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Cytochromes P450 Catalyze Both Steps of the Major Pathway of Clopidogrel Bioactivation, whereas Paraoxonase Catalyzes the Formation of a Minor Thiol Metabolite Isomer

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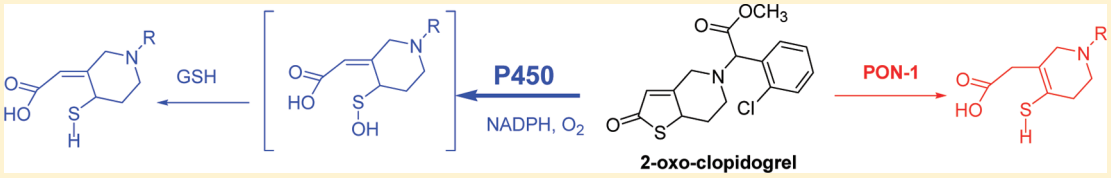
Supporting Information



ABSTRACT: The mechanism generally admitted for the bioactivation of the antithrombotic prodrug, clopidogrel, is its two-step enzymatic conversion into a biologically active thiol metabolite. The first step is a classical cytochrome P450 (P450)-dependent monooxygenation of its thiophene ring leading to 2-oxo-clopidogrel, a thiolactone metabolite. The second step was described as a P450-dependent oxidative opening of the thiolactone ring of 2-oxo-clopidogrel, with intermediate formation of a reactive sulfenic acid metabolite that is eventually reduced to the corresponding thiol **4b**. A very recent paper published in *Nat. Med.* (Bouman et al., (2011) *17*, 110) reported that the second step of clopidogrel bioactivation was not catalyzed by P450 enzymes but by paraoxonase-1(PON-1) and that PON-1 was a major determinant of clopidogrel efficacy. The results described in the present article show that there are two metabolic pathways for the opening of the thiolactone ring of 2-oxo-clopidogrel. The major one, that was previously described, results from a P450-dependent redox bioactivation of 2-oxo-clopidogrel and leads to **4b cis**, two previously reported thiol diastereomers bearing an exocyclic double bond. The second, minor one, results from a hydrolysis of 2-oxo-clopidogrel, which seems to be dependent on PON-1, and leads to an isomer of **4b cis**, **4b "endo"**, in which the double bond has migrated from an exocyclic to an endocyclic position in the piperidine ring. These results were obtained from a detailed study of the metabolism of 2-oxo-clopidogrel by human liver microsomes and human sera and analysis by HPLC-MS under conditions allowing a complete separation of the thiol metabolite isomers, either as such or after derivatization with 3′-methoxy phenacyl bromide or *N*-ethyl maleimide (NEM). These results also show that the major bioactive thiol isomer found in the plasma of clopidogrel-treated patients derives from 2-oxo-clopidogrel by the P450-dependent pathway. Finally, chemical experiments on 2-oxo-clopidogrel showed that this thiolactone is in equilibrium with its tautomer having a double bond inside the piperidine ring and that nucleophiles such as CH_3O^- preferentially react on the thioester function of this tautomer. This allowed us to understand why **4b cis** has to be formed via an oxidative opening of 2-oxo-clopidogrel thiolactone, whereas a hydrolytic opening of this thiolactone ring leads to the "endo" thiol isomer **4b "endo"**.

INTRODUCTION

The mechanism generally admitted for the bioactivation of ticlopidine **1a** and clopidogrel **1b**, two tetrahydrothienopyridine antithrombotic prodrugs, is their two-step enzymatic conversion into a biologically active thiol metabolite [1−4](Figure 1). The first step is a classical cytochrome P450 (P450)-dependent monooxygenation of their thiophene ring by NADPH and O_2 [5−8] that leads to the thiolactone metabolites **2a** and **2b**. It is mainly catalyzed by P450s 2C19 and 2B6 in the case of ticlopidine and by P450s 2C19, 1A2, and 2B6 in the case of clopidogrel.[9,10] The second step was described as a P450-dependent oxidative opening of the thiolactone ring of **2** with the eventual formation of an active thiol metabolite.[9,10] We have shown that this step was a P450-catalyzed cleavage of the thiolactone ring of metabolites **2** leading to the formation of reactive sulfenic acid intermediates **3**, which have been trapped by dimedone.[11] In the presence of glutathione (GSH) in excess, these sulfenic acids are reduced into the corresponding thiols **4**[11,12] (Figure 1). These thiol metabolites are generally considered as the pharmacologically active species that are responsible for the inhibition of the P2Y12 platelet receptor after the formation of a covalent disulfide bond with a cysteine residue of this protein.[1−3] Another possible mechanism for this inactivation of the P2Y12 receptor could be the formation of such a covalent bond from a reaction of their sulfenic acid metabolites **3** or of the glutathionyl adducts deriving from their reaction with GSH, with a cysteine SH residue of the P2Y12 platelet receptor.[11,12] The latter mechanism remains to be established.

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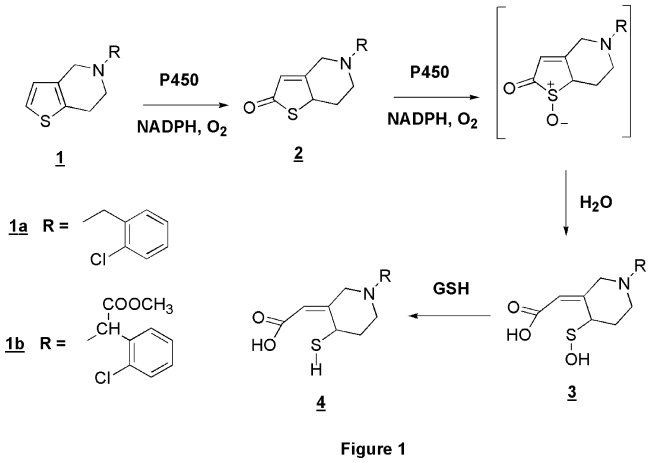

Figure 1. Different steps reported for the bioactivation of ticlopidine **1a** and clopidogrel **1b**.[11]

Pharmacokinetic, pharmacodynamic, and genetic studies provided strong evidence that *in vivo* bioactivation of clopidogrel is closely linked to the P450 system.[10,13−19] Actually, P450 2C19 was found to play a key role in clopidogrel bioactivation by contributing to both steps of this bioactivation.[9,10] A common genetic variant within the *CYP(P450) 2C19* gene, the CYP2C19*2 loss of function polymorphism, was found to be associated with an attenuated response to clopidogrel and a worse clinical outcome in patients undergoing coronary stenting and treatment with clopidogrel.[14−19]

A very recent paper published in *Nat. Med.*[20] concluded that the second step of clopidogrel bioactivation was not catalyzed by P450 enzymes but by paraoxonase-1 (PON-1) and that PON-1 was a major determinant of **1b** efficacy. It suggested that PON-1 Q192R polymorphism may have a key role for the rate of clopidogrel bioactivation and influence platelet response to this drug and the risk of stent thrombosis in **1b**-treated patients.[20] The latter results questioned our current understanding of clopidogrel bioactivation and cast doubts on a large body of previous metabolic and genetic association studies.

In order to understand the origin of these contradictory conclusions, we have reinvestigated the *in vitro* metabolism of 2-oxo-clopidogrel **2b** by human liver microsomes and human sera using HPLC techniques leading to a complete separation of the isomers of the thiol metabolite of **2b** because such a separation was not done in most studies previously reported on the metabolism of clopidogrel or 2-oxo-clopidogrel, including that of Bouman et al.[20] Actually, opening of the thiolactone ring of **2b** may lead to five thiol isomers, two *cis* diastereomers, **4b cis**, with a Z configuration of the exocyclic double bond, two *trans* diastereomers with an E configuration of the exocyclic double bond, **4b trans**, and an *"endo"* isomer, **4b "endo"**, in which the double bond has migrated from an exocyclic to an endocyclic position in the piperidine ring[21] (Figure 2). A very recent, optimized method for the specific, quantitative determination of the clopidogrel-derived thiol metabolite isomers, after derivatization of their thiol function upon treatment with 3′-methoxy phenacyl bromide, in human plasma of clopidogrel-treated subjects showed the presence of the *cis* thiol isomers as major metabolites and of the *"endo"* thiol isomer as a minor component.[21] The mode of formation of this minor *"endo"* thiol isomer was presently unknown. Moreover, the authors also showed that one of these *cis* thiol diastereomers was very active toward the platelet P2Y12 receptor and was presumably responsible for the antithrombotic activity of clopidogrel.[21]

The results on the *in vitro* metabolism of **2b** reported in this article give a detailed, complete description of the very preliminary data quite recently mentioned in a Correspondence letter[22] that we sent to the Editor of Nature Medicine as an answer to Bouman et al.[20] They show that both P450 enzymes and PON-1 do catalyze the opening of **2b** thiolactone ring but lead to different isomers of the thiol metabolite and that the

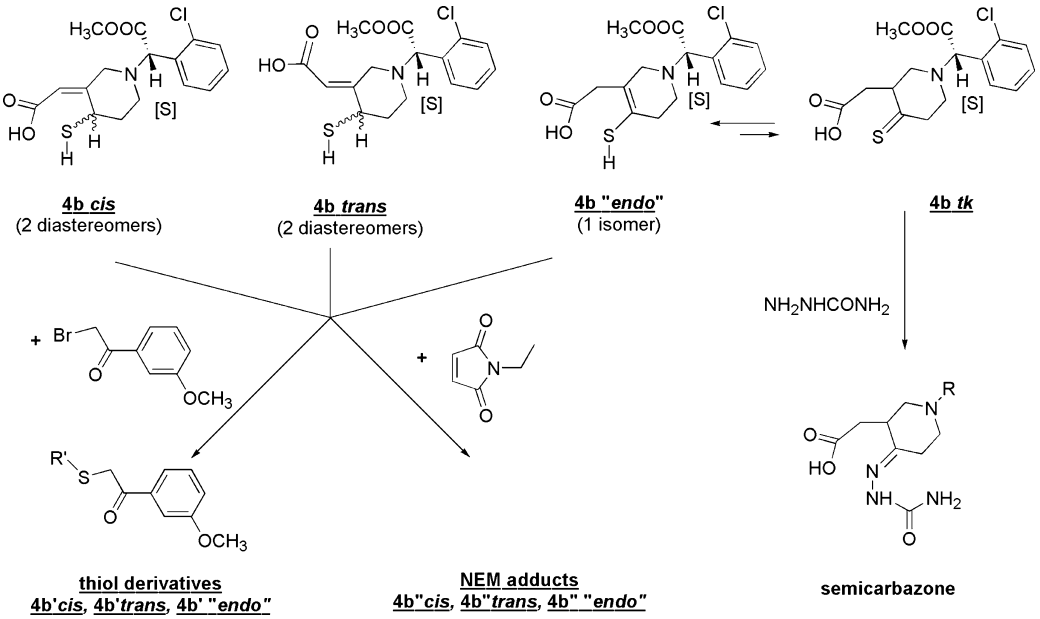

Figure 2. Different possible isomers of clopidogrel-derived thiol **4b** and derivatization from a reaction with either 3′-methoxy-phenacyl bromide or NEM. Compounds **4b cis** and **4b trans** can exist as two diastereomers as they contain two chiral carbons. The benzylic carbon has an (S) configuration, as in clopidogrel, and the carbon bearing the S atom can have an (S) or (R) configuration. The formula of the tautomer of **4b "endo"**, **4b tk**, that could exist in equilibrium with **4b "endo"** is also shown, as well as its product of reaction with semicarbazide.

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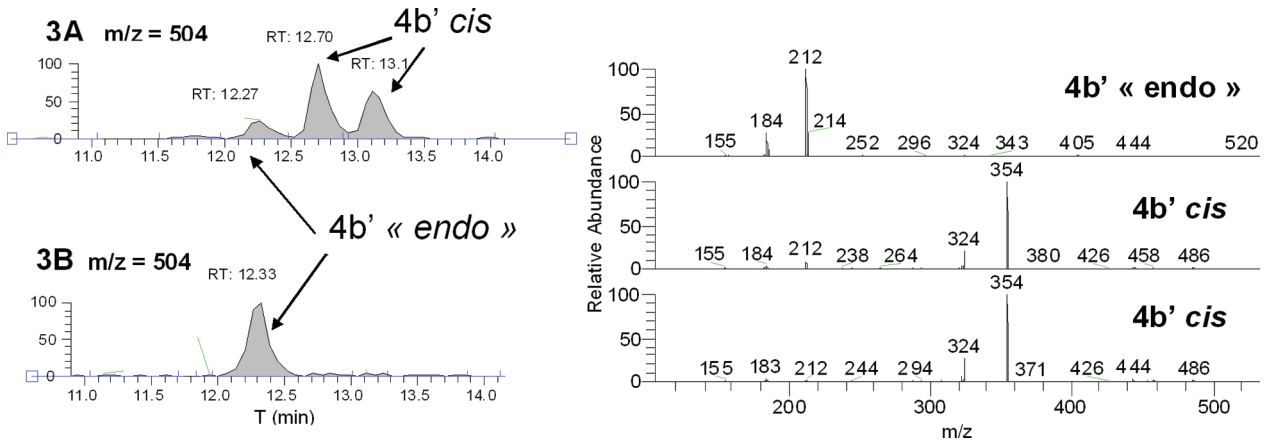

Figure 3. HPLC profiles of incubations of 2-oxo-clopidogrel with human liver microsomes with (3A) and without (3B) NADPH with MS detection after derivatization with 3′-methoxy-phenacyl bromide and MS^2 spectra (parent ion at m/z = 504) of the three derivatized thiol metabolites, **4b′cis** (2 diastereomers) and **4b′ "endo"**.

major bioactive thiol isomer found in the plasma of clopidogrel-treated subjects is formed via two P450-dependent steps. This article also provides additional data allowing us to understand why the hydrolytic, paraoxonase-dependent opening of **2b** only leads to the *"endo"* thiol isomer, whereas P450-catalyzed oxidative opening of **2b** is required to obtain the active *cis* thiol isomers.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals. [8*S*]-2-oxo-clopidogrel (the formula numerotation is in Figure 6) (SR121883), **2b**, 3′-methoxyphenacyl-*cis*-thiol (SAR206251), **4b′cis**, and 4′-bromophenacyl-*"endo"*-thiol (SAR195539) were a gift of Sanofi-Aventis (Chilly-Mazarin, France). The sample of [8*S*]-2-oxo-clopidogrel, **2b**, used in our studies was an 80:20 mixture of diastereomers, as shown by ^{1}H NMR spectroscopy (see below). All other products including enzymes were from Sigma-Aldrich (St. Quentin Fallavier, France).

Microsomal Incubations. Human microsomes (pool, 10 mg protein/mL) were obtained from BD-Gentest (Le Pont de Claix, France). Typical incubations were performed in potassium phosphate buffer (0.1M, pH 7.4) containing 2 mM $CaCl_2$, 100 mM KF, microsomes (1 mg protein/mL), **2b** (100 μM), and a reducing agent (20 mM ascorbic acid or 5 mM GSH) with or without the 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. Reactions were stopped by adding one-half volume of CH_3CN/CH_3COOH (9:1), and proteins were removed by centrifugation at 13000*g*. The KF concentration used previously[2,20] to completely inhibit the esterase-dependent hydrolysis of the methyl ester function of **2b** was 100 mM; this is why we generally used this KF concentration to have comparable data. Literature data indicated that 1 mM KF did not significantly inhibit PON-1.[23] Identical microsomal incubations were also performed with 1 and 10 mM KF to know if high KF concentrations led to inhibitory effects on PON-1 and/or P450 enzymes. These experiments showed that the P450-dependent metabolite **4b cis** formation was not affected by a change of the KF concentration from 1 to 100 mM. Formation of the PON-1-dependent metabolite, **4b "endo"**, only slightly decreased (by about 30%) upon increasing [KF] from 1 to 100 mM.

Incubations of 2-Oxo-clopidogrel with Human Sera. Typical incubations were performed in potassium phosphate buffer (0.1M, pH 7.4) containing 2 mM $CaCl_2$, 100 mM KF, human serum (1 mg protein/mL), **2b** (100 μM), and a reducing agent (20 mM ascorbic acid or 5 mM GSH), at 37 °C for 30 min. Reactions were stopped by adding one-half volume of CH_3CN/CH_3COOH (9:1) and 4 nmol of internal standard (4′-bromophenacyl-*"endo"*-thiol, SAR195539), and proteins were removed by centrifugation at 13000*g*.

HPLC-MS Studies. These studies were performed on a Surveyor HPLC instrument coupled to a LCQ Advantage ion trap mass spectrometer (Thermo, Les Ulis, France), using a Gemini C18 column (100 × 2.1 mm, 3 μm; Phenomenex) and a gradient starting at 40% B for 1 min then increasing linearly to 100% B in 15 min (A = 10 mM ammonium acetate buffer, pH 4.6, and B = CH_3CN/CH_3OH/H_2O (7:2:1)) at 200 μL/min. Mass spectra were obtained by electrospray ionization (ESI) in positive ionization mode detection under the following conditions: source parameters, sheeth gas, 20; auxiliary gas, 5; spray voltage, 4.5 kV; capillary temperature, 200 °C; and capillary voltage, 15 V; m/z range for MS recorded generally between 300 and 700 (except for exploratory experiments with a wider range of 300–800). MS^2 energy was tested between 20 and 40 eV and was generally 35 eV. For all products, the indicated molecular ions corresponded to M + H^+.

Chemical Experiments on 2b. *Reaction of **2b** in CD_3OD in the Presence of K_2CO_3.* Compound **2b** (20 mM in CD_3OD) was treated at 20 °C with a few crystals of K_2CO_3, and ^{1}H NMR spectra were recorded at t = 0, 5, 10, 15, and 25 min. An aliquot of the reaction mixture was diluted in CH_3CN/CH_3COOH/H_2O 20:9:1 and analyzed by HPLC-MS.

*Reaction of **2b** with CH_3ONa in CH_3OH.* A solution of 2 mM **2b** in CH_3OH was treated with 120 mM CH_3ONa in CH_3OH at 20 °C for 45 min. After dilution with 10 vol of 100 mM phosphate buffer at pH 7.4, the final product was analyzed by HPLC-MS, as such or after derivatization with 3′-methoxy phenacyl bromide as described above. It was purified by loading on a Seppak C18 column (Waters), washing with H_2O, eluting by CH_3OH, and analyzed by ^{1}H NMR (in CD_2Cl_2) after the evaporation of CH_3OH.

NMR Experiments. ^{1}H NMR spectra of **2b** and of **4b "endo"** methyl ester were done on a Bruker AVANCE II 500 spectrometer (500.16 MHz) at 27 °C. Chemical shifts are given in ppm relative to $(CH_3)_4Si$.

RESULTS

Metabolism of 2-Oxo-clopidogrel by Human Liver Microsomes. In order to reinvestigate the metabolic fate of **2b** in the presence of human liver microsomes or human sera, we used HPLC-MS methods allowing a complete separation of the thiol metabolite isomers, either as such or after derivatization using either 3′-methoxy- or 4′-bromo- phenacyl bromide or *N*-ethyl maleimide (NEM) that lead to more stable final metabolites.[21,24]

Analysis of the Thiol Metabolites after Derivatization with 3′-Methoxy Phenacyl Bromide. Compound **2b** (0.1 mM) was incubated for 10 min at 37 °C with human liver microsomes in the presence of NADPH, which is a required

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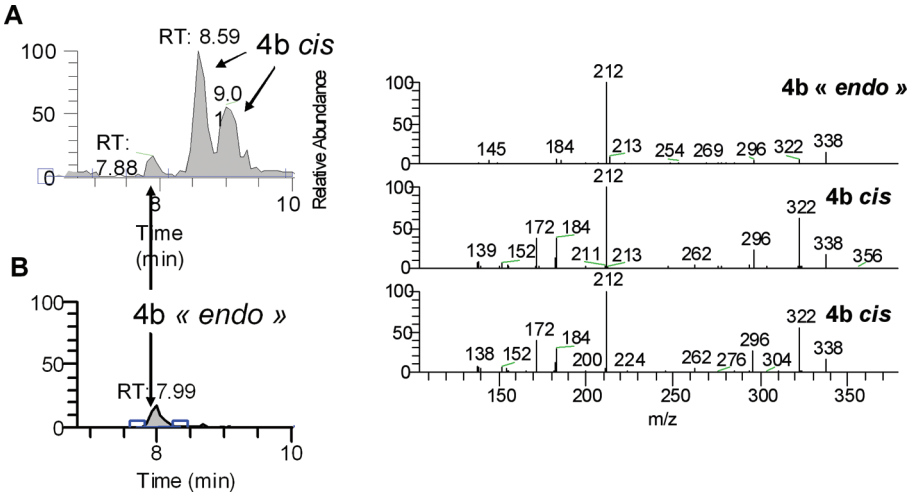

Figure 4. HPLC profiles of the incubations of 2-oxo-clopidogrel with human liver microsomes with (A) and without (B) NADPH with MS^2 detection at m/z = 358 (^{37}Cl) and MS^2 spectra (parent ion at m/z = 356 (^{35}Cl)) of the thiol metabolites **4b cis** and **4b "endo"**.

cofactor for P450-dependent activities,[25] and 100 mM KF to inhibit the esterase-dependent hydrolysis of the methyl ester function of **2b**,[2] and of a reducing agent (20 mM ascorbic acid or 5 mM GSH) to reduce the sulfenic acid intermediate **3b** into the corresponding thiol **4b**.[11,26] An HPLC-MS study of the incubate, after derivatization of the thiol metabolites by treatment with 3′-methoxy phenacyl bromide, showed the formation of three thiol isomers derivatives (Figure 3) exhibiting a molecular ion (ESI^+) corresponding to $M + H^+$ and characterized by two peaks at m/z = 504 and 506 with the ratio expected for the ^{35}Cl and ^{37}Cl isotopes. The two major thiol isomers exhibited identical MS^2 spectra, with major fragments of the molecular ion (m/z = 504) at m/z = 354 and 324 (Figure 3). Their MS^2 spectra were identical to that of an authentic sample of the 3′-methoxy acetophenone derivative of the most active *cis* thiol diastereomer, **4b′cis**.[21] Moreover, the HPLC retention time of this authentic compound was identical to that of the less polar of the two major derivatized thiol isomer metabolites. The minor derivatized thiol metabolite exhibited a different MS^2 spectrum characterized by a major fragment of the molecular ion (m/z = 504) at m/z = 212 (Figure 3). The corresponding thiol metabolite was identified at the level of its 4′-bromo acetophenone derivative, after treatment of the same microsomal incubation with 4′-bromo phenacyl bromide and comparison of its MS^2 spectrum and HPLC retention time with those of an authentic sample of the 4′-bromoacetophenone derivative of the *"endo"* thiol isomer **4b "endo"**.[21]

Analysis of the Thiol Metabolites after *in Situ* Derivatization with NEM. NEM is an interesting thiol-trapping agent because it can be used *in situ* during the metabolism of a xenobiotic by liver microsomes.[26] Incubation of **2b** with human liver microsomes was done under the above-mentioned conditions but in the presence of 1 mM NEM; the reducing agent used in this case was ascorbate in order to avoid a reaction between NEM and GSH. HPLC-MS analysis of the incubate showed the formation of three NEM-derivatized thiol metabolites with a ratio very similar to that observed after the postreaction derivatization with 3′-methoxy phenacyl bromide (see Figure 2S in Supporting Information). These three derivatized thiol metabolites, **4b″ cis** and **4b″ "endo"** (see Figure 2 for their formula), were characterized by molecular ions with two peaks at m/z = 481 and 483 (for ^{35}Cl and ^{37}Cl, respectively), as expected for NEM adducts of thiols **4b cis** (2 diastereomers) and **4b "endo"**.

Analysis of the Thiol Metabolites without Derivatization. A similar HPLC-MS study was also performed on the thiols themselves (without any derivatization) and showed the formation of three thiol isomers characterized by a MS molecular ion with two peaks at m/z = 356 and 358 (for ^{35}Cl and ^{37}Cl, respectively), in a ratio very similar to that found for their 3′-methoxyacetophenone derivatives (Figures 4 and 3). The two major thiol metabolites exhibited the same MS^2 spectrum with the main fragments of the molecular ion (m/z = 356) at m/z = 322, 296, 212, 184, and 172 (Figure 4), which was identical to that previously observed for the **4b cis** diastereomers.[2,11] The minor thiol metabolite showed a different MS^2 spectrum characterized by a main fragment at m/z = 212 (Figure 4). Thus, this analysis of the thiol metabolites was in complete agreement with the above analysis done on their 3′-methoxyacetophenone or NEM derivatives, showing that the metabolism of **2b** by NADPH-supplemented human liver microsomes mainly led to the cis thiol isomers **4b cis** and to the "endo" thiol isomer **4b "endo"** as a minor product.

The "endo" thiol isomer **4b "endo"** is a thioenol that should exist in equilibrium with its thioketone tautomer **4b tk** (Figure 2). Accordingly, microsomal incubation of **2b** under the above-mentioned conditions but in the presence of 5 mM semicarbazide, an usual reagent for ketones and thioketones,[27] led to the formation of a new product characterized by a MS molecular ion with two peaks at m/z = 397 and 399 (for ^{35}Cl and ^{37}Cl, respectively). This MS molecular ion and its MS^2 fragments at m/z = 379 (M-18, H_2O), 336 (379-43, CONH), and 324 (M-73, NNHCONH2) (see Figure 1S in Supporting Information) were in good agreement with a semicarbazone structure resulting from reaction of semicarbazide with **4b tk** (Figure 2).

Effects of the Incubation Conditions on 2-Oxo-clopidogrel Microsomal Metabolism. When identical microsomal incubations were done in the absence of NADPH, there was no formation of **4b cis**, and the only thiol isomer that could be detected was **4b "endo"** (Table 1). Moreover, the addition of 20 μM *N*-benzylimidazole, a well-

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Table 1. Formation of 4b cis and 4b "endo" upon the Metabolism of 2b by Human Liver Microsomes

conditions	(nmol/mg protein/30 min)[b] 4b cis	4b "endo"
complete system[a]	19 ± 4	2.2 ± 0.4
+ 20 μM *N*-benzyl-imidazole	0.5 ± 0.2	2.8 ± 0.4
− ascorbate	<0.1	2.3 + 0.4
− NADPH	<0.1	2.2 ± 0.4
− NADPH − $CaCl_2$	<0.1	1.0 ± 0.2
− NADPH + 500 μM paraoxon	<0.1	0.5 ± 0.2
− NADPH − $CaCl_2$ + 5 mM EDTA	<0.1	0.5 ± 0.5

[a]Complete system: incubations of 100 μM **2b** with human liver microsomes (1 mg protein/ml) in 0.1 M phosphate buffer at pH 7.4 containing 100 mM KF, 2 mM $CaCl_2$, and 20 mM ascorbic acid, for 30 min at 37 °C; analysis by HPLC-MS after derivatization with 3′-methoxy-phenacyl bromide, as described in Experimental Procedures. [b]mean values ± SD from at least 3 independent experiments.

known inhibitor of microsomal P450s,[28] to the incubation of **2b** with NADPH-supplemented microsomes almost completely inhibited the formation of the **4b cis** diastereomers, whereas it did not significantly modify the formation of **4b "endo"** (Table 1). These data indicated that the formation of the cis thiol isomers **4b cis** is P450-dependent. Since the article of Bouman et al.[20] proposed that thiol **4b** would derive from a reaction of **2b** with PON-1, identical microsomal incubations without NADPH were performed in the presence of 500 μM paraoxon, an usual substrate of PON-1.[29,30] Table 1 shows that this led to an 80% inhibition of **4b "endo"** formation. Moreover, incubations in the absence of $CaCl_2$ led to a marked decrease of **4b "endo"** formation, and addition of 5 mM ethylenediamine tetraacetate (EDTA) led to an even greater decrease of this formation (Table 1). PON-1 activity is dependent on the presence of Ca^{2+} ions.[29] Thus, the above results show that the formation of **4b cis** from **2b** is dependent on NADPH and is catalyzed by P450s, whereas that of their isomer **4b "endo"** is not dependent on NADPH and is catalyzed by an esterase such as PON-1. This conclusion was completely confirmed by the results of incubations of **2b** under conditions identical to those of the complete system of Table 1 except for the absence of a reducing agent (ascorbate or GSH). Under those conditions, one only observed the formation of **4b "endo"** (or its derivatized products with either 3′-methoxy phenacyl bromide or NEM) (line 3 of Table 1). This is in agreement with the fact that formation of **4b "endo"** from a simple hydrolysis of **2b** does not require the presence of a reducing agent contrary to that of **4b cis**, which needs the reduction of sulfenic acid **3b** (Figure 1).

Metabolism of 2-Oxo-clopidogrel by Human Sera. Incubation of 100 μM 2-oxo-clopidogrel with human serum (10 mg protein mL^{-1}), which contains PON-1 but not P450s, in the presence of 100 mM KF for 30 min at 37 °C and the study of the reaction mixture by HPLC-MS after derivatization using either 3′-methoxy phenacyl bromide or NEM under the above-described conditions showed the formation of only one thiol isomer, **4b "endo"** (Figure 2S in Supporting Information).

Chemical Hydrolysis of 2-Oxo-clopidogrel. Several chemical experiments were done to understand why a simple hydrolysis of **2b** only led to the "endo" thiol isomer **4b "endo"**. Actually, the sample of **2b** used in our studies was a 80:20 mixture of diastereomers (as shown by ^{1}H NMR spectroscopy and HPLC-MS) that could exist in equilibrium with their possible tautomer **2b′**, in which the double bond has migrated within the piperidine ring and is no longer conjugated with the keto group (Figure 5). The existence of this equilibrium was

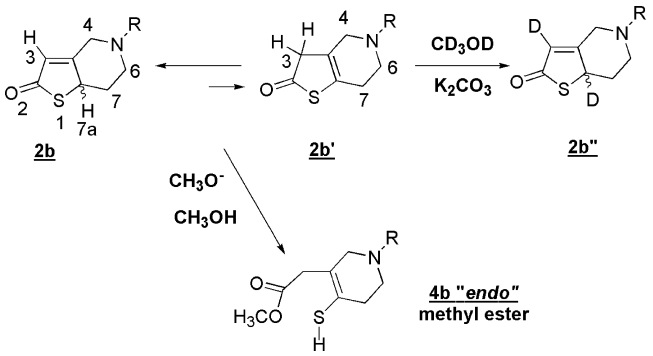

Figure 5. Equilibrium between 2-oxo-clopidogrel **2b** and its tautomer **2b′** and their reaction in CD_3OD and K_2CO_3 or with CH_3O^- in CH_3OH.

indicated by the easy exchange of the vinylic (H3) and allylic (H7a) hydrogens of **2b** with deuteriums in CD_3OD in the presence of K_2CO_3, which was shown by ^{1}H NMR spectroscopy and MS. A detailed analysis of the ^{1}H NMR spectrum of **2b** in CD_3OD, using 2D NMR methods (correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC), and heteronuclear single quantum coherence spectroscopy (HSQC)) allowed us to assign all the signals to the protons of the molecule. This NMR analysis clearly showed that **2b** was an 80:20 mixture of diastereomers (see for instance the signals of H3, H8, or H6 in Figure 6A). After the addition of K_2CO_3 to the solution of **2b** in CD_3OD, one observed a progressive disappearance of the ^{1}H NMR signals at 6.05 and 4.42 ppm of the H3 and H7a protons of **2b** and a decrease of the diastereomers ratio from 80/20 to 50/50 (Figure 6B). The final spectrum corresponded to a 50:50 mixture of the diastereomers of **2b** deuterated on carbons 3 and 7a (**2b″** in Figure 5). This was not only shown by the disappearance of the H3 and H7a signals but also by a simplification of the H7 signals, due to the loss of the coupling between H7 and H7a (Figure 6). Accordingly, the final product (characterized by two HPLC peaks corresponding to the 50:50 diastereomers mixture) exhibited an HPLC retention time identical to that of **2b** and a MS molecular ion at m/z = 340 and 342 for ^{35}Cl and ^{37}Cl, indicating that two deuterium atoms have replaced two hydrogen atoms in **2b**. This formation of **2b″** should result from a base-catalyzed migration of the double bond of **2b** leading to **2b′**, followed by a fast H/D exchange occurring at the level of the CH2 group α to the COS function of **2b′**, because of the acidity of the allylic hydrogens of this CH2 group, and a base-catalyzed migration of the **2b′** double bond with the final formation of **2b″**. During this deuteration of **2b**, an equilibration of its two diastereomers occurs, leading to the finally observed 50:50 ratio (Figure 6B).

Reaction of **2b** with CH_3ONa in CH_3OH at 20 °C for 45 min led to the opening of the **2b** thiolactone ring with the formation of only one thiol isomer, the methyl ester of **4b "endo"**, with an 80% yield. The structure of this product was established from its mass spectrum (molecular ion with two peaks at m/z = 370 and 372 for ^{35}Cl and ^{37}Cl, respectively, and a MS^2 main fragment at m/z = 212) and its ^{1}H NMR spectrum that exhibited characteristic signals at 3.7 and 3.2 ppm for the

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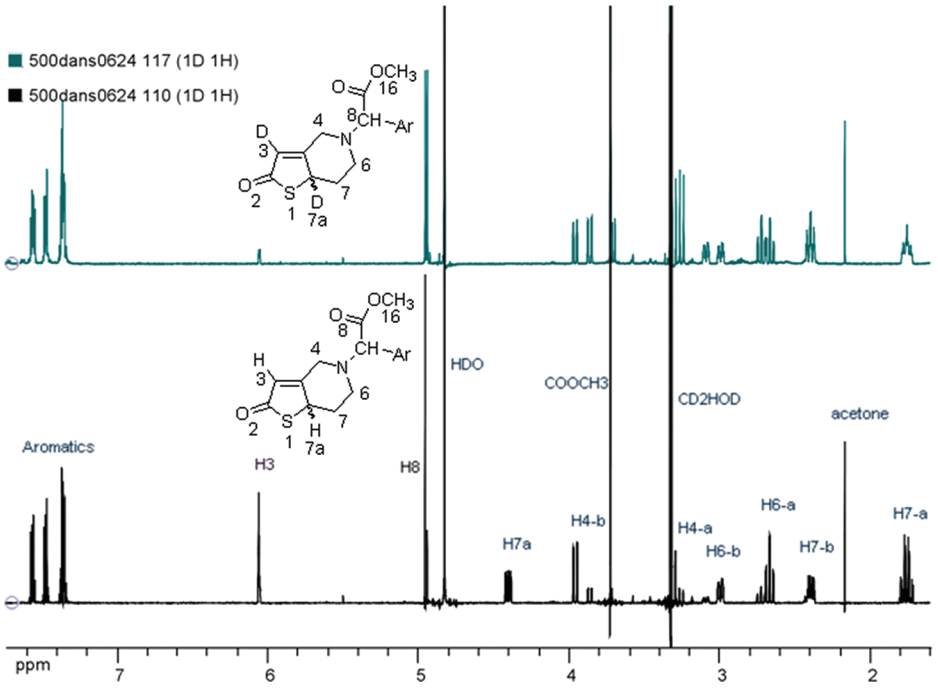

Figure 6. ^{1}H NMR spectra of 2-oxo-clopidogrel **2b** in CD_3OD at $t = 0$ (bottom spectrum) and 15 min after the addition of K_2CO_3 (top spectrum). The top spectrum shows that 15 min after the addition of K_2CO_3, deuteration at position 7a was complete (loss of the H7a signal), whereas that at position 3 was not (small H3 signal remaining). After 25 min, deuteration at positions 7a and 3 were complete (data not shown).

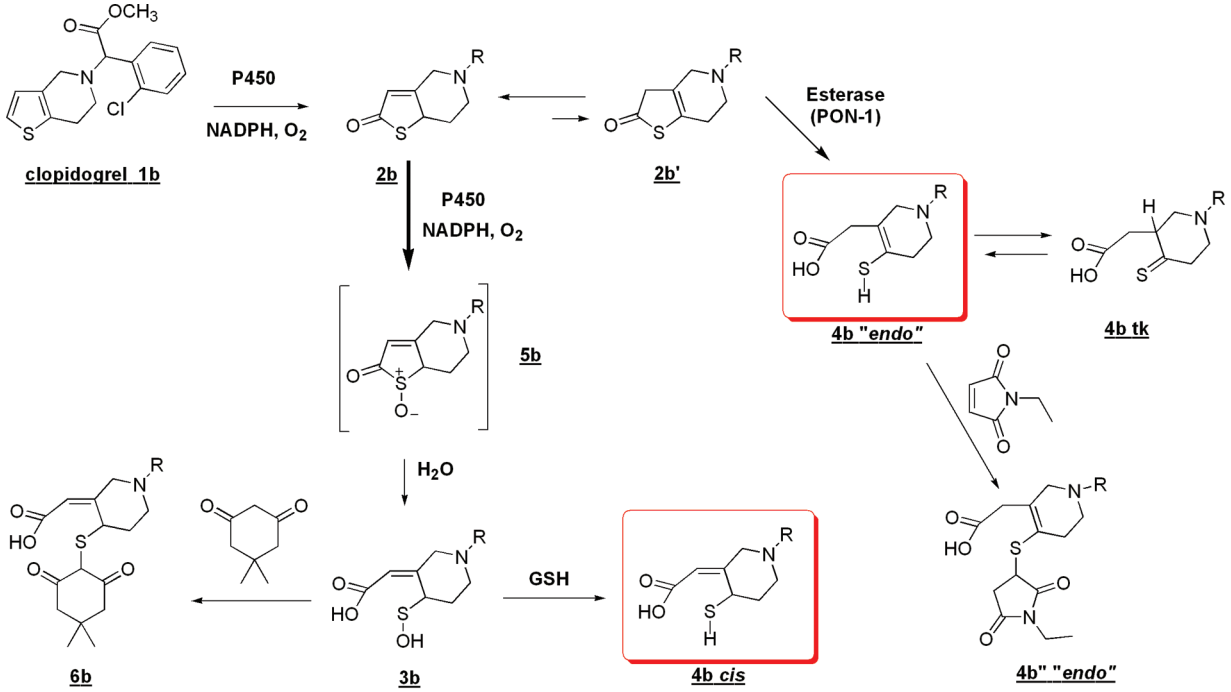

Figure 7

Figure 7. Different pathways involved in the formation of thiol metabolites **4b cis** and **4b** "**endo**" in the metabolism of clopidogrel **1b**.

allylic hydrogens α to the CO and amine groups. A detailed analysis of the ^{1}H NMR spectrum of **4b** "**endo**" methyl ester in CD_2Cl_2, using 2D NMR methods (COSY, HMBC, and HSQC) allowed us to assign all the signals to the protons of the molecule (data not shown). These data suggest that the reaction of nucleophiles such as CH_3ONa or OH^- on **2b** preferentially occurs on the thioester carbon of **2b**′, the tautomer of **2b**, which is more electrophilic than the thioester carbon of **2b** which is conjugated to a double bond.

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DISCUSSION

The aforementioned studies of the metabolism of 2-oxo-clopidogrel by human liver microsomes and human serum showed that two different pathways resulting in the opening of its thiolactone ring are occurring. The major one results from a P450- and NADPH-dependent oxidation leading to the cis thiol diastereomers **4b cis**. The second, minor one is hydrolytic and leads to the "endo" thiol isomer **4b "endo"** (Figure 7). It occurs both in liver microsomes and in human sera, depends on the presence of Ca^{2+}, and is inhibited by paraoxon (Table 1). It is presumably this metabolic way that was measured by Bouman et al.[20] These authors have compared the activities of various esterases toward **2b** and concluded that PON-1 was mainly involved in the transformation of **2b** into thiols.[20] From the presently available data, it thus seems that the formation of **4b "endo"** from **2b** would be mainly catalyzed by PON-1.

It is noteworthy that, under our incubation conditions, thiol **4b cis** was stable for at least 1 h and that thiol **4b "endo"** had a half-life of about 25 min. Moreover, under those conditions, we never observed any conversion of **4b cis** into **4b "endo"** or of **4b "endo"** into **4b cis** (data not shown).

From a chemical point of view, how is it possible to understand why **4b "endo"** derives from an hydrolysis of **2b**, while it is necessary to oxidize **2b** to produce the cis thiol isomers **4b cis**? Our studies about the chemical hydrolysis of 2-oxo-clopidogrel under basic conditions, showing that only "endo" thiol products are formed, suggest that O-nucleophiles preferentially react on the thioester carbon of the more reactive tautomer **2b′** that is in equilibrium with **2b**. This should be true for the enzymatic hydrolysis of the thioester function of 2-oxo-clopidogrel by PON-1 that would explain the formation of only **4b "endo"** in incubations of 2-oxo-clopidogrel with liver microsomes (in the absence of NADPH) and human sera. Accordingly, literature data reported that PON-1 readily catalyzed the hydrolysis of lactones, whereas it was inactive on unsaturated lactones involving a less reactive carbonyl moiety conjugated to a double bond.[30] Thus, it seems that the only way to open the thiolactone ring of 2-oxo-clopidogrel with the formation of the cis thiol isomers, **4b cis**, is to oxidize its S atom with the intermediate formation of thioester sulfoxide **5b** (Figure 7).[11] The thioester carbon of **5b** is much more reactive than that of **2b** and should rapidly react with water to give sulfenic acid **3b**, as previously reported.[11] Reduction of **3b** with GSH or ascorbic acid[26] eventually leads to **4b cis**. Formation of **3b** in P450-catalyzed metabolism of 2-oxo-clopidogrel is in agreement with a recent report showing that mechanism-based inactivation of P450 2B6 by 2-oxo-clopidogrel occurs through covalent modification of a cysteine residue CYS475.[31]

This competition between the hydrolysis of 2-oxo-clopidogrel **2b** (via **2b′**) leading to **4b "endo"** and redox activation of **2b** eventually leading to **4b cis** in NADPH-supplemented human liver microsomes was further confirmed by an incubation of 2-oxo-clopidogrel with human liver microsomes in the presence of NADPH under the above-mentioned conditions except for the absence of a reducing agent and for the presence of a trapping agent for thiols, NEM,[26] and a trapping agent for sulfenic acids, dimedone.[11,26,32] Under these conditions, the major products found by an HPLC-MS analysis were the dimedone adducts **6b** (two diastereomers in a 50:50 ratio) of the cis sulfenic acid intermediate **3b**, which were previously characterized by MS and ^1H and ^{13}C NMR spectroscopy.[11] Another product was formed in lower amounts; its MS and MS^2 characteristics showed that it was derived from the addition of the thiol function of **4b "endo"** to the activated double bond of NEM (NEM adduct **4b‴"endo"** shown in Figures 2 and 7) (data not shown). The result of this double trapping experiment completely confirmed that in the presence of NADPH-supplemented human liver microsomes and a reducing agent such as ascorbate or GSH, metabolites **4b cis and 4b "endo"** are formed from **2b** by two competing pathways, a P450-dependent oxidative one, occurring via an intermediate sulfenic acid **3b** and an esterase (PON-1)-dependent hydrolytic one, respectively. Actually, if **4b cis** could have been formed by a simple hydrolytic route, one should have observed the formation of a NEM adduct of **4b cis** (**4b″cis** of Figure 2) that was not detected.

Our results showing that the major thiol isomers formed in the metabolism of 2-oxo-clopidogrel by human liver microsomes are **4b** cis and that their formation is catalyzed by P450s are in agreement with those of several previously published studies of *in vitro* metabolism of clopidogrel or 2-oxo-clopidogrel.[2,9,11] Formation of the minor isomer **4b "endo"** in these *in vitro* systems was not reported so far presumably because either the used HPLC methods did not permit the separation of **4b cis** and **4b "endo"** and/or the used incubation conditions were not adapted for Ca^{2+}-dependent PON-1 activity (EDTA was present in most previously reported incubation media, and $CaCl_2$ was not added; see last line of Table 1). It is noteworthy that some xenobiotics, such as dietary polyphenols and fenofibrate, act as inducers of PON-1[33−36] and could have an effect on the formation of **4b "endo"** in clopidogrel-treated patients.

The conclusion concerning the P450-dependent formation of the **4b cis** metabolite is contradictory to that recently drawn by Bouman et al.[20] which excluded the fact that an oxidative P450-dependent step was involved in the second step of clopidogrel bioactivation. The latter conclusion, which was deduced from incubation experiments of **2b** with human liver microsomes, human serum, and recombinant human P450s and esterases expressed in a human embryonic kidney cell line and HPLC-MS study of the thiol metabolites, was mainly based on the following arguments: (i) from all the tested recombinant enzymes, only recombinant PON-1 and PON-3 were found to be able to catalyze the formation of thiol metabolites, whereas P450s seemed to be inactive, and (ii) inhibition of PON-1 greatly inhibited thiol formation by human liver microsomes, whereas P450 inhibitors did not.[20] Actually, the HPLC system used by Bouman et al. does not seem to separate the thiol metabolite isomers such as **4b cis** and **4b "endo"**, and it is possible that their MS method very preferentially detects **4b "endo"**. If this was the case, they would have mainly followed **4b "endo"** formation and found, as we did, that this formation is dependent on PON-1 and not on P450s.

Interestingly, the three thiol isomers that we have detected upon the metabolism of 2-oxo-clopidogrel by human liver microsomes in the presence of NADPH are those that were found to be present in the sera of patients treated with clopidogrel. Moreover, their proportions were very similar to those found in clopidogrel-treated patients.[21] This suggests that the major pathway of opening of thiolactone **2b** in humans is the P450-dependent formation of the **4b cis** diastereomers, whereas the formation of **4b "endo"** is a minor one. Previous literature data indicate that the **1b** metabolite mainly responsible for the antiplatelet activity of **1b** is one of the **4b**

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cis diastereomers.[2,21] Consequently, the bioactivation of clopidogrel should be mainly dependent on P450s. This is in agreement with several pharmacokinetic and pharmacodynamic studies showing that the bioactivation of clopidogrel is closely linked to P450s[2,9−11,13,37] and a very recent study showing that incubation of human platelets with 2-oxo-clopidogrel and baculosomes containing some human recombinant P450s led to an inhibition of platelet aggregation by ADP.[38] This is also in agreement with several data showing that a common genetic variant within the CYP 2C19 gene, the CYP 2C19*2 loss of function polymorphism, is associated with an attenuated response to clopidogrel and to a worse clinical outcome in patients undergoing coronary stenting.[14−19] Moreover, even more recently, eight independent published articles showed no association of PON-1 Q192R genotype with platelet response to clopidogrel and risk of stent thrombosis after coronary stenting,[39−46] whereas some of them confirmed the impact of the CYP 2C19*2 genotype on both platelet effect of clopidogrel and risk of coronary stenting.[39,40,42,46]

However, the antiplatelet activity of the minor metabolite **4b "endo"** was not determined yet, and its relative importance in the *in vivo* effects of clopidogrel remains to be precisely evaluated.

CONCLUSIONS

Our results show that there are two metabolic pathways for the opening of the thiolactone ring of 2-oxo-clopidogrel, the one that was previously described resulting in a P450-dependent redox bioactivation leading to **4b cis**, and a second, hydrolytic, minor one that seems to be dependent on PON-1 and leads to **4b "endo"**. Since the most bioactive metabolite of clopidogrel is one of the **4b cis** diastereomers and since those metabolites are the major thiol isomers present in the sera of clopidogrel-treated patients, both steps of clopidogrel bioactivation should be mainly dependent on P450s.

ASSOCIATED CONTENT

Supporting Information

HPLC profiles of incubations of **2b** with human serum or human liver microsomes in the presence of several trapping agents (semicarbazide, NEM and 3'-methoxy-phenacyl bromide). This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

EDTA, ethylene diamine tetraacetic acid; HPLC-MS, high performance liquid chromatography−mass spectrometry; GSH, glutathione; NEM, *N*-ethyl maleimide; PON-1, Paraoxonase 1; P450, cytochrome P450

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