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Ritonavir inhibits the two main prasugrel bioactivation pathways in vitro: a potential drug–drug interaction in HIV patients

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ABSTRACT

Prasugrel is an antiplatelet prodrug used in patients with acute coronary syndrome. Prasugrel is mainly bioactivated by cytochromes P450 3A4/5 and CYP2B6. HIV patients are at risk of cardiovascular disease, and the protease inhibitor ritonavir is a potent inhibitor of these 2 CYPs. The aim of this in vitro study was to determine the impact of ritonavir in prasugrel metabolism. Human liver microsomes (HLMs) and recombinant microsomes were used to identify the enzymes responsible for the bioactivation of prasugrel. Prasugrel concentrations of 5 to 200 μM were used for Km determination. Inhibition by ritonavir was characterized using HLMs at concentrations of 0.1 to 30 μM. Prasugrel active metabolite determination was performed with a validated liquid chromatography-mass spectrometry method. Using recombinant microsomes, prasugrel biotransformation was mainly performed by CYP2B6, CYP2D6, CYP2C19, CYP3A4, and CYP3A5. With specific inhibitors of CYP3A, CYP2B6, CYP2D6, CYP2C9, and CYP2C19, active metabolite production was decreased by 38% ± 15% with 4-(4-chlorobenzyl)pyridine (CYP2B6 inhibitor) and by 45 ± 16% with ketoconazole (CYP3A inhibitor). The Km value for prasugrel metabolism in HLMs was determined to be 92.5 μM. Ritonavir at 0.1 to 30 μM was shown to be a potent dose-dependent inhibitor of prasugrel. In this *in-vitro* study, we found a potent inhibition of prasugrel bioactivation by ritonavir compared to the specific inhibitors of CYP3A and CYP2B6 due to the simultaneous inhibition of CYP2B6 and CYP3A by ritonavir. This finding suggests a potential significant drug–drug interaction between these two drugs.

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1. Introduction

Cardiovascular diseases constitute more than 10% of deaths in human immunodeficiency virus (HIV)-infected patients [1], and recent studies have shown that cardiovascular diseases were one of the most common complications observed in HIV-infected patients [2,3]. An associative risk between HIV infection and coronary artery disease was suspected through an inflammatory process of atherosclerosis [4]. Moreover, metabolic side effects associated with antiretroviral therapy, including hypercholesterolemia, hyperglyceridemia and lipodystorphy syndrome, enhance the risk of cardiovascular events in HIV population [5].

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Cohort study compared acute myocardial infarction and cardiovascular events rates in HIV patients with non-HIV patients. It was observed that acute myocardial infarction rates were higher in HIV patients (relative risk [RR] = 1.75; $P < .0001$) with a higher frequency in women (12.71 *vs.* 4.88 to control, per 1000 person-years) [6]. Therefore, cardiovascular prevention is required in more than one-half of HIV-infected patients [7].

Ritonavir is an antiretroviral agent used in combination with other antiretroviral drugs in patients with HIV infection. Ritonavir is a peptidomimetic inhibitor of both HIV-1 and HIV-2 proteases, and is used as a booster to increase the plasma concentrations of other antiretroviral drugs by its potent CYP3A inhibition. It is known to both induce and inhibit CYP3A [8,9]. Ritonavir also showed an inhibitory potency for CYP2B6 [10].

Prasugrel is a thienopyridine antiplatelet agent recently commercialized and used for prevention of atherothrombotic events in patients with acute coronary syndrome undergoing percutaneous coronary intervention [11]. Wiviott et al. showed in a TRITON-TIMI 38 trial that prasugrel treatment was associated with significantly reduced rates of ischemic events (9.9% *vs.* 12.1%, $P < .001$, number needed to treat [NNT] = 45) compared with clopidogrel. However, an increased risk of major bleeding, including fatal bleeding (RR = 0.4 *vs.* 0.1, p = 0.002, number needed to harm [NNH] = 333), was observed [12].

Prasugrel is a prodrug transformed by hepatic carboxylesterases to form an inactive intermediate compound, which is further bioactivated by different CYPs [13] (Fig. 1). Initial studies have suggested that CYP3A is the main CYP involved in the bioactivation of prasugrel. Using recombinant CYPs, CYP3A produced the highest amount of active metabolite (AM) with a minor participation of CYP2B6, CYP2C9, CYP2C19, and CYP2D6. Moreover, the presence of ketoconazole, a CYP3A inhibitor, reduced the formation of prasugrel AM by 33% to 86% [14]. However, in a crossover randomized study, Farid et al. assessed the platelet aggregation in healthy volunteers receiving a 60-mg LD or a 15-mg MD of prasugrel with or without 400 mg ketoconazole. Ketoconazole reduced the maximum plasma concentration of prasugrel AM, but did not affect its AUC nor the antiplatelet effect [15]. The reason for this contradiction may be related to the involvement of other minor metabolic pathways (CYP2B6, CYP2C9, and/or CYP2C19) in prasugrel bioactivation when CYP3A activity is reduced. Considering the absence of studies evaluating drug–drug interaction with a multiple CYP inhibitor such as ritonavir, we investigated the effect of ritonavir on prasugrel bioactivation in vitro.

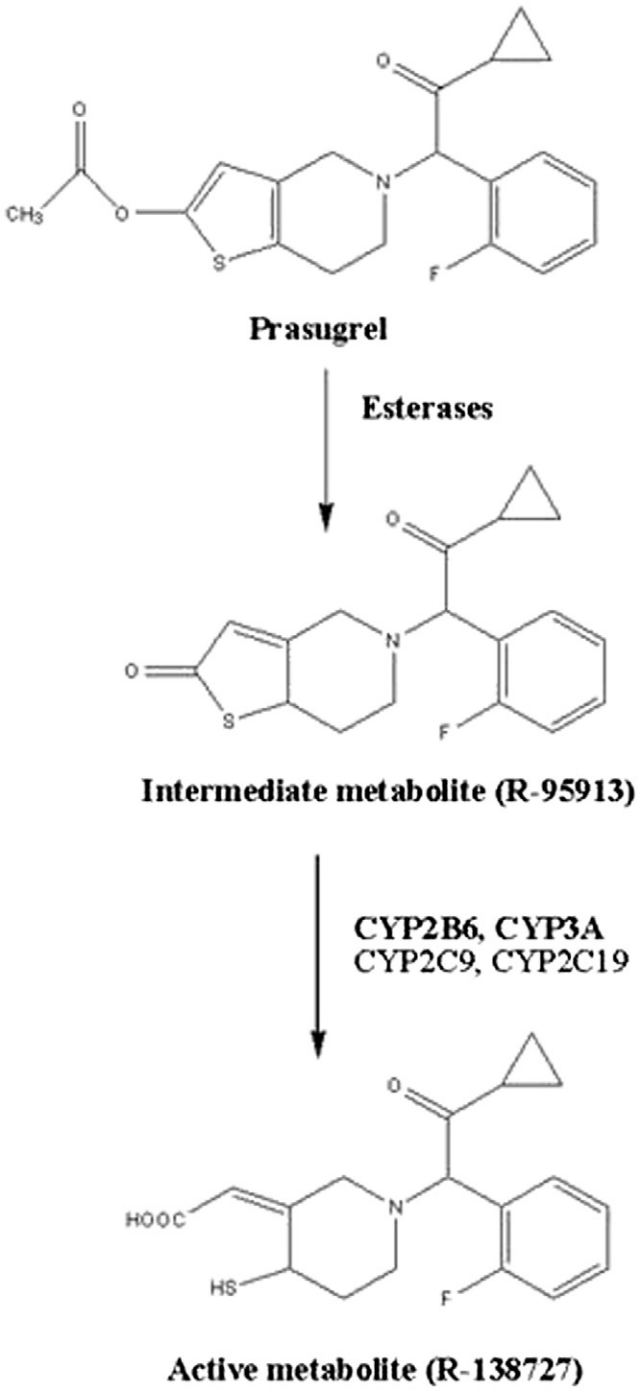

Fig. 1 – Structures of prasugrel, intermediate oxo-prasugrel metabolite, and prasugrel AM.

2. Materials and methods

2.1. *Materials*

Human liver microsomes (HLMs) were purchased from BD Gentest. Prasugrel was obtained from Toronto Research Chemicals (Ontario, Canada). Furafylline was obtained from Salford Ultrafine Chemicals and Research (Manchester, England). Sulfaphenazole was donated by Ciba-Geigy (Basel, Switzerland), and quinidine sulphate by the Pharmacy of Geneva University Hospitals. Ketoconazole, 2-bromo-3-methoxyacetophenone (BMAP), omeprazole, 4-(4-chlorobenzyl)pyridine (CBP), and the constituents of the NADPH-generating system (NADP+, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) were purchased from Sigma (Buchs, Switzerland). High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany), as were the phosphate potassium reagents used for the preparation of reaction buffer.

Ultrapure water was supplied by a Milli-Q purification unit from Millipore (Bedford, MA, US). All stock solutions were prepared in methanol at a concentration of 1 mg/mL and were stored at -20°C. The intermediate solutions were diluted from stock solutions with the reaction buffer.

2.2. Methods

2.2.1. Determination of the enzyme kinetic parameters for the formation of AM of prasugrel in recombinant CYP microsomes

The assays were performed with Supersomes of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5. Incubates contained CYP450 at 50 pmol/mL, prasugrel at varied concentrations (5, 20, 40, 80, 125, and 200 μM), and glutathione 5 mM completed with potassium phosphate buffer until 50 μL. After a preincubation of 3 min at 37°C, the reaction was initiated with a NADPH-generating system (NADP 0.4 mM, isocitrate 2 mM, $MgCl_2$ 2 mM, and isocitrate dehydrogenase 0.4 IU/mL in reaction buffer). Incubations were performed for 15 min at 37°C, and BMAP in acetonitrile was added to stop the reaction and to stabilize the AM of prasugrel. Clopidogrel at a final concentration of 0.1 μg/mL was added as the internal standard (IS). The incubates were centrifuged for 10 min at 10 000 g and diluted 5-fold in the mobile phase before injection of 15 μL in a liquid chromatography-mass spectrometry (LC-MS) system for analysis.

2.2.2. Determination of the enzyme kinetic parameters for the formation of AM of prasugrel in HLMs

All incubations were conducted under linear rate conditions. Incubates contained HLMs (0.5 mg protein/mL), prasugrel at concentrations of 5, 10, 20, 30, 40, 60, 80, 100, 150, and 175 μM, glutathione 5 mM, and potassium phosphate buffer (0.1 M, pH 7.4). Each sample was preincubated at 37°C for 3 min before addition of the NADPH-generating system (NADP 0.4 mM, isocitrate 2 mM, $MgCl_2$ 2 mM, and isocitrate dehydrogenase 0.4 IU/mL in reaction buffer) and incubated at 37°C for 15 min. After incubation, 15 mM BMAP in acetonitrile was added to each sample in ice to stop the reaction and to derivatize the thiol group of the prasugrel AM. Clopidogrel at a final concentration of 0.1 μg/mL was added as the IS. The samples were then centrifuged for 3 min at 10 000 g, and each sample was diluted 5-fold in the mobile phase before injection of 15 μL in the LC-MS system.

2.2.3 In vitro inhibition of bupropion metabolism by ritonavir

All incubations were conducted under linear rate conditions. The incubation mixtures containing microsomes (0.5 mg/mL), glutathione (5 mM), potassium phosphate buffer (0.1 M, pH 7.4), ritonavir at varied concentrations and bupropion at 20, 100, 200 and 500 μM were performed following the same procedure as described for the determination of the enzyme kinetic parameters in HLMs.

2.2.4 In vitro inhibition of prasugrel biotransformation using specific inhibitors or ritonavir in HLMs

All incubations were conducted under linear rate conditions. The incubation mixtures containing microsomes (0.5 mg/mL), CYP-specific inhibitors or ritonavir at varied concentrations, prasugrel (10 μM) glutathione (5 mM), and potassium phosphate buffer (0.1 M, pH 7.4) were performed following the same procedure as described for the determination of the enzyme kinetic parameters in HLMs.

2.2.5. Liquid Chromatography-Mass Spectrometry

LC-MS data were obtained from an Agilent 1100 Series LS system (Agilent, Palo Alto, US) coupled with an Esquire 3000 mass spectrometer (Bruker Daltonics, Billerica, PA, US). A SUPELCO C18 column (5 μm × 2.1 mm × 150 mm, Waters Company, US) with a precolumn composed by the same stationary phase (5 μm × 2.1 mm × 20 mm) were used. The LC conditions were ambient temperature and a 12-min gradient of A = ammonium formate at 20 mM and B = acetonitrile at 300 μL/min with an injection volume of 15 μL. Detection was performed using an electrospray source (ESI) in positive ionization mode with the following conditions: capillary = 4200 V, skimmer = 40 V, capillary exit = 136 V, trap drive = 40, lens 1 = -4 V, lens 2 = -55 V, nebulizer = 50 psi, dry gas = 10 L/min, and dry temperature = 350°C.

Quantification was performed in MS/MS mode using the following transitions: 332.1→177.1 for the intermediate oxo-prasugrel and 498.1→348.1 for the derivatized AM.

2.2.6. Data analysis

Each assay was performed in triplicate. The results are presented throughout as means of 3 values, and ± SD were calculated for each value. Kinetic parameters of prasugrel bioactivation were calculated according to the Michaelis-Menten equation (GraphPad Prism version 4, CA, US). Statistical differences were determined by Student's t-test, and the level of significance was set at $^{*}P < .05$, $^{**}P < .01$, or $^{***}P < .001$.

3. Results

3.1. Identification of cytochromes involved in the bioactivation of prasugrel

The prasugrel AM formation over a range of prasugrel concentrations (5–175 μm) was first determined in incubations containing HLMs. The apparent Km value was 92.5 μM (Fig. 2).

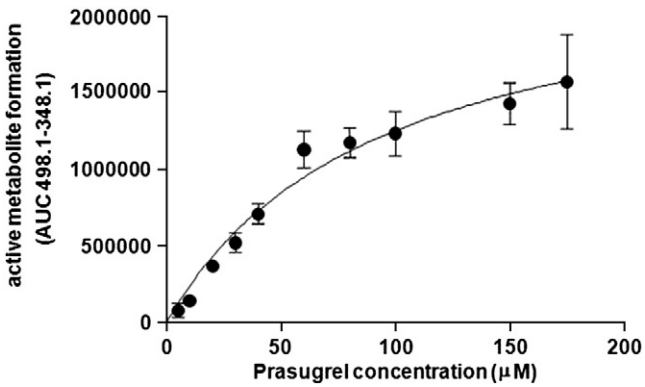

Fig. 2 – Michaelis-Menten presentation for the prasugrel AM formation versus the concentration of prasugrel in HLMs.

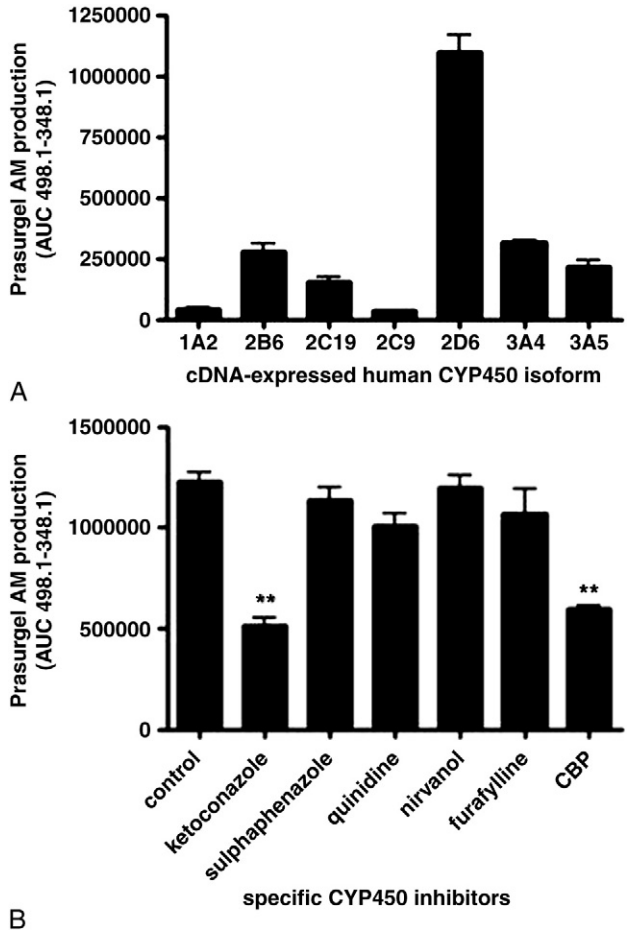

Fig. 3 – A. Prasugrel AM production mediated by cDNA-expressed CYPs. B. Effect of specific CYP450 isoform inhibitors on the production of the prasugrel AM in HLMs. $^{**}P < .01$.

Bioactivation of prasugrel using recombinant human supersomes was also evaluated after incubations containing 10 μM of prasugrel. The highest production of prasugrel AM was observed with CYP2D6, followed by CYP2B6, CYP3A4, CYP3A5 and CYP2C19; neither CYP2C9 nor CYP1A2 seemed to be involved (Fig. 3A). CYP3A is the most abundant enzyme in the liver and intestine (40%), whereas CYP2C9 represents 20%; CYP2E1 and CYP1A2 each represent 10%; and CYP2B6, CYP2C19, and CYP2D6 are present at 5% mainly in the liver [16]. Thus, we can conclude that CYP3A4/5, CYP2B6 and CYP2D6 are the most important enzymes involved in prasugrel metabolism in recombinant human supersomes, with a lower participation of CYP2C19 and CYP2C9.

These results were also confirmed by using specific inhibitors of CYP1A2 (furafylline 30 μM), CYP2B6 (CBP 1 μM), CYP2D6 (quinidine 3 μM), CYP2C9 (sulfaphenazole 5 μM), CYP2C19 (nirvanol 2.5 μM), and CYP3A4 (ketoconazole 3 μM) to determine their effects on the formation of the AM from prasugrel (20 μM) [17]. Compared with the control, AM production decreased by 38% ± 15% in the presence of CBP (a CYP2B6 inhibitor) and by 45% ± 16% with ketoconazole (a CYP3A4/5 inhibitor). Inhibitors of CYP1A2, CYP2C9, CYP2C19, and CYP2D6 did not show any significant effects on prasugrel metabolism (Fig. 3B).

3.2. *Evaluation of the inhibition of prasugrel metabolism by ritonavir*

As shown in Fig. 4, ritonavir from 0.1 to 30 μM was a dose-dependent inhibitor of each concentration of prasugrel tested (from 20 to 200 μM). Prasugrel metabolism decreased by approximately 50% in the presence of 5 μM ritonavir. The latter is known to be a potent inhibitor of CYP3A using nifedipine oxidation (IC_{50} = 0.07 μM) and terfenadine hydroxylation (IC_{50} = 0.14 μM) as metabolic probes [18]. Further

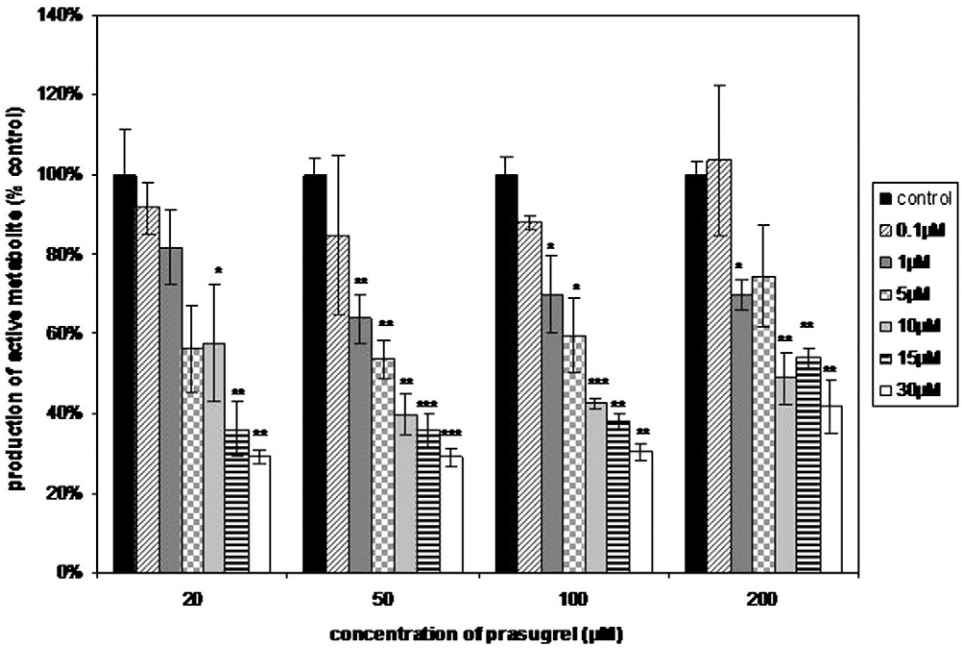

Fig. 4 – Effect of ritonavir (from 0 to 30 μM) on the production of the prasugrel AM.

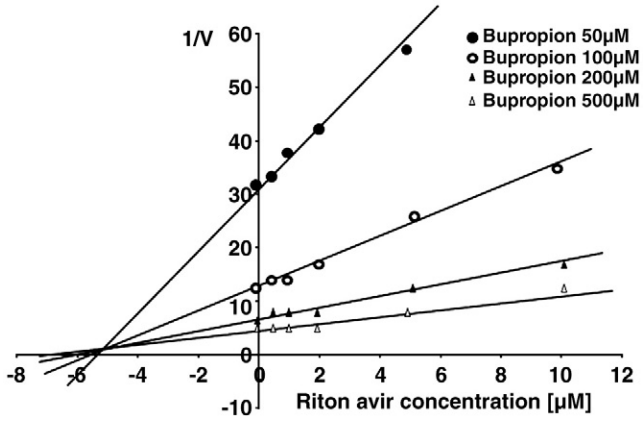

Fig. 5 – Representative Dixon plots for the inhibition of CYP2B6 catalysed bupropion hydroxylation by ritonavir.

evaluation of the effect of ritonavir on CYP2B6 activity using bupropion as a probe indicated that this enzyme was also inhibited by ritonavir (Ki = 5μM) (Fig. 5). Hence, the 2 major metabolic pathways are simultaneously inhibited, enhancing the probability of a clinical drug–drug interaction between the 2 drugs.

4. Discussion

Bioactivation of prasugrel is mainly mediated by hepatic carboxylesterases and cytochrome P450 system. The apparent Km value observed in our study using HLMs is 3-fold higher than the Km determined previously [14]. In the Rehmel study, Km was determined using the intermediate metabolite as a substrate, while in our study, prasugrel was used. Hence, the measured Km includes the first step in which carboxylesterases are mainly involved.

Using recombinant human supersomes and specific inhibitors of studied CYPs, CYP2B6 and CYP3A4/5 were clearly identified as the mean metabolic pathways involved in the bioactivation of prasugrel. Our data are in accordance with recent studies showing that prasugrel is mainly metabolized by CYP2B6 and CYP3A using HLMs and cDNA-expressed CYP isoforms [14,15,19].

After oral administration of a low dose of ritonavir (100 mg), plasma Cmax was 1 μg/mL (1.38 μM), which is higher than the K_i for CYP3A and close to the K_i for CYP2B6 [20]. Moreover, considering the nonlinear pharmacokinetics of ritonavir, an increase in the administrated dose will increase plasma concentration in a nonproportional manner. After an oral dose of 300 mg, the Cmax was 8.7 μg/mL (12 μM), which is higher than the IC_{50} for both CYP3A and CYP2B6. On the other hand, ritonavir is highly bound to plasma proteins (98%) and only its intra-hepatocyte free fraction is involved in the inhibition of CYP2B6 and CYP3A. Unfortunately, there is no data available measuring ritonavir free fraction on the hepatocyte which makes it extremely difficult to quantitatively evaluate extend of such interaction.

In a recent clinical drug–drug interaction study in which ketoconazole was coadministered with prasugrel, ketoconazole reduced the Cmax of the prasugrel AM but did neither affect its AUC nor the antiplatelet effect [21]. It must be noted that in this study, CYP2B6 was not inhibited and thus was able to potentially bioactivate prasugrel. In our study, CYP2B6 activity was also blocked by ritonavir, further reducing the bioactivation of prasugrel. In a recent pharmacogenetic study, common functional genetic variants of CYP2C19, CYP2C9, CYP2B6, CYP3A5, and CYP1A2 did not affect active drug metabolite levels, inhibition of platelet aggregation, or clinical cardiovascular event rates in persons treated with prasugrel [22]. Here again, no data were available where two or more CYPs with deficient genetic variants were present. Moreover, it is widely accepted that the correlation between the genetic polymorphism of CYP3A and its activity is poor. In fact, in a recent study, Oneda et al. demonstrated a low correlation between a CYP3A phenotyping by midazolam test and CYP3A genotyping (CYP3A4, CYP3A5, and CYP3A7 variants) [23]. In a meta-analysis from 7 clinical trials where authors assessed the correlation between CYP3A4/5 alleles and midazolam disposition, there were no differences in midazolam disposition between different genotypes, haplotypes, or diplotypes in the CYP3A cluster [24].

Ritonavir is also known to induce metabolic enzymes after long-term use. A statistically significant decrease was observed in the ethinyloestradiol AUC (-41%) and Cmax (-32%) after 30 days of ritonavir administration [18]. However, ritonavir inhibited CYP3A and increased the AUC of oral oxycodone 3-fold after administration of 300 mg of ritonavir twice daily for 4 days to healthy volunteers [25]. Hence, clinical studies are needed to evaluate the effect of ritonavir on prasugrel pharmacokinetics after long-term use. However, HIV-infected patients usually receive a wide variety of drugs in addition to their antiretroviral drug therapy. Most of them interact actively with CYPs, leading to a considerable potential for pharmacokinetic drug interactions. Hence, a clinical evaluation of such interaction in this population is extremely difficult.

5. Conclusions

Our in vitro study demonstrated that ritonavir is a potent inhibitor of CYP3A and CYP2B6, the principal prasugrel bioactivation pathways, at a clinically significant concentration. Hence, drug–drug interactions between these two drugs should be considered when antiplatelet therapy using prasugrel is considered in HIV patients. However, more clinical studies are needed to confirm the in vitro data and to evaluate the effect of ritonavir after long-term use.

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