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Bioactivation of Clopidogrel and Prasugrel: Factors Determining the Stereochemistry of the Thiol Metabolite Double Bond

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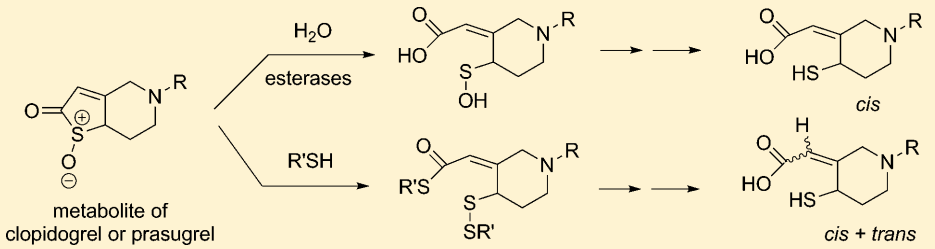

ABSTRACT: The antithrombotics of the tetrahydrothienopyridine series, clopidogrel and prasugrel, are prodrugs that must be metabolized in two steps to become pharmacologically active. The first step is the formation of a thiolactone metabolite. The second step is a further oxidation with the formation of a thiolactone sulfoxide whose hydrolytic opening leads to a sulfenic acid that is eventually reduced into the corresponding active *cis* thiol. Very few data were available on the formation of the isomer of the active *cis* thiol having a *trans* configuration of the double bond, the most striking result in that regard being that both *cis* and *trans* thiols were formed upon the metabolism of clopidogrel by human liver microsomes in the presence of glutathione (GSH), whereas only the *cis* thiol was detected in the sera of patients treated with this drug. This article shows that *trans* thiols are also formed upon the microsomal metabolism of prasugrel or its thiolactone metabolite in the presence of GSH and that metabolites having the *trans* configuration of the double bond are only formed when microsomal incubations are done in the presence of thiols, such as GSH, *N*-acetyl-cysteine, and mercaptoethanol. Intermediate formation of thioesters resulting from the reaction of GSH with the thiolactone sulfoxide metabolite appears to be responsible for *trans* thiol formation. Addition of human liver cytosol to the microsomal incubations led to a dramatic decrease of the formation of the *trans* thiol metabolites. These data suggest that cytosolic esterases would accelerate the hydrolytic opening of thiolactone sulfoxide intermediates and disfavor the formation of thioesters resulting from the reaction of these intermediates with GSH that is responsible for *trans* isomer formation. This would explain why *trans* thiols have not been detected in the sera of patients treated with clopidogrel.

INTRODUCTION

The antithrombotic drugs of the tetrahydrothienopyridine series, clopidogrel (Plavix, Iscover) **1a**, and prasugrel (Effient) **1b** (Figure 1), are prodrugs that must be metabolized *in vivo* into the corresponding pharmacologically active 4-mercapto-3-piperidinyliden acetic acid derivatives **5a** and **5b**, respectively, to exert their activity as antagonists of the platelet receptor P2Y12.[1−6] The first step of their metabolic activation leads to thiolactone metabolites **2a** and **2b**, respectively (Figure 1).[6−9] The second step of the metabolic activation of **1a** and **1b**, leading to the active metabolites **5a** and **5b**, respectively, is not a simple hydrolysis of the thioester bond of intermediate thiolactones **2a** and **2b**. Actually, it has been recently shown that such a simple hydrolysis of thiolactones **2a** and **2b**, which is catalyzed by thioesterases like paraoxonase-1, only leads to an endo isomer of **5a** and **5b** in which the double bond has migrated into the piperidine ring (**7a** and **7b** in Figure 1).[10−13] Formation of the active *cis* metabolite diastereomers **5** occurs in two steps, a P450-catalyzed oxidative opening of the thiolactone ring of **2** with the formation of intermediate sulfenic acids **4** and a reduction of **4** into the corresponding thiols **5** (Figure 1).[14−17] These electrophilic sulfenic acid intermediates are efficiently reduced by ascorbate or phosphines with quantitative formation of thiols **5**.[14−17] They are also reduced by thiols including glutathione (GSH) in two steps: a nucleophilic attack of the thiol on the electrophilic sulfur atom of the sulfenic acid function leading to mixed disulfides **6** and a reduction of these disulfides by a second thiol molecule (Figure 1).[14−19] Reduction of the disulfides can also be done by glutaredoxin and thioredoxin.[20,21] Recently, it has been shown that thiolactone sulfoxides **3** are formed as intermediates during the oxidative activation of thienopyridine antithrombotic drugs **1**. These intermediates were found to act as bis-electrophiles that were trapped by two nucleophiles, NuH (amine, thiol) and Nu′H (cyclopentane-1,3-dione (CPDH), dimedone, thiol), the first one being able to react with the CO carbon of the thiolactone sulfoxide and the second one with the sulfur atom of the intermediate sulfenic acid, **8**.[22] This led to the expected bis-adducts **9** (Figure 1) that were detected by HPLC-MS. One of them was completely

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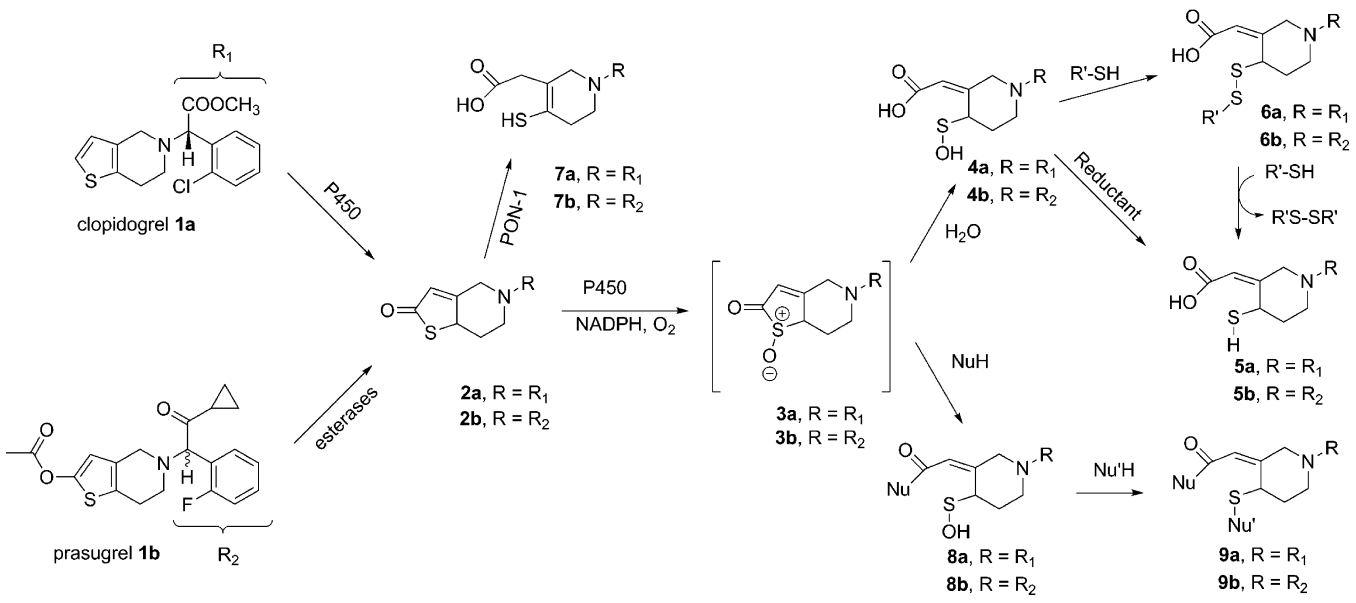

Figure 1. Detailed pathway of **1a** and **1b** bioactivation into active thiols **5a** and **5b**, and different reactions occurring upon the reaction of intermediates **3** with nucleophiles.

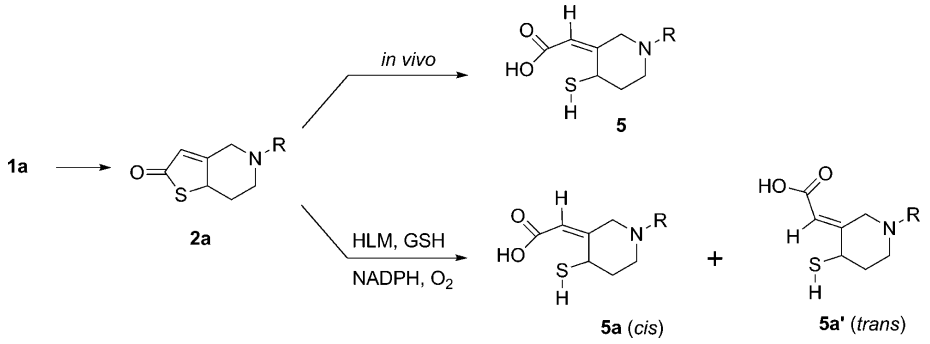

Figure 2. Different thiol stereoisomers formed upon the metabolism of **1a** *in vivo* in humans and *in vitro* in the presence of human liver microsomes, NADPH, and GSH. HLM means human liver microsomes.

characterized by ^{1}H and ^{13}C NMR spectroscopy using 1D and 2D methods.[22]

The metabolism of clopidogrel by human liver microsomes in the presence of NADPH and GSH was reported to lead to the two diastereomers of the active *cis* thiol **5a** and to the two diastereomers of its inactive isomer **5a′**, in which the double bond has a *trans* configuration (Figure 2).[1,6,19,22] Both **5a** and **5a′** were also formed when GSH was replaced with other thiols such as cysteine, *N*-acetyl-cysteine(NAC), or mercaptoethanol (ME).[18,19,22] By contrast, the metabolism of **1a** by human liver microsomes in the presence of NADPH and ascorbate only led to the *cis* thiols **5a**,[11,19] and in the sera of patients treated with **1a**, one only found these *cis* thiols **5a** (Figure 2).[23−25] In the case of prasugrel, **1b**, there was no information available in the literature about the possible formation of *trans* thiols **5b′** after *in vitro* metabolism of **1b** in the presence of human liver microsomes. These data lead to the following questions: (1) are *trans* thiol metabolites **5b′** also formed after *in vitro* metabolism of prasugrel by human liver microsomes in the presence of GSH? (2) What is the mechanism of formation of the *trans* thiols **5′**? (3) What are the factors determining the stereochemistry of the double bond of the thiol metabolites of clopidogrel *in vitro* and *in vivo*?

This article describes a study of the stereochemistry of the double bond of the thiol metabolites formed upon oxidative activation of **1a** and **1b** by human liver microsomes in the presence of various thiols or other reducing agents, with or without human liver cytosol. It shows that *trans* thiols are formed in the *in vitro* metabolism of prasugrel under conditions leading to *trans* thiols in the case of clopidogrel. It also shows that the factors controlling the stereochemistry of the thiol double bond are almost identical in the metabolism of **1a** and **1b**. Finally, it reports that the *trans* thiols are only formed when GSH or other thiols such as ME or NAC are present in the incubation medium and that their formation is strongly reduced in the presence of cytosol. These data give a first explanation for the exclusive formation of *cis* thiol **5a** in the *in vivo* metabolism of clopidogrel, and the formation of a **5a**−**5a′** mixture in the *in vitro* metabolism (human liver microsomes + GSH) of clopidogrel.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals. [7*S*]-2-Oxo-clopidogrel (SR121883), **2a**, was a gift of Sanofi-Aventis (Chilly-Mazarin, France). 2-Oxo-prasugrel base (racemate), **2b**, was obtained from Twisun Pharma (Shangai, China). All other products including enzymes were from Sigma-Aldrich (St. Quentin Fallavier, France).

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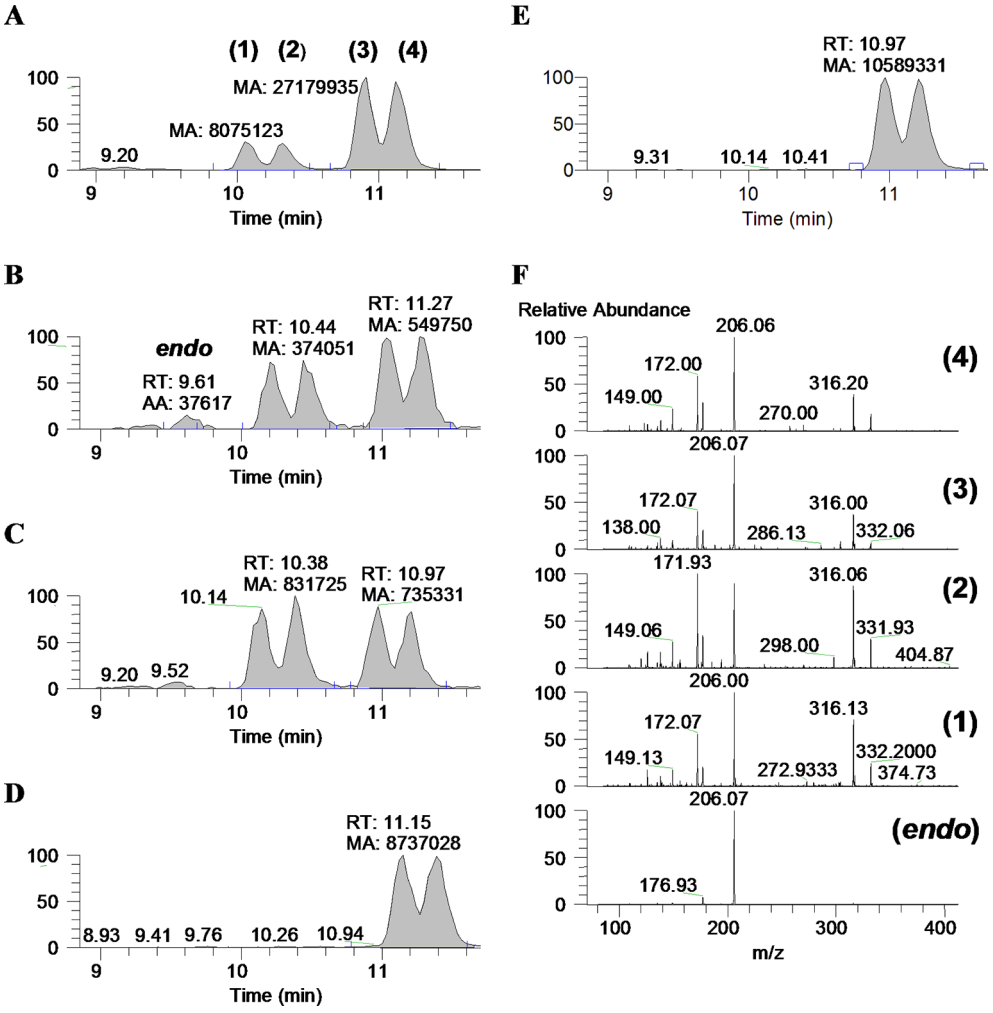

Figure 3. HPLC-MS study of thiol diastereomers **5b**′, (1) and (2), and **5 b**, (3) and (4), upon the metabolism of **2b** by human liver microsomes in the presence of various thiols or reductants. Small amounts of the endo isomer **7b** were also detected (see, for instance, panels B and C). Panels A–E show reconstructed chromatograms at m/z = 350.1216 obtained on the HPLC–HRMS system using 2 mM GSH, NAC, ME, ascorbate, or TCEP (panels A, B, C, D, and E, respectively) (MA: peak area). Panel F shows the MS^2 spectra obtained on the ion trap instrument at m/z = 350 at 25 eV for the diastereomers of thiols **5b**′ (1 and 2), **5b** (3 and 4), and for **7b**.

Microsomal Incubations. Human liver microsomes (pool, 10 mg protein/mL) and human liver cytosol (20 mg protein/mL) were obtained from BD-Gentest (Le Pont de Claix, France). Typical incubations were performed in 200 μL of potassium phosphate buffer (0.1M, pH 7.4) containing human liver microsomes (1 mg protein/mL), **2a** or **2b** (100 μM), and various nucleophiles (1 to 10 mM) with or without an NADPH generating system (1 mM NADP, 15 mM glucose-6-phosphate, and 2 u/mL of glucose-6-phosphate dehydrogenase) at 37 °C for 30 min. In the case of incubations with **2a**, 10 mM KF was added to inhibit the esterase-dependent hydrolysis of its methyl ester function.[1] Incubations performed with various KF concentrations (from 0 to 100 mM) showed that such a KF concentration variation only led to very minor changes of the **5a**′/**5a** ratio. In incubations performed in the presence of human liver cytosol, 20 μL of human liver cytosol was added. Reactions were stopped by adding one-half volume of CH_3CN containing 8% CH_3COOH. Proteins were removed by centrifugation at 13000g.

HPLC–MS Studies. HPLC–MS studies were performed on a Surveyor HPLC instrument coupled to a LCQ Advantage ion trap mass spectrometer (Thermo, Les Ulis, France), using a Shimadzu Shimpack C18 column (75 × 2.1 mm, 2.3 μm (Shimadzu, Marne La Vallée, France)), and a gradient of A+B starting at 0% B for 1 min then increasing linearly to 50% B in 13 min, then to 95% B in 1 min, and back to 0% B at 17.5 min, and equilibration at 0% B for 5.5 min (A = 2 mM ammonium acetate plus 0.2% HCOOH, pH 4.6, and B = CH_3CN/H_2O/HCOOH (980:18:2)) at 220 μL/min). Mass spectra were obtained by electrospray ionization in positive ionization mode detection (ESI^+) under the following conditions: source parameters, sheath gas 20, auxiliary gas 5, spray voltage 4.5 kV, capillary temperature 200 °C, capillary voltage 15 V, and m/z range for MS recorded generally between 300 and 700 (except for exploratory experiments with a wider range 300–1000). MS^2 energy was tested between 20 and 40 eV and was 35 eV. For all products, the indicated parent ions corresponded to $M + H^+$.

HPLC–HRMS Studies. HPLC–HRMS studies were performed on a Thermo Exactive Orbitrap spectrometer coupled to a Thermo Accela 600 HPLC (Thermo, Les Ulis France) system using the same column and gradient as those described above. ESI spray positive; tune, sheath gas 10, auxiliary gas 6, capillary temperature 250 °C, capillary voltage 65 V, tube lens 120 V, skimmer 22 V, vaporizer 300 °C; and mass range 300–1000, and HCD (higher-energy collisional dissociation) and CID (collision induced dissociation) disabled, resolution 60000.

RESULTS

Stereochemistry of the Thiol Metabolites Formed upon the Oxidation of Prasugrel by Human Liver Microsomes in the Presence of Various Reducing Agents. HPLC-MS studies of incubations of **1b** or **2b** with human liver microsomes in the presence of NADPH and 1 mM

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Chem. Res. Toxicol. XXXX, XXX, XXX–XXX

ascorbic acid led to only two products exhibiting the MS and MS^2 characteristics previously reported for the diastereomers of the *cis* thiol **5b**[15,26−28] (Figure 3D). Replacing ascorbic acid with GSH or another thiol such as cysteine, NAC, or ME, led to a clearly different thiol metabolites pattern with the appearance of two additional products (Figures 3A, B, and C) exhibiting identical MS and MS^2 characteristics (Figure 3F). An HPLC−HRMS study definitely showed that these two additional products were isomers of **5b** (Table1). Their MS^2 spectra, which were identical to those of **5b** but clearly different (Figure 3F) from that of the previously described endo isomer, **7b**, which was formed in very low amounts (Figure 3B and 3C), suggested that they were the *trans* diastereomers **5b′**. This was in agreement with the appearance of the HPLC peaks of the **5b′** diastereomers upon UV irradiation of *cis* **5b** derived from the incubation of **2b** with human liver microsomes in the presence of NADPH and ascorbate (data not shown), as UV irradiation of conjugated acids with a *cis* configuration of the double bond is known to lead to a *cis*/*trans* isomerization of this double bond.[29] This was also in complete agreement with the almost identical results previously described for the formation of both *cis* **5a** and *trans* **5a′** upon microsomal metabolism of **1a** or **2a** in the presence of GSH, NAC, or ME,[1,6,19,22] and the exclusive formation of **5a** in the presence of ascorbic acid instead of GSH.[11,19]

Table 1. HRMS Characteristics of the Main Metabolites Formed upon Incubations of 1b or 2b by Human Liver Microsomes in the Presence of GSH[a]

m/z (MH^+)			
obs.	calc.	chemical formula	structure
350.1216	350.1221	$C_{18}H_{21}FNO_3S^+$	**5b**
350.1213	350.1221	$C_{18}H_{21}FNO_3S^+$	**5b′**
655.1893	655.1902	$C_{28}H_{36}FN_4O_9S_2^+$	**6b**
944.2623	944.2635	$C_{38}H_{51}FN_7O_{14}S_3^+$	**9b**
334.1443	334.1449	$C_{18}H_{21}FNO_4^+$	alcohol[b]

[a]Conditions are described in Experimental Procedures. Mass (in Da) observed (obs.) or calculated (calc.) according to the indicated formula for the molecular ion. [b]Alcohol diastereomers resulting from the replacement of the SH group of **5b** and/or **5b′** with an OH group.

Replacement of GSH with the reductant Tris(carboxyethyl)-phosphine (TCEP) led to the exclusive formation of the *cis* diastereomers **5b** (Figure 3E), as when ascorbic acid was used instead of GSH. This result is in agreement with literature data showing that sulfenic acids are efficiently reduced by TCEP with the formation of the corresponding thiols.[17]

Metabolism of 2b by Human Liver Microsomes in the Presence of GSH. Previous studies have shown the formation of dithioethers **6a**, in addition to thiols **5a** and **5a′**, during the metabolism of **1a** by human liver microsomes in the presence of GSH or other thiols such as ME.[14,18,19] More recently, bis-adducts **9a** and **9b** (Nu = Nu′ = SCH_2CH_2OH) have also been detected during the metabolism of **1a** and **1b**, respectively, by human liver microsomes in the presence of ME.[22] These dithioethers[19] and bis-adducts[22] were a mixture of *cis* and *trans* diastereomers.

An HPLC−MS study of incubations of **2b** with human liver microsomes in the presence of NADPH and 2 mM GSH showed the formation of several GSH adducts, in addition to thiols **5b** and **5b′** (Figure 4). The major ones were characterized by peaks at m/z = 655 (Figure 4A). They exhibited MS^2 spectra (fragments at m/z = 526, 348, and 317 corresponding to a loss of pyroglutamate, GSH and SSG, respectively) (Figure 4B) and HRMS data (Table 1) in

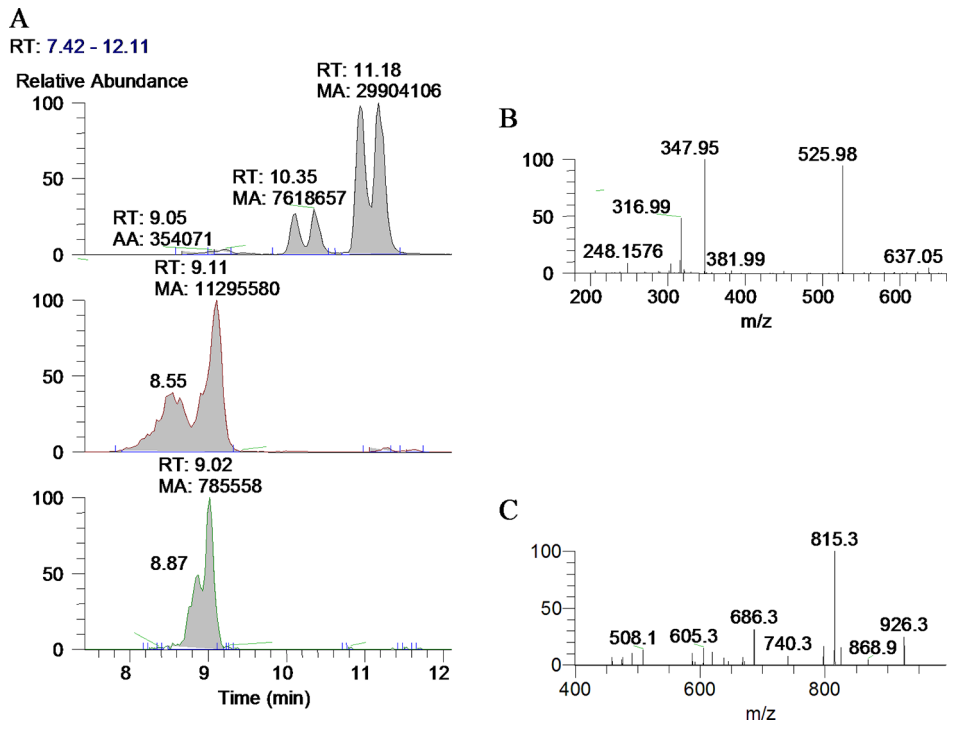

Figure 4. HPLC-MS study of the products found upon the incubation of **2b** with human liver microsomes in the presence of GSH. A: reconstructed chromatograms obtained on the HPLC−HRMS system, at m/z = 350 (upper panel), 655 (middle panel), and 944 (lower panel). B and C: MS^2 spectra at m/z = 655 and 944, respectively.

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Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

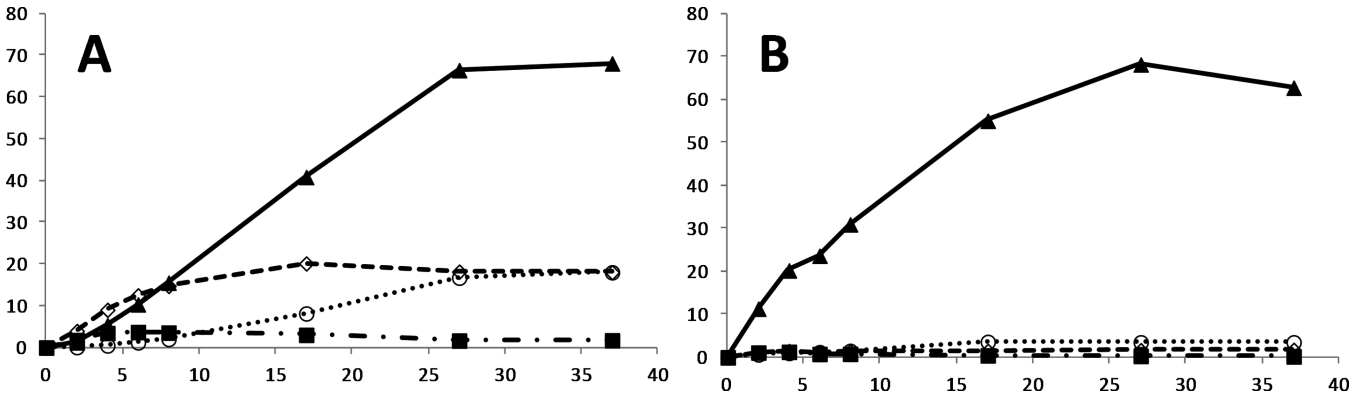

Figure 5. Kinetics of oxoprasugrel metabolism by human liver microsomes in the presence of NADPH and 2 mM GSH, without (A) or with (B) human liver cytosol. Black triangle, *cis* thiols **5b**; open diamond, monoadducts **6b**; open circle, *trans* thiols **5b**′; black square, bis-adducts **9b**. Ordinates are the areas of the HPLC−HRMS peaks of the different metabolites. In the absence of authentic samples of the metabolites for constructing calibration curves, this gave a qualitative idea of the metabolite kinetics. The indicated values are the means of two independent experiments, each in duplicate; there was less than 20% variation between the obtained values.

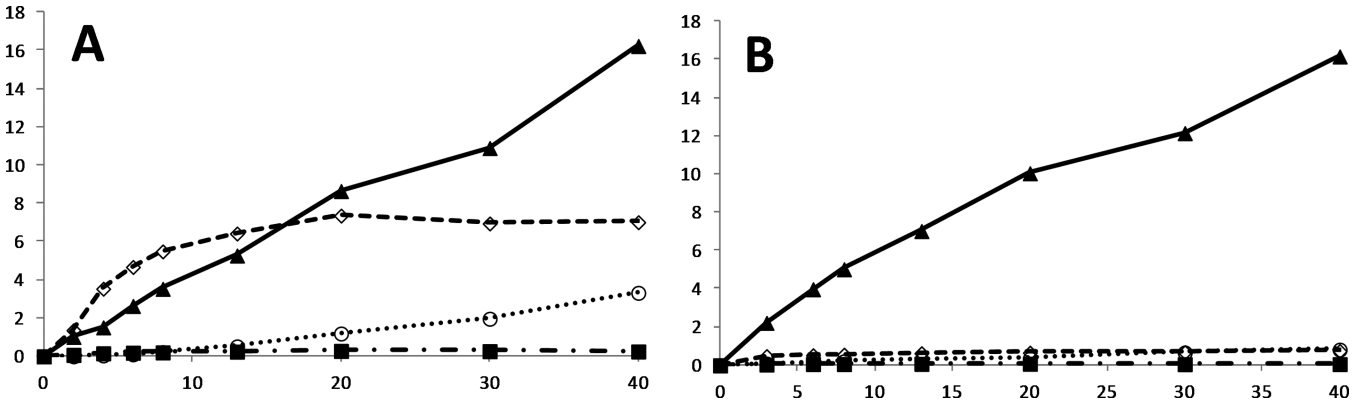

Figure 6. Kinetics of oxoclopidogrel metabolism by human liver microsomes in the presence of NADPH and 2 mM GSH, without (A) or with (B) human liver cytosol. Black triangle, *cis* thiols **5a**; open diamond, monoadducts **6a**; open circle, *trans* thiols **5a**′; black square, bis-adducts **9a**. Ordinates are the areas of the HPLC−HRMS peaks of the different metabolites (see the legend for Figure 5 concerning this point).

agreement with structure **6b** (with SR′ = SG). All components of the two broad peaks at m/z = 655 exhibited identical HRMS and MS^2 characteristics. GSH bis-adducts (m/z = 944), corresponding to the ME bis-adducts previously reported in the metabolism of **2b** by human liver microsomes in the presence of ME, were also detected. Their MS^2 (fragments at m/z = 815, 686, and 605 corresponding to the loss of pyroglutamate, 2 pyroglutamates, and GSSH, respectively, Figure 4C) and HRMS characteristics (Table 1) were in agreement with the structure of **9b** (with Nu = Nu′ = SG). However, the HPLC column used in this study, that led to a clear separation of the four *cis* and *trans* diastereomers of **6b** and **9b** (with Nu = Nu′ = SCH_2CH_2OH) observed in the metabolism of **2b** by human liver microsomes in the presence of ME,[22] did not allow us to separate the stereoisomers of **6b** and **9b** (with Nu = Nu′ = SG).

Other metabolites not containing the GS moiety were also formed in amounts comparable to those of **6b**. Their MS^2 and HRMS characteristics (m/z = 334.1443, Table 1) indicated that they were the alcohol diastereomers resulting from the replacement of the SH group of **5b** with an OH group. The formation of these metabolites upon *in vivo* metabolism of **1b** was already reported.[26,27]

cis thiols **5b** were already formed during the first minutes of the reaction, whereas significant amounts of *trans* thiols **5b**′ only appeared after 5 min (Figure 5A). After 30 min, the *cis*/*trans* thiols ratio was around 4. Dithioethers **6b** and bis-adducts **9b** (with SR′ = SG) appeared as a mixture of unresolved stereoisomers. The first ones reached a plateau after about 20 min, whereas the second ones were always formed in small amounts (Figure 5A).

Quite interestingly, very different results were observed when identical incubations were done in the presence of human liver cytosol. Under those conditions, *cis* thiols **5b** were almost the only products of the reaction, as very minor amounts of *trans* thiols **5b**′ were formed (**5b**/**5b**′ ≥ 15), and mono- and bis-GSH adducts were only formed in very low amounts (Figure 5B). The great decrease of dithioethers **6b** formation in the presence of human liver cytosol was in agreement with previous data showing that cytosolic reductases efficiently catalyze the reduction of these dithioethers into thiols **5b**.[20]

Metabolism of 2a by Human Liver Microsomes in the Presence of GSH. An analogous study of the reaction of oxoclopidogrel **2a** with human liver microsomes in the presence of NADPH and 2 mM GSH, by HPLC−MS and HPLC−HRMS, showed the formation of the same kind of metabolites. *cis* and *trans* thiols **5a** and **5a**′ and dithioethers **6a** were the major products of this reaction. They were already reported to be formed in the metabolism of **2a** by human liver microsomes in the presence of GSH.[1,6,19,22] We could also detect the minor

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Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

formation of the GSH bis-adducts **9a** (Nu = Nu′ = SG) that were characterized by MS^2 and HRMS (molecular ion MH^+ at m/z = 950.2136, calculated value 950.2132).

In a manner similar to what was observed in the case of **2b**, in the absence of human liver cytosol, *cis* and *trans* thiols **5a** and **5a′** were formed in a *cis*/*trans* ratio around 4, in addition to important amounts of dithioethers **6a** and small amounts of bis-adducts **9a** (Figure 6A), whereas, in the presence of human liver cytosol, *cis* thiols **5a** were almost the only observed products, *trans* thiols **5a′** and intermediates **6a** and **9a** (with SR′ = SG) being formed in very low amounts (Figure 6B).

DISCUSSION

The aforementioned results show for the first time that the stereochemistry of the double bond of the thiols formed upon the metabolism of **2b** by human liver microsomes in the presence of GSH or other thiols or reductants is highly similar to that previously described for the thiols derived from the metabolism of **2a** under identical conditions. In both cases, only *cis* thiols are formed in the presence of ascorbic acid or TCEP, whereas *cis* and *trans* thiols **5** and **5′** are formed in the presence of GSH or other thiols such as NAC or ME.

Table 2 summarizes the data described above or previously published about the stereochemistry of the double bond of thiols or of adducts formed upon the metabolism of **2a** or **2b** by human liver microsomes in the presence of various nucleophiles (or reductants) used to trap thiolactone sulfoxides **3** and/or sulfenic acids **4**. It clearly shows that significant amounts of *trans* isomers are only formed when thiols such as GSH, NAC, and ME are present in the incubation medium. Actually, in their absence, the monoadducts formed in the presence of dimedone, the bis-adducts **9** (Nu = NR_1R_2; Nu′ = CPD) obtained in the presence of an amine (NH_3 or piperidine) and CPDH, and thiols **5** formed in the presence of ascorbic acid or TCEP are only *cis* diastereomers. By contrast, thiols derived from **2a** and **2b** in the presence of GSH, NAC, or ME, and bis-adducts **9** derived from **2a** and **2b** in the presence of ME or NAC, are mixtures of *cis* and *trans* isomers. Moreover, it has been reported that dithioethers **6a** formed in the presence of ME were also a mixture of *cis* and *trans* isomers.[19,22]

Table 2. Stereochemistry of the Double Bond of Thiols 5 or of Various Adducts upon the Metabolism of 2a and 2b by Human Liver Microsomes in the Presence of Various Nucleophiles or Reductants

		presence of *trans* isomers[a]	
nucleophile or reductant	products	**2a**	**2b**
dimedone	dimedone adducts	- (14)	- (15)
ascorbate	thiols **5**	- (10, 11, and 18)	- (this work)
R_1R_2NH + CPDH	bis adducts **9**, (Nu = N R_1R_2, Nu′ = CPD	- (22)	- (22)
GSH	thiols **5**	+ (19)	+ (this work)
ME	thiols **5**	+ (19)	+ (this work)
	- bis adducts **9** (Nu = Nu′ = SCH_2CH_2OH)	+ (22)	+ (22)
	- **6** (R′S = SCH_2CH_2OH)	+ (19 and 22)	+ (22)

[a]- means that only products with the *cis* configuration of the double bond have been observed (*trans*/*cis* ratio <0.05); + means that *cis* and *trans* isomers were formed. The reference number of publications mentioning the formation of the indicated products are given in parentheses.

The formation of *trans* thiol diastereomers that only occurred in the presence of thiol nucleophiles could be due to the particular reactivity of intermediate thioesters **9** (Nu = Nu′ = SR′) derived from the initial reaction of thiol nucleophiles on the thioester function of thiolactone sulfoxides **3**, when compared to that of intermediate carboxylic acids or amides derived from the initial reaction of **3** with water or amines. Three mechanisms have been tentatively proposed for the *cis*–*trans* isomerization of the thioester **9** double bond.[22] They are based on the greater tendency of thioesters to give intermediate enolates, vinylidene–ketenes, or ketenes after deprotonation, elimination of GSH, or reversible Michael-type addition of the thiol on the conjugated double bond of **9**, respectively.[22] Intermediates **8** (Figure 7) also contain a thioester function and could lead to a *cis*–*trans* isomerization of their double bond. However, they also contain a very electrophilic sulfenic acid function and should only exist as transient species in very low concentrations.

The detection of only the *cis* thiol diastereomers **5a** in the sera of patients treated with clopidogrel, under conditions allowing a complete separation of the *cis* and *trans* isomers,[23−25] while high amounts of GSH are present in the hepatocytes, could be explained, at least in part, by the above data on the role of human liver cytosol on the **5a**/**5a′** ratio observed after the metabolism of **2a** by human liver microsomes in the presence of GSH. Addition of human liver cytosol to incubations of **2a** or **2b** with human liver microsomes in the presence of NADPH and GSH resulted in dramatic changes in products formation (Figures 5 and 6). Formation of *trans* thiols **5′** drastically decreased in the presence of cytosol, and one also noted a clear decrease in the formation of intermediates **6** (SR′ = SG) and **9** (Nu = Nu′ = SG). The almost exclusive formation of *cis* thiols **5a** upon the metabolism of **2a** by human liver microsomes in the presence of 2 mM GSH and human liver cytosol would explain the detection of only *cis* thiols **5a** in the sera of patients treated with clopidogrel. Globally, it seems that the presence of human liver cytosol would disfavor the formation of thioester intermediates **9** (Nu = Nu′ = SR′) that are responsible for the *trans* isomer formation. This should occur at the level of the opening of the thiolactone sulfoxide ring of intermediate **3**. In the presence of GSH (or other thiols such as ME), two nucleophiles, GSH and H_2O, are in competition for the reaction with the thioester CO group of **3** and opening of the thiolactone sulfoxide ring (Figure 7). Reaction of H_2O should lead to intermediates toward thiol **5** with a *cis* configuration of the double bond, whereas reaction with GSH (or other thiols such as ME) would lead to thioester intermediates such as **9** able to interconvert the stereochemistry of their double bond and to give a mixture of *cis* and *trans* thiols **5** and **5′**. A possible role of cytosol would be to increase the rate of opening of **3** by H_2O and thus to favor the formation of **4** relative to that of **9** (with Nu = SG). Cytosolic esterases, especially thioesterases, could be at the origin of these observed effects of cytosol. This is likely as the thioesterase PON-1 has been shown to catalyze the hydrolytic opening of the tautomer of thiolactone **2a**, in which the double bond has migrated into the piperidine ring, leading to **7a**.[10,11,13] Compound **3a** is structurally close to the tautomer of thiolactone **2a**, and its CO carbon is more electrophilic than that of the **2a** tautomer. The

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Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

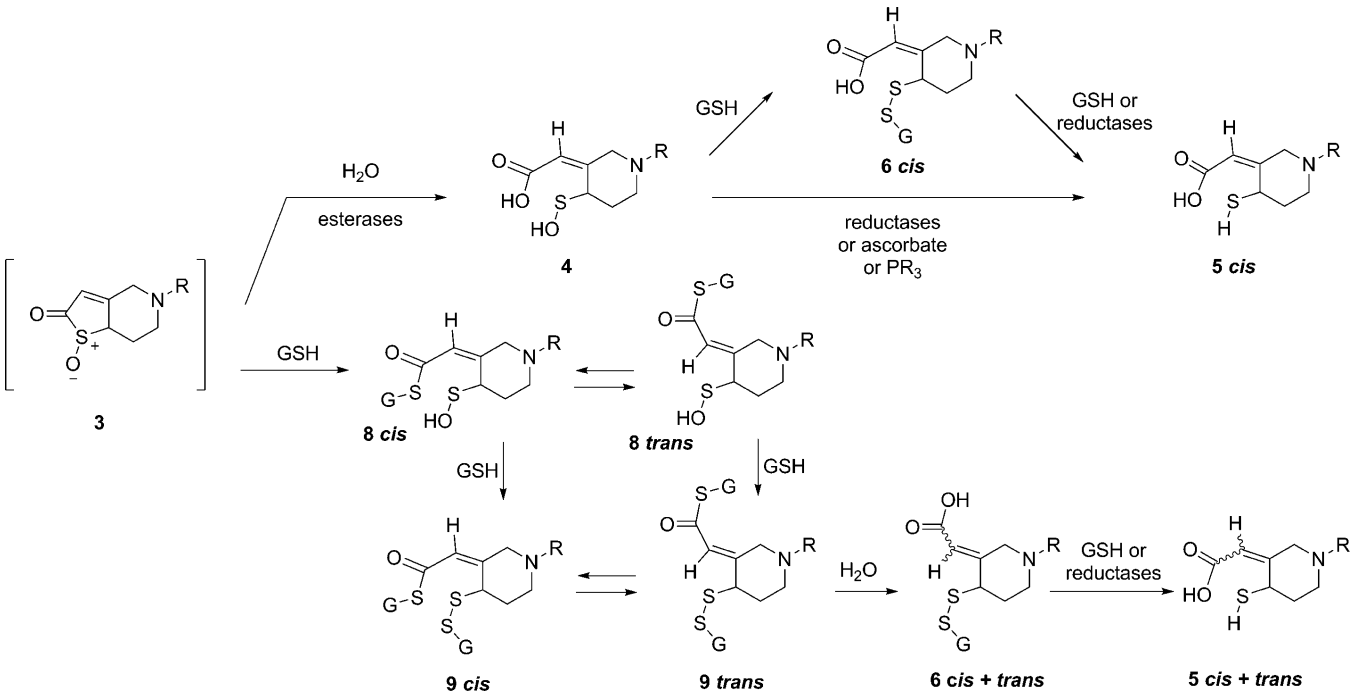

Figure 7. Competition between water and GSH for the opening of intermediate **3** explaining the different stereochemistries of the thiol **5** double bond observed upon the metabolism of **1a** and **1b** in different *in vitro* conditions or *in vivo* (in the case of **1a**).

nature of the cytosolic esterases possibly involved in the catalysis of hydrolysis of **3a** remains to be determined. Because of the very similar behavior of **2a** and **2b** in the above-described reactions, one would expect that significant amounts of *trans* thiols **5b** should not be formed *in vivo* in patients treated with **1b**.

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ABBREVIATIONS

CPDH, cyclopentane-1,3-dione; ESI^+, electron spray ionization in positive ionization mode detection; HPLC-MS, high performance liquid chromatography−mass spectrometry; HRMS, high resolution mass spectrometry; GSH, glutathione; ME, mercaptoethanol; NAC, *N*-acetyl cysteine; TCEP, tris-(carboxyethyl)-phosphine; PON-1, paraoxonase-1; P450, cytochrome P450

REFERENCES

(1) Pereillo, J. M., Maftouh, M., Andrieu, A., Uzabiaga, M. F., Fedeli, O., Savi, P., Pascal, M., Herbert, J. M., Maffrand, J. P., and Picard, C. (2002) Structure and stereochemistry of the active metabolite of clopidogrel. *Drug Metab. Dispos. 30*, 1288−1295.

(2) Sugidachi, A., Asai, F., Yoneda, K., Iwamura, R., Ogawa, T., Otsuguro, K., and Koike, H. (2001) Antiplatelet action of R-99224, an active metabolite of a novel thienopyridine-type G(i)-linked P2T antagonist, CS-747. *Br. J. Pharmacol. 132*, 47−54.

(3) Hasegawa, M., Sugidachi, A., Ogawa, T., Isobe, T., Jakubowski, J. A., and Asai, F. (2005) Stereoselective inhibition of human platelet aggregation by R-138727, the active metabolite of CS-747 (prasugrel, LY640315), a novel P2Y12 receptor inhibitor. *Thromb. Haemostasis 94*, 593−598.

(4) Testa, B., and Kramer, S. D. (2009) The biochemistry of drug metabolism–an introduction: part 5. Metabolism and bioactivity. *Chem. Biodiversity 6*, 591−684.

(5) Farid, N. A., Kurihara, A., and Wrighton, S. A. (2010) Metabolism and disposition of the thienopyridine antiplatelet drugs ticlopidine, clopidogrel, and prasugrel in humans. *J. Clin. Pharmacol. 50*, 126−142.

(6) Maffrand, J. (2012) The story of clopidogrel and its predecessor, ticlopidine: Could these major antiplatelet and antithrombotic drugs be discovered and developed today? *C. R. Chim. 15*, 737−743.

(7) Dalvie, D. K., Kalgutkar, A. S., Khojasteh-Bakht, S. C., Obach, R. S., and O'Donnell, J. P. (2002) Biotransformation reactions of five-membered aromatic heterocyclic rings. *Chem. Res. Toxicol. 15*, 269−299.

(8) Kazui, M., Nishiya, Y., Ishizuka, T., Hagihara, K., Farid, N. A., Okazaki, O., Ikeda, T., and Kurihara, A. (2009) Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite. *Drug Metab. Dispos. 38*, 92−99.

(9) Williams, E. T., Jones, K. O., Ponsler, G. D., Lowery, S. M., Perkins, E. J., Wrighton, S. A., Ruterbories, K. J., Kazui, M., and Farid, N. A. (2008) The biotransformation of prasugrel, a new thienopyridine prodrug, by the human carboxylesterases 1 and 2. *Drug Metab. Dispos. 36*, 1227−1232.

(10) Dansette, P. M., Rosi, J., Bertho, G., and Mansuy, D. (2011) Paraoxonase-1 and clopidogrel efficacy. *Nat. Med. 17*, 1040−1041.

(11) Dansette, P. M., Rosi, J., Bertho, G., and Mansuy, D. (2012) Cytochromes P450 catalyze both steps of the major pathway of clopidogrel bioactivation whereas paraoxonase catalyzes the formation of a minor thiol metabolite isomer. *Chem. Res. Toxicol. 25*, 348−356.

(12) Dansette, P. M., Rosi, J., Debernardi, J., Bertho, G., and Mansuy, D. (2012) Metabolic activation of prasugrel: nature of the two competitive pathways resulting in the opening of its thiophene ring. *Chem. Res. Toxicol. 25*, 1058−1065.

DOI: 10.1021/acs.chemrestox.5b00133
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

(13) Gong, I., Crown, N., Suen, C., Schwarz, U., Dresser, G., Knauer, M., Sugiyama, D., Degorter, M., Woolsey, S., Tirona, R., and Kim, R. (2012) Clarifying the importance of CYP2C19 and PON1 in the mechanism of clopidogrel bioactivation and in vivo antiplatelet response. *Eur. Heart J. 33*, 2856−2464.
(14) Dansette, P. M., Libraire, J., Bertho, G., and Mansuy, D. (2009) Metabolic oxidative cleavage of thioesters: evidence for the formation of sulfenic acid intermediates in the bioactivation of the antithrombotic prodrugs ticlopidine and clopidogrel. *Chem. Res. Toxicol. 22*, 369−373.
(15) Dansette, P. M., Thebault, S., Bertho, G., and Mansuy, D. (2010) Formation and fate of a sulfenic acid intermediate in the metabolic activation of the antithrombotic prodrug prasugrel. *Chem. Res. Toxicol. 23*, 1268−1274.
(16) Hagihara, K., Kazui, M., Kurihara, A., Iwabuchi, H., Ishikawa, M., Kobayashi, H., Tanaka, N., Okazaki, O., Farid, N. A., and Ikeda, T. (2010) Biotransformation of prasugrel, a novel thienopyridine antiplatelet agent, to the pharmacologically active metabolite. *Drug Metab. Dispos. 38*, 898−904.
(17) Mansuy, D., and Dansette, P. M. (2011) Sulfenic acids as reactive intermediates in xenobiotic metabolism. *Arch. Biochem. Biophys. 507*, 174−185.
(18) Zhang, H., Lau, W., and Hollenberg, P. (2012) Formation of the thiol conjugates and active metabolite of clopidogrel by human liver microsomes. *Mol. Pharmacol. 82*, 302−309.
(19) Zhang, H., Lauver, D., Lucchesi, B., and Hollenberg, P. (2013) Formation, reactivity, and anti-platelet activity of mixed disulfide conjugates of clopidogrel. *Mol. Pharmacol. 83*, 848−856.
(20) Hagihara, K., Kazui, M., Kurihara, A., Kubota, K., and Ikeda, T. (2011) Glutaredoxin and thioredoxin can be involved in producing the pharmacologically active metabolite of a thienopyridine antiplatelet agent, prasugrel. *Drug Metab. Dispos. 39*, 208−214.
(21) Hagihara, K., Kazui, M., Kurihara, A., Ikeda, T., and Izumi, T. (2012) Glutaredoxin is involved in the formation of the pharmacologically active metabolite of clopidogrel from its GSH conjugate. *Drug Metab. Dispos. 40*, 1854−1859.
(22) Dansette, P. M., Levent, D., Hessani, A., Bertho, G., and Mansuy, D. (2013) Thiolactone sulfoxides as new reactive metabolites acting as bis-electrophiles: implication in clopidogrel and prasugrel bioactivation. *Chem. Res. Toxicol. 26*, 794−802.
(23) Tuffal, G., Roy, S., Lavisse, M., Brasseur, D., Schofield, J., Delesque Touchard, N., Savi, P., Bremond, N., Rouchon, M. C., Hurbin, F., and Sultan, E. (2011) An improved method for specific and quantitative determination of the clopidogrel active metabolite isomers in human plasma. *Thromb. Haemostasis 105*, 696−705.
(24) Karazniewicz-Lada, M., Danielak, D., Tezyk, A., Zaba, C., Tuffal, G., and Gówka, F. (2012) HPLC-MS/MS method for the simultaneous determination of clopidogrel, its carboxylic acid metabolite and derivatized isomers of thiol metabolite in clinical samples. *J. Chromatogr., B 911*, 105−112.
(25) Furlong, M. T., Savant, I., Yuan, M., Scott, L., Mylott, W., Mariannino, T., Kadiyala, P., Roongta, V., and Arnold, M. E. (2013) A validated HPLC-MS/MS assay for quantifying unstable pharmacologically active metabolites of clopidogrel in human plasma: application to a clinical pharmacokinetic study. *J. Chromatogr., B 926*, 36−41.
(26) Smith, R. L., Gillespie, T. A., Rash, T. J., Kurihara, A., and Farid, N. A. (2007) Disposition and metabolic fate of prasugrel in mice, rats, and dogs. *Xenobiotica 37*, 884−901.
(27) Farid, N. A., Smith, R. L., Gillespie, T. A., Rash, T. J., Blair, P. E., Kurihara, A., and Goldberg, M. J. (2007) The disposition of prasugrel, a novel thienopyridine, in humans. *Drug Metab. Dispos. 35*, 1096−1104.
(28) Farid, N. A., McIntosh, M., Garofolo, F., Wong, E., Shwajch, A., Kennedy, M., Young, M., Sarkar, P., Kawabata, K., Takahashi, M., and Pang, H. (2007) Determination of the active and inactive metabolites of prasugrel in human plasma by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom. 21*, 169−179.
(29) Asai, F., Sudigashi, A., Ikeda, T., Kuroki, Y., Inoue, T., Iwamura, R., and Shibakawa, N. (2003) Cyclic Amino Compounds, United States Patent US 6,610,708 B1, p 94, Sankyo Company Ltd., Ube Industries Ltd., Japan.

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Chem. Res. Toxicol. XXXX, XXX, XXX−XXX