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Dissecting the Activation of Thienopyridines by Cytochromes P450 Using a Pharmacodynamic Assay In Vitro

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ABSTRACT

The thienopyridine antiplatelet drugs, such as ticlopidine, clopidogrel, and prasugrel, require activation by cytochromes P450 in vivo to effectively block platelet aggregation. The study of the metabolic activation of these compounds has been hampered by the lability and reactivity of the ring-opened active metabolite (AM) and by the numerous metabolites that can be formed in such a transformation. We have developed a novel method whereby platelets are incubated with the cytochrome P450 and the thienopyridine of interest for various amounts of time, and the effects on ADP-driven platelet aggregation are directly examined. In this way, the platelet is used as a biosensor for detection of the AM. Using this method, cytochromes P450 capable of converting clopidogrel, prasugrel, and 2-oxo-clopidogrel to metabolites that inhibit ADP-induced platelet aggregation were identified as well as which cytochromes P450 were capable of catalyzing partial reactions (e.g., conversion of 2-oxo-clopidogrel to the AM). These studies show that, in vitro, CYP3A4/5, 2C19, and 2B6 are individually capable of converting clopidogrel and prasugrel to the AM and that the cytochrome P450 preference for these two thienopyridines is very similar.

Introduction

The thienopyridine prodrugs, clopidogrel (Plavix), ticlopidine (Ticlid), and prasugrel (Effient), inhibit platelet aggregation induced by ADP. As such, these agents have been successful in prevention of the incidence of subacute thrombosis in patients who received stents and as secondary treatment of cerebrovascular disease (CAPRIE Steering Committee, 1996). These antiplatelet drugs require metabolic activation in vivo to effectively block ADP-driven platelet aggregation through the $P2Y_{12}$ receptor (Savi et al., 1994, 2001). For clopidogrel, the first step involves the oxidation of the thienopyridine ring to form an oxo intermediate methyl 2-(2-chlorophenyl)-2-(2 oxo-7,7a-dihydrothieno[3,2-*c*]pyridin-5(2*H*,4*H*,6*H*)-yl) acetate (SR-121683), which then undergoes a ring-opening reaction (Savi et al., 2000) to form several isomers of the active metabolite 2-{1-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfanyl-3-piperidinylidene) acetic acid (SR-25552) (AM) (Savi et al., 2000; Pereillo et al., 2002) in the second step. Both of these reactions have been shown to be catalyzed by various cytochromes P450. The AM is a covalent modifier of the $P2Y_{12}$ receptor on platelets (Savi et al., 2006), and different isomers have different affinities for the receptor (Savi et al., 2000; Pereillo et al., 2002). Another major metabolic transformation for clopidogrel in vivo involves the removal of the methyl ester to form clopidogrel carboxylic acid, (*R*)-2-(2-chlorphenyl)-2-(6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl) acetic acid (SR-26344) (Herbert et al., 1993; Caplain et al., 1999). SR-26334 is inactive as a $P2Y_{12}$ antagonist either directly or after exposure to cytochromes P450. Whether this is because SR-26334 is a poor substrate for cytochrome P450 oxidation or because the ring-opened version of the carboxylic acid is a poor antagonist for the platelet $P2Y_{12}$ receptor is unclear, although recent work suggests the former to be the case (Zahno et al., 2010). Metabolic activation of prasugrel, a relatively new choice for antiplatelet therapy, follows a somewhat similar pathway, which involves cleavage of the ester linkage to form the oxo intermediate [2-[2-oxo-6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl]-1-cyclopropyl-2-(2-fluorophenyl)ethanone] (R-95913) followed by ring opening to the AM, 2-[1–2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-mercapto-3-piperidinylidene acetic

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ABBREVIATIONS: SR-121683, methyl 2-(2-chlorophenyl)-2-(2 oxo-7,7a-dihydrothieno[3,2-*c*]pyridin-5(2*H*,4*H*,6*H*)-yl) acetate; AM, active metabolite; SR-26334, (*R*)-2-(2-chlorphenyl)-2-(6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl) acetic acid; SR-25552, 2-{1-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfanyl-3-piperidinylidene) acetic acid; R-95913, 2-[2-oxo-6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl]-1-cyclopropyl-2-(2-fluorophenyl) ethanone; R-138727, 2-[1–2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-mercapto-3-piperidinylidene acetic acid; LC, liquid chromatography; MS, mass spectrometry; PGE_1, prostaglandin E_1; 2MeSADP, 2-methylthioadneosine diphosphate; PRP, platelet-rich plasma; IPA, inhibition of platelet aggregation; %IPA, percentage inhibition of platelet aggregation; max %IPA, maximum percentage inhibition of PA observed after a fixed time of preincubation of platelets/P450/thienopyridine before addition of 2MeSADP (usually 25 min); PA, platelet aggregation.

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acid (R-138727) (Sugidachi et al., 2001; Hasegawa et al., 2005). A number of esterases have been shown to catalyze the ester hydrolysis (Williams et al., 2008) although cytochromes P450 could also potentially catalyze this reaction.

The study of the activation of these compounds has been hampered by the lability and reactivity of the ring-opened AM as well as by the multitude of metabolites that can be formed in vivo and in in vitro biotransformation reactions. Early studies with clopidogrel relied on the disappearance of the parent drug as an indicator of biotransformation by a particular cytochrome P450. In this way, CYP3A4 was implicated as the major cytochrome P450 responsible for bioactivation with lesser amounts of metabolic activation occurring with CYP1A2, 2B6, and 2C19 (Clarke and Waskell, 2003). However, observing the disappearance of the parent compound is not reliable when a large portion of it is converted to the acid. In vitro inhibition studies have implicated CYP2B6, 2C9, and 2C19 (Richter et al., 2004; Turpeinen et al., 2005). Although these studies indicate an interaction between cytochrome P450 and clopidogrel, the formation of the AM is not a necessary outcome of the interaction.

Polymorphisms in CYP2C19 identified through genotyping studies have supported a key role for CYP2C19 in clopidogrel activation and antiplatelet efficacy, but the existing clinical data suggest that CYP2C19 is not the only cytochrome P450 important for metabolic activation (Hulot et al., 2006; Brandt et al., 2007; Kim et al., 2008a,b; Collet et al., 2009; Mega et al., 2009; Simon et al., 2009). The development of better analytical methods for detecting the AMs of both clopidogrel and prasugrel has helped delineate which cytochromes P450 are important in thienopyridine activation. A LC-MS method for measuring the AM, which allows for determination of the concentration of the active species in plasma, has been developed (Farid et al., 2007). However, the interpretation of these results is complicated by the fact that multiple isomers are produced, and each has different affinities against the $P2Y_{12}$ receptor (Savi et al., 2000; Pereillo et al., 2002; Hasegawa et al., 2005). The recent synthesis of all four active metabolites has allowed the development of improved analytical methods that allow detection and quantitation of active metabolites in human plasma (Tuffal et al., 2011).

In this study, we sought to bypass these complications by coupling the pharmacodynamic readout of ADP-driven platelet aggregation to the in situ generation of the AM using purified cDNA expressed Baculosomes of individual cytochromes P450s. Using this assay, we identified which cytochromes P450s are capable of generating the AM from a given thienopyridine by virtue of monitoring the change in ADP-driven platelet aggregation over time.

Materials and Methods

Reagents. Prostaglandin E_1 (PGE_1), 2-methylthio-ADP (2MeSADP), ketoconazole, $NADP^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, potato apyrase grade I, and all other reagents were from Sigma-Aldrich (St. Louis, MO) unless specified. ADP was from Bio/Data Corp. (Horsham, PA), human α-thrombin was from Hematologic Technologies Inc. (Essex Junction, VT), and human fibrinogen was from Calbiochem (San Diego, CA). A stock solution of human fibrinogen at 10 mg/ml was prepared in saline and stored at −80°C in small aliquots. Vivid cytochrome P450 Baculosome screening kits, control Baculosomes, and CYP3A4/5 antibody were from Invitrogen (Carlsbad, CA). The specific activities for the cytochromes P450s used in this study, namely, CYP3A4, 2B6, 2C9, 2C19, 2D6, and 3A5 were 15.5, 0.29, 5.6, 0.84, 2.5, and 5.1 nmol · min^{-1} · mg $protein^{-1}$ (11β-hydroxytestosterone, *S*-mephenytoin, diclofenac, *S*-mephenytoin, bufuralol, and 11β-hydroxytestosterone), respectively. Clopidogrel bisulfate and 2-oxo-clopidogrel were supplied by sanofi-aventis (Bridgewater, NJ). Prasugrel was synthesized at Bristol-Myers Squibb Company (Pennington, NJ).

Preparation of Platelet Rich-Plasma. All procedures were performed at room temperature unless otherwise indicated. On the day of the experiment, 30 ml of blood was drawn from human volunteers, who gave informed consent and had been medication-free for 10 days, using venipuncture into syringes containing ACD solution (85 mM sodium citrate, 78 mM citric acid, and 110 mM D-glucose, pH 4.4). The blood/anticoagulant ratio was 6:1, respectively. PRP was prepared by centrifugation of the whole blood with 0.3 unit/ml potato apyrase grade I at 950 rpm for 12 min.

Preparation of Washed Platelets for 2-MeSADP-Induced Aggregation Measurements. PGE1 was added to the PRP to achieve a final concentration of 100 nM. The PRP was centrifuged at 2200 rpm for 6 min. The supernatant was removed, and 10 ml of modified Tyrode's buffer (10 mM Hepes, 150 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, and 0.3% human serum albumin, pH 6.8) warmed to 37°C containing 100 nM PGE1 and 0.3 unit/ml apyrase was used to resuspend the platelets. The sample was centrifuged at 2200 rpm for 6 min, and the supernatant was removed. Platelets were resuspended in modified Tyrode's buffer containing 2 mM calcium chloride and 1 mg/ml human fibrinogen so that the platelet count was between 150,000 and 350,000 platelets/μl. Platelet count was determined using a scil Vet abc Analyzer (scil Animal Care Company, Grayslake, IL). The washed platelets were maintained at 37°C until use.

Preparation of Cytochrome P450 Mixture. To prepare the cytochrome P450 mixture, the following solutions were added to a 1-ml Eppendorf tube in succession: 120 to 480 μl of cytochrome P450 Baculosomes or wild-type control Baculosomes (1 nmol/ml, stock concentration), 84 μl of 10 mM $NADP^+$ and 54 μl of regeneration system (333 mM glucose 6-phosphate and 40 U/ml glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate buffer, pH 8.0), and the appropriate amount of 20 mM Hepes (pH 8.0) buffer to bring the final volume to 760 μl. The samples were mixed well and incubated at 37°C for 10 min. This is enough for six reaction vials of washed platelets.

Incubation of Washed Platelets with Human Cytochrome P450 Baculosomes and Thienopyridine. Washed platelets (870 μl) were pipetted into separate 1-ml Eppendorf tubes. After the addition of 10 μl of thienopyridine stock solution, the contents of the tube were mixed by inversion. Generation of the AM was commenced by the addition of 120 μl of the cytochrome P450 mixture (as prepared above) to the tube containing platelets and thienopyridine. The final concentration of cytochrome P450 ranged from 20 to 80 nM, and the final concentration of $NADP^+$ was 0.1 mM in these reactions. At fixed time points after the addition of cytochrome P450 to the washed platelet suspension, 140 μl of the reaction mixture was pipetted into the wells of a clear bottom microtiter plate (Nalge Nunc International, Rochester, NY) followed by the immediate addition of 10 μl of 15 μM 2 MeSADP (final concentration 1 μM) to start the aggregation reaction. Platelet aggregation was measured by reading the optical density kinetically at 595 nm in a preheated (37°C) plate reader (SpectraMax 250; Molecular Devices, Sunnyvale, CA). The plate was mixed for 6 s before the first read, and then the plate was read every 13 s for 3 min with mixing between each reading. The slope for the linear portion of the aggregation time course was used to calculate the rate of platelet aggregation. The percentage inhibition of platelet aggregation (%IPA) was calculated using eq. 1:

$$\%\text{IPA} = (1 - (R_{rxn}/R_{ctrl})) \times 100 \quad (1)$$

where R_{rxn} equals the slope of the aggregation assay containing platelets treated with thienopyridine and active cytochrome P450

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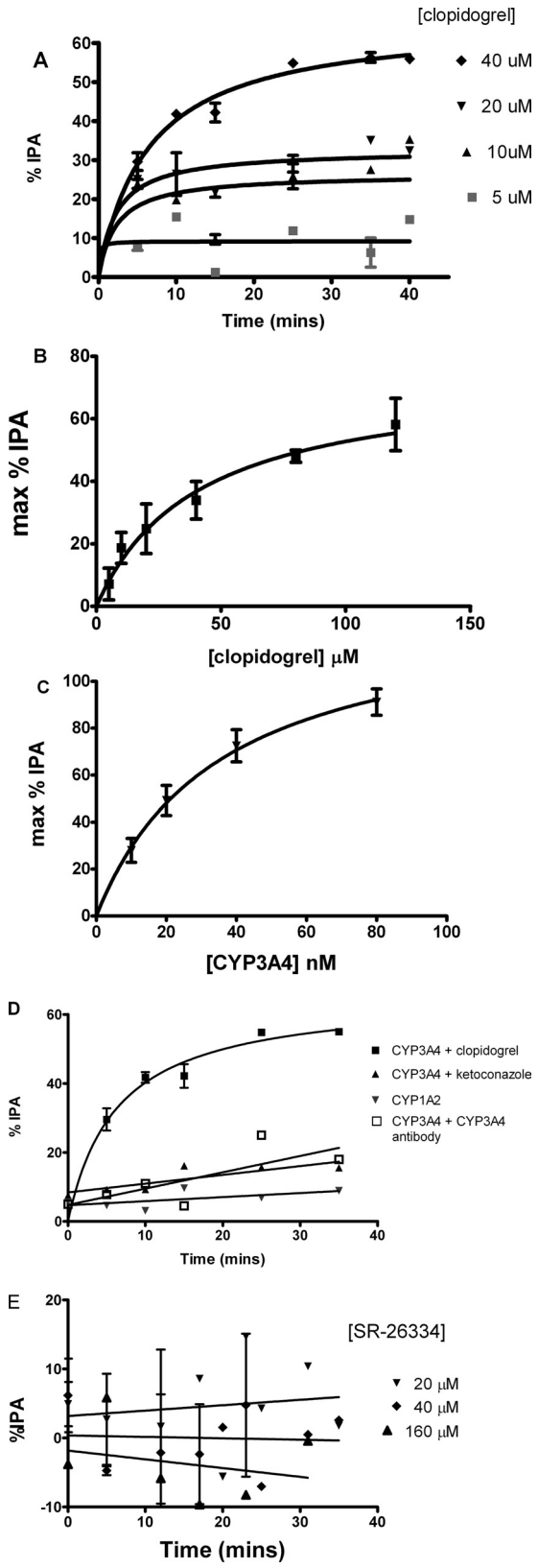

Fig. 1. Assay validation experiments. A, washed human platelets were incubated with 20 nM CYP3A4 and varying amounts of clopidogrel as indicated. Aliquots were removed at various time points and the extent of ADP-driven PA aggregation was measured upon the addition of 2 Me-SADP to a final concentration of 1 μM. The %IPA was calculated compared with a control (lacking either clopidogrel or the P450) using eq. 1 and is plotted versus time for each concentration of clopidogrel. When the clopidogrel concentration was 40 or 20 μM, the data fit well to eq. 2 with R^2 values of 0.96 and 0.87, respectively. Lower concentrations of clopidogrel show the max %IPA achieved at those concentrations. B, a replot of the data from A shows that the max %IPA achieved is dependent on the concentration of clopidogrel and fit eq. 2 with an R^2 of 0.83. Error bars represent S.D. on the mean. A minimum of three replicates were used for each data point. C, washed platelets were incubated with 40 μM clopidogrel and various concentrations of CYP3A4 as indicated. The max %IPA observed at 25 min was plotted versus concentration of CYP3A4. Error bars represent S.D. of the mean. A minimum of three replicates were used for each determination. R^2 for the fit to eq. 2 is 0.8. D, washed platelets were incubated with 40 μM clopidogrel and 20 nM CYP3A4 either in the presence or absence of 4 μM ketoconazole or the antibody against CYP3A4. For comparison purposes, this figure also contains the results of experiments in which platelets were incubated with 20 nM CYP1A2 and 40 μM clopidogrel. Only the results with active CYP3A4 fit to eq. 2 with and R^2 of 0.95. All other data on this graph were fit to a straight line for illustrative purposes. E, washed platelets were incubated with 20 nM CYP3A4 and various concentrations of the inactive metabolite of clopidogrel, SR-26334, as indicated. The effect on ADP-driven PA was measured and plotted versus the incubation time. No change in %IPA observed was measured even at high concentrations (up to 160 μM) SR-26334. Data were fit to a straight line for illustrative purposes only.

and R_{ctrl} equals the slope of the aggregation assay containing platelets treated with wild-type Baculosomes (which do not contain active P450) and thienopyridine. When ADP was used as the agonist, 10 μl of 150 μM ADP was added to 140 μl of reaction mixture to start the aggregation assay. For aggregation assays in which thrombin was used as an agonist, no fibrinogen was included in the suspension buffer, and the final concentration of human α-thrombin was 1 nM. Max %IPA was calculated in a similar manner using the %IPA observed after a 25-min incubation.

Data Handling. All data were fit using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Error bars in plots are S.D. of the mean of a minimum of three replicates. Data were fit to either a straight line or to a hyperbolic model shown in eq. 2:

$$Y = (A \cdot x)/(B + x) \quad (2)$$

where Y equals the max %IPA achieved at a given time and x equals the concentration of either the thienopyridine or cytochrome P450 used in the experiment. Except where indicated by R^2 values, curve-fitting was for illustrative purposes only. No interpretation was assigned to the values of the constants (A or B) from eq. 2. Data contained in any single plot was from experiments using the same platelets and the same cytochrome P450 reaction mixture.

Results

Assay Validation. The available literature data at the time these studies were initiated, although somewhat conflicting, had consistently indicated that CYP3A4 was capable of generating the AM of clopidogrel. To confirm this and to demonstrate the utility of the assay, we first studied the effect of clopidogrel and CYP3A4 on the inhibition of platelet aggregation under various conditions. Figure 1A shows the time course of a reaction using 20 nM CYP3A4 and varying amounts of clopidogrel. The %IPA increased with incubation time at a particular clopidogrel concentration, and the max %IPA achieved was dependent on the total concentration of clopidogrel present as replotted in Fig. 1B. The max %IPA was achieved after 25 min of incubation. A similar plot was generated when the experiment was repeated at a fixed level of clopidogrel and the amount of CYP3A4 was varied (Fig. 1C). A plot of max %IPA versus concentration of CYP3A4 showed a hyperbolic dependence of the max %IPA on the enzyme concentration. Thus, as might be expected for the

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enzymatic generation of the AM, the reaction showed a dependence on incubation time, substrate concentration, and enzyme concentration.

To further demonstrate that the reaction was dependent on CYP3A4 activity, the experiment was repeated in the presence and absence of the CYP3A4 inhibitor, ketoconazole. The results summarized in Fig. 1D show only a minimal affect on the %IPA when the reaction was conducted in the presence of ketoconazole, indicating that the change in IPA is dependent on CYP3A4 activity. Experiments conducted in the presence of 20 μg of neutralizing CYP3A4 antibody gave results similar to those conducted in the presence of ketoconazole. Inclusion of an antibody against CYP2B6 in the reaction with CYP3A4 and clopidogrel had no effect on the reaction (data not shown), indicating that the antibody effect is specific to CYP3A4. When 20 to 60 nM CYP1A2 was used, no significant change in %IPA was observed, indicating that not all cytochromes P450 are capable of catalyzing the reaction.

Figure 1E shows the results of a reaction that contained various concentrations of the inactive metabolite SR-26334 and CYP3A4. Even when the CYP3A4 concentration was raised to 80 nM and the SR-26334 concentration was increased to 160 μM, no change in %IPA was observed, indicating that the de-esterified form of clopidogrel is not converted to an AM, which can inhibit ADP-driven PA.

Results similar to those shown in Fig. 1 were obtained when ADP was used as the agonist instead of 2Me-SADP. No effect on PA was seen when thrombin was used as the agonist, indicating that the reaction products of clopidogrel and CYP3A4 are specific for ADP-driven platelet aggregation.

Reactions with Various Cytochromes P450. Once suitable reaction conditions were established, the assay was used to survey the activity of a variety of cytochrome P450 subtypes. Figure 2A shows that CYP3A4, 3A5, 2B6, and 2C9, when incubated with platelets and varying concentrations of clopidogrel, can inhibit PA to varying degrees. Each of the cytochromes P450 achieved a slightly different max %IPA at 25 min of incubation. To examine this result further, the experiment was repeated with the concentration of clopidogrel being held constant and the amount of each enzyme varied. The results shown in Fig. 2B indicate that when more enzyme was added, 100% IPA could be reached in the reaction with CYP3A4, 3A5, and 2B6. The IPA is linear with respect to CYP2C19 concentration but does not reach 100% even at very high concentrations of CYP2C19 (75 nM). In addition to the enzymes indicated in Fig. 2, CYP1A2, 2C9, and 2D6 were also tested (data not shown). Even at a high concentration of enzyme and clopidogrel (up to 80 nM and 120 μM, respectively), no change in PA was observed using these enzymes, indicating that they do not convert clopidogrel to the AM in this system.

The conversion of clopidogrel to the AM is a two-step reaction, and because we had access to the 2-oxo intermediate, we wanted to investigate whether there were some cytochromes P450 that could convert the oxo form to AM but not the parent compound. For these experiments, we examined the cytochromes P450, which did not have an effect on PA when incubated with clopidogrel and platelets. Figure 3 shows the results of these experiments. When various amounts of CYP2C9 were incubated with 40 μM 2 oxo-clopidogrel, time-dependent inhibition of platelet aggregation was observed, particularly at the 60 and 80 nM concentrations of enzyme.

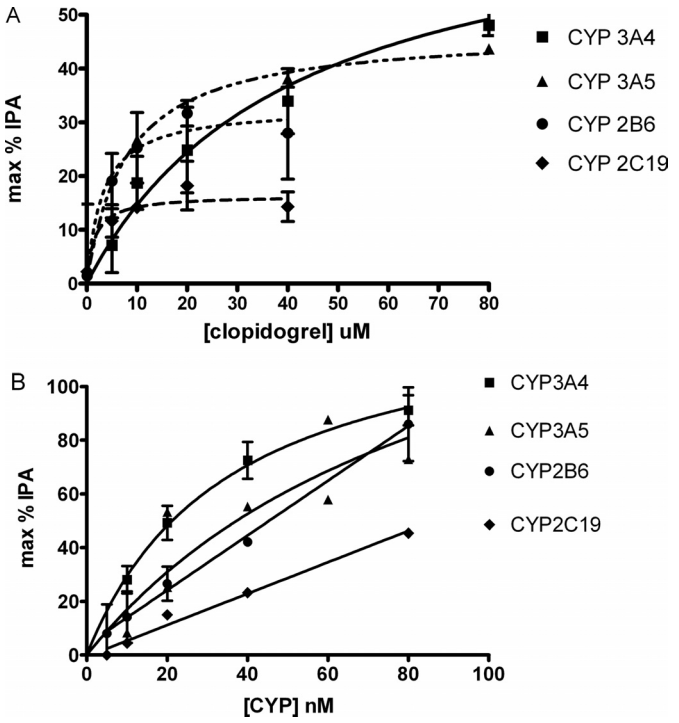

Fig. 2. The effect on ADP-driven PA by activation of clopidogrel by various cytochromes P450. A, washed human platelets were incubated with various amounts of clopidogrel and 20 nM CYP3A4, 3A5, 2B6, or 2C19. The dependence of the cytochrome P450 subtype and clopidogrel concentration on max %IPA observed is plotted. B, washed human platelets were incubated with 40 μM clopidogrel and varying concentrations of a particular cytochrome P450 subtype which showed activity in Fig. 3A. Data from experiments with CYP3A4 and CYP3A5 fit well to eq. 2 with R^2 values of 0.86. Data from experiments using CYP2B6 and CYP2C19 were fit to a straight line. The total max %IPA achieved with CYP3A4, 3A5, and 2B6 was similar; only experiments using CYP2C19 failed to reach >80% IPA.

The max %IPA varied with concentration of CYP2C9 such that max %IPA varied from 14 to 54% over the range of 40 to 80 nM CYP2C9. This result is in contrast to that observed with 80 nM CYP2C9 and 40 μM clopidogrel for which little to no inhibition of platelet aggregation was observed (max %IPA observed was 7%). In the experiments with CYP2D6 and 2 oxo-clopidogrel, time-dependent inhibition of platelet aggregation was observed at the higher levels of enzyme although the inhibition observed was the least robust of all the enzymes tested (Fig. 3B) with the max %IPA reached being 53% with 80 nM CYP2D6 and 40 μM 2 oxo-clopidogrel compared with 31% IPA with 80 nM CYP2D6 and 40 μM clopidogrel. The results with both of these enzymes indicate that 2C9 and 2D6, although poor catalysts for the production of the oxo-clopidogrel, can catalyze the conversion of the oxo intermediate to the AM. Experiments with oxo-clopidogrel and CYP1A2 did not result in inhibition of ADP-induced platelet aggregation, indicating that CYP1A2 did not catalyze the conversion of the oxo intermediate to the AM in this system. These results suggest that if any of the oxo intermediate is released by the major metabolite-producing enzymes (e.g., CYP3A4/5, 2B6, and 2C19) this product could be converted to AM not only by the main metabolizing enzymes but also by CYP2C9 and, to a lesser degree, by CYP2D6.

Experiments similar to those described above were also conducted using prasugrel. Figure 4 shows the extent of IPA achieved at different concentrations of prasugrel incubated

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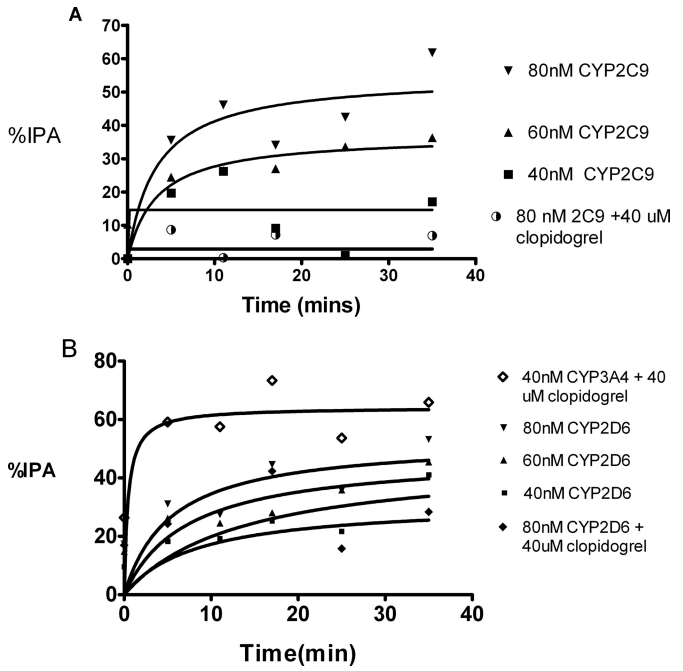

Fig. 3. The effect of incubating of 2-oxo-clopidogrel with platelets and various cytochromes P450 on the %IPA. The results overlaid on these two graphs were from experiments conducted on the same day using the same batch of washed platelets. Data were fit to either eq. 2 or a straight line for illustrative purposes. A, washed platelets were incubated with 40 μM 2-oxo-clopidogrel and various amounts of CYP2C9. The effect on %IPA with time was measured in the normal fashion. As a point of comparison, this plot also includes data from a reaction whereby 80 nM CYP2C9 and 40 μM clopidogrel were used in the incubation. B, experiment similar to that in A except that CYP2D6 was used as in place of CYP2C9. This graph also includes the results of a reaction containing 80 nM CYP2D6 and 40 μM clopidogrel as well as a reaction that used 40 nM CYP3A4 and 40 μM clopidogrel.

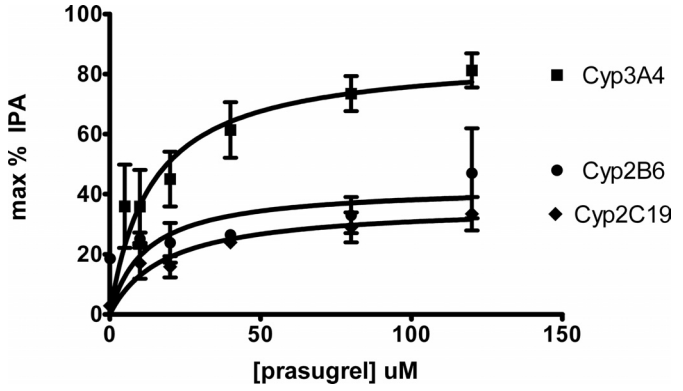

Fig. 4. Effect of incubation of washed platelets with prasugrel and different cytochrome P450 subtypes. Washed platelets were incubated with 20 nM CYP3A4, 2B6, or 2C19 and various amounts of prasugrel. Max %IPA was measured and showed a correlation with the concentration of prasugrel in the reaction. Results with CYP3A4 and CYP2C19 fit eq. 2 well with an R^2 of 0.6 and 0.75, respectively.

with CYP3A4, 2B6, and 2C19 and platelets. The profile is very similar to that seen for clopidogrel in Fig. 3A. Analogous experiments using CYP2C9, 2D6, and 1A2 did not inhibit platelet aggregation even at high levels of enzyme and prasugrel. Side-by-side experiments using the same batch of washed platelets and either clopidogrel or prasugrel with individual cytochromes P450 produced very similar profiles as shown in Fig. 5. The amount of clopidogrel (enantiomerically pure) needed to achieve the same level of IPA was

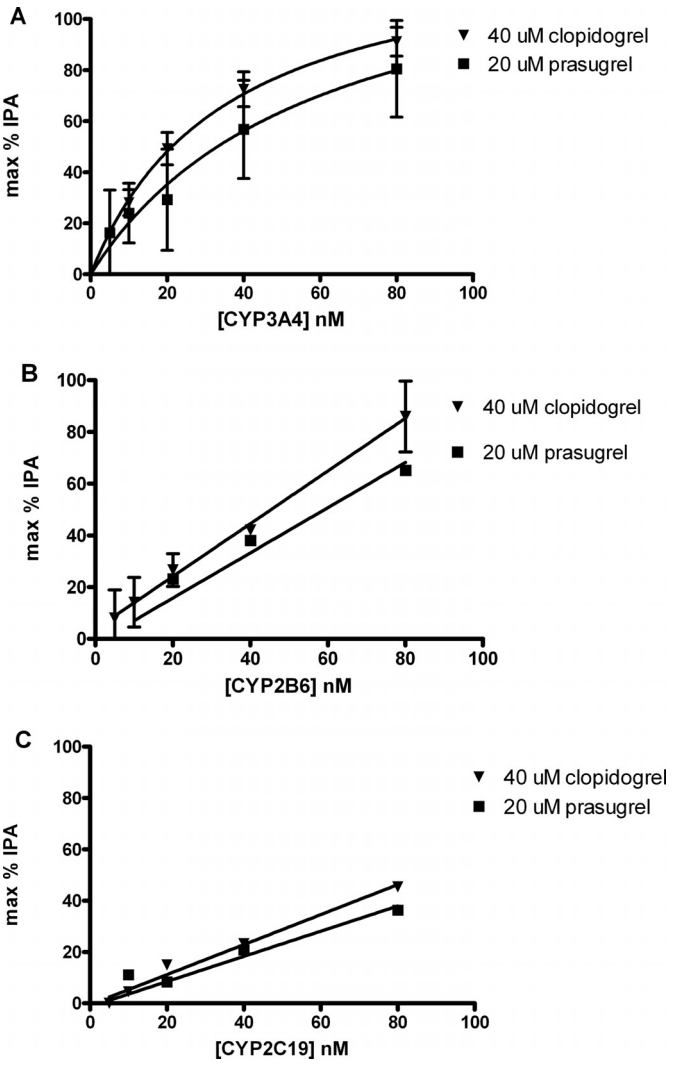

Fig. 5. Comparison of the results when platelets were incubated with either prasugrel or clopidogrel and various cytochromes P450 subtypes. These experiments were conducted on the same day using the same cytochrome P450 mix and the same platelet preparation to allow comparison. Each experiment used 20 μM prasugrel and 40 μM clopidogrel with various concentrations of cytochrome P450. A, results of platelets and thienopyridine incubated with CYP3A4. Both data sets fit to eq. 2 with R^2 values of 0.85 for clopidogrel and 0.63 for prasugrel. B and C, results when CYP2B6 or CYP2C19 was used. Data were fit to a straight line for illustrative purposes.

approximately 4 times that needed for prasugrel (racemic mixture) when the different isomeric contents of each were taken into account. The extent of inhibition that could be achieved in each reaction appears to be controlled by the cytochrome P450 catalyzing the reaction rather than to be dependent on the chemical structure of the thienopyridine.

Discussion

A novel method using platelets as a biosensor was developed to study which cytochromes P450 convert the important antiplatelet drugs clopidogrel and prasugrel to their active metabolites in vitro. Generation of the AM of the thienopyridine in situ in the presence of platelets increases the likelihood that more of the AM formed would be captured by the $P2Y_{12}$ platelet receptor before being lost through nonproductive side reactions. The method is not dependent on separa-

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tion of the numerous stereoisomers (four enantiomeric pairs have been identified for clopidogrel and prasugrel) generated by cytochrome P450 oxidation and ring opening of the thienopyridine (Pereillo et al., 2002; Hasegawa et al., 2005) and allowed us to study in finer detail the cytochrome P450 requirements for this multistep transformation.

Conditions were found whereby the thienopyridine, purified P450 Baculosomes, and washed platelets could be incubated together without changes in the ADP-induced aggregation of these platelets in the absence of thienopyridine or one of the essential components of the reaction. The reaction progress was monitored by removing aliquots of the platelet-containing reaction at various times and then measuring how the sensitivity of the platelets to ADP-induced platelet aggregation changed with the thienopyridine/cytochrome P450/platelet ratio and incubation time. In theory, with increasing incubation time, the concentration of AM should increase, the amount captured by the platelet $P2Y_{12}$ receptor should increase, and the sensitivity of the platelets to ADP-induced aggregation should decrease. This is exactly what was observed, and the occupancy of the $P2Y_{12}$ receptor by the AM was indirectly monitored by observing the decrease in ADP-induced platelet aggregation over time. The time course of the reaction as well as the max %IPA observed was shown to be dependent on the cytochrome P450 subtype and concentration of the enzyme as well as the concentration of the antiplatelet agent. The IPA was specific for the agonist ADP, indicating that only the ADP platelet receptors were affected by this reaction. Studies whereby the production of AM was measured using LC-MS required the inclusion of millimolar levels of glutathione to stabilize the AM for measurement (Pereillo et al., 2002; Kazui et al., 2010). The addition of glutathione to reactions in the current study had no effect on the amount of PA observed. Thus, its inclusion was not necessary in this system in which the platelet receptor served to capture the AM while it was being formed.

The method was used to study a number of cytochromes P450 and to identify those that were capable of converting the thienopyridine of interest to the AM. The same cytochromes P450 were identified as being capable of converting both clopidogrel and prasugrel to AM, namely, CYP3A4/5, 2B6, and 2C19. Of interest, the combination of CYP2B6 and clopidogrel was able to inhibit platelet aggregation close to 100% despite the fact that clopidogrel is a mechanism-based inhibitor of CYP2B6 in vitro (Richter et al., 2004). At a minimum, we expected to see some limiting value on the max

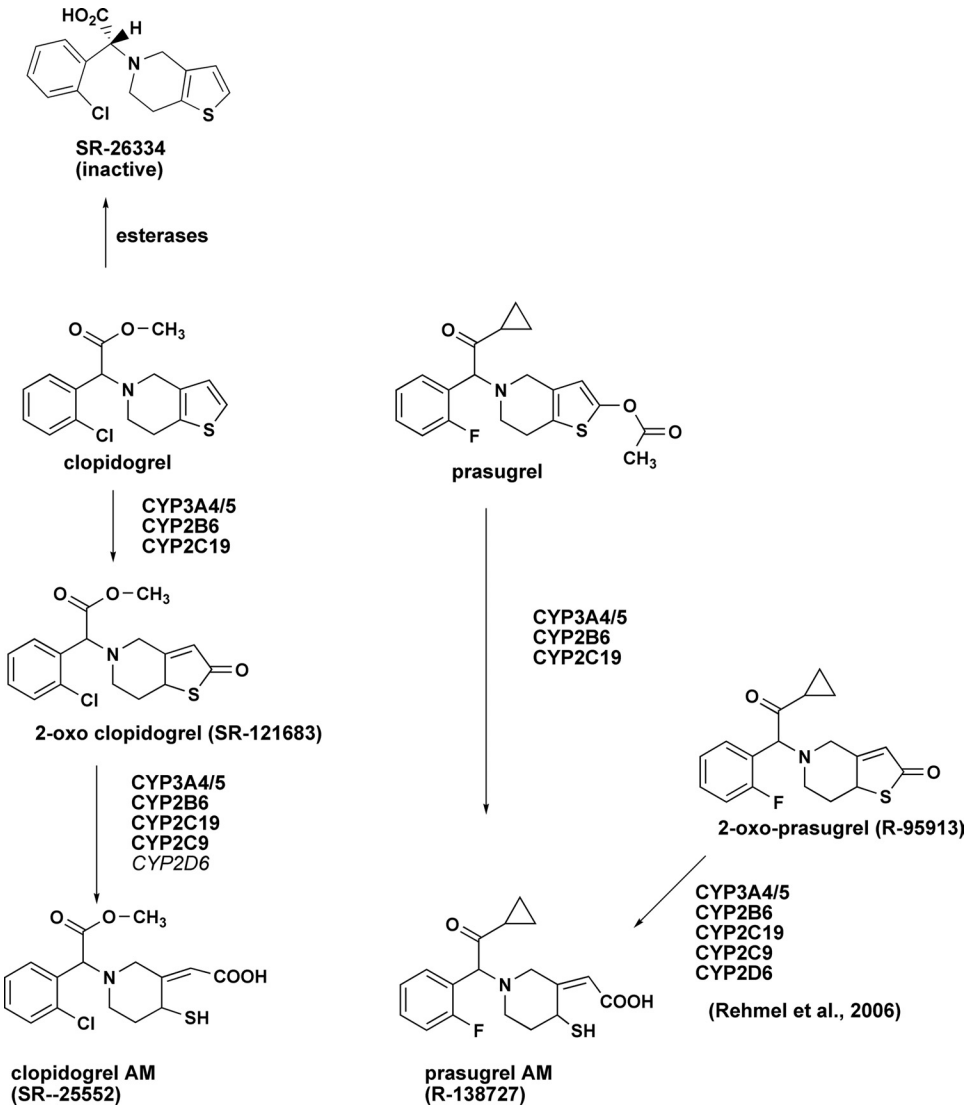

Fig. 6. Summary of thienopyridine transformation results from these experiments and comparison to the literature.

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%IPA achieved with this combination of enzyme and thienopyridine due to inactivation of the enzyme; however, this was not the case. These results suggest that the ratio of turnover to AM versus inactivation of the enzyme significantly favors the release of AM. Of the additional cytochromes P450 tested, CYP1A2, 2C9, and 2D6 did not convert either thienopyridine to AM.

Because the conversion of the parent to AM is a two-step process, we also looked for enzymes capable of converting 2-oxo-clopidogrel to AM, the second step in the conversion. Two cytochromes P450 were identified that could perform the second reaction although they were unable to convert the parent to AM, namely, CYP2C9 and 2D6. Taken together, the data using clopidogrel and oxo-clopidogrel suggest that CYP3A4, 2B6, 2C19, 2C9, and 2D6 can all catalyze the conversion of the oxo intermediate to AM. CYP1A2 could not convert the parent or the intermediate to AM in this study. Previous studies using the oxo form of prasugrel led to the same conclusions. These results are in agreement with LC-MS studies, which measured clopidogrel AM formation and showed that CYP2B6 and CYP2C19 could catalyze both steps of the reaction and that CYP2C9 could only catalyze the conversion of the oxo intermediate to AM (Kazui et al., 2010). The results of the LC-MS study and the current study differ mainly with regard to CYP3A4. In the current study, formation of the AM from clopidogrel in the presence of CYP3A4 was shown, although high concentrations of both enzyme and thienopyridine were needed, whereas the LC-MS study only saw oxo intermediate formation. Formation of the oxo intermediate alone is undetectable in the current study. On the other hand, the finding in this study with CYP3A4 and clopidogrel is consistent with other studies, which showed that CYP3A4 could convert clopidogrel to AM and that the carboxylic acid of clopidogrel is inactive in this assay (Zahno et al., 2010). Figure 6 summarizes the findings of this study and compares the results to what is known in the literature concerning oxo-prasugrel conversion.

A side-by-side comparison of the effects of clopidogrel and prasugrel with the same enzyme mixes and platelet preparations is shown in Fig. 5. A 4-fold difference in the amount of clopidogrel (single active isomer) was needed to achieve the same level of IPA as prasugrel (enantiomers) when the difference in the isomeric content of these two compounds is taken into account. Previous work has shown that the AMs of both prasugrel and clopidogrel are equipotent at the $P2Y_{12}$ receptor, so the difference in the amounts needed to achieve the same %IPA is not a reflection of a potency difference at the receptor (Pereillo et al., 2002). These overlay plots showed that the max %IPA was similar for both substrates and was merely a function of the cytochrome P450 type and depended on the concentration of substrate and enzyme. The somewhat surprising aspect of these results is that the time required to achieve a similar %IPA using either prasugrel or clopidogrel was not significantly different. It is often assumed that the conversion of prasugrel to the oxo intermediate would be significantly faster than oxidation of the thienopyridine ring of clopidogrel; however, in the absence of esterases this appears not to be the case. The fact that prasugrel does not show significantly faster inactivation of the $P2Y_{12}$ receptor when compared directly with clopidogrel suggests that the slow step in the overall process is not formation of the oxo intermediate.

These studies, although clearly showing which cytochromes P450 are capable of converting clopidogrel and prasugrel to the AM responsible for the inhibition of PA by ADP, do not indicate which enzymes are the most important in vivo. Given that multiple enzymes are capable of producing AMs either from the parent compound or oxo intermediate, identifying potential drug-drug interactions by reaction phenotyping remains a challenge.

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Authorship Contributions

Participated in research design: Abell and Liu.
Conducted experiments: Abell and Liu.
Contributed new reagents or analytic tools: Abell and Liu.
Performed data analysis: Abell and Liu.
Wrote or contributed to the writing of the manuscript: Abell and Liu.

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