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RESEARCH PAPER

Effects of drug interactions on biotransformation and antiplatelet effect of clopidogrel *in vitro*

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BACKGROUND AND PURPOSE
The conversion of clopidogrel to its active metabolite, R-130964, is a two-step cytochrome P450 (CYP)-dependent process. The current investigations were performed to characterize *in vitro* the effects of different CYP inhibitors on the biotransformation and on the antiplatelet effect of clopidogrel.

EXPERIMENTAL APPROACH
Clopidogrel biotransformation was studied using human liver microsomes (HLM) or specific CYPs and platelet aggregation using human platelets activated with ADP.

KEY RESULTS
Experiments using HLM or specific CYPs (3A4, 2C19) revealed that at clopidogrel concentrations >10 μM, CYP3A4 was primarily responsible for clopidogrel biotransformation. At a clopidogrel concentration of 40 μM, ketoconazole showed the strongest inhibitory effect on clopidogrel biotransformation and clopidogrel-associated inhibition of platelet aggregation with IC_{50} values of 0.03 ± 0.07 μM and 0.55 ± 0.06 μM respectively. Clarithromycin, another CYP3A4 inhibitor, impaired clopidogrel biotransformation and antiplatelet activity almost as effectively as ketoconazole. The CYP3A4 substrates atorvastatin and simvastatin both inhibited clopidogrel biotransformation and antiplatelet activity, less potently than ketoconazole. In contrast, pravastatin showed no inhibitory effect. As clopidogrel itself inhibited CYP2C19 at concentrations >10 μM, the CYP2C19 inhibitor lansozprazole affected clopidogrel biotransformation only at clopidogrel concentrations ≤10 μM. The carboxylate metabolite of clopidogrel was not a CYP substrate and did not affect platelet aggregation.

CONCLUSIONS AND IMPLICATIONS
At clopidogrel concentrations >10 μM, CYP3A4 is mainly responsible for clopidogrel biotransformation, whereas CYP2C19 contributes only at clopidogrel concentrations ≤10 μM. CYP2C19 inhibition by clopidogrel at concentrations >10 μM may explain the conflicting results between *in vitro* and *in vivo* investigations regarding drug interactions with clopidogrel.

Abbreviations
GSH, glutathione; HLM, human liver microsomes; IPA, inhibition of platelet aggregation; MPA, maximum platelet aggregation; PCI, percutaneous coronary intervention; PPI, proton pump inhibitor; PRP, platelet-rich plasma; (rh)CYP, (recombinant human) cytochrome P450

Introduction

The drugs currently used for the inhibition of platelet aggregation (IPA) include acetylsalicylic acid, glycoprotein IIb/IIIa receptor antagonists and the thienopyridine derivatives. The thienopyridines, in particular clopidogrel, have become standard drugs for the management of patients following percutaneous coronary intervention (PCI) and stent placement (Schulman, 2004). In addition, clopidogrel is

also used in patients after acute coronary syndromes without PCI and in aspirin intolerant patients.

Clopidogrel is a pro-drug requiring hepatic biotransformation for pharmacological activity (Pereillo *et al.*, 2002). The active metabolite of clopidogrel (R-130964) contains a thiol group, which binds irreversibly to a free cysteine in the P2Y12 receptor and blocks activation by ADP (Ding *et al.*, 2003). In humans, >85% of clopidogrel is metabolized by esterases to a carboxylic acid metabolite (clopidogrel carboxylate, SR 26334) (Caplain *et al.*, 1999; Lins *et al.*, 1999; Reist *et al.*, 2000; Taubert *et al.*, 2004), which is considered to be inactive. The remainder is converted to the active metabolite (R-130964) in a two-step, cytochrome P450 (CYP)-dependent process, proceeding via the formation of 2-oxo-clopidogrel. Initial studies suggested that CYP3A4 plays a prominent role in these metabolic steps, with lesser involvement of CYP2C19, CYP2B6, CYP1A2 and CYP2C9 (Savi *et al.*, 2000; Clarke and Waskell, 2003; Hulot *et al.*, 2006; Farid *et al.*, 2007; nomenclature follows Alexander *et al.*, 2009). A more recent *in vitro* study indicated that CYP2C19 was the most important CYP for the conversion of clopidogrel to 2-oxo-clopidogrel and CYP3A4 for the conversion of 2-oxo-clopidogrel to the active metabolite R-130964 (Kazui *et al.*, 2010).

Studies conducted *in vitro* (Clarke and Waskell, 2003) and *ex vivo* (Lau *et al.*, 2003; Neubauer *et al.*, 2003) suggested that the CYP3A4 substrate atorvastatin may attenuate the platelet inhibitory effect of clopidogrel due to interference with clopidogrel biotransformation by CYP3A4. These observations resulted in an intense debate about the clinical relevance of drug-drug interactions by lipophilic statins, drugs prescribed frequently to patients taking clopidogrel (Ratz Bravo *et al.*, 2005). This controversy remains currently unresolved, as subsequent studies investigating effects of atorvastatin and/or simvastatin on anti-platelet effects of clopidogrel failed to confirm the initial findings (Muller *et al.*, 2003; Gorchakova *et al.*, 2004; Mitsios *et al.*, 2004; Serebruany *et al.*, 2004; Vinholt *et al.*, 2005; Saw *et al.*, 2007). More recent *in vivo* studies suggest an important role for both CYP3A4 and CYP2C19 regarding biotransformation and pharmacological activity of clopidogrel. Both concomitant treatment with strong CYP3A4 inhibitors (Suh *et al.*, 2006; Farid *et al.*, 2007; Siller-Matula *et al.*, 2008) as well as a reduced activity of CYP2C19 in patients with CYP2C19 single nucleotide polymorphisms were associated with an impaired pharmacological activity of clopidogrel (Kim *et al.*, 2008; Mega *et al.*, 2009; Simon *et al.*, 2009). Several clinical studies with *ex vivo* determination of residual platelet aggregation demonstrated that CYP2C19 inhibitors such as proton pump inhibitors (PPIs) decrease the pharmacological effect of clopidogrel (Gilard *et al.*, 2008; O'Donoghue *et al.*, 2009; Price *et al.*, 2009; Zuern *et al.*, 2010). These findings are supported by two retrospective studies with clinical endpoints (death, re-hospitalization and/or re-infarction) (Ho *et al.*, 2009; Juurlink *et al.*, 2009), whereas a third clinical study failed to show an increased cardiovascular risk for patients with concomitant ingestion of clopidogrel and a PPI (O'Donoghue *et al.*, 2009).

Considering the uncertainties regarding drug interactions with clopidogrel, we undertook the current study to determine concentration-dependent effects of inhibitors and/or substrates of various CYPs on *in vitro* biotransformation and anti-platelet activity of clopidogrel.

Methods

Kinetic studies of clopidogrel or the carboxylate metabolite of clopidogrel with human liver microsomes (HLM) or rhCYP

The incubation mixture (final volume 250 µL) contained varying concentrations of clopidogrel (5–100 µM), incubation buffer (0.1 M potassium phosphate, pH 7.4), reduced glutathione (GSH; 5 mM), NADPH-regenerating system containing $MgCl_2$ (3.3 mM), $NADP^+$ (1.3 mM), glucose-6-phosphate (3.3 mM) and glucose-6-phosphate dehydrogenase (0.4 $U \cdot mL^{-1}$) and either HLM, rhCYP3A4 or rhCYP2C19. Preliminary studies were performed to determine the incubation time and protein concentrations producing a linear rate. For HLM, 10 min incubation and 0.25 $mg \cdot mL^{-1}$ protein were selected, and for rhCYP3A4 and rhCYP2C19 10 min incubation and 10 pmol $CYP450 \cdot mL^{-1}$. The concentrations of the substrates (clopidogrel or clopidogrel carboxylate) are given in the figures. The final volume of methanol (solvent for clopidogrel) did not exceed 1.0% of the total incubation volume and was identical in all incubations including controls. Each reaction mixture was equilibrated for 4 min at 37°C in a shaking thermomixer. The reaction was initiated by adding the NADPH-regenerating system and the system incubated for the respective time at 37°C. Reactions were stopped by addition of 100 µL of chilled acetonitrile (containing 6.5 µM naproxen as internal standard) and cooled on ice for 10 min. Precipitated proteins were removed by centrifugation at 10 000 g for 10 min and supernatants were analysed by HPLC as described below. The fraction of substrate metabolized was calculated as the difference between the measured and initial clopidogrel or clopidogrel

carboxylate concentration expressed as a percentage of the initial concentration.

In vitro *inhibition of clopidogrel metabolism*

Cytochrome P450 inhibition studies were performed in the presence of the respective inhibitors or substrates following the same incubation procedure as described for the kinetic experiments. Stock solutions containing inhibitors (see figures) were prepared in methanol or in water. The final volume of methanol did not exceed 1.0% of the total incubation volume and was identical in all incubations including controls. The inhibitor concentrations are given in the figures. IC_{50} values were calculated by non-linear regression analysis using the software program GraphPad Prism version 4.00 (San Diego, CA, USA).

HPLC analysis of clopidogrel and the clopidogrel carboxylate

Clopidogrel concentrations were determined using a LaChrom® high performance liquid chromatography (HPLC) system equipped with an UV detector operating at a wavelength of 235 nm, a column oven, a quaternary pump and an autosampler. The column temperature was maintained at 32°C and the injection volume was 30 µL. Separation was performed on a Nucleosil 50-5-C18 column equipped with a corresponding guard column using a gradient of solvent A (sodium phosphate 0.01 M, pH 3.0:acetonitrile; 50:50 $v{\cdot}v^{-1}$) and solvent B (sodium phosphate 0.01 M, pH 3.0:acetonitrile; 20:80 $v{\cdot}v^{-1}$). The gradient started at 80% A and 20% B for 2.5 min, changed to 100% B for 3.5 min and finally returned to the starting conditions for 4 min. The flow rate was 1 $mL{\cdot}min^{-1}$ and the total run time 10 min. The variability of the method was <10% at high and low clopidogrel concentrations. Calibration curves were performed in a concentration range of 1.0–43.0 $\mu g{\cdot}mL^{-1}$.

The carboxylate metabolite of clopidogrel was determined using the same HPLC system as described above, but with a different mobile phase. The mobile phase consisted of a gradient of solvent A (sodium phosphate 0.01 M, pH 3.0:acetonitrile; 50:50 $v{\cdot}v^{-1}$), solvent B (sodium phosphate 0.01 M, pH 3.0:acetonitrile; 20:80 $v{\cdot}v^{-1}$) and solvent C (sodium phosphate 0.01 M, pH 3.0:acetonitrile; 80:20 $v{\cdot}v^{-1}$). The gradient started at 20% A and 80% C for 2.5 min, changed to 100% B for 3.5 min and returned to the starting conditions for 4 min. The flow rate was 1 $mL{\cdot}min^{-1}$ and the total run time 10 min. Clopidogrel carboxylate was quantified by comparison with a standard curve. Variability and calibration curve range were identical to clopidogrel.

Ex vivo IPA by activated clopidogrel

Isolation of platelet-rich plasma (PRP) and platelet aggregation experiments were performed according to Born and Cross (1963). The platelet count in PRP samples was adjusted with platelet-poor plasma to 200–250 $x10^9$ platelets per L. Clopidogrel or clopidogrel carboxylate were activated in a mixture containing clopidogrel, HLM (0.25 $mg{\cdot}mL^{-1}$), incubation buffer (0.1 M potassium phosphate, pH 7.4) and NADPH-regenerating system for different periods of time. Inhibitors (dissolved in methanol; concentrations in figures) were evaporated to dryness at 37°C before addition of the same biotransformation mixture as above containing clopidogrel or clopidogrel carboxylate. In order to test the effect of GSH, biotransformation experiments were performed also in the presence of 5 mM GSH.

To assess platelet aggregation, 120 µL incubation mixture (activated clopidogrel or clopidogrel metabolite) was added to the same volume of platelets and preincubated at 37°C for 15 min. Platelet aggregation was stimulated with ADP (final concentration 2.5 µM) and recorded with an APACT4 aggregometer (LABiTec, Ahrensburg, Germany) as the maximal percentage in light transmittance of the reaction mixture. The percentage of IPA was calculated from the observed maximum platelet aggregation (MPA) as follows (Farid *et al.*, 2007):

$$IPA(\%) = \frac{(MPA_{baseline} - MPA_{postdose}) \times 100}{MPA_{baseline}}$$

Molecular modelling studies

Molecular modelling was performed on a structure of human cytochrome 3A4 (PDBcode: 1W0G). All calculations were performed on a Dell Precision 670 workstation using the program Moloc (http://www.moloc.ch). Clopidogrel was docked manually into the active site of the enzyme which was previously modified such that an oxygen atom was placed at the position completing the octahedral geometry of the central Fe^{2+} of the haem. Multiple positions of clopidogrel were tried and subsequently optimized with the force field integrated in Moloc. All atoms of the structure were considered for calculations but only substrate atoms were allowed to move. An analogous procedure was applied for the more hydrophilic clopidogrel carboxylate.

Kinetic analysis and statistics

Kinetic parameters of clopidogrel biotransformation were calculated according to the Michaelis-Menten equation using nonlinear regression (GraphPad Prism version 4.00; San Diego, CA, USA):

$$v = \frac{V_{max} \times S}{K_m + S}$$

v and S are biotransformation rate and substrate concentration, respectively, V_{max} the maximal biotransformation rate and K_m the Michaelis-Menten constant. IC_{50} values for inhibitors were calculated by non-linear regression analysis (GraphPad Prism version 4.00; San Diego, CA, USA). *P*-values were calculated using one-way analysis of variance (ANOVA) with Dunnet's multiple comparison test for post hoc analysis. Data are presented as mean ± SEM. A *P*-value <0.05 was considered to be significant.

Materials

Clopidogrel hydrogen sulphate was isolated from commercially available tablets (Plavix®, Sanofi Aventis, Geneva, Switzerland) and the carboxylate metabolite of clopidogrel was obtained by saponification of clopidogrel (ReseaChem life science, Burgdorf, Switzerland). The purity was >99% for both substances as assessed by nuclear magnetic resonance spectroscopy (NMR). Atorvastatin was obtained from Sequoia Research Products Ldt. (Pangbourne, UK). NADPH regeneration system, pooled HLM (same batch was used for all experiments), recombinant human CYP3A4 supersomes (rhCYP3A4) and rhCYP2C19 were from BD Biosciences Gentest (Woburn, MA, USA). Acetonitrile LiChrosolv for HPLC use was obtained from Merck (Darmstadt, Germany). All other chemicals used were purchased from Sigma or Fluka (Buchs, Switzerland).

Results

In vitro *metabolism of clopidogrel and clopidogrel carboxylate by HLM and rhCYP*

We used HLM and the recombinant human enzymes CYP3A4 (rhCYP3A4) and CYP2C19 (rhCYP2C19) for this purpose. Clopidogrel was metabolized in a concentration-dependent fashion following Michaelis-Menten kinetics by both HLM and rhCYPs. In the presence of HLM, the apparent K_m was 23.1 ± 3.7 μM and the V_{max} 34.1 ± 2.1 nmoles·min^{-1}·mg^{-1} protein (Figure 1A). In the presence of rhCYP3A4, the corresponding values were 45.9 ± 12.4 μM (K_m) and 0.62 ± 0.09 nmoles·min^{-1}·pmol^{-1} P450 (V_{max}) (Figure 1B).

As shown in Figure 1C, clopidogrel biotransformation by rhCYP2C19 was observed only at clopidogrel concentrations <20 μM, indicating that clopidogrel is an inhibitor of CYP2C19 at higher concentrations. Its conversion rate at 10 μM clopidogrel was estimated to be 0.07 nmoles·min^{-1}·pmol^{-1} P450, a value approximately 10 times lower than the V_{max} obtained for rhCYP3A4.

To the best of our knowledge, there are no published data regarding the possible biotransformation of clopidogrel carboxylate, the main metabolite of clopidogrel (Caplain *et al.*, 1999; Taubert *et al.*, 2004). Concentrations of clopidogrel carboxylate did not decrease measurably during incubation, in either of the two *in vitro* systems tested, indicating that clopidogrel carboxylate is metabolized neither by HLM (Figure 1D), nor by rhCYP3A4 (data not shown).

Effect of specific CYP inhibitors and CYP substrates on clopidogrel biotransformation

The inhibition of clopidogrel (40 μM) metabolism by various CYP inhibitors and substrates was investigated using HLM (Figure 2). The oxidation of clopidogrel was significantly impaired by the CYP3A4 inhibitors ketoconazole and clarithromycin at concentrations in the nanomolar range. Ciprofloxacin, a strong inhibitor of CYP1A2 and a weak inhibitor of CYP3A4 (McLellan *et al.*, 1996), reduced clopidogrel oxidation by 35% at 500 μM, but not at lower concentrations. In contrast, inhibitors of CYP2C9 (sulphaphenazole), CYP2D6 (quinidine), CYP2B6 (N,N′,N″-triethylenethiophosphoramide, thioTEPA) and CYP2C19 (omeprazole) revealed no significant effect on clopidogrel biotransformation by HML. For amiodarone, we found no significant inhibition of clopidogrel biotransformation in a concentration range of 1 to 100 μM (data only partially shown).

Due to the importance of CYP2C19 for clopidogrel biotransformation (Kim *et al.*, 2008; Mega *et al.*, 2009; Simon *et al.*, 2009), CYP2C19 inhibitors were investigated in more detail (Figure 3). At a clopidogrel concentration of 40 μM, neither the PPIs omeprazole (up to 100 μM) and lansoprazole (up to 100 μM), nor ticlopidine, inhibited clopidogrel biotransformation by HLM. In contrast, at 5 μM clopidogrel, lansoprazole affected clopidogrel biotransformation in a concentration-dependent manner, reaching significance at 100 μM.

As atorvastatin may impair clopidogrel biotransformation (Lau *et al.*, 2003), we investigated the impact of the CYP3A4 substrates atorvastatin and simvastatin on clopidogrel biotransformation by HLM. Both statins significantly inhibited clopidogrel biotransformation at the maximal concentration tested (10 μM). In contrast, pravastatin (not a CYP3A4 substrate) showed no inhibitory effect at this concentration (Figure 4).

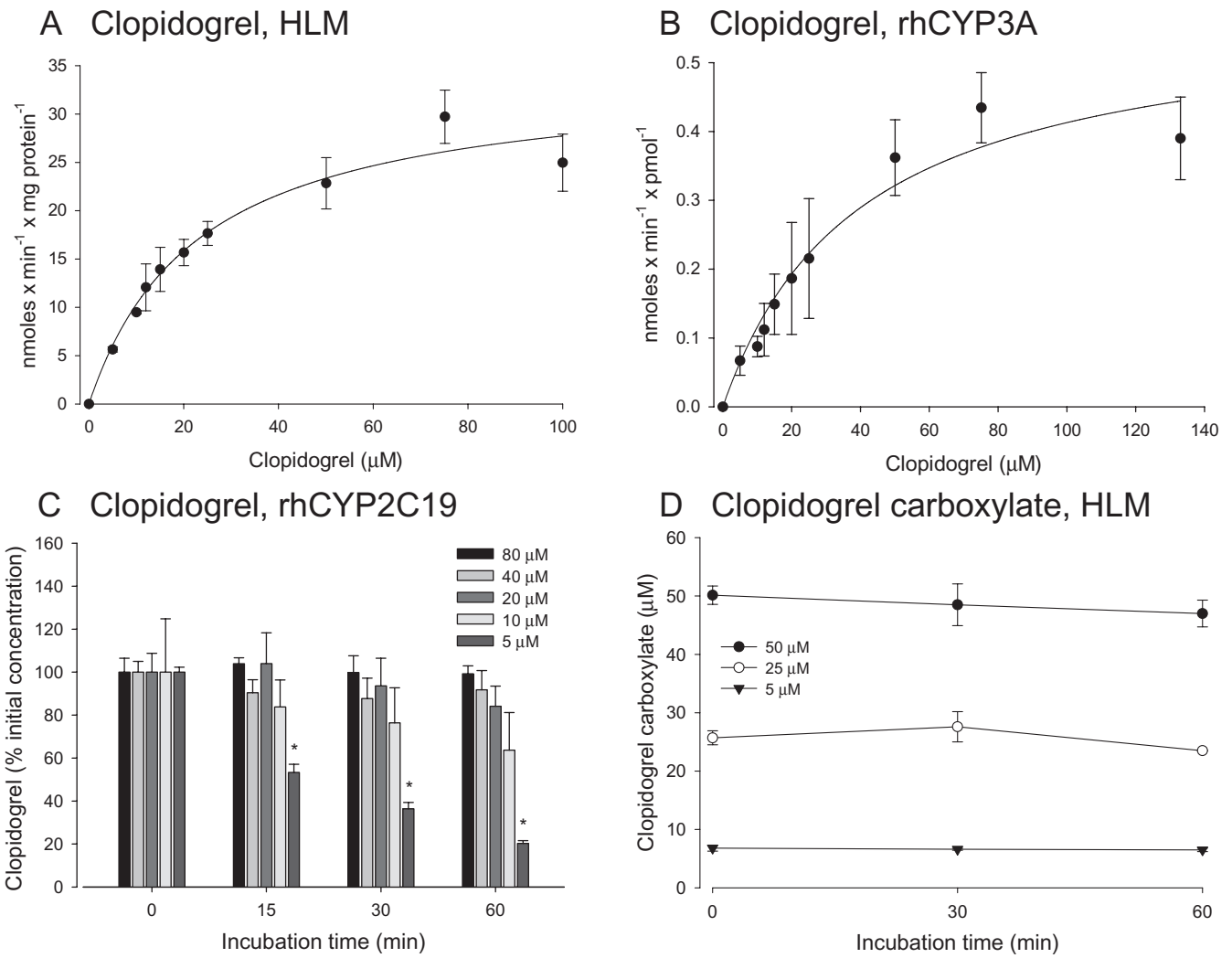

Figure 1

Biotransformation of clopidogrel and its carboxylate metabolite. Kinetic parameters for clopidogrel metabolism were determined in the presence of human liver microsomes (HLM) or supersomes (rhCYP3A4 or rhCYP2C19). Where possible, data were described using the Michaelis-Menten model. Increasing concentrations of clopidogrel (A, B, C) or of its carboxylate derivative (D) were incubated with HLM, rhCYP3A4 or rhCYP2C19 and analysed by HPLC. The biotransformation of clopidogrel in the presence of CYP3A4 showed a clear saturation and could be described by Michaelis-Menten kinetics (A, B). CYP2C19 activates clopidogrel only at concentrations <20 μM (C). The carboxylate metabolite of clopidogrel is not metabolized by HLM (D).

Inhibition of clopidogrel biotransformation by CYP inhibitors or substrates was confirmed by the determination of the corresponding IC_{50} values (Table 1). Ketoconazole showed a slightly stronger inhibitory effect (IC_{50} 0.03 μM) than clarithromycin (IC_{50} 0.33 μM). An IC_{50} for ciprofloxacin could not be determined, as 50% inhibition were not reached up to 500 μM. Simvastatin and atorvastatin both revealed dose-dependent inhibition of clopidogrel biotransformation with IC_{50} values of 1.28 μM and 16.9 μM respectively.

IPA by activated clopidogrel

Further, we developed a test system for analysing platelet aggregation in incubations containing HLM, the drugs investigated and human platelets (Figure 5A). Clopidogrel inhibited platelet aggregation concentration-dependently, reaching 67% at 200 μM. In contrast, clopidogrel without biotransformation by HLM showed no significant IPA. To demonstrate the formation of an active metabolite, we investigated the antiplatelet effect of clopidogrel in the presence of 5 mM GSH. As GSH is known to

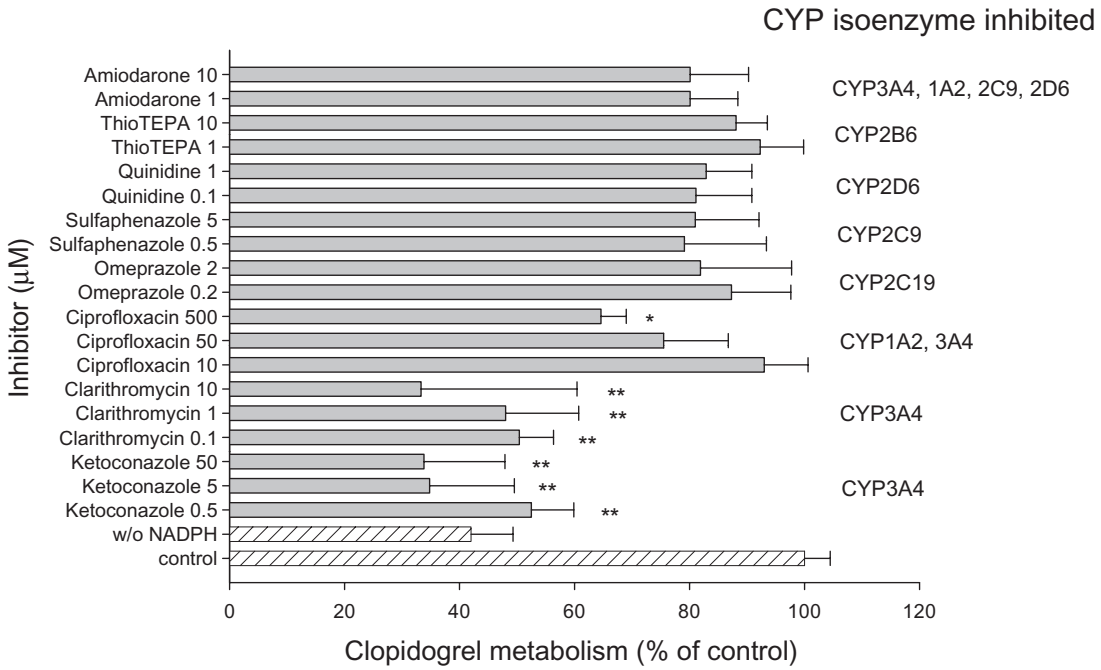

Figure 2

Effect of various CYP450 inhibitors on clopidogrel biotransformation. Clopidogrel (40 μM) was co-incubated in the presence of human liver microsomes (HLM) with different concentrations of CYP inhibitors. Data are expressed as the percentage of clopidogrel activated in the presence of the inhibitor compared with biotransformation without inhibitor (100%). Data points consist of five individual determinations. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.001$ versus control incubations.

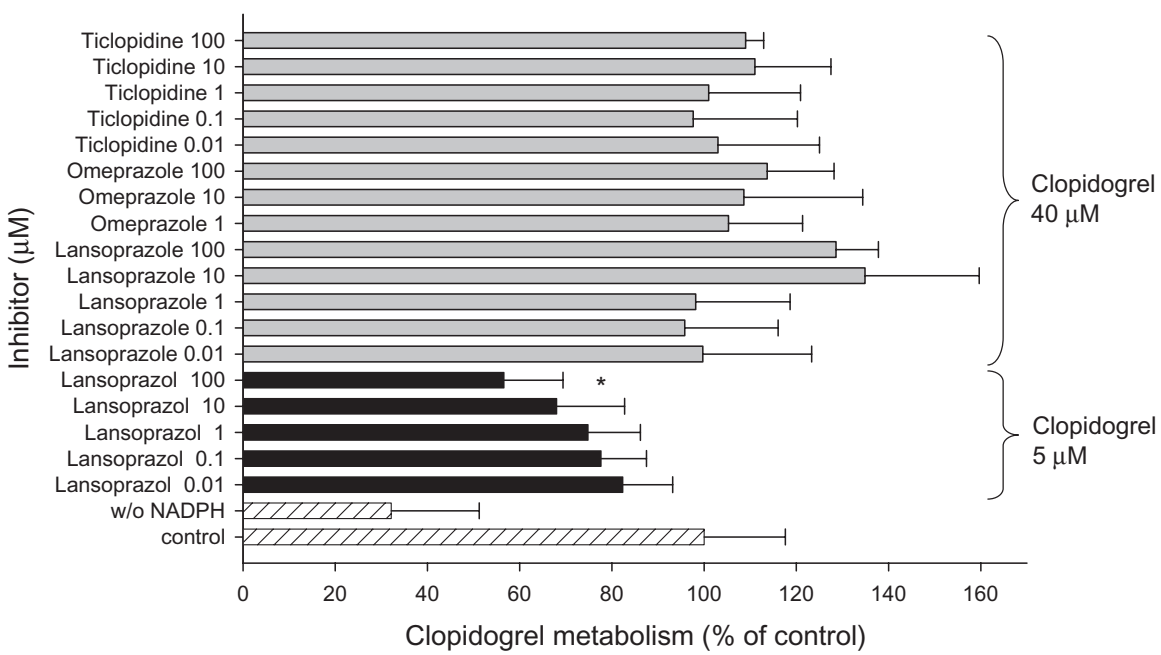

Figure 3

Effect of CYP2C19 inhibitors on clopidogrel biotransformation. Clopidogrel (40 or 5 μM) was co-incubated with different concentrations of CYP2C19 inhibitors in the presence of human liver microsomes (HLM). Data are expressed as the percentage clopidogrel biotransformation in the presence of the inhibitor compared with biotransformation without inhibitor (100%). Data points consist of three individual determinations. Data are presented as mean ± SEM. **$P < 0.001$ versus control incubations.

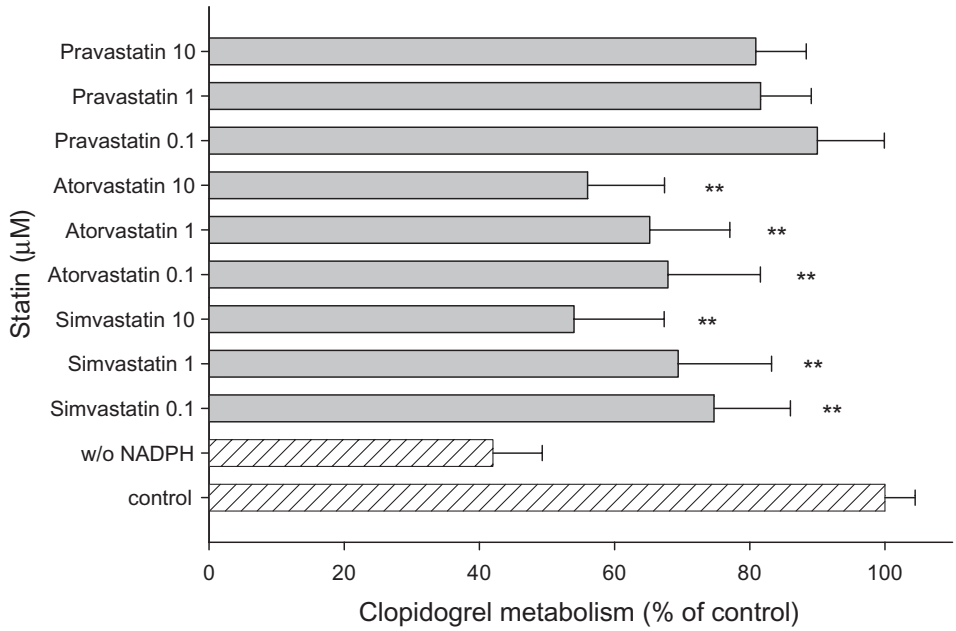

Figure 4

Effect of statins on clopidogrel biotransformation. Clopidogrel (40 μM) was co-incubated with different concentrations of statins in the presence of human liver microsomes (HLM). Data are expressed as the percentage clopidogrel biotransformation compared with the biotransformation without inhibitor (100%). Data points consist of five individual determinations. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.001$ versus control incubations.

Table 1

IC_{50} (μM) values for inhibition of clopidogrel biotransformation by human liver microsomes (HLM) and for the antiplatelet effect of clopidogrel in the presence of CYP3A4 inhibitors or substrates

	IC_{50} for biotransformation by HLM (μM)	IC_{50} for antiplatelet effect (μM)
Ketoconazole	0.03 ± 0.07	0.55 ± 0.06
Clarithromycin	0.33 ± 0.09	0.95 ± 0.04
Simvastatin	1.28 ± 0.09	1.29 ± 0.02
Atorvastatin	16.9 ± 0.3	3.83 ± 0.07

The assay conditions are described in *Methods*. The clopidogrel concentration was 40 μM for all incubations. Data are expressed as mean ± SEM of $n = 4–8$ experiments.

affect formation of and breaking of disulfide bonds in cells (Dickinson and Forman, 2002), we hypothesized that it could trap the newly formed thiol group of activated clopidogrel (Ding *et al.*, 2003). As expected, addition of GSH significantly decreased the effect of clopidogrel on platelet aggregation (Figure 5B).

Clopidogrel carboxylate has no antiplatelet effect

Clopidogrel and clopidogrel carboxylate were incubated individually with HLM for 5, 15, 30 and 60 min prior to ADP-induced platelet aggregation. As expected, clopidogrel showed a time-dependent inhibitory effect on platelet aggregation, while 80 and 200 μM clopidogrel carboxylate completely failed to inhibit platelet aggregation after incubation with HLM (Figure 6A and 200 μM not shown).

Interaction of clopidogrel with CYP3A4

In order to investigate the reason for the different binding affinity of neutral clopidogrel and its much more hydrophilic carboxylate derivative to CYP3A4, we manually docked both compounds into the active site of CYP3A4. As shown in Figure 6B, the neutral and hydrophobic clopidogrel fits smoothly into the hydrophobic active site of CYP3A4, which is optimized to recognize and bind hydrophobic substances. In contrast, the slightly smaller carboxylate metabolite contains a polar and solvated carboxylate functionality that does not bind in a productive way to the hydrophobic catalytic site of CYP3A4 (not shown).

Effect of CYP3A4 inhibitors and/or substrates on clopidogrel-associated IPA

Finally, we addressed the question whether a diminished clopidogrel biotransformation by CYP3A4 inhibitors or substrates is associated with an IPA. As shown in Table 1, ketoconazole turned out to be the

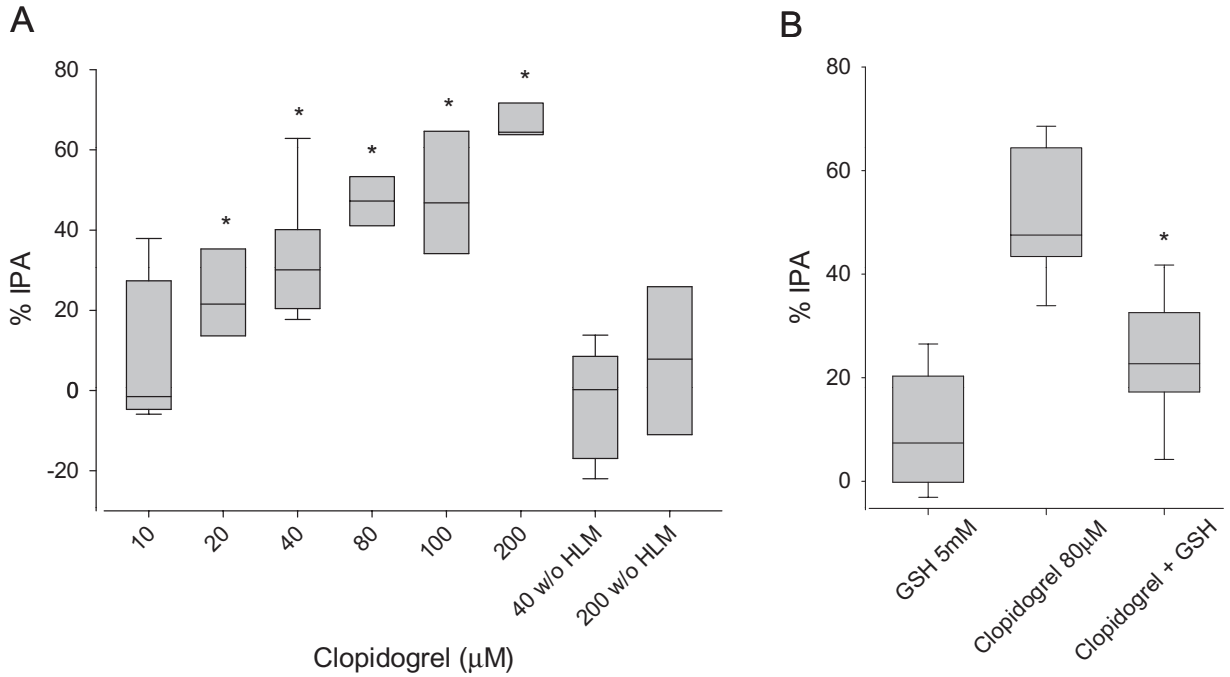

Figure 5

Effect of activated clopidogrel on platelet aggregation. (A) Increasing concentrations of clopidogrel (10–200 μM) activated by human liver microsomes (HLM) were incubated with platelet-rich plasma. Platelet aggregation was determined in response to 2.5 μM ADP by light transmittance aggregometry. Clopidogrel incubated in the absence of HLM served as a negative control. (B) In the presence of 5 mM glutathione, the effect of clopidogrel on platelet aggregation was significantly decreased. GSH itself had no effect on platelet aggregation. Results are expressed as a percentage inhibition of platelet aggregation (IPA) calculated from the maximum platelet aggregation in the presence of the solvent. Data are presented as box-plots with the median indicated by the line within the box ($n = 8$ to 12). *$P < 0.001$ versus clopidogrel 40 μM without (w/o) HLM (A) or clopidogrel 80 μM (B).

most potent inhibitor with an IC_{50} of 0.55 μM, confirming our results obtained in the biotransformation experiments. The inhibitory effect of clarithromycin was comparable (IC_{50} 0.95 μM), whereas the statins were less effective inhibitors.

Discussion and conclusions

In our studies, clopidogrel was metabolized in a concentration-dependent manner in all incubations containing CYP3A4 (Figure 1A,B), whereas supersomes containing rhCYP2C19 metabolized clopidogrel only at substrate concentrations ≤10 μM. Regarding the inhibition of CYP2C19 by clopidogrel, our data are in accordance with recent studies (Hagihara *et al.*, 2008; Nishiya *et al.*, 2009), showing that clopidogrel is a mechanism-based inhibitor of CYP2C19 with an IC_{50} in the low micromolar range. Clopidogrel is rapidly and efficiently absorbed from the GI tract (Caplain *et al.*, 1999), but more than 85% of the drug is converted to its carboxylate metabolite during the first passage across intestine and liver (Taubert *et al.*, 2004). Assuming that the maximal concentration of the carboxylate derivative of clopidogrel in plasma approximately corresponds to the maximal clopidogrel concentration in the liver (no data on the clopidogrel concentration in the liver are available), a maximal concentration of 5–20 $\mu mol \cdot L^{-1}$ is reached in hepatocytes after ingestion of 75 mg clopidogrel (Caplain *et al.*, 1999; Taubert *et al.*, 2004). Taking into account that clopidogrel inhibits CYP2C19 at concentrations >10 μM without affecting CYP3A4 and that, after oral ingestion of 75 mg, the hepatocellular clopidogrel concentration will drop below 10 μM with time, it can be expected that *in vivo* both CYP3A4 and CYP2C19 contribute to clopidogrel biotransformation. These considerations therefore help to explain why both, strong inhibitors of CYP3A4 (Suh *et al.*, 2006; Farid *et al.*, 2007; Siller-Matula *et al.*, 2008) and genetic variants of CYP2C19 (Kim *et al.*, 2008; Mega *et al.*, 2009; Simon *et al.*, 2009), are associated with impaired antiplatelet activity of clopidogrel.

In contrast to the recent study of Kazui *et al.* (2010), in the current study, clopidogrel was biotransformed not only by HML, but also by rhCYP3A4, indicating that CYP3A4 can also perform the conversion of clopidogrel to 2-oxo-

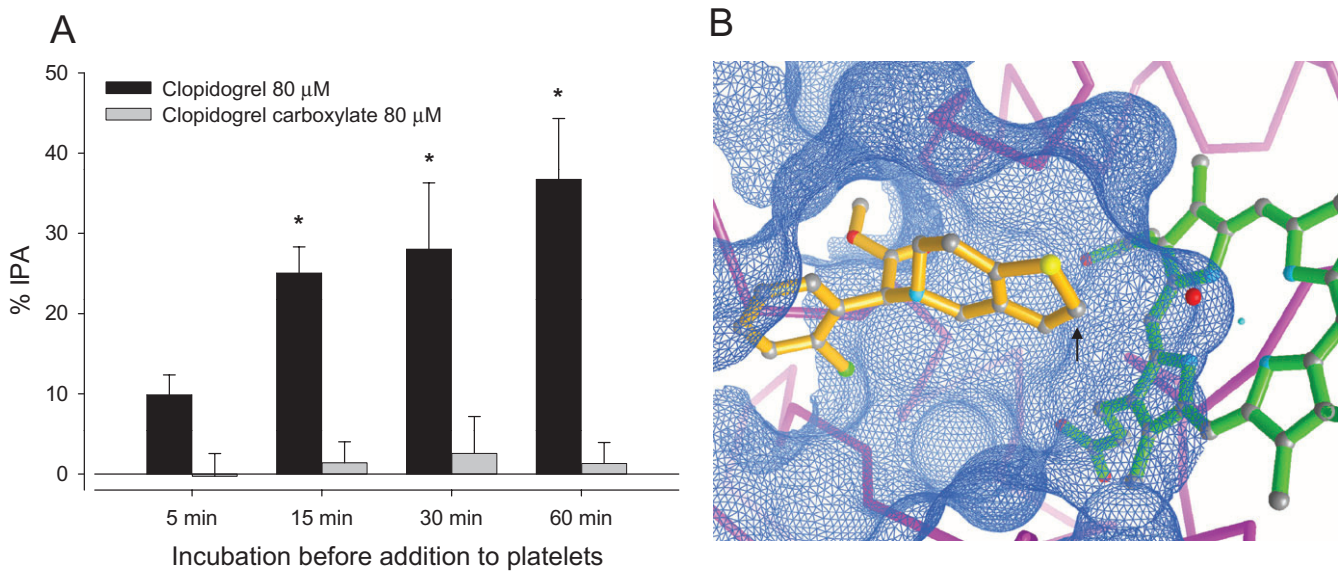

Figure 6

Time course of the effect of clopidogrel or clopidogrel carboxylate (80 μM) on platelet aggregation and interaction of clopidogrel with CYP3A4. (A) Clopidogrel or clopidogrel carboxylate were incubated with human liver microsomes (HLM) for 5, 15, 30 or 60 min. At the indicated times, aliquots were incubated with platelet-rich plasma and platelet aggregation determined in response to 2.5 μM ADP. Clopidogrel inhibited platelet biotransformation over time whereas the carboxylate derivative did not affect platelet aggregation. Results are expressed as the percentage inhibition of platelet aggregation (% IPA), calculated from the maximum platelet aggregation obtained in the presence of the vehicle (1% methanol v·v^{-1}). Data are presented as mean ± SEM ($n = 4$). *$P < 0.05$, **$P < 0.01$ clopidogrel or clopidogrel carboxylate versus incubations containing no clopidogrel (not shown). (B) Clopidogrel (orange) interacts with the active site (blue net) of CYP3A4 (backbone, magenta). A haem molecule with the Fe^{2+} (cyan dot in centre of haem) is shown in green. Note that the activated oxygen (red ball) above the Fe^{2+} of the haem is placed in an ideal position to interact with the 2-carbon of clopidogrel (arrow). In contrast, the clopidogrel carboxylate (not shown) carries a polar and solvated carboxylate group preventing it from bringing the 2-carbon close enough to the activated oxygen and the Fe^{2+} of the haem, which are crucial conditions for its subsequent biotransformation.

clopidogrel. Our data agree in this point with those of Clarke and Waskell (2003), who found that CYP3A4 and CYP3A5 are the most important CYPs for the biotransformation of clopidogrel. In the study of Kazui *et al.* (2010), CYP2C19, CYP1A2 and CYP2B6 were the most important CYPs regarding the formation of 2-oxo-clopidogrel from clopidogrel.

The good correlation between the inhibition of clopidogrel biotransformation and the antiplatelet effect by clopidogrel suggests that our systems are able to predict drug interactions with clopidogrel. In our study, ketoconazole, a potent inhibitor of CYP3A4, showed the strongest inhibitory effect on clopidogrel biotransformation and IPA. These data are in good agreement with a clinical study demonstrating that ketoconazole not only decreases clopidogrel biotransformation but also its antiplatelet activity (Farid *et al.*, 2007). Based on our data, the macrolide antibiotic clarithromycin and possibly other CYP3A4 inhibitors may have similar *in vivo* effects as ketoconazole.

Amiodarone is a drug prescribed often with clopidogrel and possibly interferes with its biotransformation (Lau *et al.*, 2004). It is mainly metabolized by CYP3A4 in humans and is an inhibitor of CYP2C9, CYP2D6 and CYP3A4 (Ohyama *et al.*, 2000). Surprisingly, amiodarone did not affect clopidogrel biotransformation in our *in vitro* system up to concentrations of 100 μM. This finding may be explained by the fact that the *in vivo* generated desethylamiodarone, the major metabolite of amiodarone, is a more potent inhibitor of human CYPs than amiodarone itself (Ohyama *et al.*, 2000). A prolonged incubation time would possibly have been necessary to generate this metabolite and detect inhibitory effects of amiodarone in our system.

Ciprofloxacin, a well-known CYP1A2 inhibitor, significantly inhibited clopidogrel biotransformation at a concentration of 500 μM, but not at lower concentrations. In the studies demonstrating inhibition of CYP1A2, ciprofloxacin concentrations in the range of 10–200 μM were used (Cherstniakova *et al.*, 2001; Karjalainen *et al.*, 2008). On the other hand, McLellan *et al.* (1996) reported that ciprofloxacin significantly decreased the activity of CYP3A4 when used at high concentrations (~2 mM), which is accordance with our findings.

Our investigations with statins are in good agreement with results presented by Lau *et al.* (2003),

Neubauer *et al.* (2003) and by Clarke and Waskell (2003). Atorvastatin and simvastatin are both metabolized by CYP3A4 and significantly inhibited clopidogrel biotransformation *in vitro*, whereas pravastatin showed no such inhibitory effect (Figure 4). Additionally, we could demonstrate that the inhibitory effect of atorvastatin and simvastatin on clopidogrel biotransformation resulted in an impaired antiplatelet effect of clopidogrel (Table 1). In agreement with our findings, Lau *et al.* demonstrated in their *ex vivo* study a dose-dependent attenuation of the clopidogrel-associated antiplatelet effects by atorvastatin (Lau *et al.*, 2003). The results of another small *ex vivo* analysis using simvastatin confirmed the occurrence of a clopidogrel-statin interaction (Neubauer *et al.*, 2003). In contrast, other studies investigating the influence of CYP3A4-metabolized statins on antiplatelet effects of clopidogrel failed to confirm these findings (Muller *et al.*, 2003; Gorchakova *et al.*, 2004; Mitsios *et al.*, 2004; Serebruany *et al.*, 2004; Vinholt *et al.*, 2005; Saw *et al.*, 2007). Taking into account our findings, the exact timepoints when clopidogrel and reversible CYP inhibitors such as statins are ingested may play a crucial role for the occurrence and possible manifestation of drug-drug interactions.

As clopidogrel inhibits CYP2C19 at concentrations >10 μM (this study and studies by Hagihara *et al.* (Hagihara *et al.*, 2008) and by Nishiya *et al.* (Nishiya *et al.*, 2009)), it could be expected that CYP2C19 inhibitors would not affect clopidogrel activation at high clopidogrel concentrations. Accordingly, the CYP2C19 inhibitors tested in the current study (ticlopidine, omeprazole and lansoprazole) revealed no inhibition of clopidogrel biotransformation at 40 μM clopidogrel. Lansoprazole, a strong inhibitor of CYP2C19 (Li *et al.*, 2004), impaired clopidogrel biotransformation only at 5 μM clopidogrel. As discussed above, in the liver of patients treated with clopidogrel, the clopidogrel concentration can be assumed to drop to levels at which inhibition of clopidogrel biotransformation by PPIs becomes potentially significant. This assumption is supported by studies showing that PPIs diminish the pharmacological effect of clopidogrel on platelet aggregation determined *ex vivo* (Gilard *et al.*, 2008; O'Donoghue *et al.*, 2009; Price *et al.*, 2009; Zuern *et al.*, 2010). Accordingly, in two retrospective studies with clinical endpoints, coadministration of PPIs was associated with an impaired outcome (Ho *et al.*, 2009; Juurlink *et al.*, 2009). Since a third retrospective, clinical study failed to show such an effect of PPIs (O'Donoghue *et al.*, 2009), the clinical relevance of the inhibition of clopidogrel biotransformation by PPIs is actually unclear, however. Nevertheless, PPIs with a strong inhibition of CYP2C19 such as lansoprazole, esomeprazole and omeprazole (Li *et al.*, 2004) should best be avoided in patients treated with clopidogrel, especially when they are treated also with CYP3A4 inhibitors.

In conclusion, we could demonstrate that CYP3A4 is the most important CYP isoenzyme for clopidogrel biotransformation at clopidogrel concentrations >10 μM, as clopidogrel inhibits CYP2C19 at high concentrations. At concentrations ≤10 μM, CYP2C19 starts to contribute to clopidogrel biotransformation and the clopidogrel biotransformation can be inhibited by PPIs. The concentration-dependent interaction pattern between CYP inhibitors, clopidogrel and CYP3A4 and CYP2C19 helps to explain the often diverging results regarding clopidogrel biotransformation and pharmacological activity between studies conducted *in vitro* and *in vivo*.

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Conflicts of interest

None of the authors declares any conflict of interest regarding this manuscript.

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