Cytochromes P450 catalyze both steps of the major pathway of Clopidogrel bioactivation.

To the Editor :

The mechanism generally admitted for the bioactivation of Clopidogrel (CLO), a tetrahydrothienopyridine anti-thrombotic prodrug, was its two-step enzymatic conversion into a biologically active thiol metabolite. The first step is a cytochrome P450 (CYP)-dependent oxidation leading to a 2-oxo-CLO thiolactone metabolite (Fig.1). The second step was described as a CYP-dependent oxidative opening of the 2-oxo-CLO thiolactone ring with the eventual formation of an active thiol metabolite [1-3]. Actually, CYP2C19 was found to play a key role in CLO bioactivation by contributing to both steps of this bioactivation [3], and a common genetic variant within the *CYP2C19* gene, the CYP2C19*2 loss of function polymorphism, was found to be associated with an attenuated response to CLO and a worse clinical outcome in patients undergoing coronary stenting [4]. A very recent paper published in *Nature Medicine* [5] reported that this second step is not catalyzed by CYP enzymes but depends on paraoxonase-1(PON-1), and that PON-1 is a major determinant of CLO efficacy. Quite recently, we have made experiments to understand the origin of these contradictory conclusions about the nature of the second step of CLO bioactivation, and found that both CYP enzymes and PON-1 do catalyze the opening of 2-oxo-CLO thiolactone but lead to different isomers of the thiol metabolite. We also found that the major active thiol isomer found in the plasma of CLO-treated subjects is formed via a CYP-dependent pathway.

Actually, opening of the thiolactone ring of 2-oxo-CLO may lead to five thiol isomers, two *cis* diastereoisomers with a Z configuration of the exocyclic double bond, two *trans* diastereoisomers with an E configuration of this double bond, and an *endo* isomer in which the double bond has migrated from an exocyclic to an endocyclic position in the piperidine ring [6] (Fig.1). A very recent, optimized method for the specific, quantitative determination of CLO-derived thiol metabolite isomers in human plasma of CLO-treated subjects showed the presence of the *cis* thiol isomers as major metabolites and of the *endo* thiol isomer as a minor component [6]. The authors also showed that one of these *cis* thiol

diastereoisomers was very active towards the platelet P2Y12 receptor, and was presumably responsible for CLO activity [6].

In order to reinvestigate the metabolic fate of 2-oxo-CLO by human liver microsomes and by human sera, we used HPLC-MS methods allowing a complete separation of the thiol metabolite isomers, either as such or after derivatization using 3'-methoxy- or 4'-bromo-phenacyl bromide [6,7]. Incubation of 2-oxo-CLO with human liver microsomes in the absence of NADPH, that is a required cofactor for CYP-dependent activities, and in the presence of KF to inhibit the esterase-dependent hydrolysis of the methyl ester function of 2-oxo-CLO, only led to the formation of the *endo* thiol isomer (Fig.2). This metabolite was completely identified after derivatization of its thiol function upon reaction with 4'-bromophenacyl bromide; its HPLC retention time and MS spectrum were identical to those of an authentic sample of the previously described [6] *endo* thiol 4'-bromoacetophenone derivative. The MS spectra of the *endo* thiol and of its 4'-bromo- and 3'-methoxy acetophenone derivatives were in complete agreement with the indicated structures (Fig. 1 and 2). Addition of paraoxon, a good, usual PON-1 substrate [8], to the incubation led to a 85% decrease of the *endo* thiol formation (data not shown), suggesting that PON-1 was involved in this formation.

When these microsomal incubations were done in the presence of NADPH, the *endo* isomer was formed in almost identical amounts but the major metabolites were now the *cis* thiol isomers, with a *cis* thiols: *endo* thiol ratio of ~ 13 (Fig.2). The *cis* thiols were completely identified at the level of their 3'-methoxyacetophenone derivatives that exhibited HPLC retention times and mass spectra identical to those of authentic samples of the previously described *cis* thiol 3'-methoxy acetophenone derivatives [6]. Addition of 10 µM N-benzyl imidazole, a well-known inhibitor of CYP enzymes [9], had no effects on *endo* thiol formation but almost completely inhibited *cis* thiols formation. These data indicated that *cis* thiols are derived from a CYP- dependent pathway (as previously described by several groups [1-3]) involving an oxidative opening of the thiolactone ring followed by the reduction of a sulfenic acid intermediate [2], whereas the *endo* thiol is derived from a PON-catalyzed hydrolysis (Fig.1). Accordingly, incubation of 2-oxo-CLO with human sera, that do not contain CYP-dependent monooxygenases but in which PON-1 is present, only led to the formation of the *endo* thiol isomer (data not shown).

As far as the measurement of CLO-derived thiol metabolites is concerned, one must note that the MS^2 spectra of the *endo* and *cis* isomers are different (see supplementary material). Thus an HPLC-MS^2 analysis of mixtures of these isomers without their complete HPLC separation,

which seems to be the case under the conditions used by Bouman et al.[5] , could lead to the preferential measurement of only one isomer, depending on the used MS conditions, and thus to misleading results.

Chemical experiments were performed in parallel to better understand the stereochemistry observed for 2-oxo-CLO hydrolysis. Treatment of 2-oxo-CLO under slightly basic conditions always led to *endo* thiol products. For instance, its reaction with CH_3ONa in CH_3OH exclusively led to the *endo* thiol methyl ester with good yields (>80%). These data suggested that enzymatic (PON-1) and chemical hydrolysis of 2-oxo-CLO only led to the *endo* thiol isomer, presumably because the conjugated carbonyl function of 2-oxo-CLO is not reactive enough to react with H_2O or HO^-. Accordingly, lactones are in general good substrates of PON-1 whereas lactones involving a carbonyl group conjugated with a double bond are not substrates [8]. By contrast the tautomer of 2-oxo-CLO with the double bond having migrated within the piperidine ring, that exists in equilibrium with its major conjugated tautomer (Fig.1), should be more reactive towards nucleophiles such as H_2O or HO^-. Their reaction on its carbonyl group should lead to the observed *endo* thiol isomer (Fig.2). Thus, the only way to go from 2-oxo-CLO to the active *cis* thiol is to increase the electrophilicity of its carbonyl group by S-oxidation, a reaction that is catalyzed by CYP enzymes [2,10]. Further hydrolysis of the reactive ketosulfoxide intermediate and reduction of the resulting sulfenic acid species eventually lead to the *cis* thiol metabolite (Fig.1) [2,10].

The ratio of the thiol isomers in the plasma of CLO-treated subjects, with the *cis* thiols as very major metabolites and the *endo* thiol as a minor metabolite [6], is highly similar to that we observed after metabolism of 2-oxo-CLO by NADPH-supplemented human liver microsomes (Fig.2). This suggests that the fate of 2-oxo-CLO *in vivo* is also mainly dependent on CYP enzymes and only to a minor extent on esterases such as PON-1. Recent data have compared the biological activity of the four *cis* and *trans* thiol diastereoisomers towards the platelet P2Y12 receptor [6]; they showed that the most active compound was one of the *cis* diastereoisomer. Taking into account the thiol isomers ratio found in the plasma of CLO-treated subjects, it appears that the anti-thrombotic activity of CLO should be mainly due to one of its *cis* thiol metabolite whose formation is dependent on CYP enzymes [1,6]. Our results are in agreement with a very recently published article showing no association of paraoxonase-1 Q192R genotype with platelet response to CLO and risk of stent thrombosis after coronary stenting [4].

The activity of the minor *endo* thiol metabolite has not been determined so far in a quantitative manner, and further experiments are required to definitely conclude about the relative importance of the CYP-dependent and PON-1-dependent 2-oxo-CLO metabolism pathways in the *in vivo* anti-platelet effects of CLO and the consequences of CYP and PON-1 polymorphisms in the medical use of CLO.

REFERENCES

1. Pereillo, J.M. et al. *Drug Metab Dispos* **30**, 1288-95 (2002).
2. Dansette, P.M., Libraire, J., Bertho, G. & Mansuy, D. *Chem Res Toxicol* **22**, 369-73 (2009).
3. Kazui, M. et al. *Drug Metab Dispos* **38**, 92-9 (2009).
4. Sibbing, D. et al. *Eur Heart J* Apr 28. [Epub ahead of print](2011) and references cited therein..
5. Bouman, H.J. et al. *Nat Med* **17**, 110-6 (2011).
6. Tuffal, G. et al. *Thromb Haemost* **105**, 696-705 (2011).
7. Takahashi, M., Pang, H., Kawabata, K., Farid, N.A. & Kurihara, A. *J Pharm Biomed Anal* **48**, 1219-24 (2008).
8. Billecke, S. et al. *Drug Metab Dispos* **28**, 1335-42 (2000).
9. Correia, M.A. & De Montellano, P.R.O. in *Cytochrome P450 Structure, Mechanism, and Biochemistry* (ed. De montellano, P.R.O.) 247-295 (Kluwer Acad/Plenum Publ., New York, 2005).
10. Mansuy, D. & Dansette, P.M. *Arch Biochem Biophys* **507**, 174-85 (2011).

Authors : Patrick M. Dansette, Julien Rosi, Gildas Bertho and Daniel Mansuy
LCBPT, CNRS UMR 8601, Université Paris Descartes, 45 Rue des Saints Pères 75270 PARIS CEDEX 06, France. E-mail : patrick.dansette@parisdescartes.fr

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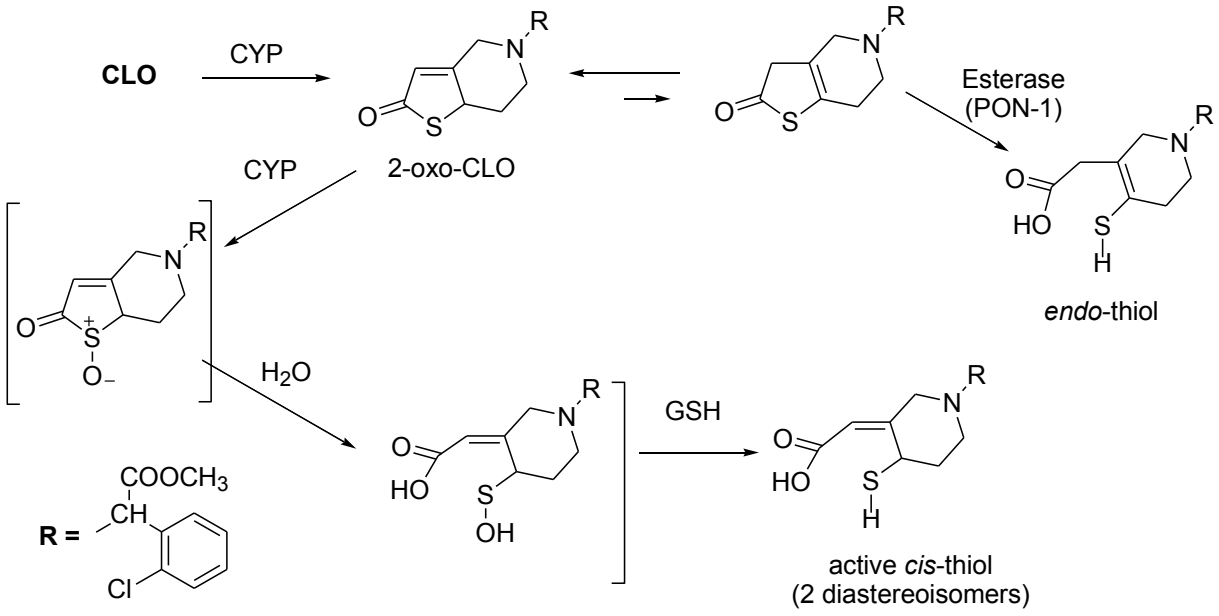

Figure 1. Bioactivation of clopidogrel (CLO) and formation of different thiol isomers by esterase- and CYP- catalyzed metabolism of 2-oxo-CLO. The *endo*-thiol should exist in equilibrium with its thioketone tautomer. GSH = glutathione.

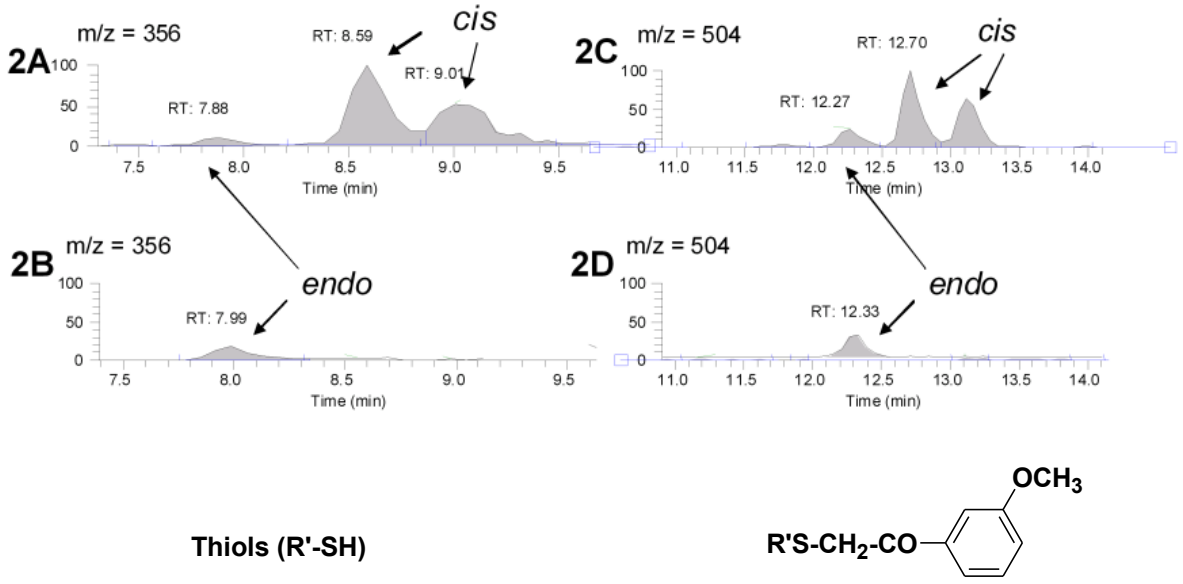

Figure 2 : HPLC profiles of incubations of 2-oxo-CLO with human liver microsomes with (2A, 2C) or without (2B, 2D) NADPH. MS detection of the thiols themselves (peaks at m/z = 356) (Fig. 2A and 2B) or of their derivatized products after treatment with 3'-methoxyphenacyl bromide (peaks at m/z= 504) (Fig. 2C and 2D). Incubations of [7S] 2-oxo-clopidogrel (100 µM) with 1mg protein/ml of pooled human liver microsomes (BD-Gentest) in 100 mM phosphate buffer pH 7.4 containing 2mM $CaCl_2$, 100 mM KF and a reducing agent (either ascorbic acid (20 mM) or glutathione (5 mM)) were done at 37°C for 30 min in absence or presence of a NADPH generating system.

Supplementary Materials

Chemicals and biochemicals : [7S] 2-oxo-clopidogrel (SR121883), 3'-methoxyphenacyl-*cis*-thiol (SAR206251) and 4'-bromophenacyl-*endo*-thiol (SAR195539) were a gift of Sanofi-Aventis. 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), 2-oxo-clopidogrel (100 μM) and a reducing agent (20 mM ascorbic acid, or 0.5 mM dithiothreitol, or 5 mM glutathione (GSH)) with or without 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. Reactions were stopped by adding one half volume of CH_3CN: CH_3COOH (9:1) and proteins were removed by centrifugation at 13000g.

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 Gemini C18 column (100 x 2 mm, 3 μm; Phenomenex), and a gradient starting at 40% B for 1 min then increasing linearly to 100% B in 15 min (A= ammonium acetate buffer (10 mM, 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, capillary voltage 15V, m/z range for MS recorded generally between 300 and 700 (except for exploratory experiments with a wider range 300-800). MS^2 energy were tested between 20 and 40 eV and were generally 35 eV.

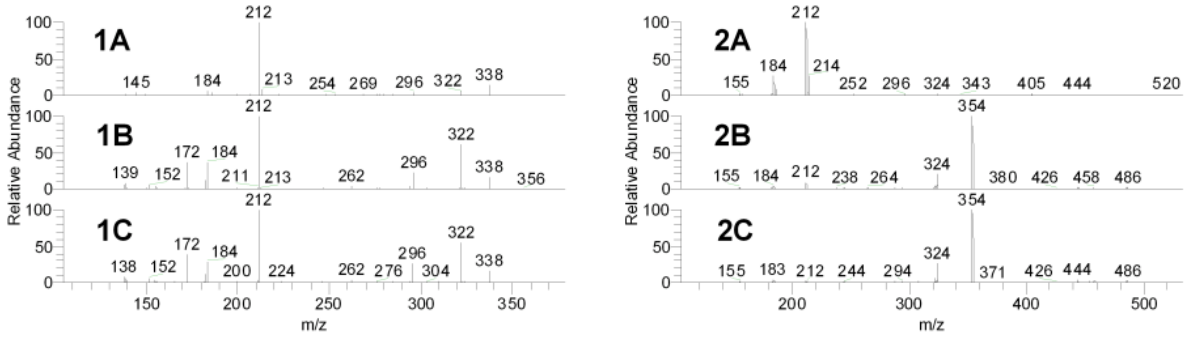

Figure S1 : MS^2 spectra of *end*o and *cis* thiols
Panel 1 shows the MS^2 spectra of the thiols at m/z = 356 (1A *endo*, 1B and 1C *cis* thiols). Panel 2 shows the MS^2 spectra of the 3'-methoxyphenacyl derivatized thiols at m/z = 504: (2A *endo* and 2B and 2C *cis* thiols).