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P-Glycoprotein Expression and Function, Relationship to Plasma and Intracellular Drug Concentrations, and

the Effects of HIV Disease

A thesis submitted to the

University of Dublin,

Trinity College

In fulfilment of the requirement

for the degree of

Doctor of Philosophy

By

Martina Joan Hennessy

MB, BAO, BCh (Hons), MRCPI

January 2006
Declaration

I declare that, except where otherwise acknowledged this thesis is entirely my own work and that it has not been submitted previously for a Higher Degree at this or any other university.

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Martina Hennessy
Summary Methods and Results

**P-glycoprotein Expression and Function:** Peripheral blood lymphocytes (PBMCs) were isolated by Ficoll density gradient centrifugation, fixed and permeabilised. P-glycoprotein (P-gp) expression was quantified by flow cytometry following staining with a P-gp specific antibody (JSB1). Vimentin and IE (nonsense antibody) were used as controls. The presence of the MDR 1 gene product was confirmed by RT-PCR. A rhodamine efflux assay was used to assess P-gp function.

**Drug Analysis:** The hypericin content of St John’s Wort was determined by HPLC with fluorescence detection. Plasma and cell associated indinavir and nelfinavir levels were assayed by high performance liquid chromatography linked to mass spectrometry. Pharmacokinetic data were analysed by non-compartmental analysis (WinNonLin).

**Effects of St John’s Wort (SJW):** In healthy volunteers (n=15) P-gp expression increased 4.2 fold from baseline in subjects treated with SJW (29.5±14.3 vs 7.0±1.9 MFI; P<0.05). There was no effect with placebo (n=7; 5.1±1.3 vs 6.0±1.9 MFI). SJW increased P-gp mediated rhodamine (Rho) efflux (reduced ratio) compared with baseline (0.12±0.04 vs 0.24±0.18; P<0.05). There was no change with placebo. Ritonavir (RTV; 5 μM) inhibited P-gp mediated efflux in both groups producing greater intracellular accumulation of Rho, however this effect was attenuated following treatment with SJW (23.9±15.3 % vs 75.4±16.4%; P<0.05).

**Plasma and intracellular Indinavir pharmacokinetics:** The mean intracellular indinavir (IDV) AUC₀⁻₈ was lower than the plasma AUC₀⁻₈ (7574±1003 vs 25060±4171 ng.ml.h⁻¹; P<0.004) in 10 patients receiving IDV plus dual nucleoside analogue therapy. Both the elimination half-life and the mean residence time of IDV intracellularly were prolonged compared with plasma (t½: 2.0±0.3 h vs 1.2±0.09 h; MRT: 3.6±0.6 h vs 2.1±0.1 h; P<0.05). For certain patients, this allowed intracellular IDV concentrations to
remain above the MEC despite poor plasma levels. Despite a lack of intracellular accumulation of IDV all patients were suppressed (HIV plasma RNA < 50 copies/ml).

**Intracellular Nelfinavir Concentrations and P-gp Expression and Function:** In 12 patients receiving nelfinavir (NFV) plus dual nucleoside analogue therapy, the intracellular NFV AUC$_{0-12}$ (mean ± s.e.) was ~9 fold higher than that of plasma (26420±63420 vs 29250±6629 ng/ml/h; P<0.001), and intracellular $C_{\text{Min}}$ and $C_0$ values for NFV were 5-6 fold higher ($C_{\text{Min}}$: 5712±2156 vs 1062±357 ng/ml; $C_0$: 15860±3662 vs 2553±539 ng/ml; P<0.0005). The intracellular NFV $C_{\text{Max}}$ was 15 fold higher than plasma (59420±13940 vs 3986±822 ng/ml; P<0.0005), while $T_{\text{Max}}$, elimination half-life and MRT were similar. There was a correlation between plasma and intracellular AUC$_{0-12}$ for NFV ($r=0.75; \ P=0.011$). Furthermore, intracellular NFV concentrations and P-gp function were correlated at baseline ($r =0.59; \ P<0.05$). Basal P-gp mediated Rho efflux was decreased in the presence of RTV (61.0±4.2% vs 25.6±5.5%; P=0.001), indicating an additional reversible efflux potential of 56.1±9.8%.

**P-gp Expression and Function in HIV Disease:** P-gp expression and function was assessed in PBMCs from HIV infected patients (n=75) and healthy controls (n=20). P-gp expression was lower (P< 0.05) in the PBMCs from treatment naive and unsuppressed HIV infected patients despite HAART (3.74±0.53 and 3.64±0.64 MFI) compared with healthy controls and patients successfully treated with HAART (6.2±0.45 and 8.33±0.59 MFI). There was an inverse relationship between P-gp expression and viral load ($r^2=0.47; \ P=0.002$). Trend analysis demonstrated that P-gp function between groups decreased in the rank order healthy controls > suppressed > unsuppressed > naïve (P<0.0001). The maximum response to RTV was similar between naïve HIV patients and healthy controls (98.7±1.7 vs 92.4± 4.2%) but was reduced (P<0.001) in both treated suppressed and unsuppressed patients (70.9±2.2 and 63.2±2.7%).
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Publications and Presentations

Full Papers:


Abstracts/Presentations/Prizes:

Hennessy M. Plasma versus intracellular indinavir concentrations in HIV positive patients. *International Conference for HIV Pharmacology* 2000;


Hennessy M. P glycoprotein expression and function: Stability over time. *Irish Journal of Medical Science* 2000;

Hennessy M, Feely J, Kelleher D, Mulcahy F & Barry M. St John's Wort modulates both expression and function of P glycoprotein in peripheral blood lymphocytes. *AIDS* 2000; **14**:P275

Hennessy M. P-glycoprotein expression and function in HIV positive patients. *Irish Journal of Medical Science* 2001;

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<td>ABC</td>
<td>ATP binding cassette</td>
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<tr>
<td>ALD</td>
<td>Adrenoleukodystrophy</td>
</tr>
<tr>
<td>APV</td>
<td>Amprenavir</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral Therapy</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the Concentration time curve</td>
</tr>
<tr>
<td>AZT</td>
<td>Azidothymidine (also called zidovudine)</td>
</tr>
<tr>
<td>$\beta_2$M</td>
<td>$\beta_2$-microglobulin</td>
</tr>
<tr>
<td>BCECF-AM</td>
<td>2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxy-methyl ester</td>
</tr>
<tr>
<td>CXCR4</td>
<td>$\alpha$-Chemokine Receptor</td>
</tr>
<tr>
<td>CCR5</td>
<td>$\beta$-Chemokine Receptor</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy deoxyribonucleic acid</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation (4)</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane conductance Regulator</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Intervals</td>
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<tr>
<td>$C_{\text{Min}}$</td>
<td>Minimum concentration</td>
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<tr>
<td>$C_{\text{Max}}$</td>
<td>Maximum concentration</td>
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<tr>
<td>$C_0$</td>
<td>Concentration at time zero</td>
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<tr>
<td>CPM</td>
<td>Copies per millilitre</td>
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<tr>
<td>$C_{\text{trough}}$</td>
<td>Trough concentration</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Cytochrome 3A4</td>
</tr>
<tr>
<td>dH$2$O</td>
<td>Distilled water</td>
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<tr>
<td>DISC</td>
<td>Death Inducing Signalling Complex</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<td>EC$_{50}$</td>
<td>Concentration required producing 50% of maximal response</td>
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<tr>
<td>$E_{\text{homo}}$</td>
<td>Energy of the highest occupied orbital</td>
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<tr>
<td>$E_{\text{max}}$</td>
<td>Concentration that produces maximal response</td>
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<td>ESI</td>
<td>Electrospray Ionisation</td>
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<td>FCS</td>
<td>Foetal Calf Serum</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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GAPDH  Glyceraldehyde-3-phosphate dehydrogenase  
GEM    Glycolipid-enriched membrane 
GP     Glycoprotein 
HAART  Highly Active Antiretroviral Therapy 
HIV    Human Immunodeficiency Virus 
HPLC  High Performance Liquid Chromatography 
IDV   Indinavir 
INFγ  Interferon-γ 
IL     Interleukin 
Kd     Dissociation Constant 
LC/MS Liquid Chromatography/Mass Spectrometry 
Log P Octanol-water partition Co-efficient 
MDR-1  Multi-drug Resistance Protein 1 
MEC   Minimum Effective Concentration 
MFI   Median Fluorescence Intensity 
mRNA Messenger Ribonucleic Acid 
MRP-1 Multidrug Resistance Protein 1 
MRT   Mean Residence Time 
MTX   Methotrexate 
NBD   Nucleotide Binding Domain 
NFV   Nelfinavir 
NK    Natural Killer (Cell) 
NRTI  Nucleoside Reverse Transcriptase Inhibitor 
NNRTI Non-nucleoside Reverse Transcriptase Inhibitor 
OABP  Organic Anion Binding Protein 
PBMC  Peripheral Blood Mononuclear Cell 
PCR   Polymerase Chain Reaction 
PI    Protease Inhibitor 
P-gp P-glycoprotein 
PKC   Protein Kinase C 
PXR   Pregnane X receptor 
Rho   Rhodamine 
RTV   Ritonavir 
RT-PCR Reverse Transcription – Polymerase Chain Reaction
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<td>RXR</td>
<td>Retinoid Xenobiotic Receptor</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SJW</td>
<td>St John's Wort</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SQV</td>
<td>Saquinavir</td>
</tr>
<tr>
<td>SXR</td>
<td>Steroid Xenobiotic Receptor</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with Antigen Processing</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>Elimination half-life of a drug</td>
</tr>
<tr>
<td>TDM</td>
<td>Therapeutic Drug Monitoring</td>
</tr>
<tr>
<td>T&lt;sub&gt;Max&lt;/sub&gt;</td>
<td>Time to maximum drug concentration</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane Domains</td>
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<tr>
<td>TNF&lt;sub&gt;α&lt;/sub&gt;</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>ZDV</td>
<td>Zidovudine (also called AZT)</td>
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Chapter 1

Introduction
1.1 Overview

A memorable review by Chris Higgins in 1992 introduced the term ABC transporters\(^{(1)}\). P-glycoprotein (P-gp) the multidrug transporter is undoubtedly the most famous and best characterised member of this superfamily, exemplifying their role in the transport of xenotoxins against steep concentration gradients at a cost of ATP hydrolysis. Over the last 25 years it has become clear that the primary function of this versatile protein is to prevent the uptake of toxic compounds from the gut into the body, and to protect vital structures such as the brain, cerebrospinal fluid, testis, foetus and bone marrow against toxins. The wide range of compounds transported by P-gp makes it a guardian for all seasons but the disadvantage of this in the modern era of targeted therapeutic intervention, is that P-gp may interfere with the delivery of drugs to target tissues. Kinetic experiments and mutagenesis studies have generated a large volume of data delineating the modus operandi of ABC transporters including P-gp, and the generation of P-gp knockout (KO) mice has facilitated the development of potent inhibitors with low toxicity, thus opening up new ways to selectively overcome interference with medical treatment. The translation of animal experiments from mice to new treatment modalities for human patients is in full swing and marks an exciting period in the field of transporter pharmacology. This chapter summarises some of the general principles governing P-gp, and aims to provide a concise overview focusing on three interrelated themes: (1) the structure of P-gp and its biochemical mechanism of action, (2) models of pump function and known substrates/chemosensitisers and (3) the control of P-gp expression and its role in health and disease. This is followed by chapter two which describes the aims and objectives of the thesis.
The main clinical relevance P-gp is in its potential effect on altered drug bioavailability. The third chapter demonstrates how a seemingly harmless herbal remedy may modulate P-gp expression and activity over time, and discusses the potential clinical relevance of this effect.

Nowhere is the relationship between drug bioavailability and therapeutic effect more important than in the treatment of HIV disease, in which achieving plasma concentrations above a critical level may determine virologic response. The fourth chapter explores this concept indirectly by assessing the relationship between plasma and intracellular concentrations of indinavir, a recognised substrate of P-gp and relates this to therapeutic responsiveness to antiretroviral drugs. Chapter five extends this to directly investigate the relationship between P-gp expression and function and plasma and intracellular nelfinavir concentrations in treated suppressed HIV positive patients, while the sixth and final chapter explores the relationship between expression and function of P-gp and surrogate markers of disease activity in a large cohort of treated compared with treatment naïve HIV patients.

1.1.1 The ABC Superfamily

The ATP binding cassette (ABC) transporters belong to a superfamily of >100 membrane transporters and channels, involved in a variety of functions including the extrusion of noxious compounds, uptake of nutrients, transport of ions and peptides, and cell signalling. ABC transporters are ubiquitously expressed across genera ranging from bacteria, yeast and plants to mammals.\(^{(1,2)}\) In man, 48 ABC transporters have been identified, and classified on the basis of phylogenetic analysis into the following subfamilies, each containing several members: ABC1 (12 members), MDR/TAP (11 members), MRP/CFT (12 members), ALD (4 members), OABP (1
member), GCN20 (3 members) and White (5 members)\(^{(3)}\). P-gp is a member of the MDR/TAP subfamily.

### 1.1.2 P-gp Isoforms

ABC transporters share a common domain organisation and considerable amino acid sequence identity, consistent with a common architecture and evolutionary origin. P-gp exists in a number of different isoforms which have a >70% sequence homology and are encoded by a small family of closely related genes. In man, P-gp is encoded by two multi-drug resistance genes (MDR), MDR1 and MDR3 (also designated MDR2), these are located on the long arm of chromosome 7 (7q21)\(^{(4,5)}\), while three genes have been identified in rodents, mdr1a, mdr1b & mdr2\(^{(6,7)}\). Of the isoforms identified, the multidrug resistant phenotype characterised by transporter-mediated substrate efflux, ultimately leading to lower intracellular substrate concentration, is associated with human MDR1 and rodent mdr1a and mdr1b isoform expression. In contrast human MDR3 and rodent mdr2 isoforms function as a phosphatidylcholine translocase, or “flippase”, mediating export of this phospholipid into bile\(^{(8,9)}\). Despite this, recent data demonstrates that human MDR3 may under certain conditions bind and transport selected MDR1 substrates, although inefficiently\(^{(10)}\). \textit{In vitro}, P-gp mediated transport is generally considered saturable, osmotically sensitive and requires ATP hydrolysis to generate a substrate concentration gradient\(^{(11)}\).

### 1.1.3 P-gp: Primary and Secondary Structure

Human P-gp is a 170 kDa polypeptide consisting of 1280 amino acids organised in two tandem repeats of 610 amino acids joined by a linker region of 60 amino acids\(^{(12,13)}\). The protein appears to have arisen by a gene duplication event, fusing 2
related demi molecules each consisting of one ABC domain and one transmembrane domain\(^{(14)}\). P-gp is synthesised in the endoplasmic reticulum as a core glycosylated intermediate with a molecular weight of 150 kDa. The carbohydrate moiety being subsequently modified in the Golgi apparatus prior to export to the cell surface\(^{(15)}\).

The secondary and tertiary structures of P-gp have not been fully elucidated due to an inability to crystallize the protein for 3 dimensional analysis, hence, there are several proposed models for its structure and composition. Models are consistent with published data and variations are not mutually exclusive, however, differences between models warrant some discussion since they underlie potential alternative mechanisms of P-gp pump function and substrate recognition.

The basic unit of an ABC transporter consists of 4 core domains\(^{(16,17)}\). The most widely accepted structural model for the human P-gp isoform was determined by hydropathic profiling of the primary amino acid sequence\(^{(18)}\). Based upon this data, P-gp is purported to consist of 2 homologous halves, each encompassing a hydrophobic domain composed of 6 membrane spanning segments (putative α-helices) separated by hydrophilic loops and one intracellular nucleotide binding domain (NBDs)\(^{(19,20)}\).

Conservation of the NBDs are central to classification and demarcation of the ABC transporter superfamily\(^{(16)}\). Furthermore, multiple alignment analysis of the primary sequence of 94 ABC domains of E. coli, has highlighted 3 short motifs that are highly conserved, the Walker A and B motifs\(^{(21)}\) which are involved in the binding and hydrolysis of ATP and an ABC signature motif (C) the function of which remains unclear\(^{(17,22)}\).

Fourier transform attenuated reflective infrared spectroscopy and circular dichroism analysis indicates that P-gp has a secondary structure composition of approximately 32-43% α-helix, 16-26% β-sheet, 15-29% β-turn and 13-26% unordered
structure\textsuperscript{(23,24)}. This is consistent with the 48\% $\alpha$-helix, 16\% $\beta$-sheet and 36\% coil structures as predicted from amino acid sequence using secondary structure prediction algorithms\textsuperscript{(24)}. Mutational analysis and cysteine cross-linking experiments have been used to design two practical models for the secondary structure of P-gp (Figure 1.1A, B).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematics depicting 2 practical models for the organisation of transmembrane spanning domains for P-gp: A) the "Linear" model and B) the "Cyclone" model. In both models, domains 6 and 12 being are shown in close proximity based upon cysteine crosslinking studies. \textit{Taken from Loo & Clarke, 1999}\textsuperscript{(25)}.}
\end{figure}

The linear model conventionally represents each half of P-gp as being symmetrically arranged within the membrane allowing parallel crosslinking of transmembrane segments (Figure 1.1A)\textsuperscript{(26,27)}. Other studies support an alternative cyclone model (Figure 1.1B) as described by Loo and Clarke in their review of the symmetry and structure of ABC transporters, this predicts a different pattern of crosslinking,
providing insight into the dynamic nature of the transmembrane segments and potential conformational changes that occur during the catalytic cycle of P-gp\(^{(25)}\).

Together, these data are consistent with P-gp existing as a single aqueous pore formed by a toroidal ring of 12 α helices, deployed in two arcs of six helices each in association with a nucleotide binding domain (Figure 1.2B).

**Figure 1.2**

A schematic of the classical 6 + 6 helical model for P-glycoprotein. A) Linearised secondary structure showing the putative α-helical transmembrane spanning domains and the nucleotide binding domains (NBD 1 & 2). B) Proposed tertiary organisation of the transmembrane spanning domains and their spatial relationship to the nucleotide binding domains. The α-helices in this schematic are arranged in a toroidal ring forming a central pore region. *Taken from Jones & George, 2000\(^{(28)}\).*
Jones has also presented data to support an alternative arrangement for the structure of P-gp based upon computer prediction algorithms, hydropathic profiling and secondary structure analysis\(^{(29)}\). In this model, P-gp is comprised of 2 membrane-embedded 16-strand \(\beta\)-barrels, attached by short loops to two 6 \(\alpha\)-helical bundles beneath each barrel. With each NBD contributing 2 \(\beta\)-strands and one \(\alpha\)-helix to the structure (Figure 1.3). The main difference between this model and those previously described is that the \(\beta\) pleated sheets form the central pore rather than the \(\alpha\) helices which are thought to lie intracellularly. The main functional consequence of this arrangement is to support a two-cylinder model of P-gp pump function as discussed later, as opposed to either the “pore” or “flippase” models of pump function.
Figure 1.3

A schematic showing a proposed alternate assembly of the N-terminal half of P-glycoprotein consisting of a transmembrane pore formed by a β-barrel below which lies the α-helical bundles. The relative positions of the ATP binding motifs are also highlighted (A, B and C boxes). *Taken from Jones & George, 1998*.

1.1.4 P-gp: Tertiary Structure

Data pertaining to the tertiary structure of P-gp has been lacking due to the difficulties in crystallising the protein for X-ray diffraction analysis. However, progress has been made towards obtaining low-resolution data at ~2.2nm resolution by electron microscopy and single particle image analysis\(^{(2,30,31)}\). The extracellular face of P-gp (Figure 1.4A) is toroidal in shape with six-fold symmetry and a diameter of approximately 10 nm and depth of approximately 8 nm. There is a large diameter opening (~5nm) that is required for the passage of substrates. While this pore is closed
at the cytoplasmic face of the membrane a cross sectional view indicates that the aqueous chamber is accessible from the lipid phase (Figure 1.4B). P-gp also has two 3 nm lobes on the cytoplasmic side of the membrane that are of an appropriate size to be consistent with that of the NBDs. Although the NBDs are positioned such that they may gate the central chamber intracellularly, this is unlikely, as the NBDs are not exposed to the extracellular milieu.

**Figure 1.4**

A 3-D reconstruction of P-gp as determined by electron microscopy and image analysis. A) A view perpendicular to the extracellular surface of the membrane showing the two transmembrane domains (TMD) and assumed lobes of the nucleotide binding domains projecting at the cytoplasmic face of the plasma membrane. The aqueous pore (P) is also indicated. B) Side view of P-gp with the approximate position of the lipid membrane represented by the horizontal dashed lines. The arrow indicates access to the central pore from the lipid membrane through an asymmetrical opening. *Taken from Rosenberg et al., 1997.*
While these studies provide important information on the 3D structure for P-gp, the controversy over the secondary and tertiary structure will only be answered by high-resolution X-ray crystallographic analysis of P-gp. Recently this problem was solved by Chang and Roth for MsbA[^34] a bacterial homologue of a multidrug resistance transporter from *Escherichia coli*. MsbA although a lipid transporter is closely related to LmrA from *Lactococcus lactis* (a multidrug transporter with a high level of sequence, topographical and functional similarity to P-gp[^35,36]), thus making it likely that there is also structural similarity between the bacterial transporters and P-gp, even though both MsbA and LmrA are half size ABC transporters functioning as heterodimers. If LmrA is introduced into animal cells it confers multidrug resistance like P-gp[^36]. The structure proposed by Chang and Roth has a cone like transmembrane domain with the top of the cone pointing out of the cell (Figure 1.5). This domain contains 12 transmembrane α helices confirming the commonly accepted structure for P-gp but refuting the recently proposed alternative suggesting the α helices lie intracellularly and supporting the two-cylinder engine model of P-gp pump function.
The structure of the MsbA lipid transporter. A view of the chamber opening of the MsbA dimer, showing it’s cone like structure containing 12 transmembrane α helices. The nucleotide-binding domains (NBDs) are positioned at the ends of the helices of the transmembrane domains (TMDs). This diagram was generated using Protein Explorer. *Taken from McKeegan et al., 2003*.\(^3\)

### 1.2 Biochemistry of Drug Transport

Radioligand binding data supports the existence of at least two substrate binding sites sufficient for drug transport within the transmembrane domains of P-gp\(^{36,38-40}\). These sites appear to be closely associated with domains 5, 6, 11 and 12\(^{41-43}\). Substrate binding sites may actually be overlapping or possibly allosterically coupled\(^{44-46}\), leading to the suggestion that there is actually only a single common site\(^{47}\). However, recent studies indicate that three or perhaps even four distinct drug binding sites exist on P-gp and these can be classified as both transport and modulating sites, which can switch between high and low affinity states for substrates/ inhibitors\(^{48,49}\).
That drug transport takes place within these regions is supported by site directed mutagenesis studies, which show that alterations of certain transmembrane domains affect drug resistance\(^{(50,51)}\).

### 1.2.1 Substrate Binding and ATPase Activity

There is unequivocal biochemical evidence to support a conformational change in P-gp upon substrate binding, including changes in epitope accessibility\(^{(52,53)}\) and vulnerability to protease degradation\(^{(54,55)}\). In fluorescence labelling experiments, Liu\(^{(56)}\) demonstrated quenching of the probe fluorescence within the NBDs following substrate binding, thereby linking drug binding site to a conformational change in the catalytic site of the NBD. Furthermore, infrared spectroscopic data have shown a change in the tertiary structure of P-gp and stimulation of the ATPase activity of P-gp by verapamil and other substrates and chemosensitisers\(^{(23)}\). Cysteine cross linking studies have shown substrate binding to initiate a conformational change in the distal NBDs\(^{(57)}\). This has been extended by a study demonstrating that binding of an ATP analogue caused the cylindrical, barrel-like structure of P-gp to be reorganised into 3 compact domains each with a diameter of 2-3 nm and a depth of 5-6 nm. This effectively opens the central pore along its length, which may allow access of hydrophobic substrates directly from the plasma membrane\(^{(58)}\).

P-gp is an unusual translocating ATPase in that the purified protein exhibits a high level of basal ATPase activity in the absence of a substrate\(^{(59-61)}\) which can be further increased by 3-4 fold *in vitro* by transport of substrates and chemosensitisers\(^{(62,63)}\). ATPase activity is associated with the two NBDs which have similar apparent affinity for ATP, and hydrolyse ATP at comparable rates\(^{(36,64,65)}\). Several groups provide evidence to support an alternating catalytic cycle mechanism in which only one NBD
hydrolyses ATP at a time\textsuperscript{(25,62,66,67)}. This is in direct contrast to the situation for the multidrug resistance related protein 1 (MRP1) a closely related human drug transporter in which inactivation of NBD1 does not completely eliminate drug transport whereas inactivation of the second NBD does\textsuperscript{(68)}. While early studies found that ATP hydrolysis was directly coupled to the substrate transport process\textsuperscript{(17,69)}, a recent study has eloquently shown conformational changes in the tertiary structure of P-gp as a result of ATP binding rather than ATP hydrolysis\textsuperscript{(58)}. Furthermore, biochemical data indicates that a reduction in substrate binding affinity to P-gp results directly from ATP binding rather than hydrolysis\textsuperscript{(30,70)}. Together, these findings support a role for ATP hydrolysis "resetting" the transporter to its initial starting conformation\textsuperscript{(71)}.

Little is known regarding the stoichiometry of nucleotide binding or how this may be altered during the catalytic cycle. Early experiments indicated that a minimum of 50 ATP molecules were hydrolysed per molecule of substrate transported\textsuperscript{(72)}. This ratio has been revised to suggest that 2-3 molecules of ATP are hydrolysed per molecule of substrate transported\textsuperscript{(73-75)}. Using a fluorescent nucleotide derivative Qu et al.\textsuperscript{(76)} demonstrated that in the absence of substrates, both nucleotide binding domains are occupied with either ATP or ADP in the resting and transition state. Release of the drug from the transporter during the catalytic cycle precedes formation of the transition state\textsuperscript{(77)}.

ATPase activity is frequently biphasic, with stimulation at low drug concentrations, and inhibition at higher concentrations. Inexplicably, some substrates inhibit rather than stimulate activity in a concentration dependent manner\textsuperscript{(11)}. This may be due to allosteric hindrance, arising from the overlap of putative stimulatory and inhibitory binding sites within P-gp\textsuperscript{(63,78)}. In addition, drug induced effects on ATPase activity
are both cell and species dependent, as vinblastine inhibits P-gp-mediated ATPase activity from Chinese hamsters\(^{(59-61)}\), but increases it in human KB cells (epithelial carcinoma cell line)\(^{(79)}\).

1.2.2 The Pore Model of Pump Function

The mechanism by which P-gp and other ABC transporters couple the hydrolysis of ATP to movement of drugs across the plasma membrane is not well defined, but several models have been proposed namely, "pore", "hydrophobic vacuum cleaner", "flippase" and "two cylinder engine".

Early models hypothesised that P-gp formed a hydrophilic pathway or pore permitting transport from the cytosol to the extracellular environment by shielding the substrate from the hydrophobic plasma membrane\(^{(80,81)}\). In support of such a model, Altenberg et al. in 1994 published data demonstrating that rhodamine 6G (a known P-gp substrate), is effluxed from the cytoplasm\(^{(82)}\). The pore model has significant limitations, since it is based upon the premise that the initial interaction between the substrate and transporter occurs from the aqueous phase, therefore precluding the well established ability of P-gp to extrude hydrophobic compounds.

1.2.3 Hydrophobic Vacuum Cleaner and Flippase Model

Both the "hydrophobic vacuum cleaner" and "flippase" models for P-gp are consistent with the proposed tertiary structure of P-gp indicating that substrates can gain access to the pore from the lipid phase\(^{(30,32)}\). P-gp is envisaged as a "hydrophobic vacuum cleaner" extracting hydrophobic compounds embedded in the inner leaf of the plasma membrane, and pumping them directly to the external aqueous medium\(^{(39)}\). This is strengthened by the observation that, LmrA a prokaryotic homologue of P-gp also
extrudes substrates from the inner leaflet of the cytoplasmic membrane\textsuperscript{(35)}, and by the data of several groups demonstrating unidirectional transportation of fluorescent P-gp substrates from the cytoplasmic leaflet of the plasma membrane to the external aqueous environment\textsuperscript{(83-86)}.

In the alternative “flippase” model (Figure 1.6), substrates are flipped from the inner leaflet of the lipid bilayer, to either the outer leaflet of the plasma membrane or directly to the extracellular environment\textsuperscript{(81)}. Both of these models may help to explain the unusually broad substrate specificity of P-gp, since the primary determinant of specificity would be the ability of a substrate to partition into the lipid bilayer appropriately, with subsequent binding site interactions being of secondary importance. Evidence supporting a “flippase” mechanism demonstrates a) that fluorescent lipids are flipped between the inner and outer leaflets of the plasma membrane in a reconstituted system\textsuperscript{(87)}, b) the Mouse P-gp homologue mdr2 catalyses ATP-dependent transbilayer movement of a fluorescent phosphatidylcholine analogue\textsuperscript{(8)} and c) in the mdr2 knock-out mouse phosphatidylcholine is not secreted into bile\textsuperscript{(9)}. 
A schematic representation of the “flippase” model for P-glycoprotein-mediated drug efflux. In this model, substrates interact with the inner leaflet of the lipid membrane prior to interaction with P-gp for transport either directly to the extracellular medium, or alternatively, are “flipped” into the outer leaflet and released into the extracellular environment. Modified from Johnstone et al., 2000[88].

1.2.4 The Two-Cylinder Engine: A Conceptual Variation

An conceptual variation of the “hydrophobic vacuum cleaner” is the “two-cylinder engine” model (Figure 1.7)[28,29], which has been proposed as a mechanism for the prokaryotic P-gp efflux pump LmrA[14]. In this model, the loops connecting the helical bundles to the β-barrels represent the both the intracellular and outward facing substrate binding sites. Upon binding one set of loops move into the lumen of the β-barrel transporting the substrate towards the extracellular environment, while “empty”
(low affinity) loops on the alternative cylinder move inward. An alternating cycle mechanism operates between the β-barrels.

Figure 1.7
A schematic representation of the “two-cylinder engine” model of P-glycoprotein-mediated drug efflux. The substrate binds between the intra-cytoplasmic loops of the transmembrane domains and is expelled from the cell by a “piston” driven efflux mechanism through the β-barrels. *Taken from Jones & George, 2000*.

1.3 P-gp Expression, Function and Regulation

1.3.1 P-gp Substrates and their Recognition

P-gp is unique in its ability to recognise and transport a plethora of diverse substrates that differ considerably in chemical structure and pharmacological action, including many clinically important substrates (Table 1.1). The interaction of substrates/
inhibitors with P-gp is a complex process that is poorly understood, and an area of great contention. Interestingly, $K_d$ (dissociation constant) values for P-gp substrates cover a 1000 fold range, indicating that P-gp can discriminate between dissimilar compounds in a measurable manner$^{11}$. While the underlying mechanism is unknown, many studies have attempted to address the molecular basis for the substrate/P-gp interaction by investigating structure-activity relationships of established P-gp substrates and inhibitors. Data from such studies have highlighted the importance of lipophilicity, hydrogen bonding potential, presence of an amine, molecular weight, size, surface area and the presence of aromatic ring structures as important determinants of substrate binding and functional effect$^{89,90}$. In other studies, utilising 3D modelling to screen for universal molecular features of $>100$ diverse P-gp substrates a commonality was the presence of a hydrogen bond acceptor (or electron donor) moiety, such as carbonyl, ether, hydroxyl or tertiary amine groups, with a defined spatial separation$^{91,92}$. Two specific spatial separation patterns were identified and classified as type I and II. The type I pattern is defined as 2 electron donor groups separated by $2.5 \pm 0.3 \text{Å}$ while the type II pattern is made up of 2 electron donor groups with a spatial separation of $4.6 \pm 0.6 \text{Å}$. A type II pattern may also contain 3 electron donor groups with the outer 2 groups being separated by $4.6 \pm 0.6 \text{Å}$. A prerequisite for substrate binding is either 1x type I or 1x type II pattern$^{91}$, with substrate transport requiring either two type I or one type I and one type II pattern$^{93}$. Analysis of the transmembrane spanning domains of P-gp indicates grouping of amino acids with hydrogen bond donor side chains on one side of the TM domain and amino acid residue with inert (non-hydrogen bonding) side chains on the opposite side, possibly adjacent to the lipid bilayer$^{91,94}$. P-gp substrates containing type II moieties induce P-gp over-expression and are associated with development of drug
resistance\(^{(91)}\); interestingly, type II patterns are also prevalent in antibiotics and drugs targeted to DNA.

Alternatively, the ability of P-gp to transport a diverse range of compounds may be explained on the basis of a "half-coupled" mechanism\(^{(95)}\). In this scheme, ATP hydrolysis is still linked to carrier transport, as in a fully coupled system, but differs in that the substrate plays a passive role, negating the requirement of a specific interaction between substrate and transporter. Interestingly, recent biochemical studies demonstrate that substrate binding alters the arrangement of the transmembrane segments in the drug-binding site, indicating that P-gp may accommodate a diverse range of substrates through a "drug-induced-fit" mechanism\(^{(96)}\).

Partitioning of substrate into the plasma membrane is the rate limiting step for binding with P-gp, with dissociation rate of the substrate/P-gp complex being controlled by the extent and strength of the hydrogen bonding within the complex\(^{(93)}\).

With regard to effects on ATPase activity, P-gp substrates are broadly classified into those compounds that: 1) stimulate ATPase activity at low concentrations but inhibit activity at high concentrations, 2) enhance ATPase in a dose dependent manner without any inhibition and 3) inhibit both basal and verapamil stimulated ATPase activity\(^{(97)}\). Interestingly, MDR modulators, which counteract resistance mechanisms through interference with drug efflux, also appear to be transport substrates.
### Table 1.1

<table>
<thead>
<tr>
<th>Clinically relevant P-gp substrates</th>
<th>HIV protease inhibitors</th>
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<tbody>
<tr>
<td><strong>Cancer drugs</strong></td>
<td><strong>Amprenavir</strong></td>
</tr>
<tr>
<td>• Doxorubicin</td>
<td>o Indinavir</td>
</tr>
<tr>
<td>• Daunorubicin</td>
<td>o Nelfinavir</td>
</tr>
<tr>
<td>• Vinblastine</td>
<td>o Ritonavir</td>
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<tr>
<td>• Vincristine</td>
<td>o Saquinavir</td>
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<tr>
<td>• Actinomycin D</td>
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<td>• Paclitaxel</td>
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<tr>
<td>• Teniposide</td>
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<td>• Etoposide</td>
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<tr>
<td><strong>Immunosuppressive drugs</strong></td>
<td><strong>Cardiac drugs</strong></td>
</tr>
<tr>
<td>• Cyclosporin A</td>
<td>o Digoxin</td>
</tr>
<tr>
<td>• Tacrolimus</td>
<td>o Quinidine</td>
</tr>
<tr>
<td><strong>Lipid-lowering agent</strong></td>
<td><strong>Anti-emetic</strong></td>
</tr>
<tr>
<td>• Lovastatin</td>
<td>o Ondansetron</td>
</tr>
<tr>
<td><strong>Antihistamine</strong></td>
<td><strong>Anti-diarrhoeal agent</strong></td>
</tr>
<tr>
<td>• Terfendaine</td>
<td>o Loperamide</td>
</tr>
<tr>
<td><strong>Steroids</strong></td>
<td><strong>Anti-gout agent</strong></td>
</tr>
<tr>
<td>• Aldosterone</td>
<td>o Colchicine</td>
</tr>
<tr>
<td>• Hydrocortisone</td>
<td><strong>Antibiotic</strong></td>
</tr>
<tr>
<td>• Cortisol</td>
<td>o Erythromycin</td>
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<tr>
<td>• Corticosterone</td>
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<td>o Ivermectin</td>
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<td><strong>Anti-tuberculous agent</strong></td>
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<td>o Rifampin</td>
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<tr>
<td></td>
<td><strong>Fluorescent dye</strong></td>
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<td>o Rhodamine-123</td>
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Modified from [www.aidsinfonyc.org/tag/science/pgptables.html](http://www.aidsinfonyc.org/tag/science/pgptables.html)

#### 1.3.2 P-gp Modulators

From a pharmacological perspective, P-gp function may be modulated as a result of competitive/ non-competitive antagonism, interference with ATP binding / hydrolysis, coupling of ATP to the transport process or by altering the drug membrane interaction\(^{(98)}\). Potential mechanisms range from direct binding to P-gp and depressing ATPase activity to inhibiting protein kinase C (PKC). Modulators such as safingol, a PKC inhibitor, regulate P-gp function through phosphorylation of the protein\(^{(99)}\),
while others compete for transport with other substrates. Competitive transport combined with favourable biophysical properties permit rapid diffusion of substrate across and recycling within the membrane bilayer\(^{(47,82)}\). In this situation, the rate of membrane equilibration may be so rapid that P-gp-mediated efflux cannot keep pace. Consequently, P-gp operates in a futile cycle, hence cells are unlikely to become resistant to such chemosensitisers\(^{(100)}\). P-gp rapidly facilitates resistance to bulky amphiphatic drugs such as paclitaxel, anthracyclines and *Vinca* alkaloids, the hydrophobic parts of these drugs allow their rapid insertion in the membrane, while the hydrophilic residues prevent rapid flipping of the drug to the inner leaflet slowing down entry into the cell. In fact for doxorubicin this takes approximately a minute, giving the P-gp ample opportunity reduce drug influx\(^{(100,101)}\). It is interesting to note that substrates which enter cells faster than P-gp can pump them out may be missed by currently available functional assays, which can be insensitive. A case in point is methotrexate (MTX), a hydrophilic negatively charged drug, considered to be unaffected by P-gp as a result of the above process. However, in cell lines deficient in MTX carrier mediated uptake P-gp may confer resistance\(^{(102)}\).

Most modulators share some common chemical features, such as aromatic ring structures, a tertiary or secondary amino group and high lipophilicity. However, some compounds such as progesterone and the flavinoids may lack some of these features yet still possess resistance-reversal activity. Recently, molecular modelling of 106 representative P-gp substrates and inhibitors showed that an effective P-gp modulator should has a log P (octanol-water partition coefficient) value of $\geq 2.92$, an 18-atom-long or longer molecular axis, a high $E_{\text{homo}}$ value (energy of the highest occupied orbital), in addition to at least one tertiary basic nitrogen atom\(^{(49)}\). In general modulators are grouped on the basis of their structural features (a) calcium or sodium
channel blockers, (b) calmodulin antagonists, (c) PKCIs, (d) flavinoid and steroidal compounds, (e) indole alkaloids and polycyclic compounds, (f) cyclic peptides and macrolide compounds, (g) miscellaneous compounds (Table 1.2).

### Table 1.2

<table>
<thead>
<tr>
<th>Clinically relevant P-gp inhibitors</th>
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<tbody>
<tr>
<td>• Cyclopropyldibenzosuberane</td>
<td>• Anti-oestrogen cancer agent</td>
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<tr>
<td>o Zosuquidar (LY335979)</td>
<td>o Tamoxifen</td>
</tr>
<tr>
<td>• Immunosuppressant</td>
<td>• Antiarrhythmic agent</td>
</tr>
<tr>
<td>o Cyclosporin A</td>
<td>o Quinidine</td>
</tr>
<tr>
<td>o Valspodar (PSC833)</td>
<td>• Antifungal agent</td>
</tr>
<tr>
<td>o HIV protease inhibitors</td>
<td>o Ketoconazole</td>
</tr>
<tr>
<td>o Ritonavir</td>
<td>• Sedative</td>
</tr>
<tr>
<td>o Saquinavir</td>
<td>o Midazolam</td>
</tr>
<tr>
<td>o Nelfinavir</td>
<td>• Acridonecarboxamide derivative</td>
</tr>
<tr>
<td>o Indinavir (?)</td>
<td>o GF120918</td>
</tr>
<tr>
<td>• Calcium channel blocker</td>
<td>• Peptide chemosensitisers</td>
</tr>
<tr>
<td>o Verapamil</td>
<td>o Reversin 121</td>
</tr>
<tr>
<td>• Progesterone antagonist</td>
<td>o Reversin 205</td>
</tr>
<tr>
<td>o Mefipristone (RU486)</td>
<td></td>
</tr>
</tbody>
</table>

Modified from [www.aidsinfonyc.org/tag/science/pgptables.html](http://www.aidsinfonyc.org/tag/science/pgptables.html)

### 1.3.3 P-gp Expression in Normal Tissues and Physiological Function

Studies demonstrate wide spread expression of P-gp in many tissues and cells. The principal location (Table 1.3) of P-gp is on the apical membranes of the epithelia, the luminal surface of the small intestine, colon, hepatocytes (canalicular membrane), capillary endothelial cells at the blood-brain and blood-testis barrier, kidney proximal tubules, placenta and on lymphocytes\(^\text{103-109}\). These cellular and tissue locations of P-gp leave little doubt that it forms part of a physiological detoxification system and is
therefore a key player in the defence of the body against amphipathic xenotoxins. In the gut mucosa it prevents entry of toxins into the body; in the blood brain barrier, placental trophoblasts, testis and bone marrow it provides protection to vital body parts; and in the gut, liver and kidney P-gp helps to eliminate toxins from the body. The detailed analysis of defence functions of P-gp has been greatly simplified by the generation of P-gp KO mice\(^{(9,110,111)}\). Mice have two ABCB1 type drug transporters that appear to do the job of a single P-gp (MDR1) in humans. Mdr1a\(^{+/−}\), Mdr1a/lb\(^{+/−}\) and the triple KO mice, has been extensively used for pharmacological studies, and to establish the effect of P-gp on oral availability and brain penetration of drugs\(^{(112,113)}\). KO mice are viable and fertile displaying no obvious phenotypic abnormalities other than sensitivity to cytotoxic agents. Interestingly, inflammatory bowel disease and has been described in P-gp KO mice\(^{(114)}\).
Table 1.3 Location and function of P-glycoprotein in Man

<table>
<thead>
<tr>
<th>Organ/Tissue</th>
<th>Site</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gut</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Colon</td>
<td>Apical (luminal) surfaces of superficial columnar epithelial cells</td>
<td>Intestinal excretion and reduced absorption of drugs and toxins</td>
</tr>
<tr>
<td>• Jejunum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver and Biliary System</td>
<td>Hepatocytes and luminal surface of biliary ductile epithelial cells</td>
<td>Hepatobiliary excretion of drugs and toxins</td>
</tr>
<tr>
<td></td>
<td>Hepatocytes</td>
<td>Regulation of cytochrome expression</td>
</tr>
<tr>
<td>Kidney</td>
<td>Apical surface of proximal tubules</td>
<td>Urinary excretion of drugs/toxins</td>
</tr>
<tr>
<td>Brain</td>
<td>Endothelial cells of cerebral capillaries</td>
<td>Contributes to blood-brain barrier, extruding drugs and toxins</td>
</tr>
<tr>
<td></td>
<td>Choroid plexus</td>
<td>Unknown</td>
</tr>
<tr>
<td>Peripheral Nerves</td>
<td>Endothelial cells of nerve capillaries</td>
<td>Contributes to blood-nerve barrier, extruding drugs and toxins</td>
</tr>
<tr>
<td>Uterus</td>
<td>Foetally-derived epithelial cells of placenta</td>
<td>Materno-fetal barrier contribution protects fetus from toxins</td>
</tr>
<tr>
<td></td>
<td>Cells of endometrial glands</td>
<td>Role in steroid production?</td>
</tr>
<tr>
<td>Testis and Ovary</td>
<td>Capillary endothelial cells</td>
<td>Contributes blood-testis/ovary barrier, gonadal protection from toxins/drugs</td>
</tr>
<tr>
<td>Immune System</td>
<td>Skin dendritic cells</td>
<td>Dendritic cell migration to lymph nodes</td>
</tr>
<tr>
<td></td>
<td>Activated lymphocytes</td>
<td>Export of interleukins and interferon γ</td>
</tr>
<tr>
<td></td>
<td>Natural killer and CD8+ T cells</td>
<td>Reduced cytolytic activity</td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>Hematopoietic stem cells</td>
<td>Removal of drugs/toxins</td>
</tr>
<tr>
<td>Adrenal Gland</td>
<td>Medulla and cortex</td>
<td>Role in steroid production?</td>
</tr>
</tbody>
</table>

Modified from www.aidsinfonyc.org/tag/science/pgptables.html
1.3.4 Regulation of P-gp Expression

The MDR1 gene is located on the long arm of chromosome 7 and consists of 28 exons that encode the 1280 amino acids encompassing P-gp. P-gp is constitutively expressed in a cell and tissue-specific manner but may be induced by environmental factors such as heat shock, cytokines, chemotherapeutic agents, UV radiation and tumour suppressor genes\(^{115-122}\). In many cell lines and human metastatic sarcomas \textit{in vivo}\(^{118,120,123-126}\), P-gp expression is increased as a result of up-regulation of MDR1 mRNA levels. However, in cell lines selected for resistance to cytotoxic agents, gene amplification and the appearance of self-replicating episomes may also underlie the increase in P-gp expression\(^{80}\).

It is generally accepted that transcriptional regulation is of primary importance with regard to the complex expression pattern of P-gp \textit{in vivo}\(^{127,128}\). However, changes in MDR1 mRNA stability, or alterations in P-gp turnover or trafficking are also implicated\(^{129-131}\). Recently up-regulation of MDR1 expression in a leukaemic cell line has been shown to occur at post-transcriptional mRNA turnover and translational regulation\(^{132}\). These data and the fact that the human MDR1 promoter region is atypical in that it does not contain a TATA promoter sequence but has multiple response elements (Figure 1.8) support a complex regulatory pattern with regard to control of P-gp expression. At present, MDR1 gene transcription can be up-regulated by several transcription factors including, GC, HSF1, Sp1, AP-1, NF-IL6, NF-Y, EGR1, YB-1 and MEF-1\(^{117,133-141}\). Furthermore, it is inhibited by cross-coupled NFκB/p65 and cFos\(^{142}\) and may be either positively or negatively regulated by the tumour suppressor protein p53\(^{121,143}\).

Recently, a steroid xenobiotic receptor (SXR) response element has been described approximately 7800 bp upstream of the initiator site of the MDR1 gene. This response
element binds a pregnane xenobiotic receptor/retinoid xenobiotic receptor α heterodimer (PXR/RXRα) leading to activation of MDR1 transcription in response to a number of xenobiotic inducers (nifedipine, mefipristone (RU486), dexamethasone and rifampicin)

In addition, SXR is also implicated in the development of multidrug resistance in SXR-expressing tumours. Interestingly, SXR nuclear receptors co-ordinately regulate drug metabolism via cytochrome P450 genes (CYP3A4, CYP2C8) and drug efflux via MDR1.

Figure 1.8
A schematic representation of the untranslated 5' regulatory region of the human MDR1 gene showing promoter elements and their relative start sites. The numbers under each promoter element indicates their position with respect to the +1 start site.

Taken from Labialle et al. 2002.

It is generally assumed that up-regulation of MDR1 mRNA is translated directly into an increase in P-gp expression and by extension P-gp function. In support of this, both MDR1 mRNA and P-gp protein content are increased in human renal carcinoma cells and rat liver cells exposed to chemical and physical stressors (heat shock and arsenic), culminating in a transient resistance to vinblastine. However, the situation is
not straight forward, as no such correlation between steady state MDR1 expression and P-gp function is noted in acute myeloid leukaemia cells, human bone marrow lymphoid cells or K562 leukaemia cells\(^{(132,147,148)}\).

1.4 P-gp and Altered Drug Disposition

The importance of P-gp in modulating the pharmacokinetics of many drugs in addition to those used in cancer therapy has become more apparent with the advent of P-gp gene knockout mice, particularly in the areas of drug transport across blood/endothelial barriers and intestinal excretion/absorption.

1.4.1 The Blood Brain Barrier (BBB)

The blood brain barrier consists of endothelial cells of brain capillaries which are linked together by tight junctions leaving no space between cells, hence drugs entering the brain must be highly lipophilic, in order to enter the BBB by passive diffusion, and be capable of circumventing the high level of P-gp present in the apical membrane of these cells. It was only through the availability of knockout mice that the important contribution of P-gp to the BBB was fully recognised. Intravenous administration of cyclosporin, digoxin and ivermectin (a neurotoxic pesticide), in mdr1a\(^{-/-}\) knockout mice, elicits a 17-100 fold increase in the brain concentrations of these agents compared with wild type controls\(^{(110,149)}\). Similar results are obtained in double knockout mice (mdr1a/1b\(^{-/-}\)) in that brain concentrations of digoxin increased approximately 19 fold compared to wild type animals. However, this was not accompanied by an increase in plasma digoxin levels, consistent with preferential accumulation in the brain (increased brain to plasma ratio)\(^{(150)}\). This is supported by an *in vitro* study in which intravenous administration of LY-335979 (Zosuqidar), a
potent P-gp inhibitor increased both brain and testicular concentrations of nelfinavir (a potent HIV protease inhibitor) up to 37- and 4-fold respectively in mice. Similar effects were also obtained for amprenavir, indinavir and saquinavir (HIV protease inhibitors). However, there was only a modest increase in plasma nelfinavir levels following LY-335979 administration which is consistent with the primary effect being to increase tissue penetration of these agents\(^{151}\). Potential consequences of enhancing brain penetration of drugs through modulation of P-gp function are highlighted in a study investigating the effects of loperamide and domperidone in mdr1a\(^{-/-}\) mice\(^{152}\). In this study, loperamide, a peripherally acting anti-diarrhoeal agent, underwent substantial (7 fold) increase in brain concentrations to elicit potent opiate-like activity compared with wild type controls. Interestingly, in the same study, the knockout mice were also more sensitive to the neuroleptic-like side effects of oral domperidone\(^{152}\).

### 1.4.2 The Maternal-Foetal Barrier

Lankas et al.\(^{(153)}\) demonstrated in 1998 that Mdr1a\(^{-/-}\) mutants displayed enhanced sensitivity to the teratogenic effects of the pesticide ivermectin, and showed that Mdr1a P-gp is present in foetus derived epithelial cells in the exchange border between the foetal and maternal circulations. P-gp in the apical border faces the maternal circulation, and hence is optimally placed to protect the foetus from incoming toxins. This work has been extended by Smit et al.\(^{(154)}\) to show that digoxin, saquinavir and paclitaxel penetrate the foetuses of Mdr1a\((-/+)/lb(-/-) knockout mice more effectively than wild type mice following IV administration.
1.4.3 P-gp and Intestinal Excretion/Absorption of Drugs

The small intestine represents the principle site of absorption for any ingested compound, whether dietary, therapeutic or toxic. The majority of drugs are well absorbed by the GI tract and are therefore given by this method since it is inexpensive, convenient and non invasive. Specific membrane transport systems and intracellular metabolising enzymes allow intestinal enterocytes to form a selective barrier to drugs and xenobiotics and are therefore critical in determining drug disposition and bioavailability. The multidrug transporter, P-gp, is ideally positioned to alter drug disposition due to its widespread constitutive expression in intestine (where it is localised to the villus tip enterocytes), kidney, liver, placenta, blood brain and blood testes barriers.

The first evidence that P-gp acts as a secretory detoxifying system to limit drug absorption came from studies in the human intestinal epithelial cell lines caco-2, HT29 and T84. Polarised apical expression in these cells was accompanied by secretory (basolateral to apical; blood to lumen) transport of the anticancer agent vinblastine which was reduced in the presence of MRK 16 monoclonal antibodies directed against P-gp, and the P-gp inhibitors verapamil and nifedipine\(^{155-157}\). Definitive data implicating P-gp in drug disposition arose from studies in Mdr knockout mice. In mdr1a\(^{\text{\textsuperscript{+/+}}}\) knockout mice, Kim et al.\(^{158}\) showed that plasma levels of the protease inhibitors, indinavir, nelfinavir and saquinavir, increased 2-5 fold compared with wild type controls following oral administration, representing increased absorption. In man, erythromycin, a inhibitor of P-gp, increases oral bioavailability of talinolol (\(\beta_1\)-selective antagonist) as the area under the curve and the maximum serum concentration obtained are increased compared to placebo\(^{159}\). Interestingly, pgp can modulate intestinal drug excretion as the excretion of
intravenously administered digoxin from the systemic circulation into the gut is
decreased to 2% in mdr1a−/− mice compared to 16% in control mice\(^{(160)}\). Similarly, the
pgp inhibitor PSC833, abolishes p-gp mediated excretion of digoxin by the intestinal
mucosa of wild-type mice\(^{(150)}\).

However, the situation may be more complex due to a possible coordinated
interaction between intestinal enzymes and efflux transporters to modulate entry of
orally ingested drugs/xenobiotics into the body\(^{(161,162)}\). This is based upon several
observations: 1) P-gp and cytochrome P450 (CYP) 3A4, a major intestinal oxidative
enzyme\(^{(163)}\), co-localize in tissues responsible for drug disposition such as small
intestine and liver\(^{(164)}\), 2) most substrates of CYP 3A4 are also substrates for P-
gp\(^{(162,165)}\), and 3) CYP3A4 and P-gp are coregulated through the nuclear receptor
steroid and xenobiotic receptor/pregnane X receptor\(^{(145)}\). Recently, Cummins et al.\(^{(166)}\)
demonstrated in vivo, that the extraction ratio of K77 (CYP3A/Pgp substrate)
decreased when Pgp is inhibited by GG918, but remained unchanged for midazolam,
an exclusive CYP3A substrate. From this the authors concluded that GG198 can
modulate the extent of intestinal metabolism by controlling drug access to the
enzyme (Figure 1.9).
Figure 1.9

Functional interaction between drug transport (P-gp) and drug metabolism (CYP3A4) in enterocytes. (1) Absorption of an orally administered drug via passive diffusion or carrier-mediated processes form the lumen of the gastrointestinal tract into the enterocyte. (2) Intestinal metabolism via CYP3A4. (3) Transport of the parent compound and / or its metabolite form the enterocyte into the gut lumen via P-glycoprotein, which is located in the apical membrane of enterocytes. (4) Translocation of drug and / or metabolite across the basal membrane of enterocytes via passive diffusion or carrier-mediated processes. *Taken from Fromm, 2003*\(^{(167)}\).
1.4.4 P-gp and Drug-Drug Interactions

Mechanisms contributing drug-drug interaction are multifactorial, and include competitive interaction between drugs for a shared metabolic pathway (eg. cytochrome P450), or as competitive substrates for a common active transport system (eg. P-gp and MDR transporters). In addition to these competitive mechanisms some drugs also show evidence of inhibiting or inducing activities on the expression and / or activity of CYP and the transporters.

One of the most common and serious drug-drug interactions in clinical practice is that between digoxin and the antiarrhythmic agent quinidine**(168)**. Administration of quinidine to patients receiving digoxin increased plasma digoxin levels by 2- to 3-fold, and frequently resulted in symptoms of digoxin toxicity. Although digoxin is not extensively metabolised in man**(169)**, it is a recognised P-gp substrate**(149,170)**. Fromm et al**(171)**, showed that quinidine is a potent inhibitor of P-gp and that both digoxin and quinidine undergo active transport in Caco-2 (intestinal epithelial cell line) cells which express P-gp in a polarised fashion. The importance of this was demonstrated in mdr1a/^−/^ knockout mice, in which quinidine increased plasma digoxin levels by approximately 75% in wild-type mice compared to less than 20% in the knockout animals**(171)**. In keeping with the in vitro and animal studies, intraluminal administration of quinidine inhibits intestinal elimination of digoxin in man**(172)**. Conversely, in healthy volunteers, rifampin decreased the digoxin AUC and caused a 3.5 fold increase in intestinal P-gp expression, which correlated with the digoxin AUC following oral administration**(173)**. Similarly, St John’s Wort has been shown to increase P-gp expression**(174)**, and decrease digoxin levels in man**(175)**. Together these studies suggest that P-gp may have even greater potential to alter drug disposition following its up-regulation when compared to its inhibition.
1.5 P-gp in Health and Disease

1.5.1 P-gp and Cellular Immunity

P-gp is detectable and functionally active in haematopoietic stem cells and in several subclasses of lymphocytes, in particular CD56 cells but also to a lesser degree in CD8, CD4, CD15, CD19 and CD14 cells\textsuperscript{(108,148,176,177)}. Within the population of CD4 cells, P-gp function is variable with some cells (approximately 25\%) displaying substantial P-gp activity, and others little. Several lines of evidence suggest that P-gp modulates effector cell function as part of an immune response, in particular the functionality of NK cells and CD8\(^+\) T lymphocytes, which constitutively express high levels of P-gp. Firstly, inhibition of P-gp expression or function using antisense oligonucleotides or P-gp inhibitors/monoclonal antibodies directed against P-gp attenuate the cytolytic activity of both NK cells and CD8\(^+\) T lymphocytes\textsuperscript{(178,179)}. Secondly, P-gp mediates cellular efflux of several cytokines including IL1\(\beta\), IL2, IL4 and IFN\(\gamma\) from activated normal lymphocytes\textsuperscript{(180-182)}. Finally, transendothelial migration of antigen presenting dendritic cells and T lymphocytes during an immune response are inhibited by verapamil (P-gp inhibitor) and monoclonal antibodies to P-gp\textsuperscript{(183)}. Taken together, it may be hypothesised that P-gp modulates immune cell migration through the regulation of chemotactic cytokine transport. However, this is a controversial area as others have demonstrated that in mdr1a/1b knockout mice cytolytic activity of NK cells and lymphocyte derived cytokine (IL2, IL4, IL10 and IFN\(\gamma\)) release\textsuperscript{(111,184)} are similar to their wild type counterparts.
1.5.2 P-gp and Programmed Cell Death

Cell survival or death is an important issue in the pharmaco-therapy of both infectious and non-infectious diseases, within which modulation of apoptosis (programmed cell death) is an active area of research\(^{(185,186)}\). Interestingly, a number of groups have shown that functional P-gp confers resistance to apoptosis induced by a range of stimuli including cytotoxic drugs, Fas ligand, TNFα, serum starvation and UV radiation\(^{(187-190)}\). This protective effect is reversed by either anti-P-gp monoclonal antibodies or verapamil. Apoptosis involves a complex signaling cascade comprising pro-apoptotic or anti-apoptotic signals, and the activation of specific proteases (e.g. caspases)\(^{(191)}\). Within this signaling cascade, P-gp has recently been shown to inhibit caspase-8 activation but not formation of the death inducing signaling complex (DISC), this activity of P-gp is dependent on ATP hydrolysis\(^{(192)}\). The relationship between P-gp and programmed cell death is complex as; P-gp while representing a ‘resistance factor’ against certain apoptotic stimuli, may also act as a ‘risk factor’ towards mitochondrial bound apoptotic stimuli. The latter may be due to modification of the electrical membrane potential\(^{(193)}\). These results uncover an additional physiological function for P-gp over-expression in promoting the survival of tumour cells.

1.5.3 P-gp and Cancer Associated Multi Drug Resistance

P-glycoprotein was the first key protein implicated in the phenomenon of multidrug resistance to the use of cytotoxic agents\(^{(13)}\). Interestingly, in vitro experiments demonstrated that P-gp over-expressing cell lines, selected on the basis of resistance to a single cytotoxic agent, were cross-resistant to a wide but well defined spectrum of structurally diverse cytotoxic drugs, embracing anthracyclines, epipodophyllotoxins,
taxanes and *Vinca alkaloids*\(^{(194-196)}\). Since this initial discovery, other ABC transport proteins, namely the multidrug resistance proteins 1 & 2 (MPR1, MRP2) from the MRP/CFTR subfamily, have also been found to confer multidrug resistance.

It is well established that renal and colonic tumours often express high basal levels of P-gp\(^{(197,198)}\), while other tumours (lung, breast and lymphoma) only express P-gp following exposure to a chemotherapeutic agent or upon relapse\(^{(199)}\).

### 1.6 P-gp and HIV Disease.

The human immunodeficiency virus (HIV) is an enveloped virus from the family retroviridae and consists of a RNA genome packaged in a protein capsid, surrounded by a lipid envelope. Presently approx 20 million people are living with HIV disease worldwide. The majority of infected persons live in Sub-Saharan Africa however the majority of HIV research has been conducted in Europe and the USA. The introduction of highly active antiretroviral therapy (HAART) in the mid 1990s led to a dramatic improvement in outcome for patients living with HIV disease, this was mainly as a result of the introduction of protease inhibitor therapy to antiretroviral regimens. Protease inhibitors interfere with the post translational modification of gag and gag-pol polyprotein precursors into their functional components such that immature non infectious virions are released. A number of important studies have demonstrated a reduction in the morbidity and mortality associated with HIV disease co-incident with the introduction of protease inhibitors\(^{(200)}\). Current standard of care antiretroviral regimens include a combination of at least three drugs including a Protease inhibitor (PI) and a nucleoside reverse transcriptase inhibitor (NRTI), or a PI and an non nucleoside reverse transcriptase inhibitor (NNRTI). Regardless of these advances, potency and durability remain critical issues for the success of HAART.
The Swiss cohort study has shown that rebound viraemia to detectable levels occurs at a rate of 20% per year in ART experienced patients switching therapy and up to 50% of patients will require treatment changes by 24 months\(^{(201)}\). Complete irradication of the virus has proved impossible because of the persistence of latently infected resting CD4\(^+\) cells harbouring replication competent HIV-1. Important pharmacological factors contributing to this include poor intracellular drug concentrations, sub-optimal infiltration of drugs into sanctuary sites such as the central nervous system, macrophages and lymphocytes, and the development of drug resistant viral mutants as a result of ongoing replication in the presence of poor drug levels. Numerous studies have shown that sub-optimal plasma PI levels are associated with poor response to therapy and it is because of this that Therapeutic Drug Monitoring (TDM) continues to be debated as part of patient management\(^{(202)}\).

### 1.6.1 P-gp Expression in PBMCs of HIV Patients

P-gp is detectable and functionally active in haematopoietic stem cells and in several subclasses of lymphocytes, in particular CD56 cells but also to a lesser degree in CD8, CD4, CD15, CD19 and CD14 cells\(^{(108,148,176,177)}\). Within the population of CD4 cells, P-gp function is variable with some cells (approximately 25%) displaying substantial P-gp activity, and others little. The expression of P-gp in lymphocytes (a main site of HIV viral replication) suggests it may play a role both the intracellular accumulation of protease inhibitors used to treat HIV or indeed in the disease process per se.

HIV-1 can induce activation of the MDR gene and increase expression of P-gp in H9 (T cell) and U937 (monocytic) cell lines, culminating in reduced intracellular accumulation of ZDV, a nucleoside reverse transcriptase inhibitor\(^{(203)}\). Similarly P-gp
expression was increased in CD4\(^+\) T cells from patients with HIV-1 compared to healthy controls\(^{(204)}\). Expression levels were highest in those with advanced disease, however rhodamine accumulation studies suggested that the P-gp expressed in this group was functionally defective\(^{(204)}\). In contrast others report that both surface expression of P-gp on CD4 and CD19 cells\(^{(177)}\) and intracellular expression in CD8\(^+\) and NK-CD16\(^+\) cells\(^{(205)}\) are lower in patients infected with HIV. Interestingly, the cellular location of P-gp in CD4\(^+\) and CD8\(^+\) lymphocytes from healthy volunteers is altered following exposure to HIV-binding glycoprotein gp120 \textit{in vitro}\(^{(205)}\). It is difficult to reconcile the data from these conflicting studies and unfortunately few clinical studies have addressed the effect of naïve and treated HIV disease on P-gp expression and function in the PBMCs of HIV infected patients.

1.6.2 Protease Inhibitors as Substrates and Modulators of P-gp

Several \textit{in vitro} and \textit{in vivo} studies have shown the HIV-1 protease inhibitors indinavir, nelfinavir, saquinavir, and ritonavir to be P-gp substrates\(^{(158,206-208)}\). While these drugs are recognised as substrates, saquinavir, ritonavir and nelfinavir also inhibit P-gp-mediated efflux, leading to intracellular accumulation of paclitaxel and vinblastine (known P-gp substrates) within P-gp expressing cells\(^{(209)}\). However, when compared with other P-gp inhibitors such as verapamil or cyclosporin, their inhibitory potential is generally weaker\(^{(207)}\).

Even within the PI drug class, there is differential potency (RTV>SQV>NFV>IDV) with respect to the ability to inhibit P-gp function in PBMCs from healthy volunteers and HIV infected patients\(^{(210,211)}\). Unsurprisingly, intracellular accumulation of NFV, SQV and RTV was reduced in CEM cells expressing P-gp (CEM\(_{VBL}\)), and SQV and RTV accumulation was attenuated in MRP1 expressing cells (CEM\(_{E1000}\)), indicating
that both P-gp and MRP1 are likely to have a role in limiting intracellular concentrations of PIs\(^{(212)}\). In a follow-up study, the same group extended their earlier findings to show that intracellular accumulation of PIs is significantly reduced in both CEM-MDR and CEM-MRP cells which express increased levels of P-gp (30 fold) and MRP (5 fold), compared to control CEM cells. Furthermore, the reduction in intracellular accumulation was reversed upon co-administration of the P-gp inhibitor GF 120918\(^{(213)}\). Together these data raise the possibility that variation in P-gp expression may have an influence on treatment outcome of HAART. Interestingly, in a HIV protease inhibitors do not inhibit P-gp-mediated efflux when applied simultaneously and competitively in the LLC-PK1:MDR1 (over-expressing MDR1) cell line\(^{(214)}\), which may indicate limited benefit in using this approach clinically to achieve increased PI concentrations in sanctuary sites such as the Blood brain barrier.

1.6.3 The Effect of P-gp on Antiretroviral Efficacy

The first early indications implicating P-gp with reduced anti-HIV efficacy came from a study showing diminished anti-viral activity of ZDV in P-gp expressing cells\(^{(215)}\). In this study, loss of anti-viral efficacy was associated with reduced intracellular concentrations of the active metabolite, ZDV-triphosphate, and restoration of intracellular ZDV concentrations following co-incubation of the cells in the presence of a P-gp inhibitor\(^{(215)}\). More recently, in a study utilising a P-gp over-expressing carcinoma cell line (KB-VI) the inhibitory potential of the protease inhibitors, ritonavir, saquinavir and indinavir was shown to be reduced\(^{(207)}\). Furthermore, intracellular accumulation of ritonavir, indinavir, saquinavir and nelfinavir is reduced in human T lymphocytes over-expressing MDR1 (CEM-MDR1) and MRP1 (CEM-MRP1)\(^{(213)}\). However, these data are at variance with the *in vitro* dye accumulation
data of Srinivas et al.\textsuperscript{(216)} which shows effective inhibition of HIV-1 viral replication by saquinavir, ritonavir, nelfinavir and indinavir in both MDR1\textsuperscript{+} (CEM/VBL100) and MRP1\textsuperscript{+} (CEM/VM-1-5) over-expressing T lymphocytic cell lines. Indeed, the IC\textsubscript{90} for each PI were similar amongst cell lines used, indicating that over-expression of MDR1 or MRP1 by T-lymphocytes is unlikely to limit the antiviral potency of the HIV PIs, although clearly it may reduce their clinical efficacy by decreasing intracellular concentration of drug.

1.6.4 The Effect of Antiretroviral Therapy on P-gp

There is increasing awareness of the importance of P-gp in limiting the bioavailability of co-administered drugs. In addition evidence that P-gp may limit first pass metabolism by CYP3A4 (by decreasing exposure of drug to the P450 enzyme in the gut) has led to investigations assessing the potential for antiretroviral drugs to induce expression of P-gp\textsuperscript{(217)}. Again data is conflicting. In a study by Depuis et al.\textsuperscript{(218)} CEM cells which over express P-gp in the presence of vinblastine and which return to baseline expression in its absence, were highly inducible in the presence of saquinavir. Similarly oral administration of amprenavir or nelfinavir to rats induces intestinal P-gp. However, In vitro only nelfinavir has been shown to induce expression of P-gp in PBMCs\textsuperscript{(219)}. With respect to the NNRTIs, nevirapine causes a concentration dependant induction of P-gp expression in LS180V cells which results in marked rhodamine accumulation\textsuperscript{(220)}. There is little convincing evidence that NRTIs induce P-gp expression.
1.6.5 P-gp Polymorphisms: Relevance to HIV Disease

The first comprehensive investigation for polymorphisms in the ABCB1 gene was by Hoffmeyer and colleagues\(^{(221)}\). 15 single nucleotide polymorphisms were identified in the coding region of which 3 entered the primary amino acid sequence of the protein. Of these only the C3435T SNP had a significant correlation with expression and function of P-gp resulting in decreased duodenal ABCB1 expression and increased digoxin levels. However the C3435T allele is located at a non coding region of the MDR1 gene and is thought not to be directly associated with P-gp expression.

To date, 28 single nucleotide polymorphisms (SNPs) have been identified at 27 different positions on the MDR1 gene\(^{(221,222)}\). While most of the SNPs are independent of one another, some may be linked, for example, the polymorphism on exon 26 appears to be related to SNPs in exon 12 (C1236T) and exon 21 (G2677T)\(^{(223)}\). There is considerable debate as to the functional importance of the known SNPs. Correlations between SNPs and either drug absorption or intestinal expression of P-gp /MDR-1 are inconsistent\(^{(223-225)}\). In a study by Kim et al.\(^{(223)}\), individuals with the T/T genotype at position 3435 was associated with increased P-gp function and hence lower plasma levels of fexofenadine (a high affinity P-gp substrate) in comparison to those with the C/C genotype. Furthermore, Uwai et al.\(^{(226)}\) found no correlation between common SNPs in the MDR1 gene and P-gp expression in normal kidney or in renal cell carcinoma of Japanese patients. These discrepancies may reflect linkage to as yet undefined polymorphisms in the promoter or enhancer regions of MDR1 or nucleotide sequences intrinsic for mRNA processing\(^{(223)}\). Differences in allele frequencies have been noted in Africans, or those of African descent, who have a lower frequency of the 3435 T/T genotype and a higher frequency of the C/C genotype than caucasians\(^{(223,227)}\). It has been suggested that genetic differences in
MDR1 genotype, may in part account for some of the interindividual variability in P-gp expression among individuals, as well as variability in patient response to drug therapy\(^{(223,224)}\).

Few studies have addressed the potential for genetic variation of P-gp expression in HIV patients and particularly regarding the importance of the C3435T polymorphism as a predictor of antiviral drug response. However, Fellay et al.\(^{(228)}\) demonstrated low MDR-1 expression in association with the MDR-1 3435TT genotype compared to either the CC or CT genotypes. There was a correlation between the CC genotype and higher nelfinavir and efavirenz plasma concentrations, while the T allele was associated with lower nelfinavir concentrations. This suggests that the T allele may be associated with increased P-gp function. However P-gp mRNA transcription was lower in the PBMCs of these patients highlighting the complexity of the interrelationship between P-gp expression and function. Interestingly, those with the T/T genotype experienced a greater recovery in CD4\(^+\) T-cell count following antiretroviral therapy containing nelfinavir\(^{(228)}\), which may be due to the protective function of P-gp on pluripotent stem cells\(^{(148)}\). Together these data raise the possibility that the level of MDR-1 transcripts in PBMCs from HIV infected patients may be depend upon allelic variants of MDR-1\(^{(228)}\), however the wide variation in sample size and antiviral regimens used in subsequent studies coupled with treatment periods ranging from one dose to 192 weeks it impossible to draw firm conclusions on this issue.

### 1.6.6 HIV Entry into Cells is there a Role for P-gp?

The human immunodeficiency virus (HIV) is an enveloped virus from the family retroviridae and consists of a RNA genome packaged in a protein capsid, surrounded by a lipid envelope. Within the lipid envelope a number of proteins including the viral
envelope protein complex (gp120/gp41) are embedded. This complex plays an important role in mediating virus entry into host cells. The envelope protein complex is synthesised as the glycosylated precursor gp160, which undergoes enzymatic cleavage by a convertase to yield a surface subunit (gp120) and a transmembrane subunit (gp41). These subunits remain non-covalently coupled and undergo oligomerisation, most likely as trimers, at the surface of the virion. HIV entry involves binding of virion-associated gp120 to both CD4 receptors and either a CXCR4 or CCR5 co-receptor on the target cells, inducing a conformational change in gp41 and initiating fusion of the virus and target cell membranes. It is interesting to note that gp41 contains a N-terminal, hydrophobic glycine-rich “fusion” peptide that is critical for membrane fusion. In light of this and since P-gp is capable of extruding hydrophobic peptides it has been postulated that P-gp may interfere with HIV entry into cells. This concept extends earlier observations that P-gp over-expression may prevent insertion of the influenza virus fusion protein (haemagglutinin-2) into a KB-VI carcinoma cell line altering infectivity of the influenza virus. Data from several studies indirectly support this hypothesis. Following P-gp over-expression virus production is attenuated in a human CD4 T-leukaemia cell line (12D7) infected with HIV-1NL-A3 (a T-trophic molecular clone of HIV-1). More recently, Speck et al. demonstrated that HIV-1 protein and virus production are approximately 70 fold lower following HIV-1HIB infection of CEM cells over-expressing P-gp. Fusion of HIV-1 with its target cells takes place at specialised regions in the membrane known as cholesterol-rich rafts or glycolipid-enriched membrane (GEM) domains. These lipid rafts are highly ordered sphingolipid and cholesterol rich structures in the outer leaflet of the plasma membrane at which CD4 and CXCR4 are reorganized into bundles as a prerequisite for efficient HIV infection.
Furthermore, P-gp is also preferentially distributed within the GEM domains, indicating that these areas of the plasma membrane may play a central role in the cellular binding and egress of HIV. A potential mechanism by which P-gp may modulate HIV-1 production has not yet been described, however, it is unlikely to involve P-gp-mediated ATP hydrolysis since virus production remains attenuated in the presence of an over expressed and non-functional ATP mutant. Nor is it likely to involve down-regulation or gross rearrangement of either the CD4 receptors or the co-receptor CXCR4 at the cell surface since P-gp over expression is not associated with changes in surface expression of these proteins. Based upon experiments designed to directly by-passed the fusion step, Lee hypothesised that P-gp over-expression interferes with HIV-1 infection at steps downstream of fusion. In contrast Speck et al. found that late post-integration steps in viral replication were not significantly affected by the expression of P-gp. Interestingly, they also found that HIV-1 infectious virus production was increased by at least 50-fold in CEM cells over-expressing MRP1, and that MRP1 was not preferentially found in GEM domains. The observation that MRP-1 is capable of increasing HIV antigen production and infectious virus at an early stage in the viral life cycle suggests an alternative mechanism to P-gp and other ABC transporters. Given that MRP is also a transporter of glutathione and its conjugates, and that low intracellular concentrations of glutathione are associated with enhanced HIV production this may reflect depletion of intracellular glutathione by MRP-1.
Chapter 2

Aims
2.1 Aims and Objectives

An overarching theme of the studies presented in this thesis is to examine in a clinically relevant way specific pharmacological aspects of the multi drug transporter P-glycoprotein. As such, the potential for P-gp to be induced or inhibited by certain drugs is considered. The effect of P-gp on the pharmacokinetics of commonly used drugs is examined, along with the effects of disease and its treatment on P-gp expression and function. Many antiretroviral drugs are either substrates or modulators of P-gp, thus the studies are conducted in either patients with HIV disease, or healthy volunteers, in order to maximise the clinical relevance of the results.

The primary aim of the thesis is to enhance our understanding of how P-gp interacts with certain drugs to alter their disposition, and to outline the potential clinical consequences of that effect. A secondary aim of the thesis is to determine the effects of disease and its treatment, on P-gp expression and function. Each of the studies included in the thesis has specific objectives which in accrual support these aims.

P-gp is differentially expressed in a variety of tissues including gut, epithelium and peripheral blood lymphocytes. Many drugs of important clinical value including methotrexate, steroids and protease inhibitors are substrates for P-gp mediated drug efflux. The disposition of these drugs and their clinical efficacy may be altered as a result of upregulation of P-gp expression or activity. The objectives of the first study are two fold, to investigate the effects of the seemingly harmless herbal remedy St Johns’ Wort on P-gp and function, and secondly to consider the P-gp inhibitory potential of ritonavir, a protease inhibitor used in the treatment of HIV disease.

The majority of antiretroviral drugs are thought to act intracellularly. For HIV disease, plasma drug concentrations are used as a surrogate marker for intracellular drug disposition because the latter has proved difficult to measure in clinical situations. The
main objective of the second study is to determine the feasibility of measuring intracellular concentrations of the protease inhibitor indinavir and to compare the plasma and intracellular pharmacokinetics of this commonly used drug in a group of HIV infected patients.

The presence of P-gp on the PBMCs of HIV infected patients suggests it may modulate intracellular drug concentrations altering efficacy. Therefore, the main objective of the third study is to test this hypothesis by investigating not only the plasma and intracellular pharmacokinetics of nelfinavir, but also P-gp expression and function in the same group of treated HIV patients. The latter two studies are placed together to demonstrate PI specific differences in relation to interactions with P-gp.

The objectives of the final study are to investigate the relationship between P-gp and HIV disease by assessing P-gp expression and function in the PBMCs of a cohort of treated (suppressed and unsuppressed), and untreated HIV infected patients. This study attempts to differentiate between the effects of the disease *per se* and the effects of long term exposure to drugs known to alter P-gp function. In addition, the potential for further modulation of P-gp function through the use of ritonavir is examined.
Chapter 3

St John’s Wort Increases Expression of P-Glycoprotein:

Implications for Drug Interactions
3.1 Introduction

St John’s Wort (*Hypericum Perforatum*) has been used for centuries as an herbal remedy. Galen (A.D. 150-200) was known to have prescribed it for menstrual disorders and it was also used in the middle ages to treat depression, acquiring its popular name due to the fact that flowering occurs on the birthday of St John the Baptist (June 24th). St John’s Wort (SJW) has recently gained popularity as “nature’s Prozac” with at least 5 randomised controlled clinical trials providing evidence that it is as effective as conventional antidepressant therapy. In Germany, it is currently the most commonly prescribed anti-depressant with more than 2.7 million prescriptions written each year. Despite the widespread use of SJW little is known about its pharmacokinetic properties and since it is usually sold as an over the counter herbal remedy it has not been subjected to the rigorous clinical testing required of other compounds. In addition, the public perception that what is “Natural” is safe has resulted in the interaction potential of SJW being largely ignored until recently. Given that 18.4% of U.S adults surveyed reported concurrent use of at least one herbal product or high dose vitamin with regular prescription medications, but over 60% did not disclose such use to their physicians the potential for SJW to interact with co-administered medications should be viewed with concern. Several recent reports suggest that SJW may produce clinically relevant interactions by promoting the metabolism of co-administered drugs such as warfarin and the oral contraceptive pill. Chronic co-administration of SJW reduced cyclosporin levels by approximately 80% resulting in the development of acute transplanted organ rejection. Similarly, administration with the HIV 1 protease inhibitor indinavir produced an 81% reduction in indinavir trough concentrations. In contrast, co-administration with digoxin produced a 30% reduction in plasma digoxin.
concentrations\(^{175,246}\). Induction of the hepatic CYP 3A4 by SJW has been implicated as the most likely mechanism for these interactions and *in vitro* studies demonstrate that SJW approximately doubles CYP3A4 expression\(^{247-249}\). In addition, *hyperforin* (the postulated active ingredient of SJW) is a potent ligand for the pregnane X receptor, an orphan nuclear receptor which regulates expression of CYP3A4\(^{250,251}\). Interestingly, the magnitude of the interaction seen in clinical reports (80%) is greater than that predicted by *in vitro* data (50%) suggesting a second interaction mechanism may exist, and since digoxin undergoes primarily renal clearance with metabolism by CYP3A4 providing a minor metabolic pathway, induction of CYP3A4 is unlikely to explain this interaction. Cyclosporin, indinavir and digoxin in addition to being substrates for CYP3A4 are also recognised as substrates for the multi drug transporter P-glycoprotein which functions as a transmembrane drug efflux pump\(^{98,149,207,252}\). We postulate that SJW may alter expression and/or function of P-glycoprotein reducing tissue or cellular concentrations of certain drugs thereby contributing to its potential for drug-drug interactions. Since lymphocytes, express functional P-glycoprotein but not functional CYP3A4 we have chosen the peripheral blood lymphocytes (PBMCs) of healthy volunteers as the model for this study.

### 3.2 Methods

#### 3.2.1 Study Design

Twenty-two healthy volunteers (13 female) participated in a single blind randomised placebo controlled trial. Following informed consent, fifteen volunteers were randomised to SJW (Good n' Natural 0.15\% standardised extract 600 mg tds) for 16 days and seven received placebo for 16 days. Smoking and concomitant medication use (including the oral contraceptive pill) was prohibited throughout the study. All
subjects were monitored for the development of adverse effects commonly associated
with SJW such as dry mouth, gastro-intestinal upset and constipation.

Venous blood samples were drawn for P-glycoprotein expression and function at
baseline, 16 days and 32 days (16 days post discontinuation of treatment). In addition,
each batch of SJW used in the study was assayed for hypericin content by a standard
HPLC method.

3.2.2 Lymphocyte Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood
(within 2 hr) by Ficoll density gradient centrifugation. In brief whole blood was
layered onto Lymphoprep (Gibco USA) and centrifuged at 1200 rpm, 4 °C for 25 min.
The buffy coat was removed, resuspended and washed twice (2000 rpm x 5 min) with
Hanks balanced salt solution without calcium and magnesium (Life Technology,
Paisley U.K.). Cells were finally resuspended in culture medium (RPMI plus
glutamine; Life technology, Paisley U.K.), supplemented with 10% foetal calf serum
(FCS; Sigma, Poole, Dorset). Cell count and viability were quantitated in a
Neubauer's chamber following staining with ethidium bromide/acridine orange. Cells
were aliquoted (1x10^6 cell.ml^-1) and reserved for P-glycoprotein expression and
function and for RNA isolation.

3.2.3 P-glycoprotein Expression

In accordance with the St Jude consensus guidelines\(^{253}\) regarding the methodology
and interpretation of P-glycoprotein expression and function the presence of P-
glycoprotein was detected by flow cytometry and confirmed by RT-PCR. Cells were
fixed in 2% paraformaldehyde (Cellfix, Becton Dickinson Mountain View C.A) for
20 min at room temperature and permeabilised with 0.05% (w/v) saponin (Sigma Aldrich). Cells were labelled with a JSB1 monoclonal antibody (Sanbio, Uden, Netherlands, Figure 3.1A) directed towards a highly conserved intracellular epitope of P-glycoprotein (30 min, at 37°C). A negative fluorescence control was performed using mouse monoclonal IE immunoglobulin (Ig)G derived from a murine hybridoma supernatant (ATCC, Manasses V.A.) HB179, 30 min, at 37°C, whereas antibody to the cytoskeletal component vimentin (Dako AS Glostrup Denmark 30 min, at 37°C) was used as a control for the permeabilisation technique. After removal of excess antibody by washing with 0.05% (w/v) saponin, cells were incubated with 100 μl rabbit anti-mouse fluorescein labelled isothiocyanate (FITC)-conjugated F(ab′)2 (1:50 dilution; Dako Glostrup AS) for 30 min, at 37°C. Finally cells were washed free of excess antibody and re-suspended in 0.5ml of paraformaldehyde before being subjected to flow cytometric analysis, and median fluorescence intensity values (MFI; a measure of expression) obtained (Figure 3.1B). The presence of MDR1 mRNA in each sample was confirmed by RT PCR as previously described.  

3.2.4 Functional Studies of Rhodamine 123 Transport: Efflux and Inhibition

PBMCs (1x10⁶) cells were loaded with rhodamine 123 (1.25 μg.ml⁻¹; Sigma) for 25 min at 37°C in RPMI 1640 supplemented with 10% FCS. Cells were washed twice in ice-cold media and incubated at 37°C for 3h in 3 ml of dye free media to allow dye efflux. At the time points baseline, T=0 (maximum loading) and three hours later T=180min (maximum efflux) an aliquot was removed and washed twice in ice-cold media (3 min x 2000rpm) before being fixed in ice-cold paraformaldehyde (Cellfix). Cellular fluorescence was determined by flow cytometric analysis and expressed as median fluorescence intensity.
3.2.5 Flow Cytometry

Lymphocytes shown in forward scatter and side scatter were electronically gated and acquired (10000 events) through the FL1 channel (expression) or the FL2 channel (function). The amount of fluorescence was plotted as a histogram of FL1 or FL2 staining within the gate. Data acquisition was performed using Cellquest software (WINMDI version 2.6) to determine median fluorescence intensity values. Parallel experiments were performed in the presence of ritonavir (5 μM), a known P-glycoprotein inhibitor. This concentration was chosen from a previously derived dose response curve for the inhibition of P-glycoprotein mediated rhodamine efflux by ritonavir in the PBMCs of healthy volunteers (unpublished data). Ritonavir was a gift from Abbott laboratories.

3.2.6 Reverse Transcription Polymerase Chain Reaction

Total cellular RNA was isolated from PBMCs by a modification of the method of Chomczynski and Sacchi using Tri Reagent (Sigma). 1st Strand cDNA was synthesised from 2 μg total RNA using random decamers and M-MLV reverse transcriptase (Reverse-iT kit, Abgene). The resultant cDNA was amplified by PCR using gene specific primers (Table 3.1) as described by Egashira et al., and a standard PCR protocol. All PCR reactions were performed in duplicate. The PCR products were electrophoresed on a 2.0% (w/v) agarose gel and stained with ethidium bromide. Gels were visualised under ultraviolet illumination, photographed and analysed on a PC using Gene Tools analysis software (Syngene).
Table 3.1 Sequences of upstream and downstream oligonucleotide primers.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward</th>
<th>Reverse</th>
<th>Tm</th>
<th>No. Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2M</td>
<td>ACCCCCACTGAAAAAG</td>
<td>ATCTTCAAACCTCCA</td>
<td>55°C</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>ATGA</td>
<td>TGATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDR-1</td>
<td>CCCATCATGCAATAG</td>
<td>GTTCAAACTTCTGCT</td>
<td>55°C</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>CAGG</td>
<td>CCTGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2.7 Hypericin Assay

The HPLC method was based upon that of Chi J.D. and Franklin M(256,257). In brief, hypericin (Fluka Chemie AG, Buchs Germany) was assayed by using a C-8 column (15 x 4.6 mm, 5 μm; column temperature: 60°C) with a Shimadzu LC-10AS pump (flow rate: 1.5 ml.min⁻¹) and a Shimadzu RF-10AXL fluorescence detector (Ex 390 nm; Em 620 nm). A standard curve was constructed using serial dilutions of a stock solution of hypericin (1 mg.ml⁻¹) prepared in DMSO and diluted accordingly with the HPLC mobile phase (0.03 M phosphate buffer (pH 7) and methanol (30:70, v/v). A 10 mg aliquot of each St John’s Wort capsule (0.15% extract) was dissolved in DMSO (1 ml) and centrifuged at 2000 rpm for 5 min. The supernatant was removed and diluted (1:100) with mobile phase prior to injection.

3.2.8 Data Analysis

P-gp expression was calculated as the difference between the median fluorescence intensities following labelling with JSB1 and IE for each sample.

P-glycoprotein function was expressed as the ratio T180/T0 (maximum efflux/maximum loading), therefore, a reduced ratio indicates enhanced drug efflux function.
The percentage of reversible efflux in the presence of ritonavir (an inhibitor) was calculated as:

\[ 100 \times \frac{R_{180} - T_{180}}{T_0 - T_{180}} \]

3.2.9 Statistical Analysis

Data were subjected to nonparametric analysis for paired (Wilcoxon), unpaired (Mann Whitney U) t tests or a one-way analysis of variance followed by multiple comparison (Dunn’s test) where appropriate. Data are expressed as mean ± SD. P<0.05 indicates statistical significance.

3.3 Results

3.3.1 P-glycoprotein Expression and Function

Fifteen subjects were initially randomised to receive SJW, of these 12 completed the study. 1 subject discontinued SJW due to adverse effects (nausea, dry mouth), and 2 subjects were withdrawn from the study because of the need for a potentially interacting medication. All 7 healthy volunteers randomised to receive placebo completed the study. We have previously established that in resting peripheral blood lymphocytes of healthy individuals, under identical experimental conditions (ie non-smoking and non medicated) that P-glycoprotein expression in vivo is stable over 2mths (baseline vs. 2 month: 6.02±1.5 vs. 6.4±2.7 MFI; 95% CI: -2.57 to 3.3) and normally distributed (KS value= 0.07; n=77; mean± SD; 7.82±3.2). In the present study, there was a mean 4.2 fold increase in P-glycoprotein expression in subjects treated with SJW for 16 days compared with baseline values (29.5±14.3 vs. 7.0±1.9 MFI P<0.05; Figure 3.2; 95% CI: 13.5 to 31.6). In contrast there was no change in P-
glycoprotein expression in the PBMCs of those treated with placebo (5.1±1.3 vs. 6.0±1.9 MFI; Figure 3.2; 3.3B; 95% CI: -0.6 to 2.4). There was large individual variability with respect to increased P-glycoprotein expression following SJW (Figure 3.3A; range: 1.09-9.06 MFI). P-glycoprotein expression had returned to baseline 16 days post discontinuation of SJW (8.5±4.8 MFI; Figure 3.2). Treatment with SJW increased P-glycoprotein mediated rhodamine efflux (reduced ratio) compared with baseline levels (0.12±0.04 vs. 0.24±0.18 (P<0.05; 95% CI: 0.01 to 0.29; Figure 3.4A). There was no change in P-glycoprotein function with placebo (0.16±0.17 vs. 0.24±0.11; 95% CI: -0.05 to 0.21; Figure 3.4B). Ritonavir (5 µM) produced significantly greater inhibition of P-glycoprotein mediated rhodamine efflux in the PBMCs of subjects before treatment with SJW compared with post treatment values (75.4±16.4 vs. 23.9±15.3%; P< 0.01; 95% CI: 43.7 to 70.1; Figure 3.5). The percentage inhibition by ritonavir was similar before and after treatment with placebo.

3.3.2 MDR1 mRNA Expression

The presence of MDR1 mRNA was confirmed by non-quantitative RT-PCR. Amplification products of the expected size were detected at 167 base pairs for MDR1 and 120 base pairs for β2 microglobulin in PBMCs in every sample collected.

3.3.4 Hypericin Assay

Duplicate analysis was performed on each of two capsules from each batch (n=3). The results were 0.15, 0.14, and 0.15% w/w hypericin for batches A, B, and C as determined against the hypericin standard; this was in agreement with the hypericin content quoted by the manufacturers product insert. The intra-day (n=4) variability for
10 \mu g.ml^{-1} and 50 \mu g.ml^{-1} was 3.1 and 2.2\% respectively. The inter-day (n=8) variability was 4.3\% and 3.5\% for 10 \mu g.ml^{-1} and 50 \mu g.ml^{-1} respectively. Standard curves were found to have correlation coefficients approximating to 0.99.
Figure 3.1

A) Plate showing intracellular staining of a peripheral blood mononuclear cell with JSB1 (a P-glycoprotein specific antibody). B) A representative flow cytometry trace depicting the median fluorescence intensity following staining for P-glycoprotein with JSB1 and vimentin (positive control) and ie (negative control).
Figure 3.2

P-glycoprotein expression in peripheral blood lymphocytes of subjects’ pre and post 16 days treatment with either St John’s Wort (600mg 0.15%; n=12) or placebo (n=7). Data are expressed as net MFI (median fluorescence intensity) following labelling with JSB1 and IE for each sample. Data are expressed as mean ± SD. * P<0.05.
Figure 3.3

P-glycoprotein expression in peripheral blood lymphocytes of individual subjects before and after 16 days treatment with either (A) St John’s Wort (600mg 0.15%; n=12) or (B) placebo (n=7). Data are expressed as net MFI (median fluorescence intensity) following labelling with JSB1 and IE for each sample. Data are expressed as mean ± SD. * P<0.05.
Figure 3.4

P-glycoprotein function expressed as the ratio of rhodamine efflux at time 0 and 180 min (T180/T0) in peripheral blood lymphocytes of subjects pre and post 16 days treatment with either (A) St John’s Wort or (B) placebo. Data are expressed as mean ± SD. * P<0.05 compared with pre St John’s Wort.
Figure 3.5

Effect of ritonavir (5 μM) on rhodamine efflux from peripheral blood lymphocytes of subjects pre and post 16 days treatment with either St John's Wort or placebo. Data are expressed as mean ± SD. * $P<0.05$ compared with pre St John’s Wort.
3.4 Discussion

P-glycoprotein is an energy dependant membrane-associated multi-drug efflux pump encoded by the MDR-1 gene on the long arm of chromosome 7. Increasing multi-drug resistance is associated with altered expression of P-glycoprotein, which then actively transports drug substrates out of the cell lowering their intracellular drug concentration and facilitating the development of drug resistance\(^{(98,258-260)}\). P-glycoprotein is differentially expressed in a variety of normal tissues including gut epithelium and peripheral blood lymphocytes\(^{(261)}\). Many drugs including methotrexate, protease inhibitors, and steroids in addition to some cytotoxic agents are known to be substrates for this drug efflux mechanism. Their disposition and metabolism will therefore be affected by its expression and activity\(^{(201,252,259,262,263)}\). A number of non-cytotoxic compounds are capable of reversing the drug efflux effect including verapamil, cyclosporin and more recently the anti HIV drug ritonavir\(^{(260,264,265)}\). Inhibition of the efflux pump may facilitate accumulation of drugs within previously resistant cells, improving drug bioavailability, intracellular concentrations and penetration into sanctuary sites such as the central nervous system\(^{(262,266)}\).

Recent reports have documented clinically relevant drug interactions between SJW and co-administered drugs such as indinavir, cyclosporin and digoxin\(^{(175,244,245)}\), attributing induction of hepatic CYP3A4 as the likely mechanism\(^{(248,249)}\). However, interactions with digoxin are unlikely to be fully explained by this mechanism, as it is not a CYP3A4 substrate. Furthermore, the discrepancy between \textit{in vitro} and clinical data suggests that a second interaction mechanism may be involved. It has been postulated that drug interactions with SJW may be mediated through P-glycoprotein\(^{(174,245)}\). The present study assesses the effects of chronic administration of
SJW on P-glycoprotein expression and function in human PBMCs. We found that chronic treatment with SJW produced a greater than 4 fold increase in expression of the multidrug transporter P-glycoprotein in the PBMCs of healthy volunteers. This was associated with enhanced drug efflux function resulting in reduced intracellular accumulation of rhodamine. Furthermore, in the presence of increased P-glycoprotein expression the inhibitory effects of ritonavir (a potent P-glycoprotein inhibitor) were attenuated(267). Our data supports the data of Durr et al. (174) which showed a 1.4 fold increase in intestinal P-glycoprotein/MDR1 expression following chronic oral administration of SJW. Furthermore, Greiner et al. (173) demonstrated a reduction in plasma digoxin concentrations following a similar inductive response to oral treatment with rifampicin suggesting P-glycoprotein induction as an alternative mechanism for drug-drug interactions. Our study provides further evidence of a second mechanism by which SJW may interact with co-administered drugs. Since P-glycoprotein and CYP3A4 and have distinct though overlapping substrates the magnitude of interactions encountered clinically may depend on whether a drug is transported mainly by P-glycoprotein (digoxin, colchicine), or metabolised by CYP3A4 (cimetidine, oral contraceptive pill), or both (indinavir, ritonavir, cyclosporin).

Our data and that of others would suggest that CYP3A4 and P-glycoprotein expression may be co-induced by SJW(248,268). Studies indicate that St John’s Wort induces hepatic drug metabolism through activation of the pregnane X receptor(250), and have identified PXR response elements in the upstream regulatory regions of these genes(251,269-272). In addition Geick et al. have recently identified a distinct DR4 nuclear receptor response element that is essential for MDR1 induction by
rifampicin\(^{(144)}\). Whether the inductive response to St John’s Wort seen in PBMCs is via an interaction with the PXR receptor was not addressed in the present study.

In recent years the therapeutic potential of P-glycoprotein modulation has been re-examined and at least one specific inhibitor (valspodar) is presently undergoing clinical trials for use in oncology\(^{(150,246)}\). Similarly low dose ritonavir has been added to some HIV antiretroviral salvage therapies in an effort to enhance efficacy\(^{(259,263,273,274)}\). Our finding that chronic administration of SJW reduces the potential of ritonavir to inhibit P-glycoprotein mediated drug efflux suggests that the clinical use of P-glycoprotein modulators such as ritonavir or valspodar (PSC833) may be limited in the presence of SJW. In conclusion, when prescribing drugs, that are substrates of P-glycoprotein, CYP3A4 or both, patients should be strongly warned against chronic co-administration of SJW since clinically significant drug-drug interactions are likely.
Chapter 4

Intracellular Indinavir pharmacokinetics In HIV Infected Patients: Comparison with Plasma Pharmacokinetics
4.1 Introduction

The HIV protease enzyme is responsible for post-translational modification of \textit{gag} and \textit{gag-pol} polyprotein precursors into their functional products\textsuperscript{(275,276)}. Inhibition of the enzyme results in the production of immature non-infectious virions and subsequent interruption of viral spread\textsuperscript{(258)}. Since the inclusion of protease inhibitors (PI) within highly active antiretroviral drug regimens (HAART) the morbidity and mortality due to HIV disease has been reduced\textsuperscript{(200)}. Despite its success, HAART still lacks sufficient potency and durability\textsuperscript{(201)}. Large cohort studies suggest that up to 20\% of patients fail to achieve adequate suppression of HIV plasma RNA. Even when this is achieved, up to 30\% of patients will experience viral rebound within 1 year\textsuperscript{(201,277)}. While loss of viral suppression is multifactorial sub-optimal drug levels whatever the cause will permit ongoing viral replication and favour the emergence of resistant virus over time.

For indinavir a relationship exists between plasma concentrations and virologic response\textsuperscript{(278-281)}. In clinical practice the aim is to obtain plasma indinavir levels above a minimum inhibitory concentration (MEC) of 100 ng.ml\textsuperscript{-1}\textsuperscript{(278,282)} throughout the dosing interval, taking into account mitigating factors that might lead to reduced free concentrations such as protein binding. It is to ensure that PI-containing regimens achieve trough concentrations above this critical value that therapeutic drug monitoring (TDM) is being debated as a part of patient management\textsuperscript{(202)}. Numerous studies have shown that sub-optimal PI levels are associated with poor virological response\textsuperscript{(278,283,284)} and recently, the ATHENA trial reported that TDM of non-boosted and boosted indinavir in treatment naïve patients improves treatment outcome\textsuperscript{(285)}. However, while Casado \textit{et al.}\textsuperscript{(286)} also showed that sub-optimal PI levels were
associated with a poorer virological response, there was no correlation between optimal drug levels and an improvement in virologic outcome.

Therapeutic discordance describes failure to achieve viral load reduction despite adequate plasma PI concentrations or alternatively viral load response regardless of inadequate plasma drug levels or poor adherence to therapy. Factors which may influence treatment failure or success, include viral resistance, lack of adherence and viral penetration into sanctuary sites\(^{(287-289)}\). The importance of pharmacological factors such as intracellular drug concentrations and drug influx/efflux mechanisms (P-glycoprotein and MRP) in determining treatment success or failure is only recently becoming apparent\(^{(207,210,252,262)}\). PIs inhibit the activity of a virally encoded protease required for the post integration assembly of infectious viral particles within HIV infected cells\(^{(276,290,291)}\). Therefore, intracellular PI concentrations are likely to be of critical importance in determining anti retroviral efficacy, and such information might be expected to contribute significantly to our understanding of therapeutic discordance in certain HIV infected individuals. To date, little is known about intracellular PI concentrations in patients, however, *in vitro* experiments with HIV transfected HeLa cells and PBMCs demonstrate that the duration of anti retroviral effect varies between PIs and occurs in the rank order saquinavir and nelfinavir > ritonavir > indinavir. Differences in antiviral kinetics were attributable to differences in intracellular concentrations of PI rather than intrinsic antiviral activity, suggesting that intracellular PI concentrations may determine both the potency and durability of antiretroviral effect\(^{(260)}\). Furthermore, the relationship between intracellular PI concentrations and the minimum effective concentration (MEC) in plasma has not been examined *in vivo*. The purpose of the present study was to assess the feasibility of measuring intracellular indinavir concentrations in HIV infected patients and to
determine the relationship between plasma and intracellular indinavir steady state pharmacokinetics following oral administration.

4.2 Methods

4.2.1 Study Protocol and Patient Selection

Written informed consent was obtained from all patients entering the study. After fasting overnight subjects attended the day care unit at a time 1 hour prior to the time of their first daily dose of indinavir. All patients (n=10) received dual nucleoside analogue therapy (Combivir: AZT 300mg / 3TC 150mg bd), and indinavir 800mg tds. Patients were included in the study if they had HIV plasma RNA levels below 50 copies.ml\(^{-1}\) for at least 3 months prior to the study and a CD4 count greater than 150x10\(^6\) cells/l. Exclusion criteria included abnormal liver function, inter-current illness or concomitant administration of medications likely to influence the metabolism of indinavir.

An intravenous cannula was inserted and blood was collected into lithium heparin tubes for determination of plasma and intracellular indinavir concentrations at baseline (time 0), 1, 2, 4, 6, and 8 h after oral administration of indinavir. An additional blood sample was taken at baseline for the determination of CD4 lymphocyte count and HIV plasma RNA levels. Patients received a standard breakfast 2 h post indinavir ingestion. All patients had full pharmacokinetic profiles determined (0-8 h/ tds).
4.2.2 Sample Preparation

Plasma was separated from whole blood (7ml) by centrifugation (5000 rpm at 4°C for 10 min), and stored at -70°C until analysis.

PBMCs were isolated from a duplicate blood sample (30 ml) by density gradient centrifugation upon direct layering onto Lymphoprep (GIBCO, Life Technology, Paisley U.K.) and centrifugation at 1200 rpm, 4°C for 25 min. The PBMCs were pelleted (2000rpm x 5min at 4°C) and washed 3 times with Hanks buffered salt solution (Life Technology, Paisley U.K.). Cells were finally resuspended in culture medium (RPMI plus glutamine, Life technology, Paisley U.K.) supplemented with 10% FCS (2 ml, GIBCO Life technology, Paisley U.K.). An aliquot (20 μl) was removed for cell count estimation. Cell viability and number were determined by staining with ethidium bromide/acridine orange and quantified in a Neubauer’s chamber using fluorescence microscopy. The remaining cells were immediately pelleted (2000 rpm x 5 min at 4°C). Intracellular indinavir was extracted in 60% aqueous methanol (1ml for 12 h). Following methanol extraction, cell debris was removed by centrifugation and the supernatant evaporated to dryness. Samples were stored at -70°C until analysis.

4.2.3 Indinavir Assay

Plasma samples were heat inactivated (58° C for 40 min) prior to analysis. Plasma samples and methanolic cell extracts were analysed by LC/MS using a Thermoquest Finnigan LCQ Duo bench top mass spectrometer operating in atmospheric pressure electrospray ionisation (ESI) mode. Samples were spiked with an internal standard (Ro31-9564) prior to extraction with diethyl ether (3 ml for 30 min). Following centrifugation (4000 rpm for 5 min) the organic layer was removed and evaporated to
dryness. Extracts were reconstituted in mobile phase for injection into the LC/MS system using an autosampler. Indinavir and the internal standard were eluted on a C18 column (Hypurity Elite 5; 5µm: 250 x 4.6 mm) using an ammonium acetate buffer (10 mM) / acetonitrile (30:70 v/v) mobile phase and a flow rate of 1.2 ml.min⁻¹. Indinavir (retention time 2.94 min) was analysed by fragmentation of the parent compound and quantification of the resulting fragments (monitoring of ions \( m/z \) 614.4, 465.3, 596.3). The internal standard (retention time 8.04 min) was monitored at \( m/z \) 674.4, 573.3, 388.2. The minimum quantifiable drug on column was 5 pg corresponding to lower limits of quantification of 125 pg.ml⁻¹ for plasma and 40 pg for a PBMC extract from 10x10⁶ cells. The inter-assay coefficients of variation were 6.9 % and 1.5 % at concentrations of 150 and 3000 ng.ml⁻¹ respectively. The intra-assay coefficients of variation were 4.5 % and 4.7 % respectively at the same concentrations.

4.2.4 Indinavir Assay Validation

Prior to the study, intracellular drug loss during the washing procedure was extensively studied using CEM cells in culture. CEM cells (1x10⁶/ml; 500µl) were incubated with \(^3\)H-indinavir (0.1µCi; 1 µM) for up to 18 h to allow drug accumulation. Cells were harvested by two methods, oil stop and cell washing (methodology analogous to PBMC sample separation). Cell harvesting by oil stop methodology was performed using 10% n-hexadecane/90% silica oil (400µl). Cell containing media (400 µl) was layered onto the oil and cell suspensions pelleted by centrifugation (14,000g; 30s). The bottom of the micro centrifuge tube containing the pellet was clipped off into a scintillation vial. The pellet was reconstituted (dH₂O; 100µl), solubilised (tissue solubiliser: glacial acetic acid: hydrogen peroxide; 2:2:1; 100µl) and incubated for 30min at 37°C.
Scintillation cocktail (4ml) was added prior to liquid scintillation counting for determination of intracellular drug accumulation. This is the exact methodology used in our previously published *in vitro* intracellular accumulation studies. Under oil-stop conditions, the intracellular concentration of indinavir was $0.80 \pm 0.4\mu M$ (mean $\pm$ s.d.; $n=9$). However, following cell washing ($x3$), the indinavir concentration was $0.7 \pm 0.2\mu M$ (mean $\pm$ s.d.; $n=9$) illustrating that approximately 12% of indinavir is lost during the entire washing and extraction procedure. Efflux of protease inhibitors from cells is minimized by performing cell isolation procedures at 4°C as drug loss due to either active transport or passive diffusion is reduced (>50% of PI was lost over 1h at 20°C, <10% was lost at 4°C).

### 4.2.5 Statistical Analysis

The intracellular concentration of indinavir was calculated on the basis of a single PBMC volume of 0.4 pl (determined by flow cytometric analysis) and total cell count. Plasma and intracellular indinavir concentration versus time data were evaluated by non-compartmental analysis (WinNonLin, version 3, Pharsight Corporation, CA USA) and the following variables determined: trough concentration ($C_{trough}$), maximum concentration ($C_{max}$), area under the curve over 8 h ($AUC_{0-8}$), time to peak concentration ($T_{max}$), elimination half life ($t/2$) and mean residence time (MRT). Differences between plasma and intracellular pharmacokinetic parameters were analysed by Mann Whitney or Wilcoxon matched pairs tests where appropriate (Graphpad Prism). The correlation between plasma and intracellular $AUC_{0-8}$ was subjected to non-parametric Spearman correlation analysis. Data are expressed as mean $\pm$ SEM, and differences considered significant at a value of $P<0.05$. 
4.3 Results

4.3.1 Patient Characteristics

Ten patients mean age 37 y (range: 26-51 y) were entered into the study (9 male/ 1 female). Seven patients cited homosexual contact as the mode of acquisition of HIV, 2 patients contracted the disease through infected blood products and 1 through occupational exposure. The mean duration of indinavir therapy was 2.4 y (range: 1.8-4.0 y). On chart review of the patients entering the study all had prior antiretroviral experience. The mean reduction in HIV plasma RNA levels following the original introduction of indinavir therapy was 120,000±1,500 copies.ml⁻¹, however, all patients had a viral load of less than 50 copies.ml⁻¹ of plasma (limit of detection, branch DNA test, Bayer, USA) for at least 3 months prior to commencing the study. In accordance with selection criteria the mean CD4 count was 420±40 x 10⁶ cells.l⁻¹ (range: 220-760 x10⁶ cells.l⁻¹).

4.3.2 Pharmacokinetic Results

Concentration time responses were fitted to a non-compartmental pharmacokinetic model. Following oral administration, the intracellular indinavir Cmax was lower than that of plasma (3245±702 ng.ml⁻¹ vs 10700±1260 ng.ml⁻¹; P<0.002; CI= 5207-9705; Figure 4.1A) while Tmax values were similar (1.6±0.2 h vs 1.2±0.1 h), as were mean intracellular and plasma trough concentrations (133.7±45 vs 136.8±54 ng.ml⁻¹ Figure 4.2). Overall, the intracellular AUC₀⁻₈ was 70 % lower than that of plasma (7574±1003 ng.ml.h⁻¹ vs 25060±4171 ng.ml.h⁻¹; P<0.004; CI=8704-26263; Figure 4.1B). Furthermore, there was little correlation between the intracellular and plasma AUC₀⁻₈ values (r=0.48; P=0.17; Figure 4.3). Interestingly the intracellular elimination half-life of indinavir was longer than that of plasma (2.0±0.3 h; vs 1.2±0.09 h;
P<0.05), as was the mean residence time of indinavir within the cell (3.6±0.6 h vs 2.1±0.14 h; P<0.01).

4.3.3 Individual Pharmacokinetic Profiles: Relationship of Trough Concentrations to the Plasma MEC (Figures 4.2, 4.4; Table 4.1)

Marked inter-individual variability in the plasma pharmacokinetics of PIs is well recognised and the Liverpool HIV pharmacology group have reported that up to 40% of patients may demonstrate trough concentrations below the plasma MEC for indinavir. In the present study we also note considerable individual variability in intracellular trough concentrations of the drug, (Figure 4.2) which appear to correlate poorly with plasma concentrations (r=0.38; P=0.28). Interestingly for some individuals (Figure 4.4 A,B) intracellular concentrations of indinavir were below the plasma MEC of 100 ng.ml\(^{-1}\) towards the end of the dosing interval despite having adequate plasma levels. For these patients, the intracellular t\(_{1/2}\) was shorter than that of plasma (0.7 and 0.8 h vs 1.7 and 1.1 h). In contrast, other patients (Figure 4.4 C,D) receiving the same combination therapy demonstrated intracellular indinavir concentrations above the plasma MEC throughout the dosing interval despite having plasma levels below 100 ng.ml\(^{-1}\). For these patients the intracellular t\(_{1/2}\) was prolonged (3.1 h and 2.1 h) compared with plasma (1.6 h and 1.4 h).
Figure 4.1
Indinavir plasma and intracellular concentration- time profiles and B) area under the curve over 8 h (AUC_{0-8}) following oral administration in 10 HIV-1 infected patients. The minimum effective concentration (MEC) for indinavir (100 ng.ml^{-1}) in plasma is indicated for comparison. Data are presented as mean ± SEM ; * P<0.004.
Figure 4.2

Individual variability in plasma and intracellular trough concentrations ($C_{\text{trough}}$) for indinavir. The short horizontal lines indicate mean values and the short dashed lines show the accepted plasma MEC for indinavir (100 ng.ml$^{-1}$).
Figure 4.3

The relationship between intracellular and plasma AUC over 8 h for 10 individual patients following oral administration of indinavir, note the weak correlation (solid line) and 95% CI (dashed lines). Data were subjected to non-parametric Spearman correlation analysis.
Figure 4.4

Individual plasma and intracellular concentration-time profiles for 4 patients following oral administration of indinavir. Dose regimens are as indicated on the graphs. The minimum effective concentration (MEC) for indinavir (100 ng.ml\(^{-1}\)) in plasma is included for comparison.
Table 4.1

A comparison of plasma and intracellular pharmacokinetics following oral administration of indinavir to patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Plasma</th>
<th>Intracellular</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>AUC₀⁻⁸ (ng.ml⁻¹.h⁻¹)</td>
<td>C_max (ng.ml⁻¹)</td>
</tr>
<tr>
<td>A</td>
<td>16403</td>
<td>9704</td>
</tr>
<tr>
<td>B</td>
<td>25205</td>
<td>8978</td>
</tr>
<tr>
<td>C</td>
<td>5860</td>
<td>4526</td>
</tr>
<tr>
<td>D</td>
<td>15321</td>
<td>9354</td>
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<tr>
<td>E</td>
<td>34652</td>
<td>12576</td>
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<td>F</td>
<td>47326</td>
<td>12053</td>
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<td>G</td>
<td>13440</td>
<td>5544</td>
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<td>H</td>
<td>42245</td>
<td>15913</td>
</tr>
<tr>
<td>I</td>
<td>22941</td>
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<td>J</td>
<td>27177</td>
<td>16983</td>
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**Abbreviations:**
- AUC₀⁻⁸: area under the curve over 8 h
- C_max: maximum concentration
- C_trough: trough concentration
- MRT: mean residence time
- t₁/₂: elimination half-life.
4.4 Discussion

The main aims of this study were to assess the feasibility of measuring intracellular indinavir concentrations in HIV-1 infected patients and compare intracellular and plasma pharmacokinetics. The goal of achieving therapeutic plasma PI concentrations may be confounded by the presence of viral resistance. To minimize the potential for drug failure due to emerging viral resistance we selected patients with a sustained viral load (VL) of less than 50 cpm since recent consensus guidelines\(^{(293)}\) recognize that the lower the nadir of plasma HIV-1 RNA levels the longer it takes for drug failure to occur\(^{(294)}\). In addition, clonal selection for drug resistant virus implies that protease inhibitor plasma MEC values are unlikely to take account of patients who are highly ART experienced, on complex drug regimens or at differing stages of the disease. Given the small number of patients in this study we made no attempt to link intracellular indinavir concentrations with long term virological response, since to do so would require a much larger patient cohort and some measure of phenotypic testing. The main findings of the present study are that there is reduced intracellular accumulation of indinavir relative to plasma with intracellular indinavir \(AUC_{0-8}\) being 70\% lower than plasma \(AUC_{0-8}\). However, intracellular elimination half-life was significantly prolonged compared with plasma, as was the MRT of indinavir within the cellular compartment. There was no difference in time to peak concentration between the groups, a finding that may have been influenced by the sampling schedule used in this study. Overall there was a poor correlation between plasma and intracellular AUC values.

This is the first study to assess intracellular PI concentrations \textit{in vivo} and is in agreement with the results of Jones \textit{et al.}\(^{(212)}\) and others\(^{(213)}\), who in experiments on cell lines found that indinavir had the least intracellular accumulation (1-4 fold) compared with nelfinavir and saquinavir (>30 fold) and ritonavir (6-10 fold). In addition, chronically HIV infected
cells and the presence of alpha 1 acid glycoprotein caused reduced accumulation of all the PIs studied.

Indinavir plasma protein binding in HIV-1 infected patients has recently been reported as approximately 60%. By pharmacologic theory, only the free fraction (0.4) is available to cross the membrane, thus if indinavir crosses by passive diffusion only, and all intracellular drug is free, then the expected intracellular ratio would be 0.4. We found the intracellular: plasma ratio to be 0.31. Given that we estimate up to 12% of the drug may efflux from the cells during the entire analytical procedure there is almost complete agreement between results. Therefore the results we have obtained for intracellular indinavir concentrations levels confirm what would be expected based on the degree of protein binding in plasma lending weight to our methodology and adding to our understanding of the disposition of this commonly used PI.

Furthermore, Nascimbeni\(^{260}\) found that the half life for antiviral activity in PBMCs in vitro was 1-2 h for indinavir compared with 3 h for ritonavir and 8 h for nelfinavir and saquinavir, data which is compatible with the intracellular elimination half life of 2.0 h for indinavir seen in the present study. Of note restoration of viral infectivity following removal of PI from the extracellular culture medium occurred in the rank order indinavir >ritonavir >saquinavir and nelfinavir. These data suggest that intracellular PI concentrations may influence both the potency and durability of antiretroviral effect\(^{260}\). Interestingly, we found that some patients demonstrate intracellular indinavir concentrations above the plasma MEC throughout the dosing period despite the presence of sub-therapeutic plasma levels of the drug. It is likely that the prolonged intracellular half life and mean residence time of indinavir within the cell contributed to this effect which may partly explain why some patients maintain adequate viral suppression despite poor compliance and dose omission. In contrast those patients who had intracellular
indinavir concentrations below the plasma MEC but acceptable plasma levels of the drug (N=2) both had shorter intracellular half-life relative to plasma. For indinavir the relationship between plasma concentrations of the drug and viral load suppression is established although there is no generally accepted therapeutic range\(^{(295)}\). Our results suggest that the relationship between intracellular drug concentrations and the plasma MEC for indinavir may also be important and warrants further study. Presently, no data is available regarding intracellular minimum effective concentrations of indinavir, or the extent of intracellular protein binding.

Lastly, we found only a weak correlation between plasma and intracellular indinavir AUC\(_{0-8}\) concentrations (r =0.48), suggesting that one cannot necessarily be used as a surrogate marker for the other.

Although plasma protein binding alone may account for lower intracellular concentrations of indinavir, other contributory factors may include intracellular protein binding and altered drug influx/efflux mechanisms including P-glycoprotein mediated drug efflux\(^{(207,252,262)}\). Indinavir is a P-glycoprotein substrate and thus may be actively transported out of the cell by this mechanism however it is not a significant inhibitor of P-glycoprotein mediated drug efflux\(^{(207,210,296)}\) a finding which may partly explain the reduced intracellular accumulation of indinavir (relative to plasma) seen in this study. In contrast other PIs such as ritonavir are recognised as potent inhibitors of P-glycoprotein function and their intracellular accumulation may be modulated through this mechanism. As such, further investigation of intracellular concentrations of PIs known to inhibit P-glycoprotein function (nelfinavir, saquinavir) is warranted, as is the measurement of cell associated indinavir concentrations in the presence of ritonavir.

In relation to interpreting the data from the present study there are several caveats. Firstly, although we have used the term “intracellular” it would be more accurate to state
cell associated" indinavir since we do not actually know if the entire drug is contained within the cytoplasmic compartment. Secondly, in the pharmacokinetic calculations an estimated cell volume of 0.4 pl (based upon flow cytometric analysis) is used. Thirdly, we have minimised drug efflux from cells by processing the samples at 4°C (slowing passive diffusion and active transport) and streamlined the cell isolation procedure by reducing delays in processing. Nevertheless, we estimate that up to a maximum of 12% of drug may efflux from the cell during the entire procedure, this should be considered when interpreting the data but is unlikely to alter our conclusion that cell associated indinavir concentrations are significantly lower than plasma levels.

In conclusion, indinavir concentrations within lymphocytes of HIV infected patients are lower than in plasma. Despite this a prolonged intracellular half-life may contribute to antiretroviral efficacy and help to maintain indinavir concentrations within the cellular compartment above the plasma MEC for indinavir throughout the dosing period. Recently published work highlights differences between protease inhibitors in terms of both the potential for intracellular accumulation\textsuperscript{(212,213)} and, the durability of antiretroviral efficacy \textit{in vitro}\textsuperscript{(260)}, we recommend that further studies are required to clarify both intracellular pharmacokinetics and the degree of intracellular accumulation of other protease inhibitors such as nelfinavir and saquinavir in a HIV infected patient population. Such information might be expected to improve our understanding of the reasons for therapeutic success or failure for certain patients.
Chapter 5

Intracellular Accumulation of Nelfinavir and its Relationship to P-glycoprotein Expression and Function in HIV Infected Patients
5.1 Introduction

Despite the success of HAART in reducing the morbidity and mortality associated with HIV disease, the ideal antiretroviral regimen remains elusive. Critically, potency and durability remain unresolved issues. In the Swiss Cohort Study rebound viraemia to detectable levels occurred at a rate of 20% per year in ART experienced patients switching therapy, while some 50% of patients required treatment changes by 24 months\(^{(201)}\). In addition, a significant number of patients require salvage therapy with mega-A RT, risking exposure to toxicity and serious drug interactions\(^{(297)}\).

The pharmacokinetic variability of PIs is well documented; with several studies demonstrating marked inter-patient variability following standard dosing regimens\(^{(273,292)}\). Despite this, plasma PI concentrations correlate with antiviral effect\(^{(298-300)}\). In the ADAM study both plasma saquinavir and nelfinavir concentrations were strongly associated with the initial rate of HIV clearance\(^{(301)}\). In clinical practice we endeavour to achieve high plasma PI concentrations throughout the dosing interval in an attempt to ensure maximal suppression of viral replication and to prevent the emergence of viral drug resistance\(^{(278,279,281)}\). Failure to achieve this goal, particularly within cellular compartments creates de facto mono or dual therapy, increasing the potential for resistant virus to seed via plasma\(^{(287)}\). Despite efforts, viral mutations reducing drug susceptibility have been demonstrated for all available PIs and are associated with treatment failure\(^{(302,303)}\). Drug resistance is not the sole contributor to antiretroviral frailty, other pharmacological factors such as intracellular drug concentrations and drug influx/efflux mechanisms such as P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) also determine the eventual disposition of PI within the cell, and therefore may have a critical role modulating therapeutic failure or success\(^{(207,210,252,262)}\).
Lymphocytes are a major site of HIV replication\(^2\), and although it is not certain that PIs must act intracellularly to be effective, nevertheless intracellular drug concentrations are likely to influence antiretroviral efficacy\(^2\). Marked differences in intracellular pharmacokinetics between PIs \textit{in vitro} have been reported, and may account for differences in antiviral kinetics over and above intrinsic antiviral activity\(^2\). \textit{In vitro} studies demonstrate intracellular accumulation occurs in the rank order nelfinavir > saquinavir / ritonavir > indinavir\(^2\). Conversely, restoration of viral infectivity following removal of drug from the culture medium occurs in reverse order\(^2\), suggesting intracellular PI concentrations may determine both the potency and durability of antiretroviral effect. Our previous work demonstrated a lack of intracellular accumulation of indinavir within PBMCs of HIV infected patients, and that intracellular values were consistent only with indinavir free drug concentrations in plasma\(^3\), though variability between PIs means such results cannot be easily extrapolated to other drugs within the class.

PIs are both substrates for and variable inhibitors of the multidrug transporter P-gp\(^1\). This raises the possibility that differential accumulation intracellularly may be associated with P-gp inhibitory potential. \textit{In vivo} studies examining this possibility are few and results confusing, being confounded by the inclusion of patients receiving multiple PIs and at various stages of disease. Thus to date, poor intracellular accumulation has been associated with both an absence of MDR-1 and P-gp over-expression\(^3\). In this study we investigate the totality of plasma and intracellular PI concentrations plus P-gp expression and function within a group of HIV infected patients fully suppressed (less than 50 copies/ml) on chronic single PI therapy with nelfinavir. Given the potential exists to alter intracellular accumulation of PIs through the use of P-gp inhibitors\(^4\),
we also assessed the capacity for ritonavir added ex vivo to further modulate P-gp function. This work highlights potential differences between PIs with respect to intracellular accumulation.

5.2 Methods

5.2.1 Study Protocol and Patient Selection.

Written informed consent was obtained from patients entering the study. Following an overnight fast, subjects attended the day care unit 1 hour prior to the time of their first daily dose of nelfinavir. All patients (n=12) received nelfinavir 1250mg bd and dual nucleoside analogue therapy (Combivir: AZT 300mg / 3TC 150mg bd) for at least 3 months prior to study. Patients were included in the study if they had HIV plasma RNA levels below 50 copies/ml for at least 3 months prior to the study and a CD4 count greater than 150x10^6 cells/l. Exclusion criteria included abnormal liver function, intercurrent illness or concomitant administration of medications likely to influence the metabolism of nelfinavir or interfere with P-gp function. The institutional ethics committee approved the study.

Blood samples were obtained for determination of plasma and intracellular nelfinavir concentrations at baseline (time 0), 1, 2, 4, 6, 8 and 12 h after oral administration of nelfinavir. Additional blood samples were taken at baseline for the determination of CD4 lymphocyte count, HIV plasma RNA levels and P-glycoprotein function. Patients received a standard breakfast with supervised ingestion of their medication. Pharmacokinetic profiles were determined over a 12 hr period. The patients entered into the study were followed with respect to CD4 counts and viral load estimation for at least 9 months after the study.
5.2.2 Sample Preparation

Plasma was separated from whole blood (7ml) by centrifugation (5000rpm at 4°C for 10 min), and stored at –80°C until analysis. PBMCs were isolated from a duplicate blood sample (30ml) by density gradient centrifugation upon direct layering onto Lymphoprep (Gibco Life Technologies, UK) and centrifugation at 1200 rpm, 4°C for 25 min. The cells were pelleted (2000rpm x 5min at 4°C) and washed with Hanks buffered salt solution (Gibco Life Technologies, UK) prior to resuspension in RPMI (plus glutamine) supplemented with 10% foetal calf serum (FCS, Gibco Life Technologies, UK). Cell count and viability were quantitated following staining with ethidium bromide/acridine orange. For the determination of intracellular nelfinavir levels, all samples were promptly processed at 4°C, with less than 1hr between phlebotomy and methanol extraction. In brief, PBMCs were immediately pelleted (2000 rpm x 5 min at 4°C) and intracellular nelfinavir extracted in 60% aqueous methanol (1ml for 12 h). Cell debris was removed by centrifugation and the supernatant evaporated to dryness. Samples were stored at –80°C until analysis.

5.2.3 Nelfinavir Assay

Plasma samples were heat inactivated (58°C for 40min) prior to analysis. Plasma samples and methanolic cell extracts were analysed by liquid chromatography mass spectrometry (LC/MS) using a Thermoquest Finnigan LCQ Duo bench top mass spectrometer operating in atmospheric pressure electrospray ionisation (ESI) mode. Samples were spiked with an internal standard (Ro31-9564; 20µl, 100 ng/ml) prior to extraction with diethyl ether (3ml for 30 min). Following centrifugation (4000 rpm for 5 min) the organic layer was removed and evaporated to dryness. Extracts were reconstituted in mobile phase for injection into the LC/MS system using an autosampler. Nelfinavir and
the internal standard were eluted on a C18 column (Hypurity Elite 5; 5μm: 250 x 4.6 mm), protected by a pre-column guard (Si 60, 5μm), using an ammonium formate buffer (20 mM) / acetonitrile (30:70 v/v) mobile phase, and a flow rate of 1.2 ml/min. Nelfinavir (retention time 6.57 min) was analysed by fragmentation of the parent compound and quantification of the resulting fragments (monitoring of ions m/z 568.3/467.2/330). The internal standard (retention time 8.67 min) was monitored at m/z 674.4/573.3/388.2. The minimum quantifiable drug on column was 5pg corresponding to a lower limit of quantification of 125 pg/ml for plasma and 3.1 ng/ml for a PBMC extract from 10x10⁶ cells. The inter-assay coefficients of variation were 9 % and 11 % at concentrations of 0.15 and 3 ng/ml, respectively. The intra-assay coefficients of variation were 4.5 % and 4.7 % respectively at the same concentrations. The data were recorded and quantified by Xcalibur software (version 1.0), programmed to recognise specific peaks and to quantify the intensity of the ion signal. Nelfinavir content was determined in the experimental samples from a standard curve using peak area: internal standard ratios.

5.2.4 P-Glycoprotein Expression

P-glycoprotein was detected by flow cytometry and MDR1 gene expression confirmed by RT-PCR. Cells were fixed in 2% paraformaldehyde (Cellfix, Becton Dickinson Mountain View C.A), permeabilised with 0.05% saponin (Sigma) and labelled with a JSB1 monoclonal antibody (Sanbio, Uden, Netherlands) directed towards a highly conserved intracellular epitope of P-glycoprotein (30 min, at 37°C). JSB1 has been widely validated as a MDR1 P-gp specific antibody. A monoclonal IgG derived from a murine hybridoma supernatant (ATCC, Manasses V.A.) HB179, served as a negative fluorescence control and antibody to the cytoskeletal component vimentin (Dako AS Glostrup Denmark) was employed as a control for the
permeabilisation technique. Excess primary antibody was removed by washing with 0.05% saponin, prior to incubation with a rabbit anti-mouse fluorescein labelled isothiocyanate (FITC)-conjugated F(ab)$_2$ (1:50 dilution; Dako Glostrup AS) for 30 min, at 37°C. Finally, cells were washed free of excess antibody and re-suspended in 0.5ml of paraformaldehyde prior to flow cytometric analysis.

### 5.2.5 P-Glycoprotein Mediated Drug Efflux Function and Inhibition.

PBMCs ($1\times10^6$ cells) reserved at baseline (T0) were loaded with rhodamine 123 (1.25 µg/ml; Sigma Aldrich, Poole, Dorset, UK) for 25 min at 37°C in RPMI 1640 supplemented with 10% FCS. Cells were washed twice in ice-cold media and incubated at 37°C for 3h in 3 ml of dye free media to allow dye efflux. At baseline (T=0: maximum loading) and three hours later (T=180min, maximum efflux) an aliquot was removed and washed twice in ice-cold media (3 min x 2000rpm) before being fixed in ice-cold paraformaldehyde (Cellfix, Becton Dickinson, UK). Cellular fluorescence was determined by flow cytometric analysis. Parallel experiments were performed in the presence of ritonavir (5 µM), a recognised P-glycoprotein inhibitor. This concentration of ritonavir was chosen for maximal inhibition from dose response curves for the inhibition of P-glycoprotein mediated rhodamine efflux in the PBMCs of healthy volunteers (data not shown).
5.2.6 Flow Cytometry

Lymphocytes shown in forward scatter and side scatter were electronically gated and acquired (10000 events) through the FL1 channel (expression) and the FL2 channel (function). The amount of fluorescence was plotted as a histogram of either FL1 (expression) or FL2 (function) staining within the gate. Data acquisition was performed using Cellquest software (WINMDI version 2.6) to determine median fluorescence intensity values. The presence of MDR-1 mRNA in each sample was confirmed by non-quantitative RT-PCR as previously described\(^{315}\).

5.2.7 Data Analysis

The intracellular concentration of nelfinavir was calculated on the basis of a single PBMC volume of 0.4 pl (determined by flow cytometric analysis and Furman et al. 1986\(^{316}\)) and total cell count. Plasma and intracellular nelfinavir concentration versus time data were evaluated by non-compartmental analysis (WinNonLin, version 3, Pharsight Corporation, CA USA) and the following variables derived: area under the curve over 12 h (AUC\(_{0-12}\)), concentration at time zero (C\(_0\)), time to peak concentration (T\(_{\text{Max}}\)), maximum concentration (C\(_{\text{Max}}\)), minimum concentration (C\(_{\text{Min}}\)), elimination half-life (t\(_{1/2}\)) and mean residence time (MRT). P-gp function was expressed as the ratio of:

\[
\frac{(T0 - T180)}{T0} = \frac{(\text{maximal.loading} - \text{maximal.efflux})}{\text{maximal.loading}}
\]

The percentage of reversible P-gp mediated rhodamine efflux in the absence or presence of ritonavir was calculated as:

\[
100x\left(\frac{T0 - T180 \pm \text{rit}}{T0}\right) = 100x\left(\frac{\text{maximal.loading} - \text{maximal.efflux} \pm \text{rit}}{\text{maximal.loading}}\right)
\]
5.2.8 Statistical Analysis

Data were subjected to non-parametric analysis and analysed by Mann Whitney or Wilcoxon signed rank tests where appropriate. The associations between plasma and intracellular AUC$_{0-12}$, and cellular accumulation of nelfinavir and P-gp function were subjected to Spearman correlation analysis. Data are expressed as mean±SEM, and a value of P<0.05 was taken to indicate significance.

5.3 Results

5.3.1 Patient Characteristics

Twelve patients all male, mean age 40±2.82 y (range: 28-59 y) were entered into the study. Seven patients cited homosexual contact as the mode of acquisition of HIV, 4 patients contracted the disease through injection drug use, and 1 patient though heterosexual contact. The mean duration of nelfinavir therapy was 3.25 y (range: 2.1-5.0 y). On chart review of the patients entering the study all had prior antiretroviral experience. All patients had a viral load of less than 50 copies/ml of plasma (limit of detection, branch DNA test, Bayer, USA) for at least 3 months prior to commencing the study, and remained suppressed during follow-up. In accordance with selection criteria the mean CD4 count was 477±78 x 10$^6$ cells/l.

5.3.2 Pharmacokinetic Analysis (Table 5.1)

Concentration time responses were subjected to non-compartmental pharmacokinetic modelling (Figure 5.1A). Overall the intracellular nelfinavir AUC$_{0-12}$ was ~9 fold higher than that of plasma (264200 ± 63420 vs 29250 ± 6629 ng/ml/h; CI: 102338-363726; P<0.001; Figure 5.1B), Intracellular C$_{\text{Min}}$ and C$_0$ values for nelfinavir were approximately 5-6 fold higher than that of plasma (C$_{\text{Min}}$: 5712 ± 2156 vs 1062 ± 357 ng/ml; CI 589-
Following oral administration, the intracellular nelfinavir $C_{\text{Max}}$ was 15 fold higher than that of plasma ($59420 \pm 13940$ vs $3986 \pm 822$ ng/ml; CI: 25882-84990; $P<0.0005$). However, plasma and intracellular $T_{\text{Max}}$ values were similar ($3.33 \pm 0.70$ vs $2.83 \pm 0.5$ hr; CI: $-0.756-1.756$). Furthermore, there was no difference in plasma and intracellular MRT or elimination half-life values ($t^{1/2}$: $4.44 \pm 0.57$ vs $4.41 \pm 0.79$ hr; CI: $-1.891-1.394$; MRT: $7.41 \pm 0.69$ vs $7.38 \pm 1.17$h). Interestingly, there was a correlation between the plasma and intracellular AUC$_{0-12}$ for nelfinavir ($r=0.75$; $P=0.011$; Figure 5.3).

### 5.3.3 P-glycoprotein Expression and Function

P-gp expression was detected and confirmed in all samples. In patients chronically treated with nelfinavir, mean P-gp expression was $8.85 \pm 1.3$ MFI, (range: 3.2-14.5 MFI) there was no correlation between P–gp expression and either intracellular AUC$_{0-12}$, ($r=-0.35$; $P=0.29$), or intracellular $C_0$ values. Basal P-gp mediated rhodamine efflux was $61.0 \pm 4.2\%$ (CI: 51.7-70.3; range: 15-57%). In the presence of ritonavir (5μM added ex vivo) cellular rhodamine efflux decreased to $25.6 \pm 5.5\%$ ($P=0.001$; Figure 5.4A) representing an additional reversible efflux potential of $56.1 \pm 9.78\%$. Interestingly, there was a correlation between intracellular nelfinavir concentrations and P-gp function at baseline (Figure 5.4B; $r=0.59$; $P<0.05$), such that higher the intracellular concentration of nelfinavir the greater the activity of the efflux pump, conversely, lower nelfinavir concentrations were associated with reduced P-gp activity.
Figure 5.1

A) Nelfinavir, plasma and intracellular concentration-time profiles. The minimum effective concentration (MEC) for nelfinavir (400 ng/ml) is indicated for comparison. B) Intracellular compared with plasma area under the curve over 12 h (AUC_{0-12}) following oral administration of nelfinavir in 11 HIV infected patients. Statistical analysis was performed using a Wilcoxon signed rank test. Data are presented as mean±SEM. *P<0.001 compared with plasma area under the curve (AUC_{0-12}).
Figure 5.2

Individual variability in plasma and intracellular minimum concentrations ($C_{Min}$) for nelfinavir. The short horizontal lines indicate mean values and the short dashed lines show the accepted plasma MEC for nelfinavir (400 ng/ml). * P<0.005.
Figure 5.3
Correlation between (Log$_{10}$ transformed) plasma and intracellular area under the curve over 12 h (AUC$_{0-12}$) for 11 individual patients following oral administration of nelfinavir. Data were subjected to non-parametric Spearman correlation analysis.
Figure 5.4

P-glycoprotein function in patients treated with nelfinavir: (A) effect of nelfinavir on p-glycoprotein mediated rhodamine-123 efflux in the absence and presence of ritonavir (5µM) in PBMCs from 11 HIV infected patients following oral administration of nelfinavir, (B) correlation between intracellular accumulation of nelfinavir and P-glycoprotein function (rhodamine efflux). Data were subjected to non-parametric Spearman correlation analysis and Wilcoxon signed rank test where appropriate. * P<0.001.
Table 5.1 Summary of Nelfinavir Pharmacokinetic Parameters

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Intracellular</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AUC_{0-12} (ng/ml/h)</strong></td>
<td>29250±6629</td>
<td>264200±63420*</td>
<td>9.0</td>
</tr>
<tr>
<td><strong>C_{Max} (ng/ml)</strong></td>
<td>3986±822</td>
<td>59420±13940*</td>
<td>14.9</td>
</tr>
<tr>
<td><strong>C_{Min} (ng/ml)</strong></td>
<td>1062±357</td>
<td>5712±2156*</td>
<td>5.4</td>
</tr>
<tr>
<td><strong>C_{0} (ng/ml)</strong></td>
<td>2553±539</td>
<td>15860±3662*</td>
<td>6.2</td>
</tr>
<tr>
<td><strong>T_{Max} (h)</strong></td>
<td>2.83±0.5</td>
<td>3.33±0.7</td>
<td>NA</td>
</tr>
<tr>
<td><strong>t%2 (h)</strong></td>
<td>4.44±0.57</td>
<td>4.41±0.79</td>
<td>NA</td>
</tr>
<tr>
<td><strong>MRT (h)</strong></td>
<td>7.41±0.69</td>
<td>7.38±1.17</td>
<td>NA</td>
</tr>
</tbody>
</table>

AUC_{0-12}: area under the concentration time curve over 12 h, C_{Max}: maximum concentration, C_{Min}: minimum concentration, C_{0}: concentration at baseline, T_{Max}: time to maximum concentration, t\%2: elimination half-life and MRT: mean residence time. NA: not applicable. Data are presented as mean±SEM. * P<0.05
5.4 Discussion

In contrast to our previous findings, the main findings of the present study are that there is significant intracellular accumulation (>8 fold) of nelfinavir in the lymphocytes of HIV infected patients relative to plasma. Furthermore, there is an excellent correlation between plasma and intracellular nelfinavir concentrations suggesting plasma levels are an acceptable indicator of cell-associated drug. Despite chronic treatment with nelfinavir, 60% of P-gp mediated dye efflux continued unhindered, with ritonavir (added ex vivo to avoid the confounding effect of combined treatment on baseline P-gp function) producing 56% additional inhibition. Given the baseline degree of accumulation, the relative clinical value of this for patients already suppressed on therapy is questionable and should be balanced against improved bioavailability as a result of CYP3A4 inhibition at the level of the gut. Interestingly, no relationship was demonstrated between P-gp expression and intracellular accumulation, but a positive correlation was found between P-gp function and intracellular nelfinavir concentrations.

The intracellular accumulation of nelfinavir noted in this study is in excess of free drug available to the cell over a dosing interval (<1% of total), suggesting the effect occurs chronically; a finding supported by the 6 fold higher intracellular C₀ values compared with plasma. The lack of differences between plasma and intracellular values for elimination half-life, MRT and T_max indicate accumulation plateaus over time. The ultimate disposition of nelfinavir within the cell remains unknown; however, it’s highly lipophilic nature would facilitate extensive binding to cellular proteins and organelles and may be principally responsible for the observed effect. Indeed, Khoo et al. detected other PIs in all subcellular fractions (cytosolic, nuclear, and mitochondrial).

Drug accumulation within the cell represents an imbalance between influx and efflux pathways, the latter being partly dependent on P-gp function. Interestingly, we found this
increased with increasing intracellular concentrations of nelfinavir, thus demonstrating P-gp activity is primarily substrate concentration dependent. This is consistent with data showing P-gp mediated efflux of Hoechst 33342 is proportional to its intracellular concentration\(^{(8)}\). The success of P-gp in lowering intracellular concentrations of a MDR drug relies on its capacity to overcome the passive influx rate of each substrate\(^{(100)}\), and the tendency of that substrate to modulate its function. Nelfinavir is both a substrate and moderate inhibitor of P-gp\(^{(207,210,211,266,306,307)}\). How do we reconcile the seemingly paradoxical observations of enhanced accumulation with enhanced P-gp pump function? Interestingly, Eytan has proposed a novel mechanism whereby, for P-gp chemosensitisers, the rate of membrane equilibration is so rapid that export out of the cell via P-gp cannot keep pace\(^{(100)}\). In essence, the transporter operates in a futile cycle so that while transport turnover is high there is no net reduction in substrate concentration. Fluorescence quenching experiments demonstrate this hypothesis holds for progesterone, which flip-flops so rapidly across the plasma membrane that no net efflux is detectable, although transport by MDR cells does occur\(^{(100)}\). It is possible this mechanism, in combination with intracellular protein binding and partitioning of drug within the plasma membrane\(^{(100)}\) accounts for both the degree of accumulation seen and the increased P-gp pump activity as assessed by rhodamine efflux. Although logic dictates that an enhanced intracellular accumulation should ultimately translate into increased free (pharmacologically active) drug within the cell, this is as yet unproven, but may be critical in determining antiretroviral effect for certain individuals.

We found no correlation between P-gp expression and intracellular accumulation of nelfinavir, suggesting for nelfinavir, the relationship between expression and function is complex. This is consistent with a previous study showing St Johns Wort increases P-gp mediated rhodamine efflux by 50% in the presence of a greater than 4 fold increase in P-
gp expression\(^{(315)}\). Intriguingly, the orphan nuclear receptor, SXR, plays a central role in regulating MDR-1 gene transcription\(^{(145,319)}\). Ritonavir binds to SXR activating its target genes and increases expression of P-gp. However, other PIs such as saquinavir are much weaker in this regard and still others, such as nelfinavir and indinavir completely fail to activate SXR\(^{(319)}\), and therefore are unlikely to up-regulate P-gp expression. This does not necessarily preclude effects on P-gp function, and may explain why Meaden et al. found a correlation between increased P-gp expression and intracellular accumulation of ritonavir but not saquinavir\(^{(308)}\) and, why we found no correlation between P-gp expression and nelfinavir accumulation within the cell.

Conventional pharmacokinetic modelling makes no allowance for factors such as P-gp drug efflux or number/affinity of binding sites, and hence may be inadequate to describe intracellular pharmacokinetics. Jang et al.\(^{(320)}\), have developed a computational model to assess the impact of these factors on intracellular drug accumulation. Their data show the most important determinant of intracellular drug levels remains the extracellular drug concentration. However, simultaneous changes in more than one parameter may alter the relative importance of other factors. Given the variability between PIs with respect to intracellular accumulation, it would be interesting to test the utility of this model for this class of drug.

We have extensively discussed the methodology used in this study, its limitations and the possibility that intracellular drug concentrations could change during processing in our previous paper investigating intracellular levels of indinavir\(^{(305)}\). Loss during processing may underestimate intracellular levels by up to 12%. This is unlikely to detract from the main findings of the study.

We interpreted plasma and intracellular nelfinavir concentrations in the context of expression and function of the multidrug transporter P-gp, in order to clarify how some
of the pharmacological factors that influence treatment failure or success integrate in a clinical setting. Only patients fully suppressed on nelfinavir were included because viral suppression to less than 50 copies/ml limits the potential for emergent drug resistance, therefore, reducing this as a confounding variable and allowing direct comparison with our previous data\(^{(305)}\). Together these studies highlight differences between PIs with respect to intracellular accumulation and complexity of the relationship between P-gp expression and function. Furthermore, our data suggest interactions with the multidrug transporter are remarkably PI specific. Further studies are warranted to assess similar variables in patients who are failing therapy and those with documented viral drug resistance.
Chapter 6

The Role of P-glycoprotein Expression and Function in HIV Disease
6.1 Introduction

Multi drug resistance (MDR) proteins such as P-glycoprotein have been shown to lower intracellular concentrations of antiretroviral drugs particularly the protease inhibitors. In patients with HIV disease, possession of an MDR phenotype characterised by enhanced transporter mediated efflux of antiretroviral drugs has almost invariably been linked with the development of cellular drug resistance and a reduction in the therapeutic efficacy of HAART\(^{(213,228)}\). Several studies have investigated the role of P-gp in resistance to antiretroviral therapy. Gollapudi and Gupta\(^{(203)}\) demonstrated that both T and monocytic cell lines expressed increased levels of P-gp following HIV infection, resulting in a significant decrease in zidovudine (ZDV) accumulation, while Antonelli \textit{et al.}\(^{(215)}\) showed that P-gp expressing cells are less susceptible to the effects of ZDV when compared with control cells. This effect could be reversed in the presence of P-gp specific inhibitors. Oral and intravenous administration of HIV protease inhibitors (Pis) to \textit{mdr1a} knockout mice results in marked elevations of both plasma and brain drug concentrations compared with control mice, suggesting that P-gp also limits systemic bioavailability and brain penetration of the Pis\(^{(158)}\). This may result in \textit{de facto} dual or mono antiretroviral drug concentrations in certain areas with the development of reservoirs of fully viable, replication competent but partially resistant HIV-1 despite seemingly adequate antiretroviral drug therapy\(^{(321-323)}\). \textit{In vitro} most of the currently available Pis are substrates and to a variable extent inhibitors of P-gp mediated efflux\(^{(207,209,210,216)}\). Expression of P-gp in lymphocytes\(^{(108,176,254)}\) suggest it modulates intracellular accumulation of Pis\(^{(216,324)}\). Therefore P-gp may decrease the effectiveness of Pis by decreasing plasma and possibly intracellular drug concentrations. However, the potential exists to reverse this process through the addition of P-gp inhibitors such as verapamil, cyclosporin, and RTV to standard therapy, enhancing intracellular and brain penetration.
of the PIs, and reducing the emergence of viral drug resistance. For clinicians much emphasis has been placed on overcoming P-gp mediated drug efflux activity through pharmacoenhancement with low dose (100mg bd) ritonavir (RTV). RTV inhibits gut wall CYP3A4 and P-gp the key enzyme and transport systems that limit the bioavailability or metabolism of other PIs. It can therefore be used to boost and maintain PI plasma concentrations of other PIs such as IDV, amprenavir (APV), SQV, and to a lesser extent NFV\(^{325}\). Boosted regimens are considered by many to represent the standard of use of PIs, particularly in the presence of viral rebound after a PI sparing regimen, or in salvage of PI-experienced patients failing therapy. The finding that human lymphocytes express non-functional CYP3A\(^{326}\), in conjunction with the data of Garaffo \textit{et al.}\(^{310}\) support the concept that RTV most likely enhances PI concentration in PBMCs through inhibition of P-gp mediated drug efflux activity. The clinical relevance of pharmacoenhancement in altering long-term outcome remains unconfirmed.

Bearing in mind that P-gp is a molecule highly conserved throughout evolution, a fact which points to an indispensable physiological role involving the protection of vital organs through regulation of cellular and tissue levels of noxious compounds\(^{80}\). It is not surprising that the complex interactions between P-gp, HIV infection and its treatment remain unclear. Published studies lack consensus, with some groups reporting a decrease in P-gp expression in lymphocytes of HIV-infected patients\(^{177,205,327}\). Additionally, Lucia\(^{327}\) demonstrated decreased P-gp mediated Rhodamine efflux and altered NK cell cytotoxicity suggesting that functional P-gp is required for normal NK cell function. In contrast, Andreana \textit{et al.}\(^{204}\) showed an increase in CD4\(^+\) P-gp expression in T cells of HIV infected patients compared with controls; however, the detected P-gp was functionally defective. More recently Speck \textit{et al.}\(^{328}\) reported differences in P-gp activity but not expression in PBMCs of HIV patients treated with nucleoside analogues.
compared with untreated controls. Few clinical studies have considered P-gp expression and function in the context of the spectrum of HIV disease, its treatment and plasma HIV RNA levels. Chaillou and colleagues\textsuperscript{309} investigating this relationship found undetectable viral load only in association with the use of a low dose RTV/PI combinations. They postulated enhanced PI accumulation within cells as a likely mechanism for this effect. However, inexplicably poor intracellular accumulation was associated with both over-expression and an absence of MDR1 in the PBMCs of HIV infected patients\textsuperscript{309}. The disparity in published studies likely reflects the combination of (1) differing analytical procedures\textsuperscript{205,313}, (2) small group numbers in primarily untreated patients, (3) difficulties in extrapolating \textit{in vitro} data to the clinical situation and (4) exclusion of the effects of treatment. Direct effects on viral replication need to be considered, in addition to the impact on plasma and intracellular drug concentrations.

In the present study we address some of these shortcomings by assessing P-gp expression and function simultaneously in a large group of both naïve and HAART treated patients at various stages of disease, assigned to clinically relevant groups. Results are analysed in the context of viral load, a surrogate marker of effective treatment, and the known effects of the PIs on P-gp function. Finally we assessed the ability for low dose RTV added \textit{ex vivo} to further modulate P-gp function in the PBMCs of both treated and untreated HIV infected patients.

6.2 Methods

6.2.1 Patient Selection and Study Design

This was a prospective study of a random sample of HIV infected patients attending the Genito-Urinary Medicine Clinic of St James Hospital in Dublin over a 6 week period. Eighty patients with HIV infection were recruited after informed consent. Age and sex
matched healthy controls (n=20) were recruited from hospital staff. Exclusion criteria included: ethnic background other than Caucasian, inter-current illness, pregnancy / lactation, or concomitant administration of drugs other than anti-retroviral medications likely to interfere with the expression and/or function of P-gp (e.g. rifampicin, cyclosporin and methothrexate).

During a routine clinic visit, suitable patients were asked to provide in addition to their routine clinic bloods for determination of lymphocyte subsets and HIV viral load, an extra 30 ml venous blood sample for measurement of P-gp expression and function in PBMCs. All samples were coded so that investigators were blinded to patient characteristics. In addition, separate investigators assessed P-gp expression and function independently. Both patients and healthy volunteers were included on each study day. Subsequent to analysis the code was broken and a retrospective chart review undertaken to assess the following patient characteristics: age, sex, stage of HIV infection (revised classification for HIV infection and expanded case definition for AIDS in adolescents and adults; Centre for Disease Control revised case definition 1993; Table 6.1), risk factor for disease acquisition, CD4 and CD8 lymphocyte subsets, viral load, drug therapy and hepatitis status (B and C). For consistency, the stage of disease assessed from the patients’ clinical notes was cross-referenced against a computerised patient database.

Patients were then assigned to one of three groups: those fully suppressed on HAART, with a viral load of less than 50 copies per ml (suppressed group), those unsuppressed despite HAART with a viral load greater than 100 copies per ml (unsuppressed group), and those with established infection but who had never received HIV therapy (naïve group).
Table 6.1

The revised classification for HIV infection and expanded case definition for AIDS in adolescents and adults (Centres for Disease Control revised case definition 1993). A) Categorisation by CD4 cell count, and B) expanded case definition.

<table>
<thead>
<tr>
<th>CD4 Cell Count</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 500/mm(^3) (&gt;29%)</td>
<td>A1</td>
<td>B1</td>
<td>C1</td>
</tr>
<tr>
<td>200 to 499/mm(^3) (14-28%)</td>
<td>A2</td>
<td>B2</td>
<td>C2</td>
</tr>
<tr>
<td>&lt; 200/mm(^3) (&lt;14%)</td>
<td>A3</td>
<td>B3</td>
<td>C3</td>
</tr>
</tbody>
</table>
**Table 6.1 (B)**

<table>
<thead>
<tr>
<th>Category A</th>
<th>Category B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic HIV infection</td>
<td>Bacillary angiomatosis</td>
</tr>
<tr>
<td>Persistent generalised lymphadenopathy</td>
<td>Oral or recurrent vulvovaginal candidiasis</td>
</tr>
<tr>
<td>Acute retroviral syndrome</td>
<td>Cervical dysplasia</td>
</tr>
<tr>
<td></td>
<td>Oral hairy leukoplakia</td>
</tr>
<tr>
<td></td>
<td>Herpes Zoster</td>
</tr>
<tr>
<td></td>
<td>Constitutional syndromes (fever of 38.5°C, diarrhoea &gt;1 month)</td>
</tr>
<tr>
<td></td>
<td>Peripheral neuropathy</td>
</tr>
<tr>
<td></td>
<td>Pelvic inflammatory disease</td>
</tr>
<tr>
<td></td>
<td>Idiopathic thrombocytopenic purpura</td>
</tr>
<tr>
<td></td>
<td>Listeriosis</td>
</tr>
</tbody>
</table>

**Category C (Aids Defining Conditions)**

| CD4 count<200 cells/ml                         | HIV encephalopathy                                                        |
| Candidiasis of oesophagus or pulmonary         | Kaposi’s sarcoma                                                          |
| Cervical cancer                                | Lymphoma                                                                  |
| Coccidiomycosis extrapulmonary                 | *Mycobacterium avium* complex or *M. Kansi*                                |
| Cryptosporidiosis                              | *Mycobacterium tuberculosis*                                               |
| Cytomegalovirus infection                      | *Pneumocystis carinii* pneumonia                                           |
| Herpes simplex with oesphageyal, pulmonary or  | Pneumonia recurrent with more than two episodes in 12 months              |
| mucocutaneous involvement of >1 month         | Progressive multifocal encephalopathy                                      |
| Histoplasmosis                                 | Toxoplasmosis                                                             |
| Isosporiasis                                   |                                                                           |
6.2.2 P-Glycoprotein Expression.

The presence of P-glycoprotein expression was detected by flow cytometry and MDR1 gene expression confirmed by RT-PCR in accordance with consensus guidelines\(^{(253)}\). Cells were fixed in 2% paraformaldehyde (Cellfix, Becton Dickinson Mountain View C.A), permeabilised with 0.05% saponin (Sigma Aldrich) and labelled with a JSB1 monoclonal antibody (Sanbio, Uden, Netherlands) directed towards a highly conserved intracellular epitope of P-glycoprotein (30 min, at 37°C). JSB1 has been widely validated as a MDR1 P-gp specific antibody\(^{(311-314)}\). A monoclonal IgG derived from a murine hybridoma supernatant (ATCC, Manasses V.A.) HB179, was used as a negative fluorescence control where as an antibody to the cytoskeletal component vimentin (Dako AS Glostrup Denmark) was employed as a control for the permeabilisation technique. Excess primary antibody was removed by washing with 0.05% saponin, prior to incubation with a rabbit anti-mouse fluorescein labelled isothiocyanate (FITC)-conjugated F(ab)\(_2\) (1:50 dilution; Dako AS Glostrup Denmark) for 30 min, at 37°C. Finally, cells were washed free of excess antibody and re-suspended in 0.5 ml of paraformaldehyde prior to flow cytometric analysis.

6.2.3 P-Glycoprotein Mediated Drug Efflux Function and Inhibition.

PBMCs (1x10\(^6\) cells) were loaded with rhodamine 123 (1.25 μg.ml\(^{-1}\); Sigma Aldrich, Poole, Dorset, UK) for 25 min at 37°C in RPMI 1640 supplemented with 10% FCS. Cells were washed twice in ice-cold media and incubated at 37°C for 3h in 3 ml of dye free media to allow dye efflux. At baseline (T=0: maximum loading) and three hours later (T=180 min, maximum efflux) an aliquot was removed and washed twice in ice-cold media (3 min x 2000 rpm) prior to being fixed in ice-cold paraformaldehyde (Cellfix,
Becton Dickinson, UK). Cellular fluorescence was determined by flow cytometric analysis. Parallel experiments were performed in the presence of RTV (5 μM; a gift from Abbott Laboratories), a recognised P-glycoprotein inhibitor. In a subgroup of patients and healthy volunteers, full dose response curves over the concentration range (RTV 0.1-31 μM) were constructed for inhibition of P-gp mediated Rho efflux by RTV.

6.2.4 Flow Cytometry:

Lymphocytes shown in forward scatter and side scatter were electronically gated and acquired (10000 events) through the FL1 channel (expression) and the FL2 channel (function). The amount of fluorescence was plotted as a histogram of either FL1 (expression) or FL2 (function) staining within the gate. Data acquisition was performed using Cellquest software (WINMDI version 2.6) to determine median fluorescence intensity values (MFI).

6.2.5 RNA Isolation and Reverse Transcription Polymerase Chain Reaction

The presence of MDR-1 and MRP mRNA in random samples (Figure 6.1) was confirmed by non-quantitative RT-PCR as previously described\(^\text{315}\). In brief, total cellular RNA was isolated by a modification of the method of Chomczynski and Sacchi\(^\text{255}\) using Tri Reagent (Sigma Aldrich). First Strand cDNA was synthesised from 2 μg total RNA using random decamers and M-MLV reverse transcriptase (Reverse-iT kit, Abgene). The resultant cDNA was amplified by PCR following a standard PCR protocol. Each PCR reaction contained 2 μl cDNA, 1 x reaction buffer, 1.5 mM MgCl\(_2\), 200 μM of each dNTP, 1.25 U DNA polymerase (Thermus 'Icelandicus', Red Hot DNA polymerase, Abgene) and between 0.5-1 μM of each gene specific primer. The gene
specific primers (Table 6.2) were based upon those previously reported in the literature\(^{(254)}\).

After an initial denaturation at 94°C for 4 min, the following cycling profile was used: denaturation at 94°C for 30 s, annealing at each suitable temperature for 40 s, and extension at 72°C for 60 s. Amplification was performed over several cycles (Table 6.2) and ended with a final extension at 72°C for 5 min and cooling to 15°C for 5 min. All PCR reactions were performed in duplicate. The PCR products were separated on a 2.0% agarose gel and stained with ethidium bromide. Gels were visualised under ultraviolet illumination, photographed and underwent non-quantitative analysed using a GeneGenius Gel documentation system with Gene Tools analysis software (Syngene). Band intensity was expressed as the target mRNA to GAPDH mRNA ratio.

### Table 6.2 Sequences of upstream and downstream oligonucleotide primers.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward</th>
<th>Reverse</th>
<th>Tm</th>
<th>No. Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>β₂M</td>
<td>ACCCCCACCTGAAAA</td>
<td>ATCTTCAAAACCTCC</td>
<td>55°C</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>AGATGA</td>
<td>ATGATG</td>
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<td></td>
</tr>
<tr>
<td>MRP</td>
<td>TGGGACTGGAATGTC</td>
<td>AGGAATATGCC</td>
<td>55°C</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>ACG</td>
<td>GACTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDR-1</td>
<td>CCCATCATGCAATA</td>
<td>GTTCAAACCTCTGC</td>
<td>55°C</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>GCAGG</td>
<td>TCCTGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.2.6 Data Analysis

P-gp function was expressed as the ratio of T180/T0 (maximum efflux /maximum loading) therefore, a reduced ratio indicates enhanced efflux function. The percentage of P-gp mediated reversible rhodamine efflux was calculated in the presence of RTV (5 μM) using the formula:

\[
100 \times \left( \frac{R_{180} - T_{180}}{T_{0} - T_{180}} \right)
\]

6.2.7 Statistical Analysis

Data were subjected to one-way analysis of variance with Newman-Keuls or Dunnett's post hoc analysis or an unpaired Students t-test where appropriate. All correlations were subjected to Pearson correlation analysis or trend analysis (Graphpad Prism, version 4.02). Data are expressed as mean±SEM, and a value of P<0.05 was taken to indicate significance.

6.3 Results

63.1 Patient Characteristics (Table 6.3)

Eighty HIV positive patients (M=54) were recruited into the study. However, at chart review four patients were excluded due to active inter-current illness and one due to concomitant drug use. The mean age for male patients was 39.4 yr, (range 20-59 yr) and for females was 32.5yr (range 19-61yr). Patient characteristics and demographics are summarised in table 6.3. As expected, the majority of patients were classified as having stage C disease. Of the 75 patients included in the study, 14 were naïve to HAART and 61 were receiving HAART. Treatment regimens included dual nucleoside reverse transcriptase inhibitors (NRTI) plus a PI (NFV= 22; IDV=10; SQV=4, and RTV alone or...
in combination with another PI; n=5), or alternatively a non-nucleoside reverse transcriptase inhibitor (NNRTI; nevirapine, efavirenz). Of the patients receiving HAART, 39 were fully suppressed with a viral load estimate of less than 50 copies /ml, while 22 patients remained unsuppressed despite HAART (mean viral load = 44309±22980). Most patients were receiving a three-drug regimen (n=55), while a few were receiving four or more drugs (n=6).

6.3.2 Effect of HIV Disease and Treatment on P-glycoprotein Expression

P-gp expression was 40% lower in naïve HIV patients compared to age matched healthy controls (3.74 ±0.53 vs 6.2 ± 0.45 MFI; P<0.05; Figure 6.2). In contrast, there was a 2.3 fold increase in P-gp expression in suppressed patients (8.33±0.59 MFI; P<0.001) compared with both unsuppressed patients and naïve patients, with no significant difference between these latter two groups, (3.64±0.64 and 3.74±0.53 MFI respectively; Figure 6.2).

There was a significant correlation between viral load and P-glycoprotein expression such that increasing viral load was associated with decreased P-gp expression (r=-0.47; P<0.01; Figure 6.3). There was no correlation between P-gp expression and stage of disease, with expression remaining higher in the PBMCs of suppressed patients, independently of this variable (Figure 6.4). Similarly P-gp expression did not correlate with any of the following variables: risk factor for disease acquisition, CD4 or CD8 lymphocyte count or hepatitis status.

With regard to HAART treated patients, expression was 3-fold higher in suppressed patients compared with unsuppressed patients, regardless of whether therapy included a PI or an NNRTI (PI suppressed vs unsuppressed: 8.56±0.77 vs 2.43±0.85 MFI; NNRTI: suppressed vs unsuppressed: 8.10±0.74 vs 3.16±0.83 MFI; P<0.05; Figure 6.5). Twenty-
two patients were receiving nelfinavir as PI therapy allowing further analysis. Within this group, P-gp expression was 70% lower in the un-suppressed compared with the suppressed patients receiving (3.04±0.94 vs 10.04±0.99 MFI P< 0.05 Figure 6.6).

6.3.4 Effect of HIV disease and Treatment on P-glycoprotein Function

P-gp function was estimated by flow cytometric analysis of intracellular accumulation of Rhodamine (Rho) at baseline and after efflux (180min), and a functional index derived (T180/T0). The higher ratio indicating reduced P-gp mediated Rho efflux (poor P-gp function). Post hoc Trend analysis demonstrated that P-gp function between groups decreased in the rank order: healthy controls > suppressed > unsuppressed > naive (P< 0.0001). P-gp function was reduced in naïve HIV patients compared with healthy controls (0.54±0.11 vs 0.19±0.03 MFI; P<0.05). However P-gp function was similar in both suppressed and un-suppressed patients receiving treatment (0.32±0.03 vs 0.41±0.06). Suppressed patients had improved function compared with naïve patients (0.32±0.03 vs 0.54±0.11; P<0.05; Figure 6.7).

With respect to P-gp function and treatment with HAART, T₀ (maximum Rho loading) was higher in patients suppressed on PI therapy compared with those suppressed on NNRTIs (32.4±3.9 vs 13.2±5.3 MFI; P=0.016), while T₀ values were similar in unsuppressed patients in either of these treatment groups. Similarly there was no difference in T₀ values between healthy controls and the naïve group (44.5±5.4 vs 34.9±4.6). P-gp mediated efflux was 40% lower in those treated with nelfinavir compared with those on indinavir therapy (0.36±0.03 vs 0.22±0.04; P<0.02; Figure 6.8). There were no differences in P-gp function between patients treated with either nevaripine or efavirenz: 0.35±0.07 vs 0.29±0.09). There was no correlation between P-gp
expression and function in PBMCs of the HIV infected patients studied (r= -0.08; P=0.54; n=75).

6.3.5 Dose Response Curves for the Effect of RTV on P-glycoprotein mediated Rho Efflux.

The response to RTV 5 μM was determined for all patients on the day of study. In selected patients from each group, and healthy volunteers full dose response curves for the effect of RTV on P-gp mediated Rho efflux function were constructed over the concentration range 0.1-31 μM (Figure 6.9). The following variables were derived following non-linear regression analysis, EC\textsubscript{50} (concentration required to produce 50% response), E\textsubscript{max} (maximal response) and the interpolated response to 5 μM RTV. The EC\textsubscript{50} for each of the groups was comparable (1.8±0.3 μM; range 1.3-2.1 μM). Similarly RTV (5 μM) produced approximately 60% reversible inhibition of P-gp mediated Rho efflux in each of the groups studied, with good agreement between the interpolated and actual response obtained for each patient on the day of study. Interestingly, the maximum response to RTV (E\textsubscript{max}) was similar between naïve HIV patients and healthy controls (98.7±1.7% vs 92.4±4.2) but was reduced (P<0.001) in both the suppressed and unsuppressed groups (70.9±2.2 and 63.2±2.7%).
Table 6.3 Patient Characteristics and Demographics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Male (n=54)</th>
<th>Female (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age ± se (y)</td>
<td>39.4 ± 20-59</td>
<td>32.5 19-61</td>
</tr>
<tr>
<td>Range (y)</td>
<td>A</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>30</td>
</tr>
<tr>
<td>HIV Stage (n)</td>
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<tr>
<td></td>
<td>Heterosexual</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Homosexual</td>
<td>23</td>
</tr>
<tr>
<td>Hepatitis Status</td>
<td>C</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>8</td>
</tr>
</tbody>
</table>

| CD4^+ Count x 10^6/l           | 461±61      | 483.9±39      | 313±77            |
| CD8^+ Count x10^6/l           | 998±91      | 1011±82.1     | 843±88           |
| Viral Load (copies/ml)        | 26380±7085  | 50            | 44309±22980      |
| Treatment (n)                 | Dual NRTI + PI (n) | 0 | 28 | 5 |
|                                | Dual NRTI + NNRTI | 0 | 11 | 11 |
|                                | Dual NRTI + NNRTI + PI  | 0 | 0 | 6 |

Abbreviations: IVDU = intravenous drug user; NRTI = nucleoside reverse transcriptase inhibitor; NNRTI = non-nucleoside reverse transcriptase inhibitor; PI = protease inhibitor
Figure 6.1

A representative gel showing the presence of β₂-microglobulin (β₂M), multidrug resistance protein 1 (MDR-1) and multidrug resistance associated protein (MRP-1) mRNA expression in random samples from HIV infected patients.
Figure 6.2

Effects of HIV disease and viral load response following treatment (suppressed: <50 copies per ml; unsuppressed >100 copies per ml) on P-gp expression in PBMCs from HIV infected patients compared with healthy controls. Data are expressed as mean±SEM. Horizontal bars depict statistical significance at P<0.05. Group sizes are: Controls, n=20; Suppressed, n=37, Unsuppressed, n=22; Naïve, n=14.
Figure 6.3

The relationship between plasma HIV RNA levels (viral load) and P-gp expression in PBMCs from all HIV infected patients. The red symbol (•) represents an average P-gp expression level for all patients with a viral load of <50 copies/ml (n=38).
Figure 6.4

The effect of HIV disease staging on P-gp expression in PBMCs of naïve and treated-suppressed (viral load: <50 copies per ml) HIV infected patients. Data are expressed as mean±SEM. Group sizes are detailed on the graph above the error bars.
Figure 6.5

Effects of therapeutic responsiveness as defined by viral load (suppressed: <50 copies per ml; unsuppressed: >100 copies per ml) on P-gp expression in PBMCs from HIV infected patients treated with either a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI). Data are expressed as mean±SEM. Horizontal bars depict statistical significance at P<0.05. Group sizes are: PI suppressed, n=27; PI unsuppressed, n=9; NNRTI suppressed, n=9; NNRTI unsuppressed, n=14.
Figure 6.6

Effects of therapeutic responsiveness as defined by viral load (suppressed: <50 copies per ml, n=17; unsuppressed: >100 copies per ml, n=5) on P-gp expression in PBMCs from HIV infected patients treated with nelfinavir (NFV) Data are expressed as mean±SEM. * P<0.05.
Figure 6.7

Effects of HIV disease and viral load response following treatment (suppressed: < 50 copies/ml; unsuppressed >100 copies/ml) on P-gp function (T180/T0) in PBMCs from HIV infected patients compared with healthy controls. Data are expressed as mean±SEM. Horizontal bars depict statistical significance at P<0.05. Group sizes are: Controls, n=20; Suppressed, n=32, Unsuppressed, n=19; Naïve, n=9.
Figure 6.8

The effect of the protease inhibitors nelfinavir (NFV; n=22) and indinavir (IDV; n=10) on P-gp function (T180/T0) in PBMCs from HIV infected patients. Data are expressed as mean±SEM. * P<0.05.
Figure 6.9

Concentration dependent changes in reversible Rhodamine (Rho) efflux (expressed as a percentage) in response to increasing concentrations of ritonavir (RTV) in PBMCs from naïve (untreated), suppressed (viral load <50 copies per ml) and unsuppressed (viral load >100 copies per ml), HIV infected patients and healthy controls. Group sizes are: Controls, n=6; Suppressed, n=11, Unsuppressed, n=4; Naïve, n=4.
6.4 Discussion

HIV entry into cells involves binding of virion-associated gp120 to CD4 receptors and either CXCR4 or CCR5 co-receptors on target cells, inducing a conformational change in gp41 and initiating fusion of the virus and target cell membranes\(^{229}\). Recent studies have postulated that P-gp expression may directly alter replication of enveloped viruses such as influenza and HIV by inhibiting membrane fusion. Raviv et al.\(^{230}\) found that P-gp over-expression blocked insertion of the influenza virus fusion protein (hemagglutinin-2) inhibiting membrane fusion and infectivity of the virus. Lee et al.\(^{231}\) reported decreased HIV-1 infectivity and a 40-fold reduction in virus production when P-gp was over-expressed in CD4\(^+\) T cell lines infected with different HIV viral strains. However, expression of CD4 and CXCR4 were not significantly altered leading to the conclusion that P-gp expression inhibited HIV-mediated membrane fusion, as well as subsequent step(s) in the HIV-1 life cycle. More recently Speck et al.\(^{232}\) confirmed and extended these findings by demonstrating a 70-fold decrease in supernatant p24 production and a 100-fold decrease in infectious HIV units, in a P-gp over-expressing lymphoblastoid cell line compared with control cells. Verapamil, an inhibitor of P-gp, reversed this effect in over expressing cells, but had little effect in control cells, thus supporting a direct role for P-gp in altering HIV p24 and infectious virus production. Fusion of HIV with its target cells takes place at specialised regions in the membrane known as glycolipid enriched membrane (GEM) domains. Interestingly P-gp is also preferentially distributed within GEM domains, highlighting that the spatial arrangement of both is such that P-gp could potentially play a role in modulating cellular binding of HIV\(^{233}\). These studies elegantly demonstrated the effects of P-gp over expression on HIV infectivity and virus production. To extend this approach we present a complementary study examining the
potential effects of HIV disease on P-gp expression and function in PBMCs of HIV infected patients.

The main findings of the present study are that P-gp expression is reduced in the PBMCs of naïve HIV infected patients, expression remains low in those unsuppressed despite HAART, but increases to above normal levels in patients who attain a viral load of less than 50 copies per ml, regardless of which drug regimen is used to achieve suppression. Overall this produces a strong inverse correlation between P-gp expression and viral load. Given that P-gp levels are lower in naïve patients compared with healthy controls, these data suggest that the effect of HIV disease *per se* may be to inhibit production of P-gp protein in PBMCs of affected patients. With effective treatment viral replication is suppressed allowing P-gp expression to increase, while therapeutical failure favours persistent viral replication and ongoing inhibition of P-gp expression. The present study therefore confirms that the relationship between P-gp expression and viral infectivity noted in recent *in vitro* studies is also seen in the PBMCs of patients with infected with HIV disease. Mechanistically this may occur at the level of gene transcription since P-gp mRNA levels have been shown to decrease significantly during SHIV infection of macaques\(^{(329)}\).

In addition to reduced expression, P-gp function was also lower (increased ratio) in the PBMCs of naïve patients compared with healthy controls and suppressed groups, a result consistent with the data of Andreana *et al.*\(^{(204)}\). However, they hypothesised that the P-gp in naïve patients with HIV disease was functionally defective on the basis of reduced Rho accumulation at baseline, and a lack of response to cyclosporin A. Interestingly in the present study despite significant differences in efflux function, there was no difference in baseline Rho accumulation between naïve patients and healthy controls. Furthermore we were able to demonstrate a similar EC\(_{50}\) and maximal repose to RTV for
both groups. These findings would suggest that P-gp mediated efflux in the PBMCs of untreated HIV patients is not defective, and that differences in function may occur primarily as a result of reduced expression in this group.

The relationship between P-gp expression and viral load persists regardless of the stage of HIV disease; furthermore, there was no correlation between P-gp expression and either CD4 or CD8 lymphocyte count, implying that the effect occurs independently of either of these factors. Overall we found no correlation between P-gp expression and P-gp mediated Rho efflux, suggesting the relationship between expression and function in HIV patients is complex and difficult to predict and may be confounded by drug therapy. Disparity between P-gp expression and function has been reported previously in haemopoietic cells, with no correlation seen between steady state MDR1 expression and P-gp function in acute myeloid leukaemia cells\cite{147}, human bone marrow lymphoid cells\cite{148} or K562 leukaemia cells\cite{132}. Similarly, we have shown that St Johns Wort increases P-gp mediated rhodamine efflux by only 50% in the presence of a greater than 4 fold increase in P-gp expression in the PBMCs of healthy volunteers\cite{315}. Interestingly, in the study by Lee et al.\cite{231} non-functional mutants of P-gp also inhibited virus production, suggesting that the relationship between P-gp expression and viral infectivity does not involve transporter-mediated efflux implying that expression and function may be differentially regulated. Several authors have suggested that PIs may be associated with alterations in P-gp expression\cite{217,330}. However, within our study population there were no differences in expression between those suppressed on either a PI or an NNRTI. Similarly, expression was three-fold higher in patients suppressed on NFV compared with those unsuppressed on exactly the same treatment, supporting the concept that it is the reduction in viral load that allows P-gp expression to return to normal levels, rather than a particular therapy. We did not assess coexisting antiretroviral drug resistance in
our study population however, recent consensus guidelines\(^{293}\) recognise that the lower the nadir of plasma HIV-1 RNA levels the longer it takes for drug failure to occur\(^{294}\). By assigning only patients with a viral load of less than 50 copies per ml to the suppressed group we attempted to minimize the potential influence of emerging viral resistance in this subgroup, though clearly drug resistance is a likely important contributor in unsuppressed patients. Similarly poor adherence is an important determinant of response to therapy. We have previously shown in a random sample of patients from our clinic, that both self-reported compliance (ie >80% of medications) and prescription redemption rates are approximately 90%\(^{331}\), this is consistent with the data of others but represents an overestimate of adherence when compared with electronic monitoring (63%)\(^{332}\). However, our intention was to present results from a random population of patients treated under normal clinical conditions.

Our data shows that P-gp function between groups decreased in the rank order: healthy >suppressed > unsuppressed > naïve. If a direct relationship existed between P-gp expression and function, then by virtue of changes in expression alone one would expect efflux to be approximately 30% higher in the suppressed group, and 40% lower in the unsuppressed group relative to healthy controls. This was not the case as P-gp function was similar in both suppressed and unsuppressed patients despite marked differences in viral load, suggesting the confounding influence of drug therapy within these groups. PIs are well recognised as both substrates\(^{158,206,296}\) and inhibitors\(^{209,210}\) of the multi-drug transporter. To assess the effect of PI therapy on P-gp mediated efflux we analysed function in patients fully suppressed on either a PI or an NNRTI, removing the influence of both viral load and altered P-gp expression. Interestingly, based on our standard functional index, which normalises to T0 and therefore takes no account of changes at baseline, there was no difference in P-gp function between groups. However when the
absolute change in Rho efflux (T0-T180 not normalised to T0) is used, patients suppressed on a PI show reduced efflux function compared with those on an NNRTI. In support of this we also found that Rho accumulation at baseline (T0) was higher in patients suppressed on a PI compared with those suppressed on an NNRTI. We have shown that NFV undergoes significant intracellular accumulation within the PBMCs of HIV infected patients responsive to therapy. Therefore, prolonged intracellular presence of a PI should increase Rho accumulation at baseline due to chronic inhibition of efflux. Furthermore, both Rho and PI should compete as substrates for P-gp efflux further enhancing this effect. Between PIs, P-gp function was reduced in those receiving NFV compared with those receiving IDV confirming previous studies showing a minimal effect of IDV on P-gp function.\(^{266}\)

RTV at low doses is increasingly used as part of boosted therapeutic regimens to enhance the bioavailability of co-administered antiretroviral agents, although the long-term benefits of this remain to be established. In this study we assessed the potential for additional RTV added \textit{ex vivo} to further modulate P-gp mediated Rho efflux in the PBMCs of each of the groups studied. The concentration of RTV chosen was that which produced approximately 65% inhibition of P-gp mediated efflux in the PBMCs of healthy volunteers (preliminary experiments) and was intended to reflect plasma concentrations similar to those expected with the use of low dose RTV. In a subgroup of patients full dose response curves for this effect were constructed. The EC\(_{50}\) of RTV was similar between all groups, as was the response to 5\(\mu\)M demonstrating that RTV was equally potent in each of the groups studied, healthy, suppressed unsuppressed or naïve. However the maximum response to RTV was significantly lower in patients receiving HAART irrespective of viral load suggesting that pre-treatment reduces but does not abolish the potential for low dose RTV to further modulate P-gp mediated drug efflux.
from cells. This represents an additional mechanism to enhance intracellular drug concentrations for both antiretroviral naive and experienced patients. However, the clinical relevance of boosting with ritonavir to enhance intracellular PI levels for patients already suppressed on therapy must be balanced with improved bioavailability as a result of CYP3A4 inhibition at the level of the gut and further studies are required to assess the relationship between intracellular PI concentrations and the durability of response.

In conclusion, and consistent with \textit{in vitro} data, we find there is a relationship between P-gp expression and HIV viral burden in the PBMCs of infected patients. HIV disease \textit{per se} appears to inhibit expression of P-gp, favouring viral infectivity. That P-gp expression is inversely related to viral load independently of the stage of disease or the therapeutic regimen used to achieve suppression, suggests that any effective antiretroviral regimen has the potential to reverse this effect restoring P-gp expression to normal levels or above. Further studies will be crucial to delineate the role of P-gp in maintaining therapeutic responsiveness, and to establish the cause and effect relationship between P-gp and viral infectivity in HIV infected patients. The P-gp expressed in HIV patients does not appear to be functionally defective; however, drug efflux function is reduced in PBMCs of patients on chronic PI therapy, in a manner consistent with their known inhibitory potential for P-gp. Ritonavir inhibits P-gp mediated drug efflux function in PBMCs of HIV infected patients in a dose dependant manner, however prior antiretroviral experience may result in a sub-maximal response. P-gp expression and function appear to be differentially regulated in the PBMCs of HIV infected patients. This may have important implications given recent evidence that non-functional P-gp (in addition to its functional counterpart) may also prevent viral entry into cells since it suggests that chronic blockade of P-gp by PIs may not necessarily preclude a cellular protective role for this highly conserved protein.
Chapter 7

Discussion
7.1 Discussion and Conclusions

The St Johns Wort (SJW) study investigates the potential for upregulation of P-gp expression on PBMCs from healthy volunteers, and considers what effect this may have on drug efflux function. SJW is widely used in the treatment of depression but concerns have been raised about its interaction potential with other drugs. Co-administration of indinavir and cyclosporin with SJW has resulted in significant reductions in trough concentrations of these drugs\(^{244,245}\). Although induction of CYP3A4 has been implicated, the magnitude of the interaction is greater than that predicted by \textit{in vitro} studies suggesting additional mechanisms of interaction exist. Since indinavir and cyclosporin are substrates for both CYP3A4 and P-gp, we hypothesised that modulation of P-gp expression and function by SJW contributes to the development of potentially harmful drug-drug interactions. In this study P-gp expression increased four fold from baseline in subjects treated with SJW, while P-gp mediated rhodamine efflux was increased by approximately 50%. Ritonavir inhibited P-gp mediated efflux in both groups (active and placebo) allowing greater intracellular accumulation of rhodamine. This effect was attenuated following treatment with SJW. We conclude from this study that chronic treatment with SJW induces P-gp expression on PBMCs, and has the potential to alter the bioavailability of co-administered drugs. This provides a alternative mechanism for SJW associated drug interactions seen in clinical practice, and accounts for the discrepancies between \textit{in vitro} and \textit{in vivo} data. In addition, the study draws attention to the fact that if the metabolic preferences of a drug are considered in isolation from its transport processes then the interaction potential of that drug may be underestimated. Depression in HIV disease is common\(^{333}\), as is undeclared self administration of herbal and alternative medicines\(^{334}\). Since P-gp and CYP3A4 have distinct though overlapping substrates, patients receiving antiretroviral drugs, many of
which are P-gp substrates, should be warned against self-medication with SJW since clinically significant drug interactions are likely, and may contribute to antiretroviral failure. The role of P-gp in reducing drug bioavailability across the gut wall is clear. Since most PIs are P-gp substrates, expression of P-gp on PBMCs suggests it may decrease intracellular PI concentrations. Lack of a reproducible method for determining intracellular PI concentrations has resulted in a paucity of \textit{in vivo} data. The main objective of the second study was to determine the feasibility of measuring intracellular indinavir concentrations in the PBMCs of HIV patients, and to compare plasma with intracellular indinavir pharmacokinetics.

The assay as described, although not without fault, is sensitive, robust and reproducible, and is a useful tool for examining the relationship between plasma and intracellular PI concentrations\textsuperscript{335}. Although, it is imperative that stringent assay conditions are adhered to. The main findings of this study were that the mean intracellular indinavir $\text{AUC}_{0-8}$ was approximately 30% that of plasma. Surprisingly, both the elimination half-life and the mean residence time of indinavir intracellularly were prolonged. Individual plasma versus intracellular time course data suggests that this may allow some patients to achieve acceptable intracellular concentrations despite sub-therapeutic plasma levels. It can be concluded that there was no significant intracellular accumulation of indinavir within the lymphocytes of HIV-1 infected patients, with intracellular concentrations merely reflecting available free drug from plasma. The clinical implication is that for indinavir, a correlation between plasma and intracellular drug concentrations cannot be taken for granted. Thus indinavir containing regimens are likely to be less forgiving of dose omission or poor concordance with medication regimens. Recently the U.K. Collaborative HIV Cohort Study Group has found that for antiretroviral naïve patients
there is an increased rate of viral rebound associated with regimens containing indinavir compared with efavirenz(336).

Following on from the indinavir study it was important to determine if absence of intracellular accumulation is specific to the PI, or a class effect. The nelfinavir study compared plasma and intracellular nelfinavir pharmacokinetics and determined the relationship of these parameters to P-gp expression and function in lymphocytes of HIV infected patients. In contrast to indinavir, nelfinavir underwent significant intracellular accumulation (9 fold) within the PBMCs of HIV infected patients. This may in part be due to extensive binding to cellular proteins, and/or related to its moderate ability to inhibit P-gp mediated drug efflux. The correlation between plasma and intracellular nelfinavir concentrations suggest that for NFV, plasma levels provide a reasonable surrogate marker of intracellular concentrations, and are therefore a useful guide in clinical practice. Intracellular accumulation of nelfinavir correlated with P-gp function (but not expression) confirming P-gp efflux activity is substrate concentration dependant. Despite elevated intracellular nelfinavir levels, the addition of ritonavir further reduced P-gp function by approximately 55%. This likely reflects the fact that the assay measured “cell associated” rather than cytosolic free drug concentrations. The study highlights both the PI specific nature of interactions between P-gp and antiretroviral drugs and also the complexity of protein binding in determining realtionships with P-gp. Therefore, it would be important that for the new antiretroviral drugs interactions with lymphocyte derived P-gp should be examined at an early stage of drug development.

Altered P-gp expression has been suggested as a potential mechanism for the development of drug resistance in HIV disease. However, little is known about the effect of HIV disease on P-gp expression and function. The last study investigates this in a cohort of naïve and ART treated HIV positive patients and shows that in a clinical setting
P-gp expression is inversely related to viral load (independent of stage of disease or CD4/CD8 count). These results support *in vitro* data showing P-gp overexpression to reduce HIV infectivity. In essence, reduced viral load, independent of the antiretroviral regimen chosen, appears to favour enhanced P-gp expression. This underlines the cellular protective role of P-gp and points the direction for further studies to investigate the effect of P-gp expression on long term therapeutic responsiveness to antiretroviral therapy. The relationship between expression and function is complex, difficult to predict and confounded by drug therapy. Despite this, inhibition of P-gp mediated efflux by co-administered drugs does not appear to preclude effects on viral load. The possibility that P-gp expression and function may be differentially regulated is interesting and raises the possibility of developing novel therapeutic targets for HIV therapy in the future. The rhodamine accumulation data from this study indicate that P-gp is functional in HIV patients, which is in contrast to earlier studies suggesting it may be defective\(^\text{231}\). With regards to ritonavir the EC\(_{50}\) for inhibition was similar across all groups, however, pre-treatment with a PI reduced the potential for further modulation of P-gp function. In clinical practice there are few data available on virological rebound rates in patients receiving ritonavir boosted PI regimens\(^\text{336}\), and the long term effect of this strategy on viral resistance patterns remains unclear. That P-gp can be both induced and inhibited is clear, as is its ability to alter extracellular and intracellular bioavailability of PIs in patients with HIV disease. What remains to be determined, and warrants further exploration is the role of P-gp in immune reconstitution following HAART, and its effect on long term outcome in HIV disease.
References


76. Qu Q, Russell PL, Sharom FJ. Stoichiometry and affinity of nucleotide binding to P-glycoprotein during the catalytic cycle. *Biochemistry.* 2003;42:1170-1177.


characterization of the drug-stimulatable ATP hydrolysis. *Proc Natl Acad Sci.*

80. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the

81. Higgins CF, Gottesman MM. Is the multidrug transporter a flippase? *Trends

82. Altenberg GA, Vanoye CG, Horton JK, Reuss L. Unidirectional fluxes of
rhodamine 123 in multidrug-resistant cells: evidence against direct drug extrusion

83. Shapiro AB, Ling V. Extraction of Hoechst 33342 from the cytoplasmic leaflet of

84. Shapiro AB, Corder AB, Ling V. P-glycoprotein-mediated Hoechst 33342 transport

85. Shapiro AB, Ling V. Transport of LDS-751 from the cytoplasmic leaflet of the
plasma membrane by the rhodamine-123-selective site of P-glycoprotein. *Eur J

86. Chen Y, Pant AC, Simon SM. P-glycoprotein does not reduce substrate
concentration from the extracellular leaflet of the plasma membrane in living cells.

87. Romsicki Y, Sharom FJ. Phospholipid flippase activity of the reconstituted P-

88. Johnstone RW, Ruefli AA, Smyth MJ. Multiple physiological functions for

89. Ueda K, Taguchi Y, Morishima M. How does P-glycoprotein recognise its

90. Stouch TR, Gudmundsson O. Progress in understanding the structure-activity

91. Seelig A. A general pattern for substrate recognition by P-glycoprotein. *Eur J


93. Seelig A, Landwojtowicz E. Structure-activity relationship of P-glycoprotein

94. Seelig A, Li Blatter X, Wohnsland F. Substrate recognition by P-glycoprotein and
the multidrug resistance-associated protein MRP1: a comparison. *Int J Clin


132. Yague E, Armesilla AL, Harrison G, Elliott J, Sardini A, Higgins CF, Raguz S. P-glycoprotein (MDR1) expression in leukaemic cells is regulated at two distinct


285. Burger DM, Hugen PWH, Draste J, Huitema A. Therapeutic drug monitoring (TDM) of nelfinavir (NFV) and indinavir (IDV) in treatment-naive patients improves therapeutic outcome after 1 year: Results from ATHENA. 1st


330. Perloff MD, von Moltke LL, Marchand JE, Greenblatt DJ. Ritonavir induces P-glycoprotein expression, multidrug resistance-associated protein (MRP1)


