*Pharmaceutical Research, Vol. 25, No. 5, May 2008 (© 2007)*
DOI: 10.1007/s11095-007-9473-3

*Research Paper*

# An Examination of the Effect of Intestinal First Pass Extraction on Intestinal Lymphatic Transport of Saquinavir in the Rat

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*Received July 27, 2007; accepted October 5, 2007; published online November 2, 2007*

***Purpose.*** To assess the impact of intestinally based efflux/elimination processes on the extent of intestinal lymphatic transport of saquinavir. To compare the relative effects of co-administration of P-gp/CYP modulators on intestinal lymphatic transport versus systemic bioavailability of saquinavir.
***Methods.*** A cremophor mixed micelle formulation of saquinavir alone, or co-administered with P-gp/CYP modulators, verapamil, ketoconazole or cyclosporine, was dosed intraduodenally in the mesenteric lymph duct cannulated anaesthetized rat model.
***Results.*** Co-administration of P-gp/CYP modulators resulted in significant increases in the extent of intestinal lymphatic transport of saquinavir. A comparison of the relative enhancement of lymphatic transport and plasma bioavailability compared to control (i.e. saquinavir alone) reveals a greater effect of verapamil and ketoconazole on the amount of drug transported by the lymphatic route, an observation consistent with a preferential targeting of saquinavir via the intestinal lymphatics. In contrast co-administration of cyclosporine increased both the extent of lymphatic transport (5.5-fold), and systemic bioavailability (4.1-fold).
***Conclusions.*** Intestinal P-gp/CYP efflux/elimination restricts saquinavir transport via the intestinal lymphatics in the rat. Targeted increases in intestinal lymphatic levels of saquinavir may be achieved by selective inhibition of intestinal P-gp and/or CYP.

**KEY WORDS:** cytochrome P450; P-glycoprotein; saquinavir; targeted intestinal lymphatic transport.

## INTRODUCTION

Saquinavir was the first protease inhibitor to be licensed for the treatment of HIV infections and, over the last decade, has become a leading component of combination chemotherapy, commonly termed highly active antiretroviral therapy (HAART) (1,2). However, the bioavailability of saquinavir is low (4%), formulation dependent and displays wide inter-individual variability. This arises as a consequence of multiple factors, including the poor solubility characteristics, but also extensive first pass metabolism, primarily mediated by cytochrome P450 (CYP) (3,4). In addition, saquinavir interacts with the apically polarized efflux pump, P-glycoprotein (P-gp), (5,6), which may also limit intestinal uptake and/or influence enterocyte based metabolism via the apical recycling hypothesis (7). Thummel et al. (1997) predicted, on the basis of *in vitro* metabolism kinetics, that the high first pass metabolism of saquinavir *in vivo* could be entirely attributed to intestinal metabolism (8). However more recent studies, both *in vitro* and *in vivo*, suggest that intestinal and hepatic metabolism may, on average, contribute equally to overall first pass extraction (9,10).

There are numerous reports indicating that the lymphoid tissue is the major storage and/or replication site of HIV *in vivo* (11,12). The clinical evidence, to date, indicates that HARRT can suppress viral replication in peripheral blood to below the limit of detection (13). However, the HIV virus appears to persist and replicate within certain type of lymphoid cells, such as memory CD4+ T lymphocytes, the majority of which are harbored within the gut associated lymphoid tissue (GALT) (14). These high viral load cells in intestinal lymphoid tissue can serve as a sanctuary site for HIV replication and hence targeted delivery of antiviral agents specifically to the intestinal lymphatics may confer therapeutic advantages. The concept of intestinal lymphatic targeting of lipophilic drugs by lipid formulation approaches is now well established (15). The effect of a lipid based formulation approach for enhancing intestinal lymphatic transport of saquinavir has previously been investigated (16). The extent of lymphatic transport however was lower than expected on the basis of the highly lipophilic nature of saquinavir (clog $P$=4.5).

There have been several studies demonstrating the impact of intestinal efflux proteins and/or enterocyte based metabolism on limiting oral bioavailability of saquinavir. The effect of specific modulators of P-gp and/or CYP, such as verapamil, ketoconazole and cyclosporine, on increasing intestinal permeability of saquinavir has been demonstrated *in vitro* in Caco 2 cell lines (5). Furthermore, a number of *in situ*/*in vivo* studies have demonstrated the effect of inhibition

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**ABBREVIATIONS:** CYP, Cytochrome P450; P-gp, P glycoprotein; SQV, Saquinavir; TG, Triglyceride.

of Pgp/CYP like processes on increasing portal and systemic blood levels of saquinavir (9,17). To this end, saquinavir therapy is now generally recommended in combination with another protease inhibitor, ritonavir, primarily on the basis of a favorable pharmacokinetic interaction (i.e. steady state saquinavir plasma concentrations increased by up to 30-fold via ritonavir mediated inhibition of first pass metabolism) (18). However, to date no study has examined the potential effects of intestinally based efflux and/or metabolism on the intestinal lymphatic absorption pathway. In the present study we hypothesized that concomitant oral administration of P-gp/CYP inhibitors might also increase the intestinal lymphatic transport of saquinavir and have tested this in the mesenteric lymph duct cannulated rat model. The administered formulation contains a P-gp/CYP modulator co-solubilized in the vehicle. The three Pgp/CYP modulators chosen for this investigation were verapamil, ketoconazole and cyclosporine. Verapamil is a potent inhibitor of P-gp activity but also acts as a substrate/inhibitor of CYP 3A4 (8,19). Ketoconazole is a well-characterized non-competitive inhibitor of CYP 3A4 (20,21). It is not believed to be a good substrate for P-gp, although it has been shown to inhibit Pgp in a vinblastine resistant cell line (KB-V1) (22), and it is likely to affect other efflux transporters (23). Cyclosporine is an effective inhibitor of P-gp (24) and also acts as a substrate/inhibitor for CYP3A (25). With the objective of achieving maximal suppression of P-gp/CYP activity in the intestine, and by comparisons with a number of in situ/in vivo studies, a final concentration of 1 mM modulator was used, equivalent to doses of 4.9, 5.3 and 12 mg/kg of verapamil, ketoconazole and cyclosporine respectively (26,27). In summary, the specific objectives of this study were threefold: (a) to demonstrate a proposed P-gp/CYP mediated effect on the intestinal lymphatic transport of saquinavir; (b) to assess the relative effects of co-administration of three P-gp/CYP modulators, verapamil, ketoconazole and cyclosporine, on intestinal lymphatic transport of saquinavir and (c) to compare the effects on lymphatic transport versus systemic bioavailability of saquinavir.

## MATERIALS AND METHODS

### Materials

Saquinavir base (Ro-31-8959/000) and internal standard (Ro 31-9564) were kindly donated by Roche Products UK. Cyclosporine A was donated by Sandoz. Cremophor EL (Polyoxyl 35 castor oil) was a gift from BASF. Ketoconazole was purchased from Tocris (UK). Verapamil, Oleic acid (*cis*-9-Octadecenoic acid) and tri-olein were obtained from Sigma (Ireland). All solvent were of HPLC grade.

### Methods

#### *Mixed Micelle Formulations*

The cremophor oleic acid mixed micelle formulations were prepared by drop wise addition of oleic acid (at 37°C with continuous stirring) over 30 min to a simple micellar solution of cremophor EL (2%w/v) dissolved in a volume of phosphate buffer (pH 7.2). All solutions were viewed in front of a strong light source in order to check that the solution was transparent, and allowed to equilibrate at room temperature for 12–24 h. Micellar solutions containing 1 mM of either verapamil, ketoconazole or cyclosporine, were allowed to equilibrate for 12 h prior to addition of saquinavir. Saquinavir (1.667 mg/ml) was added to the mixed micellar formulations 2 h prior to intraduodenal dosing. Saquinavir concentrations in all micellar solutions were verified on the day of dosing using a validated HPLC assay.

#### *Surgical Procedures*

All animal experiments were performed in accordance with EU directive 86/609 (as implemented in Ireland by Statutory Instrument 17/9) in association with BioResources unit, Trinity College, Dublin, which is registered with the Department of Health and Children. Male Wistar rats, (280–320 g) were fasted for 24 h with free access to water. The animals were anaesthetized for the duration of the experiment using 50 mg/kg sodium pentobarbital given by intraperitoneal injection. The duodenum and mesenteric lymph duct were cannulated as previously described (28). 3 ml of test micellar solutions were administered by intraduodenal infusion over 2 h (i.e. 1.5 ml/h). Normal saline was then infused at a rate of 1.5 ml/h for the remaining 6 h of the experiment to rehydrate the animal. Lymph was collected hourly for 8 h (i.e. 2 h dosing and 6 h post-dosing periods), in pre weighed cooled glass tubes containing anticoagulant (heparin). Non-lymph cannulated were sham operated in terms of the mesenteric lymph duct cannulation and had an intra-duodenal cannula (for rehydration) inserted, as described above. Serial blood samples (0.25 ml) were taken in both lymph cannulated and non-lymph cannulated animals, by cardiac puncture, at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h after commencement of drug administration. Lymph triglyceride (expressed as mg equivalents of C18 LCT) was determined using a Triglyceride Enzymatique PAP 150 assay (bioMeirieux) as described previously (16).

#### *Analytical Procedures*

Lymph and plasma concentrations of saquinavir were determined using a previously validated method for saquinavir analysis (16). Briefly, saquinavir detection was performed at $\lambda$=238 nm, using a 5-μm Megellen C8 column (250×4.6 mm, Phenomenex) stationary phase. The mobile phase consisted of a 63:37 mix of acetonitrile:ammonium acetate (10 mM) and the flow rate was 1 ml/min. Retention times for saquinavir and internal standard (Ro 31-9564) were 9 and 21 min, respectively. Lymph/plasma samples (0.2 ml) were then made basic by the addition of 50 μl of 10 M $K_2CO_3$ and extracted with diethyl ether (4 ml). The organic phase was decanted and evaporated to dryness. The residue was dissolved in HPLC mobile phase (0.15 ml), vortexed for 1 min, and 50 μl was injected onto the column. The analysis of saquinavir in plasma and lymph exhibited excellent linearity ($r^2 \geq 0.99$) over the concentration range of 25–400 ng/ml for plasma and 40–800 ng/ml for lymph. The limit of quantitation was 25 ng/nl for plasma and 40 ng/ml for lymph (CV≤20%). The extraction recoveries of saquinavir were all in excess of 93%, the recovery of internal standard was >97%.

*Determination of Saturation Solubility of Saquinavir in Triolein*

Saturation solubility of saquinavir in triolein was determined by adding excess saquinavir to 5 ml triolein in 10 ml amber glass ampoules (29). The flame-sealed vials were equilibrated in a thermostated water bath equipped with a shaker at 37°C for up to 7 days. A vial was sampled from each replicate batch every 24 h. The samples were filtered though a 0.22 μm filter, and diluted immediately with an appropriate quantity of mobile phase, to prevent precipitation and subsequently analyzed by HPLC. At least three determinations were made per sample, with a minimum of four experiments ($n$=4).

*Data Analysis*

The extent of lymphatic transport was calculated using the concentration of drug found in each lymph sample, multiplied by the volume of the lymph produced per hour, and expressed as a cumulative percentage of the dose. Plasma concentrations versus time data for saquinavir in individual rats were tested using the nonlinear curve fitting and model development program, Micromath R Scientist® for Windows® Version 1.05 (Micromath R Scientific Software). In order to calculate saquinavir bioavailability (F), the AUC obtained following intravenous (i.v.) administration of saquinavir determined in a previous study, was used (16). The AUC for saquinavir after intra-duodenal administration was obtained using the linear trapezoidal rule from time zero to the last measured time point, followed by the addition of the extrapolated tail area, calculated by dividing the last measured plasma concentration by the terminal rate constant, as determined from i.v. data. Saquinavir bioavailability was estimated from the ratio of the dose normalized AUC's after i.d. and i.v. administration as follows: $F = \left(AUC^{i.d.}_{0\to\infty h}/D^{i.d.}\right)/\left(AUC^{i.v.}_{0\to\infty h}/D^{i.v.}\right)$. In lymph-cannulated animals, saquinavir bioavailability reflects the contribution of portal absorption to systemic plasma concentrations, and not intestinal lymph transport (as mesenteric lymph was collected and removed). Bioavailability in non lymph cannulated rats, is a more accurate estimate of the extent of systemic absorption, as it reflects the contribution of both portal uptake, and transport via the lymph, on systemic plasma concentrations.

One way analysis of variance (for multiple comparisons) and student's $t$ test were used to determine the statistical significance ($p$<0.05) of calculated results between the experimental groups.

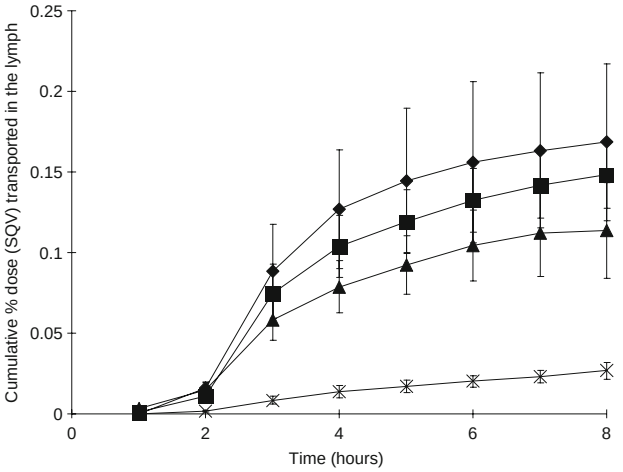


**Fig. 1.** Cumulative percent of dose of saquinavir (mean±s.e., $n$≥5) collected in intestinal lymph as a function of time. Saquinavir (5 mg) was administered intraduodenally as mixed micellar vehicle (2% Cremophor: 40 mM Oleic acid) alone (*crosses*) or containing either 1 mM verapamil (*triangles*), 1 mM cyclosporine (*squares*) or 1 mM ketoconazole (*prisms*).

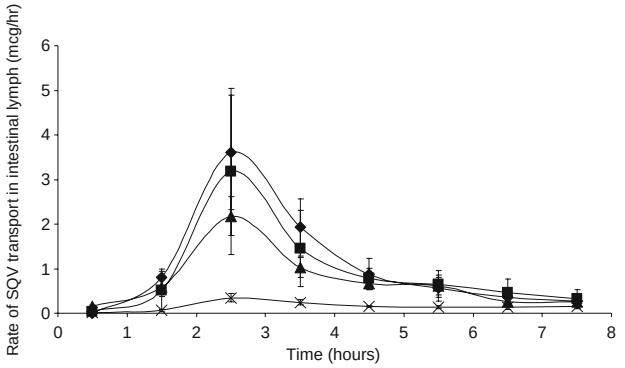


**Fig. 2.** The rate of intestinal lymphatic transport of saquinavir as a function of time (mean±s.e., $n$≥5). Saquinavir (5 mg) was administered intraduodenally as mixed micellar vehicle (2% Cremophor: 40 mM Oleic acid) alone (*crosses*) or containing either 1 mM verapamil (*triangles*), 1 mM cyclosporine (*squares*) or 1 mM ketoconazole (*prisms*).

## RESULTS

### Intestinal Lymphatic Transport of Saquinavir

The extent of lymphatic transport of saquinavir from a cremophor mixed micelle formulation was 0.027% of the administered dose, as previously reported (16). In the present study, co-administration of verapamil (1 mM), ketoconazole (1 mM) and cyclosporine (1 mM) resulted in significant increases in the cumulative extent of intestinal lymphatic transport of saquinavir, as depicted (Fig. 1). The increases in intestinal lymphatic transport were all significantly greater than control ($p$<0.05) and the rank order in the % dose administered transported in intestinal lymph over 8 h was as follows: ketoconazole (0.17%)> cyclosporine (0.15%) > verapamil (0.11%). The differences between groups treated with each modulator, however, were not significant. The rate of intestinal lymphatic transport of saquinavir, as a function of the different formulations (Fig. 2) demonstrates that peak transport occurred between 2 and 3 h for all the systems examined. The maximal rates of transport were significantly higher ($p$<0.05) for the systems containing P-gp/CYP modulators versus control (i.e. SQV alone), with a similar rank order i.e. ketoconazole > cyclosporine > verapamil. The rate profiles confirm that the increases in lymphatic transport are related to an increased drug transfer rate through enterocyte cells as a result of inhibition of intestinally based efflux and/or metabolism.

### Intestinal Lymphatic Transport of Triglycerides

Table I present a summary of the cumulative $C_{18}$ triglyceride transport and lymph flow data after intraduodenal dosing of the different formulations examined in this study. Each formulation contained the same lipid content, with an equivalent dose of 33.85 mg of oleic acid and 60 mg of cremophor EL administered to each animal. Hence any differences in lipid turnover are attributable to the effects of the co-administered modulator. The lipid turnover for the formulations containing either 1 mM verapamil or 1 mM ketoconazole was similar to the lipid turnover in the absence of a modulator i.e. saquinavir alone. However following concomitant administration of 1 mM cyclosporine, the observed triglyceride transport, as determined by the cumulative extent of recovery after 8 h, was significantly reduced ($p<0.05$). The rank order of the percentage of the administered (exogenous) dose of oleic acid (33.85 mg) recovered as re-synthesized triglyceride in intestinal lymph after 8 h, after endogenous (blank) triglyceride correction, was as follows: ketoconazole (92.13%) ≥verapamil (82.88%) > cyclosporine (36.8%). However, to assume an almost quantitative recovery of administered oleic acid is most likely incorrect, as the cremophor EL (polyethoxylated castor oil) may also be digested to liberate free fatty acids as recently reported (30,31).

### Relationship Between Saquinavir Lymphatic Transport and Triglyceride Turnover

While it appears that, at the concentrations studied, cyclosporine reduced triglyceride turnover, the extent of saquinavir lymphatic transport was not significantly affected (Fig. 1), which may imply that this formulation produced a higher loading per mg of lymph triglyceride. This was investigated by examining the relationships between saquinavir transport rate and triglyceride transport rate (Fig. 3). The slopes of these lines represent the relative concentration, or apparent loading of saquinavir, per mg of lymph triglyceride. A strong positive correlation exists between saquinavir transfer rates and the intestinal triglyceride turnover for all systems examined, confirming that saquinavir is transported into the lymph in association with triglyceride-rich lipoproteins. Co-administration of P-gp/CYP modulators produced higher apparent loadings of saquinavir in lymph triglyceride versus control (i.e. SQV alone). The higher loadings most likely reflect an improved efficiency of the drug transfer to lipoproteins within the enterocyte, following inhibition of intestinal Pgp/CYP (16).

The % loading and % of theoretical maximum loading of saquinavir in lymph triglyceride are presented in Table II. The % theoretical maximal loading of saquinavir may be calculated from the actual loading of saquinavir in intestinal triglyceride (i.e. weight of saquinavir per gram of the lymph triglyceride (g/g)), expressed as a % of the saturation solubility (g/g) of saquinavir in pure triglyceride (32). The saturation solubility of saquinavir in triolein (i.e. C18 triglyceride) was determined to be 0.2±0.04% w/w. For the SQV alone formulation, 1.35 μg of saquinavir (Fig. 1) and 43 mg of intestinal triglyceride (Table I) were recovered in intestinal lymph after 8 h. This represents an actual loading of 0.003% w/w in intestinal triglyceride or 1.5% of the theoretical maximum loading of saquinavir in pure triolein. All of the formulations containing modulators produced significant increases in the % actual loading of saquinavir per mg of lymph triglyceride, compared to control (i.e. SQV alone). Furthermore, the formulations containing cyclosporine produced the highest loadings per mg of lymph triglyceride. This effect appears to compensate for a lower triglyceride turnover observed i.e. despite a reduction in intestinal triglyceride transport, the drug concentration within the lymph triglyceride is higher. It is generally accepted that for lipophilic drugs, loadings of up to 25% theoretical maximum, can be achieved in this animal model under similar experimental conditions (32). This would suggest that the loadings of saquinavir achieved in intestinal triglyceride are not at maximal capacity.

### Saquinavir Plasma Concentrations in Lymph Cannulated and Non-lymph Cannulated Rats

The plasma concentration profiles of saquinavir in lymph-cannulated and non-lymph cannulated rats following administration of the cremophor mixed micelle formulation containing 5 mg saquinavir, with and without a series of modulators, are presented in Fig. 4. This data must be viewed with the understanding that for lymph cannulated animals, systemic plasma concentrations do not reflect contributions from saquinavir lymphatic transport, (as mesenteric lymph was collected), whereas plasma levels in the non-lymph

**Table I.** Cumulative Transport of Triglyceride into the Mesenteric Lymph (Mean±S.E.) and Cumulative Lymph Flow after 8 h as a Function of Formulation. Each Formulation was Prepared in 2% Cremophor: 40 mM Oleic Acid Mixed Micelles

| Formulation | Total mass of TG transported (mg)[b] | Mass attributable to exogenous TG (mg)[c] | Cumulative lymph flow (ml) |
|---|---|---|---|
| Saline[a] | 15.06±0.85 | | 4.45±0.35 |
| SQV alone (control) | 43.07±3.84 | 28.01±4.50 | 4.86±0.64 |
| SQV + Verapamil | 43.12±2.96 | 28.05±3.63 | 4.35±0.83 |
| SQV + Ketoconazole | 46.25±3.90 | 31.18±4.55 | 6.67±0.51 |
| SQV + Cyclosporine | 27.52±3.08* | 12.45±3.74* | 5.08±0.69 |

[a] Representing endogenous control
[b] Representing endogenous and exogenous lipid
[c] Attributable to exogenous lipid (i.e. total—endogenous)
*$p<0.05$ relative to SQV alone

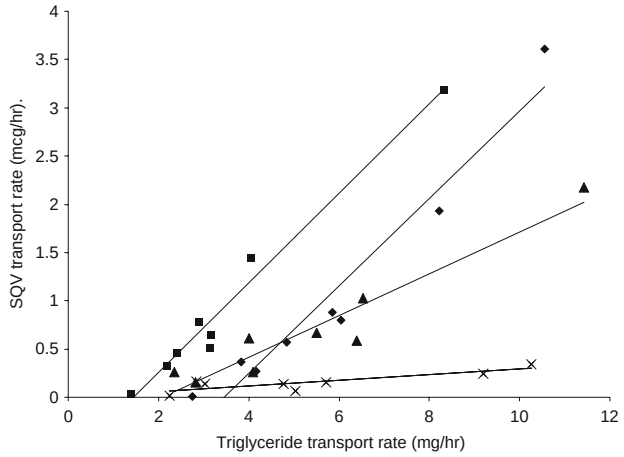


**Fig. 3.** Intestinal saquinavir transport rate (μg/h) versus triglyceride turnover (mg/h) in intestinal lymph (mean±s.e., $n \geq 5$). Details of best fit trendlines for each test formulation are as follows: SQV alone (slope=0.029, $r^2$=0.84, *crosses*), SQV + verapamil (slope=0.21, $r^2$=0.91, *triangles*), SQV + ketoconazole (slope=0.45, $r^2$=0.95, *prisms*) or SQV + cyclosporine (slope=0.46, $r^2$=0.98, *squares*).

cannulated (lymph intact) rats result from saquinavir absorption directly into the blood and indirectly into the blood via the lymph. Co-administration of cyclosporine resulted in a significant increases in saquinavir plasma concentrations ($Cmax_{plasma}$), both in lymph cannulated and non-lymph cannulated rats. However, while there is a clear trend towards higher systemic plasma concentrations of saquinavir for the groups co-administrated verapamil and ketoconazole, the differences versus control were not statistically significant. The relevant pharmacokinetic parameters for each formulation in non-lymph cannulated and lymph cannulated rats are presented in Tables III and IV, respectively. There were no significant differences between plasma concentrations in lymph cannulated and non lymph cannulated rats which confirms the lack of substantial lymphatic transport (i.e. saquinavir is primarily transported to the systemic circulation via the portal route). As previously reported the intestinal lymphatic transport contributes approximately 0.32% of the 'bioavailable' dose of saquinavir, assuming that all drug transported in the lymph is redistributed into the systemic circulation (16). In the present study the intestinal lymphatic transport contributes 0.96, 1.64 and 0.42% of the 'bioavailable' dose of saquinavir, for the groups co-administered verapamil, ketoconazole and cyclosporine respectively.

Fig. 5 shows the relative enhancement in lymphatic transport and plasma bioavailability for the various formulations relative to control (i.e. SQV alone). There is a greater relative enhancement in the extent of lymphatic transport compared to the corresponding relative enhancement in plasma bioavailability, for the formulations containing verapamil and ketoconazole. Hence, the effects of these modulators produce greater relative increases in lymphatic transport than systemic bioavailability and in this way indicate a degree of preferential targeting to the lymphatic system. Co-administration of cyclosporine appears to increase both lymphatic transport and systemic bioavailability to similar extents.

## DISCUSSION

The pathological progression of HIV infection suggests that therapeutic benefit may be gained by redirecting a proportion of the dose to the lymphatic system and the lymphoid tissue in general. The extent of intestinal lymphatic transport of saquinavir (as % dose administered) from a range of lipid based formulations was deemed to be low, considering the highly lipophilic nature of the compound as reported previously (16). It was postulated that susceptibility to the P-gp/CYP counter transport system may limit the extent of intestinal lymphatic transport of saquinavir. Co-administration of P-gp/CYP modulators significantly increased the extent of intestinal lymphatic transport. The increase in the total fraction of the administered dose transported in intestinal lymph compared to control (i.e. saquinavir administered alone) were 4.2, 5.5, and 6.3-fold higher for the verapamil, cyclosporine and ketoconazole formulations respectively. These results support roles for enterocyte based P-gp and CYP elimination processes as determinants of intestinal lymphatic transport of saquinavir. While neither of the modulators used in this study has total selectivity for either P-gp or CYP, it is generally accepted that verapamil acts primarily as a P-gp inhibitor, whereas ketoconazole acts as a potent non-competitive CYP3A4 inhibitor (33). Hence, in terms of delineating the relative roles of Pgp efflux versus enterocyte based metabolism on limiting intestinal lymphatic transport of saquinavir, it would appear that intestinal Pgp mediated efflux is at least equivalent to the enterocyte based CYP metabolism effect.

While the precise mechanisms by which lipophilic drugs gain access to the intestinal lymphatics via the enterocytes are not fully elucidated, the majority of lymphatically transported drugs are associated with the triglyceride core of the chylomicron. In the present study, the cremophor mixed micellar formulation acts as a lipid source to 'drive' intestinal lipoprotein synthesis and hence increase lipoprotein secretion by enterocytes. There is a relatively good correlation between the lymphatic triglyceride turnover and the rate of saquinavir transport in the intestinal lymph, and consequently it is reasonable to assume saquinavir is transported in association with lymph triglyceride (Fig. 3). An unexpected finding of this study was that administration of cyclosporine formulations produced a statistically significant reduction in the total extent of triglyceride recovered in intestinal lymph.

**Table II.** The Loadings of Saquinavir in Lymph Triglyceride (% w/w) as a Function of Formulation. SQV (5 mg) was Administered as Mixed Micellar Vehicle (2% Cremophor: 40 mM Oleic Acid) Alone or Containing either 1 mM Verapamil, 1 mM Ketoconazole or 1 mM Cyclosporine

| Formulation | Loading of SQV in lymph triglyceride[a] (% w/w) | % of the theoretical maximum loading in lymph triglyceride[b] |
|---|---|---|
| SQV alone | 0.003 | 1.5 |
| SQV + verapamil | 0.013 | 6.5 |
| SQV + ketoconazole | 0.018 | 9 |
| SQV + cyclosporine | 0.027 | 13.5 |

[a] Cumulative amount of SQV and triglyceride transported after 8 h
[b] Assume saturation solubility of SQV in triolein is 0.2% w/w

**Table III.** Pharmacokinetic Parameters of Saquinavir (mean % dose ±SE, $n \geq 5$) in Non-lymph Cannulated Rats as a Function of Test Formulation

| | SQV alone | SQV + Verapamil | SQV + ketoconazole | SQV + cyclosporine |
|---|---|---|---|---|
| $C\max_{plasma}$ (ng/ml) | 96.04±19.39 | 153.04±21.85 | 174.63±31.11 | 392.59*±92.09 |
| $AUC_{0\rightarrow 8\ h}$ (ng h $ml^{-1}$) | 375.48±79.40 | 610.58±123.89 | 398.38±48.76 | 1955.61*±389.08 |
| $AUC_{0\rightarrow\infty h}$ (ng h $ml^{-1}$) | 580.13±116.13 | 807.82±169.3 | 700.85±71.21 | 2379.74*±398.87 |
| $F$ (% dose) | 8.53±1.71 | 11.86±3.08 | 10.29±1.63 | 34.96 *±7.69 |
| $F_R$ | 1 | 1.39±0.46 | 1.21±0.31 | 4.10*±1.22 |

[a] $F_R$ = bioavailability relative to SQV alone
*$p<0.05$ compared to SQV alone

The triglyceride turnover appears to be unaffected by the administration of either verapamil or ketoconazole, at the concentrations used in this study (Table I). While it is unclear how these effects were manifested, the effect is undesirable not only from a lymph-targeting perspective, but also the therapeutic ramifications of a reduced triglyceride turnover are considerable. Previously, Seeballuck et al., (2003) have reported a strong correlation between excipient-mediated inhibition of intestinal lipoprotein assembly and inhibition of P-glycoprotein efflux, in Caco-2 cell lines, implying a link between the two biochemical processes (34). The cyclosporine-mediated effect observed in the current study provides further evidence, *in situ*, of a possible link between a P-gp-like mechanisms and intestinal lipoprotein production, but further investigation is required to fully elucidate the exact mechanisms involved.

The data presented in Table II confirm that the plasma concentrations of saquinavir are significantly increased following co-administration of cyclosporine, with a 4.1-fold increase in systemic bioavailability observed in non-lymph cannulated rats. By comparison, in humans co-administration of cyclosporine (75 mg) with saquinavir (600 mg) increased systemic plasma AUC of saquinavir 4.3-fold (35). It is unclear whether this reflects a more pronounced effect of cyclosporine on increasing intestinal absorption or reduced hepatic based extraction, as delineating the relative contributions of intestinal versus hepatic first pass effects would typically necessitate concurrent portal vein (p.v.) and systemic venous (s.v.) sampling. Sinko et al., (2004) reported a 10.9-fold increase in saquinavir bioavailability following co-administration of saquinavir (5 mg/kg) and cyclosporine (15 mg/kg) to the upper small intestine in the intestinally and vascular access (p.v. and s.v.) ported rabbit model (9). Interestingly the increased bioavailability reflected primarily a reduced rate of metabolism (prolonged $t_{1/2}$) rather than by increasing intestinal absorption, as $C\max$ was relatively unchanged. In particular hepatic clearance was reduced approximately twofold, confirming a pronounced effect of cyclosporine on inhibiting hepatic mediated first pass metabolism of saquinavir. The lack of a significant effect for ketoconazole on saquinavir bioavailability in the present study suggests that a higher dose of ketoconazole is required to produce a significant increase in systemic plasma levels in the rat. A threefold increase in bioavailability has been reported for saquinavir and ketoconazole in humans (36).

Many lipophilic drugs, which are candidates for intestinal lymphatic transport, are also candidates for P-gp efflux and CYP metabolism. The possible influence of inhibition of enterocyte based P-gp and/or CYP on intestinal lymphatic targeting was first discussed by O'Driscoll (37). Inhibition of P-gp and/or CYP may increase intracellular concentration and if this is achieved in tandem with an increase in

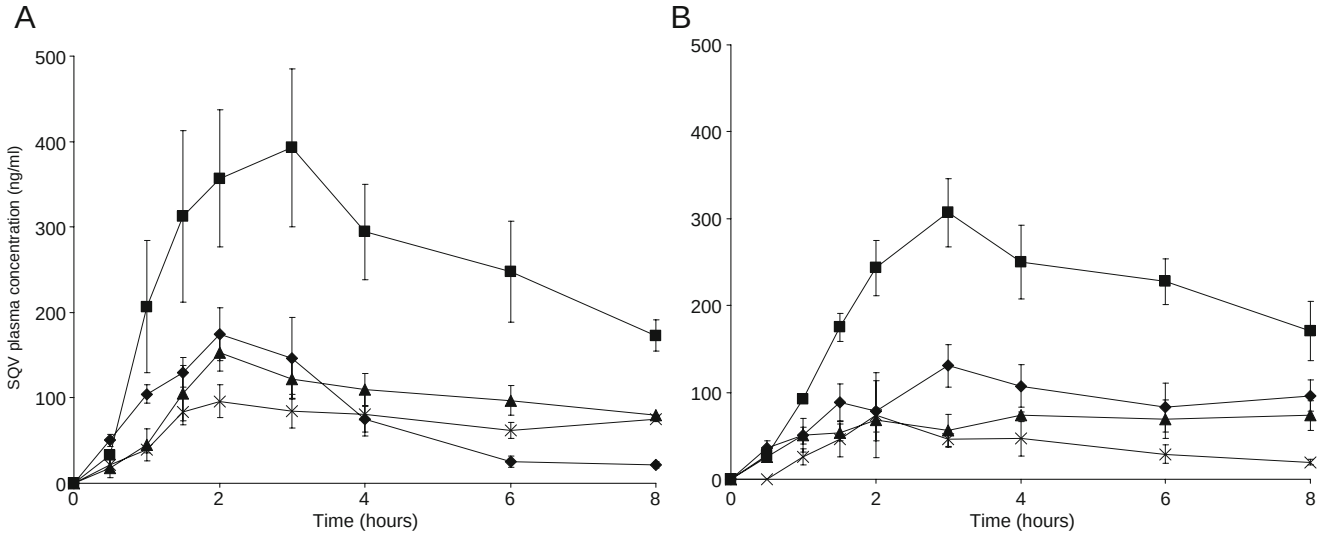


**Fig. 4.** Concentration of saquinavir in systemic plasma versus time (mean±SE, $n \geq 5$) in non-lymph cannulated (**a**) and lymph cannulated rats (**b**). Saquinavir (5 mg) was administered intraduodenally as mixed micellar vehicle (2% Cremophor: 40 mM Oleic acid) alone (*crosses*) or containing either 1 mM verapamil (*triangles*), 1 mM ketoconazole (*prisms*) or 1 mM cyclosporine (*squares*) *significantly increased ($p<0.05$) versus control (i.e. SQV alone).

**Table IV.** Intestinal Lymphatic Transport and Pharmacokinetic Parameters of Saquinavir (mean % dose ±SE, $n \geq 5$) in Lymph-cannulated Rats as a Function of Test Formulation

| | SQV alone | SQV + Verapamil | SQV + ketoconazole | SQV + cyclosporine |
|---|---|---|---|---|
| $C\text{max}_{\text{lymph}}$ (ng/ml) | 729.86±209.39 | 4967.87*±1455.71 | 9152.59*±4841.30 | 4885.26*±1269.85 |
| Lymphatic transport (% dose) | 0.027±0.005 | 0.114*±0.030 | 0.169*±0.049 | 0.148*±0.021 |
| $C\text{max}_{\text{plasma}}$ (ng/ml) | 73.82±48.88 | 73.76±7.71 | 130.64±58.79 | 306.74*±42.26 |
| $AUC_{0\rightarrow 8\ h}$ (ng h $ml^{-1}$) | 155.41±21.22 | 426.21±48.15 | 627.55±143.46 | 1050.43*±108.58 |
| $AUC_{0\rightarrow\infty h}$ (ng h $ml^{-1}$) | 339.12±89.05 | 610.16±79.76 | 809.27±179.87 | 1564.82*±147.19 |
| $F^{A}_{portal}$(% *dose*) | 4.98±1.72 | 8.96±1.60 | 11.88±3.24 | 22.99*±3.53 |
| $F^{B}_{R(portal)}$ | 1 | 1.80±0.70 | 2.39±1.05 | 4.62*±1.74 |

[a] $F_{(\text{portal})}$ = bioavailability in lymph cannulated rats, reflecting the contribution of portal absorption only, and was estimated relative to an i.v. control
[b] $F_{\text{R (portal)}}$= bioavailability relative to SQV alone
*$p<0.05$ compared to SQV alone

lipoprotein production then potential exists to increase the extent of lymphatic transport. The results of this study support this hypothesis, but also demonstrate a preferential targeting of drug via the lymphatic transport route. Verapamil and ketoconazole appear to selectively increase intestinal lymphatic transport without a comparable increase in systemic bioavailability; whereas cyclosporine appears to increase both lymphatic transport and systemic bioavailability equally (Fig. 5). There are two possible explanations for these selective effects. Firstly, inhibition of intestinal efflux/elimination may produce similar relative increases in both lymphatic transport and portal blood. However, drug carried through the portal route must pass through the liver before reaching the systemic circulation, whereas drug transport through the lymphatic route bypasses first pass metabolism. Consequently this suggests that verapamil and ketoconazole are acting primarily to reduce intestinal extraction, whereas cyclosporine reduces extraction both at the intestine and in the liver. Alternatively, at a cellular level, inhibition of intestinal Pgp/CYP may act primarily to increase drug concentrations within the cell, and in particular within the lymph lipid precursor pool (LLPP) (38). This scenario assumes that transport through the apical membrane is not rate limiting, and a modest disruption of intestinal efflux/elimination acts to increase residence time within the cell. Hence while concentration of drug within the LLPP (and the intestinal lipoproteins that are subsequently synthesized and secreted) may be elevated, portal blood concentrations may not be similarly increased due to on-going intracellular metabolism. It has been suggested that drug 'trafficked' through the intestinal lymphatic route may be less susceptible to ongoing enterocyte based metabolism (39). Furthermore, Trevaskis et al., (2006) recently reported that intracellular metabolism of halofantrine was reduced by expanding the LLPP and concluded that lipid formulations may act concurrently to enhance intestinal lymphatic transport (via stimulation of the LLPP) and reduce the rate of intracellular metabolism (38). Hence inhibition of intestinal efflux/metabolism is more likely to produce greater relative increases in lymphatic transport of lipophilic drugs than portal uptake, as is the case for verapamil and ketoconazole in this study. In contrast, in the case of cyclosporine, while the concentration of saquinavir within the LLPP may be increased, cyclosporine appears to reduce intestinal lipoprotein secretion. It is possible therefore, that assuming near-maximal saturation of the LLPP, the reduced lipoprotein secretion into intestinal lymph serves to limit the amount of drug that can be transported lymphatically. Under these conditions, portal blood absorption would be favoured, and no preferential lymphatic targeting would be seen.

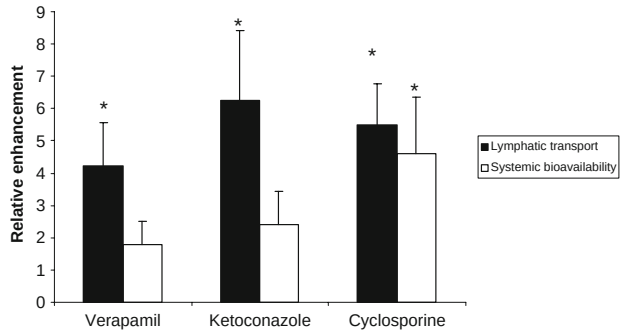


**Fig. 5.** The relative enhancements in lymphatic transport (% dose administered) and % bioavailability of saquinavir compared to control (i.e. no modulator) in lymph cannulated rats.

While any extrapolation to clinical therapy must be considered within the limitations of predicting human data from an *in situ* rat model, these findings provide a rationale for attempts to selectively target the intestinal lymphatics by concomitant administration of modulators of intestinal efflux/metabolism. Charman (2000) has previously noted that from a lymph targeting perspective, a relatively small overall extent of absolute lymphatic transport (i.e. % dose administered), may be adequate to provide an enhanced pharmacological effect (40). Given that saquinavir acts as a competitive inhibitor of HIV protease, and the assumption that increased intestinal lymphatic transport should increase local intestinal lymphoid tissue concentrations, maintenance of high concentrations, such as those observed in this study (Table III), may potentially increase its therapeutic value for treatment of HIV infection in intestinal lymphoid tissue. Furthermore, the current study suggests that increases in intestinal lymphatic transport may be achieved by inhibition of intestinal elimination processes alone. In contrast a significant increase in systemic plasma level, such as that observed with cyclosporine, may require a more pronounced effect on the liver, which may be undesirable in chronic treatment settings. In addition, any undesirable effect of therapy on triglyceride uptake would limit application of such combination-therapy.

## CONCLUSIONS

P-gp/CYP mediated intestinal first pass extraction markedly restricts the transport of saquinavir through the intestinal lymphatic route. The implications of this study are that inhibition of P-gp and/or CYP can produce selective increases in the intestinal lymphatic transport of saquinavir. Further increases in the extent of lymphatic transport of saquinavir may also be possible, for example, by using higher concentrations of modulator, or by more appropriate selection of modulators which do not reduce triglyceride turnover. These findings provide a rationale for formulation approaches to improve the intestinal lymphatic targeting of saquinavir, the proposed mechanisms for which include the following. By inhibition of intestinal Pgp/CYP like efflux/elimination processes, the concentration of drug within the enterocytes will be elevated. Coupled with the co-administration of an appropriate lipid vehicle to drive triglyceride uptake by the intestinal lymphatics, these two effects combined will serve to maintain a higher concentration of the drug in the absorbed lipoidal fraction, resulting in a higher loading of saquinavir per mg triglyceride. By contrast if the P-gp/CYP counter transport process remains fully functional, or in the absence of co-administered lipid, relatively more saquinavir will be either lost to intestinal metabolism or absorbed via the portal blood.

## ACKNOWLEDGMENTS

The authors wish to thank Dr. Hugh Wiltshire, Roche Products Ltd., U.K., for assistance with the HPLC assay.

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