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(Article begins on next page)

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Intracellular accumulation of atazanavir/ritonavir according to plasma concentrations and OATP1B1, ABCB1 and PXR genetic polymorphisms

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Abstract

Objectives The rate of accumulation of atazanavir and ritonavir within cells is still debated due to methodological limitations. Our aim was to measure peripheral blood mononuclear cell (PBMC) concentrations of atazanavir and ritonavir and investigate whether single-nucleotide polymorphisms of OATP, ABCB1, CYP3A4 and PXR genes are involved in intracellular drug penetration.

Methods HIV-positive patients administered 300 mg of atazanavir/100 mg of ritonavir were enrolled. Blood sampling was performed at the end of the dosing interval (C_{trough}). PBMC-associated and plasma atazanavir and ritonavir concentrations were measured by validated HPLC coupled with a single mass detector (HPLC–MS) and HPLC–photodiode array (PDA) methods, respectively. Cell count and mean cellular volume were determined using a Coulter counter. Genotyping was conducted using real-time PCR.

Results Thirty-five patients were enrolled. Median atazanavir and ritonavir intracellular concentrations were 1844 and 716 ng/mL, respectively. Median plasma concentrations were 645 ng/mL for atazanavir and 75 ng/mL for ritonavir, while median intracellular/plasma concentration ratios were 2.4 and 9.2, respectively. Median ritonavir intracellular concentrations were higher for OATP1B1 521 T→C TC or CC carriers and for PXR 44477 A→G AG or GG carriers. Atazanavir intracellular/plasma concentration ratios were higher in patients GG for the ABCB1 2677 G→T single-nucleotide polymorphism (SNP) compared with GT and TT groups.

Conclusions Our study showed a higher intracellular ritonavir accumulation than previously reported. Ritonavir intracellular concentrations were associated with OATP1B1 521 and PXR 44477 SNPs while intracellular atazanavir exposure was associated with the ABCB1 2677 SNP. Further clinical studies are necessary in order to confirm these data.

Introduction

The development of protease inhibitors (PIs) and their introduction in HIV infection treatment made the use of highly active antiretroviral therapy (HAART) possible. Durable virological suppression with antiviral therapy has been achieved, and HIV infection has evolved as a chronic disease (1).

Further substantial improvements occurred with the advent of pharmacoenhancement ('boosting strategy'), which consists of low-dose ritonavir in combination with another PI; this results in increased PI plasma levels, reduced inter-individual variability in plasma concentrations (pCs) and increased overall potency of the regimens (2–4). In fact, most PIs are mainly pre-systemically metabolized by CYP3A4 enzymes and then enter the systemic circulation (5). As a potent inhibitor of CYP3A4, ritonavir leads to a significant decrease in metabolism of another PI given in combination with it (6) and a consequent increase in intracellular concentrations (iCs) of that PI.

Despite the long-term efficacy of PI/ritonavir regimens, several toxicity events have been related to ritonavir and to increased plasma drug concentrations of the co-administered PIs (7). Events include gastrointestinal disturbances, lipid profile alterations, insulin resistance and central body fat accumulation

(8), although not all of these have been directly associated with plasma exposure. In addition, CYP3A4 inhibition of ritonavir is a major cause of drug-to-drug interactions with co-administered compounds (9,10).

It is known that the major target of most antiretrovirals is within HIV-infected cells. There may be a correlation between PI iCs, their activity and clinical outcomes (11–17).

Studies demonstrated that the iCs of antiretrovirals may have large inter-individual variability (11,12); furthermore, different methods have been used for determining iCs and contrasting data have been published (18,19).

Our group described the iCs of ritonavir and PIs (20) with a method based on accurate calculation using a mean cell volume individualized for each patient (18). In the above-mentioned study (20) we also observed that atazanavir was the drug with the highest intracellular accumulation ratio (ratio of intracellular to plasma concentrations) (2.4, IQR 1.7–5.1), while other PIs showed lower values; furthermore, in patients treated with atazanavir/ritonavir, the ritonavir iC was the lowest observed (716 ng/mL, IQR 495–1153 ng/mL) (20).

Intracellular penetration of PIs is associated with the physicochemical characteristics of the drugs, such as lipophilicity, ionization level and binding with plasma and transport proteins, associated with their cellular influx and efflux (21).

Our aim was to evaluate the impact of genetic polymorphisms in OATP, ABCB1, CYP3A4 and PXR genes on iCs and pCs of atazanavir and ritonavir in HIV-infected patients, and also to evaluate whether pCs could be useful to predict peripheral blood mononuclear cell (PBMC) concentrations of atazanavir and ritonavir.

Methods

Patients

HIV-positive adult (male and non-pregnant female) patients on atazanavir/ritonavir-containing HAART followed at the University Hospital Amedeo di Savoia in Torino (Italy) were considered. Patients enrolled in the study took the recommended dose of atazanavir (300 mg once daily, co-administered with 100 mg of ritonavir once daily, with food) (22). Patients with concomitant interacting drugs, self-reported adherence <95%, hepatic or renal impairment or with other clinically significant diseases were not enrolled. Sampling was performed, in accordance with the requirements of the local ethics committee, at the end of the dosing interval (C_{trough}) (time reported by patients) following written informed consent.

Measurement of plasma and PBMC antiretroviral concentrations

Blood samples were collected 24 ± 2 h after drug intake, to obtain a trough concentration (C_{trough}). Samples were collected in lithium heparin tubes (7 mL), and plasma, obtained after centrifugation at 1400 g for 10 min at +4°C, was stored at –20°C until analysis.

PBMCs were isolated and cell number and mean cellular volume were determined using a Coulter counter (18,19).

PBMC-associated and plasma atazanavir and ritonavir concentrations were measured by validated HPLC–MS and HPLC–photodiode array (PDA) methods, respectively (18–20,23). Median values of individual measurements were considered and expressed as ng/mL.

Genotyping

A venous blood sample was obtained from each patient (3 mL of EDTA), together with the sampling for pharmacokinetic analyses, after the acquisition of informed consent, as required by the local ethics committee. Whole blood was stored at -80°C and DNA extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). The purified and eluted DNA was used directly for real-time PCRs (Applied Biosystems, Foster City, CA, USA). All primers, probes and PCR conditions are available on request. The single-nucleotide polymorphisms (SNPs) evaluated are listed in Table 1.

Table 1. Correlation (expressed as *P* values) between investigated SNPs and atazanavir and ritonavir iCs and pCs, and atazanavir and ritonavir accumulation ratios

SNP	Atazanavir iC	Atazanavir pC	Ritonavir iC	Ritonavir pC	Atazanavir accumulation ratio	Ritonavir accumulation ratio
<i>OATP 521 (TC/CC)</i> rs4149056	0.307	0.298	<i>0.010</i>	<i>0.077</i>	0.637	0.937
<i>ABCB1 3435 (CC)</i> rs1045642	0.694	0.182	0.694	0.271	<i>0.050</i>	0.307
<i>ABCB1 2677 (GG)</i> rs2032582	0.370	<i>0.043</i>	0.706	0.187	<i>0.007</i>	<i>0.090</i>
<i>ABCB1 1236 (CC)</i> rs1128503	0.726	<i>0.066</i>	0.431	0.126	<i>0.073</i>	0.175
<i>PXR 44477 (AG/GG)</i> rs1523130	0.381	0.948	<i>0.020</i>	0.325	0.599	0.511
<i>PXR 63396 (CC)</i> rs2472677	0.609	0.238	0.306	0.120	0.733	0.183
<i>PXR 7635 (GA/AA)</i> rs6785049	0.597	0.571	0.880	0.186	0.970	<i>0.042</i>
<i>CYP3A4*1B (GA/AA)</i> rs2740574	0.322	0.488	0.276	0.198	0.552	0.166

P values ≤ 0.05 are shown in bold italic type and *P* values > 0.05 and ≤ 0.1 are shown in bold type.

Correlation (expressed as *P* values) between investigated SNPs and atazanavir and ritonavir iCs and pCs, and atazanavir and ritonavir accumulation ratios

Statistical analysis

For descriptive statistics, continuous variables were described as medians (25th–75th percentiles, IQR) and categorical variables as frequencies (percentages). All polymorphisms were tested for Hardy–Weinberg equilibrium by the χ^2 test in order to determine whether the observed genotype frequencies differed significantly from theoretical genotype frequencies. All data were assessed for normality using the Shapiro–Wilk test and data were compared using Kruskal–Wallis and Mann–Whitney tests. The Spearman rank correlation test was used to investigate correlations between continuous variables. Linear regression analysis was used to investigate the potential influences of different factors (genetic and demographic) on intracellular drug concentrations. Statistical analyses were performed using the SPSS software package ver. 18.0 (Chicago, IL, USA), accepting statistical significance at *P* values < 0.05 .

Results

Thirty-five patients met the inclusion criteria and were included in this analysis. The median age was 45 years (IQR 36–50 years), 23 patients (65.7%) were male, 30 patients (85.7%) were Caucasian and 5 patients (14.3%) were African.

The median weight was 72 kg (IQR 58–78 kg) with a median body mass index (BMI) of 24.6 kg/m² (IQR 21.4–27.0 kg/m²). Atazanavir and ritonavir median iCs were 1844 ng/mL (IQR 973–3334 ng/mL) and 716 ng/mL (IQR 502–1028 ng/mL), respectively. Atazanavir and ritonavir median pCs were 645 ng/mL (IQR 469–991 ng/mL) and 75 ng/mL (IQR 46–164 ng/mL) respectively. Median iC/pC ratios were 2.4 (IQR 1.5–5.0) for atazanavir and 9.2 (IQR 6.0–12.3) for ritonavir.

Atazanavir and ritonavir iCs and pCs were not associated with age, BMI, gender or ethnicity. For both drugs we observed a statistically significant direct correlation between iCs and pCs, with $P=0.004$ and $P=1.07 \times 10^{-7}$ for atazanavir and ritonavir, respectively (Table 2).

Table 2. Bivariate correlation of ritonavir and atazanavir iCs and pCs

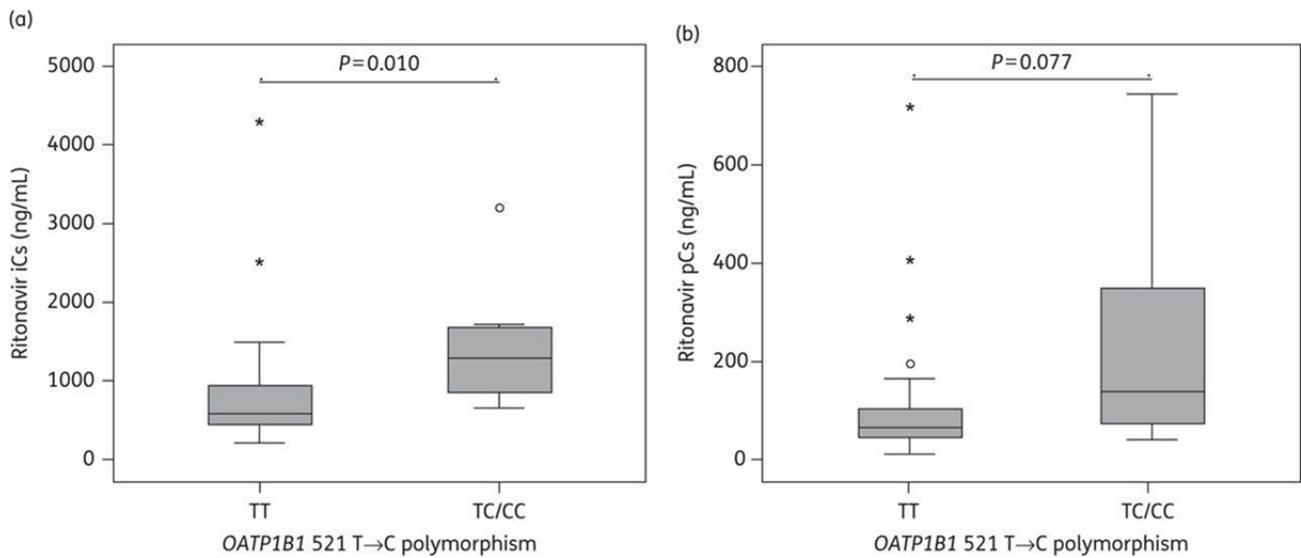
	Ritonavir pC (ng/mL)	Ritonavir iC (ng/mL)	Atazanavir pC (ng/mL)
Ritonavir iC (ng/mL)	$\rho=0.569$ $P=1.072 \times 10^{-7}$		
Atazanavir pC (ng/mL)	$\rho=0.689$ $P=4.737 \times 10^{-6}$	$\rho=0.549$ $P=0.001$	
Atazanavir iC (ng/mL)	$\rho=0.572$ $P=3.278 \times 10^{-4}$	$\rho=0.569$ $P=3.594 \times 10^{-4}$	$\rho=0.472$ $P=0.004$

Bivariate correlation of ritonavir and atazanavir iCs and pCs

All SNPs were in Hardy–Weinberg equilibrium, and the allele frequencies for all polymorphisms observed in our population were comparable to that in the Caucasian population (PubMed SNP).

Individuals with TC or CC genotypes ($n=8$) for the OATP1B1 521 SNP had a significantly higher ritonavir iC than TT genotype patients ($n=27$) [1439 ng/mL (808–1701 ng/mL) versus 860 ng/mL (431–1007 ng/mL), respectively, $P=0.010$]. The same trend, without statistical significance, was observed for the ritonavir pC (Figure 1).

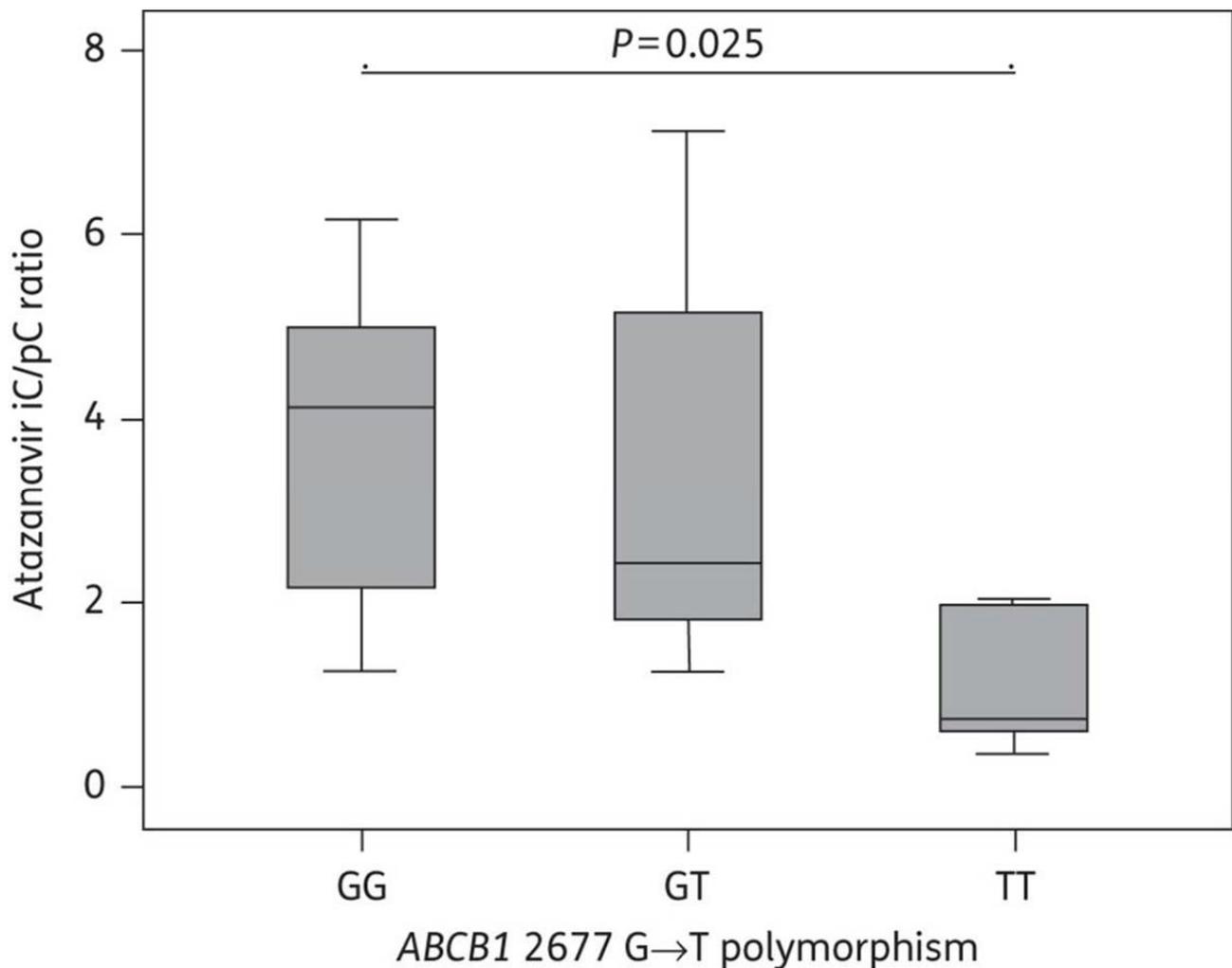
Figure 1.



Ritonavir intracellular (a) and plasma (b) concentrations (ng/mL) according to the OATP1B1 521 T→C (rs4149056) SNP. The boxes and the black lines in the boxes represent IQRs and median values, respectively. Circles and asterisks represent outlier values.

The atazanavir iC/pC ratio was higher in patients with the GG genotype ($n=17$) in ABCB1 at position 2677 compared with GT ($n=13$) and TT ($n=5$) patients [4.10 (1.99–5.18), 2.43 (1.65–5.40) and 0.75 (0.47–1.99), respectively, $P=0.025$] (Figure 2). A significant difference was observed when comparing GG/GT ($n=30$) with TT patients ($n=5$) [3.26 (1.82–5.22) and (0.75 (0.47–1.99), respectively, $P=0.007$].

Figure 2.



Atazanavir iC/pC ratio according to the ABCB1 2677 G→T (rs2032582) SNP.

Moreover, atazanavir pCs were significantly different in patients with SNPs in ABCB1 at position 2677; the GG/GT group had lower values than the TT group [577 ng/mL (418–818 ng/mL) versus 1081 ng/mL (714–6426 ng/mL), respectively, $P=0.043$]. The atazanavir iC/pC ratio was borderline associated with the ABCB1 1236 C→T ($P=0.066$ and $P=0.073$, respectively) and ABCB1 3435 C→T ($P=0.182$ and $P=0.05$) SNPs, with higher levels for C carriers.

Ritonavir iC in individuals with the AG or GG genotype ($n=29$) for the PXR 44477 polymorphism was higher compared with patients with the AA genotype ($n=6$) [1081 ng/mL (572–1153 ng/mL) versus 567 ng/mL (321–738 ng/mL), respectively, $P=0.020$]. We also observed a higher ritonavir iC/pC ratio in patients with the PXR 7635 GA or AA genotype ($n=26$) compared with GG patients ($n=9$) ($P=0.042$).

All the other evaluated polymorphisms were not associated either with plasma or intracellular atazanavir and ritonavir concentrations.

Table 3 shows that pC was the factor with the greatest influence on each intracellular drug concentration.

Table 3. Univariate and multivariate linear regression evaluation of atazanavir and ritonavir iCs

Factor	Atazanavir iC		Ritonavir iC	
	univariate <i>P</i>	multivariate <i>P</i>	univariate <i>P</i>	multivariate <i>P</i>
Sex	0.729	—	0.948	—
Ethnicity	0.394	—	0.271	—
Age	0.395	—	0.585	—
BMI	0.890	—	0.221	—
pC	0.021	0.021	<0.001	<0.001
<i>OATP1B1</i> 521 (TC/CC) rs4149056	0.391	—	0.091	0.866
<i>ABCB1</i> 3435 (CC) rs1045642	0.950	—	0.175	0.704
<i>ABCB1</i> 2677 (GG) rs2032582	0.404	—	0.615	—
<i>ABCB1</i> 1236 (CC) rs1128503	0.404	—	0.615	—
<i>PXR</i> 44477 (AG/GG) rs1523130	0.675	—	0.181	0.190
<i>PXR</i> 63396 (CC) rs2472677	0.315	—	0.726	—
<i>PXR</i> 7635 (GA/AA) rs6785049	0.677	—	0.506	—
<i>CYP3A4*1B</i> (GA/AA) rs2740574	0.244	—	0.560	—

P values that are statistically significant are shown in bold type.

Discussion

Methodological issues and the relatively large blood volume required contribute to the difficulties in studying intracellular PI concentrations in vivo. Studies of the intracellular pharmacokinetics of HIV drugs are a key element in investigating one of the putative sanctuary sites where HIV may replicate (12). However, stringent methodological procedures need to be applied and there is no standard technique for measuring intracellular exposure (12,18,19). Our group recently proposed a method of quantifying intracellular drugs using mean corpuscular volume individualized for each patient (18,19). This method allowed us to calculate PBMC concentrations accurately in ng/mL (instead of concentrations per PBMC number), to compare PBMC concentrations and pCs, and it has already been used to investigate ritonavir iCs associated with different PIs (20).

In this work our aim was to understand the relationship between several SNPs potentially involved in drug transport and metabolism and the pCs and iCs of atazanavir and ritonavir. For this purpose we evaluated SNPs in genes encoding the organic anion-transporting polypeptide (OATP), P-glycoprotein (ABCB1), cytochrome P-450 (CYP3A4) and pregnane-X receptor (PXR).

Organic anion-transporting polypeptides are encoded by the SLCO genes. These are a family of solute-carrier membrane proteins that transport many endobiotic and xenobiotic compounds into cells. Janneh et al. (24,25) have shown, in in vitro studies, that SLCO transporters are important for the influx of some PIs into T CD4+ cells and PBMCs. These transporters have been reported to have numerous functional SNPs in the SLCO1B1 gene (26–28). SNPs of OATP1B1, in particular the 521C allele, have been associated with a decrease in the transport function of PIs, both in vitro and in vivo (29–31). OATP1B1 521 T→C frequency, like ABCB1 3435 C→T, varies according to ethnic origin (27,32). Specifically, the OATP1B1 521 C→T SNP appears to be associated with reduced activity of intracellular transporters encoded by this gene (28,33,34).

We observed that the iCs of ritonavir (Figure 1a) were higher in the TC/CC group. The same trend was observed for the pCs of ritonavir (Figure 1b), although it did not reach statistical significance. Therefore, this SNP seems to affect both intracellular and plasma ritonavir concentrations, despite some authors having reported that OATP1B1 is not expressed on PBMCs (35). OATP1B1 is mainly expressed in hepatocytes, so a decrease in drug outflow could lead to decreased bile excretion, with a consequent increase in pC. Moreover, in our study a significant correlation between the iC and pC of ritonavir (Table 2) was also observed. Thus, our data seem to suggest a role for both ritonavir pCs and the OATP1B1 SNP, with neither having a definite priority role. Another possible explanation of this observation could be linkage disequilibrium of the OATP1B1 SNP with another SNP influencing intracellular atazanavir uptake and/or efflux.

P-glycoproteins are widely expressed transporters that influence the bioavailability and elimination of PIs, as well as their penetration into sanctuary sites and target cells. ABCB1 encodes a trans-membrane transporter (P-glycoprotein) that operates by exporting drugs from cells, and most PIs are substrates of this transporter. The expression and function of these efflux transporters are also modified by genetic polymorphisms of the ABCB1 gene, as well as by ritonavir (36–38). Several ABCB1 polymorphisms were identified and mutations at positions 2677 and 3435 were associated with variability of P-glycoprotein (39). Several studies have reported a relationship between the ABCB1 3435 C→T polymorphism and PI activity (40).

We observed the same trend for pC of atazanavir and its accumulation ratio for each ABCB1 polymorphism, confirming the linkage disequilibrium of these three SNPs in the ABCB1 gene (32,41,42); nevertheless, in our population only two of these SNPs (ABCB1 3435 C→T and ABCB1 2677 G→T) showed a statistically significant difference.

Atazanavir pCs and iCs showed a significant direct correlation (Tables 2 and 3). P-glycoprotein seems to influence atazanavir disposition; specifically, ABCB1 2677 TT patients showed higher atazanavir pCs compared with GG/GT patients ($P=0.043$) and the same patients showed a non-significant decrease in iC; these data agree with the lower atazanavir iC/pC ratio trend observed for the ABCB1 2677 TT genotype.

PXR is a nuclear transcription factor that, as the primary sensor of xenobiotic and endobiotic stress, influences the expression of P-glycoprotein as well as other transporters; it has been demonstrated that PXR regulates the expression of CYP3A4 and ABCB1 and is associated with atazanavir plasma exposure (T allele at position 63396) (43,44). We have observed a concordant influence of PXR polymorphisms on ritonavir ratio and iC.

Linear regression analysis, shown in Table 3, seems to demonstrate that pC could be the factor with the greatest effect on each intracellular drug concentration, but this statistical test could be affected by the low number of patients and by the use of dichotomous genetic factors.

Possible limitations of this study are the limited sample size, which did not allow specific subanalyses (effects of co-administered antiretrovirals and other drugs, different timepoints), and the cross-sectional design. Moreover, physiochemical properties have not yet been related to the transporters studied, and each PI is a substrate of specific transporters. Then, we need further studies to clarify the relevance of the properties of drugs to their intracellular penetration.

In conclusion, we were able to show a significant influence of SNPs and pCs on atazanavir and ritonavir PBMC accumulation; these observations could have an impact on the management of patients who are

candidates for atazanavir and ritonavir HIV therapy. These observations may be useful from the points of view of outcome and toxicity, in the light of recent findings.

Further studies on the clinical impact of intracellular atazanavir and ritonavir accumulation according to different genotypes are warranted in order to tailor antiretroviral therapies and to achieve HIV-1 eradication/functional cure.

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