Ottenhoff, R., van der Lugt, N. M., van Roon, M. A., Offerhaus, G. J. A., Berns, A. J. M. and Borst, P. (1993). Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* **75,** 451-62.

Staubus, A. E. and Smith, S. F. (1984). Concept of time-averaged clearance and mean residence time in Michaelis-Menten elimination system. In *Pharmacokinetics- A modern View* (ed. Benet, L. Z., Levy, G. and Ferraiolo, B. L.), pp. 411-414. New York: Plenum Press.

Su, S. F. and Huang, J. D. (1996). Inhibition of the intestinal digoxin absorption and exsorption by quinidine. *Drug Metabolism and Disposition* **24,** 142-7.

Tanigawara, Y., Okamura, N., Hirai, M., Yasuhara, M., Ueda, K., Kioka, N., Komano, T. and Hori, R. (1992). Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). *Journal of Pharmacology and Experimental Therapeutics* **263,** 840-5.

Tsuruo, T., Iida, H., Kitatani, Y., Yokota, K., Tsukagoshi, S. and Sakurai, Y. (1984). Effects of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and adriamycin in drug-resistant tumor cells. *Cancer Research* **44,** 4303-7.

Upton, R. A. (1975). Simple and reliable method for serial sampling of blood from rats. *Journal of Pharmaceutical Sciences* **64,** 112-4.

van der Graaf, W. T., de Vries, E. G., Uges, D. R., Nanninga, A. G., Meijer, C., Vellenga, E., Mulder, P. O. and Mulder, N. H. (1991). In vitro and in vivo modulation of multi-drug resistance with amiodarone. *International Journal of Cancer* **48,** 616-22.

von Moltke, L. L., Greenblatt, D. J., Schmider, J., Duan, S. X., Wright, C. E., Harmatz, J. S. and Shader, R. I. (1996). Midazolam hydroxylation by human liver microsomes

in vitro: inhibition by fluoxetine, norfluoxetine, and by azole antifungal agents. *Journal of Clinical Pharmacology* **36,** 783-91.

Wacher, V. J., Wu, C. Y. and Benet, L. Z. (1995). Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: Implications for drug delivery and activity in cancer chemotherapy. *Molecular Carcinogenesis* **13,** 129-134.

Weinhouse, E., Kaplanski, J. and Genchik, G. (1985). Plasma and tissue levels of digoxin in the rat following pretreatment with verapamil. *Research Communications in Chemical Pathology and Pharmacology* **47,** 469-72.

Weinhouse, E., Kaplanski, J., Zalzstein, E., Genchik, G., Colton, D. and Nudel, D. (1988). Mechanisms of digoxin-amiodarone interaction in the rat. *Proceedings of the Society for Experimental Biology and Medicine* **188,** 96-102.

Wirth, K. E. and Frölich, J. C. (1974). Effect of spironolactone on excretion of 3H-digoxin and its metabolites in rats. *European Journal of Pharmacology* **29,** 43-51.

Woodland, C., Verjee, Z., Giesbrecht, E., Koren, G. and Ito, S. (1997). The digoxin-propafenone interaction: characterization of a mechanism using renal tubular cell monolayers. *Journal of Pharmacology and Experimental Therapeutics* **283,** 39-45.

Wrighton, S. A. and Ring, B. J. (1994). Inhibition of human CYP3A catalyzed 1'-hydroxy midazolam formation by ketoconazole, nifedipine, erythromycin, cimetidine, and nizatidine. *Pharmaceutical Research* **11,** 921-4.

Yusa, K. and Tsuruo, T. (1989). Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and

transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Research* **49,** 5002-6.

Chapter 4

CHARACTERIZATION OF DIGOXIN TRANSPORT IN *VITRO*

4.1 Abstract

The objective of this work was to determine whether transporter(s) including Pgp, but particularly MRP1 and cMOAT, may be involved in the secretion of digoxin *in vitro*.

[^{3}H]Digoxin transepithelial secretion activity was characterized in cell culture systems. The wild-type cell lines utilized were Caco-2, MDCKI and MDCKII (distal tubule epithelial cells of Madin-Darby canine kidney) as well as LLC-PK1 (proximal tubule of porcine kidney). The transfected cell lines include MDR1-MDCK, MDCKII-MDR1 and LLC-PK1-MDR1 (MDCKI, MDCKII and LLC-PK1 cells transfected with human MDR1 cDNA, respectively), LLC-PK1-MRP1 (LLC-PK1 cells transfected with human MRP1 cDNA) and MDCKII-cMOAT (MDCKII cells transfected with human cMOAT cDNA). The integrity of the cell monolayers were measured by transepithelial electrical resistance (TEER) and [^{14}C]mannitol leakage.

Digoxin transport across cultured Caco-2 and kidney epithelial cells was found to be greater in the direction of secretion and displayed temperature-dependence. This is consistent with our hypothesis that this drug may be actively secreted in the small intestine, liver and kidney. The net basal to apical secretion of digoxin was much higher across MDR1 cDNA transfected than across wild-type LLC-PK1 epithelia. A similar inhibition pattern of digoxin transport by MRP inhibitors was observed in wild-type and MRPs transfected cells, suggesting the presence of endogenous MRP1 or an unidentified absorptive transporter in LLC-PK1 cells, and Pgp or cMOAT or an unidentified secretory transporter in MDCKII cells.

Our results indicate that multiple polarized transporters including Pgp, MRP1, MRP2 and an uncharacterized uptake or secretory transporter, are involved in secretion of digoxin, which is believed to be a typical Pgp probe.

4.2 Introduction

Digoxin, isolated initially from the leaves of *Digitalis lanata*, is the most commonly used drug for congestive heart failure (CHF) and atrial fibrillation. Even though its clinical use was introduced in 1934, its chemical structure was not finalized until 1953. Digoxin's chemical structure has both lipophilic (steroid) and hydrophobic parts. SAR suggests that lactone and steroid components are essential for activity, while the sugars modify the drug's pharmacokinetics including absorption, clearance and distribution.

The pharmacokinetics, pharmacodynamics and numerous drug interactions of digoxin have been extensively studied and reviewed (Reuning and Geraets, 1986), yet the mechanisms of its kinetics and drug interactions remain unclear. Digoxin exhibits fairly

good bioavailability, suggesting that solubility and dissolution rate are not important parameters. It is widely speculated that transporters play significant roles in digoxin pharmacokinetics.

P-glycoprotein (Pgp), named from permeability-glycoprotein (Juliano and Ling, 1976), was first discovered in Chinese Hamster Ovary (CHO) cells (Juliano and Ling, 1976). This plasma membrane glycoprotein of 1280 amino acid and about 170 kDa, functions as a transmembrane efflux pump in humans. Pgp has a total of 12 transmembrane domains in two homologous halves, with each half containing an ATP binding site (Leveille-Webster and Arias, 1995). Pgp also possess glycosylation sites on the first extra cellular loop. Recent low-resolution 2.5 nm structural data obtained by electron microscopy analysis confirmed this secondary structure (Rosenberg *et al.*, 1997). Only one Pgp (MDR1), which is located on chromosome 7 at band q21, transports drugs in humans. At least three different models (flippase, "vacuum cleaner" and "aqueous pore") have been proposed for MDR1-mediated substrate translocation (Müller and Jansen, 1998; van Veen and Konings, 1997).

Pgp not only over-expresses to confer MDR in tumor cells (Gottesman and Pastan, 1993) but is also present in normal tissues (Gottesman and Pastan, 1993). It is considered to function to reduce drug absorption (intestinal epithelium), prevent drug distribution (blood-brain barrier (BBB) and placenta) or enhance drug elimination (hepatic canalicular membrane and proximal renal tubule). In the intestine, Pgp (Thiebaut *et al.*, 1987; Watkins, 1997) has been shown to be localized at the tips of microvilli where it is believed that Pgp and CYPs behave coordinately as a counter-transport enzymatic defensive mechanism (Paine *et al.*, 1996; Watkins, 1997; Wu *et al.*, 1995).

MDR-associated protein (MRP), a 190 kDa protein, has been discovered to belong to the ABC protein family (Cole *et al.*, 1994) like Pgp. The gene of human MRP1 is localized on chromosome 16 at band p13.1. MRP1 is the only member of the MRP family, of at least six members (Kool *et al.*, 1997), shown to be associated with MDR (Kool *et al.*, 1997), but MRP1 and Pgp show a similar but not identical pattern of resistance.

MRP1 shows only 15% amino acid identity with Pgp (Lautier *et al.*, 1996), but transports many of Pgp substrates, such as vinblastine, cyclosporine (CsA) and verapamil (Holló *et al.*, 1996). Unlike Pgp that prefers lipophilic or weakly basic substrates, MRP prefers anionic compounds and products of phase II metabolism such as glutathione-conjugates (Heijn *et al.*, 1997; Shen *et al.*, 1996). MRP can transport a broad spectrum of hydrophobic anions, including the leukotriene LTC4 (substrate with the highest affinity for MRP), steroid glucuronides, bile salt derivatives, oxidized glutathione (GSSG) and GSH complexes with heavy metal oxyanions. MRP1 has been characterized as an efflux pump for drugs and glutathione-drug conjugates (Flens *et al.*, 1994).

MRP1 is found in various solid tumors as well as normal tissue (Kruh *et al.*, 1995). MRP mRNA is expressed at low levels in liver, intestine, and at higher levels in skeletal muscle, heart, kidney and lung. However, the physiological relevance of MRP in these tissues is unknown. Unlike MRP1 whose expression in the liver was found to be very low (Paulusma *et al.*, 1996), MRP2, also called cMOAT, is found almost exclusively in the liver and in lesser amounts in the small intestine and other tissues. The ATP-dependent transport of MRP substrates from liver cells into the bile is disturbed in EHBR and TR^- rats, which serve as standard models for cMOAT deficiency. The

transport properties of cMOAT have been well characterized by Ishikawa *et al.*, (1990) and Oude Elferink and Jansen (1994). Evidence suggests that MRP3 is similar to MRP2, and MRP5 is similar to MRP1. Even though the roles of MRP4 and MRP6 are unclear, homology studies suggest that they all function as GS-X pumps (Kool *et al.*, 1997).

Many *in vitro* cell culture studies have shown that digoxin is a Pgp substrate. Digoxin secretion by intestinal epithelium is likely to involve both diffusional uptake and Na^+/K^+ pump-mediated endocytosis, followed by active extrusion at the apical membrane (Cavet *et al.*, 1996). Digoxin did not compete with azidopine for binding in rat BBM or membranes prepared from the multidrug resistant CHO cell line (De Lannoy *et al.*, 1992). The specific transport of digoxin B to A transport was saturable. Metabolic inhibitors and several Pgp inhibitors reduced total and specific net B to A flux of digoxin without affecting the non-specific flux significantly (Ito *et al.*, 1993).

de Lannoy and Silverman (1992) showed accumulation of digoxin was 3 to 5-fold greater in drug sensitive parent cells than in multidrug resistant CHO cells, which was 180 times resistant to colchicine relative to drug sensitive parent cells, suggesting transport of digoxin is mediated by Pgp. Studies in LLC-PK1 cells transfected with the human MDR1 cDNA (Tanigawara *et al.*, 1992), in which transfected cells exhibited 8 fold greater net B to A transport than the host cells, and studies in LLC-PK1, L-MDR1, L-mdr1a cell lines (Schinkel *et al.*, 1995) showed that mouse mdr1a and human MDR1 Pgp actively transport digoxin.

In vivo studies in mdr1a and mdr1a/1b knockout mice (Mayer *et al.*, 1996b; Schinkel *et al.*, 1997) showed that intestinal excretion (Mayer *et al.*, 1996b), brain accumulation (Mayer *et al.*, 1996b; Schinkel *et al.*, 1996), tissue distribution and

pharmacokinetics of digoxin (Schinkel *et al.*, 1995) are strongly affected by the mouse Pgp activity.

Indomethacin has also long been known to inhibit renal clearance of many anionic xenobiotics (Smith and Benet, 1979), presumably through competition for kidney organic anion transporters (OAT). Indomethacin inhibited and thereby reversed MDR in human and murine cell lines expressing MRP (Draper *et al.*, 1997) and decreased vincristine accumulation and increased susceptibility of cell lines over-expressing MRP, but not Pgp (Kobayashi *et al.*, 1997). But indomethacin is not thought to inhibit cMOAT (Evers *et al.*, 1998).

MRP1 has been shown to be inhibited by probenecid, vinblastine and CsA in cancer cell lines with over-expressing MRP, but not Pgp (Holló *et al.*, 1996). Sulfinpyrazone and probenecid inhibited apical to basal transport of daunorubicin by MRP but not basal to apical by MDR1 (Evers *et al.*, 1996). However, sulfinpyrazone did not inhibit the MRP2 mediated secretion of vinblastine (Evers *et al.*, 1998).

Evers *et al.* (1996) reported that MRP1 was polarized on the basolateral surface of LLC-PK1 cells upon over-expression. Even though MRP1 expression results in resistance to vinblastine in cancer cells, but vinblastine efflux by MRP1 was not detectable above baseline secretion (Evers *et al.*, 1996). Recently, it has been demonstrated that cMOAT (MRP2) is expressed on the basolateral membrane of the MDCKII cells and vinblastine, previously believed to be a typical Pgp substrate, can be transported by cMOAT (Evers *et al.*, 1998).

The locations of Pgp, MRP1 and cMOAT in the liver, kidney, intestine and BBB, suggest that these transporters may play roles in digoxin absorption and disposition.

Recent recombinant techniques have allowed the use of polarized cell lines over-expressing human cDNA of specific transporters, such as Pgp. The objective of this study was to determine whether transporters other than Pgp, particularly MRP1 and cMOAT, may be involved in the secretion of digoxin *in vitro*. Studies on the temperature and concentration dependence of digoxin transport, as well as chemical inhibition studies were also undertaken.

4.3 Materials and Methods

4.3.1 Materials

Digoxin, probenecid, (±)sulfapyrazone, indomethacin and CsA were purchased from Sigma Chemical Company (St. Louis, MO). Digoxin[^{3}H(G)] (19 μCi/mmol, 1 mCi/ml) and D-[1-^{14}C]-mannitol (51.5 mCi/mmol, 0.1 mCi/ml) were purchased from DuPont NEN Life Science (Boston, MA).

Transwell-clear inserts (24mm, 0.4 μm) and 6-well companion plates (25 mm) were obtained from Fisher Scientific (Pittsburgh, PA). All cell culture media was obtained from the UCSF Cell Culture Facility (San Francisco, CA).

The 21-day Caco-2 cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The 3-day Caco-2 (Biocoat HTS) assay system was purchased from Becton Dickinson (Franklin Lakes, NJ). P-glycoprotein transfected and wild-type Madin-Darby Canine Kidney, strain I (MDR1-MDCK and MDCK, respectively) were generously provided by Dr. Ira Pastan (NIH, Bethesda, MD) (Pastan *et al.*, 1988). MRP1 (LLC-MRP1) and MRP2 (MDCKII-cMOAT), as well as the respective wild-types (LLC-PK1 and MDCK strain II) were generally provided by Dr. Piet Borst of The Netherlands Cancer Institute (Amsterdam, The Netherlands).

4.3.2 Preparation of cell culture monolayers

All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2: 95% air in medium containing 10% fetal bovine serum. Transfected and wild-type MDCK cells (strain I), were grown in Dubelco's modified Eagle's medium (DMEM), DME H21 medium (0.1 µm sterile filtered) containing 4.5 g/L glucose, 0.584 g/L L-glutamine and 3.7 g/L $NaHCO_3$. For MDR1-MDCK cells, in addition to the above the growth medium also contained 80 ng/ml colchicine.

The MII-cMOAT and wild-type MDCKII (strain II) cells were grown in DME H-21 medium (0.1 µm sterile filtered); the L-MRP1 and wild-type LLC-PK1 cells were grown in M-199 medium (0.1 µm sterile filtered) with Earle's BSS. Both media contained Penicillin-Streptomycin, 100 µg/ml streptomycin SO_4 and 100 units/ml penicillin G, as advised by Dr. Raymond Evers (Amsterdam, The Netherlands).

The 21-day Caco-2 cells were grown in Minimum Essential Medium (MEM) (0.1 µm sterile filtered) Eagle's with Earle's BSS containing 0.292 g/L L-glutamine, 1.0 g/L glucose, 2.2 g/L $NaHCO_3$, 100 µM of MEM non-essential amino acids (NEAA) and 110 µg/ml of sodium pyruvate (0.1 µm sterile filtered). The 3-day Caco-2 cells were seeded and grown in the seeding and growth cell media from Biocoat HTS assay system.

Cells grown to confluence in culture flasks were harvested with STV (Trypsin 0.05 %, Versene 0.02% in Saline A, 0.1 µm sterile filtered) containing 0.5 g/L Trypsin, 0.2 g/L EDTA, 1 g/L glucose and 0.58 g/L $NaHCO_3$, then seeded onto polycarbonate filters in 6-well cluster plates at an approximate density of 10^6 cells/insert (Figure 4-1). Studies were conducted at 4 to 5 or 5 to 6 days post seeding for the four MDCK or two LLC-PK1 cell lines, respectively. Studies were conducted at 21 days or 3 days post

seeding for Caco-2 cells. Media was changed at least twice per week for Caco-2, and media was changed once every 2 days for all of the other cell lines and always included feedings 18 to 24 hr both post-trypsinization and pre-testing.

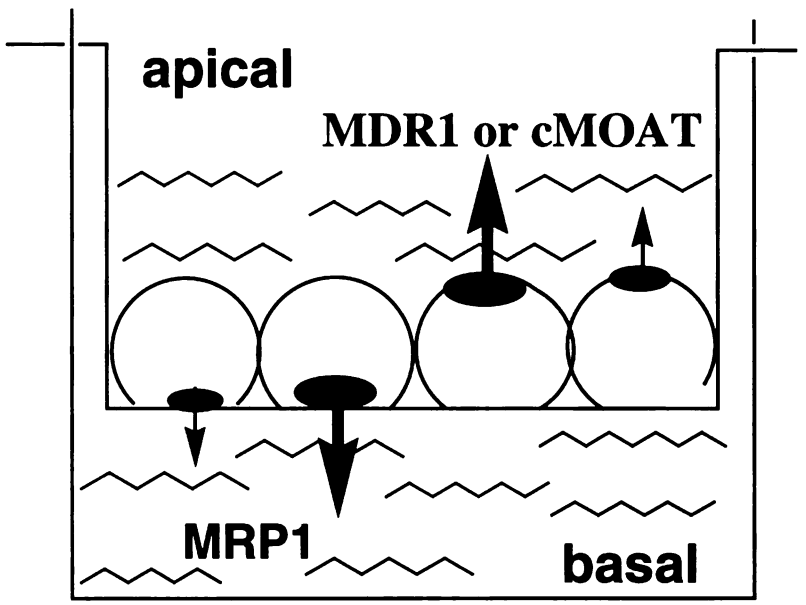


Figure 4-1. Representation of transwell system used in transport studies *in vitro*.

Studies were conducted after equilibrating the cells at 37°C for 30 min in serum free growth media (transport media), without any additional supplements (colchicine or antibiotics), and tested with 2 ml in the apical chamber and 3 ml in the basolateral chamber. Both the basolateral-to-apical and apical-to-basolateral directions were tested over a surface area of 4.2 cm^2. Transepithelial electrical resistance (TEER) was measured using a Millipore Millicell-ERS resistance system (Millipore Corporation, Bedford, MA) to assess the integrity of monolayers.

4.3.3 *Transport studies*

For transport studies, medium on either the basal or apical side of the monolayers was replaced with fresh medium containing [^{3}H]digoxin, and that on the opposite side with fresh medium alone (Figure 4-1). To examine the effects of inhibitors on the transepithelial transport of digoxin across MDR1-MDCK cell monolayers, the inhibitors were added to the same side of the monolayers along with [^{3}H]digoxin. Digoxin and inhibitors were added from concentrated DMSO solutions for a final solvent concentration in the transport media of exactly 1% and pH of approximately 7.4, and studies were conducted at 37°C (incubator shaker), unless otherwise noted.

Sampling was done without replacement every 1 hr during the experiments from the receiver and the entire solution was removed at 3 hr. The maximum volume removed during the course of an experiment was 0.15 ml, which corresponds to 10% of the total volume of the apical and 6% of the basolateral starting volumes. The data in each figure or table refers to an individual experiment with its own control from a single batch (same source vial) of cells.

4.3.4 *Data analysis*

Assuming that the cell behaves as a single membrane, active transport for digoxin only occurs in the secretory (or absorptive in the case of MRP1) direction, and passive transport is independent of direction. Statistical comparisons were performed using *t* tests (Primer of Biostatistics version 1.0, San Francisco, CA). Experimental results are presented as means ± S.D. unless otherwise noted.

4.3.5 Analytical methods

Quantitation of [^{14}C]mannitol and [^{3}H]digoxin were by liquid scintillation counting (Beckman LS1801 scintillation counter, Beckman Instruments, Inc., Palo Alto, CA).

4.4 Results

4.4.1 Bi-directional transport of digoxin

Cavet *et al.* (1996) observed a small net secretion of digoxin using Caco-2 cells. Digoxin B to A transport is greater than A to B transport in all cell lines tested. Here, diffusion studies of digoxin transport across Caco-2 cells exhibited net secretion (Figure 4-2), where mannitol passage was negligible in both directions.

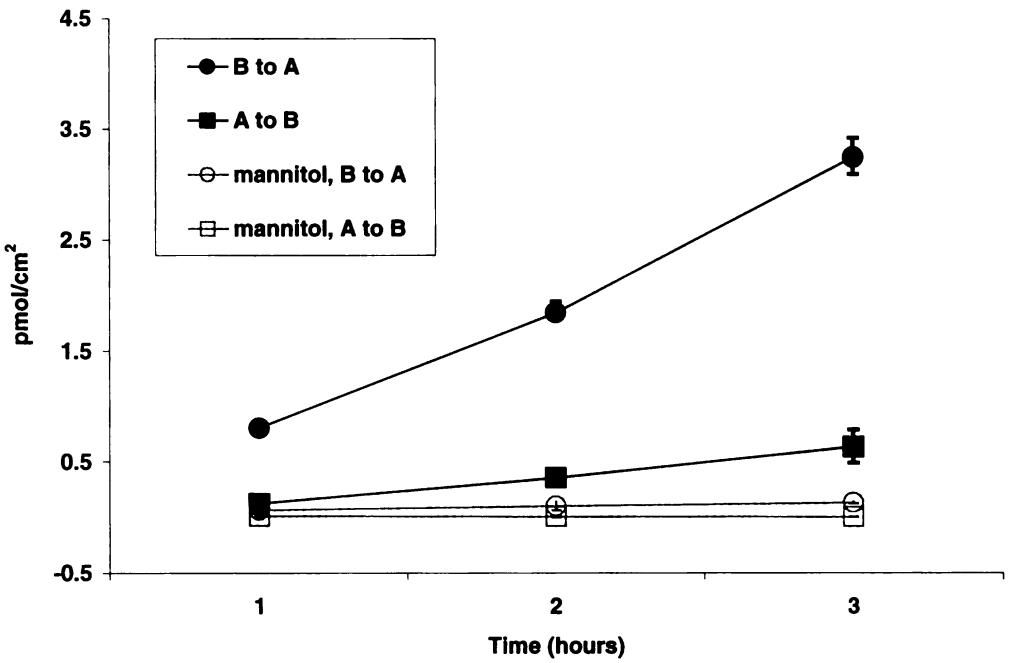


Figure 4-2. Digoxin (5 μM) and mannitol (2 μM) bi-directional transport across 21-day Caco-2 cell monolayers. Each point represents the mean ± SD (n=3).

Digoxin transport through 21 and 3 day Caco-2 cells grown on filter inserts was measured over a concentration range of 1 to 200 μM. Transport was found to be linear with time at soluble concentrations (less than 200 μM); no saturation was seen (Figure 4-3). Digoxin net B to A transport across the 21-day Caco-2 was less than across the 3-day Caco-2 cell monolayers (Figure 4-3).

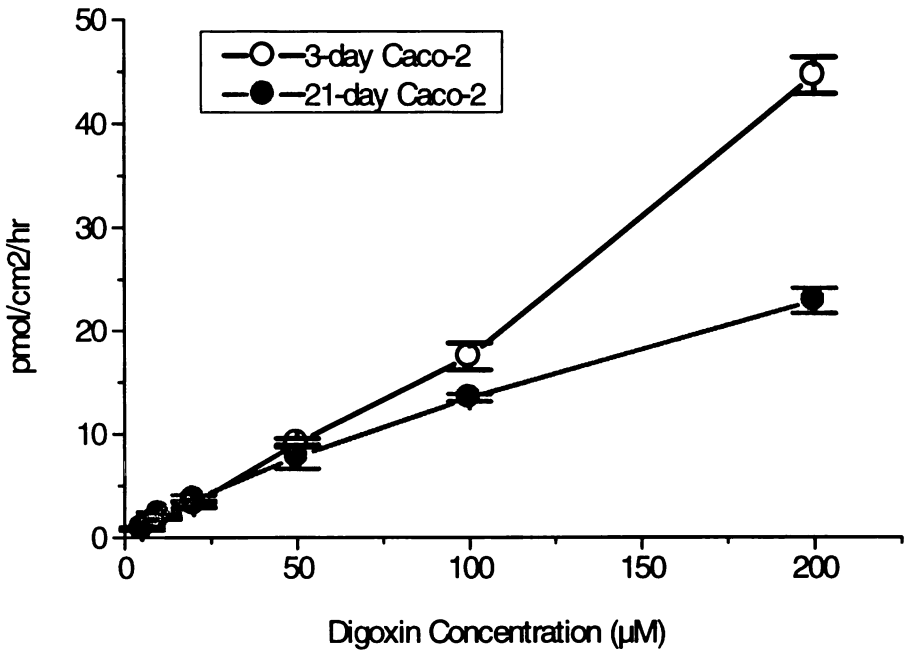


Figure 4-3. Comparisons of secretion rate of digoxin at 2 hours in 21-day and 3-day Caco-2 cells. Each point represents the mean ± SD from n=3.

4.4.2 Effect of temperature on digoxin transport

Figure 4-4 shows the effect of temperature on the net secretion of digoxin in MDCK cells. Here and in MDR1-MDCK cells, both B to A and A to B transport were reduced significantly when the temperature dropped from 37°C to 4°C. No extensive A to

B transport was observed at either 37°C or 4°C. The effect of temperature on net digoxin B to A secretion was more significant in transfected cell lines than in wild-type cell lines. These changes are consistent with carrier-mediated processes. Studies have shown that in LLC-PK1 cells, metabolic inhibitors, 2,4-DNP and sodium azide inhibited digoxin B to A transport (Ito *et al.*, 1993), suggesting digoxin transport is an active process.

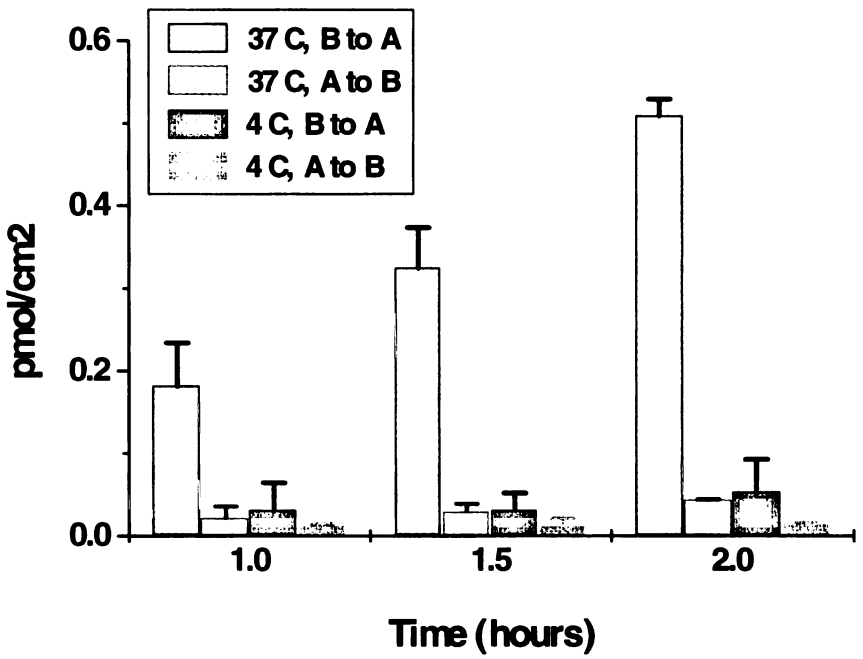


Figure 4-4. Effect of temperature on digoxin (1 μM) transport through MDCK epithelia. Each point represents the mean ± SD from n=3.

In the wild-type cells, marginal amounts of transporters if any are expressed at both the apical or basal sides of the cell monolayers. In transporter cDNA transfected cells, the transporters are over-expressed at either the apical or basal sides of the cell monolayers, dependent upon which transporter is over-expressed. By comparing the bi-

directional transport activity of drugs in these cell lines, we can determine the substrate specificity of these transporters.

4.4.3 Digoxin transport in MDR1 transfected cells

Comparison of digoxin permeation across LLC-PK1 and LLC-PK1-MDR1 cells implicated Pgp (Figure 4-5). Digoxin B to A transport was greater while A to B transport was less in LLC-PK1-MDR1 cells than in LLC-PK1 cells, suggesting that the MDR1 transporter protein was responsible for the net digoxin B to A secretion (13.4-fold in MDR1 transfected cells *vs.* 1.8-fold in wild-type cells at 2 hours). We observed a slightly higher net B to A secretion in MDR1-MDCK than MDCK cells for 50 μM of digoxin (data not shown).

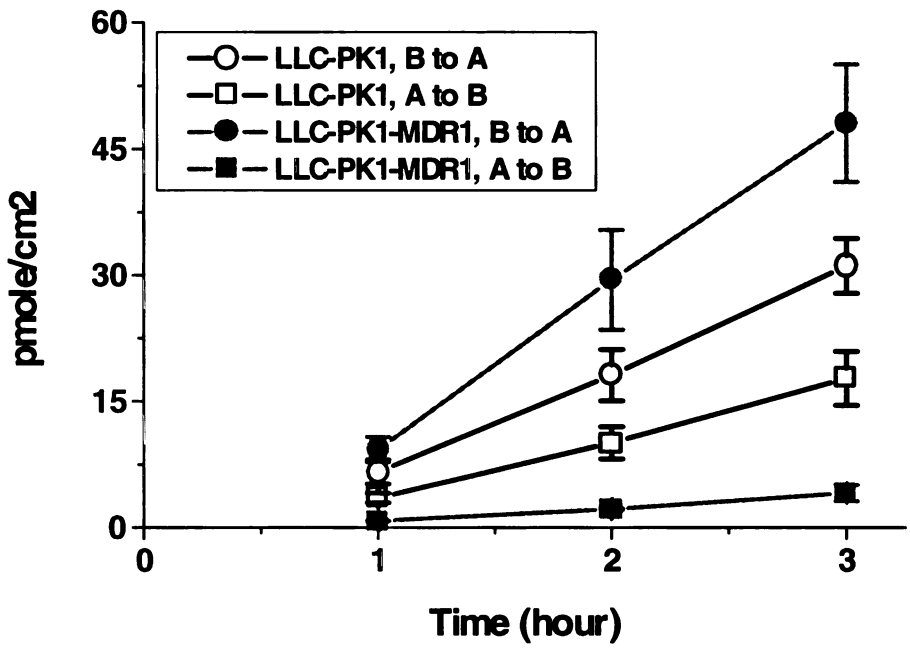


Figure 4-5. Digoxin (50 μM) bi-directional transport across LLC-PK1 and LLC-PK1-MDR1 cell monolayers. Each point represents the mean ± SD from n=3.

In MDR1 transfected cells, digoxin transport was tested for sensitivity to vinblastine, a known inhibitor of both MRPs and Pgp. 100 μM of vinblastine reduced digoxin B to A transport while increasing A to B transport, which provided indirect evidence that MDR1 can transport digoxin (Figure 4-6). At 2 hours, net digoxin B to A secretion was decreased from 13-fold in MDR1 transfected cells to 2.0-fold by vinblastine. The direct evidence that vinblastine is a MDR1 substrate will be presented in Chapter 6 (Figure 6-7).

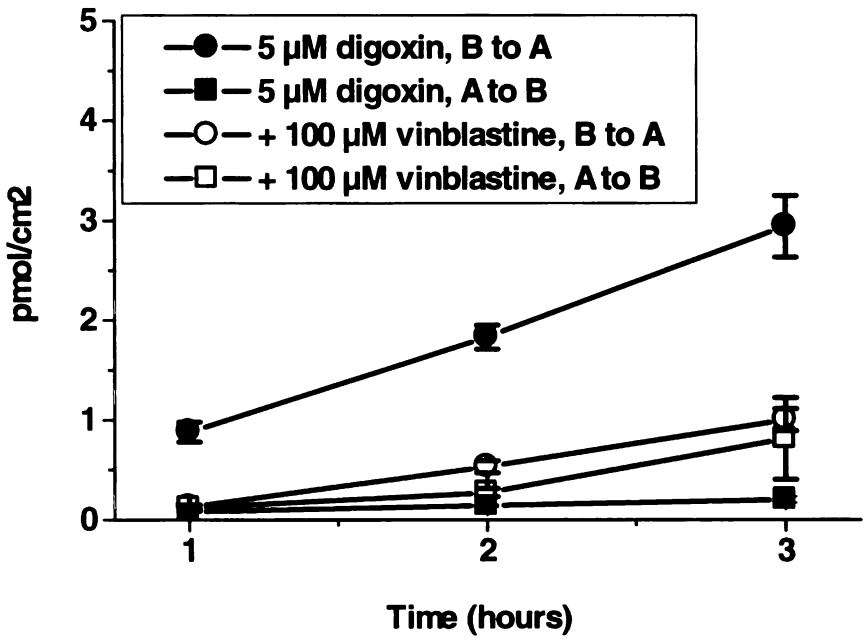


Figure 4-6. The effect of vinblastine (100 μM) on digoxin transport in MDR1-MDCK cells. Each point represents the mean ± SD from n=3.

4.4.4 Digoxin transport in cMOAT transfected cells

Net digoxin B to A secretion is almost the same in MDCKII-cMOAT cells and in the wild-type MDCKII cells (Figure 4-7). There are three possible explanations for these results. First, digoxin is not a substrate of cMOAT. Second, the endogenous Pgp in the wild-type MDCKII cells may compromise the ability to identify the cMOAT transport activity in the transfected cells, since both cMOAT and Pgp are located in the apical side of the cell monolayers and transport drug in the B to A direction. In this case, even though cMOAT is involved in digoxin transport, net digoxin B to A secretion in cMOAT transfected cells will not be greater than in wild-type cells (Figure 4-7). Third, if cMOAT is not stably over-expressed, we are unable to characterize the transport activity of cMOAT by comparing the wild-type versus the cMOAT over-expressed cell lines.

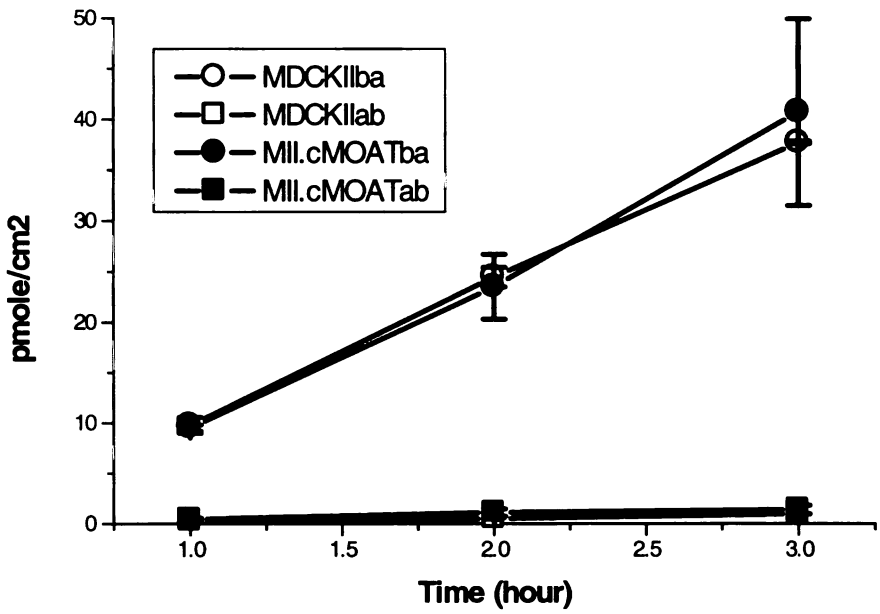


Figure 4-7. Digoxin (50 μM) bi-directional transport across MDCKII and MDCKII-cMOAT cell monolayers. Each point represents the mean ± SD from n=3.

Digoxin transport in cMOAT transfected cells was tested for sensitivity to CsA, a known inhibitor of both MRPs and Pgp. The direct evidence that CsA is a cMOAT substrate will be presented in Chapter 6 (Figure 6-5). As depicted in Figure 4-8, 100 μM of CsA reduced digoxin B to A transport and increased A to B transport. At 2 hours, net digoxin B to A secretion was decreased from 42-fold in cMOAT transfected cells to 1.7-fold by CsA.

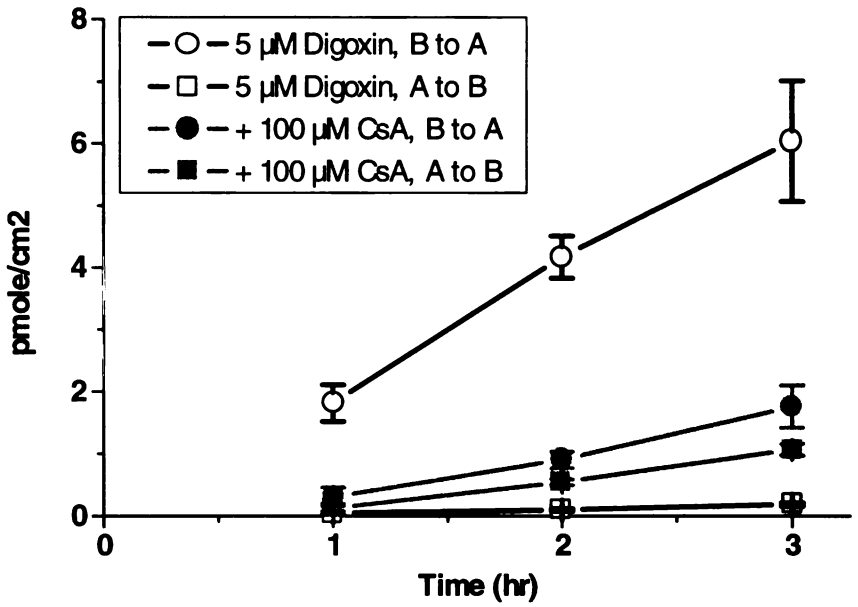


Figure 4-8. The effect of CsA (100 μM) on digoxin transport in MII-cMOAT cells. Each point represents the mean ± SD from n=3.

4.4.5 Digoxin transport in MRP1 transfected cells

In transfected LLC-PK1 cells, MRP1 is found exclusively on the basolateral membrane (Evers *et al.*, 1996), which would be expected to result in net absorption of digoxin. In LLC-PK1-MRP1 and LLC-PK1 cells, digoxin showed a net B to A secretion

(1.7 fold) even though this net secretion is slightly smaller than in the wild-type cells (1.8 fold) (Figure 4-9).

There are three possibilities for this result. First, digoxin is not a substrate of MRP1. Second, digoxin is a substrate of MRP1, but endogenous Pgp in the LLC-PK1 cells secretes digoxin in the B to A direction, opposite to the transport direction of MRP1. The transport of digoxin by Pgp over MRP1 results in a net digoxin B to A secretion. But if the dominant transporter of digoxin is MRP1, digoxin will be net secreted in the A to B direction. Lastly, MRP1 is not stably over-expressed, so it is not possible to characterize the transport activity of MRP1 by comparing the wild-type versus the MRP1 over-expressed cell lines.

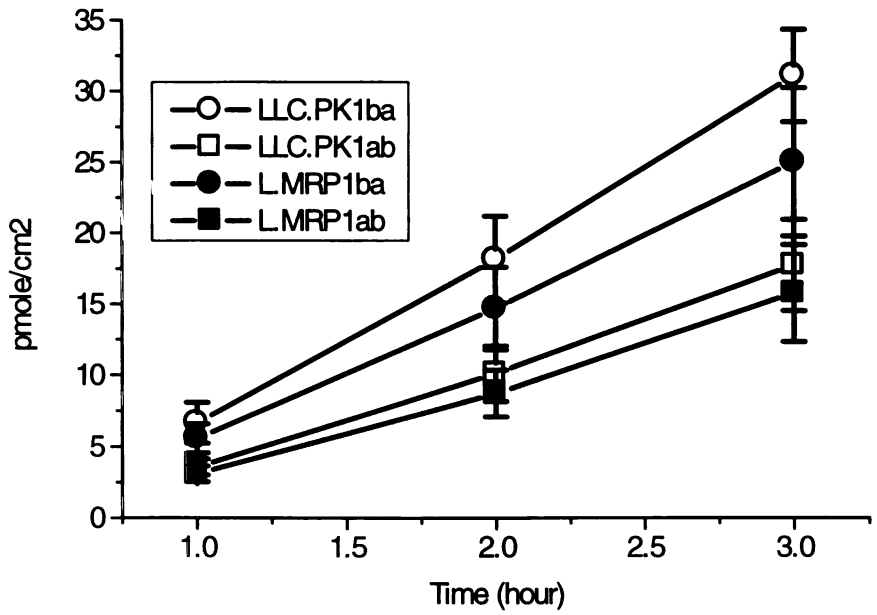


Figure 4-9. Digoxin (50 μM) bi-directional transport across LLC-PK1 and LLC-PK1-MRP1 cell monolayers. Each point represents the mean ± SD from n=3.

In LLC-PK1, LLC-MRP and LLC-MDR1 cells, sulfinpyrazone and probenecid inhibited apical to basal transport of daunorubicin by MRP, but not basal to apical by MDR1 (Evers *et al.*, 1996). Digoxin transport across MRP1 transfected cells was tested for sensitivity to inhibition by probenecid (Figure 4-10), sulfinpyrazone (Figure 4-11) and indomethacin (Figure 4-12), all known inhibitors of MRP1. In each case, digoxin B to A transport is increased while A to B transport is decreased. These results provide indirect evidence that net digoxin A to B transport was inhibited by these compounds and that digoxin is a substrate for MRP1.

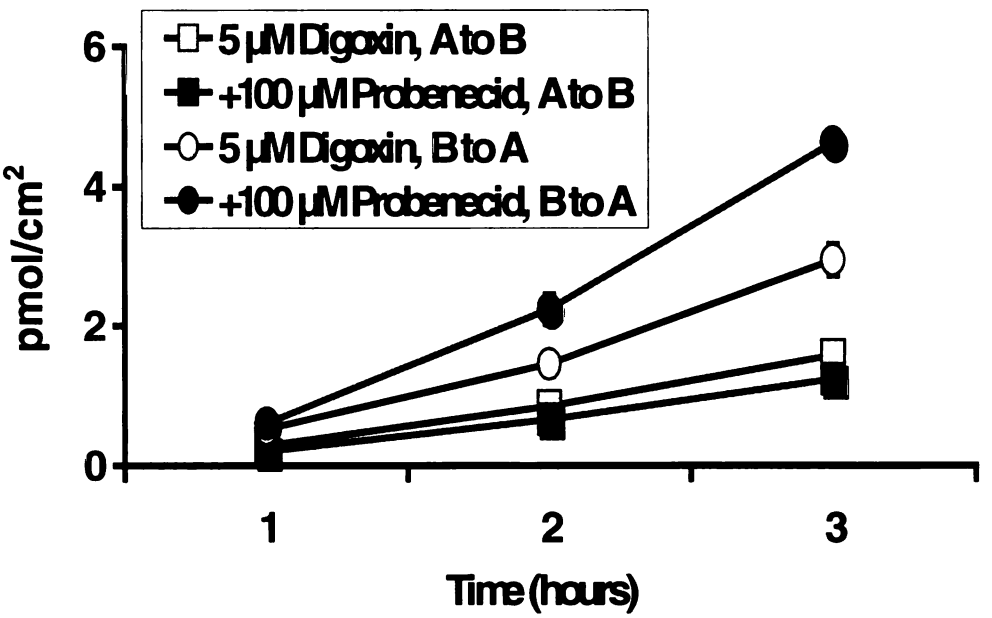


Figure 4-10. Digoxin transport in MRP1 over-expressed cells is subject to inhibition by probenecid.

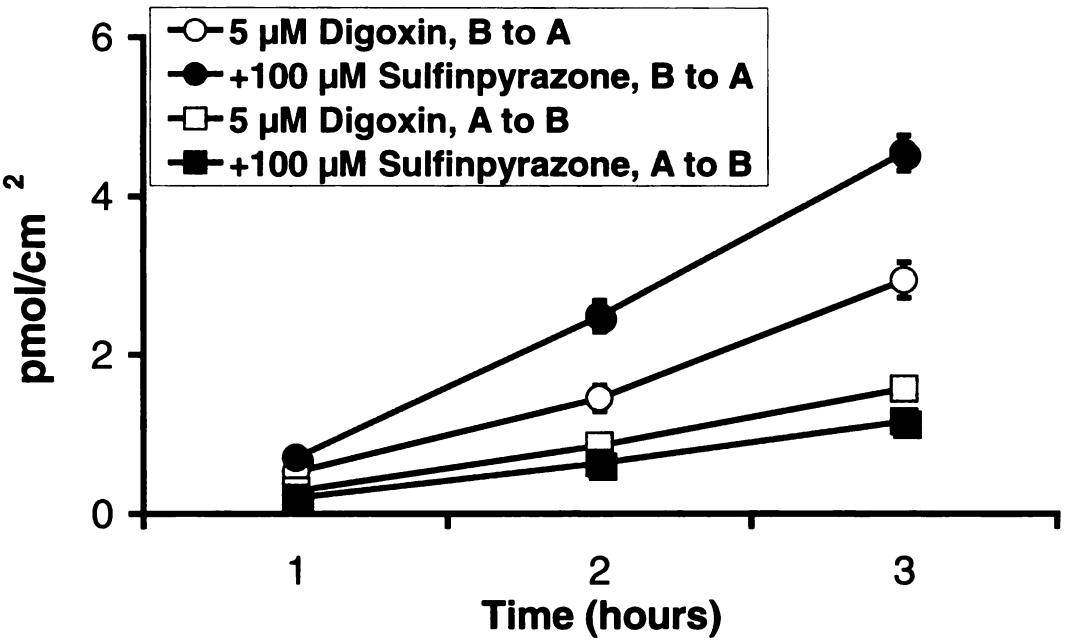


Figure 4-11. Digoxin transport in MRP1 over-expressed cells is subject to inhibition by sulfinpyrazone.

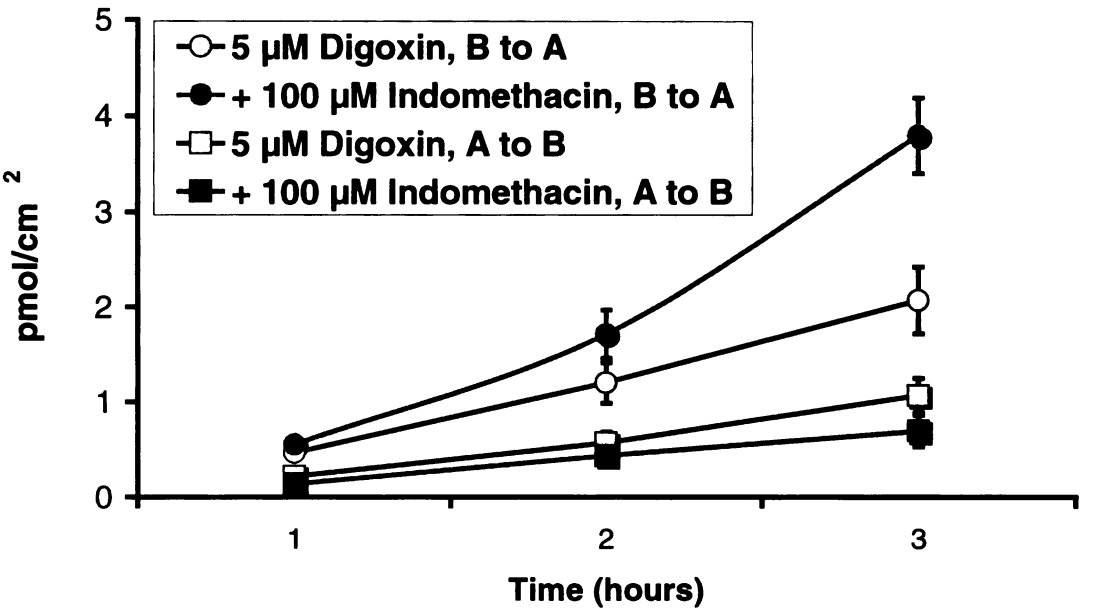


Figure 4-12. Digoxin transport in MRP1 over-expressed cells is subject to inhibition by indomethacin.

4.4.6 Digoxin transport in wild-type cells

To verify the indirect evidence that cMOAT is involved in digoxin transport, we also tested the effects of 100 μM CsA on digoxin transport in MDCKII cells, as shown in Figure 4-13. The reduction in net B to A digoxin transport by 100 μM CsA was similar in MDCKII and MII-cMOAT cells (Figures 4-8 and 4-13). Since CsA is an inhibitor of both Pgp and cMOAT, these results further suggest that a secretory flux system exists in MDCKII cells, such as Pgp or cMOAT or an unidentified transporter, which accounts for the results observed in Figure 4-7.

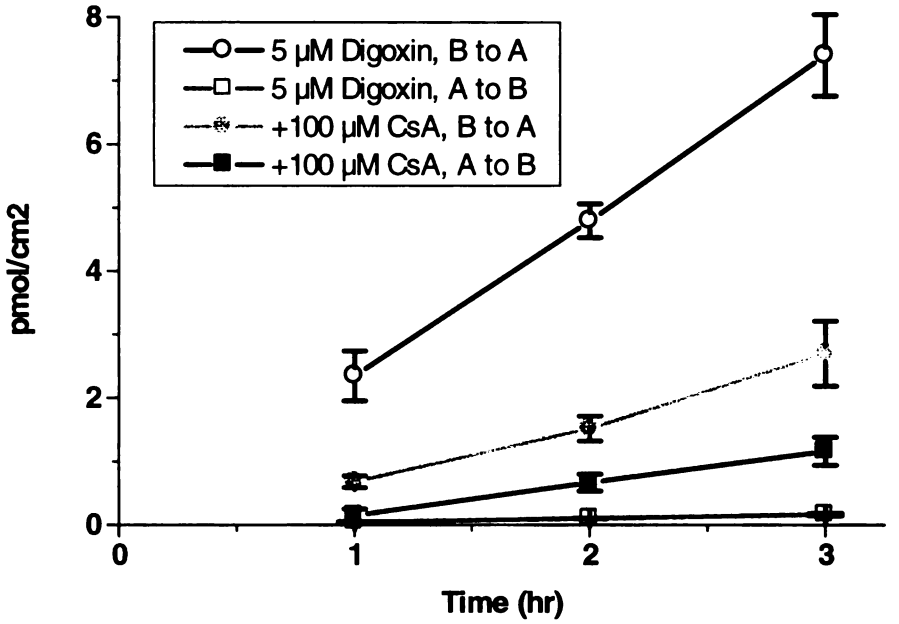
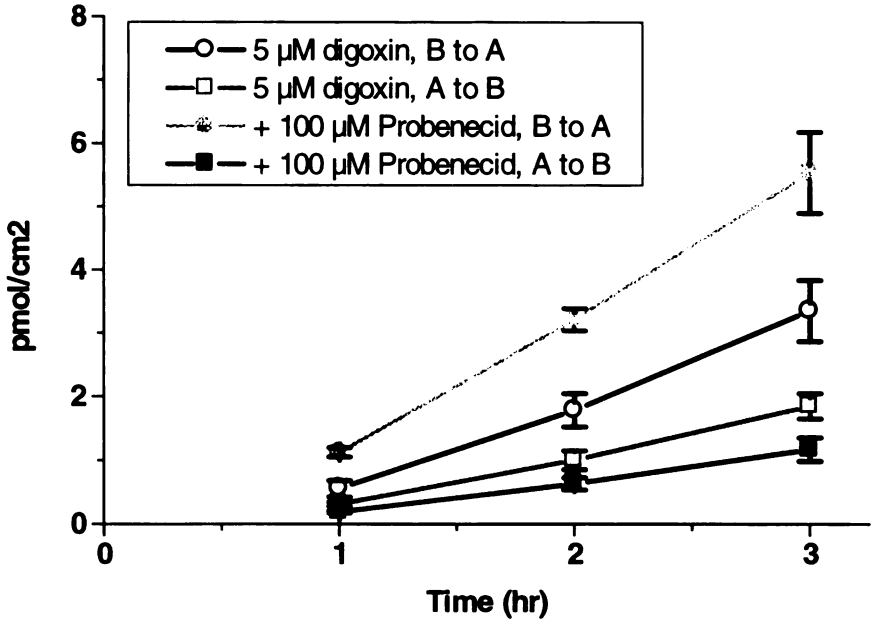
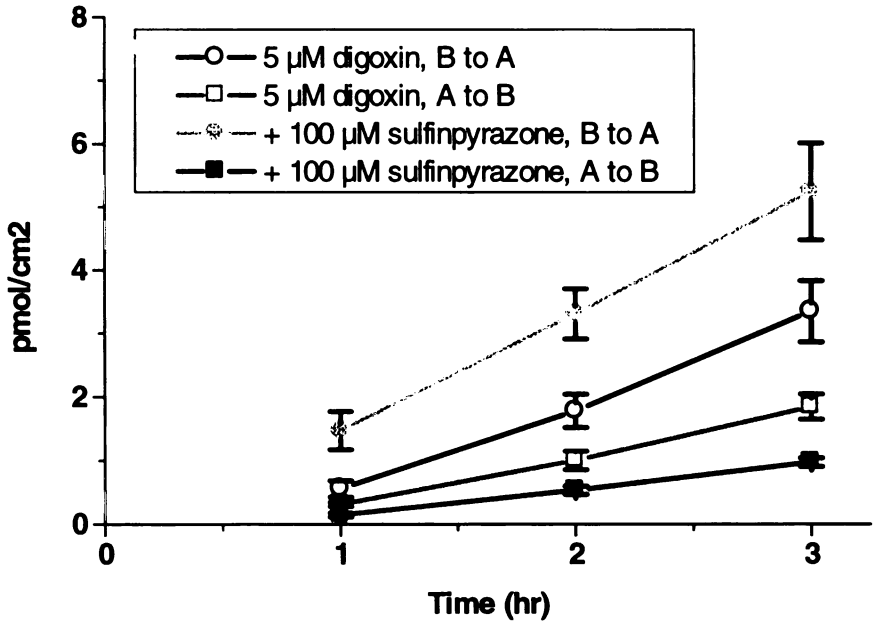


Figure 4-13. The effect of CsA (100 µM) on digoxin transport in MDCKII cells. Each point represents the mean ± SD from n=3.

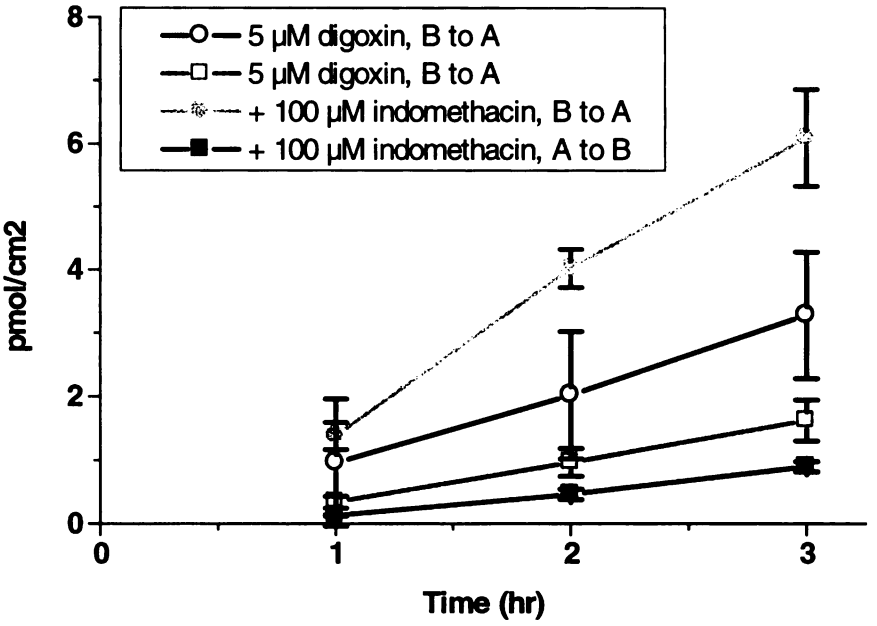
To verify that the inhibitory effects of probenecid, sulfinpyrazone and indomethacin at 100 µM on digoxin transport in MRP1 transfected cells, we carried out control studies in LLC-PK1 cells (Figures 4-14, 4-15 and 4-16). As seen in the transfected cells, probenecid, sulfinpyrazone and indomethacin all increased digoxin transport in the B to A direction and decreased transport in the A to B direction in wild-type LLC-PK1 cells.



Figures 4-14. Digoxin transport in LLC-PK1 cells is subject to inhibition by probenecid.



Figures 4-15. Digoxin transport in LLC-PK1 cells is subject to inhibition by sulfinpyrazone.



Figures 4-16. Digoxin transport in LLC-PK1 cells is subject to inhibition by indomethacin.

Evers *et al.* (1996) reported that MRP1 was polarized on the basolateral surface of LLC-PK1 cells upon over-expression. Indomethacin reversed MDR in human cell lines expressing MRP (Draper *et al.*, 1997) and increased susceptibility of cell lines over-expressing MRP, but not Pgp (Kobayashi *et al.*, 1997). MRP1 has been shown to be inhibited by probenecid in cancer cells over-expressing MRP, but not Pgp (Holló *et al.*, 1996). Sulfinpyrazone and probenecid inhibited A to B transport of daunorubicin by MRP but not B to A by MDR1 (Evers *et al.*, 1996).

Our results suggest that an unidentified endogenous absorptive transporter is present in the wild-type cells. LLC-PK1 cells may already contain an absorptive pump, such as MRP1, which can be inhibited by probenecid, sulfinpyrazone and indomethacin.

It is also possible that the transfected MRP1 cells actually are not stable. The results from these inhibition studies are consistent and further explain the non-conclusive data depicted in Figure 4-9.

4.5 Discussion

Typical TEER values (Table 4-1) varied between cell lines. MDR1-MDCKI seem to have the tightest junctions. TEERs of MDCKII cells transfected with cMOAT and MDR1 were close to the TEER values measured across filters alone (120-150 Ω/4.2 cm^2) and not different from their low resistance parental MDCK (strain II). MDCKII cells were more leaky than MDCK (strain I) cells, with LLC-PK1 exhibiting intermediate TEERs. Caco-2 cells exhibited the highest TEERs among the wild-type cells, which is consistent with the fact that these intestinal cells have tighter junctions than kidney epithelial cells. TEERs of LLC-PK1 cells transfected with MDR1 did not differ from those of the un-transfected wild-type cells, but the MRP1 transfected cells were nearly 2 or 3 times more resistant (tighter junctions).

The differences in these measurements between the transfected cell lines and their respective wild-types can not be explained. A broad range of TEER values does not correlate with the extent of drug secretion. The TEER values, which are characteristics of cell lines, are generally determined by cell structures, para-cellular and transcellular pathways of the cells. TEERs are neither a measure of expression levels nor transport activity of the cell transporters. The extent of drug secretion reflects the transport activity of either the uptake or efflux pumps in the cells, which depend on the cell types and growth conditions.

Table 4-1. Comparisons of the cell lines used in the studies.

Parent cell line	**Caco-2**		**LLC-PK1**			**MDCK**				
Species	human		porcine			canine				
Organ	intestine		kidney proximal			kidney distal				
Names	21-day	3-day	Wild-type	L-MRP1	L-MDR1	MDCKI	MDCKII	MDR1-MDCKI	MII-MDR1	MII-cMOAT
Transfected cDNA	None	None	None	Human MRP1	Human MDR1	None	None	Human MDR1	Human MDR1	Human cMOAT
Passages	24-26	24-26	12-17	9-14	NA	(76-78)*	(120-122)*	NA	NA	NA
TEER (Ω/4.2cm^2)	440-500	NA	150-180	260-500	150-160	220-320	130-150	700-1700	130-140	130-150
Sources	ATCC	ATCC	Dr. Borst	Dr. Borst	Dr. Borst	NIH	Dr. Borst	NIH	Dr. Borst	Dr. Borst

*From (Mohamed *et al.*, 1997).

NA, not available.

Digoxin transport across cultured Caco-2 and kidney epithelial cells was found to be greater in the direction of secretion. This is consistent with our hypothesis that this drug may be actively secreted in the small intestine, liver and kidney. Digoxin does not have a large paracellular component to its secretion, given that Caco-2 cells have tighter junctions than kidney epithelial cells, and no extensive B to A or A to B secretion of mannitol was observed in Caco-2 cells (Figure 4-2). These directional differences could be the result of an efflux transporter such as Pgp.

Two features of carrier-mediated transport, temperature and concentration dependence were tested in order to characterize the transport of digoxin. A great reduction in B to A directional secretion was seen for digoxin as the temperature was decreased from 37°C to 4°C (Figure 4-4), indicating that a carrier protein is likely to be involved.

Apparent K_m and V_{max} values have been reported by others (Cavet *et al.*, 1996; Ito *et al.*, 1993). The specific B to A transport of digoxin was saturable (Cavet *et al.*, 1996; Ito *et al.*, 1993), where the specific B to A flux was defined as the difference between the net transport of radiolabeled digoxin in the presence (nonspecific flux) and the absence (total flux) of unlabeled digoxin. Cavet *et al.* (1996) reported that net secretory B to A digoxin flux was concentration-dependent, but failed to reach saturation at 200 μM, the highest soluble concentrations of digoxin.

However, in our studies, the directional transport of digoxin did not display saturable dependence on concentration over the tested concentration range (Figure 4-3). The possibility that we are still in the linear range cannot be tested as digoxin is close to its limit of solubility at 200 μM. The possibility that we have greatly exceeded the K_m

even at the lowest concentrations was slim since B to A secretion was greater than A to B secretion in all cell lines tested.

Any artifacts due to the filters alone, physiochemical properties of the drug or membrane properties and the fact that fluid tends to accumulate over time into the bottom compartment (Flanagan, 1999) would tend to exaggerate A to B and reduce B to A transport. Digoxin transport across cells showed a significant secretory preference in the B to A direction, indicating active transport in the B to A direction.

Various studies have already shown that digoxin is a Pgp substrate (Cavet *et al.*, 1996; de Lannoy and Silverman, 1992; Ito *et al.*, 1993; Tanigawara *et al.*, 1992). As discussed in Chapter 1, Pgp prefers cationic and hydrophobic compounds, while MRPs prefer anionic and drug conjugates. In this chapter, three transporters, Pgp transfected into MDCKI and II, MRP2 (cMOAT) in MDCKII, MDR1 and MRP1 in LLC-PK1 cells, were studied for their ability to transport digoxin. These are the first studies to characterize transporters, other than MDR1, on digoxin.

Evers *et al.* (1996) reported that vinblastine efflux by MRP1 was not detectable above baseline secretion even though MRP1 expression results in resistance to vinblastine in cancer cells. Since MRP1 has been shown to be polarized on the basolateral surface of LLC-PK1 cells upon over-expression (Evers *et al.*, 1996), A to B transport is favored. The overall net B to A secretion of digoxin in LLC-PK1-MRP1 observed is presumably due to endogenous Pgp or cMOAT or some un-known uptake systems or efflux transporters.

Indomethacin has been known to inhibit kidney organic anion transporters (OAT) (Smith and Benet, 1979) and to reverse MDR in cells expressing MRP (Draper *et al.*,

1997). Digoxin transport was inhibited by probenecid, sulfinpyrazone and indomethacin in MRP1 transfected LLC-PK1 cells (Figures 4-10, 4-11 and 4-12), although these inhibitors do not differentiate between OAT and MRP1 transport processes. The inhibition studies of these inhibitors in LLC-PK1 cells (Figures 4-14, 4-15 and 4-16) also suggest the presence of an endogenous "MRP1 like" unidentified transporter in LLC-PK1 cells.

cMOAT (MRP2), expressed on the apical membrane upon over-expression in MDCKII cells, is not thought to be sensitive to indomethacin inhibition (Evers *et al.*, 1998). Sulfinpyrazone did not inhibit the MRP2 mediated secretion of vinblastine (Evers *et al.*, 1998). In our studies, vinblastine failed to be (See Chapter 6, Figure 6-8), but CsA was secreted by cMOAT (Chapter 6, Figure 6-5). Secretion of digoxin in the MDCKII-cMOAT cell line was found to be similar to that in the MDCKII wild-type (Figure 4-7).

CsA affected digoxin bi-directional transport similarly in both wild-type (Figure 4-13) and cMOAT transfected (Figure 4-8) MDCKII cells. Pgp can contribute to the net B to A secretion in MDCKII cells, since Pgp inhibitors including amiodarone, verapamil, vinblastine, vincristine and CsA (Figure 4-13) reduced digoxin net B to A secretion in MDCKII cells (See Chapter 6, Table 6-2).

Western blots showed that protein levels of Pgp were higher in MDR1 over-expressed cells than wild-types (data not shown). However, the protein levels of MRP transporters were not measured due to the lack of specific antibodies for the MRPs. Even though the correlation of protein levels to transport activities is not known, passage numbers of the cell lines were kept within three to avoid variability in our studies. It is

possible that these transporters were not stably over-expressed in the transfected cell lines, although we were told so by the providers.

Because renal secretion is an important route of digoxin elimination, comparisons of digoxin transport here were all made to baseline secretion in kidney or intestine derived cell lines. Perhaps *in vivo*, other endogenous renal or intestinal transporters could be masking the effects of over-expressed transporters. Our results indicate that the apparent secretion of digoxin observed in cultured MDCK (strains I and II), LLC-PK1 and Caco-2 cells, could be the result of polarized Pgp, MRP1, MRP2, some of the non-characterized uptake or secretory transporter, or some combination of these transporters.

Because of the simultaneous presence of multiple secretory and absorptive transporters in these cells and because transporters may exhibit varying degrees of susceptibility to inhibition, at this point it is not feasible to demonstrate how many transporters are responsible for net digoxin secretion in these cells. Further definitive studies using molecular biology technologies, such as transgenic cell lines (Evers *et al.*, 1996 and 1998) and "transporter knock-out" mice (Mayer *et al.*, 1996a and b) are likely required to characterize transporters that are responsible for the secretion and interactions of digoxin.

4.6 References

Cavet, M. E., West, M. and Simmons, N. L. (1996). Transport and epithelial secretion of the cardiac glycoside, digoxin, by human intestinal epithelial (Caco-2) cells. *British Journal of Pharmacology* **118,** 1389-96.

Cole, S. P., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M. and Deeley, R. G. (1994). Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Research* **54,** 5902-10.

de Lannoy, I. A. and Silverman, M. (1992). The MDR1 gene product, P-glycoprotein, mediates the transport of the cardiac glycoside, digoxin. *Biochemical and Biophysical Research Communications* **189,** 551-7.

de Lannoy, I. A., Koren, G., Klein, J., Charuk, J. and Silverman, M. (1992). Cyclosporin and quinidine inhibition of renal digoxin excretion: evidence for luminal secretion of digoxin. *American Journal of Physiology* **263,** F613-22.

Draper, M. P., Martell, R. L. and Levy, S. B. (1997). Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein. *British Journal of Cancer* **75,** 810-5.

Evers, R., Kool, M., van Deemter, L., Janssen, H., Calafat, J., Oomen, L. C., Paulusma, C. C., Oude Elferink, R. P., Baas, F., Schinkel, A. H. and Borst, P. (1998). Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. *Journal of Clinical Investigation* **101,** 1310-9.

Evers, R., Zaman, G. J., van Deemter, L., Jansen, H., Calafat, J., Oomen, L. C., Oude Elferink, R. P., Borst, P. and Schinkel, A. H. (1996). Basolateral localization and export activity of the human multidrug resistance-associated protein in polarized pig kidney cells. *Journal of Clinical Investigation* **97,** 1211-8.

Flanagan, S. D. (1999). The role of intestinal transporters in limiting bioavailabilty of diuretics: Studies with two model compounds, amiloride and furosemide. In *Department of Pharmaceutical Chemistry*, p. 65. San Francisco: University of California.

Flens, M. J., Izquierdo, M. A., Scheffer, G. L., Fritz, J. M., Meijer, C. J., Scheper, R. J. and Zaman, G. J. (1994). Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Research* **54,** 4557-63.

Gottesman, M. M. and Pastan, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual Review of Biochemistry* **62,** 385-427.

Heijn, M., Hooijberg, J. H., Scheffer, G. L., Szabó, G., Westerhoff, H. V. and Lankelma, J. (1997). Anthracyclines modulate multidrug resistance protein (MRP) mediated organic anion transport. *Biochimica et Biophysica Acta* **1326,** 12-22.

Holló, Z., Homolya, L., Hegedüs, T. and Sarkadi, B. (1996). Transport properties of the multidrug resistance-associated protein (MRP) in human tumour cells. *Febs Letters* **383,** 99-104.

Ishikawa, T., Müller, M., Klünemann, C., Schaub, T. and Keppler, D. (1990). ATP-dependent primary active transport of cysteinyl leukotrienes across liver canalicular membrane. Role of the ATP-dependent transport system for glutathione S-conjugates. *Journal of Biological Chemistry* **265,** 19279-86.

Ito, S., Koren, G., Harper, P. A. and Silverman, M. (1993). Energy-dependent transport of digoxin across renal tubular cell monolayers (LLC-PK1). *Canadian Journal of Physiology and Pharmacology* **71,** 40-7.

Juliano, R. L. and Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* **455,** 152-62.

Kobayashi, S., Okada, S., Yoshida, H. and Fujimura, S. (1997). Indomethacin enhances the cytotoxicity of VCR and ADR in human pulmonary adenocarcinoma cells. *Tohoku Journal of Experimental Medicine* **181,** 361-70.

Kool, M., de Haas, M., Scheffer, G. L., Scheper, R. J., van Eijk, M. J., Juijn, J. A., Baas, F. and Borst, P. (1997). Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Research* **57,** 3537-47.

Kruh, G. D., Gaughan, K. T., Godwin, A. and Chan, A. (1995). Expression pattern of MRP in human tissues and adult solid tumor cell lines. *Journal of the National Cancer Institute* **87,** 1256-8.

Lautier, D., Canitrot, Y., Deeley, R. G. and Cole, S. P. (1996). Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. *Biochemical Pharmacology* **52,** 967-77.

Leveille-Webster, C. R. and Arias, I. M. (1995). The biology of the P-glycoproteins. *Journal of Membrane Biology* **143,** 89-102.

Mayer, U., Wagenaar, E., Beijnen, J. H., Schinkel, A. H. and Borst, P. (1996a). Altered pharmacokinetics of (3H)-digoxin in mdrla P-glycoprotein knockout mice. *Proceedings of the American Association for Cancer Research Annual Meeting* **37,** 334.

Mayer, U., Wagenaar, E., Beijnen, J. H., Smit, J. W., Meijer, D. K., van Asperen, J., Borst, P. and Schinkel, A. H. (1996b). Substantial excretion of digoxin via the

intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. *British Journal of Pharmacology* **119,** 1038-44.

Mohamed, A., Ferguson, D., Seibert, F. S., Cai, H. M., Kartner, N., Grinstein, S., Riordan, J. R. and Lukacs, G. L. (1997). Functional expression and apical localization of the cystic fibrosis transmembrane conductance regulator in MDCK I cells. *Biochemical Journal* **322,** 259-65.

Müller, M. and Jansen, P. L. (1998). The secretory function of the liver: new aspects of hepatobiliary transport. *Journal of Hepatology* **28,** 344-54.

Oude Elferink, R. P. and Jansen, P. L. (1994). The role of the canalicular multispecific organic anion transporter in the disposal of endo- and xenobiotics. *Pharmacology and Therapeutics* **64,** 77-97.

Paine, M. F., Shen, D. D., Kunze, K. L., Perkins, J. D., Marsh, C. L., McVicar, J. P., Barr, D. M., Gillies, B. S. and Thummel, K. E. (1996). First-pass metabolism of midazolam by the human intestine. *Clinical Pharmacology and Therapeutics* **60,** 14-24.

Pastan, I., Gottesman, M. M., Ueda, K., Lovelace, E., Rutherford, A. V. and Willingham, M. C. (1988). A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. *Proceedings of the National Academy of Sciences of the United States of America* **85,** 4486-90.

Paulusma, C. C., Bosma, P. J., Zaman, G. J., Bakker, C. T., Otter, M., Scheffer, G. L., Scheper, R. J., Borst, P. and Oude Elferink, R. P. (1996). Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271,** 1126-8.

Reuning, R. H. and Geraets, D. R. (1986). Digoxin. In *Applied pharmacokinetics : principles of therapeutic drug monitoring* (ed. W. E. Evans, J. J. Schentag and W. J. Jusko), pp. 570-623. Spokane, WA.

Rosenberg, M. F., Callaghan, R., Ford, R. C. and Higgins, C. F. (1997). Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. *Journal of Biological Chemistry* **272,** 10685-94.

Schinkel, A. H., Mayer, U., Wagenaar, E., Mol, C. A., van Deemter, L., Smit, J. J., van der Valk, M. A., Voordouw, A. C., Spits, H., van Tellingen, O., Zijlmans, J. M., Fibbe, W. E. and Borst, P. (1997). Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 4028-33.

Schinkel, A. H., Wagenaar, E., Mol, C. A. and van Deemter, L. (1996). P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *Journal of Clinical Investigation* **97,** 2517-24.

Schinkel, A. H., Wagenaar, E., van Deemter, L., Mol, C. A. and Borst, P. (1995). Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *Journal of Clinical Investigation* **96,** 1698-705.

Shen, H., Paul, S., Breuninger, L. M., Ciaccio, P. J., Laing, N. M., Helt, M., Tew, K. D. and Kruh, G. D. (1996). Cellular and in vitro transport of glutathione conjugates by MRP [retracted in Biochemistry 1997 Nov 11;36(45):13972]. *Biochemistry* **35,** 5719-25.

Smith, D. E. and Benet, L. Z. (1979). Relationship between urinary excretion rate, steady-state plasma levels and diuretic response of furosemide in the rat. *Pharmacology* **19,** 301-6.

Tanigawara, Y., Okamura, N., Hirai, M., Yasuhara, M., Ueda, K., Kioka, N., Komano, T. and Hori, R. (1992). Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). *Journal of Pharmacology and Experimental Therapeutics* **263,** 840-5.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I. and Willingham, M. C. (1987). Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National Academy of Sciences of the United States of America* **84,** 7735-8.

van Veen, H. W. and Konings, W. N. (1997). Multidrug transporters from bacteria to man: similarities in structure and function. *Seminars in Cancer Biology* **8,** 183-91.

Watkins, P. B. (1997). The barrier function of CYP3A4 and P-glycoprotein in the small bowel. *Advanced Drug Delivery Reviews* **27,** 161-170.

Wu, C. Y., Benet, L. Z., Hebert, M. F., Gupta, S. K., Rowland, M., Gomez, D. Y. and Wacher, V. J. (1995). Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine. *Clinical Pharmacology and Therapeutics* **58,** 492-7.

Chapter 5

IN VITRO DIGOXIN TRANSPORT IS SUBJECT TO INHIBITION BY AZOLE ANTI-FUNGAL AGENTS

5.1 Abstract

Digoxin is a commonly used cardiac glycoside for the treatment of congestive heart failure and atrial fibrillation. Itraconazole (ICZ), ketoconazole (KCZ), miconazole (MCZ) and fluconazole (FCZ) are azole antifungal agents available in the U.S. for systemic administration. Seven cases of digoxin-itraconazole interactions have been reported. However, among these cases, therapy with KCZ or MCZ did not alter digoxin serum levels and their use did not produce side effects in the patient. The objective of this study was to determine the inhibitory effects of these azoles on digoxin transport.

[^{3}H]digoxin transport was characterized in the presence and absence of azoles in transporter over-expressed cell lines (dog or porcine kidney tubular epithelia transfected with human MDR1, MRP1 and cMOAT cDNA). In our study, azoles abolished net secretion of digoxin in Pgp, MRP1 and cMOAT transfected cells, with KCZ overall

being a better inhibitor of digoxin transport. MCZ had no effect on digoxin transport by MDR1, while the effects of MCZ and FCZ on digoxin transport in MRP1 and cMOAT over-expressed cells, respectively, were not obvious. The pharmacokinetic mechanisms of digoxin-ICZ interactions and the lack of digoxin-KCZ (FCZ or MCZ) interactions may not be caused solely by azoles' differential inhibition of digoxin transport by MDR1, MRP1 and cMOAT in the body.

5.2 Introduction

Digoxin (*Lanoxin*®) has seen widespread use for many years for the treatment of congestive heart failure and atrial fibrillation. In man, digoxin is not extensively metabolized but mainly eliminated unchanged by the kidney, in which renal tubular secretion appears to be a major route. Biliary excretion becomes more important when age or disease impairs renal excretion.

It has been shown that the tubular transport of digoxin is neither associated with the anionic or cationic transport systems nor with Na^+/K^+-ATPase (Koren *et al.*, 1986). Several studies have shown that digoxin is a Pgp substrate (Cavet *et al.*, 1996; de Lannoy and Silverman, 1992; Ito *et al.*, 1993 and Chapter 4). P-glycoprotein (Pgp), a member of highly conserved ABC transporters, is a drug efflux pump that has broad substrate specificity. It is not only over-expressed in cancer cells, but also expressed in many normal tissues at the apical sides of the epithelia. Pgp uni-directionally extrudes drugs out of cells across the apical membrane into urine, bile or intestine.

Studies in LLC-PK1 cells transfected with the human MDR1 cDNA (Tanigawara *et al.*, 1992) showed that digoxin was transported by Pgp. Similarly, investigations using perfused rat kidney (Hori *et al.*, 1993) and rat everted gut sacs (Su and Huang, 1996)

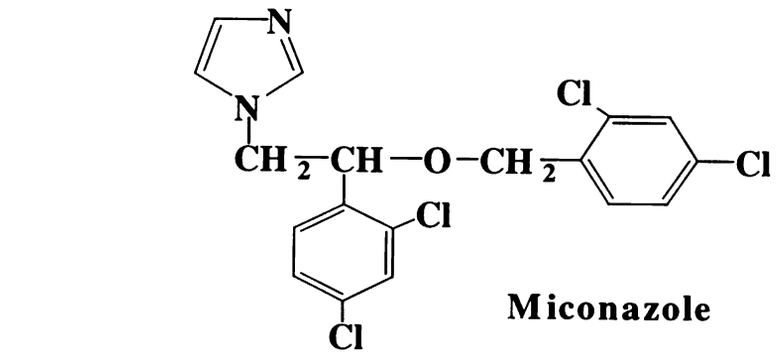
concluded that this drug was a rat Pgp substrate. Studies in mdr1a and mdr1a/1b knockout mice (Mayer *et al.*, 1996; Schinkel *et al.*, 1997) showed that intestinal excretion (Mayer *et al.*, 1996), brain accumulation (Mayer *et al.*, 1996; Schinkel *et al.*, 1996), tissue distribution and pharmacokinetics of digoxin (Schinkel *et al.*, 1995) are strongly affected by the mouse Pgp activity.

Clinically digoxin interacts with many drugs (Rodin and Johnson, 1988). In approximately 10% of patients, co-administration of erythromycin with digoxin increased serum digoxin concentrations. Pgp has been shown to play an important role in clinically relevant digoxin-drug interactions. Many drugs that interact with digoxin at the tubular level, *e.g.* verapamil (Belz *et al.*, 1983), propafenone (Belz *et al.*, 1983; Calvo *et al.*, 1989), are Pgp substrates/inhibitors (Tsuruo *et al.*, 1984; van der Graaf *et al.*, 1991; Woodland *et al.*, 1997; Yusa and Tsuruo, 1989). Indomethacin decreased vincristine accumulation and increased susceptibility of cell lines over-expressing MRP, but not Pgp (Kobayashi *et al.*, 1997). Administration of indomethacin to patients receiving a chronic therapeutic digoxin dose resulted in toxic serum digoxin concentrations (Haig and Brookfield, 1992; Jørgensen *et al.*, 1991). Since indomethacin is an inhibitor of MRPs, this suggests that MRP proteins may play roles in digoxin kinetics and digoxin-drug interactions.

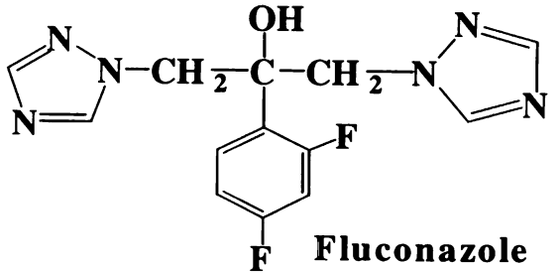
Seven cases of digoxin-itraconazole interactions have been reported (Alderman and Allcroft, 1997; Alderman and Jersmann, 1993; Cone *et al.*, 1996; Kauffman and Bagnasco, 1992; McClean and Sheehan, 1994; Meyboom *et al.*, 1994; Rex, 1992; Sachs *et al.*, 1993), in which digoxin serum concentrations reached toxic levels after concurrent administration of ICZ. In one case, therapy with KCZ did not produce side effects in the

patient while itraconazole did (Rex, 1992) and in a second study MCZ did not lead to toxicity (Alderman and Allcroft, 1997). Controlled prospective studies showed that itraconazole increases digoxin serum concentrations (Partanen *et al.*, 1996) and decreases its renal clearance (Jalava *et al.*, 1997). Itraconazole does not interfere with the fluorescence polarization immunoassay of serum digoxin (McClure *et al.*, 1995) and the pharmacokinetics of ICZ are not affected by renal impairment (Bodey, 1992). It has been hypothesized that digoxin-ICZ interactions and a lack of a digoxin-KCZ interaction are caused by ICZ and KCZ differential inhibition of digoxin transport by Pgp in the kidney (Ito and Koren, 1997; Ito *et al.*, 1994 and 1995; Koren *et al.*, 1998).

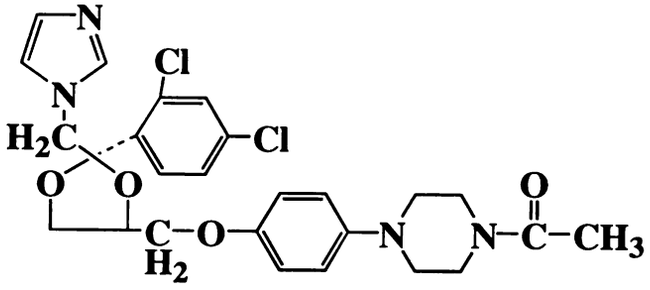
Azoles that are available for the treatment of systemic fungal infections include imidazoles (MCZ and KCZ) and tri-azoles (ICZ and FCZ) (Bodey, 1992) according to whether they contain two or three nitrogen atoms, respectively, in the five-member azole ring (Figure 5-1). Cytochrome P450 enzymes are present in most living cells; in eukaryocytic cells, they are integral components of the smooth endoplasmic reticulum or inner mitochondrial membrane. The principle mechanism of action of the azoles is to preferentially inhibit cytochrome P450 enzymes in fungal organisms (Bodey, 1992). The major enzyme inhibited by the azoles is the 14α-demethylase that is responsible for the conversion of lanosterol to ergosterol, the major component of the fungal cell membrane. One important distinction between tri-azoles and imidazoles is the greater preferential affinity of the former for fungal as compared with mammalian cytochrome P450s (Como and Dismukes, 1994). Comparisons of the pharmacokinetics for the four azole anti-fungal agents are listed in Table 5-1.



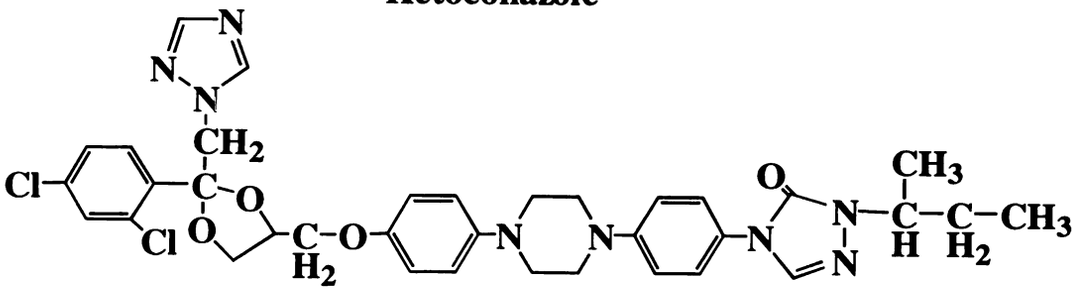
Miconazole



Fluconazole



Ketoconazole



Itraconazole

Figure 5-1. Chemical structures of azole anti-fungal agents.

The chemical structures and pharmacologic profiles of KCZ and ICZ are similar. FCZ is the only azole available as oral and intravenous preparations. Because of its comparatively small molecular size and lower lipophilicity, FCZ is only minimally metabolized in the liver and largely excreted in the urine unchanged as active drug (Table 5-1). The major concern with the use of azoles has been the potential for drug-drug interactions, and this appears to occur most frequently with KCZ (Table 5-2) (Bodey, 1992).

All four azole anti-fungal agents are inhibitors of CYP3A4 (See Chapter 3, Table 3-1). *In vitro*, KCZ exhibits a significantly greater inhibitory effect than ICZ and FCZ on CYP3A4 (Como and Dismukes, 1994). For example, the K_i's of KCZ and ICZ inhibition of formation of α-OH-midazolam from midazolam, a typical P450 3A4 but not Pgp substrate, are 3.7 nM and 0.27 μM, respectively (von Moltke *et al.*, 1996).

KCZ and ICZ are inhibitors of MDR and MRP transporters, since they affect MDR gene and/or MRP gene associated resistance. *In vitro* at concentrations that are safely used *in vivo* for treatment of fungal infection, KCZ was found to have little or no effect on the parental cell line, but overcomes resistance to vinblastine and doxorubicin in resistant cells (Siegsmund *et al.*, 1994). ICZ reversed adriamycin-resistant cells at concentrations comparable to the plasma levels achieved by the therapeutic dosages used for fungal infections; at these concentrations ICZ also partially reversed etoposide resistance in cells (Kurosawa *et al.*, 1996).

Table 5-1. Pharmacokinetics of systemic azole anti-fungal agents (Bodey, 1992; Como and Dismukes, 1994).

Azoles	**imidazoles**		**triazoles**	
	MCZ	**KCZ**	**ICZ**	**FCZ**
Brand name	Monistat IV	Nizoral	Sporanox	Diflucan
Maker	Janssen	Janssen	Janssen	Pfizer
Introduced (year)	1970s	1981	1992	1990
Route	*iv*	*p.o.*	*p.o.*	*iv, p.o.*
Recommended daily dose (mg)	400	200	200	200
F (%)	-	75[a]	>70[a]	>80
C_{peak} (µg/ml)	1.2-2.5	1.5-3.1	0.2-0.4	10.2
T_{peak} (hr)	-	1-4	4-5	2-4
Protein binding (%)	91-93	99	99.8	11
Terminal elimination $t_{1/2}$ (hr)	20-24	7-10[b]	24-42[b]	22-31
Excretion route	liver	liver	liver	kidney
Unchanged drug in urine (%)	1	2-4	<1	80

[a] The absolute F of KCZ and ICZ has not been determined because of the absence of a suitable *iv* formulation. The reported values represent the F of these agents relative to that of an oral solution in normal subjects.

[b] KCZ and ICZ exhibit dose-dependent elimination.

Table 5-2. Drug interactions, in which plasma concentrations of coadministered drugs are increased by azole antifungal agents (Como and Dismukes, 1994).

Drug involved	**Azole involved**	
	Clinically important	**Potentially clinically important**
Coumarin	KCZ	
Cyclosporine	KCZ	FCZ, ICZ
FK506	KCZ, FCZ	
Rapamycin	KCZ, FCZ	ICZ?
Digoxin		ICZ
Phenytoin	KCZ, FCZ	ICZ
Theophylline	KCZ	
Tolbutamide		KCZ, FCZ, ICZ
Terfenadine	KCZ, ICZ	
Astemizole	KCZ, ICZ	
Warfarin		KCZ, FCZ, ICZ

In vitro data demonstrated that KCZ is an inhibitor of Pgp, while FCZ has little effect (Ito and Koren, 1997). Only abstracts report that KCZ and ICZ exhibit differential inhibition of digoxin transport across MDCK cell monolayers (Ito *et al.*, 1994 and 1995). However, this data has not been yet published in the peer reviewed literature. Because digoxin is not extensively metabolized by CYP3A in humans, digoxin-ICZ interactions can not be due to CYP3A inhibition. The mechanism of the observed digoxin-ICZ

interactions is very likely a result, at least in part, of ICZ inhibition of digoxin secretory transport in the body.

In Chapter 4, we showed that digoxin may be transported by an unidentified MRP1-like absorptive transporter in addition to Pgp. The purpose of the present study is to investigate the effects of azoles on digoxin transport in MRP1, cMOAT and Pgp transporter cDNA transfected cell lines. Here, we also try to understand if the pharmacokinetic mechanisms of digoxin-ICZ interactions and the lack of digoxin-KCZ (FCZ or MCZ) interactions may be caused by differential inhibition of digoxin transport by these azoles.

5.3 Materials and Methods

5.3.1 Materials

Digoxin was purchased from Sigma Chemical Company (St. Louis, MO). KCZ and MCZ were purchased from U.S.P.C., Inc. (Rockville, MD). Janssen Pharmaceutica (Belgium) kindly provided ICZ. FCZ was extracted from Diflucan (Pfizer Inc., CT). Briefly, 200 mg FCZ injection solution was extracted with ethyl acetate, then the organic phase was evaporated to dryness in an evaporator. NMR and MS methods were used to determine the purity of FCZ. Digoxin[^{3}H(G)] (19 μCi/mmol, 1 mCi/ml) and D-[1-^{14}C]-mannitol (51.5 mCi/mmol, 0.1 mCi/ml) were purchased from DuPont NEN Life Science (Boston, MA).

Transwell-clear inserts (24mm, 0.4 μm) and 6-well companion plates (25 mm) were obtained from Fisher Scientific (Pittsburgh, PA). All cell culture media was obtained from the UCSF Cell Culture Facility (San Francisco, CA).

P-glycoprotein transfected and wild-type Madin-Darby Canine Kidney, strain I (MDR1-MDCK and MDCK, respectively) were generously provided by Dr. Ira Pastan (NIH, Bethesda, MD) (Pastan *et al.*, 1988). MRP1 (LLC-MRP1) and MRP2 (MDCKII-cMOAT), as well as the respective wild-types (LLC-PK1 and MDCK strain II) were generously provided by Dr. Piet Borst of The Netherlands Cancer Institute (Amsterdam, The Netherlands).

5.3.2 Preparation of cell culture monolayers

All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2: 95% air in medium containing 10% fetal bovine serum. Transfected and wild-type MDCK cells (strain I), were grown in Dubelco's modified Eagle's medium (DMEM), DME H21 medium (0.1 µm sterile filtered) containing 4.5 g/L glucose, 0.584 g/L L-glutamine and 3.7 g/L $NaHCO_3$. For MDR1-MDCK cells, in addition to the above, the growth medium also contained 80 ng/ml colchicine.

The MII-cMOAT and wild-type MDCKII (strain II) cells were grown in DME H-21 medium (0.1 µm sterile filtered); the L-MRP1 and wild-type LLC-PK1 cells were grown in M-199 medium (0.1 µm sterile filtered) with Earle's BSS. Both media contained Penicillin-Streptomycin, 100 µg/ml streptomycin SO_4 and 100 units/ml penicillin G, as advised by Dr. Raymond Evers (Amsterdam, The Netherlands).

Cells grown to confluence in culture flasks were harvested with STV (Trypsin 0.05 %, Versene 0.02% in Saline A, 0.1 µm sterile filtered) containing 0.5 g/L Trypsin, 0.2 g/L EDTA, 1 g/L glucose and 0.58 g/L $NaHCO_3$, then seeded onto polycarbonate filters in 6-well cluster plates at an approximate density of 10^6 cells/insert (See Chapter 4, Figure 4-1). Studies were conducted at 4 to 5 or 5 to 6 days post seeding for the four

MDCK or two LLC-PK1 cell lines, respectively. Media was changed once every 2 days for all of the cell lines and always included feedings 18 to 24 hr both post-trypsinization and pre-testing.

Studies were conducted after equilibrating the cells at 37°C for 30 min in serum free growth media (transport media), without any additional supplements (colchicine or antibiotics), and tested with 2 ml in the apical chamber and 3 ml in the basolateral chamber. Both the basolateral-to-apical and apical-to-basolateral directions were tested over a surface area of 4.2 cm^2. Transepithelial electrical resistance (TEER) was measured using a Millipore Millicell-ERS resistance system (Millipore Corporation, Bedford, MA) to assess the integrity of monolayers.

5.3.3 Transport studies

For transport studies, medium on either the basal or apical sides of the monolayers was replaced with fresh medium containing [^{3}H]digoxin, and that on the opposite side with fresh medium alone (See Chapter 4, Figure 4-1). To examine the effects of inhibitors on the transepithelial transport of digoxin across the cell monolayers, the inhibitors were added to the same side of the monolayers along with [^{3}H]digoxin. Digoxin and inhibitors were added from concentrated DMSO solutions for a final solvent concentration in the transport media of exactly 1% and pH of approximately 7.4, and studies were conducted at 37°C (incubator shaker), unless otherwise noted.

Sampling was done without replacement every 1 hr during the experiments from the receiver and the entire solution was removed at 3 hr. The maximum volume removed during the course of an experiment was 0.15 ml, which corresponds to 10% of the total volume of the apical and 6% of the basolateral starting volumes. The data in each figure

or table refers to an individual experiment with its own control from a single batch (same source vial) of cells.

5.3.4 Data analysis

Assuming that the cell behaves as a single membrane, active transport for digoxin only occurs in the secretory (or absorptive in the case of MRP1) direction, and passive transport is independent of direction. Statistical comparisons were performed using *t* tests (Primer of Biostatistics version 1.0, San Francisco, CA). Experimental results are presented as means ± S.D. unless otherwise noted.

5.3.5 Analytical Methods

Quantitation of [^{3}H]digoxin and [^{14}C]mannitol was by liquid scintillation counting (Beckman LS1801 scintillation counter, Beckman Instruments, Inc., Palo Alto, CA).

5.4 Results

We tested the effects of four azole antifungal agents on digoxin transport in transporter over-expressed cell lines *in vitro*.

5.4.1 Azoles on digoxin transport in MDR1 transfected cells

In MDR1-MDCK cells, KCZ, ICZ and FCZ all decreased digoxin net B to A secretion. The inhibition effects by azoles also showed concentration dependence (Figure 5-2 and Table 5-3). We also attempted to determine the IC_{50}'s of azole inhibition. KCZ showed good inhibition and gave an IC_{50} = 64.2 ± 7.3 μM (Figure 5-3). Because ICZ is poorly soluble in the cell medium, the maximal soluble concentrations tested are no more than 50 μM, which makes the determination of IC_{50} very unreliable. In contrast, FCZ is very soluble in the cell medium up to 1000 μM, and this azole inhibited digoxin secretion in Pgp transfected cells. MCZ, which exhibits good solubility in the study medium, failed

to inhibit digoxin transport over the wide concentration range tested, from 0-1000 μM (Table 5-3). The results rank these azoles as inhibitors of digoxin transport in MDR1-MDCK cells: KCZ>FCZ>ICZ>>MCZ.

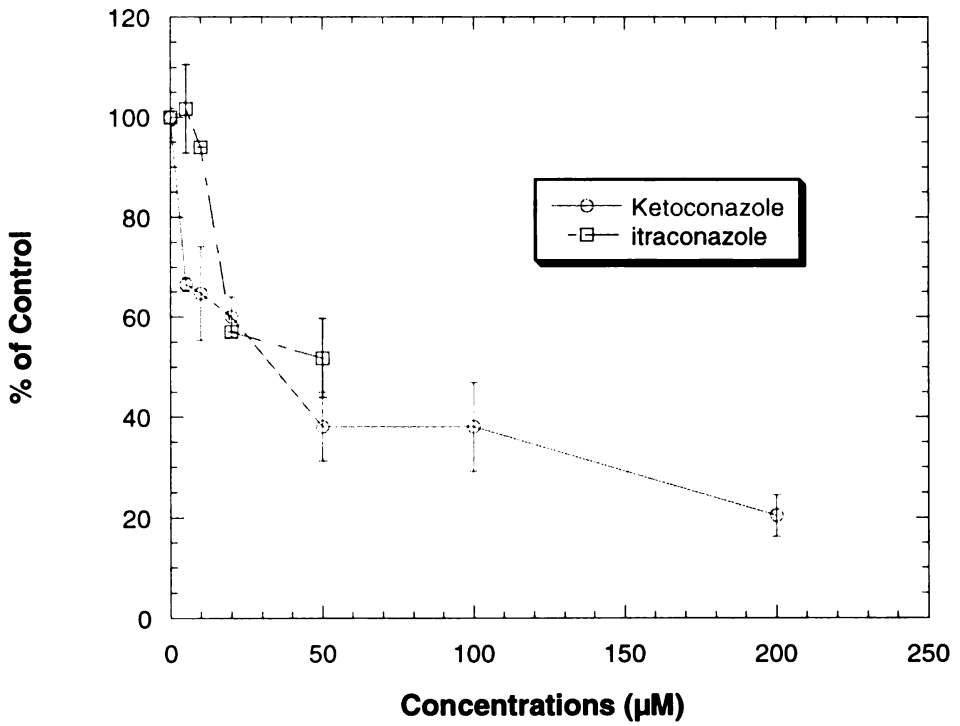


Figure 5-2. The effects of KCZ and ICZ on digoxin (50 μM) B to A transport at 1 hour in MDR1-MDCK cells.

Table 5-3. Azoles inhibition of digoxin (50 μM) B to A transport at 1 hour in MDR1-MDCK cells.

Concentrations (μM)	**% of Control (ratio of means, n=3)**			
	KCZ	**FCZ**	**ICZ**	**MCZ**
5	66	83	102	106
10	65	ND	94	ND
20	60	42	57	ND
50	38	ND	52	94
100	38	36	ND	91
200	20	ND	ND	100
500	ND	32	ND	100
1000	ND	33	ND	81

ND, not determined.

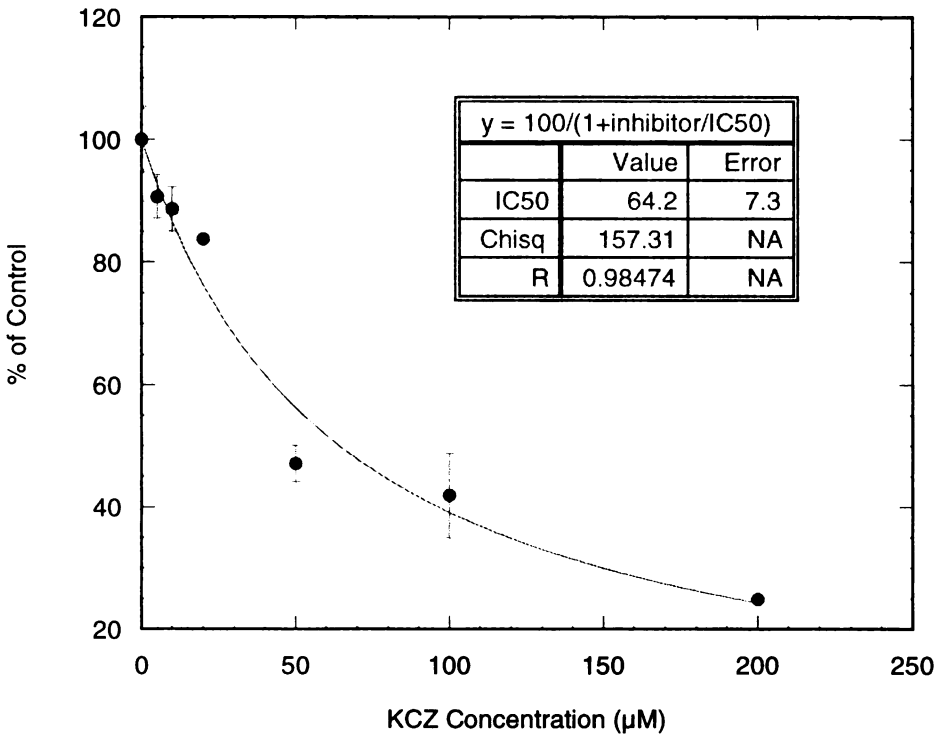


Figure 5-3. IC_{50} determination of KCZ on digoxin (50 μM) B to A transport at 3 hours in MDR1-MDCK cells.

5.4.2 Azoles on digoxin transport in MRP1 transfected cells

We tested the effects of 50 and 200 μM of azoles on digoxin transport in MRP1 transfected cells. As discussed in Chapter 4, digoxin showed a net secretion in the B to A direction, probably because endogenous Pgp or an unidentified secretory transporter co-exists with MRP1 in this cell line or MRP1 is not stably expressed. All four azoles decreased B to A transport of digoxin (50 μM) (Figures 5-4 and 5-5, Table 5-4). FCZ,

KCZ and ICZ also decreased A to B digoxin transport while MCZ slightly, but not significantly, increased digoxin A to B transport.

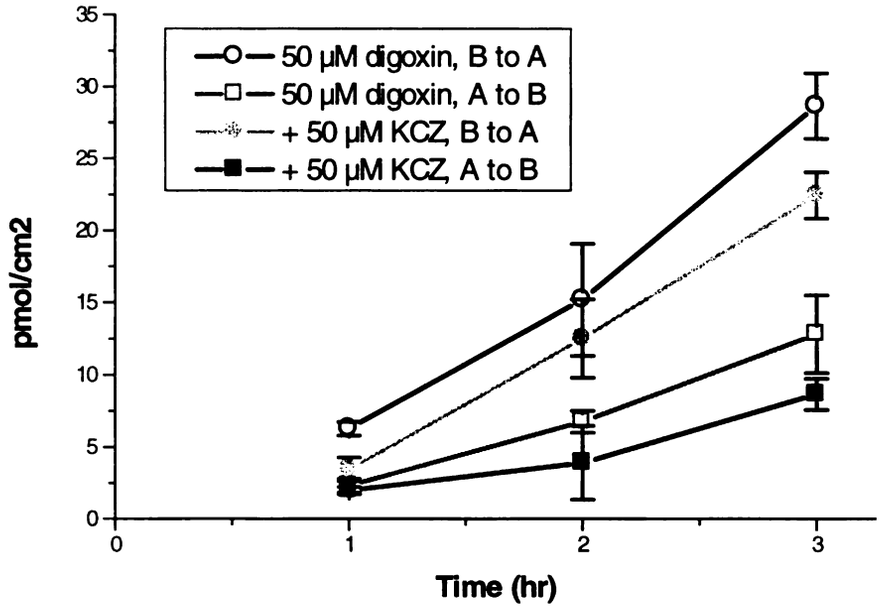


Figure 5-4. Effect of 50 μM of KCZ on digoxin (50 μM) transport in LLC-PK1-MRP1 cells.

Because MRP1 transport is in the A to B direction, we compared the decreased extent of digoxin A to B secretion. The A to B digoxin transport at 2 hours (Table 5-4) was reduced from 6.77 to 3.68, 3.87, 3.91 pmol/cm^2 by 50 μM FCZ, ICZ and KCZ, respectively. Net digoxin B to A secretion at 2 hours was 2.2 fold, which was increased to 3.2 fold and 3.4 fold by 50 μM KCZ (Figure 5-4) and ICZ, respectively (Table 5-4). However, 50 μM FCZ and MCZ decreased digoxin 2-hour net B to A secretion from 2.2 fold to 1.8 fold and 1.2 fold, respectively (Table 5-4). Considering the extents by which

azoles increase the net digoxin B to A secretion, the potency of azoles on digoxin transport in MRP1 transfected cells would be: ICZ>KCZ> FCZ>MCZ.

Table 5-4. Effects of azoles on 50 μM digoxin transport at 2 hours in LLC-PK1-MRP1 cells.

	pmol/cm^2 (means ± SD, n=3)		**Net Secretion**
	B to A	**A to B**	
Control	15.2 ± 3.9	6.77 ± 0.76	2.2
+50 μM KCZ	12.5 ± 2.7	3.91 ± 2.57	3.2
+50 μM ICZ	13.3 ± 0.8	3.87 ± 0.65	3.4
+50 μM FCZ	6.69 ± 1.62	3.68 ± 1.53	1.8
+200 μM FCZ	10.5 ± 1.0	4.87 ± 0.57	2.2
+50 μM MCZ	9.38 ± 2.23	7.80 ± 1.79	1.2

FCZ (200 μM) did not affect the net digoxin B to A secretion due to a parallel reduction of digoxin transport in both B to A and A to B directions (Figure 5-5). Net digoxin B to A secretion was 2.2 fold in both absence and presence of 200 μM FCZ.

The comparison of FCZ data at 200 μM with 50 μM cannot be easily explained (Table 5-4). The differences in A to B transport are not significant. However, the higher B to A transport at 200 μM FCZ (although less than control) appears to be real. It is known that LLC-PK1 cells have endogenous Pgp. As discussed in Chapter 4, it is possible that an unidentified absorptive transporter is present in LLC-PK1 cells or LLC-

PK1 cells already contain MRP1 or MRP1 transporters are not stably over-expressed. These multiple transporters present in the cells may exhibit varying degrees of susceptibility to inhibition by different azoles and/or at different concentration ranges, or alternatively FCZ may be toxic to the cells at a 200 μM concentration. Cell viability was not measured. However, the nonlinear bi-directional digoxin transport after 2 hours in the presence of 200 M FCZ (Figure 5-5) suggests that cells may be no longer viable.

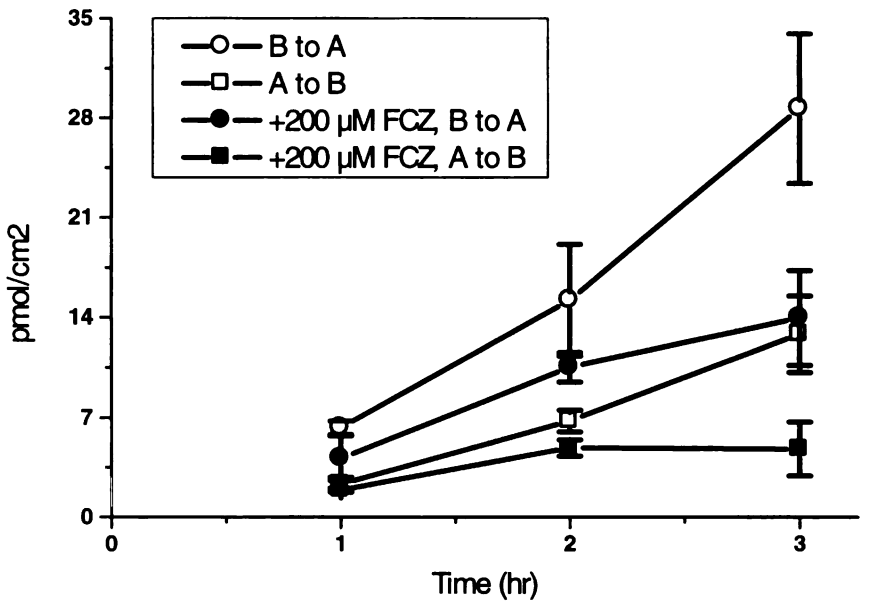


Figure 5-5. Effect of 200 μM of FCZ on digoxin (50 μM) transport in LLC-PK1-MRP1 cells.

5.4.3 Azoles on digoxin transport in cMOAT transfected cells

The effects of 50 and 200 μM of azoles were also tested on digoxin transport in cMOAT transfected cells. Four azoles all decreased B to A and increased A to B (Figure 5-6) with exception of FCZ, which did not significantly increase in the A to B direction

of digoxin (50 μM) transport (Table 5-5). As a result, net digoxin B to A secretion at 2 hours was reduced from 20.8 fold to 2.9 fold and 6.0 fold by 50 μM KCZ and ICZ, respectively (Figure 5-6 and Table 5-5). The data show that the potency of azoles on digoxin transport in cMOAT transfected cells: KCZ>ICZ>MCZ>FCZ.

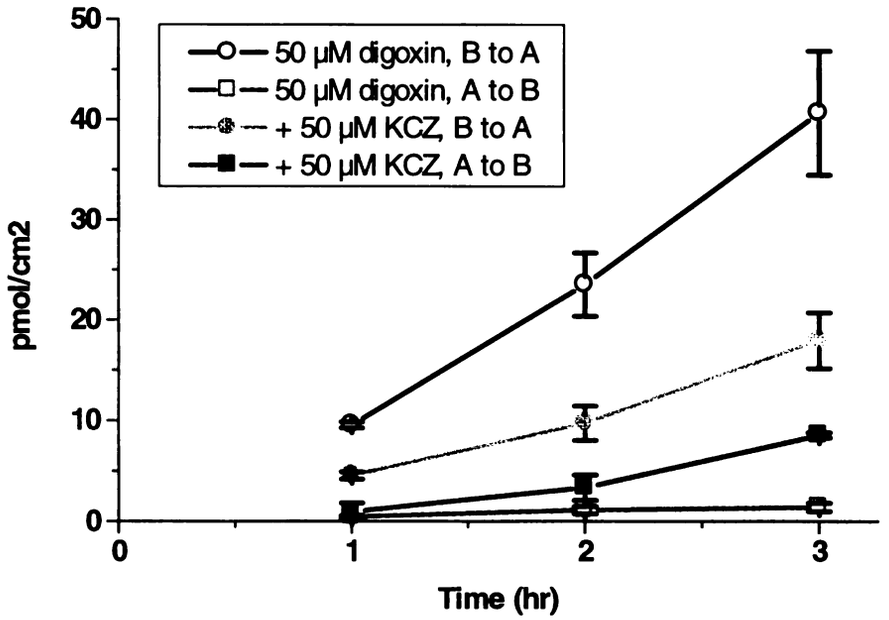


Figure 5-6. Effect of 50 μM of KCZ on digoxin (50 μM) transport in MDCKII-cMOAT cells.

Here again the comparisons of 200 versus 50 μM inhibitor concentrations require some comments. For FCZ and MCZ, B to A transport is decreased similarly from control. The increased effect of 200 μM MCZ results primarily from the increase in A to B transport. In contrast, 200 μM FCZ appears to have no effect on digoxin A to B transport. These results are in contrast to the effect of 200 μM FCZ in MRP1 transfected cells

discussed in the previous section, where a potential toxic effect of FCZ on the cells was listed as one of the possible explanations. Possible explanations for the anomalous results with 200 μM KCZ (Table 5-5) will be discussed in section 5.6.

Table 5-5. Effect of 50 μM and 200 μM of azoles (ICZ only at 50 μM) on digoxin (50 μM) transport at 2 hours in MDCKII-cMOAT cells.

	Inhibitor concentrations (μM)	**pmol/cm^2 (means ± SD, n=3)**		**Net secretion**
		B to A	**A to B**	
Control	0	30.7 ± 6.5	1.07 ± 0.33	28.7
MCZ	50	20.6 ± 2.9	2.50 ± 1.24	8.2
	200	18.1 ± 6.3	6.41 ± 4.80	2.8
FCZ	50	18.2 ± 1.0	1.86 ± 1.43	9.8
	200	18.6 ± 2.8	1.09 ± 0.32	17.0
KCZ	50	9.74 ± 1.68	3.35 ± 1.26	2.9
	200	21.0 ± 1.3	38.07 ± 10.60	0.6
ICZ	50	24.3 ± 5.2	4.09 ± 1.61	6.0

5.5 Discussion

In Chapter 4, digoxin transport across cultured intestinal and kidney epithelial cells was found to be greater in the direction of secretion. Digoxin secretion from epithelial cells was shown to be temperature-dependent and sensitive to inhibition by compounds including vinblastine.

Previous results using transfected cell lines and chemical inhibition have also indicated that digoxin is transported by Pgp, and can be secreted from MRP1 and cMOAT (MRP2) over-expressed cells. Inhibitors of non-specific OAT and MRP1 (indomethacin) or Pgp and MRP2 (CsA) were found to cause a decrease in digoxin secretion (See Chapter 4). This would have lead to the belief that MRPs were at least partially responsible for digoxin's secretion.

As demonstrated by control cell line studies in Chapter 4, an unidentified absorptive or secretory transporter may also co-exist in LLC-PK1 and MDCKII cells, respectively. Recently, a multi-specific organic anion transporting polypeptide (oatp2), a member of the oatp gene family of membrane transporters, was isolated from rat brain (Noe *et al.*, 1997) and was found to mediate high-affinity uptake of digoxin in oocytes. The digoxin active secretion observed in our studies could be the result of either primary active efflux pumps such as Pgp or MRPs, or secondary active transporters such as OATs or OCTs.

Before initiating a clinical study to test the pharmacokinetic mechanisms of the digoxin-ICZ interaction, we wanted to further investigate the potential inhibition of azoles on digoxin transport in Pgp, MRP1 and cMOAT cDNA over-expressed cell lines. The results of these *in vitro* experiments, summarized below, served as the justification for the *in vivo* human study.

Our results showed that digoxin secretion from Pgp, MRP1 and cMOAT over-expressed cells is subject to inhibition by azole anti-fungal agents *in vitro*, with KCZ appearing to be the best inhibitor overall. In addition to not showing strong inhibition of digoxin transport in MRP1 and cMOAT over-expressed cells, ICZ did not inhibit digoxin

transport by Pgp, which is not consistent with data of Ito *et al.* (1994 and 1995). One possibility for this inconsistency is that Ito *et al.* used a wild-type MDCK cell line while here an MDR1 transporter over-expressed cell line was used. In our studies, FCZ seemed to be a better inhibitor than ICZ due to the ICZ's poor solubility. MCZ only showed good inhibition of digoxin transport in MDCKII-cMOAT cells (Tables 5-4 and 5-5).

The unexplainable results in Table 5-5 are seen with 200 μM KCZ in cMOAT transfected cells. Digoxin B to A transport was less than control at 1 and 2 hours but greater than control at 3 hours, suggesting a possible toxic effect of KCZ on the cells. However, the very large A to B transport observed at 1, 2 and 3 hours is not readily explained. Unfortunately, mannitol flux was not determined for these studies. The TEER values were within the normal range for all cells tested. But it is known that TEERs do not correlate with the presence of tight junctions or transport activities of the cells. It is very possible that 200 μM KCZ opened the cell tight junction, with the resulting effect that fluid may accumulate from the apical to the basal side of the cell monolayers, resulting in a greater digoxin A to B transport than control at 1, 2 and 3 hours.

As discussed in Chapter 4, LLC-PK1 cells have endogenous Pgp, and MDCKII cells have higher levels of endogenous Pgp than LLC-PK1 cells. It is possible that an unidentified absorptive transporter is present in LLC-PK1 cells or an unidentified secretory transporter is present in MDCKII cells. Alternatively, LLC-PK1 cells already contain MRP1, or MDCKII cells already contain MRP2. Thirdly, MRP1, or MRP2 transporters might not stably over-express. These multiple transporters present in the cells may exhibit varying degrees of susceptibility to inhibition by different azoles and/or at

different concentration ranges. These can also result in the differential inhibition of digoxin transport by azoles at variable concentrations in MRP transfected cells.

There is some concern that *in vitro* cell cultures may not mimic transport systems *in vivo*. We studied transport in these cell lines for lack of a better-established alternative. Permeability of the paracellular marker mannitol was determined and TEER values were also tested during the experiments for most of cell cultures tested. Mannitol transport across cells was found to be both independent of direction and extremely low. In most cases less than 0.1% was recovered in the receiver solutions. Typical TEER values varied between cell lines (Table 4-1), as discussed in Chapter 4. The differences in TEER measurements between the transfected cell lines and their respective wild-types can not be explained, but a broad range of TEER values does not correlate with the presence of tight junctions or the extent of drug secretion in the cells.

The usual daily doses of KCZ and ICZ are 200 mg (Table 5-2), however, the C_{peak} of KCZ is 10 times higher than that of ICZ, although the KCZ $t_{1/2}$ is much shorter. Thus the AUC of KCZ and ICZ after a single dose would be very similar. Because digoxin is not extensively metabolized in humans, we ignore the inhibition effects of azoles on P450 enzymes. Our *in vitro* data do not fully support the hypothesis that digoxin-ICZ interactions and the lack of digoxin-KCZ interactions are caused by azoles differential inhibition of digoxin transport in the body, since ICZ did not show a lower K_i than KCZ on digoxin secretion from Pgp, MRP1 and cMOAT cDNA transfected cells in our studies.

But we cannot exclude the possibility that transporters are the dominant factors in digoxin kinetics and the reported cases of digoxin-ICZ interactions are caused by azole

inhibition of digoxin transport in the body, since chemical inhibition failed to implicate a single or a group of transporters due to the lack of specificity of inhibitors. It is possible that ICZ might be an irreversible inhibitor or a very potent inhibitor of some unidentified transporter in the body for digoxin. Our results suggest that multiple transporters present in the cells exhibit varying degrees of susceptibility to inhibition by different azoles and/or at different concentrations.

5.6 References

Alderman, C. P. and Allcroft, P. D. (1997). Digoxin-itraconazole interaction: possible mechanisms [see comments]. *Annals of Pharmacotherapy* **31,** 438-40.

Alderman, C. P. and Jersmann, H. P. (1993). Digoxin-itraconazole interaction [letter]. *Medical Journal of Australia* **159,** 838-9.

Belz, G. G., Doering, W., Munkes, R. and Matthews, J. (1983). Interaction between digoxin and calcium antagonists and antiarrhythmic drugs. *Clinical Pharmacology and Therapeutics* **33,** 410-7.

Bodey, G. P. (1992). Azole antifungal agents. *Clinical Infectious Diseases* **14 Suppl 1,** S161-9.

Calvo, M. V., Martin-Suarez, A., Martin Luengo, C., Avila, C., Cascon, M. and Domínguez-Gil Hurlé, A. (1989). Interaction between digoxin and propafenone. *Therapeutic Drug Monitoring* **11,** 10-5.

Cavet, M. E., West, M. and Simmons, N. L. (1996). Transport and epithelial secretion of the cardiac glycoside, digoxin, by human intestinal epithelial (Caco-2) cells. *British Journal of Pharmacology* **118,** 1389-96.

Como, J. A. and Dismukes, W. E. (1994). Oral azole drugs as systemic antifungal therapy [see comments]. *New England Journal of Medicine* **330,** 263-72.

Cone, L. A., Himelman, R. B., Hirschberg, J. N. and Hutcheson, J. W. (1996). Itraconazole-related amaurosis and vomiting due to digoxin toxicity [letter]. *Western Journal of Medicine* **165,** 322.

de Lannoy, I. A. and Silverman, M. (1992). The MDR1 gene product, P-glycoprotein, mediates the transport of the cardiac glycoside, digoxin. *Biochemical and Biophysical Research Communications* **189,** 551-7.

Evers, R., Kool, M., van Deemter, L., Janssen, H., Calafat, J., Oomen, L. C., Paulusma, C. C., Oude Elferink, R. P., Baas, F., Schinkel, A. H. and Borst, P. (1998). Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. *Journal of Clinical Investigation* **101,** 1310-9.

Haig, G. M. and Brookfield, E. G. (1992). Increase in serum digoxin concentrations after indomethacin therapy in a full-term neonate. *Pharmacotherapy* **12,** 334-6.

Hori, R., Okamura, N., Aiba, T. and Tanigawara, Y. (1993). Role of P-glycoprotein in renal tubular secretion of digoxin in the isolated perfused rat kidney. *Journal of Pharmacology and Experimental Therapeutics* **266,** 1620-5.

Ito, S. and Koren, G. (1997). Comment: possible mechanism of digoxin-itraconazole interaction [letter; comment]. *Annals of Pharmacotherapy* **31,** 1091-2.

Ito, S., Koren, G., Harper, P. A. and Silverman, M. (1993). Energy-dependent transport of digoxin across renal tubular cell monolayers (LLC-PK1). *Canadian Journal of Physiology and Pharmacology* **71,** 40-7.

Ito, S., Woodland, C. and Koren, G. (1994). Ketoconazole and itraconazole inhibit digoxin transport across renal tubular cell monolayer (MDCK). *Pediatric Research* **37,** 77A.

Ito, S., Woodland, C. and Koren, G. (1995). Characteristics of itraconazole and ketoconazole inhibition of digoxin transport across renal tubular cells. *FASEB Journal* **9,** A686.

Jalava, K. M., Partanen, J. and Neuvonen, P. J. (1997). Itraconazole decreases renal clearance of digoxin. *Therapeutic Drug Monitoring* **19,** 609-13.

Jørgensen, H. S., Christensen, H. R. and Kampmann, J. P. (1991). Interaction between digoxin and indomethacin or ibuprofen. *British Journal of Clinical Pharmacology* **31,** 108-10.

Kobayashi, S., Okada, S., Yoshida, H. and Fujimura, S. (1997). Indomethacin enhances the cytotoxicity of VCR and ADR in human pulmonary adenocarcinoma cells. *Tohoku Journal of Experimental Medicine* **181,** 361-70.

Kauffman, C. A. and Bagnasco, F. A. (1992). Digoxin toxicity associated with itraconazole therapy [letter]. *Clinical Infectious Diseases* **15,** 886-7.

Koren, G., Klein, J., MacLeod, S. and Silverman, M. (1986). Cellular mechanisms of digoxin transport and toxic interactions in the kidney. *Veterinary and Human Toxicology* **28,** 549-52.

Koren, G., Woodland, C. and Ito, S. (1998). Toxic digoxin-drug interactions: The major role of renal P-glycoprotein. *Veterinary and Human Toxicology* **40,** 45-46.

Kurosawa, M., Okabe, M., Hara, N., Kawamura, K., Suzuki, S., Sakurada, K. and Asaka, M. (1996). Reversal effect of itraconazole on adriamycin and etoposide resistance in human leukemia cells. *Annals of Hematology* **72,** 17-21.

Mayer, U., Wagenaar, E., Beijnen, J. H., Smit, J. W., Meijer, D. K., van Asperen, J., Borst, P. and Schinkel, A. H. (1996). Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. *British Journal of Pharmacology* **119,** 1038-44.

McClean, K. L. and Sheehan, G. J. (1994). Interaction between itraconazole and digoxin [letter; comment]. *Clinical Infectious Diseases* **18,** 259-60.

McClure, A. F., Alderman, C. P. and Hayball, P. J. (1995). Itraconazole does not interfere with fluorescence polarization immunoassay of serum digoxin [letter]. *Annals of Pharmacotherapy* **29,** 938-9.

Meyboom, R. H., de Jonge, K., Veentjer, H., Dekens-Konter, J. A. and de Koning, G. H. (1994). [Potentiation of digoxin by itraconazole]. *Nederlands Tijdschrift voor Geneeskunde* **138,** 2353-6.

Mohamed, A., Ferguson, D., Seibert, F. S., Cai, H. M., Kartner, N., Grinstein, S., Riordan, J. R. and Lukacs, G. L. (1997). Functional expression and apical localization of the cystic fibrosis transmembrane conductance regulator in MDCK I cells. *Biochemical Journal* **322,** 259-65.

Noe, B., Hagenbuch, B., Stieger, B. and Meier, P. J. (1997). Isolation of a multi-specific organic anion and cardiac glycoside transporter from rat brain. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 10346-50.

Partanen, J., Jalava, K. M. and Neuvonen, P. J. (1996). Itraconazole increases serum digoxin concentration. *Pharmacology and Toxicology* **79,** 274-6.

Pastan, I., Gottesman, M. M., Ueda, K., Lovelace, E., Rutherford, A. V. and Willingham, M. C. (1988). A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. *Proceedings of the National Academy of Sciences of the United States of America* **85,** 4486-90.

Rex, J. (1992). Itraconazole-digoxin interaction [letter]. *Annals of Internal Medicine* **116,** 525.

Rodin, S. M. and Johnson, B. F. (1988). Pharmacokinetic interactions with digoxin. *Clinical Pharmacokinetics* **15,** 227-44.

Sachs, M. K., Blanchard, L. M. and Green, P. J. (1993). Interaction of itraconazole and digoxin [see comments]. *Clinical Infectious Diseases* **16,** 400-3.

Schinkel, A. H., Wagenaar, E., van Deemter, L., Mol, C. A. and Borst, P. (1995). Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *Journal of Clinical Investigation* **96,** 1698-705.

Schinkel, A. H., Wagenaar, E., Mol, C. A. and van Deemter, L. (1996). P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *Journal of Clinical Investigation* **97,** 2517-24.

Siegsmund, M. J., Cardarelli, C., Aksentijevich, I., Sugimoto, Y., Pastan, I. and Gottesman, M. M. (1994). Ketoconazole effectively reverses multidrug resistance in highly resistant KB cells. *Journal of Urology* **151,** 485-91.

Su, S. F. and Huang, J. D. (1996). Inhibition of the intestinal digoxin absorption and exsorption by quinidine. *Drug Metabolism and Disposition* **24,** 142-7.

Tanigawara, Y., Okamura, N., Hirai, M., Yasuhara, M., Ueda, K., Kioka, N., Komano, T. and Hori, R. (1992). Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). *Journal of Pharmacology and Experimental Therapeutics* **263,** 840-5.

Tsuruo, T., Iida, H., Kitatani, Y., Yokota, K., Tsukagoshi, S. and Sakurai, Y. (1984). Effects of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and adriamycin in drug-resistant tumor cells. *Cancer Research* **44,** 4303-7.

van der Graaf, W. T., de Vries, E. G., Uges, D. R., Nanninga, A. G., Meijer, C., Vellenga, E., Mulder, P. O. and Mulder, N. H. (1991). In vitro and in vivo modulation of multi-drug resistance with amiodarone. *International Journal of Cancer* **48,** 616-22.

von Moltke, L. L., Greenblatt, D. J., Schmider, J., Duan, S. X., Wright, C. E., Harmatz, J. S. and Shader, R. I. (1996). Midazolam hydroxylation by human liver microsomes in vitro: inhibition by fluoxetine, norfluoxetine, and by azole antifungal agents. *Journal of Clinical Pharmacology* **36,** 783-91.

Wacher, V. J., Gomez, D. Y., Hebert, M. F., Tomlanovich, S. J. and Benet, L. Z. (1995). Differing effects of ketoconazole on the distribution of cyclosporine metabolites after oral and intravenous cyclosporine administration. *Pharmaceutical Research (New York)* **12,** S372.

Wacher, V. J., Silverman, J. A., Zhang, Y. and Benet, L. Z. (1998). Role of P-glycoprotein and cytochrome P450 3A in limiting oral absorption of peptides and peptidomimetics. *Journal of Pharmaceutical Sciences* **87,** 1322-30.

Woodland, C., Verjee, Z., Giesbrecht, E., Koren, G. and Ito, S. (1997). The digoxin-propafenone interaction: characterization of a mechanism using renal tubular cell monolayers. *Journal of Pharmacology and Experimental Therapeutics* **283,** 39-45.

Yusa, K. and Tsuruo, T. (1989). Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Research* **49,** 5002-6.

Chapter 6

CHARACTERIZATION OF TRANSPORT ACTIVITIES OF PGP, MRP1 AND CMOAT *IN VITRO*

6.1 Abstract

Most drugs that clinically interact with digoxin, *e.g.* quinidine, verapamil, CsA, and propafenone, demonstrate inhibition of Pgp *in vitro* and *in situ*, suggesting a central role for Pgp in numerous digoxin-drug interactions. However, some drugs not interacting with digoxin clinically, such as ketoconazole, are also substrates/inhibitors of Pgp and *vice versa*. The objective of this chapter was to characterize the transport activities of the various cell lines employed in our studies, using known Pgp or MRPs substrates/inhibitors and to determine the substrate specificity of MDR1 and MRPs.

Using polarized cell lines over-expressing human cDNA of specific transporters, such as Pgp, MRP1 and MRP2 (cMOAT), we have demonstrated that CsA can be

transported by MDR1 and cMOAT, suggesting substrate/inhibition specificities overlap between Pgp and MRPs. Transport activities of MDR1 can be characterized in wild-type and MDR1 transfected LLC-PK1 cells instead of MDCKII cells. Our results suggest that MDR1 interactions may cause clinical interactions between digoxin and amiodarone, CsA, verapamil or erythromycin, while MRP2 or "MRP1 like" absorptive transporter interactions might also lead to clinical digoxin-CsA or digoxin-indomethacin interactions.

6.2 Introduction

Digoxin (*Lanoxin*®), isolated initially from the leaves of *Digitalis lanata*, is the most commonly used cardiac glycoside (Antman and Smith, 1985; Heller, 1990) for the treatment of congestive heart failure and atrial fibrillation (Kelly and Smith, 1996). Tubular secretion, a major route of digoxin renal elimination (Koren, 1987), is not associated with either the anionic or cationic transport systems, nor with Na^+/K^+-ATPase (Koren *et al.*, 1986).

Many *in vitro* (Cavet *et al.*, 1996; de Lannoy and Silverman, 1992; Ito *et al.*, 1993) and *in situ* (Hori *et al.*, 1993; Su and Huang, 1996) studies have shown that digoxin is a Pgp substrate. Digoxin-drug interactions have also been studied *in vitro* in cell culture. Inhibition of Pgp-mediated drug transport has been shown to be an unifying mechanism to explain the interaction between digoxin and quinidine (Fromm *et al.*, 1999). Propafenone significantly inhibited the secretory flux of digoxin across confluent MDCK cells, but cellular digoxin accumulation did not decrease, suggesting propafenone did not prohibit digoxin from entering the cells at the basolateral side (Woodland *et al.*, 1997). In LLC-PK1 and MDR1-LLC-PK1 cells, CsA inhibited digoxin transepithelial transport mediated by human Pgp (Okamura *et al.*, 1993).

In situ animal studies further demonstrated the determinant role of Pgp in digoxin distribution and disposition. Su *et al.* studied digoxin exsorption and absorption using everted sac and single-pass perfusion, and found that quinidine may affect digoxin elimination, as well as digoxin absorption/exsorption in the gastrointestinal tract (Su and Huang, 1996).

In isolated perfused rat kidney studies, quinidine, verapamil, vinblastine, daunorubicin, 2,4-dinitrophenol (Hori *et al.*, 1993) and CsA reduced renal tubular secretion of digoxin, but digoxin did not affect CsA transport by Pgp (Okamura *et al.*, 1993). Uptake of digoxin across basolateral membrane was large and non-saturable, but the urine recovery ratio indicated net digoxin secretion. CsA and quinidine decreased digoxin urinary recovery (de Lannoy *et al.*, 1992).

P-glycoprotein (Pgp), named from permeability-glycoprotein (Juliano and Ling, 1976), not only over-expresses and confers MDR in tumor cells (Gottesman and Pastan, 1993) but also presents in several normal tissues (Gottesman and Pastan, 1993). It is considered to function to reduce drug absorption (intestinal epithelium), prevent drug distribution (blood-brain barrier (BBB) (Schinkel *et al.*, 1994) and placenta), or enhance drug elimination (hepatic canalicular membrane and proximal renal tubule). In the intestine, Pgp (Thiebaut *et al.*, 1987; Watkins, 1997) has been shown to be localized at the tips of microvilli where it is believed that Pgp and CYPs behave as a counter-transport defensive mechanism (Paine *et al.*, 1996; Watkins, 1997; Wu *et al.*, 1995).

MDR-associated protein (MRP) has been discovered, like Pgp, to belong to the ABC protein family (Cole *et al.*, 1994). MRP1 is the only member of the MRP family (Kool *et al.*, 1997), shown to be associated with MDR (Kool *et al.*, 1997). MRP1 has

been characterized as a glutathione conjugate and drug efflux pump (Flens *et al.*, 1994). MRP1 shows low amino acid identity with Pgp (Lautier *et al.*, 1996), but is functionally similar to Pgp in transporting many of the same substrates such as vinblastine, CsA and verapamil (Holló *et al.*, 1996). Unlike Pgp that prefers lipophilic or weakly basic substrates, MRP is believed to prefer anionic compounds and products of phase II metabolism such as glutathione-conjugates (Heijn *et al.*, 1997; Shen *et al.*, 1996).

MRP1 is found in various solid tumors as well as normal tissue (Kruh *et al.*, 1995). Unlike MRP1, whose expression in the liver was found to be very low (Paulusma *et al.*, 1996), MRP2, also called cMOAT, is found almost exclusively in the liver and in lesser amounts in the small intestine and other tissues. The ATP-dependent transport of MRP substrates from liver cells into the bile is disturbed in EHBR and TR^- rats, which serve as standard models for cMOAT deficiency. The transport properties of cMOAT have been well characterized by Ishikawa *et al.* (1990) and Oude Elferink and Jansen (1994).

Clinically digoxin interacts with many drugs (Rodin and Johnson, 1988). Generally, when digoxin is administered with one of these drugs, digoxin C_{peak}, AUC, F and $t_{1/2}$ increase, while CL_{nr} and CL_r decrease. Many drugs that interact with digoxin at the tubular level, *e.g.* verapamil (Belz *et al.*, 1983) and propafenone (Belz *et al.*, 1983; Calvo *et al.*, 1989), are Pgp substrates/inhibitors (Tsuruo *et al.*, 1984; van der Graaf *et al.*, 1991; Woodland *et al.*, 1997; Yusa and Tsuruo, 1989). Yet some drugs not interacting clinically with digoxin, are also inhibitors of Pgp (Koren *et al.*, 1998), such as KCZ.

Administration of indomethacin to patients on chronic digoxin treatment, a therapeutic digoxin dose resulted in toxic serum digoxin concentrations (Haig and

Brookfield, 1992; Jørgensen *et al.*, 1991). Indomethacin decreased vincristine accumulation and increased susceptibility of cell lines over-expressing MRP, but not Pgp (Kobayashi *et al.*, 1997). Sulfinpyrazone and probenecid inhibited apical to basal transport of daunorubicin by MRP but not basal to apical transport by MDR1 (Evers *et al.*, 1996). Results from Chapter 4 using those agents as inhibitors, have demonstrated that the secretion of digoxin is mediated by transporters, at least, including Pgp and an unidentified absorptive transporter in LLC-PK1 cells. Furthermore, digoxin secretion in Pgp and MRPs transfected cells is subject to chemical inhibition by azole antifungal agents (See Chapter 5). These data suggest that MRP proteins may play roles in digoxin kinetics and digoxin-drug interactions.

Vinblastine, previously believed to be a typical Pgp substrate (Horio *et al.*, 1988; Doige and Sharom, 1992), can be transported by cMOAT (Evers *et al.*, 1998). Three patients while taking erythromycin, experienced severe toxicity when co-administered vinblastine (Tobe *et al.*, 1995) or digoxin (Lindenbaum *et al.*, 1981a; Lindenbaum *et al.*, 1981b). Digoxin-ICZ but not digoxin-KCZ interactions have been reported (See Chapter 2, Table 2-4). Transient increases in plasma quinidine concentrations occur during quinidine-KCZ therapy (McNulty *et al.*, 1989). In a double-blind, randomized two-phase crossover study, ICZ increased quinidine peak concentration, AUC and $t_{1/2}$, decreased CL_r while unaffecting creatinine CL (Kaukonen *et al.*, 1997). These results suggest that competition for Pgp and/or MRPs among substrates of these transporters may cause clinical drug interactions.

Recently, a novel multi-specific organic anion transporting polypeptide (oatp2), homologous to other members of the oatp gene family of membrane transporters, has

been isolated from rat brain (Noe *et al.*, 1997) and was found to mediate high-affinity uptake of digoxin (K_m = 0.24 μM) in functional expression studies in oocytes. On the basis of Northern blot analysis, oatp2 is highly expressed in brain, liver, and kidney but not in heart, spleen, lung, skeletal muscle, and testes. This indicates that oatp2 may play an especially important role in the brain accumulation and toxicity of digoxin and in the hepatobiliary and renal excretion of cardiac glycosides from the body. These results provide further support that substrate specificity may overlap between substrates of Pgp and MRP transporters.

The role of a transporter in the disposition of a drug is dependent upon: (1) expression levels and tissue distribution of the transporter; (2) cellular localization, such as apical or basal-lateral membrane, of the transporter; (3) affinity of the transporter for the drug; and (4) other transporters that interact with the drug. The tissue distribution and cellular localization of various MDR transporters were listed in Table 1-7 (See Chapter 1). In this chapter, using polarized cell lines over-expressing human cDNA of specific transporters, such as Pgp in MDCKI and II, MRP2 (cMOAT) in MDCKII, MDR1 and MRP1 in LLC-PK1 cells, we characterize the transport activities of the various cell lines used in our studies, determine the substrate specificity of MDR1 and MRPs, as well as study the involvement of these transporters in clinical digoxin interactions.

6.3 Materials and Methods

6.3.1 Materials

β-Estradiol-17-(β-D-glucuronide) sodium salt, etoposide, glutathione, doxorubicin hydrochloride, daunorubicin hydrochloride, indomethacin, erythromycin,

CsA, vinblastine sulfate salt, vincristine sulfate salt and (±)verapamil were purchased from Sigma Chemical Company (St. Louis, MO).

Digoxin[^{3}H(G)] (19 μCi/mmol, 1 mCi/ml), D-[1-^{14}C]-mannitol (51.5 mCi/mmol, 0.1 mCi/ml), D-[1-^{3}H(N)]-mannitol (19.7 Ci/mmol, 1 mCi/ml), verapamil hydrochloride[N-methyl-^{3}H] (84 Ci/mmol, 1 mCi/ml), indomethacin[2-^{14}C] (22.3 mCi/mmol, 0.1 mCi/ml), erythromycin[N-methyl-^{14}C] (55 mCi/mmol, 0.1 mCi/ml), glutathione[glycine-2-^{3}H] (44.8 Ci/mmol, 1 mCi/ml), [estradiol-6,7-^{3}H(N)]estradiol-17β-D-glucuronide (55 Ci/mmol, 1 mCi/ml) and [^{3}H(G)]daunomycin (2.2 Ci/mmol, 1 mCi/ml) were purchased from DuPont NEN Life Science (Boston, MA).

[G-^{3}H]vincristine sulphate (5.7 Ci/mmol, 50 μCi/200 μl), [G-^{3}H]vinblastine sulphate (12.5 Ci/mmol, 250 μCi/ml) and [mebmt-β-^{3}H]CsA (8 Ci/mmol, 1mCi/ml) were purchased from Amersham Life Sciences (Piscataway, NJ).

Transwell-clear inserts (24mm, 0.4 μm) and 6-well companion plates (25 mm) were purchased from Fisher Scientific (Pittsburgh, PA). All cell culture media was obtained from the UCSF Cell Culture Facility (San Francisco, CA).

The 21-day Caco-2 cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The 3-day Caco-2 (Biocoat HTS) assay system was purchased from Becton Dickinson (Franklin Lakes, NJ). P-glycoprotein transfected and wild-type Madin-Darby Canine Kidney, strain I (MDR1-MDCK and MDCK, respectively) were generously provided by Dr. Ira Pastan (NIH, Bethesda, MD) (Pastan *et al.*, 1988). MRP1 (LLC-MRP1) and MRP2 (MDCKII-cMOAT), as well as the respective wild-type cell lines (LLC-PK1 and MDCK strain II), were generously provided by Dr. Piet Borst of The Netherlands Cancer Institute (Amsterdam, The Netherlands).

6.3.2 *Preparation of cell culture monolayers*

All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2: 95% air in medium containing 10% fetal bovine serum. Transfected and wild-type MDCK cells (strain I), were grown in Dubelco's modified Eagle's medium (DMEM), DME H21 medium (0.1 μm sterile filtered) containing 4.5 g/L glucose, 0.584 g/L L-glutamine and 3.7 g/L $NaHCO_3$. For MDR1-MDCK cells, in addition to the above, the growth medium also contained 80 ng/ml colchicine.

The MII-cMOAT and wild-type MDCKII (strain II) cells were grown in DME H-21 medium (0.1 μm sterile filtered); the L-MRP1 and wild-type LLC-PK1 cells were grown in M-199 medium (0.1 μm sterile filtered) with Earle's BSS. Both media contained penicillin-streptomycin, 100 μg/ml streptomycin SO_4 and 100 units/ml penicillin G, as advised by Dr. Raymond Evers (Amsterdam, The Netherlands).

The 21-day Caco-2 cells were grown in Minimum Essential Medium (MEM) (0.1 μm sterile filtered) Eagle's with Earle's BSS containing 0.292 g/L L-glutamine, 1.0 g/L glucose, 2.2 g/L $NaHCO_3$, 100 μM of MEM non-essential amino acids (NEAA) and 110 μg/ml of sodium pyruvate (0.1 μm sterile filtered). The 3-day Caco-2 cells were seeded and grown in the seeding and growth cell media from Biocoat HTS assay system.

Cells grown to confluence in culture flasks were harvested with STV (Trypsin 0.05 %, Versene 0.02% in Saline A, 0.1 μm sterile filtered) containing 0.5 g/L Trypsin, 0.2 g/L EDTA, 1 g/L glucose and 0.58 g/L $NaHCO_3$, then seeded onto polycarbonate filters in 6-well cluster plates at an approximate density of 10^6 cells/insert (See Chapter 4, Figure 4-1). Studies were conducted at 4 to 5 or 5 to 6 days post seeding for the four MDCK or two LLC-PK1 cell lines, respectively. Studies were conducted at 21 days or 3

days post seeding for Caco-2 cells. Media was changed at least twice per week for Caco-2, and media was changed once every 2 days for all of the other cell lines and always included feedings 18 to 24 hr both post-trypsinization and pre-testing.

Studies were conducted after equilibrating the cells at 37°C for 30 min in serum free growth media (transport media), without any additional supplements (colchicine or antibiotics), and tested with 2 ml in the apical chamber and 3 ml in the basolateral chamber. Both the basolateral-to-apical and apical-to-basolateral directions were tested over a surface area of 4.2 cm^2. Transepithelial electrical resistance (TEER) was measured using a Millipore Millicell-ERS resistance system (Millipore Corporation, Bedford, MA) to assess the integrity of monolayers.

6.3.3 Transport studies

For transport studies, medium on either the basal or apical side of the monolayers was replaced with fresh medium containing [^{3}H]digoxin, and that on the opposite side with fresh medium alone (See Chapter 4, Figure 4-1). To examine the effects of inhibitors on the transepithelial transport of digoxin across MDR1-MDCK cell monolayers, the inhibitors were added to the same side of the monolayers along with [^{3}H]digoxin. Digoxin and inhibitors were added from concentrated DMSO solutions to a final solvent concentration in the transport media of exactly 1% and pH of approximately 7.4, and studies were conducted at 37°C (incubator shaker), unless otherwise noted.

Sampling was done without replacement every 1 hr during the experiments from the receiver and the entire solution was removed at 3 hr. The maximum volume removed during the course of an experiment was 0.15 ml, which corresponds to 10% of the total volume of the apical and 6% of the basolateral starting volumes. The data in each figure

or table refers to an individual experiment with its own control from a single batch (same source vial) of cells.

6.3.4 Analytical methods

Quantitation of [^{14}C]mannitol, [^{3}H]digoxin, [^{3}H]vinblastine, [^{3}H]CsA [^{3}H]verapamil, [^{14}C]indomethacin, [^{14}C]erythromycin, [^{3}H]glutathione, [^{3}H]estradiol-17β-D-glucuronide, [^{3}H]vincristine, [^{3}H]vinblastine was carried out by liquid scintillation counting (Beckman LS1801 scintillation counter, Beckman Instruments, Inc., Palo Alto, CA).

6.4 Results

Part I-- Drugs, which interact with digoxin clinically, were tested for substrate/inhibition activity in Pgp, MRP1 and cMOAT over-expressed cells.

6.4.1 Wild-type cell lines

Endogenous Pgp is known to be present in wild-type cell lines including Caco-2, MDCK and LLC-PK1. As previously shown in Chapter 4 for digoxin in Caco-2 (Figures 4-2 and 4-3) and MDCK cells (Figure 4-4), CsA also shows a preferable B to A secretion in Caco-2 cells (Figure 6-1).

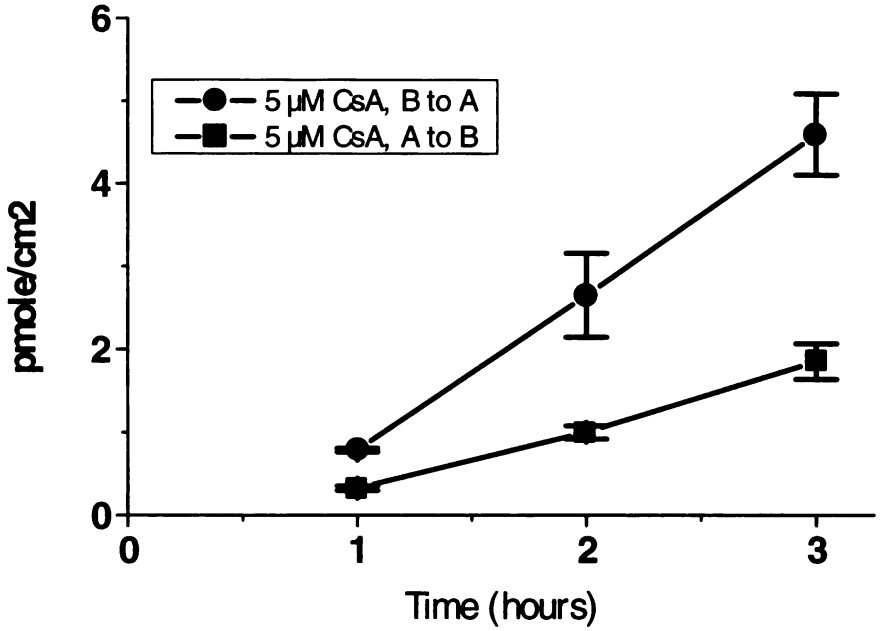


Figure 6-1. Bi-directional secretion of CsA (5 µM) across 21-day Caco-2 cell monolayers.

6.4.2 Transport activity of MDR1 transfected cells

Western blot studies have indicated that MDCK cells (Strain I and II) have higher endogenous Pgp than LLC-PK1 cells. LLC-PK1 and LLC-PK1-MDR1 seemed to be a very good pair of cell lines to assess the transport activity of Pgp, as demonstrated for digoxin (See Chapter 4, Figure 4-5), erythromycin (Figure 6-2), vinblastine (Figure 6-7).

Back in the 1970's, it was believed that the interactions between digoxin and erythromycin result from erythromycin inhibition of gut flora. That is, the intestinal bacteria *Eubacterium lentum* (Dobkin *et al.*, 1982), which metabolizes digoxin to dihydro-digoxin (Lindenbaum *et al.*, 1981a and b). Our data (Figures 4-5 and 6-2)

suggest that both digoxin and erythromycin are Pgp substrates and thus the digoxin-erythromycin interaction may be caused by a Pgp interaction in the gut.

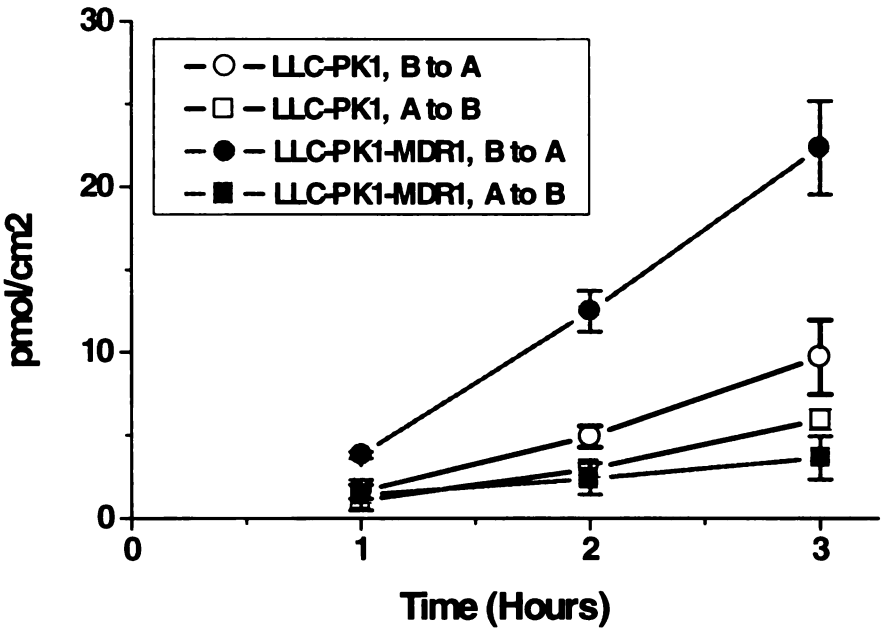


Figure 6-2. Bi-directional secretion of erythromycin (20 μM) in wild-type and MDR1 over-expressed LLC-PK1 cells.

6.4.3 Inhibition of digoxin transport in MDR1 transfected cells

We tested the ability of various drugs that clinically interact with digoxin, to inhibit digoxin transport in MDR1 over-expressed cells. In MDR1-MDCK cells, verapamil (Figure 6-3) at 100 μM, abolished 5 μM of digoxin secretion by Pgp due to increasing A to B and decreasing B to A transport. Amiodarone decreased digoxin B to A transport but had a negligible effect on A to B transport (Figure 6-4). At 2 hours, net digoxin B to A secretion was reduced from 13.3 fold to 2.5 fold and 4.7 fold by

verapamil and amiodarone, respectively. This suggests that the *in vivo* interactions between digoxin and verapamil or amiodarone could result from Pgp interactions.

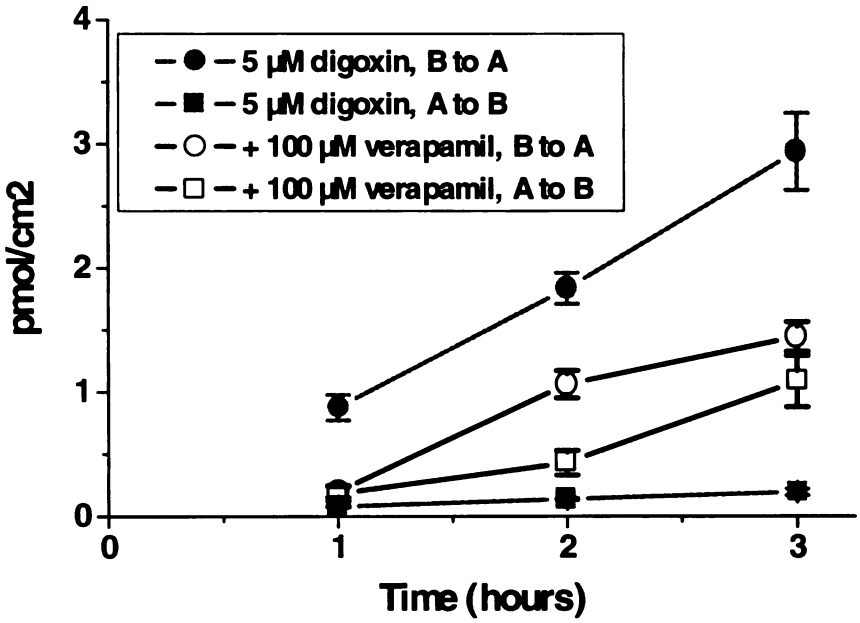


Figure 6-3. Digoxin transport by Pgp across MDR1-MDCK cell monolayers is subject to inhibition by verapamil.

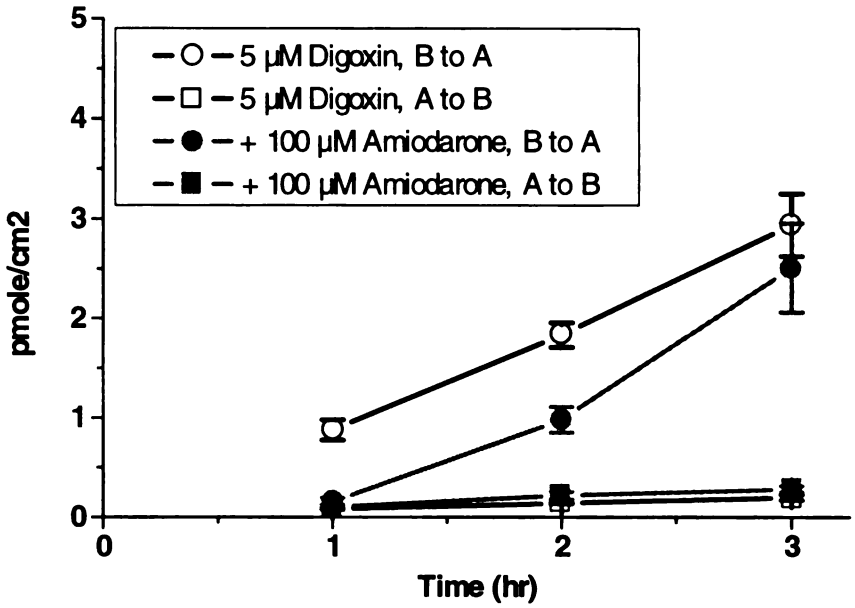


Figure 6-4. Digoxin transport by Pgp across MDR1-MDCK cell monolayers is subject to inhibition by amiodarone.

6.4.4 Transport activity of MRPs transfected cells

Vinblastine was found to be secreted by both MDCKII wild-type and MDCKII-cMOAT cells (Evers *et al.*, 1998). Permeability of MRP2 transfected cells to drugs, such as digoxin (See Chapter 4, Figure 4-7) and vinblastine, did not differ from that observed for our studies in wild-type cells. The fact that drugs failed to exhibit higher net B to A secretion in MII-cMOAT cells (also true for MII-MDR1) than in MDCKII cells suggest that MDCKII cells have higher endogenous amounts of Pgp than LLC-PK1 cells, since drugs such as digoxin (See Chapter 4, Figure 4-5) showed higher net B to A secretion in LLC-PK1-MDR1 cells than in LLC-PK1 cells.

CsA interacts with digoxin clinically, and has been shown to inhibit digoxin net B to A secretion in cMOAT over-expressed cells (See Chapter 4, Figure 4-8). Here, a higher net B to A secretion was seen for CsA in cMOAT over-expressed cells than in wild-type (Figure 6-5), although the transport in the A to B direction was almost the same in these two cell lines, suggesting that CsA is a substrate of cMOAT.

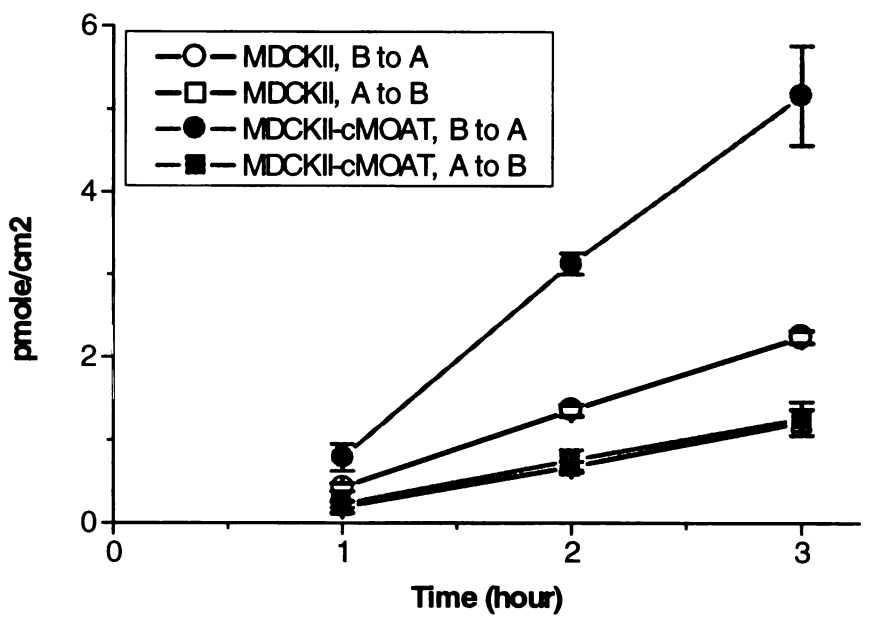


Figure 6-5. Bi-directional secretion of CsA (5 μM) in wild-type and cMOAT over-expressed MDCKII cells.

Similar to digoxin (See Chapter 4, Figure 4-10), CsA exhibited a lower A to B transport in MRP1 over-expressed cells than in the wild-type cells (Figure 6-6). But there is a difference between digoxin and CsA. Net B to A secretion of digoxin was higher than that of CsA in both wild-type and MRP1 cells, and for CsA this net B to A secretion was

lower in MRP1 over-expressed cells than in wild-type cells. This suggests wild-type and MRPs cells have efflux transporters that transport CsA and digoxin in the B to A direction, and that CsA has a higher affinity than digoxin for an A to B uptake pump, such as MRP1.

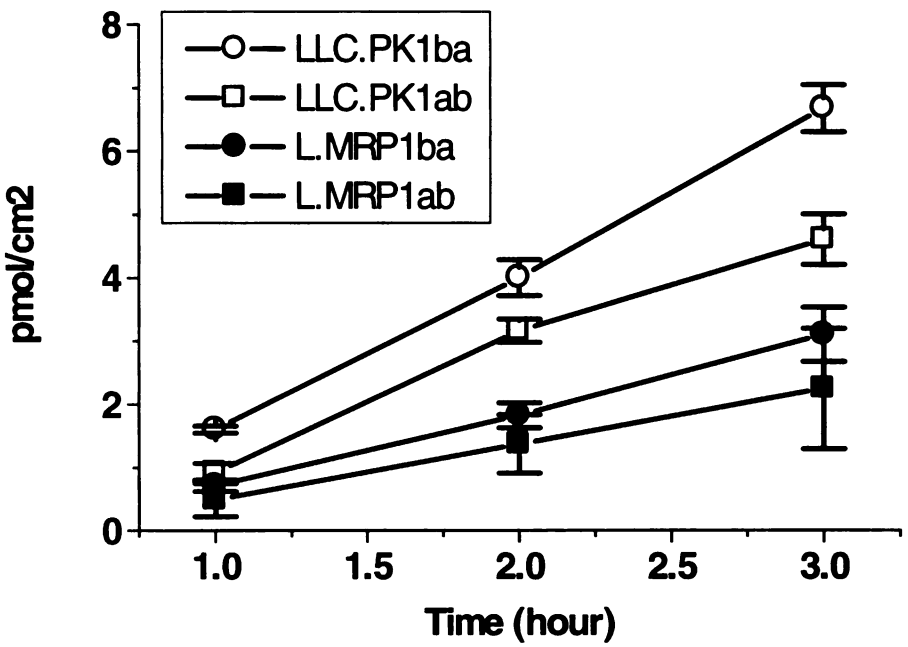


Figure 6-6. Bi-directional secretion of CsA (5 μM) in wild-type and MRP1 over-expressed LLC-PK1 cells.

6.4.5 Inhibition of digoxin transport in MRPs transfected cells

As shown in Chapter 4, indomethacin at 100 μM (See Chapter 4, Figures 4-13 and 4-16) decreased A to B and increased B to A digoxin transport in wild-type and MRP1 over-expressed LLC-PK1 cells. CsA is secreted by cMOAT (Figure 6-5) and 100 μM CsA (See Chapter 4, Figure 4-8) decreased B to A and increased A to B digoxin

transport in MDCKII-cMOAT cells. This suggests that the interactions between digoxin and indomethacin or CsA, could be an unidentified absorptive transorpter or cMOAT interactions, respectively.

Part II-- Drugs, which do not interact with digoxin clinically, were tested for substrate/inhibition activity in Pgp, MRP1 and cMOAT over-expressed cells.

6.4.6 Further validation of our cell lines

Because vinblastine has been reported to be a substrate for both Pgp and MRP, we used vinblastine as a model compound to further validate our transport systems. As shown in Figure 6-7, vinblastine showed a greater net B to A secretion in Pgp over-expressed cells than in wild-type cells, confirming that vinblastine is a substrate of Pgp. Similarly, in our studies, net digoxin B to A secretion at 2 hours in MDR1-MDCK cells was reduced from 13.1 fold to 2.0 fold by vinblastine (See Chapter 4, Figure 4-6).

It has been demonstrated that vinblastine, previously believed to be a typical Pgp substrate, can be transported to the apical side of cell monolayers by cMOAT (Evers *et al.*, 1998). However, we failed to confirm this result in MDCKII and MDCKII-cMOAT cells (Figure 6-8), which is similar to our results with digoxin (See Chapter 4, Figure 4-7). A positive result for CsA in these cell lines (Figure 6-5) indicated that CsA probably has a higher affinity than vinblastine and digoxin for cMOAT, leading to less interaction with the endogenous Pgp in the wild-type MDCKII cells.

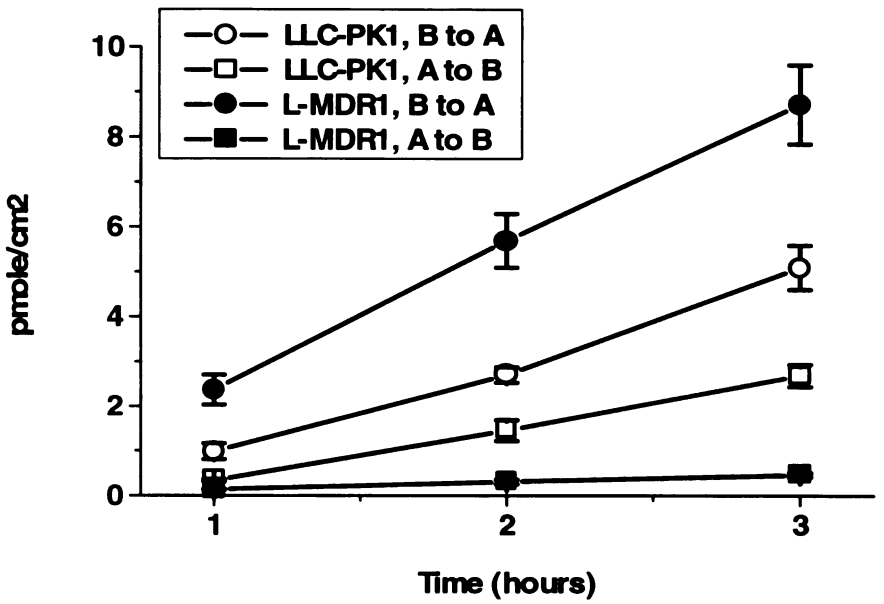


Figure 6-7. Bi-directional secretion of vinblastine (5 μM) in wild-type and MDR1 over-expressed LLC-PK1 cells.

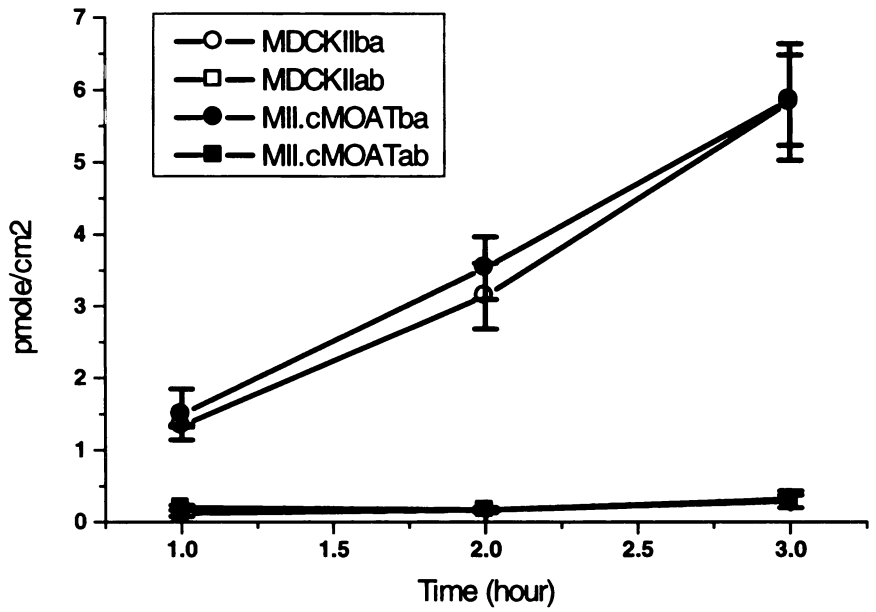


Figure 6-8. Bi-directional secretion of vinblastine (5 μM) in wild-type and cMOAT over-expressed MDCKII cells.

6.4.7 *Comparing the various cell lines in terms of inhibition activities*

Thus far we have shown the inhibition effects of 100 μM verapamil (Figure 6-3), amiodarone (Figure 6-4) and vinblastine (Chapter 4, Figure 4-6) on 5 μM digoxin transport in MDR1-MDCK cells. The inhibition effects of 100 μM amiodarone, vinblastine and verapamil, as well as CsA and vincristine, on 5 μM digoxin bidirectional transport were compared in MDR1-MDCK, LLC-PK1-MDR1 and MDCKII-MDR1 cells (Table 6-1).

There is some discrepancy between these MDR1 over-expressed cell lines (Table 6-1). For MDR1-MDCK and MDCKII-MDR1 cells, all of the inhibitors decreased B to A and increased A to B transport with a decrease in ratios of B to A over A to B transport. This is not true in LLC-PK1-MDR1 cells for B to A transport, where all of the inhibitors caused B to A transport to increase. However, since A to B transport increased relatively more, the ratio of B to A over A to B transport decreased in all cases.

The relative potency of the inhibitors on digoxin transport across the two MDR1 transfected MDCK cell lines is almost identical. In MDR1-MDCK cells, CsA>vinblastine>verapamil>amiodarone>vincristine; in MDCKII-MDR1 cells, CsA>verapamil>vinblastine>amiodarone>vincristine. In LLC-PK1-MDR1 cells, there is a reversal of vinblastine and CsA, vinblastine>verapamil>CsA>amiodarone>vincristine. Overall, CsA, vinblastine and verapamil are potent, while amiodarone and vincristine are less potent inhibitors.

Table 6-1. Comparison of the inhibition activities of 100 μM inhibitors on 5 μM digoxin bidirectional transport at 2 hours in three MDR1 over-expressed cell lines.

Inhibitors	MDR1-MDCK			MDCKII-MDR1			LLC-PK1-MDR1		
	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B
control (no inhibitor)	1.83 ± 0.12	0.14 ± 0.01	13.1	2.96 ± 0.34	0.06 ± 0.02	49.3	2.97 ± 0.75	0.22 ± 0.06	13.5
amiodarone	0.98 ± 0.13	0.21 ± 0.04	4.7	2.92 ± 0.29	0.09 ± 0.02	32.4	4.48 ± 0.66	0.64 ± 0.13	7.0
CsA	0.46 ± 0.21	0.35 ± 0.07	1.1	1.27 ± 0.15	0.26 ± 0.09	4.9	3.37 ± 0.31	0.83 ± 0.14	4.1
vincristine	1.29 ± 0.51	0.22 ± 0.14	5.9	2.53 ± 0.37	0.06 ± 0.01	42.2	4.69 ± 0.56	0.45 ± 0.07	10.4
vinblastine	0.53 ± 0.06	0.27 ± 0.04	2.0	1.51 ± 0.11	0.18 ± 0.06	8.4	3.76 ± 0.71	2.56 ± 0.65	1.5
verapamil	1.06 ± 0.11	0.43 ± 0.10	2.5	2.15 ± 0.19	0.33 ± 0.14	6.5	3.50 ± 0.34	1.01 ± 0.09	3.5

Ratio B-A/A-B, calculated by the means of B to A transport divided by the means of A to B transport.

The effects of these five inhibitors on 5 μM digoxin bi-directional transport in wild-type LLC-PK1 and MDCKII cells are listed in Table 6-2. The potency of the inhibitors on digoxin bi-directional transport in wild-type kidney epithelial cells can be ranked as: CsA>vinblastine>verapamil>amiodarone>vincristine for MDCKII cells, similar to that found for the MDR1 transfected cells (Table 6-1). The data in LLC-PK1 cells appears difficult to explain, since the ratio of B to A over A to B transport is increased by inhibitors (Table 6-2). However, this is consistent with our results in Chapter 4, in which an unidentified absorptive transporter exists in the LLC-PK1 cell line. It is possible that increased B to A and decreased A to B transport results from inhibition of this unidentified absorptive transporter in LLC-PK1 cells (Table 6-2).

Digoxin exhibits net secretion in the B to A direction in wild-type cells (Table 6-2) and MDR1 over-expressed cells (Table 6-1) as observed in all control values. The extent of digoxin B to A secretion is the least in LLC-PK1 cells, and the largest in wild-type and MDR1 transfected MDCKII cells, suggesting that less endogenous Pgp or a non-characterized efflux pump or an unidentified absorptive transporter exist in LLC-PK1 cells. The relative potency of inhibitors on digoxin transport is the same in MDR1-MDCK and in MDCKII cells. However, the fact that in the presence of inhibitors digoxin still exhibits net secretion in the B to A direction in both wild-type (Table 6-2) and transfected cells (Table 6-1), indicates that some un-known uptake or efflux transporters in the cell lines can not be inhibited by these inhibitors.

Table 6-2. Comparison of the inhibition activities of 100 μM inhibitors on 5 μM digoxin bidirectional transport at 2 hours in two wild-type kidney cell lines.

Inhibitors	MDCKII			LLC-PK1		
	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B
Control (no inhibitor)	4.80 ± 0.27	0.11 ± 0.01	43.6	2.02 ± 0.91	0.97 ± 0.09	2.1
amiodarone	3.15 ± 0.20	0.19 ± 0.03	16.6	3.57 ± 0.44	1.05 ± 0.24	3.4
CsA	1.52 ± 0.20	0.67 ± 0.13	2.3	2.82 ± 0.22	0.63 ± 0.17	4.5
vincristine	2.46 ± 0.22	0.07 ± 0.02	35.1	4.94 ± 0.74	0.62 ± 0.15	8.0
vinblastine	1.40 ± 0.24	0.57 ± 0.43	2.5	2.87 ± 0.41	0.64 ± 0.23	4.5
verapamil	1.91 ± 0.17	0.65 ± 0.20	2.9	4.40 ± 0.06	0.63 ± 0.19	7.0

6.4.8 Comparing the various cell lines in terms of substrate activities

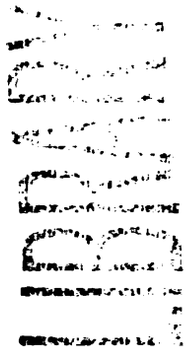
The bi-directional transport of 5 μM digoxin in various cell lines is presented in Table 6-3.

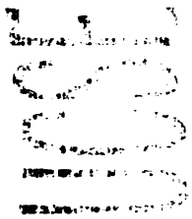
Table 6-3. Comparison of bidirectional transport for 5 μM digoxin at 2 hours in various cell lines.

Cell lines	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A /A-B
MDCKII	2.62 ± 0.08	0.08 ± 0.01	32.8
MDCKII-MDR1	2.66 ± 0.30	0.04 ± 0.01	66.5
MDCKII-cMOAT	2.34 ± 0.19	0.10 ± 0.01	23.4
MDCKI	ND		
MDR1-MDCK(I)	1.83 ± 0.12	0.14 ± 0.01	13.1
LLC-PK1	1.29 ± 0.18	0.68 ± 0.04	1.9
LLC-PK1-MDR1	2.97 ± 0.75	0.22 ± 0.06	13.5
LLC-PK1-MRP1	1.09 ± 0.22	0.62 ± 0.10	1.8

ND, not determined.

As each experiment included controls, it is apparent that different control values are reported in different tables for 5 μM digoxin transport in the same cell line. Digoxin B to A transport from MDCKII cells in Table 6-2 was 1.8 fold the value in Table 6-3, while digoxin B to A and A to B transport from LLC-PK1 cells in Table 6-2 were 1.6 and 1.4 fold the values in Table 6-3, respectively. Ratio of digoxin B to A over A to B transport in MDCKII-MDR1 cells was 49.3 in Table 6-1, but was 66.5 in Table 6-3, resulting from variability of transport in both directions.





Digoxin transport in wild-type MDCKI cells (control for MDR1-MDCK transfected cells) was not tested. The digoxin bi-directional transport in MDCKII-MDR1 and wild-type MDCKII cells suggests that digoxin transport did not differ significantly between wild-type and MDR1 transfected MDCKII cells. The net B to A secretion of digoxin in MDR1 transfected LLC-PK1 cells was much greater than in wild-type LLC-PK1 cells, consistent with digoxin being a Pgp substrate. The net B to A secretion of digoxin was not significantly different in cMOAT or MRP1 transfected cells from that observed in wild-type MDCKII or LLC-PK1 cells, respectively.

6.4.9 Inhibition/substrate specificity overlaps between MDR1 and MRPs

The effects of 100 μM indomethacin (Figure 4-13), probenecid (Figure 4-11), and sulfinpyrazone (Figure 4-12) on digoxin transport in LLC-PK1-MRP1 cells were previously presented in Chapter 4. The values at 2 hours from these studies are given here in Table 6-4. Increased B to A and decreased A to B transport of digoxin by these inhibitors in MRP1 transfected and wild-type LLC-PK1 cells suggest that an unidentified absorptive "MRP1-like" transporter is present in this wild-type cell line (See Chapter 4). However, net digoxin secretion in the B to A direction in the absence and presence of these inhibitors in MRP1 transfected and wild-type LLC-PK1 cells indicate that an uncharacterized efflux transporter (if not Pgp) that cannot be inhibited by these inhibitors, might exist in LLC-PK1 cells as well.

Here in Table 6-2, digoxin B to A transport was 2.1 fold and A to B transport was 1.5 fold higher than the values from LLC-PK1 cells in Table 6-4. The comparisons of the controls for LLC-PK1 cells in Table 6-2 and 6-3 have been discussed in section 6.4.8. The variability in the ratio of digoxin B to A over A to B transport from LLC-PK1 cells

in three different studies was 2.1, 1.9 and 1.5 as noted in Tables 6-2, 6-3 and 6-4. In LLC-PK1-MRP1 cells 1.6 fold and 1.4 fold higher B to A and A to B transport reported in Table 6-4, compared to the respective values in Table 6-3, resulted in ratios of digoxin B to A over A to B transport increasing to 2.1 in Table 6-4 compared to 1.8 in Table 6-3.

The effects of 100 μM estradiol-17β-D-glucuronide on digoxin bi-directional transport in MRP1 and MRP2 transfected cells are illustrated in Tables 6-4 and 6-6, respectively. The controls for 5 μM digoxin transport from MDCKII cells listed in Table 6-6 are from the same study reported in Table 6-3. In the presence of estradiol-17β-D-glucuronide, digoxin B to A transport was increased in both LLC-PK1 and MDCKII cells (Tables 6-4 and 6-6), while B to A transport was increased in LLC-PK1-MRP1 cells (Table 6-4). Neither digoxin B to A or A to B transport was changed in MDCKII-cMOAT cells compared to control (Table 6-6).

Since MRP1 and cMOAT prefer anionic drugs and their conjugates, we investigated if indomethacin and estradiol-17β-D-glucuronide are substrates of MRP1 (Table 6-5) and cMOAT (Table 6-7). Net B to A secretion of estradiol-17β-D-glucuronide was slightly more in MRP1 over-expressed cells than in wild-type cells mainly due to increased B to A transport (Table 6-5). Net B to A transport of estradiol-17β-D-glucuronide was the same in wild-type and cMOAT transfected MDCKII cells (Table 6-7), resulting from increased transport in both B to A and A to B directions. Bi-directional transport of indomethacin was not significantly different in cMOAT or MRP1 transfected cells from wild-type MDCKII or LLC-PK1 cells, respectively.

Table 6-4. Comparison of 100 μM inhibitors on 5 μM digoxin bidirectional transport at 2 hours in wild-type and MRP1 transfected LLC-PK1 cell lines.

Inhibitors	LLC-PK1			LLC-PK1-MRP1		
	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B
Control (no inhibitor)	0.95 ± 0.22	0.63 ± 0.07	1.5	1.77 ± 0.49	0.85 ± 0.12	2.1
Estradiol-17β-D-glucuronide	1.97 ± 0.15	0.94 ± 0.57	2.1	2.48 ± 1.57	0.86 ± 0.08	2.9
Indomethacin	4.02 ± 0.06	0.47 ± 0.08	8.6	1.72 ± 0.25	0.43 ± 0.11	4.0
Probenecid	3.21 ± 0.18	0.64 ± 0.09	5.0	2.24 ± 0.51	0.66 ± 0.15	3.4
Sulfinpyrazone	3.31 ± 0.40	0.53 ± 0.07	6.2	2.49 ± 0.30	0.64 ± 0.06	3.9

Table 6-5. Comparison of 20 μM substrate bidirectional transport at 2 hours in wild-type and MRP1 transfected LLC-PK1 cell lines.

Substrates	LLC-PK1			LLC-PK1-MRP1		
	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B
Estradiol-17β-D-glucuronide	1.03 ± 0.08	1.02 ± 0.09	1.0	1.63 ± 0.11	1.22 ± 0.11	1.3
Indomethacin	27.3 ± 2.15	13.2 ± 1.15	2.1	24.6 ± 2.31	14.6 ± 0.63	1.7

Table 6-6. Comparison of inhibition effects of estradiol-17β-D-glucuronide on 5 μM digoxin bidirectional transport in wild-type and cMOAT transfected MDCKII cell lines at 2 hours.

Inhibitors	MDCKII			MDCKII-cMOAT		
	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B
Control (no inhibitor)	2.62 ± 0.08	0.08 ± 0.01	32.8	2.34 ± 0.19	0.10 ± 0.01	23.4
Estradiol-17β-D-glucuronide	4.19 ± 0.55	0.10 ± 0.02	31.7	2.50 ± 0.26	0.11 ± 0.03	22.7

Table 6-7. Comparison of substrate specificity of estradiol-17β-D-glucuronide and indomethacin in wild-type and cMOAT transfected MDCKII cell lines at 2 hours.

Substrate	MDCKII			MDCKII-cMOAT		
	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B	B-A ($pmol/cm^2$)	A-B ($pmol/cm^2$)	Ratio B-A/A-B
Estradiol-17β-D-glucuronide	0.76 ± 0.04	0.44 ± 0.06	1.7	1.74 ± 0.17	0.97 ± 0.11	1.8
Indomethacin	21.5 ± 1.48	12.7 ± 1.61	1.7	23.3 ± 2.62	13.1 ± 1.52	1.8

Table 6-8. Substrates and/or inhibitors of CYP3A, Pgp and MRPs.

Drugs	CYP3A	MDR1	MRP1	cMOAT	Drugs	CYP3A	MDR1	MRP1	cMOAT
Antiarrhythmics					Antibiotics				
Amiodarone	S	I	I?		Daunomycin		S		
Digoxin	S	S, I	S?	S?	Doxorubicin		I		I?
Quinidine	S	I			**Erythromycin**	S	S, I		
Calcium Channel Blockers					Immuno-suppressants				
Verapamil					**Cyclosporine**	S	S, I	S, I?	S
Anticancer drugs	S, I	S, I	I?		NSAIDs				
Etoposide					**Indomethacin**		I	S, I?	
Vinblastine					Antifungals				
Vincristine	S	S			**Fluconazole**	I	I	I^A	I^B
Antigout Agents	S, I	S, I	I?	S?	**Itraconazole**	I	I	I^A	I
Colchicine	S, I	S, I	I?		**Ketoconazole**	I	I	I^A	I
Probenecid					**Miconazole**	I			I
Sulfinpyrazone	S	S			Others				
Hormones			I?	I?	**Estradiol-17β-D-**		S	I?	
β-Estradiol			I?	I?	**glucuronide**				
					Glutathione		S	S?	
		I	I?	I?					

[A] only showed positive results on digoxin transport in the A to B direction.
[B] only showed positive results on digoxin transport in the B to A direction.
Bolded drugs are those, of which results have been presented in figures and/or tables.

As listed in Table 6-8, our results expand the overlaps between substrates/inhibitors of CYP3A enzymes and transporters Pgp, MRP1 and cMOAT.

6.5 Discussion

MRP and Pgp transporters are ABC efflux transporters, with low homology between each other. MRPs are capable of transporting many anions and drug conjugates. At least six different MRPs have been identified, but only MRP1 has been demonstrated to convey MDR.

For each experiment in our studies, 5 μM digoxin bi-directional transport was carried out as a control in all cell lines tested. As discussed in the sections 6.4.8 and 6.4.9, differences were seen in the same cell line from study to study. Many possible causes can lead to such differences, *e.g.* passage numbers of the cells, the days on which the cells are grown, the media the cells are fed, the transwell system used for transport, temperature effects or variability of operation procedures during the experiments. To be consistent, the control values are reported in different tables for each set of studies carried out at the same time (Tables 6-1 to 6-4 and 6-6).

The inhibition discrepancy of inhibitors between the different MDR1 transfected cells (Table 6-1) could be due to the different amounts of endogenous Pgp or the differential presence of MRPs or some unknown uptake or efflux pumps in the parent cells. The different amounts of MDR1 over-expressed in the transfected cell lines can also cause the inconsistency.

Digoxin is secreted in the B to A direction in both wild-type and MDR1 transfected cell lines (Tables 6-1 and 6-2). The data suggest that MDCKII cells have higher levels of endogenous Pgp or an unidentified efflux transporter. Transport activities

of MDR1 can be characterized in wild-type and MDR1 transfected LLC-PK1 cells, instead of in wild-type and MDR1 transfected MDCKII cells.

CsA, vinblastine and verapamil are more potent inhibitors than amiodarone and vincristine on digoxin transport by Pgp (Table 6-1), even though the rank order of their relative inhibition potency differ within the three MDR1 transfected cell lines. CsA and vinblastine almost exhibited "perfect" inhibition by decreasing ratios of B to A over A to B transport of digoxin to 1.1 and 1.5 in MDR1-MDCK and LLC-PK1-MDR1 cells, respectively (Table 6-1). However, in most cell lines tested, digoxin was secreted in the B to A direction even when the inhibitors were present. The inhibition data in Tables 6-1 and 6-2 support our speculation (See Chapter 4) that an unidentified secretive transporter that can not be inhibited by many of the inhibitors tested, exists in the wild-type cells, especially in MDCKII cells.

A to B transport should be favored in LLC-PK1-MRP1 cells, since MRP1 has been shown to be polarized on the basolateral surface of LLC-PK1 cells upon over-expression (Evers *et al.*, 1996). Even though MRP1 expression results in resistance to vinblastine in cancer cells, Evers *et al.* (1996) reported that vinblastine efflux by MRP1 was not detectable above baseline secretion.

We tested vinblastine and CsA in these cell lines for lack of a better control. Net CsA B to A secretion in MRP1 transfected cells was slightly lower than in wild-type cells, indicating that MRP1 is involved, but certainly not the dominant transporter (Figure 6-6), since no net A to B secretion was seen for drugs including digoxin (Figure 4-9) in wild-type and MRP1 over-expressing LLC-PK1 cells.

Indomethacin has long been known to inhibit renal clearance of many anionic drugs (Smith and Benet, 1979), presumably through competition for kidney organic anion transporters (OAT). It has been shown that indomethacin inhibited and thereby reversed MDR in human and murine cell lines expressing MRP (Draper *et al.*, 1997) and decreased vincristine accumulation and increased susceptibility of cell lines over-expressing MRP, but not Pgp (Kobayashi *et al.*, 1997). But cMOAT (MRP2) is not thought to be sensitive to indomethacin inhibition (Evers *et al.*, 1998).

Sulfinpyrazone and probenecid inhibited apical to basal transport of daunorubicin by MRP but not basal to apical by MDR1 (Evers *et al.*, 1996). MRP1 has been shown to be inhibited by probenecid, vinblastine and CsA in cancer lines over-expressing MRP, but not Pgp (Holló *et al.*, 1996). Sulfinpyrazone did not inhibit the MRP2 mediated secretion of vinblastine (Evers *et al.*, 1998), but was found to decrease secretion of digoxin in MRP1 over-expressed cells (See Chapter 4, Figure 4-12).

Probenecid, sulfinpyrazone and indomethacin are inhibitors of digoxin transport in MRP1 over-expressed cells (Figures 4-11, 4-12 and 4-13). The inhibition studies of probenecid, sulfinpyrazone and indomethacin observed in LLC-PK1 cells (Figures 4-14, 4-15 and 4-16) suggest the presence of an unidentified endogenous "MRP1 like" absorptive transporter. The overall net B to A secretion of digoxin in LLC-PK1-MRP1 observed is presumably due to endogenous Pgp or cMOAT or some un-known uptake or efflux transporters (Table 6-3).

cMOAT (MRP2), expressed on the apical membrane of MDCKII cells upon over-expression, has been shown to result in secretion of vinblastine (Evers *et al.*, 1998). However, we observed a similar B to A secretion of vinblastine in both wild-type and

cMOAT over-expressing cells (Figure 6-8), but CsA showed a greater net B to A secretion in cMOAT transfected cells than in wild-type MDCKII cells (Figure 6-5). Secretion of digoxin in the MDCKII-cMOAT cell line was found to be similar to in the MDCKII wild-type cell line (Figure 4-7). Pgp contributes to net B to A secretion of digoxin in MDCKII cells, since in this chapter we verified that Pgp inhibitors such as verapamil, vinblastine and CsA reduced net digoxin B to A secretion in MDCKII cells (Table 6-2).

Digoxin B to A transport was increased in LLC-PK1 and MDCKII cells (Tables 6-4 and 6-6), A to B transport was increased in LLC-PK1 cells (Table 6-4) by 100 μM estradiol-17β-D-glucuronide, while others remained the same. This suggests the inhibition of endogenous MRP1 and/or an unidentified absorptive transporter in wild-type LLC-PK1 or MDCKII cells, the inhibition of endogenous of Pgp and/or a non-characterized efflux transporter in wild-type LLC-PK1 cells by estradiol-17β-D-glucuronide. Because of the possibility that multiple transporters may exist in parent LLC-PK1 and MDCKII cell lines, we cannot conclude that either estradiol-17β-D-glucuronide or indomethacin is a substrate of MRP1 or MRP2 (Tables 6-5 and 6-7) from our studies.

Although Pgp prefers lipophilic or weakly basic substrates, MRP prefers anionic compounds and products of phase II metabolism such as glutathione-conjugates, MRPs and Pgp function similarly in transporting many of the same substrates such as verapamil, vinblastine and cyclosporine (CsA) (Holló *et al.*, 1996). The fact that CsA can be transported by MDR1 and cMOAT (Figure 6-5) in our studies, further support the overlaps of substrate/inhibition specificities between Pgp and MRPs (Table 6-8). Our

data of the involvement of MRP2 in digoxin-CsA interaction is also consistent with a recent finding (Noe *et al.*, 1997) that a novel multi-specific organic anion transporting polypeptide (oatp2) from rat brain, mediates high-affinity uptake of digoxin (K_m = 0.24 μM).

Back in the 1970's, it was hypothesized that digoxin-erythromycin interactions were caused by erythromycin inhibiting intestinal flora enzymes, therefore reducing digoxin gut metabolism (Lindenbaum *et al.*, 1981a and b). Our studies indicated that erythromycin is a Pgp substrate (Figure 6-2), and the mechanism of digoxin-erythromycin interactions can be competition of these two drugs for gut Pgp. Our results suggest that MDR1 interactions may cause clinical interactions between digoxin and amiodarone, CsA, verapamil or erythromycin, while MRP2 or "MRP1 like" absorptive transporter interactions might also lead to clinical digoxin-CsA or digoxin-indomethacin interactions.

Cell line providers claimed that these transporters were stably over-expressed in the transfected cell lines and Western blots showed that protein levels of transporters were higher in transporter over-expressed cells than in wild-types (data not shown). However, it is not clear that the protein levels correlate to transport activities. The cell lines used in our studies were within three passages to avoid variability and/or loss of transporter expression.

Here, transport studies were all carried out in kidney or intestine derived cell lines. Perhaps *in vivo*, other endogenous renal or intestinal transporters could be masking the effects of over-expressed transporters. Because of the simultaneous presence of multiple secretory and absorptive transporters in these cells and because transporters may

exhibit varying degrees of susceptibility to inhibition, it is not feasible at this point to identify the substrate or inhibition specificity of individual transporters. Further definitive studies using molecular biology technologies and "transporter knock-out" mice (Mayer *et al.*, 1996a and b) are likely required to elucidate the role of transport processes in the kinetics and interactions of drugs, such as digoxin.

6.6 References

Antman, E. M. and Smith, T. W. (1985). Digitalis toxicity. *Annual Review of Medicine* **36,** 357-67.

Belz, G. G., Doering, W., Munkes, R. and Matthews, J. (1983). Interaction between digoxin and calcium antagonists and antiarrhythmic drugs. *Clinical Pharmacology and Therapeutics* **33,** 410-7.

Calvo, M. V., Martin-Suarez, A., Martin Luengo, C., Avila, C., Cascon, M. and Domínguez-Gil Hurlé, A. (1989). Interaction between digoxin and propafenone. *Therapeutic Drug Monitoring* **11,** 10-5.

Cavet, M. E., West, M. and Simmons, N. L. (1996). Transport and epithelial secretion of the cardiac glycoside, digoxin, by human intestinal epithelial (Caco-2) cells. *British Journal of Pharmacology* **118,** 1389-96.

Cole, S. P., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M. and Deeley, R. G. (1994). Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Research* **54,** 5902-10.

de Lannoy, I. A. and Silverman, M. (1992). The MDR1 gene product, P-glycoprotein, mediates the transport of the cardiac glycoside, digoxin. *Biochemical and Biophysical Research Communications* **189,** 551-7.

de Lannoy, I. A., Koren, G., Klein, J., Charuk, J. and Silverman, M. (1992). Cyclosporin and quinidine inhibition of renal digoxin excretion: evidence for luminal secretion of digoxin. *American Journal of Physiology* **263,** F613-22.

Dobkin, J. F., Saha, J. R., Butler, V. P., Jr., Neu, H. C. and Lindenbaum, J. (1982). Inactivation of digoxin by Eubacterium lentum, an anaerobe of the human gut flora. *Transactions of the Association of American Physicians* **95,** 22-9.

Doige, C. A. and Sharom, F. J. (1992). Transport properties of P-glycoprotein in plasma membrane vesicles from multidrug-resistant Chinese hamster ovary cells. *Biochimica et Biophysica Acta* **1109,** 161-71.

Draper, M. P., Martell, R. L. and Levy, S. B. (1997). Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein. *British Journal of Cancer* **75,** 810-5.

Evers, R., Kool, M., van Deemter, L., Janssen, H., Calafat, J., Oomen, L. C., Paulusma, C. C., Oude Elferink, R. P., Baas, F., Schinkel, A. H. and Borst, P. (1998). Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. *Journal of Clinical Investigation* **101,** 1310-9.

Evers, R., Zaman, G. J., van Deemter, L., Jansen, H., Calafat, J., Oomen, L. C., Oude Elferink, R. P., Borst, P. and Schinkel, A. H. (1996). Basolateral localization and export activity of the human multidrug resistance-associated protein in polarized pig kidney cells. *Journal of Clinical Investigation* **97,** 1211-8.

Flens, M. J., Izquierdo, M. A., Scheffer, G. L., Fritz, J. M., Meijer, C. J., Scheper, R. J. and Zaman, G. J. (1994). Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Research* **54,** 4557-63.

Fromm, M. F., Kim, R. B., Stein, C. M., Wilkinson, G. R. and Roden, D. M. (1999). Inhibition of P-glycoprotein-mediated drug transport: A unifying mechanism to explain the interaction between digoxin and quinidine [see comments]. *Circulation* **99,** 552-7.

Gottesman, M. M. and Pastan, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual Review of Biochemistry* **62,** 385-427.

Haig, G. M. and Brookfield, E. G. (1992). Increase in serum digoxin concentrations after indomethacin therapy in a full-term neonate. *Pharmacotherapy* **12,** 334-6.

Heijn, M., Hooijberg, J. H., Scheffer, G. L., Szabó, G., Westerhoff, H. V. and Lankelma, J. (1997). Anthracyclines modulate multidrug resistance protein (MRP) mediated organic anion transport. *Biochimica et Biophysica Acta* **1326,** 12-22.

Heller, M. (1990). Cardiac glycosides. New/old ideas about old drugs. *Biochemical Pharmacology* **40,** 919-25.

Holló, Z., Homolya, L., Hegedüs, T. and Sarkadi, B. (1996). Transport properties of the multidrug resistance-associated protein (MRP) in human tumour cells. *FEBS Letters* **383,** 99-104.

Hori, R., Okamura, N., Aiba, T. and Tanigawara, Y. (1993). Role of P-glycoprotein in renal tubular secretion of digoxin in the isolated perfused rat kidney. *Journal of Pharmacology and Experimental Therapeutics* **266,** 1620-5.

Horio, M., Gottesman, M. M. and Pastan, I. (1988). ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proceedings of the National Academy of Sciences of the United States of America* **85,** 3580-4.

Ishikawa, T., Müller, M., Klünemann, C., Schaub, T. and Keppler, D. (1990). ATP-dependent primary active transport of cysteinyl leukotrienes across liver canalicular membrane. Role of the ATP-dependent transport system for glutathione S-conjugates. *Journal of Biological Chemistry* **265,** 19279-86.

Ito, S., Koren, G., Harper, P. A. and Silverman, M. (1993). Energy-dependent transport of digoxin across renal tubular cell monolayers (LLC-PK1). *Canadian Journal of Physiology and Pharmacology* **71,** 40-7.

Jørgensen, H. S., Christensen, H. R. and Kampmann, J. P. (1991). Interaction between digoxin and indomethacin or ibuprofen. *British Journal of Clinical Pharmacology* **31,** 108-10.

Juliano, R. L. and Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta* **455,** 152-62.

Kaukonen, K. M., Olkkola, K. T. and Neuvonen, P. J. (1997). Itraconazole increases plasma concentrations of quinidine. *Clinical Pharmacology and Therapeutics* **62,** 510-7.

Kelly, R. A. and Smith, T. W. (1996). Pharmacological treatment of heart failure. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (eds. Hardman, J. G. and Limbird, L. E.), pp. 809-838. New York: McGraw-Hill.

Kobayashi, S., Okada, S., Yoshida, H. and Fujimura, S. (1997). Indomethacin enhances the cytotoxicity of VCR and ADR in human pulmonary adenocarcinoma cells. *Tohoku Journal of Experimental Medicine* **181,** 361-70.

Kool, M., de Haas, M., Scheffer, G. L., Scheper, R. J., van Eijk, M. J., Juijn, J. A., Baas, F. and Borst, P. (1997). Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Research* **57,** 3537-47.

Koren, G. (1987). Clinical pharmacokinetic significance of the renal tubular secretion of digoxin. *Clinical Pharmacokinetics* **13,** 334-43.

Koren, G., Klein, J., MacLeod, S. and Silverman, M. (1986). Cellular mechanisms of digoxin transport and toxic interactions in the kidney. *Veterinary and Human Toxicology* **28,** 549-52.

Koren, G., Woodland, C. and Ito, S. (1998). Toxic digoxin-drug interactions: the major role of renal P-glycoprotein. *Veterinary and Human Toxicology* **40,** 45-6.

Kruh, G. D., Gaughan, K. T., Godwin, A. and Chan, A. (1995). Expression pattern of MRP in human tissues and adult solid tumor cell lines. *Journal of the National Cancer Institute* **87,** 1256-8.

Lautier, D., Canitrot, Y., Deeley, R. G. and Cole, S. P. (1996). Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. *Biochemical Pharmacology* **52,** 967-77.

Lindenbaum, J., Rund, D. G., Butler, V. P., Jr., Tse-Eng, D. and Saha, J. R. (1981a). Inactivation of digoxin by the gut flora: reversal by antibiotic therapy. *New England Journal of Medicine* **305,** 789-94.

Lindenbaum, J., Tse-Eng, D., Butler, V. P., Jr. and Rund, D. G. (1981b). Urinary excretion of reduced metabolites of digoxin. *American Journal of Medicine* **71,** 67-74.

Mayer, U., Wagenaar, E., Beijnen, J. H., Schinkel, A. H. and Borst, P. (1996a). Altered pharmacokinetics of (^{3}H)-digoxin in mdrla P-glycoprotein knockout mice. *Proceedings of the American Association for Cancer Research Annual Meeting* **37,** 334.

Mayer, U., Wagenaar, E., Beijnen, J. H., Smit, J. W., Meijer, D. K., van Asperen, J., Borst, P. and Schinkel, A. H. (1996b). Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. *British Journal of Pharmacology* **119,** 1038-44.

McNulty, R. M., Lazor, J. A. and Sketch, M. (1989). Transient increase in plasma quinidine concentrations during ketoconazole-quinidine therapy. *Clinical Pharmacy* **8,** 222-5.

Noe, B., Hagenbuch, B., Stieger, B. and Meier, P. J. (1997). Isolation of a multispecific organic anion and cardiac glycoside transporter from rat brain. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 10346-50.

Okamura, N., Hirai, M., Tanigawara, Y., Tanaka, K., Yasuhara, M., Ueda, K., Komano, T. and Hori, R. (1993). Digoxin-cyclosporin A interaction: modulation of the multidrug transporter P-glycoprotein in the kidney. *Journal of Pharmacology and Experimental Therapeutics* **266,** 1614-9.

Oude Elferink, R. P. and Jansen, P. L. (1994). The role of the canalicular multispecific organic anion transporter in the disposal of endo- and xenobiotics. *Pharmacology and Therapeutics* **64,** 77-97.

Paine, M. F., Shen, D. D., Kunze, K. L., Perkins, J. D., Marsh, C. L., McVicar, J. P., Barr, D. M., Gillies, B. S. and Thummel, K. E. (1996). First-pass metabolism of midazolam by the human intestine. *Clinical Pharmacology and Therapeutics* **60,** 14-24.

Pastan, I., Gottesman, M. M., Ueda, K., Lovelace, E., Rutherford, A. V. and Willingham, M. C. (1988). A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. *Proceedings of the National Academy of Sciences of the United States of America* **85,** 4486-90.

Paulusma, C. C., Bosma, P. J., Zaman, G. J., Bakker, C. T., Otter, M., Scheffer, G. L., Scheper, R. J., Borst, P. and Oude Elferink, R. P. (1996). Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271,** 1126-8.

Rodin, S. M. and Johnson, B. F. (1988). Pharmacokinetic interactions with digoxin. *Clinical Pharmacokinetics* **15,** 227-44.

Schinkel, A. H., Smit, J. J., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Mol, C. A., van der Valk, M. A., Robanus-Maandag, E. C., te Riele, H. P. and et al. (1994). Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77,** 491-502.

Shen, H., Paul, S., Breuninger, L. M., Ciaccio, P. J., Laing, N. M., Helt, M., Tew, K. D. and Kruh, G. D. (1996). Cellular and in vitro transport of glutathione conjugates by MRP [retracted in Biochemistry 1997 Nov 11;36(45):13972]. *Biochemistry* **35,** 5719-25.

Smith, D. E. and Benet, L. Z. (1979). Relationship between urinary excretion rate, steady-state plasma levels and diuretic response of furosemide in the rat. *Pharmacology* **19,** 301-6.

Su, S. F. and Huang, J. D. (1996). Inhibition of the intestinal digoxin absorption and exsorption by quinidine. *Drug Metabolism and Disposition* **24,** 142-7.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I. and Willingham, M. C. (1987). Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National Academy of Sciences of the United States of America* **84,** 7735-8.

Tobe, S. W., Siu, L. L., Jamal, S. A., Skorecki, K. L., Murphy, G. F. and Warner, E. (1995). Vinblastine and erythromycin: an unrecognized serious drug interaction [see comments]. *Cancer Chemotherapy and Pharmacology* **35,** 188-90.

Tsuruo, T., Iida, H., Kitatani, Y., Yokota, K., Tsukagoshi, S. and Sakurai, Y. (1984). Effects of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and adriamycin in drug-resistant tumor cells. *Cancer Research* **44,** 4303-7.

van der Graaf, W. T., de Vries, E. G., Uges, D. R., Nanninga, A. G., Meijer, C., Vellenga, E., Mulder, P. O. and Mulder, N. H. (1991). In vitro and in vivo modulation of

multi-drug resistance with amiodarone. *International Journal of Cancer* **48,** 616-22.

Watkins, P. B. (1997). The barrier function of CYP3A4 and P-glycoprotein in the small bowel. *Advanced Drug Delivery Reviews* **27,** 161-170.

Woodland, C., Verjee, Z., Giesbrecht, E., Koren, G. and Ito, S. (1997). The digoxin-propafenone interaction: characterization of a mechanism using renal tubular cell monolayers. *Journal of Pharmacology and Experimental Therapeutics* **283,** 39-45.

Wu, C. Y., Benet, L. Z., Hebert, M. F., Gupta, S. K., Rowland, M., Gomez, D. Y. and Wacher, V. J. (1995). Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine. *Clinical Pharmacology and Therapeutics* **58,** 492-7.

Yusa, K. and Tsuruo, T. (1989). Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Research* **49,** 5002-6.

Chapter 7

EFFECTS OF KETOCONAZOLE AND ITRACONAZOLE ON DIGOXIN PHARMACOKINETICS IN HEALTHY VOLUNTEERS

7.1 Abstract

The objective of this study was to evaluate the effects of the azole anti-fungal agents, ketoconazole and itraconazole, potent inhibitors of CYP3A4 and Pgp, on the pharmacokinetic profile of digoxin, a substrate of human Pgp.

The pharmacokinetics of digoxin were studied in 6 healthy volunteers (3 female and 3 male subjects) in an open-label, randomized, six-period interaction study. Each subject received a single dose of digoxin alone (0.25 mg orally or intravenously) and with concomitant KCZ or ICZ (200 mg orally), in which KCZ or ICZ and digoxin were dosed simultaneously. Digoxin concentrations in plasma and urine as well as KCZ or ICZ

concentrations in plasma were determined using validated HPLC/MS/MS assays. Estimated pharmacokinetic parameters of digoxin and azoles in plasma (mean ± SD) were calculated using non-compartmental methods by WinNonlin software.

Concomitant azoles increased *iv* digoxin C_{max} by 2-fold (KCZ) and 3-fold (ICZ), increased $AUC_{0\text{-}120}$ and $AUC_{0\text{-}\infty}$, decreased CL, V_{ss}, $t_{1/2,\lambda z}$ and MRT. In the presence of azoles, *p.o.* digoxin C_{max}, $AUC_{0\text{-}120}$ and $AUC_{0\text{-}\infty}$ increased with parallel decreases in T_{max}, while $t_{1/2,\lambda z}$ and AUMC/AUC increased. KCZ seemed to have a more pronounced effect than ICZ on oral digoxin pharmacokinetics (C_{max} and $AUC_{0\text{-}\infty}$ increased more than 2-fold by KCZ). However, administration of digoxin did not significantly affect pharmacokinetic parameters of azoles.

The data demonstrate that all digoxin pharmacokinetic parameters were significantly affected in the presence of KCZ or ICZ. Results from our studies suggest that the changes in exposure to digoxin were attributable to the effects of azoles on digoxin absorption as well as the effects on digoxin elimination. The parallel increase in AUC for *iv* and *p.o.* administration of digoxin implicated the major effects of ICZ on digoxin elimination. A local inhibitory effect by KCZ on digoxin intestinal Pgp/MRP-mediated efflux could explain the marked increase in oral digoxin plasma concentrations and decrease in digoxin T_{max} in the presence of KCZ.

7.2 Introduction

Digoxin, a cardiac glycoside, also known as *Lanoxin*®, was originally isolated from leaves of *Digitalis lanata*. Digoxin is the most commonly used drug for congestive heart failure (CHF) and atrial fibrillation. Digoxin is predominately eliminated in the urine as unchanged drug. Renal clearance accounts for most of total digoxin body

clearance, and exceeds creatinine and inulin clearances. Non-renal excretion of digoxin and/or digoxin metabolites includes hepatic metabolism, biliary excretion, intestinal secretion and subsequent fecal elimination of digoxin and/or metabolites that are not reabsorbed. The total fecal excretion of radioactivity averaged 11 % after an *iv* dose and 20% after a *p.o.* dose (Reuning and Geraets, 1986).

It has been shown that the tubular transport of digoxin is not associated with the anionic (Koren *et al.*, 1986) or cationic transport systems (Koren *et al.*, 1986), nor Na^+/K^+-ATPase (Koren *et al.*, 1986). As described in Chapter 4, the permeation of digoxin across kidney epithelial and Caco-2 monolayers *in vitro*, is greatest in the direction of secretion suggesting that active secretion may occur *in vivo*.

It has been shown by several studies that digoxin is a Pgp substrate (Cavet *et al.*, 1996; de Lannoy and Silverman, 1992; Ito *et al.*, 1993; Tanigawara *et al.*, 1992). Investigations using perfused rat kidney (Hori *et al.*, 1993) and rat everted gut sacs (Su and Huang, 1996) concluded that Pgp played an important role in some digoxin-drug interactions. Studies in mdr1a and mdr1a/1b knockout mice (Mayer *et al.*, 1996; Schinkel *et al.*, 1997) showed that intestinal excretion (Mayer *et al.*, 1996), brain accumulation (Mayer *et al.*, 1996; Schinkel *et al.*, 1996), tissue distribution and pharmacokinetics of digoxin (Schinkel *et al.*, 1995) are strongly affected by the mouse Pgp activity.

Drug interactions have been observed between digoxin and many other compounds such as indomethacin (Haig and Brookfield, 1992), resulting in decreased renal clearance and increased plasma concentrations of digoxin. Seven cases of digoxin-ICZ interactions have been reported (See Chapter 2, Table 2-4) (Alderman and Allcroft, 1997; Alderman and Jersmann, 1993; Cone *et al.*, 1996; Kauffman and Bagnasco, 1992;

McClean and Sheehan, 1994; Meyboom *et al.*, 1994; Rex, 1992; Sachs *et al.*, 1993), in which digoxin serum concentrations reached toxic levels after concurrent administration of ICZ. However, no interaction between digoxin and KCZ has been reported. Therapy with KCZ (Rex, 1992) did not produce side effects, nor did concomitant MCZ (Alderman and Allcroft, 1997) lead to toxicity in the same patient where ICZ did.

The major concern with the use of azole antifungal agents has been the potential for drug-drug interactions. In general, the potential for drug-drug interactions occurs most frequently with KCZ (Bodey, 1992). KCZ, ICZ and FCZ are potent inhibitors of cytochrome P450 3A4. KCZ has a significantly greater *in vitro* inhibitory effect than ICZ and FCZ on CYP3A4 (Como and Dismukes, 1994). The K_i values for KCZ and ICZ inhibition of formation of α-OH-midazolam from midazolam, a typical P450 3A4 but not a Pgp substrate, are 3.7 nM and 0.27 μM, respectively (von Moltke *et al.*, 1996). The order of *in vivo* inhibitory potency of azole antifungal agents following typical therapeutic regimens is consistent with their *in vitro* K_i values for CYP3A inhibition, *i.e.* KCZ > ICZ > FCZ, with MCZ having essentially no effect (Baciewicz and Baciewicz, 1993; Gillum *et al.*, 1993).

KCZ and ICZ can reverse MDR. KCZ was found to overcome resistance to vinblastine and doxorubicin in resistant cells *in vitro* at the concentrations safely used *in vivo* for treatment of fungal infection (Siegsmund *et al.*, 1994), but had little or no effect on parental cells. ICZ reversed adriamycin-resistant cells in dosage compatible to the plasma levels achieved by the therapeutic dosages used for the treatment of fungal infections. ICZ also partially reversed etoposide resistance in both parental and resistant cells. The results suggested involvement of ICZ in MDR gene and/or MRP gene

associated resistance (Kurosawa *et al.*, 1996). KCZ and ICZ have been shown to inhibit digoxin transport across MDCK cells, with ICZ more potent than KCZ (Ito *et al.*, 1994 and 1995), while FCZ has little effect (Ito and Koren, 1997). It was hypothesized that the digoxin-ICZ interactions and a lack of digoxin-KCZ interaction are caused by azoles' differential inhibition of digoxin secretion by Pgp in the kidney (Ito and Koren, 1997; Ito *et al.*, 1994 and 1995; Koren *et al.*, 1998).

We have demonstrated that digoxin is not extensively metabolized by CYP3A in humans (See Chapter 3), and that in addition to Pgp, MRP1 and cMOAT, ATP-dependent drug efflux systems found in many tissues (Benet *et al.*, 1996; Kruh *et al.*, 1995) may also possibly be involved in digoxin transport (See Chapter 4). Our studies using transporter cDNA transfected cell lines indicated that azole anti-fungal agents abolished net secretion of digoxin in Pgp, MRP1 and cMOAT transfected cells, with KCZ showing a higher inhibitory effect than ICZ (See Chapter 5). MCZ did not affect digoxin transport by MDR1, while no obvious effects of MCZ and FCZ on digoxin transport were observed in MRP1 and cMOAT over-expressed cells, respectively. We hypothesize that co-administration of digoxin and these azoles results in significantly higher digoxin plasma concentrations and an increased incidence of digoxin toxicity caused, at least in part, by inhibition of digoxin secretion, but not inhibition of CYP3A in the body by azoles.

Two clinical studies have been conducted by others to understand the pharmacokinetic mechanisms of digoxin-ICZ interactions (Jalava *et al.*, 1997; Partanen *et al.*, 1996). In one study, ten volunteers received 0.25 mg digoxin orally once daily for 20 days. The subjects were randomized to receive concomitantly either 200 mg ICZ or

placebo orally for the first 10 days, ICZ was changed to placebo and *vice versa* for the next 10 days. Plasma measurements on day 10 after dosing ICZ yielded increased digoxin concentrations 12 hr after drug administration in each subject from 1.0 ± 0.1 nmol/L (placebo phase) to 1.8 ± 0.1 nmol/L (ICZ phase) (Partanen *et al.*, 1996). In the other study, a double-blind, randomized, two-phase crossover study, ten healthy volunteers received either 200 mg ICZ or placebo orally once daily for 5 days. On day 3, each volunteer ingested a single 0.5 mg oral dose of digoxin. $AUC_{0\rightarrow72}$ was 50% higher, CL_r decreased about 20%, but C_{max} and $t_{1/2}$ were not significantly increased by ICZ (Jalava *et al.*, 1997).

Understanding the mechanisms underlying the kinetics of drugs would help pharmacologists and drug researchers develop new and more dependable drugs. The extent and clinical significance of potential drug interactions observed *in vitro* must be assessed in clinical studies. Therefore, it was the aim of this study to investigate the effects of KCZ and ICZ on the kinetics of digoxin in healthy volunteers and to understand the pharmacokinetic mechanisms of digoxin-ICZ interactions and lack of digoxin-KCZ (FCZ or MCZ) interactions.

7.3 Subjects and methods

7.3.1 Materials

All dispensed medications were obtained through the UCSF-Stanford Health Care In-patient Pharmacy at the University of California, San Francisco, Moffit Hospital. Digoxin (*Lanoxin*®) was given either as a 0.25 mg (*iv)* by intravenous infusion over a three minute interval or orally (*p.o.*) as 0.25 mg tablets. KCZ (*Nizoral*®) and ICZ

(*Sporanox*®) were given as 200 mg tablets *p.o.* White polypropylene scintillation vials (20 ml, caps attached) were purchased from Fisher Scientific (Pittsburgh, PA).

7.3.2 Subjects

The study was approved by the Committee on Human Research (CHR) and the General Clinical Research Center (GCRC) of the University of California, San Francisco and carried out and monitored in the latter facility. All study subjects gave their signed informed consent and were free to withdraw from the study at any time. Subjects were considered to have completed the study if they went through six study periods and the final study evaluation, as well as any post-study assessments that were deemed clinically necessary. Subjects received monetary compensation for their participation.

Subjects in our clinical study included three males and three females, age 21-38 years old, height 168.0 to 179.1 cm and weighing 51 to 92 kg. The individual subject details are given in Table 7-1. The participants were randomized into six treatment sequences with code to the sequence pattern given in Table 7-2. All subjects had normal physical, and blood and urine screening results, testing negative for the HIV virus and drugs of abuse.

Table 7-1. Participants in this study.

Subjects	Ethnicity	Gender	Sequence	Age (years)	Ht (cm)	Wt (kg)
1	Hispanic & Caucasian	F	BADCFE	34	174.0	66.2
2	Hispanic	M	ABFECD	35	168.0	51.0
3	Caucasian	F	ABEFCD	29	179.1	90.7
4	Caucasian	M	BACDFE	38	173 .0	74.8
5	Caucasian	F	ABDCFE	25	168.5	69.4
6	Caucasian	M	BAEFCD	21	173.5	92.0

Table 7-2. Sequence codes for digoxin and azole treatments in volunteers.

Sequence code	Route of digoxin and azoles
A	i.v. digoxin
B	p.o. digoxin
C	i.v. digoxin with p.o. itraconazole
D	p.o. digoxin with p.o. itraconazole
E	i.v. digoxin with p.o. ketoconazole
F	p.o. digoxin with p.o. ketoconazole.

7.3.3 Study design

Six healthy volunteers were studied in an open label, 6-way crossover, randomized trial, with each subject receiving all six treatments (Table 7-2) in one of the six sequences shown in Table 7-1.

This was a 6-period study where the subjects received each of the dose administrations at 2-week intervals. The total length of the study was about 12 weeks, including one 2-hour screening visit, six 13 hour-hospital days and 36 blood-sample draws of ½ hour each (6 doses x 6 blood samples x ½ hour), which totals 98 hours for blood sampling and screening.

7.3.4 Inclusion/exclusion criteria

Before acceptance into the study, each subject underwent a complete pre-study evaluation within 2 weeks before study drug administration. Healthy male and female subjects were included when they were 20-40 years old, had normal renal function and body weights were within ±20% of their ideal body weight. Subjects were non-smokers without abnormal physical findings, with all laboratory values within the normal limits. Female subjects had to have a negative pregnancy test and, if of reproductive potential, had to be able to practice a reliable, non-hormonal form of contraception. Except for the

study drugs, no other drugs were allowed during the study period. Subjects were asked to refrain from alcohol and caffeine during the whole study period.

Subjects were not eligible for the study if they had active medical problems, had abnormal physical findings and laboratory values, used recreational or prescriptions drugs or over-the-counter drugs (including birth control pills), except occasional acetaminophen or vitamins. Subjects were excluded if they had a history of asthma or allergies (food or drug), especially clinically significant allergic conditions or hypersensitivity especially to digoxin or azole antifungal agents. Subjects were not enrolled when they had a positive test for human immuno-deficiency virus (HIV), or had a positive drug of abuse screen or pregnant or lactating or smoke tobacco. Subjects were not accepted when they were unable to understand consent forms or unable to follow instructions or unable to abstain from ethanol or caffeine throughout the study.

7.3.5 Study procedures

Subjects were admitted as outpatients for 13-hour visits on each of the six study days, and subjects were fasted overnight from the evening prior to each study day (10 hours prior to digoxin dosing). Each study day was followed by a 14-day washout.

On each of the six dosing days, subjects were admitted to the clinical research center and, after a brief physical examination including vital signs, an indwelling catheter was inserted in the subject's arm. At 8 am, subjects received a dose of 0.25 mg of digoxin either by mouth or intravenously. Two weeks later, the alternate route was used to give 0.25 mg of digoxin, so if the first dose was by mouth the next dose was intravenously. During the four subsequent study periods, a *p.o.* dose of itraconazole (*Sporanox*®) 200-mg co-administered with the digoxin dose (*p.o.* or *iv*), and a *p.o.* dose of ketoconazole

(*Nizoral*®) 200 mg co-administered with the digoxin dose (*p.o.* or *iv*) were given by randomized sequences (Table 7-1) at 2-week intervals.

Subjects were served standardized meals starting 4 hr after drug administration. During the whole study period, subjects were not allowed to drink grapefruit juice, alcohol or more than 300 mg·day^{-1} caffeine. Concomitant medication or smoking was prohibited. After the 12 hr-blood draw subjects were discharged and returned to the study unit for subsequent blood draws and returning urine collections on the following 5 days. For each dosing, blood samples (10 ml each) were drawn into heparinized tubes, after digoxin administration at the following times: 0, and 30 minutes, and 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96 and 120 hours. Blood samples were placed on ice and centrifuged at 0°C, and plasma was separated immediately. Plasma was collected in duplicates for each of time points. Urine samples of 0-4, 4-8, and 8-12, 12-24, 24-36, 36-48, 48-72, 72-96 and 96-120 hours were collected and approximately three tubes with 20 ml of each urine sample were stored and the remaining urine was discarded. Plasma and urine samples were frozen at –80°C until analysis. After the last study sample was collected, each subject had another physical examination and a complete laboratory evaluation.

7.3.6 Plasma and urine sample analysis

All analytical methods were validated and samples were analyzed following good laboratory practice (GLP) guidelines. Plasma and urine samples were analyzed for digoxin and azoles by validated HPLC/MS/MS methods.

Sample preparations:

Sample extraction methods for digoxin and azoles were modified from those previously published for KCZ (Badcock, 1984; Turner *et al.*, 1986), ICZ (Badcock, 1990;

Warnock *et al.*, 1988) and digoxin (Tracqui *et al.*, 1997). Oleandrin was used as the internal standard (IS) for digoxin, and propanolol was the internal standard for ketoconazole or itraconazole.

Digoxin was extracted from plasma and urine with 95% methylene chloride in isopropanol (IPA). Methylene chloride (lower) layer was evaporated to dryness under N_2, then reconstitute in mobile phase containing 50% acetonitrile (ACN) and 0.1% trifluoracetic acid (TFA) prior to injection onto the column. Acetonitrile was added to plasma samples containing ketoconazole to precipitate protein, which was removed following centrifugation. Methyl-terbutyl ether (MTBE) was added to plasma samples containing itraconazole. After centrifugation for 10 min at 300 rpm, the organic layer was evaporated to dryness under N_2. The residues were reconstituted with mobile phase (90% ACN and 0.06% TFA) before injection onto the column.

Instruments:

The conditions for analysis of digoxin and azoles in human plasma and urine samples are listed in Table 7-3.

7.3.7 Statistical methods

A sample size justification analysis using Sigma Stat was performed. A projected difference in means of trough levels of the interactions of digoxin with itraconazole increasing by 2.5 ng/ml (as observed previously for oral dosing), standard deviation of 1.7, power 0.8 and alpha 0.05, showed that six subjects were adequate.

Comparisons between treatments were performed using paired t-test or ANOVA. Statistically significant differences corresponded to a α-level of 0.05 and when the 90%-confidence interval was outside of the acceptance limits.

Table 7-3. Instrumental conditions for analysis of KCZ and ICZ in human plasma and digoxin in human plasma and urine.

	Digoxin	KCZ	ICZ
Computer	Macintosh Quadra 800	Macintosh Quadra 800	Macintosh Quadra 800
Injector	Waters Intelligent Sample Processor 717 Plus	Waters Intelligent Sample Processor 717 Plus	Waters Intelligent Sample Processor 717 Plus
Detector	LC/MS/MS PE Sciex API III Plus	LC/MS/MS PE Sciex API III Plus	LC/MS/MS PE Sciex API III Plus
Ionization	APCI/positive ionization	APCI/positive ionization	APCI/positive ionization
MRM mass scanning	782-651 for digoxin 571-373 for oleandrin (IS)	531-488 for KCZ 260-182 for propanolol (IS)	707-392 for ICZ 260-182 for propanolol (IS)
Pump	Shimadzu LC-10 AD	Shimadzu LC-10 AD	Shimadzu LC-10 AD
Column	C_{18}, 50 x 4.6 mm, 5 µm	Hypersil Silica, 50 x 4.6 mm, 3 µm	Silica, 50 x 4.6 mm, 5 µm
Flow rate	1 ml/min	1 ml/min	1 ml/min
Mobile phase	50% ACN and 0.1% TFA	85% ACN, 0.08% TFA and 2mM NH_4Ac	90% ACN and 0.06% TFA

7.3.8 Pharmacokinetics

Plasma data:

The pharmacokinetic parameters C_{max}, $AUC_{0\text{-}120}$, $AUC_{0\text{-}\infty}$, $t_{1/2,\lambda z}$, CL, V_{ss}, MRT (*iv*) and T_{max} (*p.o.*) were determined based on non-compartmental methods using WinNonlin-Pro™ 1.5 (Pharsight Corporation, Cary, NC). The values for C_{max} and the time to reach C_{max} (T_{max}) were also estimated directly from the data. Total clearances (CL) were calculated by CL=D/AUC (*iv*) or CL/F=D/AUC (*p.o.*). Bioavailability (F) obtained by the following formula where values oral and *iv* are compared for each of the three phases.

$$F = AUC_{p.o.} \times D_{iv}/AUC_{iv} \times D_{p.o.}$$

MRT was calculated as AUMC/AUC for the three *iv* doses, and AUMC/AUC (the sum of MRT plus the mean absorption time, MAT) is reported for the oral doses.

7.4 Results

7.4.1 Safety and adverse events

No adverse events were reported during the whole study period. After administration of digoxin without or with concomitant administration of azoles no abnormal laboratory values were observed.

7.4.2 Pharmacokinetics

There were marked increases in digoxin exposure in plasma (Table 7-4) as reflected by mean ratios (with/without KCZ) for C_{max} 2.8 (*p.o.*) and 2.0 (*iv*) and for $AUC_{0-\infty}$ values 2.4 (*p.o.*) and 1.2 (*iv*). The changes by ICZ for C_{max} were 1.3 (*p.o.*) and 3.0 (*iv*) and 1.5 (*p.o.* and *i.v.*) for $AUC_{0-\infty}$ values.

Digoxin CL and V_{ss} were decreased in the presence of azoles for *iv* administration of digoxin (Table 7-4). The digoxin/KCZ interaction yielded a 22% decrease in CL for digoxin. ICZ decreased digoxin CL by 28%. The digoxin/KCZ interaction yielded a 43% decrease in V_{ss} for digoxin. ICZ decreased digoxin V_{ss} by 42%.

T_{max} was decreased by azoles. Digoxin (*p.o.*) T_{max} was 40% (KCZ) and 25% (ICZ) shorter with than without azoles. Digoxin bioavailability was 0.71, which was consistent with literature values (See Chapter 2, Table 2-1). There appeared to be no change in the presence of ICZ, but the calculated F was greater than 1 when KCZ was co-administered (Table 7-4).

Surprisingly, azoles shortened MRT by 35% (ICZ) and 30% (KCZ) and shortened $t_{1/2,\lambda z}$ by 23% (ICZ) and 20% (KCZ) for *iv* digoxin. However, in the presence of azoles,

$t_{1/2,\lambda z}$ and AUMC/AUC of *p.o.* digoxin were increased compared to control (Table 7-4). AUMC/AUC increased by 1.4 fold (ICZ) and 1.3fold (KCZ), while $t_{1/2,\lambda z}$ increased by 1.3 fold (ICZ) and 1.1 fold (KCZ).

Urine digoxin measurements and calculations of renal clearance, as well as bioavailability determinations, were not completed at the time of writing of this thesis, but will be included in the publications resulting from this work.

Table 7-4. Comparison of digoxin pharmacokinetic parameters in healthy volunteers (n=6) receiving a single 0.25 mg oral or intravenous dose of digoxin alone or with concomitant administration of 200 mg oral doses of ketoconazole or itraconazole.

Treatments		T_{max} (hr)	C_{max} (ng/ml)	$T_{1/2,\lambda z}$ (hr)	AUC_{0-120} (ng·h/ml)	$AUC_{0-\infty}$ (ng·h/ml)	CL (L/h)	V_{ss} (L)	AUMC/AUC (hr)	F
***iv* Digoxin**	**Mean**		3.92	25.7	12.55	16.33	20.3	477	33.9	
	SD		1.72	15.8	5.83	9.24	12.1	97	25.1	
***iv* Digoxin + ICZ**	**Mean**		11.9	19.8	22.09	24.88	14.6	278	22.1	
	SD		13.5	12.7	11.66	12.42	12.3	188	11.4	
***iv* Digoxin + KCZ**	**Mean**		7.78*	20.5	21.49*	19.25	15.9	271	23.7	
	SD		3.33	14.7	13.23	9.23	8.2	186	19.7	
***p.o.* Digoxin**	**Mean**	1.33	1.16	22.9	7.97	11.00			30.0	0.71
	SD	0.75	0.74	17.3	5.91	7.44			20.4	0.33
***p.o.* Digoxin + ICZ**	**Mean**	1.00	1.55	29.5	11.33	16.72			42.0	0.67
	SD	0.55	0.54	32.9	6.23	11.71			44.8	0.31
***p.o.* Digoxin + KCZ**	**Mean**	0.80	3.26	26.3	18.93	26.39			40.1	1.67
	SD	0.67	2.26	16.2	11.56	16.39			27.7	1.66

* Six subjects were included in the calculations, while other parameters were determined from five subjects only.

The pharmacokinetic parameters of azoles in six healthy volunteers receiving a single 200 mg oral dose of azole with concomitant administration of 0.25 mg intravenous and oral dose of digoxin, are listed in Table 7-5 for KCZ and ICZ.

T_{max} of KCZ (Table 7-5) was 1.5 hours when co-administered with an oral or *iv* dose of digoxin, C_{max} and terminal $t_{1/2}$ were 2280 ng/ml and 5.8 hours with a concomitant *iv* dose of digoxin. When co-administered with an oral dose of digoxin, C_{max} and terminal $t_{1/2}$ of KCZ were 2330 ng/ml and 6.3 hours, respectively. T_{max} of ICZ (Table 7-5) was 3 hours with concomitant oral or *iv* digoxin. C_{max} was 72 ng/ml (*iv* digoxin) and 96 ng/ml (*p.o.* digoxin), while terminal $t_{1/2}$ was 12.0 hours (*iv* digoxin) and 15.9 hours (*p.o.* digoxin).

C_{max} of KCZ was about 30 fold higher than that of ICZ, while the terminal $t_{1/2}$ of ICZ was 2 to 3 fold longer than that of KCZ. T_{max} values for ICZ were twice those of KCZ. $AUC_{0-\infty}$ values of KCZ were 9.8 fold (*iv* digoxin) and 7.5 fold (*p.o.* digoxin) higher than those of ICZ. The AUMC/AUC for KCZ and ICZ were 3.6-4.0 hours (*iv-p.o.*) and 20-24 hours, respectively. This indicates that the residence time of ICZ in the body was as much as 5 to 6 fold longer than that of KCZ (Table 7-5).

The route of digoxin administration did not seem to affect KCZ or ICZ parameters significantly (Table 7-5). The kinetic parameters of azoles determined from our studies in the presence of digoxin are close to those reported in the literature (See Chapter 5, Table 5-1).

Table 7-5. Pharmacokinetic parameters of ketoconazole and itraconazole in healthy volunteers (n=6) receiving a single 200 mg oral dose with concomitant administration of 0.25 mg intravenous or oral doses of digoxin.

Digoxin dose	Parameters	Ketoconazole		Itraconazole	
		Means	SD	Means	SD
iv	T_{max} (hr)	1.5	0.5	3.3	1.6
	C_{max} (ng/ml)	2280	1090	72.3	47.8
	$T_{1/2, \lambda z}$ (hr)	5.8	7.3	12.0	6.0
	AUC_{0-120} (ng·hr/ml)	8950	4450	877	712
	$AUC_{0-\infty}$ (ng·hr/ml)	8970	4460	911	692
	V_z/F (L)	334	426	5090	2150
	CL/F (L/hr)	129	269	402	354
	AUMC/AUC (hr)	3.6	0.4	20.0	9.0
oral	T_{max} (hr)	1.5	1.3	3.2	1.3
	C_{max} (ng/ml)	2330	1370	95.7	60.7
	$T_{1/2, \lambda z}$ (hr)	6.3	5.4	15.9	5.2
	AUC_{0-120} (ng·hr/ml)	9990	5890	1300	1140
	$AUC_{0-\infty}$ (ng·hr/ml)	10000	5880	1340	1130
	V_z/F (L)	406	636	6050	4220
	CL/F (L/hr)	35.1	31.7	273	204
	AUMC/AUC (hr)	4.0	1.0	24.1	8.4

7.5 Discussion

In Chapter 3, we have demonstrated that digoxin is a substrate of rat CYP3A, but not extensively metabolized in human liver microsomes. KCZ and ICZ inhibited digoxin metabolism by CYP3A in rat liver microsomes (See Chapter 3). The fact that concentrations of digoxin metabolites in plasma were very low from our studies, further confirmed that CYP3A enzymes do not extensively metabolize digoxin in humans.

Digoxin has been shown to be a Pgp substrate (See Chapter 4), indicating that digoxin is undergoing significant Pgp-mediated intestinal efflux from the tissue mucosa into the lumen. Our studies using transporter cDNA transfected cell lines indicated in addition to Pgp, MRPs and an unidentified absorptive or secretive transporter may possibly be involved in digoxin transport (See Chapter 4). *In vitro*, it has been demonstrated that digoxin transport in Pgp, MRP1 and cMOAT transfected cells is subject to inhibition by azole anti-fungal agents, with KCZ being a stronger inhibitor than ICZ (See Chapter 5).

Six healthy volunteers were studied in an open label, 6-way crossover, randomized trial, in which each subject was given an oral and *iv* dose of digoxin. Mean values for CL and V_{ss} of *iv* digoxin were 30 L/hr and 477 L, respectively. The mean $t_{1/2,\lambda z}$ was 26 hours for *iv* and 23 hours for *p.o.* digoxin. The mean absolute bioavailability determined from our studies was 0.71. C_{max} from *p.o.* digoxin was 1.16 ng/ml, which was more than 3 fold lower than that from *iv* digoxin, 3.92 ng/ml. Mean T_{max} of *p.o.* digoxin was determined be 1.33 hours.

The literature values (See Chapter 2, Table 2-1) for digoxin CL, V_{ss}, F and T_{max} are 11.34 L/hr, 469 L, 0.70 (tablet) and 1.5 – 5 hours. The values for V_{ss}, F and T_{max}

obtained from our studies are consistent with the parameters in the literature. However, CL here was much higher, due to the lower $AUC_{0-\infty}$ values. Recall, however, that we utilized a specific LC/MS/MS method *versus* the immuno- and radioimmunoassays previously employed clinically (See Chapter 2, Table 2-3), which also measure the active bis and mono-digitoxiside metabolites. C_{max} of *p.o.* digoxin in our subjects was within the therapeutic window of digoxin (0.8 to 2.0 ng/ml). The 23-26 hour half-lives determined in our study are shorter than the reported 39 hours in Table 2-1, possibly again reflecting our inability to measure levels over the same time interval employed with the non-specific clinical assays.

Because CL was calculated as Dose/$AUC_{0-\infty}$ (*iv*), the values for CL may be more skewed since the extrapolation with the shorter half-life still accounts for more than 20% of the total AUC. It is interesting, however, that the V_{ss} values determined here are similar to the reported literature results, since this calculation also depends on extrapolations using the shorter half-lives. Possibly because the non-compartmental V_{ss} depends on extrapolations in both the numerator (AUMC) and the denominator (AUC^2), the half-life differences are minimized. Large variability of the data, similar to that reported in the literature was observed in our study. Only the trends in the parameters can be summarized here since no detailed statistics has been carried out, as yet, for our data, and only 6 subjects were studied.

Azoles affected the kinetics of *iv* digoxin, The decrease in CL and increase in AUC of *iv* digoxin by azoles (Table 7-4) indicates the inhibitory effects of azoles on digoxin elimination. No apparent difference in the effects of KCZ and ICZ on *iv* digoxin, in terms of the multiple digoxin parameters determined, was observed. Although the

variability was great, the decreases in digoxin CL seems real. A decrease in digoxin CL is consistent with our hypothesis. We speculate that the secretive transporters of digoxin are inhibited by azoles in kidney and/or liver, thereby decreasing digoxin CL from a combination of decreased CL_r and CL_{nr}.

The decrease in V_{ss} also appears to be real, and is consistent with what has been observed with the digoxin-KCZ interaction in rats (See Chapter 3, Table 3-2). Aging or diseases such as renal failure caused digoxin V_{ss} to decrease in patients (See Chapter 2, Table 2-2). A decreased physical volume of tissue (V_T) and/or an increased fraction unbound in the tissues (f_{uT}) can lead to a decreased V_{ss}. The MRT for *iv* digoxin was increased in the digoxin- KCZ interaction study in rats (See Chapter 3, Table 3-2). However here, MRT and terminal half-lives were decreased by azoles in our subjects. A decrease in MRT was seen due to the fact that the decrease in V_{ss} was more than the decrease in CL. The decreases in both parameters seem real although large variability was observed. This change is not consistent with our original hypothesis, and must reflect our lack of understanding of how the azoles could cause a larger change in V_{ss} than CL.

Compared to digoxin pharmacokinetics without azoles, simultaneous co-administration of KCZ and ICZ (Table 7-4) in our subjects affected the kinetics of *p.o.* digoxin. Azoles increase $AUC_{0\text{-}120}$ and $AUC_{0\text{-}\infty}$ of *p.o.* digoxin. The consistent changes in AUC and C_{max} suggest that the increases are real. Comparisons of AUC and C_{max} changes suggest that KCZ had a stronger effect than ICZ on oral administration of digoxin, which is also consistent with the extent of T_{max} changes.

No apparent change in F values of digoxin was observed in the presence of ICZ compared to control. Co-administration of ICZ led to a parallel increase (1.5 fold) in

AUC for *p.o.* and *iv* administrations of digoxin (Table 7-4), implicating that the major inhibitory effects of ICZ were on digoxin elimination. The F value for digoxin determined in the presence of KCZ exceeded 1. The standard deviation (SD) for $AUC_{0\text{-}120}$ of *p.o.* digoxin with KCZ is double that for the SD with ICZ, while the SDs for the *iv* $AUC_{0\text{-}120}$ values are almost the same. We have little confidence in the F value with KCZ *versus* that with ICZ, since the SD for digoxin F with KCZ is equal to the mean value and is much greater than that for F with ICZ. However, it would appear that KCZ does have some effect on digoxin absorption.

There is no difference in the mean MRT value for *iv* digoxin (34 hours) and the AUMC/AUC value of *p.o.* digoxin (30 hours), although theoretically the latter has to be larger. Here again the large SDs may hide any differences. Azoles decreased the MRT values for *iv* digoxin by 30%, while increasing the values for AUMC/AUC after *p.o.* digoxin. Because of the large SDs of AUMC/AUC values, we cannot conclude that the MAT of digoxin really increases in the presence of ICZ or KCZ. Digoxin MAT with KCZ are very similar to that with ICZ. T_{max} of digoxin was decreased from 1.33 hours to 1 hour by ICZ and to 0.8 hour by KCZ. If intestinal Pgp/MRPs-mediated efflux of digoxin was inhibited by azoles as we hypothesized, we would expect a parallel decrease for T_{max} and MAT. The large variability in our study may explain the inconsistency between the T_{max} and MAT changes.

From our study, KCZ had a 8-10 fold higher AUC but shorter terminal half-life (2-3 fold) and T_{max} (2.2 fold) than ICZ (Table 7-5). The literature values of C_{peak} for KCZ and ICZ are 1500-3100 and 200-400 (ng/ml), while T_{peak} values are 1-4 (KCZ) and 4-5 hours (ICZ). The terminal half-lives for KCZ and ICZ are 7-10 and 24-42 hours,

respectively. Comparing azole parameters determined here with those in the literature for azoles alone (See Chapter 5, Table 5-1), suggests that co-administration of digoxin and the routes of digoxin administration did not affect azole pharmacokinetic parameters.

Previously, poor oral bioavailability was mainly attributed to poor solubility in gastrointestinal fluids, poor permeability through the mucosal membrane and/or extensive hepatic first pass metabolism, and drug interactions for CYP enzymes were generally assumed to take place mainly in the liver. Recently it has been recognized for drugs that are CYP3A and Pgp/MRP substrates, CYP3A mediated drug metabolism in the intestine and Pgp/MRP counter-transport processes significantly contribute to poor oral bioavailability and play an important role in drug interactions (Wacher *et al.*, 1995; Wu *et al.*, 1995).

A more than 8-fold inter-individual variability in Pgp expression in the small intestine has been described (Brattström *et al.*, 1997; Lown *et al.*, 1997). It can be expected that variability of intestinal Pgp plays a major role in the inter-individual variability of the extent of the digoxin-azole interactions observed, especially the digoxin-KCZ interaction for *p.o.* digoxin. Large variability was also observed in our study, as usually seen in clinical studies.

Because several factors including concomitant drugs, kidney function and other diseases, present in patients may impact drug interactions and complicate establishment of a valid cause-effect relationship, healthy volunteers instead of heart patients were chosen as subjects in our clinical study. Subjects in our studies were fasted 10 hours before dosing, since it has been reported that meals decrease C_{peak} and increase T_{peak} of digoxin (Greenblatt *et al.*, 1974). Since aging can cause digoxin distribution (Lewis,

1992) and elimination (Lewis, 1992; Wettrell, 1977; Wettrell and Andersson, 1977) to decrease, only subjects 20-40 years old were recruited in our study. Gender and racial differences may exist but were not obvious in our study due to the limited numbers of subjects included.

The doses of digoxin and azoles selected for this study were the standards used in patients, based on: (1) the pharmacokinetics and safety of digoxin previously determined in healthy volunteers; (2) the known transport of digoxin in Pgp and MRPs transfected cells; (3) the known inhibition effects of ICZ and KCZ on digoxin transport in Pgp and MRPs transfected cells; and (4) the observed effect of ICZ on the digoxin bioavailability and clearance. Single digoxin doses were well tolerated by most subjects, although digoxin exposure was increased during the azole study periods.

Confidence in our conclusions from this *in vivo* study is limited due to the great variability reflected by the large SD of our results. However, the trends in the kinetic parameters of digoxin and azoles suggest that pharmacokinetic interactions occur between digoxin and azoles, and the extent of digoxin-KCZ interaction, although highly variable, was greater than the digoxin-ICZ interaction. This is consistent with our *in vitro* results that KCZ is a stronger inhibitor than ICZ on digoxin transport by Pgp and in MRPs transfected cells (See Chapter 5). Comparisons of results from *iv* and *p.o.* digoxin suggest that the major effects of ICZ on digoxin were on digoxin elimination, while KCZ affected the rate and extent of digoxin absorption more than ICZ did.

Results from our clinical study support our hypothesis that co-administration of digoxin and these azoles results in higher digoxin plasma concentrations, at least in part, by inhibition of digoxin secretion in intestine, kidney and liver, but not inhibition of

CYP3A by azoles. Therefore, it can be expected that the main mechanisms underlying the digoxin/azole interaction in our study is inhibition of digoxin intestinal, biliary and renal Pgp/MRP-mediated efflux by azoles.

The digoxin-KCZ interaction in our study is much greater than could be expected from previous reports describing digoxin-ICZ interactions. One of the possibilities for reported digoxin-ICZ cases and a lack of digoxin-KCZ interaction in the literature may result from the less frequent use of concomitant KCZ with digoxin in patients than with ICZ. The increased digoxin exposure caused by ketoconazole suggests that co-administration with KCZ will require digoxin dose reduction in some patients. Because of the large variability of the digoxin-azole interactions, a general recommendation for digoxin dose reduction in patients who require concomitant azoles could not be established and digoxin doses may require individual adjustment according to plasma concentrations.

The individual renal clearances will be determined from the periodic urine collections from our subjects. Information about CL and CL_r should help us to better understand the pharmacokinetic mechanisms of digoxin-azole interactions. At this point, we have not been able to identify individual transporters for the substrate specificity of digoxin and/or the inhibition specificity of azoles (Chapters 4, 5 and 6). Because many transporters have not yet been discovered and cloned, the tissue distributions and cellular locations of some known transporters have not been well characterized. Additional investigations are needed to elucidate the pharmacokinetic mechanisms of digoxin-azole interactions.

Our single-dose study in healthy volunteers demonstrates a good correlation between our *in vitro* and *in vivo* results. The results from this thesis work implicate that drugs that have been identified as substrates and/or inhibitors of transporters *in vitro*, should be cautiously co-administered with another known substrate and/or inhibitor of transporters in patients unless a relevant drug interaction has been excluded by a clinical drug interaction study. Greater concern should be given to those drugs that interact with both multi-drug transporters and cytochrome P450 enzymes, especially CYP3A.

7.6 References

Alderman, C. P. and Allcroft, P. D. (1997). Digoxin-itraconazole interaction: possible mechanisms [see comments]. *Annals of Pharmacotherapy* **31,** 438-40.

Alderman, C. P. and Jersmann, H. P. (1993). Digoxin-itraconazole interaction [letter]. *Medical Journal of Australia* **159,** 838-9.

Baciewicz, A. M. and Baciewicz, F. A., Jr. (1993). Ketoconazole and fluconazole drug interactions [see comments]. *Archives of Internal Medicine* **153,** 1970-6.

Badcock, N. R. (1984). Micro-determination of ketoconazole in plasma or serum by high-performance liquid chromatography. *Journal of Chromatography* **306,** 436-40.

Badcock, N. R. (1990). Micro-scale method for itraconazole in plasma by reversed-phase high-performance liquid chromatography. *Journal of Chromatography* **525,** 478-83.

Benet, L. Z., Kroetz, D. L. and Sheiner, L. B. (1996). The dynamics of drug absorption, distribution and elimination. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (eds. Hardman, J. G. and Limbird, L. E.), pp. 3-27. New York: McGraw-Hill.

Bodey, G. P. (1992). Azole antifungal agents. *Clinical Infectious Diseases* **14 Suppl 1,** S161-9.

Brattström, C., Säwe, J., Tydén, G., Herlenius, G., Claesson, K., Zimmerman, J. and Groth, C. G. (1997). Kinetics and dynamics of single oral doses of sirolimus in sixteen renal transplant recipients. *Therapeutic Drug Monitoring* **19,** 397-406.

Cavet, M. E., West, M. and Simmons, N. L. (1996). Transport and epithelial secretion of the cardiac glycoside, digoxin, by human intestinal epithelial (Caco-2) cells. *British Journal of Pharmacology* **118,** 1389-96.

Como, J. A. and Dismukes, W. E. (1994). Oral azole drugs as systemic antifungal therapy [see comments]. *New England Journal of Medicine* **330,** 263-72.

Cone, L. A., Himelman, R. B., Hirschberg, J. N. and Hutcheson, J. W. (1996). Itraconazole-related amaurosis and vomiting due to digoxin toxicity [letter]. *Western Journal of Medicine* **165,** 322.

de Lannoy, I. A. and Silverman, M. (1992). The MDR1 gene product, P-glycoprotein, mediates the transport of the cardiac glycoside, digoxin. *Biochemical and Biophysical Research Communications* **189,** 551-7.

Gillum, J. G., Israel, D. S. and Polk, R. E. (1993). Pharmacokinetic drug interactions with antimicrobial agents. *Clinical Pharmacokinetics* **25,** 450-82.

Greenblatt, D. J., Duhme, D. W., Koch-Weser, J. and Smith, T. W. (1974). Bioavailability of digoxin tablets and elixir in the fasting and postprandial states. *Clinical Pharmacology and Therapeutics* **16,** 444-8.

Haig, G. M. and Brookfield, E. G. (1992). Increase in serum digoxin concentrations after indomethacin therapy in a full-term neonate. *Pharmacotherapy* **12,** 334-6.

Hori, R., Okamura, N., Aiba, T. and Tanigawara, Y. (1993). Role of P-glycoprotein in renal tubular secretion of digoxin in the isolated perfused rat kidney. *Journal of Pharmacology and Experimental Therapeutics* **266,** 1620-5.

Ito, S. and Koren, G. (1997). Comment: possible mechanism of digoxin-itraconazole interaction [letter; comment]. *Annals of Pharmacotherapy* **31,** 1091-2.

Ito, S., Koren, G., Harper, P. A. and Silverman, M. (1993). Energy-dependent transport of digoxin across renal tubular cell monolayers (LLC-PK1). *Canadian Journal of Physiology and Pharmacology* **71,** 40-7.

Ito, S., Woodland, C. and Koren, G. (1994). Ketoconazole and itraconazole inhibit digoxin transport across renal tubular cell monolayer (MDCK). *Pediatric Research* **37,** 77A.

Ito, S., Woodland, C. and Koren, G. (1995). Characteristics of itraconazole and ketoconazole inhibition of digoxin transport across renal tubular cells. *FASEB Journal* **9,** A686.

Jalava, K. M., Partanen, J. and Neuvonen, P. J. (1997). Itraconazole decreases renal clearance of digoxin. *Therapeutic Drug Monitoring* **19,** 609-13.

Kauffman, C. A. and Bagnasco, F. A. (1992). Digoxin toxicity associated with itraconazole therapy [letter]. *Clinical Infectious Diseases* **15,** 886-7.

Koren, G., Klein, J., MacLeod, S. and Silverman, M. (1986). Cellular mechanisms of digoxin transport and toxic interactions in the kidney. *Veterinary and Human Toxicology* **28,** 549-52.

Koren, G., Woodland, C. and Ito, S. (1998). Toxic digoxin-drug interactions: The major role of renal P-glycoprotein. *Veterinary and Human Toxicology* **40,** 45-46.

Kruh, G. D., Gaughan, K. T., Godwin, A. and Chan, A. (1995). Expression pattern of MRP in human tissues and adult solid tumor cell lines. *Journal of the National Cancer Institute* **87,** 1256-8.

Kurosawa, M., Okabe, M., Hara, N., Kawamura, K., Suzuki, S., Sakurada, K. and Asaka, M. (1996). Reversal effect of itraconazole on adriamycin and etoposide resistance in human leukemia cells. *Annals of Hematology* **72,** 17-21.

Lewis, R. P. (1992). Clinical use of serum digoxin concentrations. *American Journal of Cardiology* **69,** 97G-106G; discussion 106G.

Lown, K. S., Mayo, R. R., Leichtman, A. B., Hsiao, H. L., Turgeon, D. K., Schmiedlin-Ren, P., Brown, M. B., Guo, W., Rossi, S. J., Benet, L. Z. and Watkins, P. B. (1997). Role of intestinal P-glycoprotein (mdr1) in inter-patient variation in the oral bioavailability of cyclosporine. *Clinical Pharmacology and Therapeutics* **62,** 248-60.

Mayer, U., Wagenaar, E., Beijnen, J. H., Smit, J. W., Meijer, D. K., van Asperen, J., Borst, P. and Schinkel, A. H. (1996). Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr 1a P-glycoprotein. *British Journal of Pharmacology* **119,** 1038-44.

McClean, K. L. and Sheehan, G. J. (1994). Interaction between itraconazole and digoxin [letter; comment]. *Clinical Infectious Diseases* **18,** 259-60.

Meyboom, R. H., de Jonge, K., Veentjer, H., Dekens-Konter, J. A. and de Koning, G. H. (1994). [Potentiation of digoxin by itraconazole]. *Nederlands Tijdschrift voor Geneeskunde* **138,** 2353-6.

Partanen, J., Jalava, K. M. and Neuvonen, P. J. (1996). Itraconazole increases serum digoxin concentration. *Pharmacology and Toxicology* **79,** 274-6.

Reuning, R. H. and Geraets, D. R. (1986). Digoxin. In *Applied pharmacokinetics : principles of therapeutic drug monitoring* (eds. Evans, W. E., Schentag, J. J. and Jusko, W. J.), pp. 570-623. Spokane, WA.

Rex, J. (1992). Itraconazole-digoxin interaction [letter]. *Annals of Internal Medicine* **116,** 525.

Sachs, M. K., Blanchard, L. M. and Green, P. J. (1993). Interaction of itraconazole and digoxin [see comments]. *Clinical Infectious Diseases* **16,** 400-3.

Schinkel, A. H., Mayer, U., Wagenaar, E., Mol, C. A., van Deemter, L., Smit, J. J., van der Valk, M. A., Voordouw, A. C., Spits, H., van Tellingen, O., Zijlmans, J. M., Fibbe, W. E. and Borst, P. (1997). Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 4028-33.

Schinkel, A. H., Wagenaar, E., Mol, C. A. and van Deemter, L. (1996). P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *Journal of Clinical Investigation* **97,** 2517-24.

Schinkel, A. H., Wagenaar, E., van Deemter, L., Mol, C. A. and Borst, P. (1995). Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *Journal of Clinical Investigation* **96,** 1698-705.

Siegsmund, M. J., Cardarelli, C., Aksentijevich, I., Sugimoto, Y., Pastan, I. and Gottesman, M. M. (1994). Ketoconazole effectively reverses multidrug resistance in highly resistant KB cells. *Journal of Urology* **151,** 485-91.

Su, S. F. and Huang, J. D. (1996). Inhibition of the intestinal digoxin absorption and exsorption by quinidine. *Drug Metabolism and Disposition* **24,** 142-7.

Tanigawara, Y., Okamura, N., Hirai, M., Yasuhara, M., Ueda, K., Kioka, N., Komano, T. and Hori, R. (1992). Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). *Journal of Pharmacology and Experimental Therapeutics* **263,** 840-5.

Tracqui, A., Kintz, P., Ludes, B. and Mangin, P. (1997). High-performance liquid chromatography-ionspray mass spectrometry for the specific determination of digoxin and some related cardiac glycosides in human plasma. *Journal of Chromatography. B, Biomedical Sciences and Applications* **692,** 101-9.

Turner, C. A., Turner, A. and Warnock, D. W. (1986). High performance liquid chromatographic determination of ketoconazole in human serum. *Journal of Antimicrobial Chemotherapy* **18,** 757-63.

von Moltke, L. L., Greenblatt, D. J., Schmider, J., Duan, S. X., Wright, C. E., Harmatz, J. S. and Shader, R. I. (1996). Midazolam hydroxylation by human liver microsomes in vitro: inhibition by fluoxetine, norfluoxetine, and by azole antifungal agents. *Journal of Clinical Pharmacology* **36,** 783-91.

Wacher, V. J., Wu, C. Y. and Benet, L. Z. (1995). Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: Implications for

drug delivery and activity in cancer chemotherapy. *Molecular Carcinogenesis* **13,** 129-134.

Warnock, D. W., Turner, A. and Burke, J. (1988). Comparison of high performance liquid chromatographic and micro-biological methods for determination of itraconazole. *Journal of Antimicrobial Chemotherapy* **21,** 93-100.

Wettrell, G. (1977). Distribution and elimination of digoxin in infants. *European Journal of Clinical Pharmacology* **11,** 329-35.

Wettrell, G. and Andersson, K. E. (1977). Clinical pharmacokinetics of digoxin in infants. *Clinical Pharmacokinetics* **2,** 17-31.Haig, G. M. and Brookfield, E. G. (1992). Increase in serum digoxin concentrations after indomethacin therapy in a full-term neonate. *Pharmacotherapy* **12,** 334-6.

Wu, C. Y., Benet, L. Z., Hebert, M. F., Gupta, S. K., Rowland, M., Gomez, D. Y. and Wacher, V. J. (1995). Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine. *Clinical Pharmacology and Therapeutics* **58,** 492-7.

Chapter 8

CONCLUSIONS AND PERSPECTIVES

The recognition of CYP3A and Pgp (and other ABC transporters) as major factors limiting the bioavailability of drugs (Chapter 1) could, by specific inhibition of these processes, improve the bioavailability and reduce inter- and intra-variability for numerous orally administered drugs. This research was initiated in an effort to find a typical Pgp substrate to investigate the role of the Pgp transport process in drug kinetics and interactions.

By searching the literature, digoxin, a Pgp substrate, was selected as a probe since this drug undergoes very limited (and CYP-independent) metabolism in humans (Chapter 2). The pharmacokinetic mechanisms of digoxin-ICZ interactions and a lack of digoxin-KCZ or MCZ or FCZ interactions were investigated *in vitro*, then eventually tested *in vivo* in a clinical study, in which, the effects of KCZ and ICZ on digoxin kinetics were compared.

Digoxin secretion *in vitro* from kidney (LLC-PK1 and MDCK) and intestinal (Caco-2) epithelial cells did demonstrate temperature (Chapter 4) and chemical inhibition (Chapters 4, 5 and 6), suggesting secretion in a carrier-mediated manner. The flux of digoxin across these cells increased linearly over a wide range of concentration values, indicating that the apparent K_m of secretion in these cells is large relative to digoxin's solubility (Chapter 4).

Epithelial cell lines transfected with the human transporters Pgp, MRP1 and MRP2 were used in an attempt to characterize the individulal transporters responsible for digoxin secretion (Chapters 4, 5 and 6). Only digoxin secretion in the MDR1 transfected cell line varied from that in the wild-type LLC-PK1 cells. Inhibition of digoxin secretion in MRPs transfected cells by indomethacin, probenecid, sulfinpyrazone and CsA, substrates of Pgp and cMOAT, did show that it seemed likely that OAT or MRP1 and MRP2 could play a role in the secretion of digoxin. However, a similar inhibition pattern of digoxin transport by these agents in the wild-type cells suggests that an unidentified absorptive or secretive transporter is likely involved in digoxin secretion from wild-type and MRPs transfected cells (Chapter 4).

Our studies (Chapter 6) have shown that drugs long known to have pharmacokinetic interactions with digoxin when co-administered, such as verapamil and erythromycin, are actively secreted across cells by transporters such as Pgp. In our *in vitro* study, azole anti-fungal agents exhibited differential inhibition of digoxin transport by Pgp and in MRPs over-expressed cells, with KCZ overall being a better inhibitor than ICZ > FCZ > MCZ (Chapter 5).

Prior to a human clinical study, the rat was tested to predict the effects of azoles on digoxin pharmacokinetics and drug interactions. Salphati *et al.* had demonstrated that digoxin (Dg3) undergoes a sequential metabolism, which corresponds to the cleavage of its sugar residues to form Dg2, Dg1 and Dg0. The *in vitro* metabolic profile reflected the metabolic pattern observed *in vivo*, in which Dg2 is the main metabolite present in rat after administration of Dg3 because the cleavage of Dg2 to form Dg1 was a very slow step.

Dg3 metabolism in rat and human liver microsomes was compared (Chapter 3). No extensive Dg2 was formed after incubating Dg3 with human liver microsomes. Azole antifungal agents are inhibitors of CYP3A4 with KCZ > ICZ > FCZ, while MCZ essentially showed no effect. ICZ and KCZ, markedly reduced formation of Dg2 from Dg3 in rat liver microsomes. In addition to chemical inhibitors specific to CYP3A enzymes, antibodies raised to rat CYP3A2 produced a strong inhibition. However, inhibitors of other CYPs and antibodies to other CYPs did not show any effect.

In rats, given oral and *iv* doses of digoxin with a concomitant oral dose of KCZ, a marked increase of digoxin AUC was observed. Digoxin absorption was elevated while digoxin elimination was reduced by KCZ (Chapter 3). Although this study did not allow us to differentiate the effects of KCZ on Pgp and CYP3A in terms of bioavailability and elimination, the decrease in MAT is consistent with an inhibition of rat intestinal Pgp.

Pgp and CYP3A genes of rats have >70% similarities with human Pgp and CYP3A, but the gender-dependent responses of Pgp and CYP3A to drugs are very different in rats than in humans. Male rats have higher CYP3A than female rats, while Pgp expression was higher in female than in male rats. This suggests that the rat is not a good animal model for predicting kinetics and drug interactions of digoxin in humans.

The results from our clinical study (Chapter 7) indicate that both the absorption and elimination of digoxin were affected by KCZ and ICZ. The major site of the KCZ/digoxin interaction appeared to be the small intestinal mucosa and the major effect of ICZ was on digoxin elimination. The fact that KCZ changed digoxin kinetic parameters to a greater extent than ICZ, is consistent with our *in vitro* results that KCZ is a better inhibitor of CYP3A, Pgp and MRPs than ICZ. No significant amounts of digoxin

metabolites were detected in all plasma samples from subjects, supporting the hypothesis that CYP3A did not play a role in digoxin-azoles interactions in humans.

Using chemical inhibitors has led us to suggest the existence of novel or uncharacterized transporters for digoxin. This approach, however, is insufficient to identify specific transporters of digoxin due to the overlap of substrates and inhibitors. The results from this thesis work implicate that Pgp, MRPs and CYP3A play complementary roles in drug pharmacokinetics and interactions, although each possess independent mechanisms of response to xenobiotics. The *in vitro* or *in vivo* differentiation and quantitation of each of these proteins would require the use of specific substrates or inhibitors.

The findings reported here suggest that drugs that have been identified *in vitro* as substrates or inhibitors of transporters or enzymes, should be cautiously co-administered in patients with another known substrate or inhibitor of the same protein, and greater concern should be given to drugs that interact with both multidrug transporters and CYP3A. However, quantitative *in vitro* to *in vivo* extrapolation is still a challenging task, especially in predicting the importance of transporters *in vivo*. Use of a knock-out animal and molecular techniques could provide more definitive answers. More work is still needed to characterize or even discover many new transporters, as well as to determine the cellular locations and tissue distribution of these transporters.