Metabolic activation of clopidogrel: *in vitro* data provide conflicting evidence for the contributions of CYP2C19 and PON1

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Abstract: The recent report that clopidogrel efficacy may be more dependent on paraoxonase-1 (PON1) than on cytochrome P450 2C19 (CYP2C19) activity raises questions about the roles of these and other enzymes in clopidogrel activation. To provide insight into the emerging PON1 *versus* CYP2C19 debate, this commentary summarizes the clinical evidence on the pharmacokinetic determinants of clopidogrel efficacy. We then review the *in vitro* studies investigating the enzymes involved in clopidogrel activation, and comment on their strengths and limitations. There is agreement amongst *in vitro* studies regarding the involvement of CYP1A2 and CYP2B6 in the metabolism of clopidogrel to 2-oxo-clopidogrel. However, the evidence for other CYP enzymes in the first activation step (e.g. CYP2C19 and CYP3A4) is inconsistent and dependent on the *in vitro* test system and laboratory. All major drug metabolizing CYP enzymes are capable of converting 2-oxo-clopidogrel to sulfenic acid intermediates that subsequently form the active thiol metabolite. However, the extent of CYP involvement in this second step has been challenged, and new evidence suggests that CYP-independent hydrolytic cleavage of the thioester bond may be more important than oxidative metabolism.

Keywords: antiplatelet, clopidogrel, cytochrome P450 2C19, metabolic activation, paraoxonase-1, pharmacogenomics

Introduction

The antiplatelet drug clopidogrel is used for the management and prophylaxis of cardiovascular and cerebrovascular thromboembolic events. Clopidogrel is a ‘blockbuster’ drug – the second biggest selling drug worldwide in 2009 with US\$8.6 billion in sales [Winslow, 2010]. Despite its widespread use, large interindividual variability in clopidogrel response has been reported. Patients who do not respond to clopidogrel are at increased risk of thromboembolic complications [Sofi *et al.* 2010], whereas those who respond too well are at increased risk of bleeding [Sibbing *et al.* 2010]. This variability has motivated several groups to study the factors that influence clopidogrel efficacy. Most research to date has focused on genetic polymorphism of enzymes and transporters involved in clopidogrel disposition and metabolic activation. Indeed, clopidogrel is now a frequently cited example of the potential power of pharmacogenomics in clinical practice [Relling and Klein, 2011].

Metabolism of clopidogrel

Clopidogrel is a prodrug that requires metabolic activation in two steps (Figure 1). The majority of the clopidogrel dose (>85%) is metabolized to inactive clopidogrel-carboxylic acid by human carboxylesterase 1 (CES1) [Tang *et al.* 2006]. The remainder is metabolized to 2-oxo-clopidogrel, which is then converted either to inactive 2-oxo-clopidogrel-carboxylic acid via ester hydrolysis or to the active thiol metabolite, the moiety that irreversibly inhibits the binding of adenosine diphosphate to platelet P2Y₁₂ receptors [Savi *et al.* 2000]. The active thiol metabolite is subsequently hydrolysed to an inactive carboxylic acid derivative by carboxylesterases [Tang *et al.* 2006].

Clinical evidence for CYP2C19 as a determinant of clopidogrel efficacy

Although many cytochrome P450 enzymes (CYP) have been implicated in the metabolic activation of clopidogrel, CYP2C19 is generally considered to be the most important. This view is

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Figure 1. Pathways of clopidogrel metabolism. Evidence for the enzymes involved in these pathways is reviewed in the text.

supported by a considerable body of clinical evidence implicating *CYP2C19* genotype as a major determinant of active thiol metabolite exposure, antiplatelet effect, and clinical outcomes [Holmes *et al.* 2010]. A recent meta-analysis of patients treated with clopidogrel after percutaneous coronary intervention showed that those with loss-of-function *CYP2C19* alleles were at significantly increased risk of adverse cardiovascular events, particularly stent thrombosis [Mega *et al.* 2010]. Conversely, emerging data suggest that *CYP2C19**17, an allelic variant resulting in greater *CYP2C19* expression, is associated with

increased bleeding [Sibbing *et al.* 2010]. These findings are supported by a genome-wide association study that identified the *CYP2C19* locus on chromosome 10 as important in clopidogrel efficacy [Shuldiner *et al.* 2009]. Changes in *CYP2C19*-mediated activation also provide a mechanistic explanation for several pharmacokinetic drug–drug interactions with clopidogrel (e.g. omeprazole), the clinical significance of which is the subject of ongoing debate [Bates *et al.* 2011]. Based on the evidence for the effect of *CYP2C19* genotype on clopidogrel efficacy, the US Food and Drug Administration

(FDA)-approved product information for clopidogrel was revised in March 2010 to include a 'boxed warning' that highlights potential lack of response in CYP2C19 poor metabolizers.

Clinical evidence for PON1 as a determinant of clopidogrel efficacy

More recently, Bouman and colleagues reported data in *Nature Medicine* that challenge the current understanding of clopidogrel pharmacogenomics and the role of CYP in its metabolic activation [Bouman *et al.* 2011]. In a series of biochemical, clinical and epidemiological studies, paraoxonase-1 (PON1) was shown to be the rate-limiting enzyme in clopidogrel metabolic activation. In a case-cohort study of patients who underwent percutaneous coronary intervention and received clopidogrel, *PON1* QR192 heterozygotes and QQ192 homozygotes had reduced plasma PON1 activity, lower plasma concentrations of active thiol metabolite, lower platelet inhibition, and a 4- and 12-fold increased incidence of stent thrombosis respectively compared with homozygous wild-type *PON1* (RR192) patients. Statistically significant associations were not found for variant genotypes of *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A5* or the efflux transporter *ABCB1*. As part of the same report, the importance of PON1 was supported by similar findings in a prospective cohort study of almost 2000 patients with acute coronary syndromes. Despite early excitement about the potential significance of this work [Polasek, 2011; Topol and Schork, 2011], the effect of PON1 on clopidogrel efficacy found in the study by Bouman and colleagues has recently been refuted. In an equivalent case-cohort study, Sibbing and colleagues showed no difference in *PON1* Q192R genotype frequencies between stent thrombosis cases and controls, whereas the *CYP2C19**2 genotype distributions were significantly different between the groups [Sibbing *et al.* 2011]. It should also be noted that the genome-wide association study on clopidogrel efficacy did not identify single nucleotide polymorphisms surrounding or including the *PON1* locus – only the area surrounding the CYP2C enzyme cluster was associated with diminished clopidogrel response [Shuldiner *et al.* 2009].

The emerging CYP2C19 versus PON1 debate

There are now two standpoints on the metabolic activation of clopidogrel and its efficacy in the management of thrombus-related cardiovascular and cerebrovascular events – the established role

of CYP2C19 *versus* the recent data on PON1. In view of this apparent discrepancy, and while waiting for more clinical evidence, we reviewed the *in vitro* evidence for involvement of these two enzymes. *In vitro* studies are applied routinely in research and pharmaceutical industry laboratories to identify the enzymes involved in drug metabolism, a process referred to as 'reaction phenotyping' [Harper and Brassil, 2008]. This can include the following: changes to metabolism in liver microsomes from animals pretreated with enzyme-selective inactivators or inducers; correlations between the rates of metabolism with prototypic enzyme-selective activities in a panel of human liver microsomes (HLMs); the degree of inhibition by enzyme-selective inhibitors or substrates with HLMs (or hepatocytes) as the enzyme source; and the relative activities of a panel of recombinant human enzymes towards the drug. Taken together, these approaches are considered to predict with a high degree of certainty the enzyme(s) involved in the metabolism of any given compound, although the effect of CYP enzyme selective chemical inhibitors (or antibodies) is generally considered 'diagnostic' [Miners, 2002]. The remainder of this report is a review and commentary on the *in vitro* studies conducted so far to determine the enzymes involved in clopidogrel activation.

In vitro studies on the metabolic activation of clopidogrel

Studies by Sanofi

Early work on the metabolic activation of clopidogrel was conducted at Sanofi (now Sanofi-Aventis). Although the liver had been identified previously as the site of clopidogrel activation [Savi *et al.* 1992], the first study to demonstrate CYP involvement was performed in rats using a selective inactivator/inducer approach [Savi *et al.* 1994]. The *in vitro* part of this work showed that clopidogrel was metabolized more rapidly using liver microsomes prepared from rats pretreated with β -naphthoflavone, an inducer of CYP1A enzymes. Antibodies against CYP2B and CYP3A enzymes had no effect on clopidogrel metabolism, whereas those against CYP1A significantly reduced the rate of metabolism. These results, together with significantly greater inhibition of platelet aggregation in rats given clopidogrel after receiving β -naphthoflavone or 3-methylcholanthrene (another CYP1A inducer), supported the conclusion that CYP1A enzymes

are the major enzymes involved in clopidogrel activation in the rat [Savi *et al.* 1994].

Notwithstanding the importance of this first study, it should be noted that clopidogrel biotransformation was measured as the depletion of drug in incubation mixtures rather than by measuring metabolite formation. It is therefore not possible to assign particular CYP to each activation step. Another limitation of this early work is the use of rat liver microsomes (RLMs) as the enzyme source. Considerable differences exist between the expression and substrate selectivities of human and rat CYP [Nedelcheva and Gut, 1994], so caution is necessary when extrapolating such findings to humans. In particular, a role for the CYP2C subfamily could not be investigated because female rats were used and selective inhibitors and inducers of CYP2C enzymes were not available at the time [Savi *et al.* 1994]. CYP2C11, the major rat CYP2C enzyme constitutively expressed in male rats, is known to metabolize S-mephenytoin [Yasumori *et al.* 1993], a selective 'probe' substrate of CYP2C19 in humans [Wrighton *et al.* 1993].

Studies to elucidate the chemical structure of the active metabolite of clopidogrel were published a decade ago [Pereillo *et al.* 2002; Savi *et al.* 2000]. In these studies, 2-oxo-clopidogrel was incubated with HLMs to generate sufficient amounts of metabolites for analysis (note the use of HLMs for the first time with clopidogrel rather than RLMs, however CYP2C19 genotype was not reported). NADPH, a cofactor required for CYP activity but not PON activity *in vitro* [Valiyaveetil *et al.* 2011], was included in all incubations. Thus, differentiating between NADPH-dependent (CYP) and NADPH-independent metabolism (potentially PON) in the second activation step of clopidogrel is not possible based on these data [Pereillo *et al.* 2002; Savi *et al.* 2000]. This is not a criticism, however, since these studies were designed to identify chemical structure rather than the enzymes involved in active metabolite formation. Interestingly though, in proposing the now familiar scheme of sequential clopidogrel activation (Figure 1), the authors refer only to the first step as CYP-dependent, while the second step is simply referred to as hydrolysis [Savi *et al.* 2000]. Experiments in the absence of NADPH were not reported, so exactly why the second step was not considered to be dependent on CYP activity is unclear.

Implicating CYP3A rather than CYP1A

In response to clinical reports that CYP3A inhibitors and inducers could alter the antiplatelet activity of clopidogrel [Lau *et al.* 2003, 2000], Clarke and Waskell (2003) used several *in vitro* test systems in an attempt to identify the CYP enzymes involved in its activation. These included the use of RLMs from animals pretreated with dexamethasone (CYP3A inducer) or β -naphthoflavone (CYP1A inducer) and microsomes from baculovirus-infected insect cells or human β -lymphoblast cells that were engineered to express individual recombinant human CYP enzymes. In experiments that quantified the depletion of clopidogrel in incubation mixtures, results with RLMs contradicted those previously reported by Savi and colleagues using the same approach [Savi *et al.* 1994]. Compared with controls, the rate of clopidogrel metabolism was increased by dexamethasone pretreatment and decreased by β -naphthoflavone pretreatment, suggesting a role for CYP3A but not CYP1A [Clarke and Waskell, 2003]. The reasons for the conflicting data between the two studies are not clear, but may involve the use of different rats (female Sprague Dawley *versus* male Fisher, respectively) or disparate dosing regimens of the CYP inducers. In screening experiments using insect or human B lymphoblast cells as the CYP enzyme source, Clarke and Waskell showed that CYP3A4 and CYP3A5 produced greatest depletion of substrate [Clarke and Waskell, 2003]. Minor, albeit statistically significant, depletion was also observed for CYP1A2 and CYP2B6. CYP1A1, CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 essentially lacked activity towards clopidogrel. Quantification of clopidogrel depletion by individual CYP in this setting provides useful information on the enzymes involved in the first activation step. However, rates of clopidogrel depletion normalized for the P450 content of enzyme preparations (e.g. $\eta\text{mol/min}/\eta\text{mol P450}$) were provided only for CYP1A2, CYP2B6, CYP3A4 and CYP3A5, meaning it is not possible to compare specific activities for all the CYP enzymes investigated. Data are further complicated by the use of multiple expression systems that provide different P450 to NADPH cytochrome P450 oxidoreductase (CPR) ratios. Furthermore, as noted previously, studying the depletion of clopidogrel *in vitro* provides no insight into the conversion of 2-oxo-clopidogrel to the active thiol metabolite.

Studies by Daiichi Sankyo and Eli Lilly

The first *in vitro* data showing CYP2C19 involvement in the metabolic activation of clopidogrel appear to have been published in abstract form by Kurihara and colleagues [Kurihara *et al.* 2005]. This was followed by a presentation at the American Heart Association Scientific Sessions in November 2008 [Hagihara *et al.* 2008]. Both abstracts are from the laboratories of Daiichi Sankyo and Eli Lilly, the manufacturers of the antiplatelet drug prasugrel. Full reports containing these data were not published until after prasugrel was approved by the US FDA in July 2009 [Kazui *et al.* 2010; Hagihara *et al.* 2009]. The advances made by these studies result from accurate quantification of clopidogrel's metabolites, made possible by superior analytical approaches.

Hagihara and colleagues performed correlation studies to investigate the relationships between human liver microsomal enzyme activities and the first step in clopidogrel activation [Hagihara *et al.* 2009]. Microsomal preparations from 20 human livers (two had no CYP2C19 activity) were used, and data expressed as the mean of individual results. Initial experiments measured the depletion of clopidogrel in incubation mixtures. These were followed by experiments to estimate the formation rate of 2-oxo-clopidogrel, taken as the sum of the formation rates for 2-oxo-clopidogrel, 2-oxo-clopidogrel-carboxylic acid, and active thiol metabolite (this approach takes into account the sequential metabolism of 2-oxo-clopidogrel; Figure 1). Not surprisingly, the depletion of clopidogrel in incubations of HLMs was best correlated with CES1 activity ($R^2=0.4028$). However, with the exception of CYP2D6, associations were also found for all CYP enzymes tested. Notably, the R^2 for CYP2C19 was 0.0938. Unfortunately, the relationships between estimated 2-oxo-clopidogrel formation rate and CYP activities in HLMs were shown only for CYP2B6, CYP1A2 and CYP2C19 (Figure 6 in the original publication; CYP2C19 $R^2=0.1300$). Relationships for the other CYP enzymes implicated in clopidogrel activation were not reported. For example, after CES1, CYP2C9 activity was most strongly correlated with clopidogrel depletion in the initial experiments but no data on CYP2C9 activity and 2-oxo-clopidogrel formation rate were provided. The explanation offered for this was based on the investigator's previous abstract indicating that of a panel of 11 recombinant CYP enzymes

only CYP2B6, CYP1A2 and CYP2C19 were capable of generating 2-oxo-clopidogrel [Kurihara *et al.* 2005] (later published by Kazui and colleagues, see below [Kazui *et al.* 2010]). This may have obscured the complete picture of the CYP enzymes involved in clopidogrel activation by HLMs. Based on these data alone, it is also difficult to be convinced of a major role for CYP2C19 when the correlations with clopidogrel depletion and 2-oxo-clopidogrel formation rate in HLMs were so weak, with R^2 values of 0.0938 and 0.1300, respectively.

The same research team later published a more comprehensive analysis of clopidogrel activation [Kazui *et al.* 2010]. Studies were conducted to determine the kinetic constants (K_m and V_{max}) for metabolite formation using baculovirus-infected insect cells (Supersomes) as the recombinant CYP enzyme source. This approach is considered more informative than measuring the depletion of the drug alone, since it allows for the *in vitro* intrinsic clearance ($CL_{int} = V_{max}/K_m$) of each metabolic pathway to be determined. The fraction of metabolite formed by individual enzymes (fm) can then be estimated. As described above for the corresponding abstract [Kurihara *et al.* 2005], only Supersomes expressing CYP2B6, CYP1A2 and CYP2C19 were able to form 2-oxo-clopidogrel, with estimated fm_{CYP} values of 0.19, 0.36 and 0.45, respectively. Here, involvement of CYP1A2 and CYP2B6 was consistent with data from Clarke and Waskell [Clarke and Waskell, 2003], whereas the lack of CYP3A4 involvement but detection of CYP2C19 activity was different. These anomalies may arise from the different experimental approaches; substrate depletion *versus* metabolite formation. Another contributing factor may be the short incubation time (1 min) employed by Kazui and colleagues, which may possibly avoid 'knocking out' CYP2C19 through mechanism-based inactivation [Nishiya *et al.* 2009]. In the experiments using 2-oxo-clopidogrel as substrate, CYP2C9, CYP2C19, CYP2B6 and CYP3A4 formed the active thiol metabolite using Supersomes or human β -lymphoblast cells as the CYP enzyme source. The kinetic analysis with Supersomes gave respective fm_{CYP} values of 0.07, 0.20, 0.33 and 0.40. To investigate the CYP involved in clopidogrel activation in HLMs (pooled HLMs from 10 donors with no information on CYP2C19 activity), CYP-selective monoclonal antibodies and chemical inhibitors were employed. The level of inhibition in the presence

of these inhibitors corresponded well to the fm_{CYP} values from the kinetic experiments with recombinant CYP. This provides considerably more robust evidence for the involvement of CYP2C19 than the previous correlation results in HLMs [Hagihara *et al.* 2009]. However, the shortcoming of these data is the failure to use a full range of CYP-selective chemical inhibitors, the standard approach [Elliot *et al.* 2007] – only inhibitors for the CYP enzymes identified in the recombinant experiments were tested. Thus, the role of other CYP enzymes in the metabolic activation of clopidogrel in HLMs may potentially have been overlooked.

Identification of sulfenic acid intermediates

Mansuy and colleagues studied the second step of clopidogrel activation to determine whether reactive intermediates could be formed during the cleavage of the thioester – that is, cleavage of the CO-S bond of 2-oxo-clopidogrel [Dansette *et al.* 2009]. This was done to provide a more detailed mechanism for the formation of active thiol metabolite. Potential formation of reactive electrophilic intermediates also has toxicological consequences, since they can react with protein nucleophiles with possible formation of protein adducts. Incubations of 2-oxo-clopidogrel with RLMs or HLMs in the presence of dimedone (a C-nucleophile used as a trapping agent) resulted in the formation of a dimedone-adduct consistent with the trapping of a sulfenic acid intermediate. This was NADPH-dependent, almost completely abolished by nonselective CYP inhibitors, and readily carried out by baculovirus/insect cells expressing the major drug metabolizing CYP enzymes (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5). Importantly, the sulfenic acid intermediate of clopidogrel was shown to react with thiol nucleophiles (e.g. glutathione) to form mixed dithioesters that subsequently generate the active thiol metabolite. While this confirms that CYP can be involved in the second activation step of clopidogrel, NADPH dependence was demonstrated only for the generation of the sulfenic acid intermediate rather than the active thiol metabolite itself – that is, the initial reaction in the second activation step to form intermediate but not the subsequent reaction with thiol nucleophiles. It is therefore not possible from these data to exclude a NADPH-independent direct pathway for active metabolite formation (e.g. non-CYP).

Experiments published by Bouman and colleagues in 2011

The *in vitro* experiments conducted by Bouman and colleagues in support of their clinical studies used recombinant enzymes expressed in a human embryonic kidney cell line (HEK293), HLMs (presumably pooled) and human serum as the enzyme sources [Boumann *et al.* 2011]. Measuring the kinetics of metabolite formation across a panel of 34 recombinant enzymes, the conversion of clopidogrel to 2-oxo-clopidogrel was catalysed by CYP with the rank order CYP3A4 > CYP3A5 > CYP2B6 > CYP1A2 > CYP1A1 ~ CYP2E1 ~ CYP2A6. In contrast to Kazui and colleagues, recombinant CYP2C19 lacked activity toward clopidogrel. Using the same panel of recombinant enzymes, only PON1 and PON3 catalysed the metabolism of 2-oxo-clopidogrel to the active thiol metabolite. This finding is very intriguing given the previous *in vitro* studies (described above) showing the involvement of various CYP in the second activation step. However, consistent with their reaction phenotyping data, Bouman and colleagues also showed that selective inhibition of CYP2C19 in HLMs had no effect on the rates of clopidogrel metabolite formation, whereas selective inhibition of CYP3A and PON1 inhibited the first and second activation steps, respectively. Although the data are not shown in the paper, incubations of 2-oxo-clopidogrel with HLMs in the presence of NADPH generated active thiol metabolite at the same rate as incubations conducted in the absence of NADPH (see caption to supplementary Figure 3 of Bouman and colleagues' article) [Bouman *et al.* 2011]. Taken together, these results suggest that CYP are not involved in the second activation step of clopidogrel. Although some disparity between *in vitro* test systems may be expected, the exact reasons why these new data contradict some previous work (e.g. Kazui and colleagues) are unclear.

Despite this, there are a number of strengths and limitations of the *in vitro* studies by Bouman and colleagues that should be noted. Experiments controlled for known limitations of the experimental system in several ways. Suitable 'probe' substrates were included as positive controls for each recombinant enzyme during activity screening. Also, for enzymes showing no catalytic activity toward clopidogrel or 2-oxo-clopidogrel, negative results were confirmed by measuring the recovery of substrate after extended incubations (up to 120 min) at high substrate

concentrations (>95% recovery in all cases). Although expression of CYP enzymes was assessed by quantifying mRNA relative to GADPH, this does not provide a measure of holo-P450 (i.e. active CYP protein) and hence comparison of catalytic efficiencies (as $V_{\max}/K_m$) and *in vitro-in vivo* scaling is not valid. Similarly, variability in the P450 to CPR ratios appears not to have been assessed. Another potential weakness of this work is the inhibition of carboxylesterases and acetyl- and butyr-cholinesterase in the HLM studies, done presumably to increase the amount of clopidogrel available for metabolic activation—that is, shutting down the horizontal pathways in Figure 1. It is unclear what impact, if any, this approach may have on the key finding of CYP independence in the second activation step.

Conclusions

Early *in vitro* studies implicate CYP1A2 and CYP3A4 but not CYP2C19 in the metabolic activation of clopidogrel. These studies do not differentiate between the two activation steps. The role of CYP2C19 in clopidogrel metabolic activation *in vitro* appears only to have been demonstrated by the manufacturers of prasugrel. Correlation studies in HLMs provide weak evidence for CYP2C19 in the first activation step. However, studies using recombinant CYP enzymes and CYP-selective inhibitors/antibodies indicate that CYP2C19 may catalyse approximately 45% and 20% of the first and second steps of the activation pathway, respectively. This is not supported using HEK293 cells as the enzyme source, where CYP2C19 exhibited no activity towards clopidogrel or 2-oxo-clopidogrel. Thus, agreement between *in vitro* studies exists only for the involvement of CYP1A2 and CYP2B6 in the first step of clopidogrel activation. All major drug-metabolizing CYP appear able to convert 2-oxo-clopidogrel to sulfenic acid intermediates that subsequently form the active thiol metabolite. However, the exact role of CYP in the second step of clopidogrel activation is still unclear, since recent evidence shows CYP-independent hydrolytic cleavage of the thioester bond by the esterases PON1 and PON3 [Bouman *et al.* 2011].

Despite the apparently conflicting *in vitro* data, it should be emphasized that the relationship between CYP2C19 genotype and clopidogrel response has been demonstrated in many clinical studies. With the patent for clopidogrel expiring,

more patients in developing countries are likely to be treated, possibly revealing even wider interindividual variability in response, while others in affiliate societies may be treated unnecessarily with more expensive alternative drugs. While the data of Kazui and colleagues strongly support a role for CYP2C19 in 2-oxo-clopidogrel formation and its subsequent activation [Kazui *et al.* 2010], other studies suggest CYP2C19 may not contribute to clopidogrel metabolic activation. In this regard, the data from Bouman and colleagues are particularly noteworthy [Bouman *et al.* 2011]. As the CYP2C19 *versus* PON1 story continues to unfold, further *in vitro* studies using a range of test systems may be warranted to confirm the enzymes involved in clopidogrel metabolic activation.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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