POTENT MECHANISM-BASED INHIBITION OF HUMAN CYP2B6 BY CLOPIDOGREL AND TICLOPIDINE

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Suicide inhibition of CYP2B6 by clopidogrel

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D-702, [2-(3,4-dimethoxy-phenyl)-8-(4-hydroxy-3-methoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile]; D-703, [2-(4-hydroxy-3-methoxyphenyl)-8-(3,4-dimethoxy-phenyl)-6-methyl-2-isopropyl-6-azaoctanitrile]; ESI, electrospray ionisation; HPLC, high-performance liquid chromatography.
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Abstract:

The thienopyridine derivates, ticlopidine and clopidogrel, are inhibitors of ADP-induced platelet aggregation. Pharmacological activity of these prodrugs depends on cytochrome P450 (CYP)-dependent oxidation to the active antithrombotic agent. In this study we investigated the interaction potential of clopidogrel and ticlopidine by using human liver microsomes and recombinantly expressed CYP isoforms. Both clopidogrel and ticlopidine inhibited CYP2B6 with highest potency and CYP2C19 with lower potency. Clopidogrel also inhibited CYP2C9 and ticlopidine also inhibited CYP1A2 with lower potency. Inhibition of CYP2B6 was time- and concentration-dependent, and as shown by dialysis experiments, it was irreversible and dependent on NADPH, suggesting a mechanism-based mode of action. Inactivation was of non-pseudo-first-order type with maximal rates of inactivation (K_{inact}) for clopidogrel and ticlopidine in microsomes (recombinant CYP2B6) of 0.35 min^{-1} (1.5 min^{-1}) and 0.5 min^{-1} (0.8 min^{-1}), respectively, and half-maximal inactivator concentrations (K_I) were 0.5 µM (1.1 µM) for clopidogrel and 0.2 µM (0.8 µM) for ticlopidine. Inhibition was attenuated by the presence of alternative active site ligands but not by nucleophilic trapping agents or reactive oxygen scavengers, further supporting mechanism-based action. A chemical mechanism is discussed based on the known metabolic activation of clopidogrel and on the finding that hemoprotein integrity of recombinant CYP2B6 was not affected by irreversible inhibition. These results suggest the possibility of drug interactions between thienopyridine derivates and drug-substrates of CYP2B6 and CYP2C19.
Thrombolytic drugs are widely used in patients with coronary heart disease (CHD). These include anticoagulants and antiplatelet compounds like acetylsalicylic acid, glycoprotein IIb/IIIa receptor antagonists and the thienopyridine derivates, ticlopidine and clopidogrel. The latter substance is being used more often today, because it shows a more rapid onset of action and a lower incidence of adverse effects such as neutropenia and thrombotic thrombocytopenic purpura compared to ticlopidine (Kam et al., 2003). A further increase in the use of clopidogrel may be expected for the treatment of patients with acute coronary syndrome since clinical trials showed advantageous effects of clopidogrel in combination with acetylsalicylic acid (Mehta et al., 2001).

Both ticlopidine and clopidogrel are not active in vitro and require hepatic biotransformation for pharmacologic activity, which is inhibition of ADP-induced platelet aggregation (Savi et al., 1992). The metabolic activation of clopidogrel has been investigated in detail. Whereas the majority of clopidogrel is hydrolyzed by esterases to an inactive carboxylic acid derivative, microsomal cytochromes P450 (CYP) were shown to catalyze the oxidation of the thiophene ring to 2-oxoclopidogrel (Savi et al., 2000). This intermediate is further activated by hydrolytic opening of the thiophene ring to the final active metabolite, which contains a free thiol group that is able to block the P2Y12 ADP-receptor on platelets by forming a disulfide bond with a cysteine residue, thus preventing the binding of ADP to the receptor (Savi et al., 2000). The P450 isoenzyme responsible for clopidogrel activation was initially suggested to be CYP1A2 (Savi et al., 1994), but subsequent studies indicated that CYP3A4 is the most active isozyme in human liver with lower but still significant amounts metabolized by CYP1A2 and CYP2B6 (Clarke et al., 2002). A major contribution of CYP3A to metabolic clopidogrel activation was confirmed by a clinical study which demonstrated attenuation of the antiplatelet activity of clopidogrel by the CYP3A4 substrate, atorvastatin, and modulation...
of the inhibitory effect by inhibitors and inducers of CYP3A4, suggesting competitive inhibition of clopidogrel activation by the alternative substrate (Lau et al., 2003).

Several sulfur-containing substances with structural similarities to clopidogrel were shown to be irreversible inhibitors of cytochromes P450. The thiophene derivative, ticrynafen (tienilic acid), was intensively investigated because it induced immunoallergic hepatitis in a subset of patients who developed so-called anti-LKM2 (liver-kidney microsome antibody type 2) autoantibodies. It was shown that these inhibitory autoantibodies were directed against CYP2C9, the primary P450 isozyme involved in the liver metabolism of the drug (Beaune et al., 1987). Covalent modification of CYP2C9 protein by ticrynafen was found to be the cause for irreversible enzyme inhibition and presumably for initiation of immunoallergic reactions against changed autoepitopes (Lopez-Garcia MP et al., 1994). Another thiophene derivative, the thienopyridine ticlopidine, which differs structurally from clopidogrel only by the absence of a carboxymethylgroup, caused drug interactions with substrates of CYP2C19 like phenytoin (Donahue et al., 1997) and omeprazole (Tateishi et al., 1999). The drug was subsequently shown to be a selective mechanism-based inhibitor of CYP2C19 (Ha-Duaong et al., 2001). Metabolic activation of ticlopidine has however not been studied in comparable detail to clopidogrel.

Because of the structural similarities between clopidogrel and ticlopidine on the one side, and ticrynafen on the other side, we hypothesized that irreversible and possibly rather selective interactions with CYP enzymes may also occur with clopidogrel. Until present, neither in vitro investigations nor in vivo clinical studies with the aim to evaluate the drug interaction profile of this substance have been performed. The aim of this study was, therefore, to investigate the inhibitory potential of clopidogrel towards human drug metabolizing
cytochromes P450. We also applied structural homology modeling of the involved CYPs (Bathelt et al., 2002) to study interactions with clopidogrel on a molecular level.
Materials and Methods

Chemicals. Clopidogrel was kindly provided by Sanofi-Synthelabo (Ambores, France). Ticlopidine, NADP+, NADPH, diethyldithiocarbamate (DDC), sulfaphenazole, superoxide dismutase (SOD), N-acetylcysteine (N-Ac), DMSO, glutathione (GSH), 7-ethoxycoumarin, coumarin, umbelliferone, quinidine and sodium hydrosulfite were purchased from Sigma (Taufkirchen, Germany). Furafylline was a kind gift from U. Fuhr (University of Cologne, Germany). Glycerine was purchased from Roth (Karlsruhe, Germany) and Emulgen 911 was provided by Kao-Atlas (Tokyo, Japan). Glucose-6-phosphate (GP) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Glucose-6-phosphate-dehydrogenase (GPD) was purchased from Calbiochem (Schwalbach, Germany). Ketoconazole was obtained from Ultrafine Chemicals (Manchester, England). Thiopeta was obtained from Wyeth Pharma GmbH (Muenster, Germany). S-Mephenytoin was a kind gift from U. A. Meyer (Biozentrum, Basel, Switzerland). Propafenone, varapamil and metabolites were obtained from Knoll (Ludwigshafen, Germany). Paroxetine was obtained from SmithKline Beecham (Munich, Germany).

Cytochromes P450 and Human Liver Microsomes. Recombinant CYPs co-expressed with NADPH-P450 oxidoreductase (OR) in insect cells (supersomes™) were purchased from GENTEST (Woburn, MA, USA). Human liver microsomes were prepared from surgically removed liver tissue as described previously (Lang et al., 2001). The study was approved by the ethics committees of the Medical Faculties of the Charité, Humboldt-University Berlin, and of the University of Tübingen, and written informed consent was obtained from each volunteer as well as from each patient.

Chemical Syntheses of Bupropion Hydrochloride, Hydroxybupropion Hydrochloride and [3H3]-Hydroxybupropion Hydrochloride. Bupropion hydrochloride, hydroxybupropion...
hydrochloride and the internal standard \(^{2}\text{H}_{3}\)-hydroxybupropion hydrochloride were synthesized using a modification of the method described by Mehta and Raleigh (1974). In brief, 3-chloropropiophenone or \([3',3',3']^{2}\text{H}_{3}\)-3-chloropropiophenone was brominated and the product used for amination with an excess of the corresponding amine. The resulting raw products were purified by preparing the hydrochlorides and recrystallization from 2-propanol/isoctane. Details will be published elsewhere.

**Chemical Syntheses of 4’- Hydroxymephenytoin** (3-methyl-5-ethyl-5-(4-hydroxyphenyl)-hydantoin) and \([^{2}\text{H}_{3}]\)-4’- Hydroxymephenytoin (3-methyl-5-ethyl-5-(4-hydroxyphenyl)-hydantoin). 5-Ethyl-5-(4-hydroxyphenyl)-hydantoin was prepared from 4-hydroxypropiophenone, potassium cyanide, and ammonium carbonate in 50% ethanol under pressure at 110°C for 24 hours. Methylation of 5-ethyl-5-(4-hydroxyphenyl)-hydantoin with dimethyl sulfate or \([^{3}\text{H}_{6}]\)-dimethyl sulfate and sodium hydroxide in ethanol resulted in 4’-hydroxymephenytoin and \([^{2}\text{H}_{3}]\)-4’-hydroxymephenytoin, respectively.

**Chemical Synthesis of \([^{2}\text{H}_{5}]\)-5-Ethyl-5-phenyl-hydantoin** (\(^{2}\text{H}_{5}\)-nirvanol). \([^{2}\text{H}_{6}]\)-Benzene was acylated with propionyl chloride and aluminum chloride in 1,2-dichloroethane to obtain \([^{2}\text{H}_{5}]\)-propiophenone which was used to synthesize \([^{2}\text{H}_{5}]\)-5-ethyl-5-phenyl-hydantoin with potassium cyanide and ammonium carbonate.

**Determination of Catalytic CYP Activities.** Details for each assay are described below. All assays were performed with recombinant CYPs (2.5–5 pmol) or human liver microsomes (50-100 µg protein) in final volume of 250 µl with inhibitors as indicated. After equilibrating the reaction mixture at 37°C for 3 min, preincubation with inhibitors was started by adding 25 µl of 10-fold concentrated NADPH-regenerating system (final concentrations, 5 mM MgCl₂, 4 mM glucose 6-phosphate, 0.5 mM NADP⁺ and 4 U/ml glucose 6-phosphate dehydrogenase)
and performed for the indicated times at 37°C. Subsequently, enzyme reactions were started by the addition of substrate. Reactions were terminated and processed as described below.

**Bupropion-hydroxylation** was performed with CYP2B6 or human liver microsomes at concentrations indicated in 0.1 M sodium phosphate buffer pH 7.4, and with thiotepa (10 µM) as a CYP2B6 control inhibitor (Rae et al., 2002). Enzyme reactions were carried out using 500 µM bupropion with different incubation times for different assays. The reactions were stopped by adding 50 µl 1 N HCl. After addition of the internal standard d₃-OH-bupropion (100 pmol), the samples were centrifuged at 16,000 g for 5 min. The supernatant was directly injected into the HPLC-system. The metabolite hydroxy-bupropion was separated and detected by HPLC-ESI-mass spectrometry using a HPLC system (HP 1100, Agilent Technologies, Waldbronn, Germany) equipped with a Prontosil-C18 AQ column (150x3 mm, 3 µM particle size, Bischoff, Leonberg, Germany) and a mass spectrometer (Agilent Technologies, Waldbronn, Germany). Elution was performed with a gradient of 16 % (1 % acetic acid/water) and 84 % (1 % acetic acid/acetonitrile) to 45 %/55 % from 0 to 16 min. The dynamic range for detection of hydroxybupropion was 1 to 500 pmol per incubation and assay accuracy over the calibration range was < 8%. Formation of hydroxybupropion was linear with time up to 30 min and linear with protein between 5 and 200 µg of microsomal protein.

**Verapamil O-demethylation and Verapamil N-demethylation** were assayed as described by von Richter et al. (2000). The reaction buffer consisted of 50 mM potassium phosphate buffer, pH 7.4, and 30 mM MgCl₂. Incubations were carried out as described above using 100 µM verapamil and 30 min incubation time with recombinant CYP2C8 for verapamil O-demethylation and 10 min incubation time with CYP3A4 for verapamil N-demethylation. Ketoconazole (100 µM for CYP 2C8, 10 µM for CYP3A4) was used as control inhibitor (in 1
% or 0.1 % DMSO, respectively as vehicle control). The reactions were stopped by the addition of 1.7 ml cold ethanol. After addition of the internal standard [²H₃]norverapamil (50 pmol), samples were vortexed, centrifuged at 16,000 g for 5 min and the supernatant was dried under nitrogen and finally dissolved in 150 µl mobile phase. Analysis of the metabolites D-703, D-702 and norverapamil was performed by HPLC-ESI-mass spectrometry as described for bupropion-hydroxylation assay using a LUNA C8 column (150x3 mm I.D., 5µm particle size, Penomenex, Aschaffenburg, Germany) The metabolites were separated with 5mM ammonium acetate pH 4.2 / acetonitrile as the mobile phase run with a gradient from 29:71 to 50:50 within a run time of 15 min. The dynamic range for detection of verapamil metabolites was 1 to 500 pmol per incubation and assay accuracy over the calibration range was < 14%.

*Propafenone-5-hydroxylation* was analyzed according to Hofmann et al. (2000) in 0.1 M sodium phosphate buffer, pH 7.4, using 5 µM quinidine as control inhibitor. Propafenone concentration was 2 µM and incubations were performed for 20 min. The reactions were stopped by adding 1.7 ml of cold ethanol. After addition of 200 pmol [²H₇]-5-hydroxypropafenone as internal standard, samples were vortexed, centrifuged at 16,000 g for 5 min and the supernatant was dried under nitrogen and finally disssolved in 150 µl mobile phase (70% 12 mM ammonium acetate / 30% acetonitrile). The metabolite 5-hydroxy-propafenone was seperated and detected by HPLC-ESI-mass spectrometry, essentially as described for the verapamil assays using an endcapped Lichrospher RP-18 column (150x3 mm I.D., 5µm particle size, Merck, Darmstadt, Germany). The dynamic range for detection of hydroxybupropion was 5 to 500 pmol per incubation and assay accuracy over the calibration range was < 10%.
S-mephenytoin-N-demethylation and S-mephenytoin-4'-hydroxylation were carried out in 0.1 M sodium phosphate buffer pH 7.4. Control inhibitors were 10 µM sulfaphenazole for S-mephenytoin-N-demethylation (CYP2C9) and 100 µM ketoconazole (1 % DMSO as vehicle) for S-mephenytoin-4'-hydroxylation (CYP2C19). S-mephenytoin concentrations were 1 mM and 200 µM, respectively. The reactions were stopped with 1.7 ml cold ethanol after 30 and 15 min, respectively. [2H3]-4'-OH-S-mephenytoin and [2H5]-nirvanol (100 pmol /each) were added as internal standards. The samples were vortexed and centrifuged at 16,000 rpm for 5 min. The supernatant was dried under nitrogen and finally dissolved in 150 µl of mobile phase consisting of 30 % acetonitrile / 70 % water. The metabolites were analyzed by HPLC-ESI-mass spectrometry using a column as described under propafenone-5-hydroxylation assay. The dynamic range for detection of hydroxybupropion was 1 to 500 pmol per incubation and assay accuracy over the calibration range was < 12%.

7-Ethoxycoumarin O-deethylation was assayed as described by Yamazaki et al. (1999A). Determination of CYP1A2 activity was carried out with 10 µM ethoxycoumarin and CYP2E1 activity was determined using 200 µM ethoxycoumarin. Diethyldithiocarbamate (10 µM) and furafylline (10 µM) were used as control inhibitors for CYP2E1 and CYP1A2, respectively. The formation of umbelliferone was determined fluorometrically with a Wallac 1420 Victor spectrophotometer (Turku, Finland) set at 460 nm using an excitation wavelength of 355 nm. The dynamic range for detection of hydroxybupropion was 5 to 500 pmol per incubation and assay accuracy over the calibration range was < 14%.

Coumarin-7-hydroxylation reactions were performed according to the protocol of GENTEST with minor modifications (GENTEST, Woburn, MA) (http://www.gentest.com/products/tissue_frac/prod_inserts/hlm_meth.shtm). Briefly, the incubation mixture consisted of 200 µM coumarin in 100 mM Tris pH 7.5 and diethyldithiocarbamate (100 µM) was used as
control inhibitor. The reactions were stopped after 15 min with 100 µl of aqueous 20 % trichloroacetic (w/v) acid. The samples were vortexed, centrifuged at 16,000 rpm for 5 min and 100 µl of supernatant was diluted in 1.9 ml of 100 mM Tris buffer pH 9.0. The formation of umbelliferone was determined fluorometrically as described above. The dynamic range for detection of hydroxybupropion was 1 to 500 pmol per incubation and assay accuracy over the calibration range was < 15%.

Inhibition Studies in Human Liver Microsomes. Human liver microsomes (50 –100 µg), bupropion (500 µM) and either clopidogrel or ticlopidine (0.1 - 10 µM) were equilibrated in 0.1 N sodium phosphate buffer, pH 7.4, at 37°C for 3 min. After addition of 25 µl NADPH-regenerating system, reactions were allowed to proceed for 15 min and analyzed as described for the bupropion hydroxylase assay. The effect of nucleophilic trapping agents (10 mM gluthathione or N-acetylcysteine) or scavengers of reactive oxygen species (0.1 % DMSO or 1000 Units of superoxide dismutase) was tested by adding these compounds at the indicated concentrations prior to incubation with inhibitors. Substrate protection was analyzed accordingly with 7-ethoxycoumarin (1 mM) and 0.5 µM clopidogrel or ticlopidine (controls without inhibitors) in the incubation mixture and determination of residual bupropion hydroxylase activity as described above. Substrate protection with the inhibitor paroxetine (50 µM) and 0.5 µM clopidogrel or ticlopidine (controls without inhibitors) was analyzed accordingly after removal of paroxetine as CYP2B6 inhibitor by extensive dialysis as described below.

Dialysis Experiments. Human liver microsomes (100 µg, containing 4.4 pmol CYP2B6, as determined by Western Blot [Lang et al., 2001]) were incubated with or without 10 µM of clopidogrel or ticlopidine and with or without NADPH-regenerating system for 15 min as described for the inactivation assay. The samples were then immediately dialyzed against 0.1
M sodium phosphate buffer, pH 7.4 (3 x 2 l, 2 h each) at 4°C in QuixSep micro dialyzer capsules and a regenerated cellulose tubular membrane with molecular mass cut-off of 12,000 Da (Roth GmbH, Karlsruhe, Germany). Bupropion hydroxylase activity was then determined with 500 µM bupropion and 15 min incubation time as described.

**P450 Reduced CO-Difference Spectroscopy.** Recombinant CYP2B6 (0.6 nM, obtained by expressing wild-type CYP2B6 cDNA in insect cells; details will be published elsewhere) and OR (0.6 nM; purified from rat liver) were incubated in 0.1 N sodium phosphate buffer pH 7.4 in the presence or absence of clopidogrel (10 µM) or ticlopidine (10 µM), respectively. Inhibition was started by adding NADPH-regenerating system. Controls were incubated without NADPH-regenerating system. The reactions were allowed to proceed for 15 min and were then stopped with 1.75 ml quenching buffer (0.1 M sodium phosphate buffer, pH 7.4, 10 % glycerol, 0.5 % Emulgen 911). Dithionite was added, the samples were gently bubbled with CO for 15 sec. and the reduced carbonyl spectrum was recorded between 400 and 500 nm on an Unicam UV/VIS spectrophotometer (Thermo Nicolet, Cambridge, UK). Before termination with quenching buffer, 25 µl samples were taken to determine bupropion hydroxylase activity as described above.

**Kinetic Inhibition Studies with Clopidogrel and Ticlopidine.** All incubations were carried out at 37°C with either recombinant CYP2B6 + OR supersomes (Gentest) or 100 µg human liver microsomes. The samples were equilibrated with different concentrations of clopidogrel and ticlopidine (ranging from 0.05 to 1 µM) for 3 min at 37°C and after the addition of NADPH-regenerating system, the samples were incubated for 0 to 15 min as indicated. Subsequently, 25 µl of the preincubation mixture was transferred to 225 µl of enzyme activity assay mixture, consisting of 0.1 M sodium phosphate buffer, pH 7.4, 500 µM bupropion and NADPH-regenerating system, prewarmed to 37°C. After 6 min of incubation, the reactions
were stopped with 50 µl 1 N HCl. After the addition of 100 pmol of the internal standard, d3-
hydroxy-bupropion, the samples were vortexed and centrifuged for 5 min (16,000 g). The
supernatant was directly injected into the HPLC-system (HP 1100, Agilent Technologies,
Waldbronn Germany) and analyzed as described for bupropion hydroxylation assay.

**Homology Modelling and Docking**

For modelling and docking experiments of CYP2B6 and CYP2C19, previously developed
homology models were used (Bathelt et al., 2002). The docking of clopidogrel into CYP2B6
was done by using AutoDock 3.05. (Morris et al., 1998). Charges were assigned by AMBER
(University of California, San Francisco). The structure of the ligand was calculated using
Gaussian98 (Gaussian Inc, Pittsburgh, PA) with a Hartree-Fock method and the 6-31G* basis
set. For docking, mass-centered grid maps for the active site were generated at 0.18 Å spacing
and 126x126x126 grid points. The Lamarckian genetic algorithm (LGA) and the pseudo-Solis
and Wets methods were applied for minimization. The number of generations was set to
27000. Random starting positions, orientations, and torsions were used for the ligand. 1000
runs were performed with a maximum number of 1.5 · 10^6 energy evaluations. A two-step
procedure was used for classification of the results of each job. First, the calculated free
energy was used to rank the docked conformations. Then all docked conformations with the
lowest distance from the heme oxygen to the hydrogen at position 2 of the thiophene ring
were extracted.

**Data Analysis.** Enzyme kinetic data were analyzed according to the method of Silverman et
al. (1995). The half-time of enzyme inactivation (t1/2) was calculated from the initial slopes of
the remaining enzyme activity, plotted semi-logarithmically against the preincubation time.
The half-time of enzyme inactivation thus obtained was plotted against the reciprocal of the
respective clopidogrel and ticlopidine concentrations (Kitz-Wolson plot). The concentration
required for half-maximal inactivation ($K_I$) and the maximum inactivation rate constant ($k_{\text{inact}}$) were determined from the intercepts on the abscissa and ordinate, respectively.
Results

Effect of Clopidogrel and Ticlopidine on P450 Monooxygenase Activities. Initial experiments were performed to confirm the inhibitory effect of ticlopidine on CYP2C19 (Ha-Duong et al., 2001) and to investigate whether the structure-related clopidogrel had a similar effect. Indeed, incubations of recombinant CYP2C19 with 1µM of clopidogrel or ticlopidine reduced S-mephenytoin-4’-hydroxylation by 60 % and 69 %, respectively (Fig.1). To determine the selectivity of this inhibition towards catalytic activities representing other drug metabolizing CYPs, recombinant supersomes were analysed with appropriate assays using CYP isoform-specific inhibitors as positive controls (Table 1). Surprisingly, recombinant CYP2B6 was even more potently inhibited than CYP2C19. Bupropion hydroxylation, a specific marker reaction for CYP2B6, was inhibited by both clopidogrel and ticlopidine by more than 90 % at 1 µM concentration. Incubations of human liver microsomes with 1 µM clopidogrel or ticlopidine and NADPH-regenerating system also reduced bupropion hydroxylase activity by up to 98 % (data not shown). Control incubations with NADPH-regenerating system in the absence of inhibitor showed an approximately 20 % loss of enzyme activity during 30 min incubation time which was not prevented by the addition of catalase (data not shown). At 10 µM, both bupropion-hydroxylation and S-mephenytoin-4’-hydroxylation were almost completely inhibited by both substances. A difference in selectivity between clopidogrel and ticlopidine (10 µM) was observed in that the former also inhibited CYP2C9 by 53 %, whereas the latter inhibited CYP1A2 by 51 % (Fig.1). Other CYPs were still not markedly affected at this concentration.

Mechanism-Based Inactivation of CYP2B6 by Clopidogrel and Ticlopidine. Kinetic experiments in human liver microsomes revealed unusual inhibition kinetics not explained by reversible inhibition mechanisms (data not shown). In particular, we observed that inactivation of bupropion-hydroxylation was concentration- and time-dependent if
microsomes were preincubated with clopidogrel and ticlopidine. To investigate whether the inactivation was the result of an irreversible mechanism, we performed dialysis experiments in which liver microsomes were incubated with both compounds in the presence or absence of NADPH-regenerating system. As shown in Fig. 2, extensive dialysis of samples incubated with inhibitor (10 µM) and NADPH-regenerating system did not lead to recovery of bupropion-hydroxylase activity whereas samples incubated with inhibitor in the absence of NADPH-regenerating system regained full activity compared to controls without inhibitor. Furthermore, figure 3 shows that inhibition of CYP2B6 by the thienopyridines was attenuated by the presence of alternative active site ligands. Addition of the competitive inhibitor, paroxetine (50 µM; Hesse et al., 2000), to the primary incubation mixture together with clopidogrel (0.5 µM) completely protected the enzyme from being inhibited, whereas less effective protection was observed towards ticlopidine as the inhibitor. The alternative substrate, 7-ethoxycoumarin, showed the opposite selectivity, namely higher protective effect towards ticlopidine (Fig. 3). Taken together these results demonstrated unequivocally the irreversible and mechanism-based inhibition type by both thienopyridine derivates. To further characterize the inactivation of CYP2B6, the effect of the nucleophilic trapping agents glutathione (10 mM) and N-acetylcysteine (10 mM) and the effect of the reactive oxygen scavengers DMSO (0.1 %) and superoxide dismutase (1000 Units) was tested by incubating human liver microsomes (50 µg) and inhibitors (10 µM) as described in the Materials and Methods section. None of the agents had a measurable effect on CYP2B6 inhibition caused by clopidogrel and ticlopidine (data not shown). To analyze whether inhibition of bupropion hydroxylase activity was due to destruction of the heme moiety, we recorded reduced CO-difference spectra of recombinant CYP2B6 incubated with clopidogrel and ticlopidine in the presence or absence of NADPH-regenerating system. There was no visible destruction of the heme component compared to controls without inhibitors under conditions where enzymatic activity was completely blocked (data not shown).
**Kinetic Analysis of Inactivation of Bupropion-Hydroxylation.** Detailed kinetic investigation of mechanism-based inhibition reactions requires separation of the inactivation step from observation of substrate metabolism, which is usually achieved by dialysis or by diluting the incubation mixtures. We therefore determined residual bupropion hydroxylase activity in 10-fold diluted incubation mixtures after preincubation with inhibitor (see Method section). Inactivation was time- and concentration-dependent in both cases, proceeded very rapidly and showed saturation. Figure 4 shows the microsomal inactivation kinetics of clopidogrel and ticlopidine, respectively, at various concentrations of inhibitor. Inactivator concentrations for half-maximal inactivation ($K_i$) due to mechanism-based enzyme inhibition were calculated to be 0.5 µM for clopidogrel and 0.2 µM for ticlopidine by transferring the data into a Kitz-Wilson plot (inserts in figures 4 and 5). The inactivation process was of a non-pseudo-first-order type. $K_{\text{inact}}$, the maximal rate of inactivation at saturating concentrations of clopidogrel and ticlopidine was 0.35 min$^{-1}$ and 0.5 min$^{-1}$, respectively. Accordingly, the time required for half of the enzyme molecules to be inactivated ($t_{1/2}$) was 2 min and 1.4 min, respectively. Figure 5 shows the inactivation of recombinant CYP2B6 by clopidogrel and ticlopidine, respectively, which was very similar to that in human liver microsomes and was analyzed as described above. $K_i$ were 1.1 µM and 0.8 µM, respectively. $K_{\text{inact}}$ for clopidogrel and ticlopidine were 1.5 min$^{-1}$ and 0.8 min$^{-1}$ and $t_{1/2}$ were 0.5 min and 0.9 min, respectively.
Discussion

The present in-vitro study provides the first data on the cytochrome P450 interaction profile of the platelet aggregation inhibitor, clopidogrel, and demonstrates that it acts as a potent mechanism-based inhibitor of human CYP2B6. In addition, the structure-related derivative ticlopidine was shown to inhibit CYP2B6 by a similar mechanism and potency. These conclusions are based on experiments with human liver microsomes and with recombinant CYPs in which bupropion hydroxylation was measured as CYP2B6-selective marker enzyme activity (Faucette et al., 2000; Hesse et al., 2000). The specific findings were that inhibition of bupropion hydroxylation by clopidogrel and by ticlopidine was 1) time- and concentration-dependent; 2) NADPH-dependent; 3) irreversible; 4) not affected by scavengers of reactive oxygen species or by nucleophilic trapping agents; 5) reduced by the presence of alternative active site ligands, and 6) not associated by a decrease in spectrally detected P450.

Investigation of the inhibition profiles of the two drugs included the nine CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, representing the major drug-metabolizing CYPs in human liver. In addition to CYP2B6, CYP2C19 was also inhibited by both substances, albeit at lower potency. Whereas mechanism-based inhibition of CYP2C19 by ticlopidine has been reported before, the more potent inhibition of CYP2B6 by this substance had been overlooked in that study because CYP2B6 was not included (Ha-Duong, 2001). Differences between clopidogrel and ticlopidine with respect to the CYP-inhibition profile were only observed at higher concentrations in that the former also inhibited CYP2C9, whereas the latter inhibited CYP1A2, but these inhibitions did not exceed about 50% at 10 µM inhibitor concentration, compared to over 90% inhibition of CYP2B6 at 1 µM concentration (Figure 1).

Time- and concentration-dependent inactivation of bupropion hydroxylase activity in human liver microsomes and in recombinant CYP2B6-expressing insect cell membranes proceeded with almost identical characteristics. Inactivation proceeded very rapidly within the first few
minutes and slowed down thereafter, probably due to consumption of the inhibitor by metabolism or by hydrolysis. The irreversible and NADPH-dependent nature of inhibition was demonstrated by dialysis experiments. Inhibition of bupropion hydroxylation occurred only in the presence of NADPH during incubation with the inhibitor, strongly suggesting that catalytic turnover was required for inhibition (Fig. 2). Although these experiments were performed with microsomal protein, it is unlikely that another CYP than 2B6 is involved in the activation of the inhibitor, because recombinant 2B6 was inhibited with practically the same potency as the microsomal enzyme (Figures 4 and 5) and because the microsomal inhibition was not affected by scavengers of reactive oxygen species or by nucleophilic trapping agents. Further evidence for the involvement of the CYP2B6 active site in the activation of the thienopyridine inhibitors is provided by the observation that alternative active site ligands effectively attenuated their inhibitory potency. An interesting observation was made in that the competitive CYP2B6 inhibitor, paroxetine, was a more potent attenuator of clopidogrel-mediated inhibition, whereas the alternative substrate, 7-ethoxycoumarin, had a more potent effect on ticlopidine-mediated inhibition. It is conceivable that these differences reflect subtle differences between these substances regarding their steric occupation of the enzyme’s active site.

Spectral analysis of recombinant CYP2B6 did not indicate any destruction of the heme moiety during inhibition (data not shown), suggesting that alkylation of the apoprotein should be the major event leading to inhibition. Although the precise mechanism remains to be investigated, reasonable speculations are possible on the basis of hemoprotein stability and in analogy to the known mechanism of irreversible inhibition of the P2Y12-ADP-receptor. The initial step in the microsomal activation of clopidogrel was shown to be cytochrome P450-dependent oxidation to 2-oxo-clopidogrel (Savi et al., 2000). The major contribution to this step appears to be catalyzed by CYP3A4, although only indirect evidence was provided in a recent study.
(Clarke and Waskell, 2003). Despite not being active in vitro, antiaggregating activity of 2-oxo-clopidogrel was demonstrated ex vivo suggesting that it represents an intermediate which can be converted to the active metabolite. The structure of the active metabolite was finally determined to be a hydrolyzed derivative with an opened thiophene ring and a highly reactive thiol function (Figure 6; Pereillo et al., 2002) which blocks the receptor by forming disulfide bonds with extracellular cysteine residues (Ding et al., 2003). By analogy, a similar mechanism may explain cytochrome P450 inhibition by thienopyridines. The first step could be conversion to the 2-oxo-derivative by the CYP. This intermediate may then form a disulfide bond with an available cysteine, either following hydrolysis of the 2-oxo-derivative like in the case of ADP-receptor alkylation, or, alternatively, after another cycle of CYP-dependent oxidation (Fig. 6). This mechanism was shown to be consistent with protein homology modelling results. Docking of clopidogrel into the binding site of CYP2B6 resulted in a number of complexes with similar energy between –8 and –9 kcal/mol. In 1% of the complexes, clopidogrel was oriented with the hydrogen in the 2-position of the thiophene ring at a distance between 3.0 and 3.5 Å to the reactive oxygen of the heme (Fig. 7). Because this position is chemically highly reactive, a small percentage of correctly oriented species would be sufficient for a highly regioselective oxidation. The product 2-oxo-clopidogrel would leave the active site through the substrate access channel. Interestingly, comparative modelling showed that in both CYP2B6 and CYP2C19, the metabolite would get into tight contact to a cysteine located near the substrate channel (not shown). Of course, other reaction mechanisms, which may involve thiophene S-oxidation or epoxidation, for example, can not be excluded at this time.

Other potent mechanism-based inhibitors of CYP2B6 include a series of xanthates which are however not in clinical use (Yanev et al., 1999), 17-α-ethynylestradiol (Kent et al., 2001), certain methyladamantane derivatives (Stiborova et al., 2002), and phencyclidine.
Potent competitive inhibitors are the antiretroviral drugs ritonavir, efavirenz, and nelfinavir (Hesse et al., 2001) and the triethylenethiophosphoramide thioTEPA (Rae et al., 2002). Compared to these inhibitors, the results of this study revealed clopidogrel and ticlopidine as the most potent inhibitors of CYP2B6 known to date with $K_I$ values for the microsomal inactivation of about 0.5 and 0.2 µM, respectively. It is therefore important to consider the pharmacological consequences of this finding. Clinically relevant interactions between substrates of CYP2C19 and ticlopidine have already been reported, although CYP2C19 is inhibited with lower potency (Donahue et al., 1997, Tateishi et al., 1999). The pharmacological significance of CYP2B6 has long remained unrecognized, in part due to the lack of suitable probes (Ekins et al., 1999). Furthermore, the content of CYP2B6 in human liver was recently shown to be much higher than previously estimated (Gervot et al., 1999, Lang et al., 2000). The growing list of clinically relevant substrates of CYP2B6 includes the antidepressant and antismoking agent bupropion, which is almost exclusively metabolized by this isozyme (Hesse et al, 2000); the antineoplastic agents cyclophosphamide and ifosfamide, the former of which is metabolically activated mainly by CYP2B6 with some contributions of CYPs 3A4 and 2C9 (Roy et al., 1999), whereas the latter is being deactivated (Granvil et al., 1999). CYP2B6 has also been shown to catalyze the major route of metabolism for the anesthetics propofol (Court et al., 2001) and ketamine (Yanagihara et al., 2001), the MAO-B inhibitor selegiline (Hildestrand et al., 2001), and the antiretroviral agent efavirenz (Ward et al., 2003). CYP2B6 also contributes to the metabolism of environmental toxicants and substances of abuse like nicotine and others (Yamazaki et al., 1999B). Since these drugs are widely used, drug interactions with clopidogrel or ticlopidine may not be uncommon, although none have been reported to date to our knowledge. At least one example of a clinically relevant drug interaction involving CYP2B6 has however been described. The anticancer drug thioTEPA was shown to cause a significant reduction in the plasma levels of the active metabolite of cyclophosphamide when administered first (Huijtena et al., 2000). As
mentioned above, thioTEPA was found to be a potent and selective inhibitor of CYP2B6 (Rae et al., 2002).

In conclusion, we found that the two thienopyridines, clopidogrel and ticlopidine, are highly potent, irreversible inhibitors of CYP2B6. We provided strong evidence that inhibition involves a mechanism-based process and we suggested a chemical mechanism which has to be investigated in further detail in future experiments. Since clopidogrel and ticlopidine are amongst the most potent CYP2B6 inhibitors known today, these findings may be of clinical relevance. In addition, these substances may be useful as in vitro or in vivo probes to estimate the relative contribution of CYP2B6 to drug metabolism.
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References


Footnotes

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Legends to figures

Figure 1: Selectivity of inhibition by clopidogrel and ticlopidine in recombinant CYP enzymes. Cytochrome P450 enzymes recombinantly coexpressed with OR in insect cells (supersomes™) were incubated with 1 µM (hatched bars) or 10 µM (filled bars) of clopidogrel (A) or ticlopidine (B). CYP activities were determined using appropriate marker drug assays as detailed in Table 1. For each CYP, control activity measured in the absence of inhibitor was set at 100%. Data represent the means of two independent experiments.

Figure 2: Mechanism-based irreversible inhibition of microsomal bupropion hydroxylation. Human liver microsomes (100 µg) were incubated for 15 min with NADPH-regenerating system and 10 µM of clopidogrel or ticlopidine, respectively. Controls were incubated without inhibitor and with or without NADPH-regenerating system, as indicated. After extensive dialysis, residual bupropion hydroxylase activity was determined. The means of two independent experiments are shown.

Figure 3: Substrate protection by alternative active site ligands. Human liver microsomes (100 µg) were incubated with either clopidogrel (C, hatched bars; 0.5 µM) or ticlopidine (T, open bars; 0.5 µM) in 0.1 N sodium phosphate buffer, pH 7.4, at 37°C for 3 min in the presence or absence of either paroxetine (P; 50 µM) or 7-ethoxycoumarine (E; 1 mM). After addition of 25 µl NADPH-regenerating system, reactions were allowed to proceed for 15 min. Residual bupropion hydroxylase activity was determined as described in the Method section. Results represent the means of two independent experiments.

Figure 4: Kinetics of inactivation of microsomal bupropion hydroxylation by different concentrations of clopidogrel (A) and ticlopidine (B). For details on incubation and determination of activity...
see Materials and Methods section. Human liver microsomes (100 µg) were incubated for the indicated times in the presence of NADPH-generating system and clopidogrel or ticlopidine as indicated: ◆, 0 µM; ■, 0.05 µM; ▲, 0.1 µM; ●, 0.2 µM; □, 0.3 µM; Δ, 0.4 µM; ○, 0.5 µM; ◊, 1 µM. The inserts show the corresponding Kitz-Wilson plots.

**Figure 5:**
Kinetics of inactivation of recombinant CYP2B6 by different concentrations of clopidogrel (A) and ticlopidine (B), respectively. For details on incubation and determination of activity see Materials and Methods section. SupersomesTM (5 pmol) from insect cells expressing CYP2B6 were incubated for indicated periods in the presence of NADPH-generating system and clopidogrel or ticlopidine as indicated: ◆, 0 µM; ■, 0.05 µM; ▲, 0.1 µM; ●, 0.2 µM; □, 0.3 µM; Δ, 0.4 µM; ○, 0.5 µM; ◊, 1 µM. The inserts show the corresponding Kitz-Wilson plots.

**Figure 6:** Possible mechanism for irreversible inhibition of CYP2B6 by clopidogrel. The hypothetical mechanism is based on a) the known metabolic activation and pharmacological action of clopidogrel (left part), and b) the observation that inhibition is not accompanied by heme destruction. Alternative pathways leading to the inactivated CYP may proceed via hydrolysis or via an additional oxidative step catalyzed by the CYP.

**Figure 7:** Clopidogrel docked to the active site of CYP2B6 in an orientation favorable for oxidation of the 2-position of the thiophene ring. Stick model of the substrate (yellow), heme, and residues which form the substrate binding pocket.
**TABLE 1:**

Summary of incubation conditions for cytochrome P450 marker assays

<table>
<thead>
<tr>
<th>CYP</th>
<th>Reaction</th>
<th>Substrate concentration</th>
<th>Control inhibitor</th>
<th>Inhibitor concentration</th>
<th>Residual activity¹,²,³</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>7-Ethoxycoumarin O-deethylation</td>
<td>10 µM</td>
<td>Furafylline</td>
<td>10 µM</td>
<td>8.4 %</td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>200 µM</td>
<td>Diethyldithiocarbamate</td>
<td>100 µM</td>
<td>30.7 %</td>
</tr>
<tr>
<td>2B6</td>
<td>Bupropion hydroxylation</td>
<td>50 µM</td>
<td>Thiotepa</td>
<td>10 µM</td>
<td>11.8 %</td>
</tr>
<tr>
<td>2C8</td>
<td>Verapamil O-demethylation</td>
<td>100 µM</td>
<td>Ketoconazole</td>
<td>100 µM</td>
<td>n.d.²</td>
</tr>
<tr>
<td>2C9</td>
<td>S-Mephenytoin N-demethylation</td>
<td>10 µM</td>
<td>Sulfaphenazole</td>
<td>10 µM</td>
<td>34.7 %</td>
</tr>
<tr>
<td>2C19</td>
<td>S-Mephenytoin 4'-hydroxylation</td>
<td>200 µM</td>
<td>Ketoconazole</td>
<td>100 µM</td>
<td>41.7 %</td>
</tr>
<tr>
<td>2D6</td>
<td>Propafenone 5-hydroxylation</td>
<td>2 µM</td>
<td>Quinidine</td>
<td>5 µM</td>
<td>40.7 %</td>
</tr>
<tr>
<td>2E1</td>
<td>7-Ethoxycoumarin O-deethylation</td>
<td>200 µM</td>
<td>Diethyldithiocarbamate</td>
<td>1 µM</td>
<td>1.2 %</td>
</tr>
<tr>
<td>3A4</td>
<td>Verapamil N-demethylation</td>
<td>100 µM</td>
<td>Ketoconazole</td>
<td>100 µM</td>
<td>18.2 %</td>
</tr>
</tbody>
</table>

¹ Assay conditions were as described under Materials and Methods.

² Results are means of two to four independent experiments; n.d., not detectable.

³ Control activity without inhibitor was set at 100%
Figure 1A

Residual enzyme activity (% control activity)

0  50  100

1A2  2A6  2B6  2C8  2C9  2C19  2D6  2E1  3A4
Figure 1B

Residual enzyme activity (% control activity)

B

0 50 100 150

1A2 2A6 2B6 2C8 2C9 2C19 2D6 2E1 3A4
Figure 2
Figure 3
Figure 4B

The figure shows a graph with the following axes:

- **Log % residual activity** is plotted on the y-axis.
- **Preincubation time (min)** is plotted on the x-axis.
- **1/ticlopidine (µM⁻¹)** is plotted as an inset on the lower right corner of the graph.

The graph illustrates the relationship between log % residual activity and preincubation time at different concentrations of ticlopidine.
Figure 5A

![Graph showing the relationship between log residual activity and preincubation time, with data points indicating the effect of clopidogrel concentration.](image-url)
Figure 5B
Fig. 6

pharmacologic mechanism  possible inhibition mechanism
Figure 7