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Both P-gp and MRP2 mediate transport of Lopinavir, a protease inhibitor

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ABSTRACT

Polarized epithelial non-human (canine) cell lines stably transfected with human or murine complementary DNA (cDNA) encoding for various efflux transporters (P-gp/MDR1, MRP1, MRP2, and Bcrp1) were used to study transepithelial transport of Lopinavir (LVR) and compare results with the MDCKII-Wild type cells. These transmembrane proteins cause multidrug resistance by decreasing the total intracellular accumulation of drugs. Lopinavir efflux was directional and was completely inhibited by MK-571, a selective MRP family inhibitor in the MDCKII-MRP2 cell line. Similarly, LVR efflux was also inhibited by P-gp inhibitors P-gp 4008 and GF120918 in the MDCKII-MDR1 cell line. The efflux ratios (Efflux rate/ Influx rate) of LVR in the absence of any efflux inhibitors in the MDCK-Wild type, MDCKII-MDR1, MDCKII-MRP1, MDCKII-MRP2 and MDCKII-Bcrp1 cell monolayers were 1.32, 4.91, 1.26 and 2.89 respectively. The MDCKII-MDR1 and MDCKII-MRP2 cells have significantly increased LVR efflux ratio relative to the parental cells due to the apically directed transport by MDR1 and MRP2 respectively. The efflux ratios in MRP2 and MDR1 transfected cell lines were close to unity in the presence of MK-571 and P-gp 4008 respectively; indicating that LVR efflux by MRP2 and P-gp was completely inhibited by their selective inhibitors. MDCKII-MRP1 cells did not exhibit a significant reduction in the LVR efflux relative to the parental cells, indicating that LVR is not a good substrate for MRP1. Transport studies across MDCKII-Bcrp1 cells indicated that LVR is not transported by Bcrp1 and is not a substrate for this efflux protein. In conclusion, this study presents direct evidence that LVR is effluxed by both P-gp and MRP2 which may contribute to its poor oral bioavailability and limited penetration into the CNS.

***Keywords**:* MDCKII-MDR1, MDCKII-MRP2, MDCKII-MRP1, MDCKII-Bcrp1, MDCKII-WT, P-glycoprotein (P-gp), multidrug resistance protein (MRP), breast cancer resistance protein (BCRP), Lopinavir (LVR), uptake, transport, permeability, efflux ratio (ER)

1. INTRODUCTION

HIV protease inhibitors (PIs) have revolutionized the treatment of HIV infection (Roberts et al., 1990; Vacca et al., 1994; Danner et al., 1995; Kempf et al., 1995; Patick et al., 1996). Due to limited oral bioavailability and poor pharmacokinetics of many of the currently available PIs, additional efforts have been made to design more potent PIs with improved pharmacokinetic properties. Lopinavir, an analog of ritonavir (RVR) is a potent inhibitor of wild type and mutant HIV protease (Ki =1.3-28 pM) (Kumar et al., 2004). Its molecular formula is: C37H48N4O5, and its molecular weight is 628.80. Its structural formula is outlined in Fig.1. The LVR: RVR combination (KALETRA) has been shown to be effective in the treatment of HIV infection and is approved for clinical use (Hurst and Faulds, 2000; Miller, 2000). LVR, as such, is extensively metabolized

by CYP3A4 and produces low systemic availability when administered alone. RVR potently inhibits CYP3A4 and is used in combination with LVR to enhance the systemic exposure of LVR. This combination results in LVR concentrations that greatly exceed those necessary in vitro to inhibit both wild-type and PI-resistant HIV isolates. (FDA – Kaletra Prescribing Information, 10/06/04, p. 3. Available at: http://www.fda.gov/cder/foi/label/2004/21226s014, 21251s010lbl.pdf). The current once-a-day prescribed dose for KALETRA is a 800mg/200mg (LVR: RVR) tablet or 10 ml of 80mg/20 mg (LVR: RVR) oral solution which contains 42.4% v/v alcohol. The advantages of prescribing KALETRA over other PIs include once daily dosing and evidence of better in-vitro activity against HIV. The disadvantages include low aqueous solubility of LVR, accounting for high amount of alcohol present in the liquid dosage form and high plasma protein binding (98-99%) at steady state. A number of clinically important drug interactions have been reported with LVR: RVR necessitating dosage adjustments of LVR: RVR and/or the interacting drugs. Several co administered drugs are also contraindicated in patients receiving the co formulation (Cvetkovic and Goa, 2003).

The low oral bioavailability of LVR was attributed to high first-pass metabolism (Kumar et al., 2004). High first pass metabolism can also occur due to intestinal efflux which can lead to increased exposure time to metabolizing enzymes (Wacher et al., 1995; Wacher et al., 2001; Katragadda et al., 2005). We have hypothesized that the low oral bioavailability of LVR and possibly limited brain penetration could be in part due to efflux of LVR by several efflux pumps such as P-gp, MRPs and BCRP present on intestinal epithelial and blood capillary endothelial cells. A single report on the interaction of LVR with P-gp has been published (Vishnuvardhan et al., 2003) which suggests that LVR induces P-gp upon long term exposure and inhibits P-gp on acute exposure. From this report, it appears that LVR is a substrate for P-gp but its interactions with other adenosine triphosphate-binding cassette (ABC) efflux transporters such as multidrug-resistance related proteins (MRPs) and breast cancer resistance protein (BCRP) have not yet been investigated. Therefore, it is important to delineate quantitatively if the efflux transporters can restrict, at least in part, the permeation of LVR at both the intestinal and blood brain barrier (BBB) membranes.

Numerous drug transporters are expressed in the human intestine epithelial and the brain endothelial cells, of which the ABC transporters have been extensively characterized. ABC transporters comprise one of the largest membrane bound protein families. These proteins transport substrates against a concentration gradient with ATP hydrolysis as a driving force across the membrane. Based on diverse structural characteristics and broad substrate specificity, ABC transport proteins alter the intracellular concentration of a

variety of therapeutically relevant compounds and cytotoxic agents. The efflux pumps serve as natural defense mechanisms, influence bioavailability and disposition of various structurally unrelated compounds (Hoffmann and Kroemer, 2004). P-glycoprotein (P-gp), a multiple drug resistant (MDR) gene product, is a 170-kDa transmembrane protein. It transports a wide range of compounds, including anticancer drugs, steroids, calcium channel blockers and antihistamines (Endicott and Ling, 1989; Borst et al., 1993; Germann et al., 1993; Borst et al., 2000; Pal and Mitra, 2006). P-gp-mediated efflux reduces the intracellular accumulation of these compounds, thereby diminishing drug efficacy. In the case of cytotoxic drugs, this leads to enhanced cell survival (Kim et al., 1998). P-gp is present on the apical membrane of many absorptive epithelial and endothelial cells. In the brain, P-gp is known to be localized on the subapical side of the choroid plexus epithelia , on the luminal membrane of brain microvessel endothelial cells and on the albuminal side of the BBB on adjacent astrocyte foot processes (Sakata et al., 1994; Ohnishi et al., 1995; Schinkel, 1999; Lin, 2004). Because of its localization and distribution, P-gp limits the oral absorption and bioavailability of PIs across intestine, brain, testis and placenta (Kim et al., 1998; Polli et al., 1999; Smit et al., 1999; Choo et al., 2000; Huisman et al., 2001; Huisman et al., 2002).

Recent studies have demonstrated that the PIs are also substrates for the multidrug resistance associated proteins (MRPs), belonging to the same ABC transporter family (Huisman et al., 2002; Bachmeier et al., 2005). MRPs are 190 kD proteins responsible for transport of drugs across lipid membranes from inside to outside the cell. The expression and localization of MRPs requires delineation of drug interactions with these efflux transporters. So far, eight MRP homologs have been identified for ABC proteins, MRP1-8. Human MRP1 and MRP2 have been known to be involved in efflux of many therapeutic agents such as antineoplastic agents, fluroquinolones and PIs like SQV, RVR and IVR (Bakos et al., 2000; Naruhashi et al., 2002; Letourneau et al., 2005; Pal and Mitra, 2006). MRP1 is a widely expressed transporter. When present in epithelial cells, this protein is found primarily in the basolateral membrane (Hipfner et al., 1999). High levels of MRP1 are present in the epithelium of the choroid plexus (Wijnholds et al., 2000) and in astrocytes (Decleves et al., 2000). MRP1 expression is also observed in primary cultured bovine brain microvessel endothelial cells (Zhang et al., 2004) and in rat brain parenchyma and microvessels (Regina et al., 1998) . However, it has been reported that MRP1 does not mediate substantial polarized transport of saquinavir (SQV), RVR and indinavir (IVR) in MDCKII-MRP1 cells (Huisman et al., 2002). In contrast to MRP1, MRP2 is localized on the apical membrane of several epithelia (Konig et al., 1999; Miller et al., 2000; St-Pierre et al., 2000). Its expression is found in liver canaliculi, renal proximal tubules, gut enterocytes, syncytiotrophoblast cells of the placenta

and possibly brain capillaries (Keppler and Kartenbeck, 1996; Schaub et al., 1997; St-Pierre et al., 2000; Zhang et al., 2000). Functionally it is similar to P-gp mediated elimination of toxic compounds in gut and placenta (Kruh and Belinsky, 2003). In rats, MRP2 contributes to hepatobiliary, intestinal and renal excretion and to the reduction of oral availability of its substrates (Dietrich et al., 2001; Dietrich et al., 2001). It has been fairly established that MRP2 also effluxes PIs (Huisman et al., 2002; Williams et al., 2002).

The human breast cancer resistance protein (BCRP/MXR) is a relatively new ABC efflux transporter, originally cloned from drug-selected human cancer cell lines and human placenta (Allikmets et al., 1998; Miyake et al., 1999; Doyle and Ross, 2003). Like P-gp, BCRP confers high levels of resistance to anthracyclines, mitoxantrone, and the camptothecins by enhancing drug efflux from the cell to extracellular space (Litman et al., 2000; Bates et al., 2001; Ejendal and Hrycyna, 2002). In addition to its role in inducing drug resistance, BCRP actively transports structurally diverse organic compounds , conjugated or unconjugated, such as estrone-3-sulfate, 17β-estradiol 17-(β-D-glucuronide), and methotrexate (Chen et al., 2003; Imai et al., 2003; Suzuki et al., 2003; Volk and Schneider, 2003). BCRP is prominently expressed in placental syncytiotrophoblasts, in the epithelium of the small intestine, and in the liver canalicular membrane (Maliepaard et al., 2001; Doyle and Ross, 2003). In fact, BCRP is also expressed in larger amounts than P-gp in the intestine (Taipalensuu et al., 2001). This strategic and substantial tissue localization of BCRP implies that it also functions as a protective drug efflux pump. A recent clinical study has shown that inhibition of BCRP significantly increases the oral bioavailability of topotecan from 40 to 97% (Kruijtzer et al., 2002). Thus, BCRP also plays a crucial role in determining absorption, distribution, and elimination of drugs that are substrates for this transporter. The expression of a BCRP homologue, known as brain multidrug resistance protein (BMRP), has also been reported in porcine brain capillary endothelial cells (Eisenblatter et al., 2003). Both BCRP and BMRP possess one half of the MDR1 P-gp structure with only six transmembrane domains and one ATP binding domain (Doyle et al., 1998). In addition to this structural similarity, most known substrates for BCRP/BMRP are similar to P-gp (i.e., hydrophobic, amphiphilic xenobiotics), suggesting that PIs may also interact with BCRP/BMRP (Litman et al., 2001; Doyle and Ross, 2003). In fact, results from a recent study suggest that saquinavir, ritonavir and nelfinavir may serve as inhibitors of BCRP (Gupta et al., 2004).

Thus, cellular efflux by ABC transporters, such as P-gp and MRPs, can play an important role in lowering intestinal absorption and brain penetration of LVR. Because these efflux transporters are oriented in the secretory (i.e., out of the organ or tissue) direction, high efflux will lead to lower net absorption for LVR. Sub-therapeutic

concentrations of PIs in the sanctuary sites like brain, testes and bone-marrow may cause persistence of viral infections leading to drug resistance (Williams and Sinko, 1999)**.** So far, no study has been undertaken to determine whether LVR interacts with these efflux pumps. Therefore, the purpose of this study is to assess the affinity of LVR for the efflux transporters using a well-defined system consisting of polarized non-human (canine) MDCKII cells, singly transfected with human MDR1, human MRP1/MRP2 complementary DNA (cDNA) or murine Bcrp1 cDNA and also to delineate quantitatively whether efflux limits permeation of LVR across intestinal and BBB absorptive cells.

2. MATERIALS AND METHODS

2.1. MATERIALS

Unlabeled (ulb) Lopinavir, Fumitremorgin-C and GF120918 were generous gifts from Abbott Laboratories Inc; National Institutes of Health AIDS Research and Reference Reagent Program (National Institutes of Health, Bethesda, MD) and GlaxoSmithKline Ltd respectively. [3H] Lopinavir (1Ci/mmol) and [3H] Mitoxantrone (4Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA). P-gp 4008 and MK-571 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Biomol (Plymouth meeting, PA, USA) respectively. High-performance liquid chromatography grade DMSO and methanol were obtained from Fisher Scientific Co. (Pittsburgh, PA, USA) and were used as solvents for preparing stock solutions of all drugs and inhibitors. Trypsin-EDTA solution, Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 medium and Minimum Essential Medium (MEM) were obtained from Invitrogen (Carlsbad, CA, USA) and fetal bovine serum (FBS) was obtained from Atlanta biologicals (Lawrenceville, GA, USA) respectively. Transwell plates were procured from Corning Costar Corp (Cambridge, MA, USA). All other chemicals were commercial products of reagent or enzymatic grade and were obtained from Fisher Scientific or Sigma Chemicals.

2.2. METHODS

2.2.1. CELL CULTURE

Studies were performed with wild-type Madin-Darby canine kidney cells type II (MDCKII/WT, a well-characterized, polarized epithelial cell line) and stable MDCKII transfectants overexpressing hMRP1 (MDCKII-MRP1 cells), hMRP2 (MDCKII-MRP2 cells), hP-gp/MDR1 (MDCKII-MDR1 cells) and murine Bcrp1 (MDCKII-Bcrp1 cells) (generously provided by Drs. A. Schinkel and P. Borst, The Netherlands Cancer Institute, Amsterdam). These cell lines were selected as these were the first known polarized epithelial cells that stably express these transporters, enabling direct comparison of their functional behaviors without the confounding effects of multiple transporters expressed in cell lines such as Caco-2 (Evers et al., 1997; Evers et al., 1998; Kool et al., 1999; Evers et al., 2000; Jonker et al., 2000; Wijnholds et al., 2000). Studies were performed with the following cell passage numbers from our initial stock: MDCKII-WT (passages

30 to 40), MDCKII-MRP1 (passages 5 to 25), MDCKII-MRP2 (passages 5 to 25), MDCKII-MDR1 (passages 5 to 15) and MDCKII-Bcrp1 (passages 1-5). MDCKII cell lines were cultured in T-75 flasks with DMEM (with high glucose and glutamine concentrations) supplemented with 10% FBS, 1% nonessential amino acids, penicillin 100 µg/ml and streptomycin 100 µg/ml. The medium was changed every alternate day; cells were harvested and passaged via trypsinization at 80 to 90% confluence (about 4 days of growth). Cells were also grown on collagen coated Transwell inserts (12-mm) with transparent polyester membranes. Transwell inserts were coated with type 1 rat tail collagen (100 µg/cm^2), equilibrated with medium, and seeded at a density of 25,000 cells/cm^2. Following seeding, medium was changed every alternate day, and transport or uptake studies were performed after 5-7 days.

2.2.2. TRANSPORT STUDIES

LVR transport was evaluated with monolayers of each cell line. All transport studies were performed with Dulbeco's modified phosphate-buffered saline (DPBS) containing glucose (1 g/liter) and HEPES (20mM) at pH 7.4. LVR and other drug solutions were prepared immediately prior to initiating a transport study. LVR was dissolved in methanol (not exceeding 2% v/v as the final concentration) and inhibitors were separately dissolved in DMSO (not exceeding 2% v/v as the final concentration) to prepare a stock solution and then diluted with DPBS to the specified working concentrations. Control solutions also contained the same amount of methanol/DMSO as in the drug solutions. Volumes of test solutions added were 0.5 and 1.5 ml, for apical (A) and basolateral (B) chambers respectively. Prior to testing, cultured monolayers were rinsed and equilibrated for 30 min with DPBS. While the drug solution was added either in the donor A or B chambers, the receiving chamber contained only DPBS. Samples (100 µl) were withdrawn from the receiving chamber at predetermined time points (30, 60, 90, 120, 150 and 180 min) and replaced with equal volume of DPBS to maintain sink conditions. Samples were transferred to scintillation vials containing 5 ml of scintillation fluid, and radioactivity was analyzed using a Beckman Scintillation Counter (LS6500). All transport studies were performed at least in triplicates ($n=3$) per treatment group. Representative results are presented for each study.

2.2.3. DELINEATION OF SPECIFIC EFFLUX TRANSPORT

P-gp mediated transporter function was selectively inhibited with P-gp 4008 (P4), a specific inhibitor of P-gp (Lee et al., 2004). For specific experiments, GF120918 (GF), a specific inhibitor of P glycoprotein and BCRP only, was used to inhibit P-gp (Hyafil et al., 1993). GF is found to modulate cells expressing Pgp and BCRP (de Bruin et al., 1999; Wallstab et al., 1999). MRP family transporters were selectively inhibited with MK-571, a specific leukotriene D4 (LTD4) receptor antagonist. MK-571, specifically inhibits at least MRP1 and MRP2, but not P-gp. MK-571 has been

shown to sensitize MRP1- and MRP2-expressing cell lines (Gekeler et al., 1995; van Aubel et al., 1998; Chen et al., 1999). Specific BCRP efflux inhibitor fumitremorgin-C (FC) was used to inhibit Bcrp1 mediated transport (Shukla et al., 2006). A concentration dependent study was performed with increasing concentrations of inhibitors, MK-571 in MDCKII-MRP2 cells, P4 and GF in MDCKII-MDR1 cells and FC in MDCKII-Bcrp1 cells, which demonstrated maximal inhibition at 100 μM, 50 μM and 2 μM and 10 μM respectively (Tables 3, 4 and 5).
To chemically inhibit MRPs in MDCKII-MRP transfected cell lines, the cell monolayers were equilibrated with MK-571 (100 μM in transport buffer) for 30 min prior to initiating an experiment to delineate the function of MRPs in LVR transport. Similarly, P4 (50 μM) and FC (10 μM) were added to inhibit P-gp and Bcrp1 in MDCKII-MDR1 and MDCKII-Bcrp1 cells respectively. The LVR (0.5 μCi/ml) solutions used in these transport studies also contained the respective inhibitors.

2.2.4. UPTAKE STUDIES

2.2.4.1.INHIBITION OF BCRP1-MEDIATED EFFLUX OF [3H] MITOXANTRONE (MX)

Mitoxantrone is a fluorescent compound and a well known BCRP substrate. Direct efflux of [3H] MX in MDCKII-Bcrp1 cells in the presence and absence of unlabeled LVR was measured to examine whether LVR inhibits Bcrp1-mediated efflux of [3H] MX. Briefly, cells were incubated with [3H] LVR (0.5μCi/ml) and various concentrations of unlabeled LVR (0-25 μM) or FC (10μM), in 1 ml of incubation buffer (DPBS pH 7.4.) for 30 min at 37°C. Cells were then washed once with ice-cold PBS, and resuspended in 1 ml of incubation buffer for 1 h at 37°C. Efflux was terminated by washing once in ice-cold PBS. The cell pellet was lysed with 1 ml of 1% SDS and 500 μl of the lysate was utilized for scintillation counting. Values were normalized to protein concentration that was measured using the remaining lysate by the modified Lowry assay. Intracellular [3H] MX was calculated based on the radioactivity associated with the cells and expressed as picomoles of MX per mg of protein.

2.3. DATA ANALYSIS

Linear regression of the amounts transported as a function of time yielded the rate of transport across the cell monolayer (dM/dt). Rate divided by the cross-sectional area available for transport (Kempf et al.) generated the steady state flux as shown in Eq. 1.,
Flux = (dM/dt)/ A Eq. 1
Permeability was calculated by normalizing the steady state flux to the donor drug concentration (C_d) according to Eq. 2., Permeability = Flux/C_d Eq. 2
Efflux ratio (ER) was calculated by dividing the BL-AP permeability by AP-BL permeability as shown in Eq.3.,
Efflux ratio = BL-AP permeability/AP-BL permeability Eq. 3

2.4. STATISTICAL ANALYSIS

All experiments were conducted at least in quadruplicate (n=4) and results are expressed as mean ± SD. Statistical comparison of mean values were performed using one-way analysis of variance (ANOVA) or Student *t* test (Graph Pad INSTAT, version 3.1). $*P < 0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. [3H] LVR transport across the MDCKII-WT cell line

[3H] LVR transport across the MDCKII-WT cells was determined in the absence and presence of various combinations of inhibitors. This study enabled us to compare the [3H] LVR permeabilities across the parental and the transfected cell lines in the absence and presence of specific efflux inhibitors. LVR permeabilities across MDCKII-WT cells are summarized in Table 1. Directional transport of [3H] LVR across MDCKII-WT cells in the absence of efflux inhibitors was very low (Fig.2a). As a result, there was not a significant difference in the permeability of [3H] LVR in the absence or presence of efflux inhibitors (Fig.2b) in both A and B directions as compared to control, indicating minimal expressions of efflux transporters in MDCKII-WT cells. Efflux ratios of [3H] LVR in MDCKII-WT were 1.32 in the absence of any inhibitor and 1.10, 1.08, 1.27 and 1.07 in the presence of P-gp inhibitor (P4), MRP inhibitor (MK), BCRP inhibitor (FC) and all the three inhibitors combined (P4+FC+MK) respectively (Table 1). Therefore, it can be easily concluded from this study that a very minimal level of active efflux transporters is expressed in the MDCKII-WT cell line.

3.2. [3H] LVR transport across MDCKII-MDR1 cells

LVR permeabilities and the respective ERs obtained in the MDCKII-MDR1 transfected cell line are presented in Table 2. MDCKII-MDR1 cells exhibited significant directional transport of [3H] LVR in the B-A direction. LVR B-A transport was significantly higher (5 fold) as compared to A-B transport (Figs.2c and d). A concentration dependent inhibition of LVR transport indicated that P4 at 50μM concentration was optimal for inhibition of P-gp mediated efflux in MDCKII-MDR1 cells. In the presence of P4 (50μM), the A-B and B-A transport of [3H] LVR was almost similar with an ER close to 1.03 as compared to 4.91 for the control. This apically directed MDR1 transport of [3H] LVR was substantially inhibited by P-gp inhibitor, P4. We further challenged LVR transport with another P-gp inhibitor, GF120918, which also inhibits BCRP mediated efflux. At 2μM concentration, GF exhibited an ER of 1.06, a number similar to P4 thereby causing a complete blockade of LVR efflux.

3.3. [3H] LVR transport across MDCKII-MRP2 cells

LVR permeabilities and the respective ERs obtained in the MDCKII-MRP2 transfected cell line are presented in Table 3. MDCKII-MRP2 cells also exhibited significant directional transport

of [3H] LVR. B-A transport of [3H] LVR was significantly higher (3 fold) relative to A-B transport (Figs.2e and f). A concentration dependent inhibition of LVR transport indicated that 100 μM MK-571 produced complete inhibition of MRP mediated efflux. ER for [3H] LVR transport was found to be 2.9 in the absence of any inhibitor and 1.0 in the presence of MK-571 (100 μM), a MRP inhibitor. Therefore, MK-571 at 100 μM was capable of completely blocking apically directed MRP2 mediated transport of [3H] LVR.

3.4. [3H] LVR transport across MDCKII-MRP1 cells

LVR permeabilities and respective ERs obtained in the MDCKII-MRP1 transfected cell line are noted in Table 4. MDCKII-MRP1 cells did not exhibit significant directional ransport of [3H] LVP (Figs.2g and h). ER for [3H] LVR transport was found to be 1.26 in the absence of any inhibitor, and 1.02 in the presence of 100 μM MK-571. Although the difference in the ER values for LVR in the presence and absence of MK-571 (0.24) are statistically significant, these values in MDCKII-MRP1 cells are not so significantly different for P-gp (3.88) and MRP2 (1.9) cell lines. These results indicate that LVR is not a good substrate for MRP1.

3.5. [3H] LVR transport across MDCKII-Bcrp1 cells

LVR permeabilities and respective ERs obtained in the MDCKII-Bcrp1 transfected cell line are summarized in Table 5. To determine the optimal concentration of FC for inhibition of Bcrp1 mediated efflux, a concentration dependent transport study was carried out with [3H] MX, a classic substrate for BCRP efflux pump. At 10μM FC concentration, MX exhibited an ER of 1.14 relative to 2.45 for control. Hence, this concentration was selected for all further inhibition experiments involving Bcrp1 mediated efflux. No significant directional transport of [3H] LVR was observed in MDCKII-Bcrp1 cell monolayers (Figs.2i and j). ER values of [3H] LVR were 1.08 and 1.17 in the absence and in the presence of BCRP inhibitor respectively. These values are not significantly different suggesting that LVR is probably not transported by Bcrp1. Also, to confirm our observation that LVR is not a substrate for Bcrp1, cellular accumulation of [3H] MX was measured in MDCKII-Bcrp1 cells in the presence of unlabeled LVR and FC (Fig.3). Cellular uptake of MX was elevated in the presence of BCRP efflux inhibitor, but unlabeled LVR did not show any competitive uptake with MX. Therefore, this data substantiates our early conclusion that LVR is not a good substrate for Bcrp1.

3.6. Effects of multidrug resistance proteins on [3H] LVR transport

Effects of multidrug resistance proteins on [3H] LVR transport across all transfected MDCKII cell lines (MDCKII-MDR1, MDCKII-MRP2, MDCKII-MRP1 and MDCKII-Bcrp1) were compared with MDCKII-WT cell line (Fig.2). MDCK cells form a polarized monolayer in which the rate of basolaterally or apically directed translocation can be measured after

adding compounds to the A or B side of the monolayer. Passive translocation will be similar in both directions. Since these efflux transporters excrete compounds from within the cell to extracellular space, the direction of transport will depend on the subcellular localization of such transporters in polarized cells. Thus MRP1, being localized on the basolateral membrane should transport in the B direction. Conversely MRP2, Bcrp1 and P-gp being localized on the apical membrane should transport in the apical direction. MDCKII-MDR1 and MDCKII-MRP2 cells exhibited significant directional transport of [3H] LVR in the B-A direction (Figs.1c and 1e) as compared to MDCKII-WT cells, indicating that these efflux transporters are present on the apical membrane. The differences in ER values of [3H] LVR transport across MDCKII-WT cells (1.32) relative to MDR1 (4.91) and MRP2 (2.89) transfected cell lines are highly significant. Such differences imply an increased efflux of [3H] LVR by MDR1 (P-gp) and MRP2 respectively due to enhanced expression of these transporters in these cell lines. The ER values in MDCKII-MDR1 and MDCKII-MRP2 cell lines were reduced to almost 1.0 in presence of efflux inhibitors, which suggest that LVR is a substrate for both P-gp and MRP2 efflux proteins. However, MRP1 and Bcrp1 did not produce substantial differences in directional transport of [3H] LVR (Figs.1g and 1i) as compared to MDCKII-WT cells, which confirms that LVR is not a good substrate for MRP1 and Bcrp1 efflux transporters. Differences in ER values (ER values with maximal inhibitory concentrations subtracted from the control ER values) among MDCKII-MDR1 and MDCKII-MRP2 cell lines were compared and the affinity of LVR for these efflux pumps was postulated. These differences were 1.9 for MRP2 and 3.88 for P-gp. Thus, from our studies, it appears that LVR has more affinity for P-gp than MRP2.

4. CONCLUSION

Our results demonstrate for the first time that LVR is extruded from absorptive cells by P-gp/MDR1 and MRP2 and this efflux can be prevented by specific inhibitors such as P4, GF120918 for P-gp and MK571 for MRP2. This behavior was similar to other PIs like SQV, RVR and IVR which were also shown to interact with P-gp and MRP2 (Huisman et al., 2002). LVR did not interact significantly with MRP1. P-gp and MRP2 are present apically in the epithelial cells of intestine, kidney, BBB and other tissues which can lead to significant efflux of LVR causing diminished oral absorption like other PIs (Williams and Sinko, 1999). It has been reported earlier that several PIs like SQV, RVR and nelfinavir (NFV) are inhibitors of BCRP efflux transporter and amprenavir (APV) and IVR are not (Gupta et al., 2004). Our results indicate no substantial difference in the ER values of LVR in MDCKII-Bcrp1 cells in the presence and absence of FC, a specific and potent BCRP efflux inhibitor. These results clearly indicate that LVR is not a substrate of Bcrp1 and does not interact with Bcrp1.

Since specific inhibitors of P-gp and MRP2 significantly elevated the transport of LVR in the transfected cell lines, there is no doubt that these efflux transporters may not allow sufficient intracellular accumulation of LVR thereby reducing anti-HIV efficacy. Intestinal P-gp is localised extensively on the villus tip of enterocytes (Hunter et al., 1993)(i.e., the main site of absorption for orally administered compounds (Katragadda et al., 2005)). It is, therefore, ideally positioned to limit the absorption of compounds by pumping them back into lumen. Since it is known that P-gp limits the oral bioavailability and BBB penetration of PIs like SQV, NFV and IVR (Kim et al., 1998), it can be easily be conceived that P-gp might also play an important role in the disposition of LVR. Since, MRP2 is also now known to localize in the apical membrane of polarized cells, it is possible that MRP2 also play a synergistic role along with P-gp in the extrusion of LVR from the cells.

Potential interaction between efflux transporters in the gut and CYP3A4 metabolizing enzymes may be a source of variation associated with LVR absorption and distribution (Williams and Sinko, 1999). In human, CYP3A4 is the principal enzyme involved in the hepatic and intestinal drug metabolism, and there is a striking overlap of substrate specificites among CYP3A4, P-gp and MRPs. The coordinated function of both CYP3A and P-gp, MRPs can dramatically lower oral bioavailability of compounds which are substrates for both (van Asperen et al., 1997; Wacher et al., 1998) and this may also be true for LVR.

From these results it may be postulated that inhibitors of efflux transporters can help PIs to achieve therapeutic concentrations in blood and sanctuary sites like brain and testes. To find a single inhibitor which can overcome all efflux transporters is not an easy task. Use of multiple inhibitors concomitantly with LVR for such purpose may lead to toxicity. Therefore, prodrug derivatization of LVR may be an effective strategy to bypass P-gp and MRP2 mediated efflux. Our laboratory has previously reported that peptide prodrug derivatization of SQV such as Val-Val-SQV and Gly-Val-SQV can enhance transcellular permeability across MDCKII-MDR1 cells as compared to the parent drug (Jain et al., 2005). These prodrugs are taken up by peptide influx transporters expressed on the outer leaflet of cellular membranes and avoid recognition by efflux pumps. Hence, our future work would be to design prodrugs of LVR enhancing both solubility and permeability across gut and BBB.

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Both P-gp and MRP2 mediate transport of Lopinavir, a protease inhibitor

Figure 1: Structure of Lopinavir

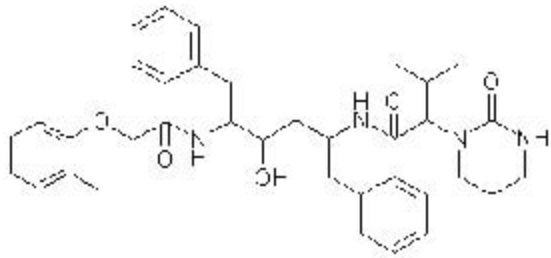

Figure 3: Intracellular accumulation of [3H] MX in MDCKII-Bcrp1 cells

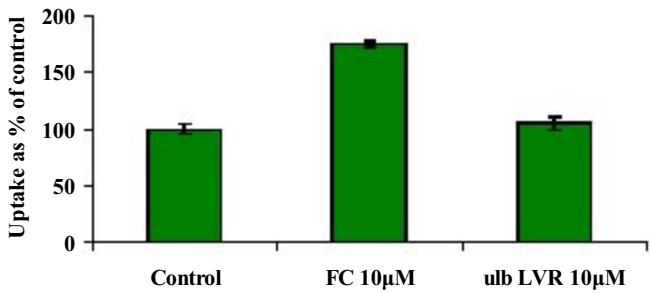

Figure 2: Transport of [3H] Lopinavir across MDCKII-WT as compared to the transfected cell lines of [3H] MX in MDCKII-Bcrp1 cells

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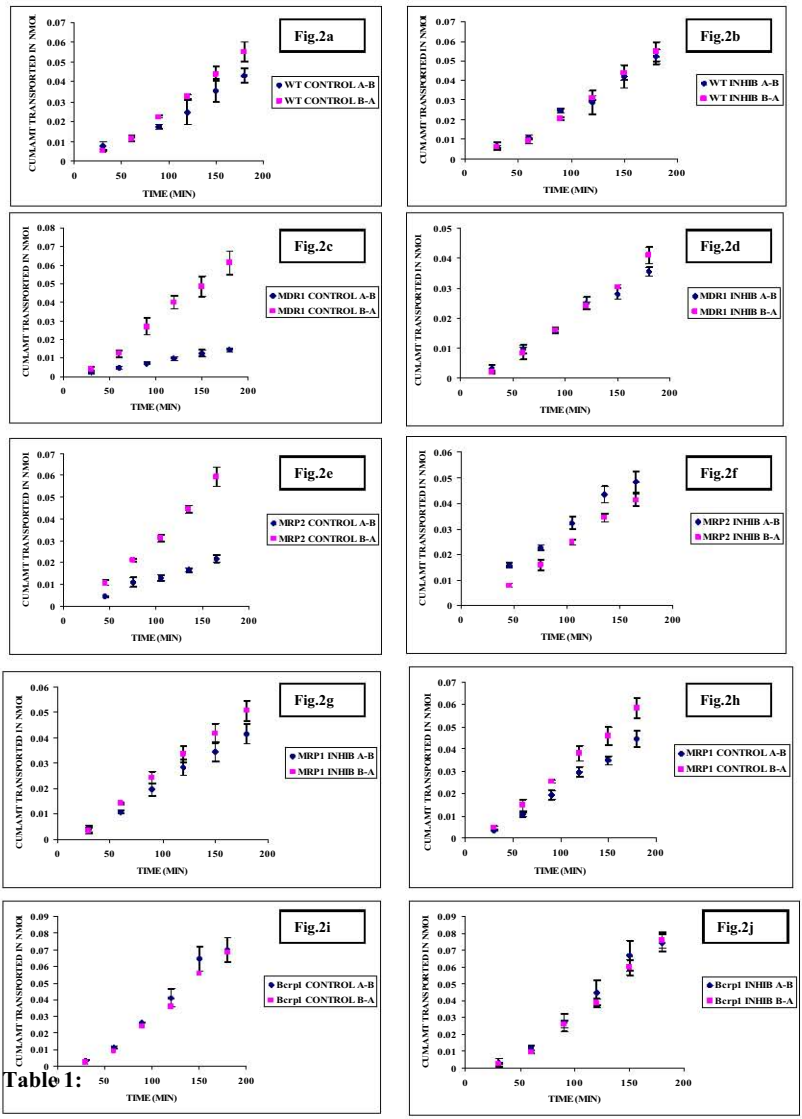

Table 1:

Permeability and Efflux ratios of 3H LVR across MDCKII-WT cells			
	A-B Permeability * 10^6 cm/sec	B-A Permeability * 10^6 cm/sec	Efflux ratio
Control	4.31 ± 0.50	5.70 ± 0.49	1.32
P4 (50µM)	5.67 ± 0.47	6.28 ± 0.61	1.10
MK571 (100µM)	5.29 ± 0.48	5.72 ± 0.41	1.08
FC (10µM)	4.25 ± 0.30	5.39 ± 0.14	1.27
MK571+P4+FC	5.26 ± 0.68	5.64 ± 0.34	1.07
ulb LVR 2.5µM	3.85 ± 0.52	5.02 ± 0.44	1.30
ulb LVR 5µM	4.05 ± 0.37	4.97 ± 0.18	1.23
ulb LVR 10µM	4.96 ± 0.20	5.94 ± 0.20	1.20
ulb LVR 25µM	5.21 ± 0.16	5.82 ± 0.52	1.12

Table 2:

Permeability and Efflux ratios of 3H LVR across MDCKII-MDR1 cells			
	A-B Permeability * 10^6 cm/sec	B-A Permeability * 10^6 cm/sec	Efflux ratio
Control	1.32 ± 0.08	6.49 ± 0.76	4.91
P4 (10µM)	3.20 ± 0.64	4.55 ± 0.30	1.40
P4 (25µM)	3.60 ± 0.06	4.26 ± 0.23	1.18
P4 (50µM)	4.60 ± 0.15	4.78 ± 0.11	1.03
GF (0.5µM)	4.61 ± 0.59	6.70 ± 0.51	1.46
GF (1µM)	5.64 ± 0.39	7.28 ± 0.39	1.25
GF (2µM)	6.32 ± 0.45	6.70 ± 0.37	1.06
ulb LVR 2.5µM	6.62 ± 0.44	8.75 ± 0.28	1.32
ulb LVR 5µM	7.90 ± 0.41	9.37 ± 0.99	1.18
ulb LVR 10µM	6.70 ± 0.31	7.85 ± 0.23	1.12
ulb LVR 25µM	5.55 ± 0.18	6.06 ± 0.28	1.09

Table 3:

Permeability and Efflux ratios of 3H LVR across MDCKII-MRP2 cells			
	A-B Permeability * 10^6 cm/sec	B-A Permeability * 10^6 cm/sec	Efflux ratio
Control	2.32 ± 0.13	6.73 ± 0.45	2.9
MK571(50µM)	3.08 ± 0.14	4.39 ± 0.23	1.52
MK571(100µM)	4.89 ± 0.28	4.90 ± 0.28	1
MK571(200µM)	4.60 ± 0.15	4.78 ± 0.11	1.02
ulb LVR 1µM	3.45 ± 0.26	4.70 ± 0.24	1.36
ulb LVR 2.5µM	4.46 ± 0.27	5.72 ± 0.25	1.23
ulb LVR 5µM	4.49 ± 0.20	5.74 ± 0.20	1.28
ulb LVR 10µM	4.50 ± 0.04	5.22 ± 0.31	1.16
ulb LVR 25µM	1.88 ± 0.06	2.0 ± 0.32	1.09

Table 4:

Permeability and Efflux ratios of 3H LVR across MDCKII-MRP1 cells			
	A-B Permeability * 10^6 cm/sec	B-A Permeability * 10^6 cm/sec	Efflux ratio
Control	4.54 ± 0.27	5.72 ± 0.25	1.26
MK571(50μM)	4.71 ± 0.14	5.28 ± 0.23	1.12
MK571(100μM)	5.09 ± 0.28	5.20 ± 0.43	1.02
MK571(200μM)	4.97 ± 0.52	5.05 ± 0.34	1.02
ulb LVR 2.5μM	3.32 ± 0.27	4.62 ± 0.25	1.40
ulb LVR 5μM	3.45 ± 0.26	4.70 ± 0.24	1.34
ulb LVR 10μM	4.99 ± 0.16	5.74 ± 0.26	1.15
ulb LVR 25μM	4.49 ± 0.20	5.74 ± 0.20	1.08

Table 5:

Permeability and Efflux ratios of 3H LVR across MDCKII-Bcrp1 cells			
	A-B Permeability * 10^6 cm/sec	B-A Permeability * 10^6 cm/sec	Efflux ratio
Control (3H MX)	3.03 ± 0.28	7.42 ± 0.60	2.45
FC 1μM (3H MX)	5.06 ± 0.54	7.59 ± 0.79	1.52
FC 5μM (3H MX)	6.00 ± 0.58	8.44 ± 0.72	1.4
FC 10μM (3H MX)	9.11 ± 0.98	7.97 ± 0.72	1.14
Control (3H LVR)	7.64 ± 0.91	7.65 ± 0.63	1.08
FC+ MK571+P4 (3H LVR)	8.53 ± 0.28	8.42 ± 0.60	1.17

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