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RESEARCH PAPER

The paraoxonase-1 pathway is not a major bioactivation pathway of clopidogrel *in vitro*

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BACKGROUND AND PURPOSE

Clopidogrel is a prodrug bioactivated by cytochrome P450s (CYPs). More recently, paraoxonase-1 (PON1) has been proposed as a major contributor to clopidogrel metabolism. The purpose of this study was to assess the relative contribution of CYPs and PON1 to clopidogrel metabolism *in vitro*.

EXPERIMENTAL APPROACH

Clopidogrel metabolism was studied in human serum, recombinant PON1 enzyme (rePON1), pooled human liver microsomes (HLMs), HLMs with the *CYP2C19*1/*1* genotype and HLMs with the *CYP2C19*2/*2* genotype. Inhibition studies were also performed using specific CYP inhibitors and antibodies. Clopidogrel and its metabolites were measured using LC/MS/MS method.

KEY RESULTS

PON1 activity was highest in the human serum and there was no difference in PON1 activity between any of the HLM groups. The production of clopidogrel's active metabolite (clopidogrel-AM) from 2-oxo-clopidogrel in pooled HLMs was approximately 500 times that in serum. When 2-oxo-clopidogrel was incubated with rePON1, clopidogrel-AM was not detected. Clopidogrel-AM production from 2-oxo-clopidogrel was lower in *CYP2C19*2/*2* HLMs compared with *CYP2C19*1/*1* HLMs, while PON1 activity in HLMs with both genotypes was similar. Moreover, incubation with inhibitors of CYP3A, CYP2B6 and CYP2C19 significantly reduced clopidogrel bioactivation while a PON1 inhibitor, EDTA, had only a weak inhibitory effect.

CONCLUSION AND IMPLICATIONS

This *in vitro* study shows that the contribution of PON1 to clopidogrel metabolism is limited at clinically relevant concentrations. Moreover, CYP2C19, CYP2B6 and CYP3A play important roles in the bioactivation of clopidogrel.

Abbreviations

BMAP, 2-bromo-3′-methoxyacetophenone; CBP, 4-(4-chlorobenzyl) pyridine; clopidogrel-AM, clopidogrel active metabolite; CYP, cytochrome P450; HLMs, human liver microsomes; MAB, monoclonal antibody; PON1, paraoxonase-1; rePON, recombinant paraoxonase-1

Introduction

Clopidogrel, a thienopyridine derivative, is widely used as an antiplatelet agent (Quinn and Fitzgerald, 1999) for the secondary prevention of thrombosis in patients with acute coronary syndrome and stent implantation (Kolansky *et al.*, 2000). Clopidogrel is a prodrug mainly bioactivated by cytochrome P450s (CYPs). Approximately 85% of clopidogrel is transformed by hepatic carboxylesterase 1 into an inactive carboxylic acid while 15% is metabolized into an active metabolite in two steps. The first step consists of oxidation of the thiophene ring of clopidogrel to produce 2-oxo-clopidogrel. This is followed by the opening of the thiophene group of 2-oxo-clopidogrel, resulting in the pharmacologically active molecule (Savi *et al.*, 2000; Hagihara *et al.*, 2008; Gurbel *et al.*, 2009) (Figure 1).

After activation, the drug works by impeding the linkage of ADP to its platelet receptor. The thiol group links to the cysteine residue of the $P2Y_{12}$ ADP receptor with a disulfide bond. This reduces the ability of ADP to link to the receptor, inhibiting the activation of glycoprotein GIIb/IIIa complex, which normally initiates clot formation (Simon *et al.*, 2009).

Patients vary widely in their response to clopidogrel (Gurbel *et al.*, 2003; Ford, 2009) and a poor biological response has been repeatedly associated with an increased risk of recurrent ischaemic events (Combescure *et al.*, 2010). This variability is probably multifactorial, including both genetic and environmental factors (Ancrenaz *et al.*, 2010). However, the level of expression and activity of the CYPs, necessary for the bioactivation of clopidogrel, seems to play a crucial role. CYP polymorphisms could explain part of the variability in response to clopidogrel (Mega *et al.*, 2009). Few studies have assessed the complete clopidogrel metabolism, probably because of the chemical instability of the pharmacologically active metabolite. One study demonstrated the importance of the CYP1A subfamily in clopidogrel metabolism by measuring platelet aggregation after induction of various CYP isoforms in rat hepatic microsomes (Savi *et al.*, 1994). Using different inducers and inhibitors that modulate the anti-aggregating effect of clopidogrel, other studies have shown that CYP3A plays a role in clopidogrel metabolism (Lau *et al.*, 2003; 2004a). The involvement of CYP2C19 in clopidogrel metabolism has been suggested by recent pharmacogenetic studies showing that carriers of the *CYP2C19* loss of function alleles *CYP2C19*2* or **3* are often resistant to clopidogrel treatment (Hulot *et al.*, 2006; Fontana *et al.*, 2007; Ford, 2009; Shuldiner *et al.*, 2009). Moreover, clinical studies have shown that significant pharmacokinetic drug–drug interactions mediated by CYPs can occur, such as with proton pump inhibitors (Gilard *et al.*, 2008; Juurlink, 2009; Sibbing *et al.*, 2009). Kazui *et al.* assessed the function of CYP in clopidogrel metabolism *in vitro* and showed that CYP1A2, 2B6 and 2C19 are involved in the first step of clopidogrel metabolism, whereas CYP3A4, 2C9, 2C19 and 2B6 are principally involved in the second step (Kazui *et al.*, 2010).

Paraoxonase-1 (PON1) is a calcium-dependent esterase synthesized primarily in the liver and secreted into the blood, where it is associated with high-density lipoprotein particles (Mackness *et al.*, 1991). Recently, PON1 was proposed as an important contributor to the biotransformation of 2-oxo-clopidogrel into the clopidogrel-AM (Bouman *et al.*, 2011). However, *in vivo* studies did not confirm these findings, as polymorphisms responsible for loss of PON1 activity were not correlated with increased frequency of cardiovascular events

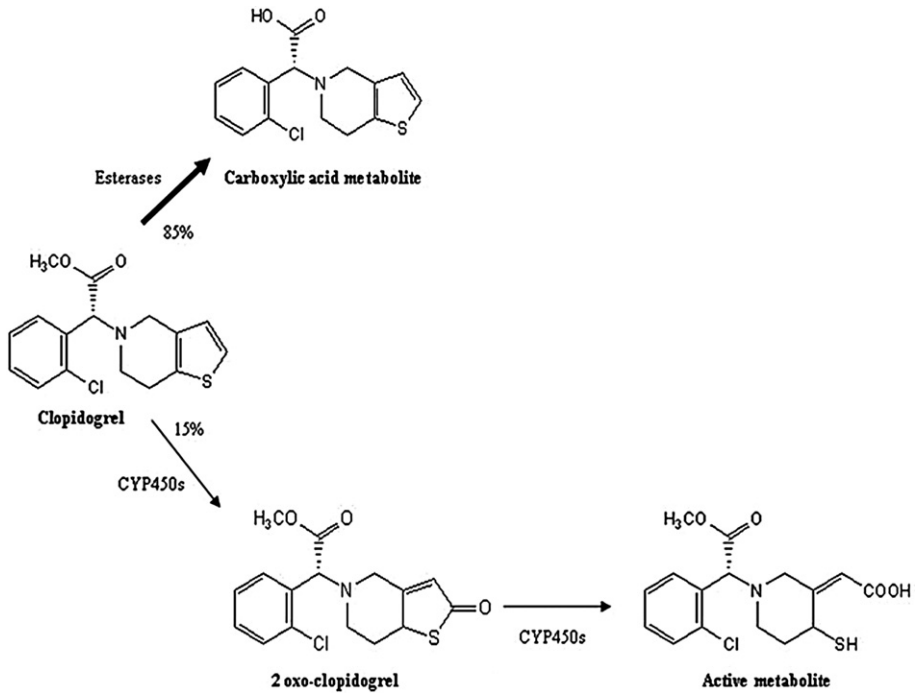

Figure 1

Pathways of clopidogrel metabolism.

in cohorts of cardiovascular patients treated with clopidogrel. Therefore, the involvement of PON1 in clopidogrel metabolism remains unclear (Fontana *et al.*, 2011; Hulot *et al.*, 2011; Sibbing *et al.*, 2011; Trenk *et al.*, 2011). A better understanding of clopidogrel metabolism is needed to predict drug–drug interactions and the responses of individual patients to clopidogrel treatment. We undertook this study to determine the relative involvement of CYPs and PON1 in the bioactivation of clopidogrel *in vitro*.

Methods

Reagents

Clopidogrel, ketoconazole, 2-bromo-3′-methoxyacetophenone (BMAP), omeprazole, 4-(4-chlorobenzyl)-pyridine (CBP) and the constituents of the NADPH-generating system (NADP+, isocitric acid, isocitrate dehydrogenase) were purchased from Sigma (Buchs, Switzerland). 2-Oxo-clopidogrel was purchased from Toronto Research Chemicals (North York, Canada). A derivative of clopidogrel-AM was purchased from Labforce (Nunningen, Switzerland). Furafylline was obtained from Salford Ultrafine Chemicals and Research (Manchester, England); sulfaphenazole was donated by Ciba-Geigy (Basel, Switzerland) and quinidine sulfate was donated by the Pharmacy of the Geneva University Hospitals. HPLC grade acetonitrile, methanol and potassium phosphate were supplied by Merck (Darmstadt, Germany). Ultra-pure water was supplied by a Milli-Q RG purification unit from Millipore (Bedford, MA, USA).

All stock solutions were prepared in methanol at a concentration of 1 mg·mL^{-1} and were stored at –20°C. The intermediate solutions were diluted from stock solutions with the incubation buffer.

Pooled HLMs and recombinant microsomes for CYP1A2, 2B6, 2C9, 2C19, 2D6, 3A4 and 3A5 (supersomes) were purchased from BD Gentest (Basel, Switzerland).

Microsomes with low or high activity for CYP2C19 (*CYP2C19*2/*2* or *CYP2C19*1/*1* genotype, respectively) were purchased from Tebu Bio (Offenbach, Germany).

Determination of kinetic parameters of S-mephenytoin in genotyped CYP2C19 microsomes

S-Mephenytoin was used as probe for the determination of CYP2C19 activity. We incubated 0.5 mg protein·mL^{-1} *CYP2C19*1/*1* HLMs or CYP2C19**2/*2* HLMs with different concentrations of S-mephenytoin (0, 10, 20, 30, 50, 100, 150 and 250 μM) in 0.1 M potassium phosphate buffer at pH 7.4. Mixtures were pre-incubated for 3 min at 37°C before the reaction was initiated by the addition of the NADPH-generating system (1 mM NADP, 5 mM isocitrate, 5 mM $MgCl_2$ and 1 UI·mL^{-1} isocitrate dehydrogenase in reaction buffer). After 40 min incubation at 37°C, the reaction was stopped with acetonitrile that contained 100 ng·mL^{-1} OH-mephenytoin-D_3 as an internal standard. After centrifugation at 10 000× *g* for 3 min at room temperature, the supernatants were diluted 1:5 in the mobile phase before injection of 10 μL diluted sample into the LC/MS/MS.

Determination of kinetic parameters of clopidogrel in HLMs with CYP2C19*1/*1 and CYP2C19*2/*2 genotypes

The kinetic parameters for the production of both 2-oxo-clopidogrel from clopidogrel and the clopidogrel-AM from 2-oxo-clopidogrel were evaluated in *CYP2C19*1/*1* HLMs and *CYP2C19*2/*2* HLMs. Clopidogrel or 2-oxo-clopidogrel at increasing concentrations (0, 0.5, 1, 5, 10, 50 and 100 μM) in incubation buffer (0.1 M potassium phosphate, pH 7.4) was added to samples containing 0.5 mg protein·mL^{-1} microsomes with 5 mM NaF to inhibit esterases activity and 5 mM glutathione for 3 min at 37°C. Then, we added the NADPH-generating system and incubated them for 30 min at 37°C. The reaction was stopped and the clopidogrel-AM was stabilized by adding 30 mM BMAP in acetonitrile. After centrifugation at 10 000× *g* for 3 min, supernatants were diluted 1:5 in the mobile phase before injection of 10 μL sample into the LC/MS/MS system.

Inhibition of clopidogrel metabolism by PON1 and CYP isoform-specific inhibitors

The metabolism of clopidogrel was investigated at 37°C under linear conditions. We prepared 0.5 mg microsomal protein·mL^{-1} CYP2C19-genotyped HLMs suspensions in reaction buffer (0.1 M phosphate potassium at pH 7.4). Samples were pre-incubated for 3 min at 37°C with 10 μM clopidogrel or 2-oxo-clopidogrel, 5 mM NaF, 5 mM glutathione, reaction buffer and CYP-specific inhibitors.

The specific CYP inhibitors (Dierks *et al.*, 2001) used in this study were 3 μM ketoconazole for CYP3A, 3 μM quinidine for CYP2D6, 5 μM sulfaphenazole for CYP2C9, 30 μM furafylline for CYP1A2, 50 μM omeprazole for CYP2C19 and 1 μM 4-(4-chlorobenzyl) pyridine for CYP2B6 (Korhonen *et al.*, 2007; Gallemann *et al.*, 2010). The PON1 inhibitor used is this study was 1 mM EDTA (Gonzalvo *et al.*, 1997). The reaction was initiated by the addition of 100 μL of NADPH-generating system and solutions were incubated for 30 min at 37°C. The reaction was stopped by the addition of 30 mM BMAP in acetonitrile. Samples were then analysed as described previously.

Inhibition of clopidogrel metabolism in human liver microsomes by monoclonal antibodies against CYP450s

Pooled HLMs (100 μg protein) were pre-incubated with monoclonal antibody (MAB)-CYP3A4 or MAB-CYP2C19 (from 0 to 10 μL) in ice for 20 min. We then added 10 μM 2-oxo-clopidogrel and 0.1 M NADPH-generating system in phosphate buffer (pH 7.4). After incubation for 30 min at 37°C, the samples were analysed as described previously.

Incubations with recombinant paraoxonase-1

Recombinant paraoxonase (rePON1) was expressed in *Escherichia coli* and isolated as described previously (Deakin *et al.*, 2007). Isolated rePON1 was stored in Tris buffer (50 mM Tris, pH 7.8, 20 mM $CaCl_2$ and 0.05% Tergitol). rePON1 activity was tested with phenylacetate as substrate. We incubated 0.1 M 2-oxo-clopidogrel with rePON1 (0 – 100 μg) for 20 min

in Tris buffer (50 mM Tris, pH 7.8, with 2 mM $CaCl_2$). Sample analysis was performed as described previously.

Paraoxonase activity

Phenylacetate is a well-known specific substrate for PON1 (Eckerson *et al.*, 1983; Smolen *et al.*, 1991). PON1 activity was measured as described previously (Blatter Garin *et al.*, 1994). Incubations of serum, supersomes or microsomal fractions with 4 mM phenylacetate in Tris buffer were performed at room temperature.

PON1 activity is reported as $U{\cdot}mL^{-1}$ for serum and $U{\cdot}mg^{-1}$ of protein for microsomal fractions and was calculated from the molar absorptivity coefficient of the phenol produced ($1310\ M^{-1}{\cdot}cm^{-1}$). One unit of arylesterase activity was defined as 1 μmol phenylacetate hydrolyzed min^{-1}. In order to correct for spontaneous substrate breakdown, we simultaneously ran blanks.

LC/MS/MS parameters

LC/MS/MS data were obtained from an Agilent 1100 Series LS system (Palo Alto, CA, USA) coupled with an API 4000 triple quadrupole mass spectrometer (AB Sciex, Concord, Canada). Chromatography was performed on a Phenomenex Kinetex C18 analytical column (50 mm × 2.1 mm, 2.6 μm; Torrance, CA, USA) preceded by a 0.5 μm KrudKatcher ultra in-line filter.

Mobile phase A contained water and mobile phase B contained acetonitrile. Initial conditions were 95:5 ($v{\cdot}v^{-1}$) mobile phase A : B. Following sample injection (10 μL), elution was performed by means of a gradient from 5 to 90% mobile phase B over 3 min, followed by 5% mobile phase B, held for 7 min. The column was then re-equilibrated to initial conditions. Mobile phase flow rate was maintained at $0.5\ mL{\cdot}min^{-1}$, and chromatography was performed at 20°C. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with positive ion electrospray ionization. The MRM transitions were 322.1→212.1, 338.1→183.1 and 504.1→354.1 for clopidogrel, 2-oxo-clopidogrel and the clopidogrel-AM respectively.

Statistical analysis

The results are presented as means of triplicate results ± SD. Differences between means were determined by Student's independent *t*-tests using SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA). P values < 0.05 were considered statistically significant. Graphic representations of data were created using GraphPad Prism version 4.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

*Paraoxonase activity in supersomes, pooled HLMs, CYP2C19*1/*1 HLMs and CYP2C19*2/*2 HLMs*

PON1 activity (mean ± SD) was 295 ± 28.5 $U{\cdot}mL^{-1}$, 2.01 ± 0.1 $U{\cdot}mg^{-1}$, 1.99 ± 0.04 $U{\cdot}mg^{-1}$ and 2.0 ± 0.04 $U{\cdot}mg^{-1}$ in human serum, pooled HLMs, *CYP2C19*1/*1* HLMs and *CYP2C19*2/*2* HLMs, respectively. No PON1 activity was detectable in any of the supersomes.

PON1 activity was high in serum and there was no difference in PON1 between any of the HLM groups.

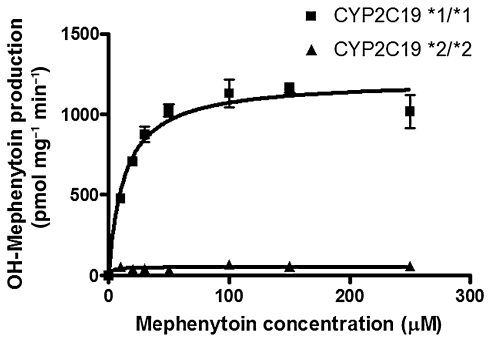

Figure 2

Kinetic analysis of OH-mephenytoin formation from mephenytoin in CYP2C19-genotyped human liver microsomes. Data are mean ± SD of three independent data points.

*CYP2C19 activity assessment in CYP2C19*1/*1 and CYP2C19*2/*2 HLMs*

OH-mephenytoin from S-mephenytoin in *CYP2C19*1/*1* HLMs was 20 times that in *CYP2C19*2/*2* HLMs (V_{max} = 1212 ± 31.3 $pmol{\cdot}min^{-1}{\cdot}mg^{-1}$ protein; CI95: 1147–1277 vs. V_{max} = 52.7 ± 3.85 $pmol{\cdot}min^{-1}{\cdot}mg^{-1}$ protein; CI95: 44.7–60.7) as shown in Figure 2. These results confirm that there is a good correlation between genotype and CYP2C19 activity when S-mephenytoin is used as a probe drug.

*Effect of the CYP2C19*2/*2 polymorphism on clopidogrel metabolism*

Kinetic parameters of clopidogrel or 2-oxo-clopidogrel biotransformation were determined using HLMs genotyped for CYP2C19 (Figure 3). For both steps of the biotransformation of clopidogrel, V_{max} values were higher and K_m values were lower in *CYP2C19*1/*1* HLMs than in *CYP2C19*2/*2* HLMs. For the first step, *CYP2C19*1/*1* HLMs had V_{max} = 2428 ± 106.1 $pmol{\cdot}min^{-1}{\cdot}mg^{-1}$ and K_m = 41.6 ± 4.1 μM, while *CYP2C19*2/*2* HLMs had V_{max} = 2081 ± 200.5 $pmol{\cdot}min^{-1}{\cdot}mg^{-1}$ and K_m = 61.4 ± 12 μM. During transformation from 2-oxo-clopidogrel to clopidogrel-AM, V_{max} was higher and K_m was lower in *CYP2C19*1/*1* HLMs than in *CYP2C19*2/*2* genotype, respectively (V_{max} = 18.4 ± 0.96 $pmol{\cdot}min^{-1}{\cdot}mg^{-1}$ vs. 4.1 ± 0.08 $pmol{\cdot}min^{-1}{\cdot}mg^{-1}$; K_m = 5.1 ± 1.1 μM vs. 10.4 ± 0.66 μM). These findings imply that CYP2C19 played an important role in the two steps of the bioactivation of clopidogrel.

Effect of specific CYP-chemical inhibitors on clopidogrel metabolism

To assess the clopidogrel metabolic pathways, clopidogrel or 2-oxo-clopidogrel was incubated in the presence of specific CYP inhibitors (Figure 4). For the transformation of 2-oxo-clopidogrel to clopidogrel-AM, ketoconazole and CBP were the most potent inhibitors. Ketoconazole and CBP inhibited

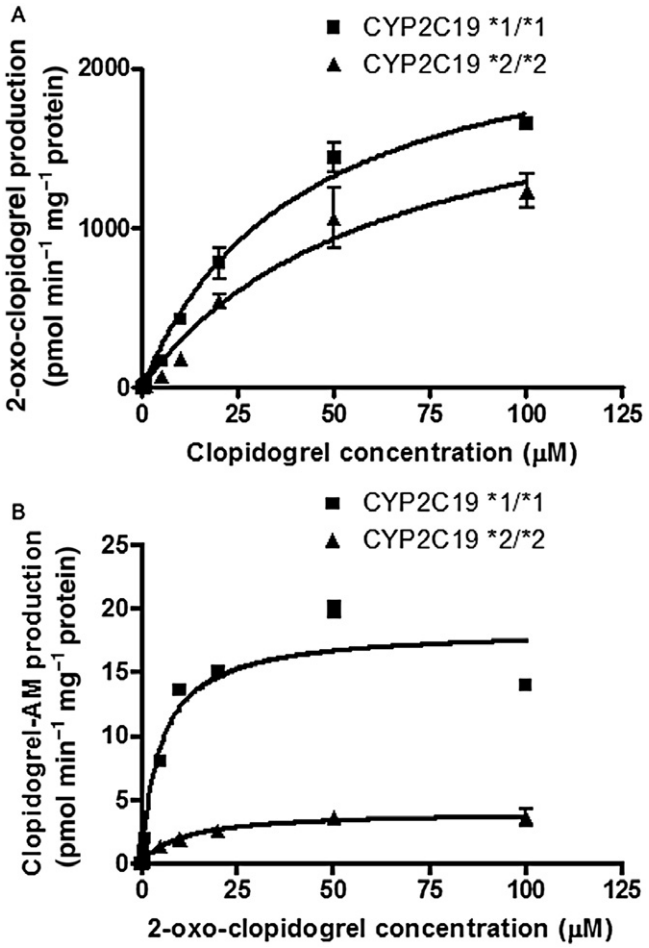

Figure 3

Kinetic analysis of 2-oxo-clopidogrel production from clopidogrel (A) and clopidogrel-AM production from 2-oxo-clopidogrel (B) in human liver microsomes (HLMs) with *CYP2C19*1/*1* and *CYP2C19*2/*2* genotypes. Data are mean ± SD of three independent data points.

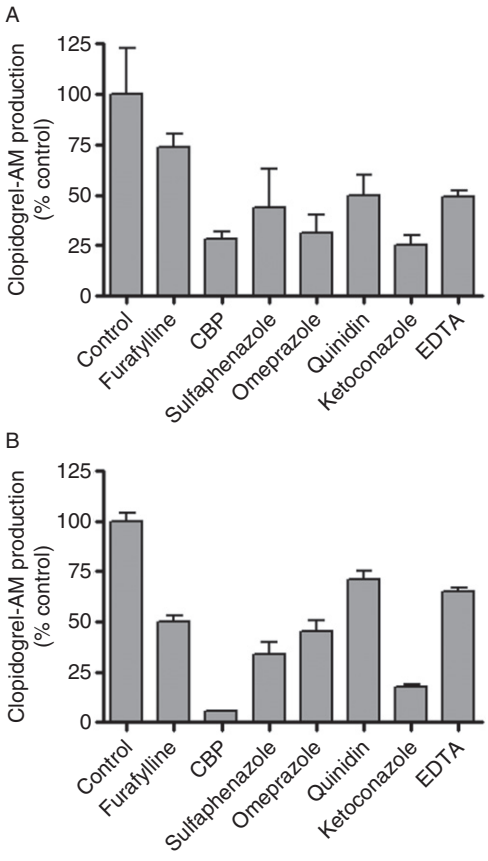

Figure 4

Inhibitory effects of cytochrome P450 (CYP) and paraoxonase (PON) inhibitors on the metabolic pathways from 2-oxo-clopidogrel to clopidogrel-AM (A) and from clopidogrel to clopidogrel-AM (B) in human liver microsomes (HLMs) with 10 μM clopidogrel or 10 μM 2-oxo-clopidogrel. Each column represents the mean ± SD of three independent data points.

clopidogrel-AM formation by 75 ± 5% and 72 ± 4%, respectively, in pooled HLMs. Omeprazole and EDTA inhibited the second pathway by 69 ± 9% and 51 ± 3%, respectively. For the transformation of clopidogrel to clopidogrel-AM representing the complete metabolic pathway, ketoconazole and CBP were also potent inhibitors with 82 ± 0.7% and 94 ± 0.2% inhibition, respectively. Omeprazole and sulfaphenazole inhibited this pathway by 55 ± 5.8% and 66 ± 5.5%, respectively. EDTA inhibited the complete metabolism in pooled HLMs by 35 ± 2%.

None of these CYP-specific inhibitors affected PON1 activity when tested with phenylacetate, whereas EDTA inhibited the production of phenol by 67 ± 9% (data not shown).

Effect of antibodies against CYP3A4 and CYP2C19 on 2-oxo-clopidogrel metabolism

The effect of MAB against CYP3A4 and CYP2C19 on the second step of clopidogrel metabolism was investigated in pooled HLMs. When 10 μL of each MAB was incubated with 100 μg microsomal proteins and 0.1 M 2-oxo-clopidogrel, clopidogrel-AM production decreased by 9 ± 3% ($P < 0.05$) for MAB-CYP2C19 and 49 ± 4% ($P < 0.005$) for MAB-CYP3A4. This confirms that CYP2C19 and CYP3A4 are involved in the production of clopidogrel-AM from 2-oxo-clopidogrel, and that CYP3A4 is especially important in this process (Figure 5).

Clopidogrel and 2-oxo-clopidogrel metabolism in human liver microsomes and in human serum

Clopidogrel and 2-oxo-clopidogrel were incubated with human serum and HLMs. PON1 activity in serum was adjusted to that of the HLMs by an appropriate dilution in phosphate buffer, pH 7.4. We measured both 2-oxo-clopidogrel production from clopidogrel and clopidogrel-AM production from 2-oxo-clopidogrel. Clopidogrel metabolites from both steps were only produced at clinically relevant concentrations in HLMs. In the first step, 2-oxo-clopidogrel production from 100 μM clopidogrel in HLMs was more than

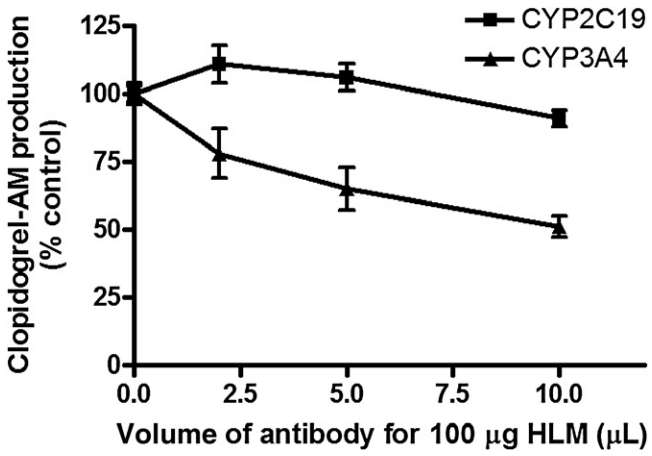

Figure 5

Active metabolite production from 2-oxo-clopidogrel 10 μM in human liver microsomes (HLM) incubated with increasing quantities of anti-CYP3A4 and anti-CYP2C19 antibodies. Data are mean ± SD of three independent data points.

180 times that in serum. Clopidogrel-AM production from 100 μM 2-oxo-clopidogrel in HLMs was about 500 times that in serum (Figure 6).

These data are consistent with the major involvement of CYPs in clopidogrel metabolism with a limited contribution of PON1.

2-Oxo-clopidogrel metabolism using recombinant PON1

PON1 activity of recombinant (re)PON1 was tested with phenylacetate as substrate and 2-oxo-clopidogrel was incubated with rePON1. Clopidogrel-AM was not detected in these conditions even though PON1 activity in rePON was very high (35 kU·mg^{-1} protein) (Figure 7).

Discussion and conclusions

PON1 is unlikely to be involved in the bioactivation of clopidogrel. In fact, the amount of clopidogrel-AM was higher in *CYP2C19*1/*1* HLMs than in *CYP2C19*2/*2* HLMs, while PON1 activity was similar in both HLMs (Figure 3). Moreover, no significant clopidogrel-AM production was observed using human serum with high PON1 activity, nor was it produced when incubated with rePON1. When PON1 was inhibited by EDTA, only a weak inhibition of clopidogrel-AM production was observed. However, significant inhibition of clopidogrel-AM production was observed when CYP-specific inhibitors of CYP2B6, CYP3A and CYP2C19 were used. The involvement of CYP3A in clopidogrel metabolism was confirmed by a dose-dependent inhibition of clopidogrel-AM production with antibodies against CYP3A. With antibodies against CYP2C19, the inhibition of clopidogrel-AM production was seen only with a high dose of antibody. Kazui *et al.* reported that supersomes have different contributions to clopidogrel metabolism (Kazui *et al.*, 2010). In our study,

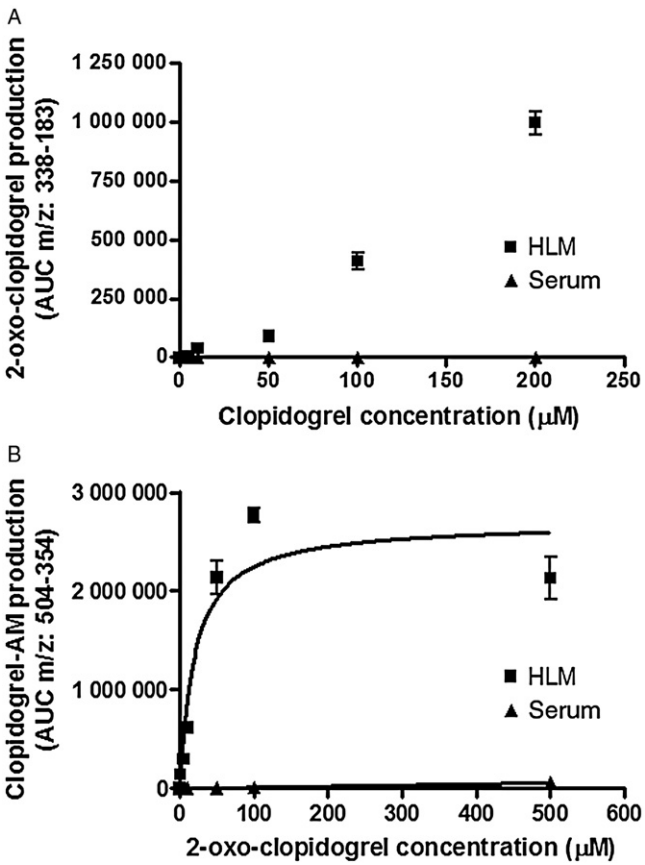

Figure 6

Kinetic analysis of 2-oxo-clopidogrel production from clopidogrel (A) and clopidogrel-AM production from 2-oxo-clopidogrel (B) in human serum and pooled human liver microsomes (HLMs) with adjusted paraoxonase 1 (PON1) activity. Data are mean ± SD of three independent data points.

similar data were obtained for the involvement of CYPs in clopidogrel metabolism whereas no PON1 activity was detected in the supersomes tested. Our results also contrast with a recent report of primary mediation of the second step of clopidogrel bioactivation by PON1 (Bouman *et al.*, 2011). Our data do agree, however, with other recent *in vitro* (Dansette *et al.*, 2011) and *in vivo* data (Fontana *et al.*, 2011; Hulot *et al.*, 2011; Sibbing *et al.*, 2011; Trenk *et al.*, 2011). For example, Hulot *et al.* found no significant association between PON1 Q192R or L55M and clopidogrel-AM formation or the antiplatelet response to clopidogrel by using a stereoselective method that provided good resolution between thiol isomers (Hulot *et al.*, 2011).

Furthermore, incubations of 2-oxo-clopidogrel with HLMs, in the absence of the NADPH required as a cofactor for CYP-dependent activities, led to the formation of the non-active clopidogrel metabolite isomer (Dansette *et al.*, 2011). In this study, PON1 was clearly involved in clopidogrel metabolism, but the resulting metabolite was a minor inactive isomer compared with the active metabolite produced by cytochromes P450. Two metabolic pathways were proposed for the opening of the thiolactone ring of 2-oxo-clopidogrel.

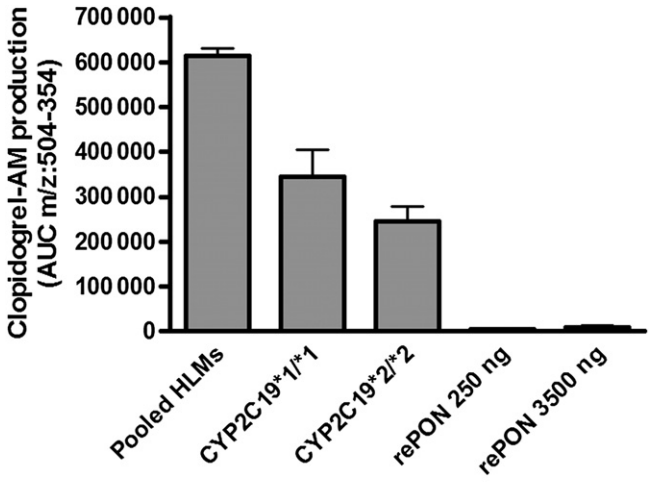

Figure 7

Comparison of the production of clopidogrel-AM in pooled human liver microsomes (HLMs), HLMs with *CYP2C19*1/*1* and *CYP2C19*2/*2* genotypes, and recombinant PON1 (rePON) at two different concentrations. Data are mean ± SD of three independent data points.

A major metabolic pathway was CYP450 dependant and leads to a cis thiol isomer whereas a minor pathway was the hydrolysis of 2-oxo-clopidogrel mediated probably by PON1 leading to the endo thiol isomer. Therefore, it is suggested that the analytical method used by Bouman *et al*. (2011) was not sufficiently selective to distinguish among the different isomers of the active metabolite, and that it preferentially detected the inactive metabolite produced by the PON1. Moreover, clopidogrel and 2-oxo clopidogrel concentrations used in the *in vitro* study from Bouman *et al*. were 10- to 100-fold higher compared with the commonly used concentrations *in vitro* (1–10 μM), which probably allowed these authors to detect high levels of the inactive isomer from the PON1 enzyme. In our study, a non-stereoselective method was used for clopidogrel-AM determination. Therefore, the thiol isomers were not separated, which probably explains the weak inhibition observed with EDTA. In fact, EDTA inhibited the production of the minor non-active clopidogrel metabolite isomer induced by PON1.

Clinical differences between PON1 gene variants observed by Bouman *et al*. may be due to a clopidogrel-unrelated mechanism as suggested by Wheeler *et al*. (2004). In fact, PON1 plays an important role in atherogenesis by protecting low-density lipoprotein from oxidation, which may partly explain the trend towards an association of the PON1-Q192R variant with higher cardiovascular risk.

Drug–drug interactions involving CYPs, like those between clopidogrel and proton pump inhibitors, have been associated with a high risk of acute thrombotic events (Gilard *et al*., 2008; Siller-Matula *et al*., 2009; Simon *et al*., 2009). Even though clopidogrel has been used for many years, its metabolism is not still completely understood. By exploring the decline in clopidogrel levels in plasma or by measuring the formation of 2-oxo-clopidogrel, initial investigations into this topic have shown that different CYPs are involved in clopidogrel metabolism (Savi *et al*., 1994; Clarke and Waskell, 2003).

However, these measures are only partly representative of its complete metabolism. Clopidogrel is mainly biotransformed by CYP3A4, 2C9, 2C19 and 2B6 to the pharmacologically active metabolite (Kazui *et al*., 2010). Savi *et al*. have shown that the subfamily CYP1A is involved in the bioactivation of clopidogrel by measuring the anti-aggregating activity of clopidogrel (Savi *et al*., 1994). We confirmed this latter finding by demonstrating that furafylline inhibited the metabolism of clopidogrel. The results of the CLARITY-TIMI 28-randomized clinical trial provided further evidence of CYP1A involvement in clopidogrel metabolism. This trial involved only smokers with myocardial infarction who were classified according to their smoking intensity. The results indicated that smoking, which induces CYP1A2, enhances the beneficial effect of clopidogrel on angiographic and clinical outcomes like myocardial infarction or urgent revascularization (Desai *et al*., 2009). CYP3A was also described as a principal cytochrome involved in the metabolism of clopidogrel because of the dose-dependent inhibition of the anti-aggregating properties of clopidogrel on platelets in the presence of atorvastatin, a CYP3A substrate (Clarke and Waskell, 2003; Lau *et al*., 2003).

We found CYP2C19 to be involved in both steps of clopidogrel bioactivation and this could explain the widely debated reports of interactions between clopidogrel and proton pump inhibitors. In the present study, the decrease in clopidogrel-AM production in HLMs with the *CYP2C19*2/*2* genotype clearly demonstrated the involvement of CYP2C19 in clopidogrel bioactivation.

Using CYP specific chemical inhibitors in HLMs, we demonstrated that CYP3A plays an important role in clopidogrel metabolism as do CYP2B6 and CYP2C19. It is well known that there is no correlation between CYP3A4 and CYP3A5 alleles and enzymes activity. Indeed, Oneda *et al*. showed a weak correlation between CYP3A phenotypes, measured using midazolam as a substrate, and CYP3A4, CYP3A5 and CYP3A7 variants. In a meta-analysis from seven clinical trials, the correlation between CYP3A4/5 alleles and midazolam disposition was assessed. There was no difference in midazolam disposition between different CYP3A4/5 genotypes. The authors concluded that environmental factors explain the majority of CYP3A activity variations. Therefore, a correlation between the response to clopidogrel and CYP3A activity should be performed by phenotyping with a specific probe drug such as midazolam (Oneda *et al*., 2009).

Farid *et al*. (2007) showed a significant decrease in the AUC and C_{max} of the clopidogrel active metabolite (29% and 61%, respectively) during the maintaining dose in the presence of ketoconazole. Ketoconazole also reduced the inhibition of platelet aggregation by 28% and 33% during the loading and maintaining dose, respectively (Farid *et al*., 2007). Moreover, Lau *et al*. have shown, by measuring the platelet aggregation, that the addition of the CYP3A4 substrate atorvastatin to clopidogrel produced a dose-dependent reduction of the anti-aggregating effect of clopidogrel in patients with a coronary stent ($P < 0.0001$) (Lau *et al*., 2004a). In another study, the same group showed an inverse correlation between platelet aggregation and CYP3A4 activity

($P = 0.003$) (Lau *et al.*, 2004b). These data support an involvement of CYP3A in clopidogrel metabolism.

In the liver, CYP3A represents about 40% of all cytochromes and is also present in the intestine. Other important CYP families are CYP2C and CYP1A2 (Shimada *et al.*, 1994). Given the results observed for clopidogrel metabolism where CYP1A2, CYP2B6, CYP2C19 and CYP3A4/5 were the primary CYP involved, the effect of a deficiency, inhibition or induction of any one of these CYP might affect biological and clinical responses to clopidogrel.

In conclusion, we confirmed with this *in vitro* study that 2-oxo-clopidogrel is bioactivated mainly by CYP3A4/5, CYP2B6, and to a lesser extent by CYP2C19 and CYP2C9. No significant involvement of PON1 in the bioactivation of clopidogrel *in vitro* was observed at clinically relevant concentrations. In fact, the production of clopidogrel-AM in HLMs was more than 100 times that in serum and there was no observable production in rePON1. Moreover, the inhibition of clopidogrel metabolism by CYP-specific inhibitors is consistent with a predominant role of CYPs in its metabolism.

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Conflict of interest

None of the authors declare any conflict of interest.

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