In Vitro Evaluation of Potential Drug-Drug Interactions with Ticagrelor: 
Cytochrome P450 Reaction Phenotyping, Inhibition, Induction and Differential Kinetics

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Running title

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ABSTRACT:

Ticagrelor is an orally administered, antiplatelet agent that inhibits ADP’s prothrombotic effects on the platelet by antagonizing the P2Y₁₂ receptor. Ticagrelor is a reversibly binding direct-acting P2Y₁₂ antagonist and does not require metabolic activation to achieve its antiplatelet effect. CYP3A4 and CYP3A5 appear to be the enzymes predominantly responsible for the formation of the ticagrelor active and inactive metabolites, AR-C124910XX and AR-C133913XX. The apparent K_m values in human liver microsomes are 27.0 and 38.8 µM, with V_max values of 730 and 417 pmol/min/mg for AR-C124910XX and AR-C133913XX, respectively. Ticagrelor moderately inhibited CYP2C9 activity in human liver microsomes with an IC₅₀ of 10.5 µM, while exhibiting little or no inhibition of CYP1A2, CYP2B6, CYP2C8, CYP2C19, CYP2D6 and CYP2E1. In human liver microsomes, ticagrelor inhibited midazolam 4-hydroxylation with an IC₅₀ of 8.2 µM, while activating 1’-hydroxylation of midazolam. Studies with recombinant enzymes suggested that cytochrome b5 and CYP3A4 interactions play a significant role in this differential kinetic behavior. Evaluated in fresh human hepatocytes at concentration up to 20 µM, ticagrelor was not an inducer of CYP1A2 or CYP3A4. Though ticagrelor exhibited a tendency for CYP2B6 and CYP2C9 induction, its potential to cause drug interactions via the induction of these enzymes is low when considering its exposure at a therapeutic dose.
INTRODUCTION

Ticagrelor (Fig. 1) is the first of a new class of antiplatelet agents known as cyclo-pentyl-triazolo-pyrimidines (CPTPs). Ticagrelor inhibits ADP’s prothrombotic effects on the platelet by antagonizing the P2Y₁₂ receptor. Ticagrelor nearly completely inhibited ADP-induced platelet aggregation ex vivo (Husted et al., 2006), and ticagrelor demonstrated a dose-dependent inhibition of platelet aggregation in humans, resulting in 79% and 95% inhibition by 90 mg bid and 180 mg bid, respectively (Storey et al., 2007). Ticagrelor is differentiated from thienopyridine antiplatelet agents such as ticlopidine, clopidogrel and prasugrel, in several important ways. Ticagrelor is an orally active reversibly binding antagonist and does not require metabolic transformation to inhibit P2Y₁₂. Conversely, the thienopyridines do require metabolic activation and bind irreversibly to the P2Y₁₂ receptor. These characteristics may make ticagrelor a drug with faster onset and offset of action, compared to clopidogrel, with less inter-patient variability in plasma concentrations and antiplatelet effects (Gurbel et al., 2009).

In addition to the active parent compound, AR-C124910XX (a major metabolite of ticagrelor) has also been found to antagonize the P2Y₁₂ receptor at approximately equal potency. After a 100 mg b.i.d. dose in humans, ticagrelor reaches steady state Cₘₐₓ of about 1.5 μM, while AR-C124910XX circulates in plasma at about one third the concentration of the parent drug (van Giezen and Humphries, 2005; Husted et al., 2006). Ticagrelor and AR-C124910XX were observed as the predominant components in plasma and feces in six human subjects receiving single oral dose of ¹⁴C-ticagrelor. Recoveries of ticagrelor and AR-C124910XX in urine were both below 1% of the administered dose. The major components in urine were an inactive metabolite AR-C133913XX and its glucuronide conjugate (Teng et al., 2010).
Ticlopidine, the first thienopyridine antiplatelet agent, is mainly metabolized by CYP3A4 and CYP2C19 (Dalvie and O’Connell, 2004). Multiple CYP enzymes, including CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4, are responsible for the metabolism of clopidogrel (Kazui et al., 2010) while CYP3A4 and CYP2B6 have been identified as major contributors to the metabolism of prasugrel (Rehmel et al., 2006). Ticlopidine, clopidogrel and prasugrel have been shown to be mechanism-based inhibitors of CYP2B6 (Richter et al., 2004; Nishiya et al., 2009) and ticlopidine was also demonstrated as a mechanism-based inhibitor of CYP2C19 (Ha-Duong et al., 2001). Unlike reversible inhibitors, mechanism-based inhibition of P450 enzymes can result in time-extended and less predictable changes in exposure to other ‘victim’ drugs that are subject to metabolism by the inactivated enzyme because the inactivated P450 enzyme can only be replaced by newly synthesized protein.

The current studies were performed to characterize the P450 enzymes that are responsible for the metabolism of ticagrelor to its major active (AR-C124910XX) and inactive (AR-C133913XX) metabolites. In addition, ticagrelor was evaluated in vitro for its potential to inhibit, inactivate or induce major human P450 enzymes.
Material and Methods

Materials. Chemicals were obtained from Sigma-Aldrich (St Louis, MO), Toronto Research Chem (North York, Canada), BD Gentest (Woburn, MA) or Inveresk (Scotland, UK). Ticagrelor, AR-C124910XX, AR-C133913XX and omeprazole were obtained from the AstraZeneca compound collection. Pooled human liver microsomes (HLM) were purchased from BD Gentest (Woburn, MA) or prepared within AstraZeneca (Södertälje, Sweden) or by Inveresk (Scotland, UK). Microsomes prepared from insect cells infected with recombinant baculovirus containing a cDNA insert for individual human P450 enzyme or cytochrome b5 (Cyt b5) were obtained from BD Gentest. All human recombinant P450 enzymes, except CYP1A2, CYP2D6 and CYP3A5, were co-expressed with Cyt b5. CYP3A4, with and without co-expression of Cyt b5, was used in this study.

Identification of the P450 Enzymes that Metabolize Ticagrelor to AR-C124910XX and AR-C133913XX. In vitro incubations of ticagrelor in HLM to evaluate AR-C124910XX and AR-C133913XX formation were conducted under linear conditions with respect to incubation time and microsomal protein concentration. Ticagrelor at 3 μM was incubated with 0.5 mg/mL HLM at 37°C either alone or with selective CYP inhibitors, furafylline (2 and 10 μM), sulfaphenazole (1 and 5 μM), omeprazole (15 and 50 μM), quinidine (0.2 and 1 μM), and ketoconazole (0.2 and 1 μM). Incubation mixtures also contained 100 mM pH 7.4 potassium phosphate buffer, 5 mM MgCl₂ and a NADPH re-generating system containing 1.3 mM NADP, 3.3 mM of glucose-6-phosphate and 0.4 unit/mL of glucose-6-phosphate dehydrogenase. Due to its inhibitory mechanism, furafylline was pre-incubated with microsomal protein and NADPH re-generating system for 15 minutes to increase its effect on CYP1A2. All incubations were conducted in duplicate. Reactions were terminated after 30 min incubation by the addition of...
equal volume of 0.1% formic acid in acetonitrile and samples were quantified by liquid chromatography mass spectrometry (LC-MS). The formations of AR-C124910XX and AR-C133913XX in the presence of inhibitors were compared to metabolites formed in their absence.

Microsomes expressing individual human cytochrome P450 enzymes, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5 at 150 pmol/mL, were incubated with ticagrelor to evaluate the individual P450 contribution to the metabolism of AR-C124910XX and AR-C133913XX. Incubation conditions and sample preparation were similar as described above. The ticagrelor metabolites formed in the presence of individual P450 were compared to the formation in microsomes from vector control Sf9 membranes.

Enzyme kinetics for the formation of AR-C124910XX and AR-C133913XX were evaluated in HLM (0.5 mg/mL), and in recombinant CYP3A4 and CYP3A5 enzymes (150 pmol/mL). Ticagrelor was incubated for 30 min in the concentration range of 1 to 50 µM.

Separation of ticagrelor, AR-C124910XX and AR-C133913XX was performed by reverse phase HPLC utilizing a Phenomenex Synergi Max-RP column (4 µm, 3 × 30 mm) with 5 min gradient at 0.6 mL/min consisting of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Compounds were quantified on Finnigan AQA single quadrupole mass spectrometer (ThermoQuest, San Jose, CA) using selected ion monitoring (SIM) mode with ion m/z at 523.0 for ticagrelor, 479.0 for AR-C124910XX and 371.0 for AR-C133913XX.

Kinetic constants for the formation of AR-C124910XX and AR-C133913XX in HLM, recombinant CYP3A4 and CYP3A5 were obtained by fitting either Michaelis-Menten kinetics (v= V_max [S]/(K_m + [S])) or sigmodial kinetics (v=V_max [S]^n/(S_{50}^n + [S]^n)) to experimental data using nonlinear regression (Prism 4, GraphPad Software Inc., San Diego, CA).
CYP Inhibition by Ticagrelor. The potential to cause drug-drug interactions by inhibiting the major human P450 enzymes was evaluated by co-incubation of ticagrelor with selective P450 probe substrates in HLM (Table 1). The formation of metabolites, resorufin, 2-hydroxybupropion, 6α-hydroxyplacitaxel, 4’-hydroxydiclofenac, 4’-hydroxymephenytoin, dextrorphan and 6-hydroxychlorzoxazone was quantified as specific P450 markers for the activities of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1, respectively. The formation of 1’-hydroxymidazolam and 6β-hydroxytestosterone were quantified as markers of the activity of CYP3A4/5. Ticagrelor was co-incubated at concentrations up to 50 µM in each P450 assay in duplicate and the respective metabolite formation of probe substrates was compared to the vehicle solvent control incubations. Samples were analyzed by fluorescent detection (Burke et al., 1985), on-line radio detection (Sonderfan et al., 1987) or Applied Biosystems Sciex API 3000 or API 4000 mass spectrometry (Li et al., 2010). The CYP inhibition results were expressed as percent inhibition of the control enzyme activity. Where the inhibition exceeded 50% of the control activity, an IC50 value was estimated using non-linear regression analysis (XL Fit 4.2.2 software, IDBS, Surrey, UK).

Interaction between Midazolam and Ticagrelor in Recombinant CYP3A4 and CYP3A5. The interaction between midazolam and ticagrelor in CYP3A4 and CYP3A5 was further investigated using individual recombinant enzymes with varying ratios of Cyt b5. Midazolam at 3 µM was incubated with different concentration of ticagrelor (0.07~50 µM) in the presence of 1 mM NADPH, 50 pmol/mL of CYP3A4 or CYP3A5 and Cyt b5. Four different Cyt b5 experimental conditions were evaluated, (1) 0 pmol/mL Cyt b5, (2) 150 pmol/mL of Cyt b5, (3) 500 pmol/mL Cyt b5 and (4) 150 pmol/mL of heat denatured Cyt b5, which resulted in the P450 to Cyt b5 ratio of 1:0, 1:3, 1:10, 1:3 denatured, respectively. Denatured Cyt b5 was
achieved by pre-treatment at 45°C for 5 minutes. Vector control protein was added to ensure constant total protein concentration in all incubations. Reactions were initiated with the addition of NADPH (1 mM), and terminated after 5 min incubation. Formations of 1’- and 4-hydroxymidazolam were evaluated as markers for CYP3A4 and CYP3A5 activities. An IC$_{50}$ value was estimated where the inhibition exceeded 50% of the control activity.

**Time-dependent Inhibition of CYP3A4/5 and CYP2B6 by Ticagrelor.** The time-dependent inhibition of CYP3A4/5 activities by ticagrelor was evaluated by pre-incubating 3 µM of ticagrelor at 37°C with 2 mg/mL HLM, 1 mM NADPH in 100 mM phosphate buffer for 0, 3, 10, 20, and 30 min. Verapamil, tested at 10 µM, was also incubated separately as a positive control. As a negative control experiment, HLM and NADPH were pre-incubated in buffer in the absence of test compound. An aliquot of 20 µL was removed from pre-incubation tube at each time point and added to a secondary incubation tube (180 µL) containing 3 µM of midazolam and 1 mM of NADPH. In this way, the test compounds were diluted 10-fold to reduce the potential reversible inhibition and to observe any decrease in P450 activity due to enzyme inactivation. After 5 min of the secondary incubation, reactions were terminated.

The time-dependent inhibition of CYP2B6 activity by ticagrelor was also evaluated similarly as for CYP3A4/5. The pre-incubation was performed at 10 µM of ticagrelor in the presence of 1 mg/mL HLM. At various time points, aliquots of the pre-incubation mixtures were added to secondary incubations in order to provide 10-fold dilution of test compound. The secondary incubations containing 120 µM of bupropion and 1 mM of NADPH were terminated after 15 min incubation. Ticlopidine at 1 µM was used as positive control.

The formation of 1’- and 4-hydroxymidazolam and hydroxybupropion was used as marker for CYP3A4/5 and CYP2B6 activities, respectively. P450 activities after pre-incubation
with ticagrelor were compared to activities following incubation with vehicle solvent (1% methanol) and without pre-incubation. The natural logarithm of percent inhibition of the control enzyme activity by the respective compound was plotted with pre-incubation time. Inactivation rate ($k_{obs}$) was calculated as a negative slope.

**CYP Induction by Ticagrelor.** The potential of ticagrelor to induce the expression of P450 enzymes was investigated in primary cultures of freshly isolated human hepatocytes from 3 individual donors. Hepatocytes were treated once daily for three consecutive days with ticagrelor (0.2, 2 and 20 µM) or positive control inducers, β-naphthoflavone (BNF, 20 µM), phenobarbital (PB, 2 mM) and rifampin (RIF, 20 µM), as well as a solvent vehicle control (0.1% DMSO). Hepatocytes were harvested after 72 hours treatment and microsomes were prepared by two-speed centrifugations. P450 enzyme induction was assessed by microsomal assay using probe substrates phenacetin (200 µM), bupropion (250 µM), diclofenac (100 µM) and testosterone (200 µM) for CYP1A2, CYP2B6, CYP2C9 and CYP3A4 catalytic activities, respectively. Metabolite of probe substrate for each enzyme was analyzed using HPLC with UV absorbance (Stresser et al., 2004). Fold of induction was calculated as the ratio of activity from ticagrelor or positive control to activity from vehicle control. The extent of enzyme activity induction, expressed as a percentage of the positive control effect, was calculated as follows:

$$\frac{\text{Activity of ticagrelor treated cells} - \text{Activity of vehicle control}}{\text{Activity of positive control} - \text{Activity of vehicle control}} \times 100$$

The mRNA expression for each P450 isoform was determined using Taqman™ RT-PCR analysis (McGinnity et al., 2009). Threshold cycle ($C_T$) values (the fractional cycle number at which the fluorescence passes the fixed threshold) were first determined. Then $\Delta C_T$ of each target gene sample was calculated by subtracting the $C_T$ from its corresponding endogenous control, β-actin or glyceraldehyde-3 phosphate dehydrogenase (GAPDH). The $\Delta \Delta C_T$ was then
determined for the positive control inducer or ticagrelor by subtracting the ΔCt from phosphate-buffered saline. Fold induction was determined by the calculation of $2^{\Delta\Delta CT}$. The extent of mRNA induction, expressed as a percentage of the positive control effect, was calculated as follows:

$$\frac{\text{Fold induction from ticagrelor} - 1}{\text{Fold induction from positive control} - 1} \times 100$$
Results

Identification of Enzymes Responsible for the Metabolism of Ticagrelor. Ticagrelor was incubated with various human recombinant P450 enzymes to assess the contribution of individual enzyme to the metabolism of ticagrelor. The formation of AR-C124910XX and AR-C133913XX is presented in Fig. 2. AR-C133913XX was not detected in vector-control, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19 or CYP2D6 incubations. CYP3A5 generated a low amount of AR-C133913XX whereas the profile in the CYP3A4 incubation showed significant AR-C133913XX formation. Small amounts of AR-C124910XX were detected in the incubations with CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19 or CYP2D6; however, similar amounts were also detected in vector control incubations, indicating these P450 enzymes are unlikely to be responsible for the metabolism of AR-C124910XX. In contrast, significant amounts of AR-C124910XX were detected in both CYP3A4 and CYP3A5 incubations. In addition, metabolites AR-C124910XX and AR-C133913XX were not observed after ticagrelor incubations with human recombinant CYP2E1 and CYP2C8 (data not shown).

The formation of AR-C124910XX and AR-C133913XX from ticagrelor in the presence and absence of selective P450 inhibitors in HLM was also evaluated to confirm the results from recombinant enzymes (Fig. 3). More than 95% of AR-C124910XX and AR-C133913XX formation was inhibited by 1 μM of ketoconazole, the selective CYP3A4/5 inhibitor. Other P450 inhibitors, including furafylline for CYP1A2, sulfaphenazole for CYP2C9, and quinidine for CYP2D6, demonstrated little impact on the metabolism of ticagrelor to AR-C124910XX and AR-C133913XX. Omeprazole, a CYP2C19 substrate/inhibitor, exhibited ~40% inhibition for both metabolites at 50 μM. However, the inhibition may be due to the lack of specificity, as omeprazole has also been shown to inhibit CYP3A4/5 mediated 1’-hydroxymidazolam formation.
in HLM (Li et al., 2004). When both recombinant enzyme and chemical inhibition results are considered together, it appears that CYP2C19 provides little or no contribution to the metabolism of ticagrelor to AR-C124910XX or AR-C133913XX.

The kinetics of AR-C124910XX and AR-C133913XX formation by HLM exhibited Michaelis-Menten kinetics (Fig. 4) with apparent $K_m$ values of $27.0 \pm 2.5$ and $38.8 \pm 1.8 \mu M$, $V_{max}$ values of $730 \pm 34$ and $417 \pm 11$ pmol/min/mg protein, respectively. The in vitro intrinsic clearances ($V_{max}/K_m$) for AR-C124910XX and AR-C133913XX by HLM were $27.0$ and $10.7 \mu L/min/mg$ protein, respectively. The enzyme kinetics of ticagrelor metabolism was further evaluated using human recombinant CYP3A4 and CYP3A5. Sigmoidal kinetics and Michaelis-Menten kinetics were observed for AR-C124910XX formation by CYP3A4 and CYP3A5 with apparent $S_{50}$ and $K_m$ values of $11.0$ and $5.36 \mu M$, respectively (Table 2). Both the AR-C133913XX formation by CYP3A4 and CYP3A5 could be described by Michaelis-Menten kinetics with apparent $K_m$ values of $41.3 \mu M$ and $>50 \mu M$, respectively (Table 2).

**CYP Inhibition by Ticagrelor.** The inhibition of human P450 enzymes by ticagrelor was evaluated using HLM and selective P450 probes (Table 1). Ticagrelor at $50 \mu M$, exhibited 0%, 38% and 12% of inhibition towards CYP1A2, CYP2C8 and CYP2C19 activities, respectively. Ticagrelor weakly inhibited CYP2B6 and CYP2D6 activity with apparent $IC_{50}$ values of $40.0$ and $26.7 \mu M$, respectively. Ticagrelor inhibited CYP2C9 activity with an $IC_{50}$ of $10.5 \mu M$ (Table 3). Due to well-documented substrate-dependent interactions, multiple probe substrates were used to evaluate the inhibition of CYP3A4/5 activities. Testosterone 6β-hydroxylation was partially inhibited by ticagrelor, with 23-30% of inhibition at concentration range of 5-50 µM. Co-incubation of ticagrelor with midazolam appeared to activate 1’-hydroxylation and moderately inhibit 4-hydroxylation (Fig. 5) with an apparent $IC_{50}$ of $8.2 \mu M$. 


The maximal activation of 1’-hydroxylation (152%) was observed at 5.6 µM of ticagrelor in vitro, with some decrease effect at higher ticagrelor concentrations.

Ticagrelor did not cause time-dependent inhibition of 1’-hydroxy (Fig. 6A) or 4-hydroxy (data not shown) midazolam formation in HLM. Verapamil, a known CYP3A inactivator, demonstrated an inactivation rate of 0.04 min⁻¹ at 10 µM (Fig. 5A). No time-dependent inhibition of CYP2B6 activity by ticagrelor was observed, while 1 µM ticlopidine inactivated buprobion hydroxylation at a rate of 0.08 min⁻¹ (Fig. 6B).

**In Vitro Interaction between Ticagrelor and Midazolam.** The interactions between ticagrelor and midazolam in CYP3A4 and CYP3A5 were studied using the recombinant enzymes and Cyt b5 addition. In the presence of Cyt b5, the formation of 1’-hydroxymidazolam in CYP3A4 appeared to be activated by ticagrelor. This activation exhibited a maximum increase in metabolism rate at 5.6 µM of ticagrelor, the same concentration as observed in HLM (Fig. 5 and Fig. 7A). Different ratios of CYP3A4 to Cyt b5 (1:3 vs. 1:10) in the incubation did not change the level of activation of 1’-hydroxylation although this activation was not observed in the absence of Cyt b5 or when denatured Cyt b5 was added to the incubation. The availability of Cyt b5 had little impact on the observed inhibition of midazolam 4-hydroxylation by ticagrelor in recombinant CYP3A4 (Fig. 7B). No activation of either midazolam hydroxylation catalyzed by CYP3A5 was observed under any tested condition. Ticagrelor weakly inhibited 1’-hydroxylation by CYP3A5 with IC₅₀ values greater than 50 µM (Fig. 7C), and moderately inhibited 4-hydroxylation with apparent IC₅₀ values of 3.1 to 6.3 µM in different ratio of CYP3A5 and Cyt b5 (Fig. 7D).

**CYP Induction by Ticagrelor.** The potential of ticagrelor to induce expression of P450 enzymes was evaluated using fresh human hepatocytes. Cytotoxicity assay using tetrazole MTT...
and visual inspection showed no apparent reduction in hepatocyte viability following treatment with ticagrelor at concentration up to 20 µM.

BNF, PB, RIF, and RIF caused marked induction of CYP1A2 activity (5.2 to 56 fold), CYP2B6 activity (19 to 31 fold), CYP2C9 activity (5.0 to 18-fold) and CYP3A4 activity (15 to 84 fold), respectively, in hepatocytes from three individual donors (Fig. 8). No induction of CYP1A2 activity was observed following 0.2, 2 or 20 µM ticagrelor treatment. CYP1A2 activity data correlated reasonably well with mRNA (Fig. 9). No induction of CYP1A1 mRNA expression was observed after ticagrelor treatment at any concentration, while BNF increased CYP1A1 mRNA levels by 8.9 fold, when evaluated in one of the three donor cells.

CYP2B6 activity was increased 2.1 to 3.6 fold by 20 µM ticagrelor treatment, or an average of 13.4% of the PB response. Less than 3% of the PB induced CYP2B6 activity was observed following 0.2 and 2 µM ticagrelor treatment (Fig. 8). Less than 3-fold CYP2B6 mRNA expression increase was observed in hepatocyte donors treated with 0.2, 2, 20 µM ticagrelor, or below 10% of PB response (Fig. 9), similar to CYP2B6 activity changes. A small concentration-related increase in CYP2C9 activity was observed with 0.2, 2 and 20 µM ticagrelor treatment in all 3 preparations of human hepatocyte cultures. The responses observed with 20 µM ticagrelor treatment were in the range of 36.5% to 55.9% of the RIF response (Fig. 8). However, no induction of CYP2C9 mRNA expression with treatment of ticagrelor was observed at any concentration tested in any donor (Fig. 9). No induction of CYP3A4 activity was observed in any of the 3 human hepatocytes preparations after ticagrelor treatment at concentrations up to 20 µM (Fig. 8). CYP3A4 mRNA expression did increase after treatment with ticagrelor, but was below 15% of the RIF response (Fig. 9).
Discussion.

Ticagrelor is an orally active antiplatelet agent. AR-C124910XX is the major active metabolite observed in plasma, while AR-C133913XX is an inactive metabolite mainly found in urine. In the current study, the P450 enzymes involved in the conversion of ticagrelor to AR-C124910 and AR-C133913 in vitro were identified. Another purpose of this study was to explore the potential of ticagrelor to cause drug-drug interactions resulting from inhibition, inactivation or induction of the human major P450 enzymes.

CYP3A4 and CYP3A5 appear to be the enzymes mainly responsible for the formation of the active ticagrelor metabolite, AR-C124910XX. CYP3A4 is also likely to be the major enzyme that forms AR-C133913XX, with less contribution from CYP3A5. Other P450 enzymes are likely to be much less important in the metabolism of ticagrelor. CYP3A4 is the predominant human hepatic and intestinal P450 isoform and is responsible for the metabolism of about 50% of clinically used drugs (Guengerich, 1999). CYP3A5 is polymorphic, where the CYP3A5*3 and CYP3A5*6 alleles are the variants that cause alternative splicing and protein truncation resulting in the absence of CYP3A5 in certain subjects (Kuehl et al., 2001). CYP3A5 contributes about 17% of total hepatic CYP3A content in about 10% of Caucasians (Westlind-Johnsson et al., 2003). Drugs that modulate CYP3A4 and CYP3A5 activities (such as ketoconazole and diltiazem) and genetic differences in CYP3A5 expression in human volunteers and patients might affect the clearance of ticagrelor in vivo.

Ticagrelor moderately inhibited only CYP2C9 among the eight P450 enzymes evaluated in this study, with apparent IC50 of 10.5 µM. The potential for in vivo drug-drug interaction against CYP2C9 substrates was accessed using the [I]/Ki approach according to FDA draft guidance for drug interaction studies (US FDA, 2006) where [I] was the maximum concentration...
of total drug in the systemic circulation at steady-state ($C_{\text{max}}$) to provide a worst-case prediction. After a 100 mg b.i.d. dose in humans, ticagrelor resulted in steady state $C_{\text{max}}$ concentration of 1.5 µM (Husted et al., 2006). $C_{\text{max}}/K_i$ was calculated for each CYP enzyme assuming competitive or noncompetitive inhibition, i.e. $K_i=IC_{50}/2$ (Table 3). $C_{\text{max}}/K_i$ is 0.28 for CYP2C9. As this value is between 0.1 and 1, it is possible for ticagrelor to cause CYP2C9 inhibition \textit{in vivo}. Therefore, a clinical drug-drug interaction trial was conducted between ticagrelor and the CYP2C9 substrate, tolbutamide. The results of this clinical study demonstrated that there was no significant effect of steady-state ticagrelor on the pharmacokinetics of single-dose tolbutamide or its metabolite, 4-hydroxytolbutamide (unpublished data). $C_{\text{max}}/K_i$ values were less than 0.1 for CYP1A2, CYP2B6, CYP2C8, CYP2C19, CYP2E1 and 0.11 for CYP2D6, suggesting that ticagrelor has low tendency to inhibit these enzymes \textit{in vivo}.

P450 induction demonstrates significant interspecies differences and primary human hepatocytes are the preferred model for enzyme induction studies as they relate to prediction of drug-drug interactions (LeCluyse, 2001). The current study demonstrated that ticagrelor is not an inducer of CYP1A2 or CYP3A4. Although ticagrelor caused more than a 2-fold induction in CYP2B6, the relative response did not exceed 15% of positive control response in any of the three donors, indicating ticagrelor is a weak inducer of CYP2B6. Even though a concentration-related CYP2C9 activity increase was observed in primary human hepatocyte cultures, only treatment at 20 µM ticagrelor exhibited 35.5% to 55.9% of rifampicin response, while treatment at 0.2 and 2 µM exhibited less than 15% of positive control response. Considering 20 µM is about 13 times the $C_{\text{max}}$ concentration \textit{in vivo}, the potential of ticagrelor at pharmacologically relevant concentrations to cause CYP2C9 or CYP2B6 inductive drug interactions is low.
Current antiplatelet agents, ticlopidine, clopidogrel and prasugrel, have been reported *in vitro* as mechanism-based inhibitors of CYP2B6 (Richter et al., 2004; Nishiya et al., 2009), which were further confirmed with clinical studies. Four days of 75 mg clopidogrel q.d. pretreatment increased area under curve (AUC) of bupropion by 60% in healthy volunteers, while 4 days of 250 mg ticlopidine b.i.d. pretreatment increased AUC of bupropion by 85% (Turpeinen et al., 2005). A clinical study also showed that a 60-mg prasugrel loading dose, followed by a 10-mg daily maintenance dose for 10 days pretreatment increased AUC of bupropion by 18% and decreased AUC of hydroxybupropion by 23% (Farid et al., 2008). Our study showed that ticagrelor was not a mechanism-based inhibitor of CYP2B6 and is not likely to cause interactions with drugs primarily metabolized by CYP2B6 *in vivo*.

Complex interactions were observed between ticagrelor and different CYP3A4/5 substrates. Ticagrelor exhibited partial inhibition towards testosterone 6β-hydroxylation, activation of 1’-hydroxylation and inhibition of 4-hydroxylation midazolam in HLM. A similar differential kinetic pattern was observed in recombinant CYP3A4 in the presence of Cyt b5, but not in recombinant CYP3A5. *In vitro* activation of P450 enzymes is not uncommon (Zhang et al., 2004; Hummel et al., 2004), though regioselective heterotropic cooperativity has mainly been observed for CYP3A4. Testosterone has been reported to inhibit 1’-hydroxylation of midazolam and stimulate 4-hydroxylation pathway in HLM and recombinant CYP3A4 (Wang et al., 2000). Similar heterotropic activation/inhibition by testosterone towards midazolam and other benzodiazepines has also been demonstrated in intact human hepatocytes (Hallifax et al., 2008), suggesting the activation effect of testosterone was not an artifact associated only with microsomes. A number of models including multiple binding domains/sites and multiple conformers (Ueng et al., 1997; Korzekwa et al., 1998; Koley et al., 1995) have been proposed to
describe these experimental observations. However, these hypotheses mainly focus on the heterotropic cooperativity characteristics caused by an effector, rather than by another protein. Cyt b5 usually acts as the source of the second electron in the P450 catalytic cycle, which enhances the activity of cytochrome P450 enzymes (Schenkman and Jansson, 2003). Cyt b5 has also been reported to decrease CYP3A4 substrate inhibition by triazolam (Schrag and Wienkers, 2001), or induce positive cooperativity of CYP3A4 in the metabolism of pyrene (Jushchyshyn et al., 2005). In addition, Cyt b5 has also been demonstrated to modulate a CYP3A4 conformational change to stimulate 7-benzyloxyquinoline metabolism (Kumar et al., 2005). Our study has revealed that the addition of Cyt b5 resulted in CYP3A4 activation in the metabolism of 1’-hydroxymidazolam. However, the full appreciation of the complex interaction between ticagrelor and midazolam in CYP3A4 was beyond the scope of the current work.

In summary, the results of the current in vitro studies suggest ticagrelor is mainly metabolized by CYP3A4 and CYP3A5 and has the possibility to be inhibited when co-administrated with potent CYP3A4/5 inhibitors. Ticagrelor demonstrated little potential to inhibit or induce the major human P450 enzymes except the observation of a moderate inhibition of CYP2C9 activity in vitro. Based on these in vitro observations, clinical interaction studies between ticagrelor and CYP3A4/5 inhibitors (ketoconazole, diltiazem), inducer (rifampin), substrate (midazolam), or CYP2C9 substrate (tolbutamide) have been conducted in healthy volunteers. The results of these clinical drug-drug interaction studies are generally in the good agreement with the in vitro observations obtained in this study, but will be reported separately.
Acknowledgments.

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Authorship Contributions

Participated in research design: Zhou, Andersson, Grimm.

Conducted experiments: Zhou.

Performed data analysis: Zhou, Andersson, Grimm.

Wrote or contributed to the writing of the manuscript: Zhou, Andersson, Grimm.
References


Legends for figures

Figure 1. Chemical structures of ticagrelor and its major metabolites AR-C124910XX and AR-C133913XX. Refer to Teng et al. 2010 for detail ticagrelor metabolic pathways.

Figure 2. Formation of AR-C124910XX and AR-C133913XX from ticagrelor (3 µM) incubation in individual human recombinant P450 enzyme. Each bar represents the mean activity from duplicate measurements.

Figure 3. Effect of P450 selective chemical inhibitors on the formation of AR-C124910XX and AR-C133913XX in pooled human liver microsomes. Each bar represents the mean percentage activity relative to vehicle control from duplicate measurements. Furafylline was pre-incubated to increase its inhibitory effect on CYP1A2.

Figure 4. Kinetic analysis of the formation of AR-C124910XX and AR-C133913XX by pooled human liver microsomes. Ticagrelor was incubated at concentrations in the range of 1~50 µM in duplicate.

Figure 5. Effect of ticagrelor on the formation of 1'-hydroxymidazolam and 4'-hydroxymidazolam in pooled human liver microsomes. Each data point represents the average activity from duplicate experiments.

Figure 6. Effect of pre-incubation of ticagrelor with pooled human liver microsomes on the activity of CYP3A4/5 (A) or CYP2B6 (B). Verapamil and ticolpidine were used as positive controls for CYP3A4/5 and CYP2B6 inactivation.

Figure 7. Interactions between ticagrelor and midazolam in CYP3A4 and CYP3A5 in the absence or presence of cytochrome b5. (A) 1'-hydroxymidazolam formation in CYP3A4; (B) 4'-hydroxymidazolam formation in CYP3A4; (C) 1'-hydroxymidazolam formation in CYP3A5; (D) 4-hydroxymidazolam formation in CYP3A5. CYP3A:Cyt b5(1:0) is represented by ■; CYP3A:Cyt b5(1:3) represented by □; CYP3A:Cyt b5 (1:10) represented by ♦; and
CY3A: denatured Cyt b5 (1:3) represented by ◊. Estimated IC₅₀ values are shown right to the panels. Each data point represents the average activity from duplicate experiments.

**Figure 8.** Average microsomal enzyme activity of CYP1A2, CYP2B6, CYP2C9 and CYP3A4 in primary human hepatocyte cultures treated with ticagrelor (0.2, 2, 20 µM) or positive control inducers (20 µM β-naphthoflavone (BNF), 2 mM phenobarbital (PB) or 20 µM rifampicin (RIF)) from three donors using P450 selective probe drugs.

**Figure 9.** Average CYP1A2, CYP2B6, CYP2C9 and CYP3A4 mRNA expression in primary human hepatocyte cultures treated with ticagrelor (0.2, 2, 20 µM) or positive control inducers (20 µM β-naphthoflavone (BNF), 2 mM phenobarbital (PB) or 20 µM rifampicin (RIF)) from three donors, except CYP3A4 from two donors as the third donor exhibited an extreme potent positive response (>6000 fold) of CYP3A4 mRNA expression to the positive control rifampicin.
Table 1. Incubation conditions for CYP inhibition assays.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
<th>Substrate</th>
<th>Incubation concentration</th>
<th>Incubation time</th>
<th>HLM concentration</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Ethoxyresorufin $O$-dealkylase</td>
<td>5 µM</td>
<td>suitable</td>
<td></td>
<td>0.25 mg/mL</td>
<td>Fluorescent</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion 2-hydroxylase</td>
<td>70 µM</td>
<td>10 min</td>
<td></td>
<td>0.2 mg/mL</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Paclitaxel 6α-hydroxylase</td>
<td>10 µM</td>
<td>10 min</td>
<td></td>
<td>0.05 mg/mL</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac 4’-hydroxylase</td>
<td>5 µM</td>
<td>15 min</td>
<td></td>
<td>0.2 mg/mL</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-Mephenytoin 4’-hydroxylase</td>
<td>200 µM</td>
<td>30 min</td>
<td></td>
<td>2 mg/mL</td>
<td>Radioactivity</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan $O$-demethylase</td>
<td>5 µM</td>
<td>15 min</td>
<td></td>
<td>0.2 mg/mL</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorzoxazone 6-hydroxylase</td>
<td>100 µM</td>
<td>30 min</td>
<td></td>
<td>2 mg/mL</td>
<td>Radioactivity</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Midazolam 1’- and 4- hydroxylase</td>
<td>3 µM</td>
<td>5 min</td>
<td></td>
<td>0.2 mg/mL</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td></td>
<td>Testosterone 6β-hydroxylase</td>
<td>150 µM</td>
<td>15 min</td>
<td></td>
<td>2 mg/mL</td>
<td>Radioactivity</td>
</tr>
</tbody>
</table>
Table 2. Enzyme kinetics of ticagrelor metabolite formation in recombinant CYP3A4 and CYP3A5 enzymes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Formation of AR-C124910XX</th>
<th>Formation of AR-C133913XX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP3A4</td>
<td>CYP3A5</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (pmol/min/pmol)</td>
<td>2.17±0.31</td>
<td>0.38±0.03</td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>11.0±2.5</td>
<td>5.36±1.27</td>
</tr>
<tr>
<td>$n$&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hill factor, others were fitted using Michaelis-Menten equation

<sup>b</sup> Estimated $K_m$ value is above the highest concentration tested (50 μM).
Table 3. *In vitro* inhibition of ticagrelor towards P450 selective metabolic pathways and prediction of *in vivo* inhibition potential.

<table>
<thead>
<tr>
<th>Selective pathway</th>
<th>IC$_{50}$ (µM)</th>
<th>C$<em>{max}$/K$</em>{i}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2 resorufin</td>
<td>&gt;50</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>CYP2B6 2-hydroxybupropion</td>
<td>40.0</td>
<td>0.07</td>
</tr>
<tr>
<td>CYP2C8 6α-hydroxypaclitaxel</td>
<td>&gt;50</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>CYP2C9 4’-hydroxydiclofenac</td>
<td>10.5</td>
<td>0.28</td>
</tr>
<tr>
<td>CYP2C19 4’-hydroxymephenytoin</td>
<td>&gt;50</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>CYP2D6 dextrophan</td>
<td>26.7</td>
<td>0.11</td>
</tr>
<tr>
<td>CYP2E1 6-hydroxychloroxazone</td>
<td>&gt;50</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>CYP3A4/5 1’-hydroxymidazolam</td>
<td>Apparent activation</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>4-hydroxymidazolam</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>6β-hydroxytestosterone</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

NA not applicable
Figure 2

Metabolite formation (pmol/min/pmol)

- AR-C124910XX
- AR-C133913XX

CYP Ctrl, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5
Figure 3

The graph shows the percent of control activity for various compounds after different treatments. The x-axis represents different compounds and concentrations, while the y-axis represents the percent of control activity. The compounds include MeOH, Furafylline 2μM, Furafylline 10μM, MeOH, Sulfaphenazole 1μM, Sulfaphenazole 5μM, Omeprazole 15μM, Omeprazole 50μM, Quinidine 0.2μM, Quinidine 1μM, Ketoconazole 0.2μM, and Ketoconazole 1μM. The data is represented by two sets of bars, one for AR-C124910XX and one for AR-C133913XX.
Figure 4

![Graph showing metabolite formation rate vs. Ticagrelor concentration. The graph compares the rates for AR-C124910XX and AR-C133913XX.](image)
Figure 5

- **1'-hydroxymidazolam**
- **4-hydroxymidazolam**
Figure 6

(A) CYP3A4/5

(B) CYP2B6
Figure 8

Phenacetin O-dealkylation

- Vehicle control
- 0.2 μM Ticagrelor
- 2 μM Ticagrelor
- 20 μM Ticagrelor
- 20 μM BNF

Fold increase

Bupropion hydroxylation

- Vehicle control
- 0.2 μM Ticagrelor
- 2 μM Ticagrelor
- 20 μM Ticagrelor
- 2 mM PB

Fold increase

Diclofenac 4'-hydroxylation

- Vehicle control
- 0.2 μM Ticagrelor
- 2 μM Ticagrelor
- 20 μM Ticagrelor
- 20 μM RIF

Fold increase

Testosterone 6β-hydroxylation

- Vehicle control
- 0.2 μM Ticagrelor
- 2 μM Ticagrelor
- 20 μM Ticagrelor
- 20 μM RIF

Fold increase
Figure 9

CYP1A2

Vehicle control
0.2 µM Ticagrelor
2 µM Ticagrelor
20 µM Ticagrelor
20 µM BNF

Fold increase

CYP2B6

Vehicle control
0.2 µM Ticagrelor
2 µM Ticagrelor
20 µM Ticagrelor
2 mM PB

Fold increase

CYP2C9

Vehicle control
0.2 µM Ticagrelor
2 µM Ticagrelor
20 µM Ticagrelor
20 µM RIF

Fold increase

CYP3A4

Vehicle control
0.2 µM Ticagrelor
2 µM Ticagrelor
20 µM Ticagrelor
20 µM RIF

Fold increase