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Original article

P-glycoprotein, multidrug resistance-associated proteins and human organic anion transporting polypeptide influence the intracellular accumulation of atazanavir

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Background: Drug efflux (for example, P-glycoprotein [P-gp], multidrug resistance-associated proteins [MRPs] and breast cancer resistance protein [BCRP]) and influx (for example, human organic anion transporting polypeptide [hOCTP] or human organic anion transporting polypeptide [hOATP]) transporters alter the cellular concentrations of some HIV protease inhibitors (HPIs). Here, we studied the lipophilicity and uptake of [^{3}H]-atazanavir (ATV) in CEM (parental), CEM_{VBL} (P-gp-overexpressing), CEM_{E1000} (MRP1-overexpressing) and peripheral blood mononuclear cells (PBMCs), and evaluate the effects of modulators of drug transporters on uptake.

Methods: Lipophilicity was measured by octanol/saline partition method. The influence of influx/efflux transporters on uptake was evaluated in the absence and presence of inhibitors of P-gp (GPV031), P-gp/BCRP (tariquidar and GF120918), P-gp/MRP1 (dipyridamole and daidzein), MRP1/2 (frusemide and genistein), hOATP/hOCTP (estrone-3-sulfate [E-3-S]) and hOATP/hOCTP/MRP (probenecid). The effects of a number of HPIs on uptake were also evaluated. Data from digitonin permeabilized cells allowed the evaluation of the contribution of cellular binding to total drug uptake, whereas the inhibitory effect of ATV on P-gp was assessed by daunomycin efflux/uptake assays.

Results: [^{3}H]-ATV is lipophilic and accumulates in the cultured cells as follows: CEM>CEM_{E1000}>CEM_{VBL}. Tariquidar, GF120918 and daidzein significantly increased the uptake of [^{3}H]-ATV in the cultured cells. By contrast, only daidzein and tipranavir significantly increased uptake in PBMCs, with tariquidar and frusemide devoid of effects, whereas dipyridamole, E-3-S, GPV031 and genistein significantly decreased accumulation. ATV inhibits P-gp activity; manipulation of uptake with digitonin suggests binding of [^{3}H]-ATV to P-gp.

Conclusions: [^{3}H]-ATV is lipophilic, a P-gp, MRP and hOATP substrate and an inhibitor of P-gp. Concomitant administration of ATV with drugs and dietary components (for example, daidzein and genistein) that interact with these transporters could alter its pharmacokinetics.

Introduction

HIV protease inhibitors (HPIs) in combination with other antiretrovirals (for example, reverse transcriptase inhibitors) play a pivotal role in the management of HIV-infected patients. The therapeutic effects of these drugs is dependent on their cellular accumulation, which is in part affected by the expression of ATP binding cassette family of drug efflux transporters, such as P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP) [1–3]. In addition, there are some known anionic and cationic transporters, such as human organic anion transporting polypeptide (hOATP) and human organic cation transporters (hOCTs), respectively, which mediate the influx/elimination of many clinically important drugs and xenobiotics, and these have been shown to limit the accumulation of some HPIs [2,4–6].

All these transporters are differentially expressed in the liver, kidney, circulating lymphocytes, brain, testes and intestines – sites important in drug disposition, or which might act as sanctuary for the replicating virus and/or support viral replication. Clearly, low intracellular/plasma drug concentration or altered distribution within these sites could affect clinical outcome.

The transporters work in concert to control the bioavailability of clinically important drugs and endogenous compounds. Thus, together with the cytochrome P450 enzymes, these transporters are important determinants of drug–drug interactions, drug efficacy and toxicity [7]. Indeed, previous studies have shown the oral availability, brain, and fetal penetration of saquinavir (SQV) to be limited by P-gp [8,9]. Similarly, the intracellular accumulations of a number of HPIs have been shown to be limited by P-gp, MRP1, MRP2 and BCRP [1,2,8–19]. There is ample evidence that some HPIs also induce and inhibit the expression of drug efflux transporters, such as P-gp, MRP1/2 and BCRP [12,16,20–25]. However, the role of BCRP in the efflux of HPIs is equivocal [1,3,24–27]; there is evidence that BCRP induces resistance to nucleoside reverse transcriptase inhibitors [27].

We have shown that the intracellular concentrations of HPIs are amenable to manipulation by coadministration with inhibitors of drug efflux transporters [1,2,13]. A potential use of such agents would be to enhance intestinal drug absorption and increase drug penetration to biologically important protective barriers, such as the blood–brain, blood–cerebrospinal fluid, maternal–fetal barriers, testes, and within circulating lymphocytes that might be impenetrable to these drugs. To this end, because ritonavir-mediated inhibition of cytochrome P450 (CYP3A4/CYP2D6) and possibly P-gp has been shown to increase exposure to coadministered HPIs, ritonavir (RTV)-boosted HPIs are a standard component of antiretroviral regimen [28]. Interestingly, we and others have recently shown that other HPIs, such as atazanavir (ATV), can pharmacoenhance the intracellular accumulation and area under the curve (AUC) of coadministed HPIs [2,29]. Published evidence to date suggests that ATV might be a substrate, or induces or inhibits P-gp, MRP and BCRP activities [2,3,23,25,30,31], but does not alter lymphocyte expression of P-gp, and MRP1 [3,31]. The current study aims to characterize the determinants of the cellular transport and sequestration of [^{3}H]-ATV in cultured CD4$^+$ T-cells and in primary human lymphocytes – an important site to study the transport and intracellular concentration of antiretrovirals. Experiments were designed to understand the lipophilicity and sequestration of [^{3}H]-ATV within cells and the efflux and influx pumps relevant for the vectorial transport and retention of the drug.

Methods

Reagents

[^{3}H]-ATV and [^{14}C]-mannitol (specific activities 3.1 Ci/mmol and 50 mCi/mmol, respectively) were purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA). RTV and lopinavir (LPV) were obtained from Abbott Laboratories (North Chicago, IL, USA); nelfinavir (NFV), indinavir (IDV), SQV, amprenavir (APV), ATV and tipranavir (TPV) were obtained from Agouron Pharmaceuticals (San Diego, CA, USA), Merck (Rahway, NJ, USA), Roche (Welwyn Garden City, UK), Glaxo Wellcome (Middlesex, UK), Bristol–Myers Squibb (Hounslow, UK) and Boehringer Ingelheim (Berkshire, UK), respectively. Tariquidar was donated by Xenova Group Plc (Berkshire, UK). The peripheral blood mononuclear cells (PBMCs) were isolated from blood buffy coats, which were obtained from the Regional Blood Transfusion Centre (Liverpool, UK). CEM (parental), CEM_{VBL} (VBL) and CEM_{E1000} (E1000) cell lines were from R Davey (Bill Walsh Cancer Research Laboratories, Royal North Shore Hospital, Sydney, Australia). Lymphoprep was purchased from Axis Shields (Oslo, Norway). GPV031 was a gift from G Ecker (Department of Medicinal Chemistry, University of Vienna, Vienna, Austria). All other chemicals were supplied by Sigma Chemical Co. (Poole, UK).

Cell culture

The cultured cells used in this study were CEM (a CD4$^+$ T-cell line), VBL (P-gp overexpressing; selected using vinblastine) and E1000 (MRP-1 overexpressing; selected using epirubicin). The expression of these transporters has been previously validated in our laboratory [13]. The cells were maintained at 37°C, 5% CO_2 in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS).

Isolation of PBMCs

PBMCs were isolated from blood buffy coats using Lymphoprep according to the manufacturer's instructions.

Octanol/saline partition coefficient

As the cellular association of a drug within target cells is a composite of active influx/efflux, passive diffusion and ion trapping, the lipophilicity of [^{3}H]-ATV was measured as described previously [6] to establish if simple physiochemical characteristics were major determinants of intracellular accumulation.

Transport of [^{3}H]-ATV and the effects of specific inhibitors on transport in CEM and CEM_{VBL} cells

Initial experiments examined the optimal concentrations of inhibitors for maximal inhibitory effects on Pgp, MRP and hOATP activity. Cells (CEM and its variant

cells) were incubated in the absence or presence of varying concentrations of XR9576 (0.01–1 μM; P-gp/BCRP inhibitor [32–34]), MK571, dipyridamole (1–100 μM; P-gp/MRP inhibitor [35,36]), frusemide (1–100 μM; MRP1/2 inhibitor [1], GF120918 (1–100 μM; P-gp/BCRP inhibitor [13,37]), GPV031 (1–100 μM; P-gp inhibitor [38]), probenecid (1–100 μM; MRP/hOATP [14,39] and E-3-S (1–100 μM; hOATP/hOCTP inhibitor [6,40]) as described [1].

In subsequent experiments, the transport and accumulation of [^{3}H]-ATV (20 nM) was measured by incubating the cells (2.5×10^6 cells/ml) in the absence and presence of fixed concentrations of inhibitors: tariquidar (1 μM), frusemide (50 μM), dipyridamole (50 μM), E-3-S (30 and 100 μM), probenecid (30 and 100 μM), GPV031 (30 and 100 μM) and GF120918 (50 μM). We also investigated the interaction of soy-derived phytoestrogens daidzein and genistein (at 1 and 5 mM) known to interact with P-gp, MRP, BCRP and hOATP [41–47] on the cellular accumulation ratio (CAR) of [^{3}H]-ATV. The interaction of the HPIs, APV, IDV, LPV, NFV, RTV, SQV and TPV (all at 10 and 30 μM) on the transport and accumulation of [^{3}H]-ATV was also investigated in CEM and its variant cells. All experiments were carried out in RPMI-1640 media supplemented with 10% FBS as described previously [1]. The amount of drug associated with the cells was quantified by taking a ratio of the amount of drug in the cell pellets to the amount of drug in a similar volume of the bathing media after incubation, taking cell volume (1 pl) into consideration. This ratio was defined as the CAR.

Accumulation of [^{3}H]-ATV in isolated PBMCs and the effects of modulators of accumulation

The assay was carried out essentially as described above. Briefly, isolated PBMCs (5×10^6 cells/ml; cell volume 0.4 pl) were incubated in the absence or presence of fixed concentrations of the inhibitors: tariquidar (1 μM), GF120918 (50 μM), frusemide (50 μM), dipyridamole (50 μM), GPV031 (30 and 100 μM), E-3-S (30 and 100 μM), and APV, IDV, LPV, NFV, RTV, SQV and TPV (all at 10 and 30 μM). In addition, we investigated the interaction of daidzein and genistein (1 and 5 mM, respectively) on the CAR of [^{3}H]-ATV.

Effects of digitonin permeabilization on the transport of [^{3}H]-ATV

In order to characterize the energy dependence and accumulation of [^{3}H]-ATV, drug transport assays were carried out in cells pretreated without or with 20 μM digitonin as described previously [48] followed by steady-state drug accumulation assay. Cells were incubated with 20 μM digitonin prior to the addition of [^{3}H]-ATV (20 nM), after which cells were incubated for a further 15 min. In other experiments, the cells were permeabilized by further incubating the incubation mixture with 20 μM digitonin for 5 min once steady-state was reached (15 min).

Effects of ATV on daunomycin accumulation and efflux in cultured cells

Daunomycin is a substrate of P-gp and its accumulation and efflux were performed as described previously [38]. Concentration–response curves were fitted to the data points using non-linear least-squares and 50% effective concentration values were calculated as described previously [38].

Statistical analyses

Data are expressed as mean ±SD and the distribution of data was assessed using the Shapiro–Wilk test. Statistically significant differences between controls and drug-treated samples were further assessed using the Mann–Whitney U test of the StatsDirect statistical software (version 2.6.3; Cheshire, UK). Differences between means were considered to be significant for $P<0.05$.

Results

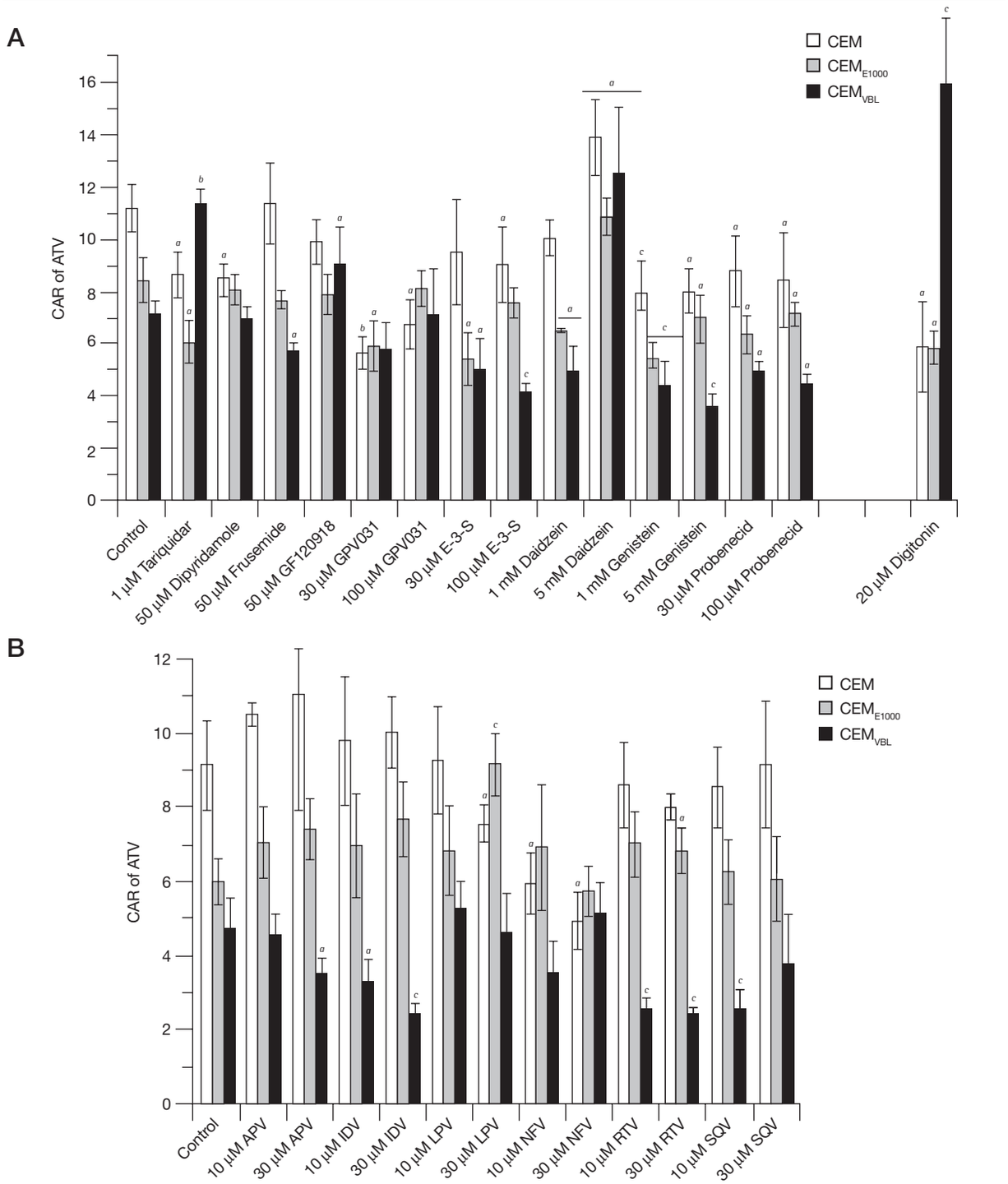
Octanol/saline partition coefficient

The octanol/saline partition coefficient for [^{14}C]-mannitol (used as control) and [^{3}H]-ATV were 0.002 ±0.001 and 29.90 ±4.12, respectively.

Transport of [^{3}H]-ATV and the effects of inhibitors of drug efflux/influx transporters and digitonin on accumulation

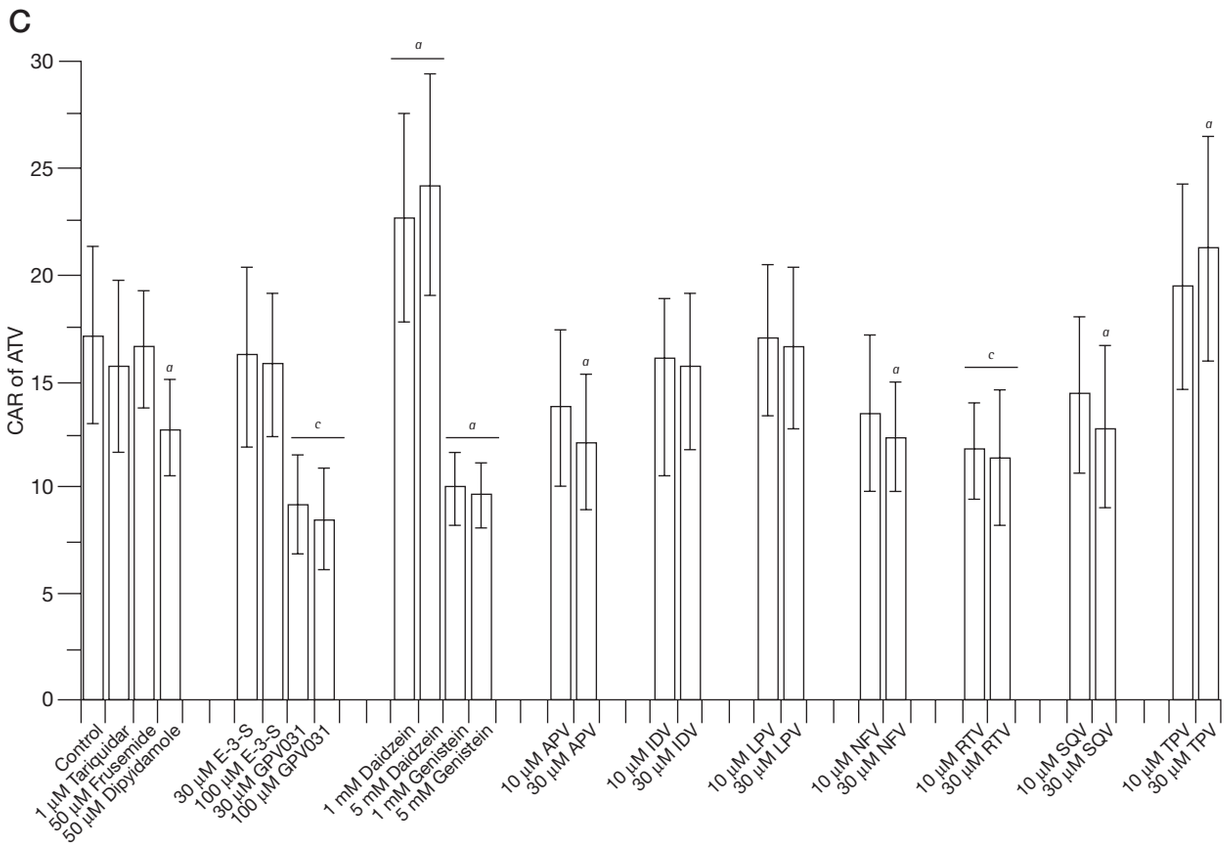
As can be seen in Figure 1A, we observed differential accumulation of [^{3}H]-ATV in the CEM, E1000 and VBL cells with the baseline CAR of CEM, E1000 and VBL cells being 11.2 ±0.9, 8.4 ±0.9 and 7.0 ±0.6, respectively. Tariquidar, dipyridamole and GPV031 (at 30 μM) significantly decreased ($P\leq0.05$) the CAR of ATV in the CEM cells. Similarly, tariquidar and GPV031 (at 30 μM), and daidzein (at 1 mM), significantly ($P\leq0.05$) decreased the CAR of [^{3}H]-ATV in the E1000 cells. Genistein significantly ($P\leq0.05$) decreased the CAR of [^{3}H]-ATV in the CEM and its variant cells. Tariquidar and GF120918 significantly ($P\leq0.05$) increased the CAR of [^{3}H]-ATV in the VBL cells, and daidzein (at 5 mM) increased the CAR of [^{3}H]-ATV in CEM and its variant cells ($P<0.05$). Frusemide, daidzein (at 1 mM) and genistein significantly ($P\leq0.01$) decreased the CAR of [^{3}H]-ATV in the VBL cells. E-3-S (at 30 μM) reduced the CAR of [^{3}H]-ATV in E1000 and VBL cells ($P<0.05$), with 100 μM E-3-S causing a significant ($P\leq0.01$) decrease in the CAR of [^{3}H]-ATV in CEM and VBL cells. To characterize the contribution of specific cellular binding to total the accumulation of ATV, we investigated the effects of digitonin permeabilization on

Figure 1. Effects of modulators of drug transporters, digitonin and HPIs on the accumulation of [^{3}H]-atazanavir in CEM, CEM_{VBL}, CEM_{E1000} and PBMCs



Effects of **(A)** modulators of drug transporters (tariquidar, dipyridamole, frusemide, GF120918, GPV031, daidzein, genistein, estrone-3-sulphate [E-3-S] and probenecid), digitonin and **(B)** HIV protease inhibitors (HPIs; amprenavir [APV], indinavir [IDV], lopinavir [LPV], nelfinavir [NFV], ritonavir [RTV] and saquinavir [SQV]) on the accumulation of [^{3}H]-ATV in CEM, CEM_{E1000} and CEM_{VBL} cells. **(C)** Modulators of drug transporters (tariquidar, frusemide, dipyridamole, E-3-S, GPV031, daidzein and genistein) and HPIs (APV, IDV, LPV, NFV, SQV and tipranavir [TPV]) on the accumulation of [^{3}H]-ATV in isolated peripheral blood mononuclear cells (PBMCs) from blood buffy coats. Bars indicate mean $\pm$SD (n=4, with 4 independent observations from cultured CEM and its variant cells and n=6, with 4 independent observations from each buffy coat PBMC sample). Results are expressed as cellular accumulation ratio (CAR), which is the ratio of the amount of [^{3}H]-ATV associated with the cell pellets to the amount in a similar volume of media after incubation. P-values of $^aP<0.05$, $^bP<0.001$ and $^cP<0.01$ indicate statistically significant differences (increases/decreases) in the CAR of [^{3}H]-ATV as a result of coincubation with the compounds compared with control. Data were analysed by the Shapiro–Wilk test followed by the Kruskal–Wallis test.

Figure 1. Continued



accumulation. Here, cells were either initially permeabilized with 20 μM digitonin prior to the accumulation studies or subsequent to the accumulation studies. We observed that permeabilization of CEM and E1000 cells with digitonin significantly ($P<0.05$) reduced the accumulation of [^{3}H]-ATV in these cells by 47% and 30%, respectively. Unexpectedly, permeabilization of VBL cells with digitonin caused a significant ($P<0.01$) increase in accumulation (from 7.0 ±0.6 to 15.9 ±2.6; Figure 1A). Identical results were obtained in experiments in which the effect of digitonin was assessed subsequent to the accumulation studies (data not shown).

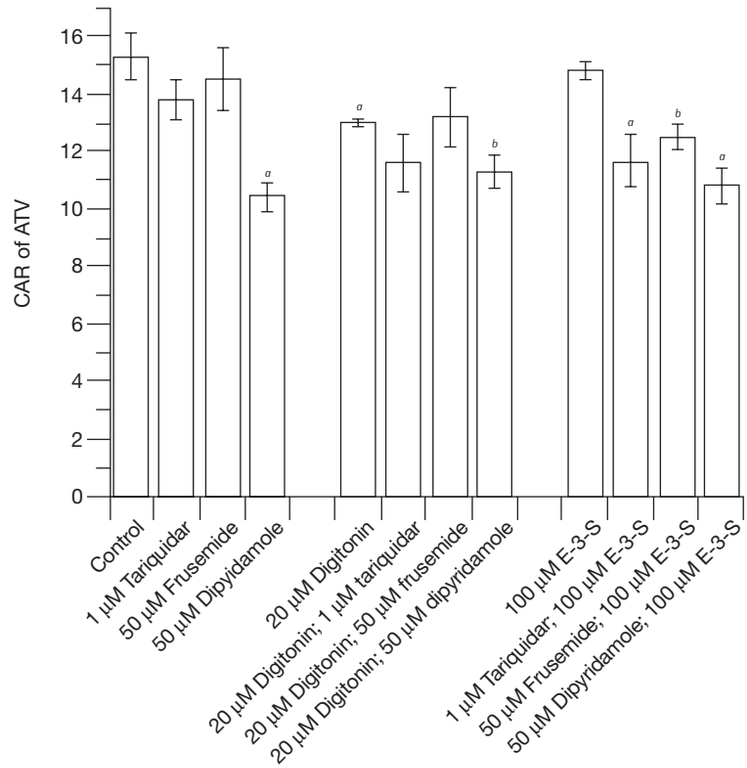
Effects of various HPIs on the accumulation of [^{3}H]-ATV

RTV is used to boost the plasma concentration of a number of HPIs. Here, we investigated whether RTV and other HPIs could modulate the transport and accumulation of [^{3}H]-ATV in the CEM and its variant cells. Of the HPIs tested, only LPV (at 30 μM) significantly ($P<0.01$) increased the association of [^{3}H]-ATV to the E1000 cells. We observed differential accumulation of [^{3}H]-ATV in CEM, E1000 and VBL cells, with the following rank order: CEM>E1000>VBL. LPV and NFV (at 30 and 10 μM, respectively) significantly ($P<0.05$) decreased the accumulation of [^{3}H]-ATV in the CEM cells. By contrast, LPV and RTV (at 30 μM) increased the accumulation of [^{3}H]-ATV in the E1000 cells. In the VBL cells, the accumulation of [^{3}H]-ATV was significantly ($P<0.01$) reduced by APV, IDV and RTV (at 30 μM). The uricosuric agent, probenecid (at 100 μM) significantly ($P<0.05$) increased the accumulation of [^{3}H]-ATV in the E1000 cells, but had no effects on accumulation in CEM and VBL cells (Figure 1B).

Transport of [^{3}H]-ATV in PBMCs and the effects of drug efflux inhibitors and HPIs on accumulation

We observed variable accumulation of [^{3}H]-ATV in the PBMCs ranging from 13.6 ±1.5 to 24.9 ±1.9. We previously showed that PBMCs express P-gp, MRP, BCRP and hOATP [1,6]. Dipyridamole, E-3-S and GPV031 caused a modest decrease in accumulation (Figure 1C). Interestingly, daidzein significantly increased ($P<0.05$), whereas genistein caused a significant ($P<0.01$) decrease in the CAR of [^{3}H]-ATV. Unexpectedly, of all the HPIs tested, only TPV (at 30 μM) caused a modest ($P<0.05$) increase in the CAR of [^{3}H]-ATV. APV, NFV, RTV and

Figure 2. Effects of modulators of drug transporters and digitonin, alone and in combination, on the accumulation of [^{3}H]-ATV in PBMCs



Data were tested for normality of distribution by the Shapiro–Wilk test followed by a parametric paired *t*-test. Each bar represents the mean ±SD (n=3, with 4 incubations per treatment). ^aP<0.01 and ^bP<0.05 compared with respective controls. ATV, atazanavir; CAR, cellular accumulation ratio; E-3-S, estrone-3-sulphate; PBMCs, peripheral blood mononuclear cells.

SQV caused a decrease (P<0.05) in the CAR of [^{3}H]-ATV, either at both of the concentrations tested or at higher concentrations.

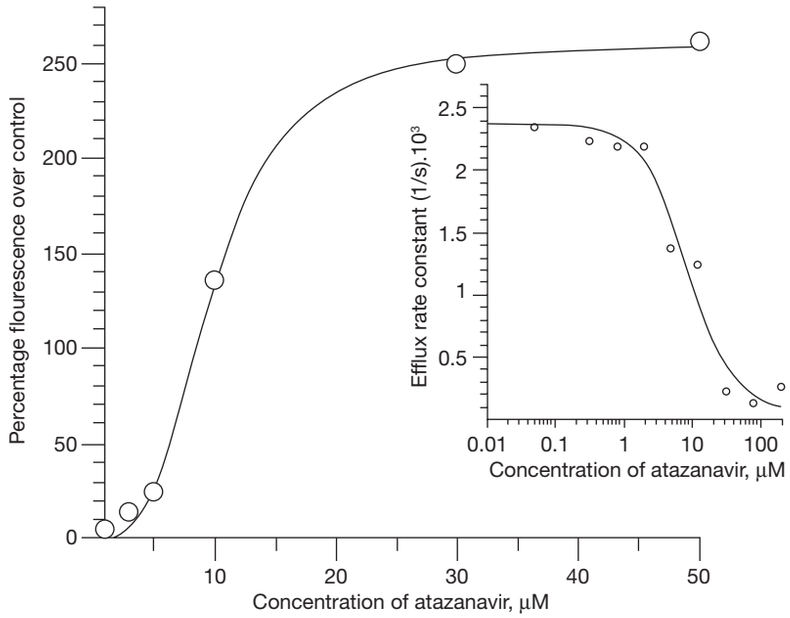
Effects of digitonin permeabilization on the transport of [^{3}H]-ATV

Here, cells were either initially permeabilized with 20 μM digitonin prior to the accumulation studies (in the absence or presence of inhibitors) or subsequently permeabilized upon carrying out the accumulation studies in the absence or presence of the inhibitors at steady state. As shown previously (Figure 1C), tariquidar and frusemide did not alter the accumulation of [^{3}H]-ATV, whereas dipyridamole significantly (P<0.01) decreased the accumulation of [^{3}H]-ATV (Figure 2). Permeabilization of the cells with digitonin significantly reduced (P<0.01) the accumulation of [^{3}H]-ATV (from 15.3 ±0.8 to 13.0 ±0.1). Of the digitonin-pretreated cells that were subsequently coincubated with tariquidar or frusemide, we observed no significant decrease in the CAR of [^{3}H]-ATV compared with digitonin-treated samples. By contrast, there was a significant (P<0.05) decrease in the accumulation of [^{3}H]-ATV in cells pretreated with digitonin followed by dipyridamole over cells treated with digitonin alone. Identical results were obtained with cells that were initially pretreated with the inhibitors and subsequently permeabilized with digitonin (data not shown). We also investigated the effects of E-3-S (alone and in the presence of tariquidar-, frusemide- or dipyridamole-treated cells) on the accumulation of [^{3}H]-ATV. E-3-S alone did not alter the CAR of [^{3}H]-ATV. However, there was a significant (P≤0.01) decrease in the CAR of [^{3}H]-ATV in cells pretreated with tariquidar, dipyridamole and frusemide, followed by subsequent E-3-S coincubation (Figure 2).

Atazanavir increases the accumulation and decreases the efflux of daunomycin in the cultured cells

ATV increased the accumulation and decreased the efflux of daunomycin in a concentration-dependent manner, with a 50% inhibitory concentration of 9.8 μM

Figure 3. Concentration-dependent effects of atazanavir on the accumulation and efflux of daunomycin in CEM_{VBL} cells



Concentration-dependent effects of atazanavir (0–50 μM) on the accumulation and efflux (inset; tariquidar at 0–200 μM) of daunomycin in CEM_{VBL} cells. Initial efflux rates were determined from the slope of linear regression line fitted to mean fluorescence intensity values determined after 1, 2, 3 and 4 min. Initial daunomycin efflux rates are given as a percentage of the fluorescence values determined for the zero time point. Data points represent the mean of two independently performed experiments. The 50% inhibitory concentration ~~IC~~$_{50}$ values for the inhibition of efflux (8.5 μM; inset) and enhancement of accumulation (9.8 μM; inset) of daunomycin were calculated from the concentration–response curves using Grafit (Erithacus Software Ltd, Surrey, UK).

and 8.5 μM, respectively (Figure 3). However, ATV had no effects on the accumulation and efflux of daunomycin in the CEM line (data not shown).

Discussion

Here, we investigated the contribution of lipophilicity, efflux/influx mechanisms and embedding/sequestration in cell membranes to the accumulation of [^{3}H]-ATV. We observed [^{3}H]-ATV to be approximately 7× less lipophilic than [^{14}C]-LPV, but 1.7× more lipophilic than [^{3}H]-SQV [6]. The octanol/saline partition coefficient determined for [^{14}C]-mannitol (our positive control) was similar to that reported previously [49].

Drug transporters are important determinants of variable drug accumulation, drug–drug interactions, efficacy, resistance and toxicity. There is ample evidence that some HPIs are substrates, inhibitors and inducers of drug transporters such as P-gp, MRP1/2, BCRP and hOATP/hOCTP [1,2,4,7–25,50]. However, the role of drug transporters in the accumulation of ATV has not been well documented [2,3,25,30,31]. In order to characterize the transport profile of ATV, we studied its accumulation in cultured cells and in primary human cells, with known transporter expression profiles [1,13]. These data provide evidence that ATV is a substrate of P-gp, and MRP1. This stems from the observations that there was differential accumulation of [^{3}H]-ATV between CEM and its variant P-gp- and MRP1-overexpressing lines and that inhibitors of P-gp/BCRP (that is, tariquidar and GF120918) and P-gp/MRP (daidzein) increased the accumulation of [^{3}H]-ATV in CEM and its variant cells. However, in contrast to the data obtained on other HPIs [1,2,13–15,51], the observation that tariquidar, dipyridamole, frusemide, GF120918 and GPV031 all significantly decreased or did not alter the accumulation of [^{3}H]-ATV in some or all of the cells (Figure 1A) was unexpected (Figure 1A and 1C). Although we found no direct evidence that the influx of HPIs are sensitive to these drugs, and given that none of the drugs (at the concentrations and incubation times employed) are cytotoxic to the cells (data not shown), we postulate that the observed effects might be the result of inhibition of influx, competition for accumulation, or displacement of [^{3}H]-ATV from cell membranes. Indeed, there is evidence that dipyridamole and frusemide influence the fluxes or exchange of Na^+, K^+, Ca^{2+}, Na^+/H^+ and Cl^-/HCO_3^- [52,53]. Given that E-3-S, a substrate/inhibitor of hOATP/hOCT [4,54], significantly decreased the accumulation of [^{3}H]-ATV, it is plausible that the accumulation of [^{3}H]-ATV might be linked to the fluxes/exchange of these cations. Despite the recent observation that tariquidar does not inhibit

hOATP1B3-mediated uptake of paclitaxel [55], it could also be possible that the effects of tariquidar on some of the cells might be the result of inhibition of an influx transporter not described elsewhere.

As with the cultured CEM and its variant cells, the effects of inhibitors of drug transport proteins on the intracellular accumulation of drugs in PBMCs might depend on the relative expression of the transporters. We recently showed the expression of several drug efflux transporters on PBMCs and demonstrated that the accumulation of HPIs is amenable to modulation by inhibitors of drug efflux and influx transporters [1,2,6]. Here, inhibitors of drug efflux transporters, such as tariquidar, dipyridamole, frusemide and E-3-S, produced contrasting results to that measured for SQV and LPV [1,2]. The following possibilities might explain these observations: reduced cell surface expression of the efflux and influx transporters; important drug–drug interactions; the transporter profile of ATV differs from other HPIs – ATV is acid-sensitive to absorption, inhibits hepatic uridine 5′-diphospho-glucuronosyltransferase and induces CYPs [56,57]; and identification of some additional properties of some of the inhibitors used, such as frusemide and dipyridamole, might have potential to inhibit drug uptake transporters. Clearly, the decrease in the accumulation of [^{3}H]-ATV by drugs such as frusemide, dipyridamole and GPV031 (all known inhibitors of efflux transporters) warrants further investigations.

Soy-derived phytoestrogens, such as daidzein and genistein, have been reported to interact with some major drug transporters, including P-gp, MRP, BCRP and hOATP [42–47,58]. However, their interactions with HPIs are largely undefined. We observed that daidzein had dual effects, with the agents causing modest but significant decrease (at 1 mM) and increase (at 5 mM) in accumulation of [^{3}H]-ATV ($P<0.05$) in the cultured cells. In PBMCs, genistein decreased the accumulation of [^{3}H]-ATV, whereas daidzein increased it. The observed effect of genistein is in agreement with the evidence that it inhibits an influx transporter, showing that it inhibits hOATP1B1-mediated uptake of [^{3}H]-dehydroepiandrosterone sulfate [45]. Furthermore, it has also been shown that genistein induces the depolarization of membrane potential [59], which might ultimately prevent the cellular accumulation of [^{3}H]-ATV. As serum concentrations of phytoestrogens could be high [60], these data suggest that intake of soy tablets and or diets rich in soy might influence the accumulation, efficacy and toxicity of ATV. Taken together, these data suggest that these phytoestrogens are both inhibitors of influx and efflux transporters and that ATV might be a substrate of transporters that are affected by these phytoestrogens [42–47,58].

The evidence that E-3-S significantly decreased the accumulation of [^{3}H]-ATV in the cultured cells suggests that ATV, like SQV and LPV [6] is also a substrate of hOATP. Although we observed that E-3-S alone did not decrease the accumulation of [^{3}H]-ATV in the PBMCs (Figure 1C and 2), contrary to the lack of effects of tariquidar and frusemide alone (Figure 1C and 2), coincubating tariquidar-, frusemide- and dipyridamole-treated cells with E-3-S significantly decreased the accumulation of [^{3}H]-ATV (Figure 2). These observations indicate the potential for significant drug–drug and drug–endogenous-substance interactions. We also observed that ATV enhances both P-gp-mediated accumulation and inhibits P-gp-mediated efflux of daunomycin in VBL cells (Figure 3). This is in some agreement with previous studies, which show that although ATV does not induce P-gp or MRP expression [31,61], it is an inhibitor of P-gp (and MRP [2,23,30] and is also able to increase the plasma concentration of coadministered HPIs [29] via blunting the activities of P-gp/MRP [2] and possibly cytochrome P450 enzymes [30].

RTV and other HPIs, including ATV, have been shown to modulate the plasma and intracellular concentrations of other HPIs [2,28,29] via inhibition of P-gp/MRP and cytochrome P450. Of the HPIs tested, only LPV and RTV (at 30 μM) increased the accumulation of [^{3}H]-ATV in E1000 cells (Figure 1B), whereas TPV (at 30 μM) significantly increased accumulation of [^{3}H]-ATV in PBMCs (Figure 1C). There is evidence that some antiretrovirals, such as IDV, RTV, SQV and efavirenz, are potent inhibitors of uptake transporters (for example, hOATP1B1) or hOCTs [5,62,63]. Some of the HPIs (for example, APV, IDV and SQV) reduced the accumulation of ATV in the P-gp overexpressing VBL cells (Figure 1B and 1C). In a previous investigation, we observed that of the hOATP isoforms studied, only VBL cells express hOATP1A2 [6]. Given that some of these HPIs are also substrates of hOATP [6], it is probably that the inhibitory effects of some of the HPIs on the accumulation of ATV are a direct result of their competition for entry into the cells with ATV. Overall, these observations plus that observed with probenecid suggest that ATV might be a substrate of uptake transporters. Thus its coadministration with some HPIs or probenecid might result in alterations in its cellular accumulation

Investigations that aim to characterize the role of drug transporters and the influence of drug embedding/sequestering into cell membranes on the accumulation of [^{3}H]-ATV in the cells using digitonin produced interesting, but unexpected, findings. This manipulation decreased CEM-, E1000- and PBMC-associated [^{3}H]-ATV by 47%, 30% and 15%, respectively. However, a similar manipulation led to a significant increase in VBL-associated [^{3}H]-ATV, suggesting the possibility that [^{3}H]-ATV binds differentially to cells expressing different and varying amounts of transporters, with the drug binding more avidly to P-gp- than MRP-overexpressing

cells. Another possibility is that [^{3}H]-ATV might not be efficiently transported by P-gp and MRP. Coincubations containing digitonin-treated cells followed by subsequent treatment with tariquidar, frusemide or dipyridamole were without any additional inhibitory effects over that observed with the inhibitors alone, suggesting lack of interaction between the coadministered agents.

In conclusion, we found that ATV is lipophilic, is a substrate of P-gp, MRP and hOATPs and is an inhibitor of P-gp, making its disposition susceptible to alteration by coadministered drugs and dietary components that interact with these transporters.

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Disclosure statement

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