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Thiolactone Sulfoxides as New Reactive Metabolites Acting as Bis-Electrophiles: Implication in Clopidogrel and Prasugrel Bioactivation

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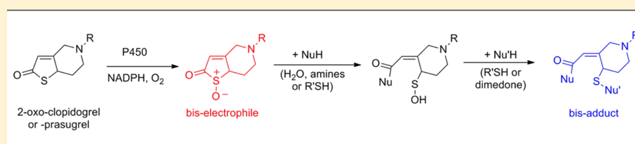
Supporting Information



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 cytochrome P450 (P450)-dependent oxidation of this thiolactone resulting in the formation of a sulfenic acid that is eventually reduced into the corresponding active thiol. It has been postulated that the sulfenic acid metabolite resulted from a nucleophilic attack of water on a highly reactive thiolactone sulfoxide derived from P450-dependent oxidation of the thiolactone primary metabolite. The data described in the present article are in complete agreement with this proposition as they show that it was possible to trap these thiolactone sulfoxides by a series of nucleophiles such as amines, thiols, or cyclopentane-1,3-dione (CPDH), an equivalent of dimedone that is used as a sulfenic acid trapping agent. HPLC-MS studies showed that various bis-adducts having incorporated two nucleophile molecules were formed in these reactions. One of them that resulted from the oxidation of 2-oxo-prasugrel by human liver microsomes in the presence of ethanolamine and CPDH was isolated and completely characterized by ^{1}H and ^{13}C NMR spectroscopy in addition to MS and MS^2 spectrometry. All metabolites derived from an attack of H_2O or an amine at the CO carbon of the intermediate thiolactone sulfoxide existed as a mixture of two diastereomers having a *cis* configuration of the double bond, whereas those formed in the presence of thiols appeared as a mixture of four diastereomers with a *cis* or *trans* configuration of the double bond. This led us to propose tentative mechanisms for the previously reported formation of *trans* isomers of the active thiol metabolite of clopidogrel upon microsomal metabolism of this antithrombotic in the presence of thiols. The results described in this article showed that thiolactone sulfoxides are formed as reactive metabolites during the metabolism of clopidogrel and prasugrel and are able to react as bis-electrophiles with a variety of nucleophiles. The possible implications of the formation of these reactive metabolites in the pharmacological and/or secondary toxic effects of these drugs remain to be studied.

INTRODUCTION

The antithrombotic drugs of the tetrahydrothienopyridine series, ticlopidine (Ticlid) **1a**, clopidogrel (Plavix, Iscover) **1b**, and prasugrel (Effient) **1c** (Figure 1), are prodrugs that must be metabolized *in vivo* into the corresponding pharmacologically active 4-mercapto-3-piperidinyliden acetic acid derivatives **3a**, **3b**, and **3c**, respectively, to exert their activity as antagonists of the platelet receptor P2Y12.[1−7] Their metabolic activation occurs in two steps. In the case of ticlopidine and clopidogrel, the first step is a cytochrome P450 (P450)-dependent hydroxylation of their thiophene ring by NADPH and O_2 leading to thiolactone metabolites **2a** and **2b**, respectively (Figure 1).[6−11] In the case of prasugrel, the first step is the hydrolysis of its ester function leading to thiolactone **2c**, which seems to be mainly catalyzed by the hCE$_2$ enzyme in humans.[12] The second step of metabolic activation of **1a**, **1b**, and **1c** leading to the active metabolites **3a**, **3b**, and **3c**, respectively, is not a simple hydrolysis of the thioester bond of intermediate thiolactones **2a**, **2b**, and **2c**. Actually, it has been recently shown[13−16] that such a simple hydrolysis of thiolactones **2b** and **2c**, which is catalyzed by thioesterases such as paraoxonase-1, only leads to an *endo* isomer of **3b** and **3c** in which the double bond has migrated into the piperidine ring (Figure 1). Formation of the active *cis* metabolites **3** from thiolactones **2** 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 **3** (Figure 2).[17−19] Formation of sulfenic acids **4** has been established after trapping with dimedone and complete characterization of the corresponding adducts **7** by MS and ^{1}H and ^{13}C NMR spectroscopy.[17,18] These electrophilic intermediates are efficiently reduced by ascorbate or phosphines with quantitative formation of thiols **3**.[13−15,18] 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 a mixed disulfide **5**[17,18,20,21] and a reduction of this disulfide by a second thiol molecule (Figure 2).[17,18,20,21] Reduction of the disulfide can also been done by glutaredoxin and thioredoxin.[22,23] It is noteworthy that the metabolism of **1b** and **1c** by human liver microsomes in the presence of NADPH and either dimedone

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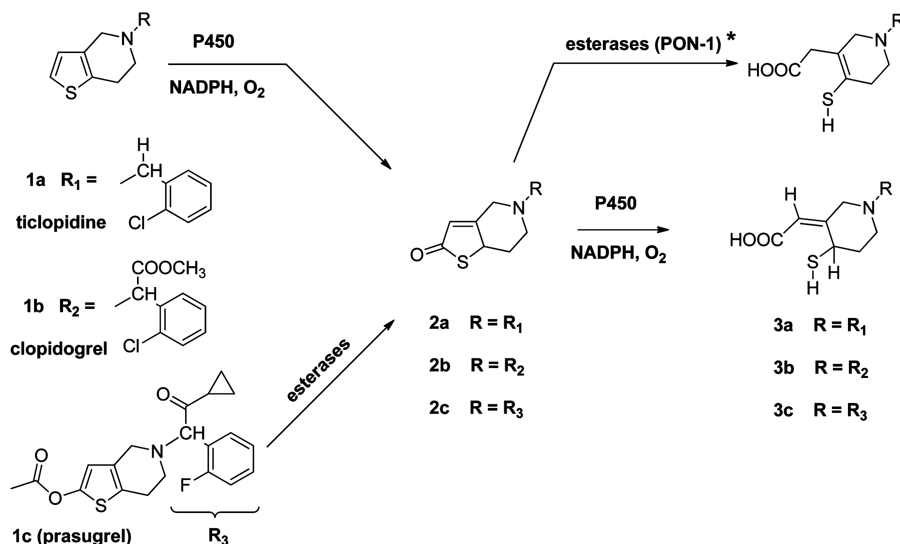

Figure 1. Metabolic activation of tetrahydrothienopyridine antithrombotic drugs into pharmacologically active *cis* thiols. (*) The esterase (PON-1)-dependent formation of the *endo* isomers was only described in the case of **1b** and **1c**.

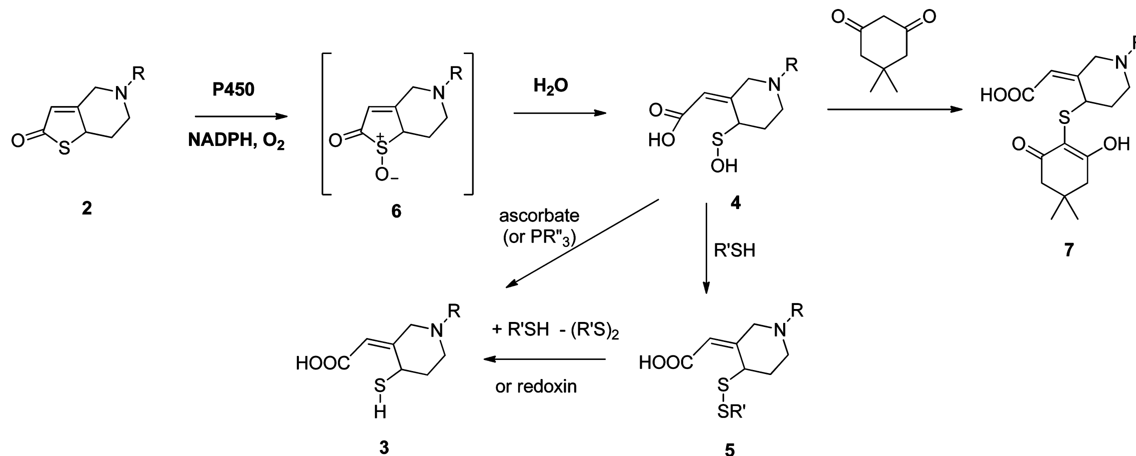

Figure 2. Detailed mechanism proposed for the formation of active metabolites **3** upon the metabolism of thiolactones **2**, via the postulated intermediate thiolactone sulfoxides **6**. Compounds **3**, **5**, and **7** derived from clopidogrel and prasugrel can exist as two diastereomers, as they contain two chiral carbons (the carbons bound to the S and N atoms).

or ascorbate to trap or to reduce intermediate sulfenic acids **4b** and **4c** only leads to the two diastereomers of either **7b** and **7c**[17,18] or of **3b**[13,14,21] having a *cis* configuration of the double bond. Similarly, only these two *cis* diastereomers **3b** were found in the sera of patients treated with **1b**, whereas the corresponding stereoisomers with a *trans* configuration of the double bond could not be detected.[16,24,25] However, when the metabolism of **1b** by human liver microsomes and NADPH occurred in the presence of various thiols such as GSH, mercaptoethanol, or *N*-acetylcysteine (NAcCysH), the formation of **3b** or **5b** was not so stereoselective as a mixture of *cis* and *trans* stereoisomers was obtained.[2,7,21] The origin of the *trans* isomers only formed in the microsomal metabolism of **1b** in the presence of thiols remains to be determined.

It has been postulated that the formation of sulfenic acids **4** from the P450-catalyzed oxidation of thiolactones **2** would result from an S-oxidation of **2** followed by a fast reaction of the highly electrophilic CO carbon of the intermediate thiolactone sulfoxide **6** with water.[17,18] The resulting opening of the thiolactone sulfoxide ring should selectively lead to the *cis* sulfenic acid **4** and eventually to the *cis* active thiol **3**. Are these highly reactive thiolactone sulfoxides only transient intermediates formed in the active site of P450s, or are they stable enough to get out from this active site and react with cell components? How do they react with nucleophiles that could be present in their environment? In order to answer these questions, we have tried to trap them with various nucleophiles. Actually, in most cases, we have used a mixture of two nucleophiles, NuH and Nu′H, the first one to react with the CO carbon of the thiolactone sulfoxide and the second one to react with the sulfur atom of the intermediate sulfenic acid, and looked for the formation of bis-adducts **8** having incorporated Nu and Nu′ (Figure 3). The following data show that incubation of 2-oxo-prasugrel or -clopidogrel, **2c** and **2b**, with human liver microsomes in the presence of NADPH, O_2, and various nucleophiles NuH and Nu′H leads in most cases to the expected bis-adducts **8** that were detected by HPLC-MS. One of them was completely characterized by ^1H and ^{13}C NMR spectroscopy using 1D and 2D methods. These results show that thiolactone sulfoxides do form during the oxidative activation of thienopyridine antithrombotic drugs and are released in the incubation medium. They rapidly react with

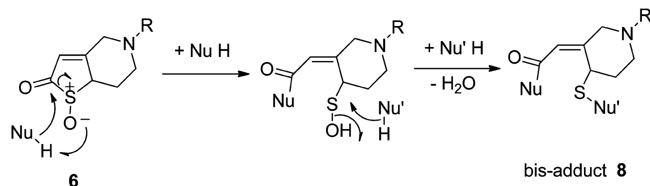

Figure 3. Possible formation of bis-adducts **8** from trapping of thiolactone sulfoxides **6** by nucleophiles.

nucleophiles and act as bis-electrophiles leading to a variety of bis-adducts. This suggests that these new reactive metabolites might be formed in the cell and react with nucleophilic cell components such as proteins or nucleic acids, with possible formation of covalent adducts including cross-linked adducts. Finally, the data reported in this article led us to tentatively propose an explanation for the formation of *trans* isomers of **3** and **5** upon the microsomal metabolism of **1** in the presence of thiols.

EXPERIMENTAL PROCEDURES

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

Microsomal Incubations. Human liver microsomes (pool, 10 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 microsomes (1 mg protein/mL), **2c** or **2b** (100 μM), and various nucleophiles (1 to 10 mM) with or without a NADPH generating system (1 mM NADP, 15 mM glucose-6-phosphate, 2 u/mL of glucose-6-phosphate dehydrogenase) at 37 °C for 30 min. For each nucleophile, a range of concentrations was tested in order to determine the optimal concentration for bis-adduct **8** formation. In the case of incubations with **2b**, 10 mM KF was added to inhibit the esterase-dependent hydrolysis of its methyl ester function.[2] Reactions were stopped by adding one-half volume of CH_3CN containing 8% CH_3COOH. 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 Shimadzu Shimpack C18 column (75 × 2.1 mm, 2.3 μm (Shimadzu, Marne La Vallée, France)), and a gradient A + B starting at 0% B for 1 min then increasing linearly to 100% B in 15 min (A = 2 mM ammonium acetate plus 0.2% HCOOH, pH 4.6, and B = $CH_3CN/H_2O/HCOOH$ (980:18:2)) at 250 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; 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 $M + H^+$.

Preparation of Bis-Adduct 8c1. Semipreparative incubations of 300 μM **2c** in phosphate buffer (100 mM, pH 7.4) in a total volume of 60 mL were performed with liver microsomes of rats pretreated for 7 days with 1g/L phenobarbital in drinking water (1 mg protein/mL) in the presence of a NADPH generating system (15 mM glucose-6-phosphate, 1 mM NADP, and 2 u/mL of glucose-6-phosphate dehydrogenase), 10 mM ethanolamine, and 2 mM CPDH for 1 h at 37 °C. Then 20 μL of CH_3COOH/mL incubation was added, and the medium was centrifuged at 3000*g* for 10 min. The supernatant was loaded on a SepPak C18 classic column (Waters, St Quentin en Yvelines, France). After washing with 2 mL of water, the metabolites were eluted with 2 mL of CH_3OH. After concentrating under vacuum,

Table 1. Comparison of the ^{1}H and ^{13}C NMR Characteristics of the Two Diastereomers (Isomers a and b) of Bis-Adduct 8c1 and of the Previously Described Dimedone Adduct 7c Diastereomers (Isomers a and b)[18a]

Compound **7c** (diastereomers **a** and **b**) (from ref. 18)					Adduct **8c1** (diastereomers **a** and **b**)				
Carbon	δ ^{1}H CD_3CN/H_2O Isomer a	δ ^{13}C CD_3CN/H_2O Isomer a	δ ^{1}H CD_3CN/H_2O Isomer b	δ ^{13}C CD_3CN/H_2O Isomer b	Carbon	δ ^{1}H CD_3CN Isomer a	δ ^{13}C CD_3CN Isomer a	δ ^{1}H CD_3CN Isomer b	δ ^{13}C CD_3CN Isomer b
2	2.85 ; 3.27	53.5	3.0 ; 3.44	55	2	3.2, 3.7	56	3.2, 3.7	56
4	4.85	41	4.85	41	4	4.93	41.9	4.97	42
5	1.70 ; 1.93	29.5	1.66 ; 1.87	29.3	5	1.85, 2.08	25.6	1.85, 2.08	25.6
6	2.65 ; 2.70	46.9	2.51 ; 2.54	44.5	6	2.69, 2.84	45.7	2.75	46.3
7	4.61	71.7	4.57	71.9	7	4.97	72.5	4.92	72.5
10	7.28	130.2	7.28	130.2	10	7.46	132	7.46	132
11	7.08	122.1	7.08	122.1	11	7.25	117	7.25	117
12	7.14	124.1	7.14	124.1	12	7.30	126	7.30	126
13	7.36	130.9	7.36	130.9	13	7.46	132	7.46	132
14	5.46	118.2	5.36	118.6	14	5.65	122	5.64	120.9
17, 18	2.19	45.9	2.19	45.9	17, 18	2.39, 2.41	31.8	2.39, 2.41	31.8
21	2.22	17.6	2.22	17.6	21	2.18	19.3	2.18	19.3
22, 23	0.74 ; 0.87	10.8	0.74 ; 0.87	10.8	22, 23	0.74, 0.80	12.6	0.74, 0.80	12.6
---					24	3.3, 3.4	42.7	3.3, 3.4	42.7
---					25	3.6	59.8	3.6	59.8

[a]Chemical shifts (δ) are in ppm relative to $(CH_3)_4Si$.

Table 2. MS and MS^2 Characteristics of Metabolites 8c and 8b Derived from Microsomal Oxidation of 2c and 2b in the Presence of Either an Amine and CPDH or a Thiol

starting thiolactone	8c and 8b metabolites	MS (m/z)	MS^2 (m/z)[c]
2c	Nu = $NHCH_2CH_2OH$; Nu′ = CPD	**8c1**: 489	391 (−CPDH), 428 (−$NH_2CH_2CH_2OH$), 471 (−H_2O)
2b	Nu = $NHCH_2CH_2OH$; Nu′ = CPD	**8b1**: 495[a]/497	397 (−CPDH), 434 (−$NH_2CH_2CH_2OH$), 365 (−CPDSH)
2c	Nu = piperidine-H; Nu′ = CPD	**8c4**: 513	415 (−CPDH), 428 (−piperidine), 330 (−CPDH −piperidine)
2b	Nu = piperidine-H; Nu′ = CPD	**8b4**: 519[a]/521	421 (−CPDH), 434 (−piperidine),
2c	Nu = NH_2; Nu′ = CPD	**8c5**: 445	347 (−CPDH), 315 (−CPDSH), 428 (−NH_3)
2b	Nu = NH_2; Nu′ = CPD	**8b5**: 451[a]/453	353 (−CPDH), 321 (−CPDSH), 434 (−NH_3)
2c	Nu = OH; Nu′ = CPD	**8c2**: 446	317 (−CPDS), 316 (−CPDSH), 428 (−H_2O), 400 (−H_2O −CO)
2b	Nu = OH; Nu′ = CPD	**8b2**: 452[a]/454	323 (−CPDS), 322 (−CPDSH), 434 (−H_2O), 406 (−H_2O −CO)
2c	Nu = Nu′ = CPD	**8c3**: 526	428 (−CPDH), 400 (−CPDH −CO)
2c	Nu = Nu′ = NAcCys[b]	**8c6**: 656	493 (−NAcCysH), 461 (−NAcCysSH)
2b	Nu = Nu′ = NAcCys[b]	**8b6**: 662[a]/664	499 (−NAcCysH), 467 (−NAcCysSH)
2c	Nu = Nu′ = SCH_2CH_2OH[b]	**8c7**: 486	377 (−$SSCH_2CH_2OH$), 408 (−$HSCH_2CH_2OH$)
2b	Nu = Nu′ = SCH_2CH_2OH[b]	**8b7**: 492[a]/494	383 (−$SSCH_2CH_2OH$), 414 (−$HSCH_2CH_2OH$)

[a]Molecular ions of bis-adducts derived from **2b** appeared as two peaks separated by 2 mass units in the ratio expected for compounds containing a Cl atom. MS^2 data were derived from fragmentation of the ion corresponding to the ^{35}Cl isotope. [b]These bis-adducts formed in the presence of a thiol appeared as a mixture of 4 stereoisomers (presumably 2 diastereomers with a *cis* configuration of the double bond and two diastereomers with a *trans* configuration of the double bond). [c]The first mentioned peak is the most intense one.

the mixture was purified by 5 repetitive HPLC separations on a Hypersil Mos column (250 × 4.6 mm, 5 μm) using a 20 min gradient of (A) ammonium acetate (0.1M, pH 4.6) to (B) $CH_3CN/CH_3OH/H_2O$ (7:2:1) at 1 mL/min. Collected fractions were lyophilized after neutralization with NH_4HCO_3, dissolved in 400 μL of CD_3CN, and transferred to Shigumi tubes (Sigma-Aldrich, St Quentin Fallavier, France) for NMR analysis.

NMR Experiments. ^1H NMR spectra of **8c1** were done on a Bruker Avance 500 spectrometer (500 MHz) at 27 °C. Chemical shifts are given in ppm relative to $(CH_3)_4Si$.

RESULTS

Oxidation of 2-Oxo-prasugrel, 2c, with Human Liver Microsomes in the Presence of Ethanolamine and Cyclopentane-1,3-dione (CPDH).

Incubation of 0.1 mM **2c** with human liver microsomes was done for 30 min at 37 °C in the presence of NADPH, which is a required cofactor for P450-dependent activities,[26] 10 mM ethanolamine as a potential nucleophile (NuH) to react with the COS carbon of thiolactone sulfoxide **6c**, and 2 mM CPDH, an equivalent of dimedone that is more soluble in water, in order to trap intermediate sulfenic acids (see Figure 3).[27] An HPLC-MS study of the incubate showed the formation of three pairs of metabolites (each pair of components in a 1:1 ratio) having incorporated the CPDH and/or ethanolamine moieties. The two metabolites of the major pair (15% yield relative to **2c**; about 50% of all compounds having incorporated the CPDH and/or ethanolamine moieties) exhibited identical mass spectra with a parent ion (ESI^+) corresponding to M + H^+ and characterized by a peak at m/z = 489, as expected for the bis-adduct **8c1** with Nu = $NHCH_2CH_2OH$ and Nu′ = CPD. Their MS^2 spectra obtained from fragmentation of the ion at m/z = 489 were also identical and exhibited a major peak at m/z = 391, as expected for the loss of CPDH, and two minor peaks at 471 (loss of H_2O) and 428 (loss of ethanolamine) (Table 2).

Identical experiments performed with phenobarbital-treated rat liver microsomes instead of human liver microsomes gave very similar results with the major formation of bis-adducts **8c1**. After semipreparative incubations of **2c** with these rat liver microsomes and purification by preparative HPLC, a detailed analysis of the ^1H NMR spectrum of a mixture of metabolites **8c1**, using two-dimensional (2D) NMR methods [total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC)], allowed us to assign all the signals to the protons of the molecules and also to find the ^{13}C chemical shifts of most carbons of the molecules (Table 1).

These NMR data were found to be very similar to those of the previously described dimedone adduct **7c** diastereomers that are formed during microsomal oxidation of **1c** or **2c** in the presence of dimedone.[18] They were in complete agreement with the structure shown for the two diastereomers of bis-adduct **8c1** in Table 1. They include characteristic ^1H NMR signals at 5.64 and 5.65 ppm and ^{13}C NMR signals at 120.9 and 122 ppm, for the vinylic CH moiety, and at 4.93 and 4.97 ppm and 41.9 and 42 ppm for the CH moiety bearing the S atom. The ^1H and ^{13}C NMR signals of the $NHCH_2CH_2OH$ moiety coming from ethanolamine were found at 3.3 and 3.4 and at 42.7 ppm for the CH_2 group in α position to the NH function, and at 3.6 and 59.8 ppm for the CH_2 in α position to the OH function (Table 1).

The two minor 1:1 pairs of metabolites had only incorporated the CPDH moiety. Those of the pair corresponding to about 60% of all metabolites having only incorporated the CPDH moiety exhibited identical mass spectra with a parent ion (ESI^+) corresponding to M + H^+ and characterized by a peak at m/z = 446. Their MS^2 spectra were also identical and exhibited a major peak at m/z = 317 corresponding to the loss of a CPDS fragment and another peak at m/z = 428 corresponding to the loss of H_2O (the ion chromatogram and MS and MS^2 spectra of these metabolites and the assignments of their fragmentation pattern are shown in Supporting Information). These data indicated that these two metabolites were the two diastereomers of the CPDH adduct **8c2** with Nu = OH and Nu′ = CPD (Table 2 and Figure 3), the equivalent of the previously described dimedone mono-adduct **7c**.[18] The metabolites of the third pair exhibited identical mass spectra with a parent ion (ESI^+) at m/e = 526, corresponding to the incorporation of two CPDH moieties. Their MS^2 spectra were also identical with two peaks at m/z = 428 (major) and 400 corresponding to the loss of CPDH and CPDH + CO, respectively (Table 2; their ion chromatogram and MS and MS^2 spectra are shown in Supporting Information). These MS

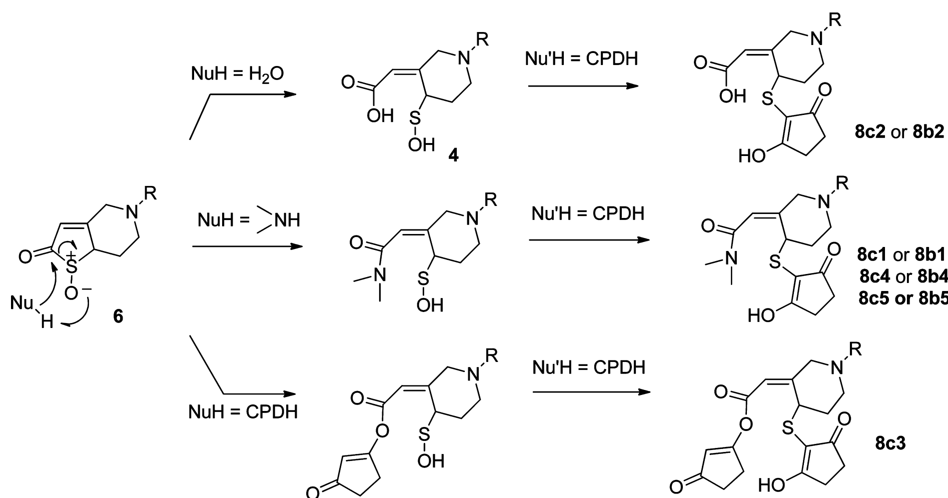

Figure 4. Reactions of thiolactone sulfoxides **6** with H_2O, amines, or CPDH itself, in the presence of CPDH in order to trap intermediate sulfenic acids. The structure of **8c3** is only tentatively proposed; an isomeric structure resulting from an initial C-acylation of CPDH (instead of an O-acylation, as shown in the figure) is also possible.

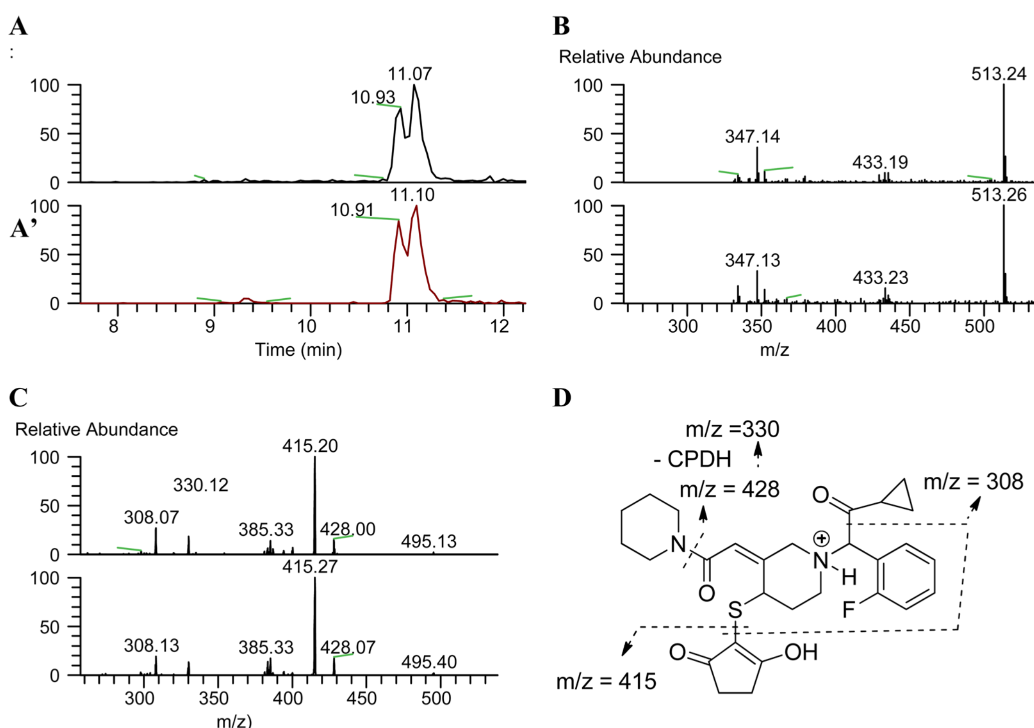

Figure 5. Extracted ion chromatogram and MS and MS^2 spectra of the two bis-adduct **8c4** diastereomers formed during metabolism of **2c** by human liver microsomes in the presence of NADPH, 2 mM CPDH, and 5 mM piperidine (conditions in the Experimental Procedures section). (A) Extracted ion chromatogram at m/z = 513 (MS); (A′) extracted ion chromatogram of collision induced dissociation at m/z = 513 (MS^2); (B) MS spectra of the two **8c4** peaks; (C) MS^2 spectra of the ion at m/z 513 for the two diastereomers; (D) assignments for the fragmentation pattern shown in Figure 5C.

and MS^2 data indicated that these two metabolites were the two diastereomers of **8c3**, a bis-adduct having incorporated two CPDH moieties (Figure 4).

The above data indicated that the three adducts **8c1**, **8c2**, and **8c3** observed in microsomal incubations of **2c** in the presence of ethanolamine and CPDH should result from a competition beween the three nucleophiles $NH_2CH_2CH_2OH$, H_2O, and CPDH for the initial attack of the electrophilic CO carbon of **6c** thiolactone sulfoxide ring (Figure 4). Accordingly, identical microsomal incubations of **2c** without ethanolamine only led to adducts **8c2** and **8c3**.

Oxidation of 2c with Human Liver Microsomes in the Presence of Other *N*-Nucleophiles and CPDH. Oxidation of **2c** with human liver microsomes in the presence of NADPH, CPDH, and other *N*-nucleophiles, such as piperidine or ammonia, led to similar results with formation of the expected new bis-adducts **8c** with Nu′ = CPD and Nu = $N(CH_2)_5$ or NH_2 in addition to the mono CPDH adduct **8c2** with Nu = OH and Nu′ = CPD. The HPLC-MS, MS, and MS^2 characteristics of the 1:1 pair of diastereomers of the bis-adduct obtained in the presence of piperidine (molecular ions at m/z = 513 and fragments at 415 and 428 corresponding to

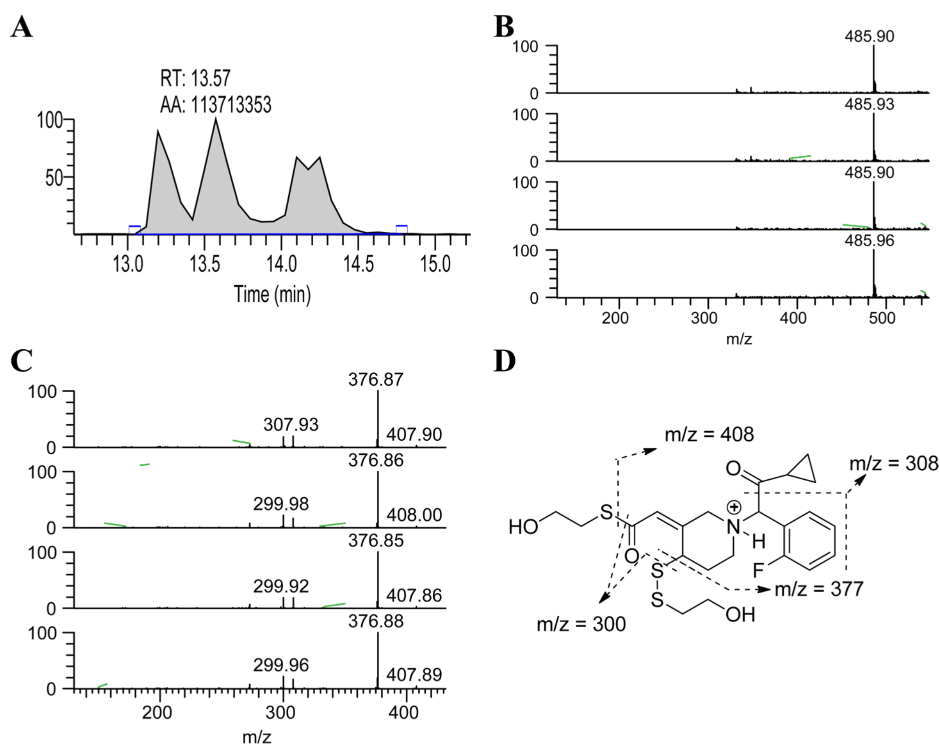

Figure 6. Extracted ion chromatogram and MS and MS[2] spectra of bis-adduct **8c7** isomers formed during the metabolism of **2c** by human liver microsomes in the presence of NADPH and 5 mM mercaptoethanol (conditions are in the Experimental Procedures section). (A) Extracted ion chromatogram at m/z = 486 (MS); (B) MS spectra of the four isomers of **8c7**; (C) MS[2] spectra of the ion at m/z = 486 of the four isomers; (D) assignments for the fragmentation pattern shown in Figure 6C.

the loss of CPDH and piperidine, respectively) are shown in Figure 5. They were in complete agreement with the expected structure **8c4**. Similar results indicating the formation of bis-adducts **8c5** were obtained when using NH_3 as a nucleophile (Table 2).

Oxidation of 2-Oxo-clopidogrel, 2b, with Human Liver Microsomes in the Presence of Various *N*-Nucleophiles and CPDH. All the above-described experiments performed on **2c** were also done on **2b**, under identical conditions, and gave almost identical results, with the formation of the corresponding bis-adducts **8b1**, **8b4**, and **8b5** in addition to monoadduct **8b2**, the equivalent of previously reported **7b**,[17] in which the dimedone moiety was replaced with CPD. The MS and MS[2] characteristics of these adducts derived from **2b** corresponded well to those of the adducts derived from **2c** (Table 2).

Oxidation of 2b with Human Liver Microsomes in the Presence of Thiols. Oxidation of **2b** with human liver microsomes in the presence of NADPH and 2 mM *N*-acetylcysteine (NAcCysH) mainly led, as reported previously,[17,20,21,23] to thiol **3b** and dithioether **5b** (Figure 2, with R′S = NAcCys). However, a careful study of the incubation mixture by HPLC-MS showed the additional, minor formation of four products exhibiting the same MS, characterized by a molecular ion MH^+ at m/z = 662 and 664 (with an isotope ratio indicative of the presence of a chlorine atom), and MS[2] fragments of the peak at 662 at m/z = 499 and 467 corresponding to the loss of a NAcCysH and a NAcCysSH moiety, respectively (Table 2). These data were in agreement with structure **8b6** in which Nu = Nu′ = $SCH_2CH(COOH)$-$NHCOCH_3$, resulting from the incorporation of two *N*-acetylcysteine moieties upon reaction of NAcCysH with thiolactone sulfoxide **6b**. Thiol **3b** and dithioether **5b** (with R′S = NAcCys) derived from a reaction of **2b** with human liver microsomes in the presence of NADPH and NAcCysH also existed as a mixture of four stereoisomers in agreement with literature data.[21] It was previously reported that these four stereoisomers consisted of a pair of diastereomers having a *cis* configuration of the double bond and a pair of diastereomers having a *trans* configuration of the double bond.[21] The detection of four peaks exhibiting a molecular ion at m/z = 662/664 suggested that the bis-adduct **8b6** also existed as a mixture of such *cis* and *trans* diastereomers.

Very similar results were obtained from the HPLC-MS study of the reaction mixture resulting from incubations of **2b** with human liver microsomes in the presence of NADPH and mercaptoethanol. This study showed the formation of a mixture of four stereoisomers of a bis-adduct exhibiting the MS and MS[2] characteristics expected for **8b7** (Table 2), in addition to previously described **3b** and **5b** (Figure 2, with R′S = SCH_2CH_2OH).[17,20,21]

Oxidation of 2c with Human Liver Microsomes in the Presence of Thiols. Microsomal incubation of **2c** instead of **2b** in the presence of NAcCysH or mercaptoethanol under conditions identical to those of the previous paragraph led to very similar results, with the major formation of thiol **3c** and dithioether **5c** (with R′S = NAcCys or SCH_2CH_2OH), as previously reported,[18] and the minor formation of bis-adducts whose MS and MS[2] characteristics corresponded well to structures **8c6** and **8c7** (Table 2). All these metabolites, **3c**, **5c**, **8c6**, and **8c7**, formed in the presence of a thiol, appeared as a mixture of four stereoisomers (see for instance Figure 6 for the case of **8c7**), as in the case of the above-described microsomal oxidation of **2b** in the presence of thiols.

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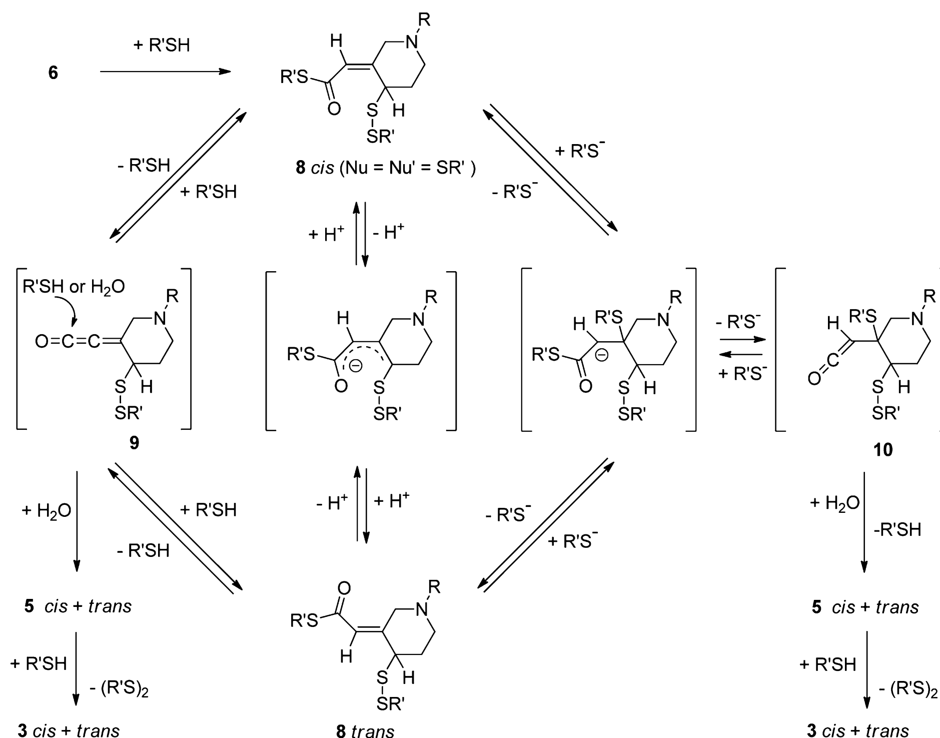

Figure 7. Possible mechanisms explaining the formation of *trans* metabolites in the microsomal metabolism of **2b** only in the presence of thiols.

■ DISCUSSION

It was previously proposed[17,18] that monoadducts **7** or **5** formed during the metabolism of thiolactones **2** by human liver microsomes in the presence of NADPH and either dimedone or a thiol were derived from two successive reactions of the electrophilic thiolactone sulfoxide intermediates, **6**, resulting from P450-dependent oxidation of **2** (Figure 2). The first one would be a nucleophilic attack of H_2O on the CO carbon of **6** resulting in the formation of sulfenic acid **4**. The second one is a nucleophilic attack of either dimedone or a thiol on the electrophilic S atom of **4**. The aforementioned data completely confirmed this mechanism by showing that such reactions of **2b** or **2c** with human liver microsomes, NADPH, and an equivalent of dimedone, CPDH, performed in the presence of a N-nucleophile such as ethanolamine, piperidine, or ammonia, led to the expected bis-adducts **8b1** or **8c1**, **8b4** or **8c4**, and **8b5** or **8c5**, respectively. These bis-adducts were formed in addition to **8b2** or **8c2**, the equivalent of **7b** or **7c** in which dimedone was replaced with CPDH, as they derived from a competition between the N-nucleophile and H_2O for the initial attack on the CO carbon of **6b** or **6c** (Figure 4). Bis-adducts **8b1**, **8c1**, **8b4**, **8c4**, **8b5**, and **8c5** were identified by their characteristic MS molecular peak and MS^2 fragments (Table 2). The structure of one of them, **8c1**, was completely established by ^1H and ^{13}C NMR spectroscopy (Table 1) using several 2D NMR techniques.

In all the microsomal reactions of **2c** in the presence of CPDH, the formation of a minor product, **8c3**, was observed. It should derive from the initial attack of CPDH, acting as a C- or O-nucleophile, on the CO carbon of **6c**, in competition with H_2O or the N-nucleophile. Its MS and MS^2 characteristics did not allow us to conclude between a structure resulting from an initial attack of a CPDH oxygen atom resulting in the eventual formation of an enol ester (as shown in Figure 4) and of a CPDH carbon atom that would result in the formation of a conjugated enol diketone.

Microsomal oxidations of **2c** or **2b** in the presence of thiols, R′SH, that were shown previously to lead to thiol **3c** or **3b** and dithioethers **5c** or **5b**[17,18,20−23,28] were reinvestigated in order to detect bis-adducts such as **8c** or **8b** with Nu = Nu′ = SR′. Reactions in the presence of NAcCysH or mercaptoethanol did mainly lead to **3c** or **3b** and **5c** or **5b** as major products, but minor amounts of the expected bis-adducts **8c6** or **8b6** and **8c7** or **8b7**, resulting from an initial attack on the CO carbon of **6c** or **6b** by R′SH, were also detected by HPLC-MS (Table 2).

As far as the stereochemistry of **2b** and **2c** metabolites are concerned, it is noteworthy that monoadducts **8b2** and **8c2**, **7b** and **7c**, their equivalents with dimedone instead of CPDH, and all bis-adducts **8b1**, **8c1**, **8b4**, **8c4**, **8b5**, and **8c5** that were formed upon microsomal oxidations in the presence of water and amine nucleophiles and were derived from carboxylic acid- or amide-sulfenic acid precursors (Figure 4) only consisted of a pair of diastereomers (see for instance Figure 5 and Figures S1 and S2 in Supporting Information) with a *cis* configuration of the double bond. Moreover, the metabolism of **2b** and **2c** by human liver microsomes in the presence of NADPH and ascorbate as a reducing agent also only leads to the formation of *cis* **3b**[13,14,21] and **3c**.[15] By contrast, all thiols **3b** and **3c**, dithioethers **5b** and **5c**, and bis-adducts **8b6** and **8c6**, as well as **8b7** and **8c7**, that were formed in microsomal oxidations in the presence of thiol nucleophiles, such as NAcCysSH or mercaptoethanol, existed as mixtures of four stereoisomers (see for instance Figure 6). The formation of diastereomers with a *trans* configuration of the double bond, in addition to the *cis* diastereomers, in microsomal oxidations of **2b** in the presence of thiols was previously reported in the case of **3b**[2,21] and **5b**.[21] Thus, it seems likely that the four stereoisomers observed in the case of **8b6**, **8c6**, **8b7**, and **8c7** also are two *cis* and two *trans* diastereomers.

The mechanism of formation of those *trans* diastereomers remains to be determined. However, their formation only in the presence of thiols could be due to the particular reactivity of intermediate thioesters **8** (Nu = Nu′ = SR′) derived from the initial reaction of thiol nucleophiles with thiolactone sulfoxides **6**, when compared to that of intermediate carboxylic acids or amides derived from the initial reaction of **6** with water or amines. At least three mechanisms taking into account the particular reactivity of intermediate thioesters can be proposed to explain the formation of *trans* isomers only in the presence of thiols. Deprotonation and formation of an enolate ion is easier from thioesters than from carboxylic acids, esters, or amides.[29−31] Such an enolization (Figure 7) would lead to a *cis*−*trans* isomerization of the double bond. Another possible mechanism of *trans* isomer formation could be related to the greater tendency of thioesters to form the corresponding ketenes by an elimination reaction (of R′SH) than carboxylic acids or amides (by elimination of H_2O or of an amine). Such an elimination of R′SH on bis-adducts **8** (with Nu = Nu′ = SR′) would lead to vinylidene-ketene **9** (Figure 7). The CO carbon of this intermediate should rapidly react with the nucleophiles available in the medium, R′SH or H_2O. Protonation of the resulting vinylic carbanion should lead to a mixture of *cis* and *trans* bis-adducts **8** (with Nu = Nu′ = SR′) or monoadducts **5**, and eventually to *cis* and *trans* thiols **3**, after the reduction of **5** by R′SH in excess. A third possible mechanism would be a reversible Michael-type addition of R′SH on the conjugated thioester (Figure 7). Elimination of the $R'S^-$ group introduced by the Michael-type addition would directly lead to *cis* and *trans* **8** (Nu = Nu′ = SR′), whereas elimination of the $R'S^-$ group of the thioester function would lead to ketene **10** (Figure 7). Reaction of **10** with R′SH or H_2O would lead to *cis* and *trans* thioesters **8** (Nu = Nu′ = SR′) or *cis* and *trans* dithioethers **5**, respectively, after the elimination of the tertiary R′S group. Further experiments are required to establish a detailed mechanism for the formation of *trans* isomers in the microsomal metabolism of **2b** or **2c** in the presence of thiols.

Coming back to the aforementioned data about the detection of several bis-adducts from microsomal oxidation of **2b** and **2c**, these data not only confirm the intermediate formation of thiolactone sulfoxides **6** in the bioactivation of thienopyridine antithrombotics but also indicate that these reactive intermediates are released in the medium and can react with a variety of N- and S-nucleophiles added to the incubate. They constitute a new class of reactive metabolites acting as bis-electrophiles whose reactions with nucleophiles may lead to a variety of bis-adducts. Actually, the formation of analogous reactive metabolites, thiazolidinedione sulfoxides, has been proposed in the metabolism of glitazones on the basis of nucleophilic trapping experiments.[32,33]

During the preparation of this article, an article appeared indicating that the metabolism of **2b** by human liver microsomes in the presence of GSH led to products that were proposed to derive from the attack of H_2O or GSH either on the carbonyl C-atom of intermediate **6b** or on the allylic, tertiary C-atom of **6b**.[34] However, all the reported final metabolites were monoadducts.[34]

The reactions of thiolactone sulfoxide bis-electrophiles **6** with protein or nucleic acid nucleophiles could lead to the covalent binding of their precursors **1** to cell components. Moreover, as bis-electrophiles, they could lead to cross-linking of proteins or nucleic acids. Such reactions could be at least in part involved in the mechanism-based inhibition of P450s by ticlopidine, clopidogrel, and prasugrel,[9,35−39] and in the pharmacological and/or secondary toxic effects of these compounds.[40] This remains to be established.

ASSOCIATED CONTENT

Supporting Information

MS and MS^2 spectra, fragmentation analysis, and extracted ion chromatograms of compounds **8c2** and **8c3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CPDH, cyclopentane-1,3-dione; HPLC-MS, high performance liquid chromatography−mass spectrometry; GSH, glutathione; NAcCysH, *N*-acetyl cysteine; PON-1, paraoxonase-1; P450, cytochrome P450

REFERENCES

(1) Yoneda, K., Iwamura, R., Kishi, H., Mizukami, Y., Mogami, K., and Kobayashi, S. (2004) Identification of the active metabolite of ticlopidine from rat in vitro metabolites. *Br. J. Pharmacol. 142*, 551−557.

(2) 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.

(3) 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.

(4) 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.

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

(6) 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.

(7) 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.

(8) Tuong, A., Bouyssou, A., Paret, J., and Cuong, T. G. (1981) Metabolism of ticlopidine in rats: identification and quantitative determination of some its metabolites in plasma, urine and bile. *Eur. J. Drug Metab. Pharmacokinet. 6*, 91−98.

(9) Ha-Duong, N. T., Dijols, S., Macherey, A. C., Goldstein, J. A., Dansette, P. M., and Mansuy, D. (2001) Ticlopidine as a selective mechanism-based inhibitor of human cytochrome P450 2C19. *Biochemistry 40*, 12112−12122.

(10) 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.

(11) 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.

(12) 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.

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

(14) 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.

(15) Dansette, P., 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.

(16) 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.

(17) 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.

(18) 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.

(19) Mansuy, D., and Dansette, P. M. (2011) Sulfenic acids as reactive intermediates in xenobiotic metabolism. *Arch. Biochem. Biophys. 507*, 174−185.

(20) 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.

(21) 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.

(22) 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.

(23) 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.

(24) 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.

(25) 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.

(26) Ortiz de Montellano, P. R. (2005) *Cytochrome P450 Structure, Mechanism, and Biochemistry*, 3rd ed., Kluwer, Academic, Plenum Publ.: New York.

(27) Qian, J., Klomsiri, C., Wright, M., King, S., Tsang, A., Poole, L., and Furdui, C. (2011) Simple synthesis of 1,3-cyclopentanedione derived probes for labeling sulfenic acid proteins. *Chem. Commun. (Cambridge, U.K.) 47*, 9203−9205.

(28) 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.

(29) Bordwell, F., and Fried, H. (1991) Heterocyclic aromatic anions with 4n + 2 π-electrons. *J. Org. Chem. 56*, 4218−4223.

(30) Um, P., and Drueckhammer, D. (1998) Dynamic enzymatic resolution of thioesters. *J. Am. Chem. Soc. 120*, 5605−5610.

(31) Alonso, D., Kitagaki, S., Utsumi, N., and Barbas, C., III (2008) Towards organocatalytic polyketide synthases with diverse electrophile scope: Trifluoroethyl thioesters as nucleophiles in organocatalytic Michael reactions and beyond. *Angew. Chem., Int. Ed. 47*, 4588−4591.

(32) Kassahun, K., Pearson, P., Tang, W., McIntosh, I., Leung, K., Elmore, C., Dean, D., Wang, R., Doss, G., and Baillie, T. (2001) Studies on the metabolism of troglitazone to reactive intermediates in vitro and in vivo. Evidence for novel biotransformation Pathways involving quinone methide formation and thiazolidinedione ring scission. *Chem. Res. Toxicol. 14*, 62−70.

(33) Reddy, V., Karanam, B., Gruber, W., Wallace, M., Vincent, S., Franklin, R., and Baillie, T. (2005) Mechanistic studies on the metabolic scission of thiazolidinedione derivatives to acyclic thiols. *Chem. Res. Toxicol. 18*, 880−888.

(34) Zhu, Y., and Zhou, J. (2013) In Vitro Biotransformation Studies of 2-Oxo-clopidogrel: Multiple Thiolactone Ring-Opening Pathways Further Attenuate Prodrug Activation. *Chem. Res. Toxicol. 26*, 179−190.

(35) Richter, T., Mürdter, T., Heinkele, G., Pleiss, J., Tatzel, S., Schwab, M., Eichelbaum, M., and Zanger, U. (2004) Potent mechanism-based inhibition of human CYP2B6 by clopidogrel and ticlopidine. *J. Pharmacol. Exp. Ther. 308*, 189−197.

(36) Hagihara, K., Nishiya, Y., Kurihara, A., Kazui, M., Farid, N. A., and Ikeda, T. (2008) Comparison of human cytochrome P450 inhibition by the thienopyridines prasugrel, clopidogrel, and ticlopidine. *Drug Metab. Pharmacokinet. 23*, 412−420.

(37) Nishiya, Y., Hagihara, K., Ito, T., Tajima, M., Miura, S., Kurihara, A., Farid, N., and Ikeda, T. (2009) Mechanism-based inhibition of human cytochrome P450 2B6 by ticlopidine, clopidogrel, and the thiolactone metabolite of prasugrel. *Drug Metab. Dispos. 37*, 589−593.

(38) Nishiya, Y., Hagihara, K., Kurihara, A., Okudaira, N., Farid, N., Okazaki, O., and Ikeda, T. (2009) Comparison of mechanism-based inhibition of human cytochrome P450 2C19 by ticlopidine, clopidogrel, and prasugrel. *Xenobiotica 39*, 836−843.

(39) Zhang, H., Amunugama, H., Ney, S., Cooper, N., and Hollenberg, P. F. (2011) Mechanism-based Inactivation of human cytochrome P450 2B6 by clopidogrel: Involvement of both covalent modification of cysteinyl residue 475 and loss of heme. *Mol. Pharmacol. 80*, 839−847.

(40) Lokhandwala, J., Best, P., Henry, Y., and Berger, P. (2011) Allergic reactions to clopidogrel and cross-reactivity to other agents. *Curr. Allergy Asthma Rep. 11*, 52−57.