

**ANTIPLATELET AGENTS ASPIRIN AND CLOPIDOGREL ARE
HYDROLYZED BY DISTINCT CARBOXYLESTERASES AND CLOPIDOGREL IS
TRANSESTERIFICATED IN THE PRESENCE OF ETHYL ALCOHOL**

Man Tang, Madhu Mukundan, Jian Yang, Nathan Charpentier, Edward L. LeCluyse
Chris Black, Dongfang Yang, Deshi Shi and Bingfang Yan

*Department of Biomedical and Pharmaceutical Sciences
University of Rhode Island
Kingston, RI 02881*

Affiliations: Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island
Kingston, RI 02881 (MT, MM, JY, NC, DY, DS, BY); CellzDirect, 1624 Headway Circle Suite 100,
Austin, TX 78754 (ELL, CB)

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Address Correspondence to: Dr. Bingfang Yan
Department of Biomedical and Pharmaceutical Sciences
University of Rhode Island
Kingston, RI 02881

Phone: (401) 874-5032
Fax: (401) 874-5048
E-mail: byan@uri.edu

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Abbreviations: CYP, cytochrome P450; HCE, human carboxylesterase; IS, internal standard; HPLC, high performance liquid chromatography; MTT, 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide.

ABSTRACT

Aspirin (acetylsalicylic acid) and clopidogrel are two major anti-thrombogenic agents that are widely used for the treatment and prevention of cerebro- and cardio-vascular conditions such as stroke. Combined use produces enhanced therapeutic effect. Aspirin and clopidogrel both are esters, and hydrolysis leads to decreased or inactivated therapeutic activity. The aim of the study was to determine whether aspirin and clopidogrel are hydrolyzed by the same enzyme(s), thus reciprocally prolong the anti-thrombogenic activity. In order to test this possibility, microsomes from the liver and intestine were assayed for the hydrolysis of aspirin and clopidogrel. In contrary to the hypothesis, aspirin and clopidogrel were hydrolyzed in a tissue-differential manner. Liver microsomes hydrolyzed both drugs, whereas intestinal microsomes hydrolyzed aspirin only. Consistent with the tissue distribution of two carboxylesterases HCE1 and HCE2, recombinant HCE1 hydrolyzed clopidogrel, whereas recombinant HCE2 hydrolyzed aspirin. In addition, hydrolysis of clopidogrel among liver samples was correlated well with the level of HCE1, and hydrolysis of aspirin with HCE2. Certain natural variants differed from the wild-type enzymes on the hydrolysis of aspirin or clopidogrel. In the presence of ethyl alcohol, clopidogrel is converted to ethyl clopidogrel. Carboxylesterases are important pharmacological determinants for drugs containing ester linkages, and exhibit a large inter-individual variation. The isoform-specific hydrolysis of aspirin and clopidogrel suggests that these two anti-thrombogenic agents may have pharmacokinetic interactions with different sets of ester drugs, and the altered hydrolysis by polymorphic mutants provides a molecular explanation to the inter-individual variation.

INTRODUCTION

Platelets are non-nucleated and represent the smallest cellular component in the blood (Gawaz et al., 2005). These cells, also called thrombocytes, play essential roles in thrombogenesis. During thrombogenesis, platelets undergo aggregation, and aggregated platelets are constituents of thrombi (Gawaz et al., 2005). Platelet aggregation is coordinated by several signaling pathways. Adenosine diphosphate (ADP), for example, activates purinergic receptor P2Y₁₂, evokes morphological changes of platelets and initiates platelet aggregation (Oury et al., 2006). Likewise, thrombogenic molecule thromboxane A₂ activates prostaglandin/thromboxane receptors and induces platelet aggregation and vasoconstriction (Huang et al., 2004). Formation of blood plug or clot is critical in maintaining the continuity of blood vessels and controlling bleeding. However, excessive thrombogenesis is recognized as one of the primary factors involved in the development of cardio- and cerebro-vascular diseases such as heart attack and stroke (Grosser et al., 2006; Lindsberg and Grau, 2003). Therefore, anti-thrombotic therapy has been increasingly used for prophylactic purposes among people who have an increased tendency of thrombogenesis.

Aspirin and clopidogrel are two widely used anti-thrombogenic agents, and co-administration produces enhanced therapeutic effect in many clinical conditions, particularly in high-risk patients with acute coronary syndromes (Cooke et al., 2006). Aspirin and clopidogrel differ on the mechanism of action. Clopidogrel targets the P2Y₁₂ receptor (Savi and Herbert, 2005), whereas aspirin inhibits cyclooxygenase activity (Wu, 2003), an enzymatic action critical in the production of thromboxane A₂. In both cases, covalent modifications are involved, leading to irreversible inhibition. Aspirin acetylates the active-site serine-530 of cyclooxygenases, however, clopidogrel does not directly modify its target P2Y₁₂ receptor (Savi and Herbert, 2005). Instead, clopidogrel is in vivo converted by CYP enzymes and eventually becomes a reactive thiol metabolite (Savi et al., 2000). It is the thiol metabolite that forms disulfide bond with cysteines in the P2Y₁₂ receptor and blocks its signaling.

Aspirin and clopidogrel, on the other hand, share an important chemical feature: both contain a single ester linkage (Needs and Brooks, 1985; 2002). More importantly, hydrolysis has profound pharmacological significance. Clopidogrel is hydrolyzed to clopidogrel carboxylate, and this metabolite does not have any therapeutic activity (Caplain et al., 1999). Salicylic acid, the hydrolytic metabolite of aspirin, on the other hand, retains the inhibitory ability against cyclooxygenases (Hinz et al., 2000). However, the inhibition is competitive and significantly less potent (only 10% of aspirin). It appears that hydrolysis of both drugs takes place in the gastrointestinal track and/or the liver, because the hydrolytic metabolites, immediately following oral administration, are the predominant forms in the systemic circulation (Heestermans et al., 2006; Kees et al., 1996). In addition, the overall hydrolysis of both drugs exhibits a large inter-individual variation (Lares-Asseff et al., 2004; Slugg et al., 2000), suggesting that the enzyme(s) for the hydrolysis is expressed differentially depending on individuals or there are polymorphic variants for this enzyme.

The liver expresses two major carboxylesterases including HCE1 and HCE2 (human carboxylesterase), whereas the gastrointestinal tract expresses predominately HCE2 (Kroetz et al., 1993; Pindel et al., 1997; Schwer et al., 1997). In addition, both enzymes are expressed lower in young infants (Pope et al., 2005). The aim of this study was to determine whether HCE1 and HCE2 are involved in the hydrolysis of aspirin and clopidogrel. While liver microsomes rapidly hydrolyzed both esters, the intestinal microsomes hydrolyzed aspirin only. Recombinant HCE1 but not HCE2 hydrolyzed clopidogrel, whereas aspirin was primarily hydrolyzed by HCE2. Clopidogrel profoundly inhibited cell proliferation, and the cytotoxic effect was completely abolished by transfection of HCE1. In the presence of ethyl alcohol, clopidogrel is converted to ethyl clopidogrel. These findings provide mechanistic insight into how individual carboxylesterase is involved in the pharmacological determination and toxicity of these two widely used anti-platelet agents.

MATERIALS AND METHODS

Chemicals and supplies

Aspirin, MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) and *para*-nitrophenyl-acetate were from Sigma (St. Louis, MO). Clopidogrel bisulfate was purchased from ChemPacific (Baltimore, MD). Clopidogrel carboxylate was purchased from Toronto Research Chemicals (Canada). The goat anti-rabbit-IgG conjugated with alkaline phosphatase was from Pierce (Rockford, IL). Cell culture media, LipofectAMINE and Plus Reagent were purchased from Invitrogen (Carlsbad, CA). Human liver microsomes were from CellDirect (Austin, TX). Human intestinal microsomes were from BD Bioscience (San Diego, CA). Normal human plasma was from Rhode Island Blood Bank (Providence, RI). Unless otherwise indicated, all other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Enzymatic assays (hydrolysis and transesterification)

Hydrolysis of aspirin was carried out at 37°C in a total volume of 100 µl. Samples (100 µg protein) were prepared in 50 µl phosphate buffer (0.1 M, pH 7.4) and then mixed with an equal volume of aspirin (usually 2 mM) in the same buffer. The incubations lasted for 10-120 min depending on the samples and the reactions were terminated with 150 µl of acetonitrile containing acetaminophen (38 ng/ml) as the IS (internal standard). The reaction mixtures were subjected to centrifugation for 15 min at 4°C (13,000 g). Hydrolysis of clopidogrel was carried out similarly as that of aspirin. Samples (12-25 µg protein) were prepared in 50 µl reaction buffer Tris-HCl (50 mM, pH 7.4), mixed with an equal volume of clopidogrel (usually 200 µM) in the same buffer, and incubated at 37°C for 20 min. The reactions were terminated and centrifuged as described for the hydrolysis of aspirin, however, naproxen (0.5 µg/ml) served the IS. The transesterification of clopidogrel was conducted similarly as the hydrolysis of this compound. However, the substrate was first mixed with ethyl alcohol at various concentrations (0-200 mM) before added to the samples (liver microsomes and lysates of HCE1-transfected cells). It should be noted that

the amount of proteins and incubation time varied depending on samples, which ensured the formation of the products was within the linear range. Typically, hydrolysis of aspirin (1 mM) with 25 μ g liver microsomes was linear time up to 60 min, whereas hydrolysis of clopidogrel (100 μ M) with 10 μ g liver microsomes up to 40 min.

High performance liquid chromatography (HPLC)

The formation of the hydrolytic metabolites was determined by HPLC (Hitachi-300) with a Chromolith SpeedROD column RP-18e (Merck, Germany). The supernatants (10-30 μ l) of the reaction mixtures were injected and separated by an isocratic (for aspirin) or gradient (clopidogrel) mobile phase. The isocratic mobile phase consisted of 12 % methanol and 0.25 % acetate acid at pH 3.9. The gradient mobile phase consisted of 4-21% acetonitrile (v/v) for the first 4 min and 21-70% for second 4 min. The flow rate for both aspirin and clopidogrel was 2 ml/min and the metabolites were detected by a diode array detector at 238 nm (salicylic acid) or 230 nm (clopidogrel carboxylate). All quantifications were performed using peak area ratios and calibration curves generated from the corresponding internal control, otherwise specified. The standard curves were generated with various concentrations of salicylic acid (0.8 to 110 μ g/ml) or clopidogrel carboxylate (0.48 to 61.2 μ g/ml). In both cases, the correlation coefficient consistently exceeded 0.9995. The inter-day values of relative standard deviation were less than 1.5%. The chemical identity of ethyl clopidogrel was confirmed in 60:40 acetonitrile:Tris-HCl (pH 7.4, 20 mM) by electrospray mass spectrometric analysis (PE Biosystems Marine API 2000 time of flight).

Plasmid constructs and site-directed mutagenesis

Expression constructs encoding human (HCE1 and HCE2) and rat carboxylesterases (hydrolase A and hydrolase B) were described elsewhere (Xie et al., 2002). Natural variants of HCE1 and HCE2 were prepared by site-directed mutagenesis as described previously (Song et al., 2005). Briefly,

complementary oligonucleotides were synthesized to introduce a substitution. The primers were annealed to the corresponding wild type construct (accession number of HCE1: L07764; accession number of HCE2 U60553) and subjected to a thermocycler for a total of 15 cycles. The resultant PCR-amplified constructs were then digested with Dpn I to remove the non-mutated parent construct. The mutated PCR-amplified constructs were used to transform XL1-Blue bacteria. All mutated constructs were subjected to sequencing analysis to confirm the desired mutation being made without secondary mutations.

Transfection

Human embryonic kidney cells (293T) were plated at a density of 60% in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. After reaching 80% confluence, cells were transfected by LipofectAMINE and Plus Reagent. A plasmid construct or the empty vector (4 µg/per 100 mm dish) was initially mixed with 20 µl Plus Reagent diluted in 750 µl serum-free medium for 15 min, and then mixed with 30 µl LipofectAMINE Reagent diluted in 5 ml serum-free medium for 15 min. The final transfection complexes were added to a monolayer of 293T cells. After a 3 h-incubation, the medium was replaced by normal culture medium and incubated for 48 h in a 37°C humidified incubator with 5.0% CO₂. Cells were rinsed and harvested in 1.5 ml Tris-HCl buffer (50 mM, pH 7.4). The cell suspension was sonicated and cell debris was removed by centrifugation at 13,000 g for 15 min at 4°C. The supernatant was assayed for hydrolytic activity toward clopidogrel, aspirin or *para*-nitrophenylacetate.

Cytotoxicity assay

Cells (293T) were cultured in 6-well plates and transfected with the vector or a construct encoding HCE1 or HCE2 as described above (1 µg/per well). After a 12-h incubation, the cells were collected and seeded into 96-well plates at a density of 10,000/well. After additional 12-h incubation, the medium was replaced with reduced serum medium (1%) containing aspirin or clopidogrel at various concentrations, and the treatment lasted for 36 h. MTT was then added to each well at a final concentration of 1 mg/ml.

After 4-h incubation at 37°C, the medium was gently decanted, and DMSO (150 µl/well) was added to dissolve formazan product. The optical density (OD) was determined at 570 nm, and the final OD values were expressed by subtracting the background reading (no seeded cells).

Other assays

Protein concentration was determined with Micro BCA Reagents (Pierce) as described by the manufacturer. Antibodies against distinct carboxylesterases were described elsewhere (Xie et al., 2002; Zhu et al., 2000). The relative intensity of Western blots was densitometrically quantified by KODAK 1D Image Analysis Software. Data are presented as mean \pm SD of at least three separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Comparisons between two values were made with Student's test at $p < 0.05$.

RESULTS

Aspirin is hydrolyzed by liver and intestinal microsomes but clopidogrel by liver microsomes only

Aspirin and clopidogrel both contain a single ester bond. Following oral administration, predominant hydrolytic metabolites are present in the systemic circulation (Heestermans et al., 2006; Kees et al., 1996). Such a rapid conversion suggests that effective first-pass elimination occurs with both clopidogrel and aspirin. The initial effort was made to determine whether both agents are comparably hydrolyzed by microsomes from the liver and the gastrointestinal track, two tissues that constitute the first-pass elimination of therapeutic agents. Microsomes were used because they contain the highest carboxyl-esterase activity (Morgan et al., 1994). In addition to microsomes, plasma from health individuals was included as well. The metabolism was monitored by HPLC, and Fig. 1A shows the representative chromatograms. For aspirin metabolism, the retention time of acetaminophen (IS), salicylic acid and aspirin was 1.04, 2.43 and 4.00 min, respectively. For clopidogrel hydrolysis, the retention time of naproxen (IS), clopidogrel carboxylate, and clopidogrel was 1.92, 3.85, and 8.75 min, respectively.

The hydrolysis of aspirin and clopidogrel was determined with pooled samples (at least from 5 individuals). Both aspirin and clopidogrel were rapidly hydrolyzed by liver microsomes (Fig. 1B). In contrast, intestinal microsomes hydrolyzed aspirin only (Fig. 1B). Plasma, on the other hand, hydrolyzed neither clopidogrel nor aspirin, although evident hydrolysis was detected toward *para*-nitrophenylacetate (Fig. 1B). These tissues differ markedly on the levels of HCE1 and HCE2. Both HCE1 and HCE2 are abundantly expressed in the liver (Kroetz et al., 1993; Schwer et al., 1997), whereas the gastrointestinal track expresses predominately HCE2 (Schwer et al., 1997). Neither HCE1 nor HCE2 is present in the plasma (Li et al., 2005). Therefore, the differential hydrolysis of aspirin and clopidogrel by these samples suggest that aspirin is a preferable substrate of HCE2, whereas clopidogrel is a preferable substrate of HCE1.

Hydrolysis by recombinant carboxylesterase HCE1 and HCE2

In order to definitively establish the catalytic preference of HCE1 and HCE2 toward clopidogrel and aspirin, hydrolysis was determined with recombinant HCE1 and HCE2. In addition, recombinant hydrolase A and B, two rat carboxylesterases, were included. Hydrolase A and B are generally considered as rat counterparts of HCE1 and HCE2, respectively (Kroetz et al., 1993; Morgan et al., 1994; Schwer et al., 1997), although rats express more forms of carboxylesterases than humans (based on the searching of the respective genome database). Consistent with the predication, lysates from HCE1- or hydrolase A-transfected cells were highly active toward clopidogrel, whereas lysates from HCE2 or hydrolase B-transfected cells were highly active toward aspirin (Fig. 2A). No hydrolysis of clopidogrel was detected with the lysates from HCE2 or hydrolase B-transfected cells (Fig. 2A). In contrast, some hydrolysis of aspirin (less than 5% of that by HCE2 or hydrolase B) was detected with the lysates from HCE1- or hydrolase A-transfected cells. Western analysis confirmed comparable expression of carboxylesterases in respectively transfected cells (data not shown).

In order to gain kinetic insight, parameters such as K_m were determined with recombinant HCE1 and HCE2. The hydrolytic rate was determined as a function of substrate concentrations. Aspirin was assayed from 0.025 to 5.00 mM, whereas clopidogrel was assayed from 3 to 300 μ M. Both HCE1 and HCE2 were used for the kinetic study on aspirin, but the kinetic parameters on clopidogrel hydrolysis were determined with HCE1 only. As shown in Fig. 2B, hydrolysis of aspirin by both HCE1 and HCE2 yielded a linear Lineweaver-Burk plot. However, the apparent K_m values varied markedly (0.36 mM for HCE2 and 2.03 mM for HCE1). Likewise, hydrolysis of clopidogrel by HCE1 yielded a linear Lineweaver-Burk plot (Fig. 2C) with the apparent K_m value of 59 μ M. Liver microsomes produced similar kinetic parameters as recombinant HCE1 and HCE2 with aspirin hydrolysis being a two-enzyme system while clopidogrel being a single enzyme system (data not shown).

Individual variation of clopidogrel and aspirin hydrolysis

Liver microsomes contain the highest carboxylesterase activity (Morgan et al., 1994), and the kinetic similarity between liver microsomes and recombinant HCE1 and HCE2 suggests that these two carboxylesterases determine the overall hepatic hydrolysis of clopidogrel and aspirin, respectively. We next examined the inter-individual variability of hepatic hydrolysis toward each drug and determined whether the variability was correlated with the variability on the expression of HCE1 or HCE2. As shown in Fig. 3A, all microsomal samples hydrolyzed clopidogrel, however, the overall activity varied by as many as 5 fold (column 6 versus 7) (Fig. 3A). When the hydrolytic rate was plotted against the immunostaining intensity of HCE1, all data-points were scattered closely along the regression line except one outlier (labeled with an arrow). The overall correlation coefficient was 0.7454 (Middle of Fig. 3A). The coefficient, however, was further improved (up to 0.9095), when this outlier (lane 7, arrowed) was excluded (Bottom of Fig. 3A). As for the hydrolysis of aspirin, the overall individual variation was markedly less (< 3 fold) (Top of Fig. 3B). When the hydrolytic rate was plotted against the immunostaining intensity of HCE2, the data-points were scattering with a correlation coefficient of 0.2947 only (Middle of Fig. 3B). However, the coefficient was improved to 0.8864, when two outliers (lanes 4 and 7, arrowed) were excluded (Bottom of Fig. 3B).

Hydrolysis of aspirin and clopidogrel by natural variants

The correlation study identified several outliers, which likely represent individuals expressing a polymorphistic variant of HCE1 or HCE2. Molecular cloning studies from this and other laboratories have revealed several HCE1 and HCE2 polymorphistic variants (Kubo et al., 2005; Marsh et al., 2004; Zhao et al., 2005), and the database of single nucleotide polymorphism from NCBI lists many natural variants of these enzymes as well. As an initial effort to establish the significance of natural variants of carboxylesterases on drug metabolism, we tested some of them for the altered hydrolysis toward aspirin or clopidogrel. As a control, variants were tested for the hydrolysis of standard substrate *para*-nitrophenylacetate.

The results on the hydrolysis by natural variants are summarized in Fig. 4. All HCE1 variants except HCE1_{C70F} showed similar activity as wild-type HCE1 on the hydrolysis of clopidogrel (Fig. 4A). HCE1_{C70F}, on the other hand, was expressed to a much lower level (Bottom of Fig. 4A). Increased amounts of the lysates from HCE1_{C70F}-transfected cells were assayed, and little hydrolysis was consistently observed, suggesting that this variant, in addition to decreased expression, is catalytically inefficient (Song et al., 2004). Among the HCE2 variants tested, three showed some decreases on the hydrolysis of aspirin with the maximum decrease being 40% (HCE2_{A139T}) (Fig. 4B). In contrast, the alteration on the hydrolysis of *para*-nitrophenylacetate varied depending on a variant. HCE2_{R180H} showed increased hydrolysis, whereas the hydrolysis was decreased with HCE2_{F485V}. In contrast to HCE1 variants, all HCE2 variants were expressed to a comparable extent (Bottom of Fig. 4B). We are in the process of preparing more natural variants of carboxylesterases, which will serve as tools to fully evaluate the pharmacological significance of polymorphisms of these enzymes.

Hydrolysis of clopidogrel represents detoxication

Altered hydrolysis of aspirin and clopidogrel may have profound clinical consequence in terms of anti-platelet aggregation, given the fact that hydrolytic metabolites of both aspirin and clopidogrel have either decreased or no therapeutic activity. Next, we examined whether hydrolysis of either drug has any toxicological significance. The hydrolytic metabolites of both aspirin and clopidogrel are carboxylate, thus the rate of uptake likely affects their cytotoxicity. To minimize the effect of uptake, we examined the toxicity in cells where the production of the carboxylate metabolites occurred intracellularly and was regulated by transfection of HCE1 and HCE2. The transfected cells were seeded into 96-well plates and treated with the respective parent compound at various concentrations. The cytotoxicity was monitored for cell viability by MTT assay and microscopic examination.

The results on the toxicity study are summarized in Fig. 5. Cells transfected with HCE2 showed marked decreases on the cell viability, and the decrease was significant even when clopidogrel was assayed as

low as 3 μ M (Fig. 5A). Similar decrease was detected in the cells transfected with the vector (data not shown). In contrast, cells transfected by HCE1 showed no changes on the cell viability. Under the bright field, HCE2 transfected cells were rounded and shrank, and the nuclei were condensed (Right of Fig. 5B). In contrast, HCE1 transfected cells were spread and the projects were well extended (Left of Fig. 5B). These results suggest that clopidogrel is more toxic than its metabolite, thus hydrolysis represents detoxication. The hydrolysis and expression of HCE1 or HCE2 were confirmed as shown in Fig. 5C. In contrast, no evident toxicity was detected with aspirin even at 2 mM, whether or not cells were transfected with a carboxylesterase construct or the corresponding vector (data not shown).

Clopidogrel was converted to ethyl clopidogrel in the presence of ethyl alcohol

In addition to catalyzing hydrolytic reaction, HCE1 and hydrolase A have been shown to catalyze transesterification (Brzezinski et al., 1994; Morgan et al., 1994). Clopidogrel is a methyl ester, and likely converted to ethyl clopidogrel in the presence of ethyl alcohol. To test this possibility, lysates from HCE1 transfected cells were incubated with clopidogrel in the presence of ethyl alcohol. The formation of ethyl clopidogrel was monitored by HPLC and LC-MS. Fig. 6A shows the representative chromatograms from the incubation in the presence of ethyl alcohol (200 mM). Two peaks representing clopidogrel metabolites were identified, and they corresponded to clopidogrel carboxylate and ethyl clopidogrel, respectively (Top of Fig. 6A). Ethyl clopidogrel had a slightly prolonged retention time compared with the parent drug (9.07 versus 8.75 min). The mass of ethyl clopidogrel was confirmed to be 335, consistent with the combined mass of clopidogrel and CH₂ (321 + 14) (Bottom of Fig. 6A).

We next examined the formation of ethyl clopidogrel as a function of ethyl alcohol. This is of significance, because it specifies whether the formation of ethyl clopidogrel varies depending on the amount of alcohol consumption. No ethyl clopidogrel was available to precisely quantify the amount of formed ethyl clopidogrel. Preliminary study demonstrated that all samples (no matter how much ethyl alcohol was added) yielded very similar totality of the areas of the peaks corresponding to clopidogrel,

ethyl clopidogrel and the carboxylate, suggesting that these compounds have similar UV-absorption profile. Therefore, percentages of a peak area (e.g., from clopidogrel) over the area totaled from three peaks were used for the relative changes on clopidogrel, ethyl clopidogrel and clopidogrel carboxylate.

As shown in Fig. 6B, increased ethyl alcohol proportionally increased the formation of ethyl clopidogrel accompanied by proportional decreases on the production of clopidogrel carboxylate, the hydrolytic metabolite. Interestingly, the parent drug was also increased as increasing amounts of ethyl alcohol were used, suggesting that the total metabolism of clopidogrel (hydrolysis and transesterification) is decreased in the presence of ethyl alcohol. In order to determine whether the ratio of hydrolysis over transesterification is constant, several individual liver samples were assayed and the formation of clopidogrel carboxylate and ethyl clopidogrel was simultaneously monitored. As shown in Fig. 6C, all samples produced both hydrolytic and transesterification metabolites. However, the formation of hydrolytic metabolite exhibited a larger variation than that of ethyl clopidogrel. Interestingly, samples (e.g., column 1 versus 3) produced comparable levels of ethyl clopidogrel, but differed markedly on the level of clopidogrel carboxylate.

DISCUSSION

Aspirin and clopidogrel are two widely used anti-thrombogenic agents, and co-administration produces enhanced therapeutic effect (Cooke et al., 2006). Aspirin and clopidogrel both contain a single ester bond, and hydrolysis leads to decreased or inactivated anti-platelet activity. Carboxylesterases HCE1 and HCE2 represent two major hydrolases that metabolize drugs and other xenobiotics. The objectives of this study were to determine whether both carboxylesterases are involved in the hydrolysis of aspirin and clopidogrel, and whether polymorphic variants differ from the corresponding wild-type enzymes on the hydrolysis. In this study, we have demonstrated that hydrolysis of aspirin and clopidogrel occurs in an isozyme-specific manner. Clopidogrel is hydrolyzed by HCE1, whereas aspirin is hydrolyzed predominately by HCE2. Certain natural variants show altered hydrolytic activity. While aspirin undergoes hydrolytic biotransformation, clopidogrel undergoes both hydrolysis and transesterification depending on the presence of ethyl alcohol (Fig. 7). Cells exposed to clopidogrel show marked decreases on the viability, but the cytotoxic effect is abolished by transfection of HCE1. Therefore, hydrolysis of clopidogrel has both pharmacological and toxicological significance.

Although there are many hydrolytic enzymes, HCE1 and HCE2 likely represent the primary enzymes that hydrolyze clopidogrel and aspirin, respectively. Several lines of evidence support this notion. First, at any time-points following oral administration, the hydrolytic metabolites are the predominant forms in the systemic circulation (Heestermans et al., 2006; Kees et al., 1996). Such a rapid conversion suggests that the hydrolysis takes place effectively in the gastrointestinal track and the liver, where HCE1 and HCE2 are abundantly expressed (Kroetz et al., 1993; Pindel et al., 1997; Schwer et al., 1997). Second, in this study, we have demonstrated that recombinant HCE1 is highly active toward clopidogrel, and recombinant HCE2 is highly active toward aspirin (Fig. 2). In addition, among individual liver samples, hydrolysis of clopidogrel is correlated with the level of HCE1, and hydrolysis of aspirin is correlated with the level of HCE2 (Fig. 3). Third, recombinant HCE1 and HCE2 produce similar kinetic parameters as liver microsomes in hydrolyzing clopidogrel and aspirin (Fig. 2B), respectively, suggesting that these two

enzymes represent the primary hydrolytic activity in liver microsomes toward each drug. Several other enzymes such as butyrylcholinesterase have been shown to hydrolyze aspirin (Masson et al., 1998). However, these enzymes have a K_m value of millimolar level, therefore, HCE2 ($K_m = 360 \mu\text{M}$, Fig. 2C) is the most kinetically favorable hydrolase toward aspirin.

The hydrolytic preference by HCE1 and HCE2 is likely related to the sizes of the acid and alcohol moieties of ester molecules. HCE1 preferably hydrolyzes esters with a larger acid moiety, whereas the opposite is true with HCE2. Based on the molecular weights, the acid moiety (clopidogrel carboxylate) of clopidogrel is 10 times as big as the alcohol moiety (methanol), therefore, it is hydrolyzed by HCE1 but not HCE2. In contrast, the acid moiety of aspirin is much smaller than the alcohol moiety (molecular weight ratio: 60 *versus* 138), thus aspirin is predominately hydrolyzed by HCE2. The alcohol/acid sizes-based preference applies well to compounds with multiple ester bonds as well. Psychomotor stimulant cocaine, for example, contains two ester bonds, and complete hydrolysis produces ecgonine, methanol and benzoic acid (Brzezinski et al., 1994). Among these products, ecgonine is the largest and acts as the acid moiety relatively to methanol but the alcohol moiety relatively to benzoic acid. Consistent with the alcohol/acid sizes-based preference, HCE1 hydrolyzes ecgonine-methyl ester, whereas HCE2 benzoate-ecgoninyl ester (Brzezinski et al., 1994; Pindel et al., 1997). In addition to the relative sizes, the shapes of respective moieties likely contribute to the hydrolytic preference as well, particularly when the acid and alcohol moieties have similar molecular weights.

The isozyme-specific hydrolysis of aspirin and clopidogrel suggests that they have pharmacokinetic interactions with different sets of ester drugs. Aspirin may interact with anti-cancer agent irinotecan and protease inhibitor nafamostat, both of them are predominantly hydrolyzed by HCE2 (Wu et al., 2002; Yamaori et al., 2006). Likewise, clopidogrel may interact with anti-influenza agent oseltamivir, which is hydrolyzed by HCE1 but not HCE2 (Shi et al., 2006). Although aspirin and clopidogrel exerts pharmacodynamic interaction through anti-clotting effect (Cooke et al., 2006), their isozyme-specific

hydrolysis provides the molecular basis for the lack of hydrolytic interaction. However, they may have metabolizing interactions based on other types of enzyme systems, particularly the CYP system. In humans, low-dose aspirin has been shown to significantly increase CYP3A4 activity (Chen et al., 2003). On the other hand, clopidogrel is a prodrug, and only the oxidized metabolite containing a reactive thiol blocks the signaling of P2Y₁₂ receptor and confers anti-platelet activity (Savi et al., 2000). Given the fact that oxidation of clopidogrel is catalyzed by CYP3A4 and CYP3A5 (Save et al., 2000), increased CYP3A4 activity by low-dose aspirin likely increases the activation of clopidogrel, thus enhances the efficacy of this anti-platelet prodrug. Although the clinical significance remains to be established regarding aspirin-increased CYP3A4 activity on clopidogrel activation, it has been reported that altered activities on CYP3A4 or CYP3A5 have profound effect on the clinical outcomes of clopidogrel (Lau et al., 2004; Suh et al., 2006).

The hepatic predominance on the hydrolysis of clopidogrel provides an important pharmacological advantage for this prodrug, particularly on the generation of the active metabolite. Both CYP3A4 and CYP3A5 are highly expressed in the gastrointestinal track. As a result, clopidogrel is activated in the gastrointestinal track before being hydrolyzed in the liver. It remains to be determined whether the active metabolite is a substrate of HCE1, and whether hydrolysis of the active metabolite represents inactivation. It is likely that the active metabolite generated in the gastrointestinal track covalently modifies the P2Y₁₂ receptor and exerts its therapeutic effect in the portal vein. On the other hand, lack of gastrointestinal hydrolysis of clopidogrel may have toxicological significance. In this study, we have shown that the parent drug but not the hydrolytic metabolite is cytotoxic (Fig. 5). Presence of high concentration of clopidogrel in the gastrointestinal track may damage the mucosa, particularly when a large loading dose is administered.

In the presence of ethyl alcohol, significant amount of clopidogrel is converted to ethyl clopidogrel. The formation of ethyl clopidogrel is proportionally increased as higher amounts of ethyl alcohol are used

(Fig. 6B). However, prolonged incubation leads to disappearance of ethyl clopidogrel accompanied by increased hydrolytic metabolite (data not shown), suggesting that ethyl clopidogrel is a substrate of HCE1 as well. The extent of transesterification in humans remains to be determined. Based on the legal limit of alcohol consumption (~25 mM), it is expected that ethyl clopidogrel is ~15% of the hydrolytic metabolite (Fig. 6B). However, the formation of ethyl clopidogrel is likely increased substantially, because the concentration of alcohol in the liver is expectedly higher than that in the blood concentration, particularly at the time right after consumption. Interestingly, aspirin has been shown to inhibit alcohol dehydrogenase, a major enzyme for the first-pass elimination of ethyl alcohol (Gentry et al., 1999). Therefore, co-presence of clopidogrel, aspirin and alcohol likely increases further the formation of ethyl clopidogrel.

The pharmacological potential of ethyl clopidogrel remains to be established. Initial study has shown that ethyl alcohol increases the cytotoxic effect of clopidogrel (unpublished data), pointing to enhanced biological activity. On the other hand, ethyl alcohol prolongs the presence of the parent drug (Fig. 6B), which may have profound pharmacological consequence. Based on the values of C_{\max} , the concentration of clopidogrel (parent drug) is 4 times as much as the active metabolite (oxidized clopidogrel), and the concentration of the hydrolytic metabolite (clopidogrel carboxylate) is approximately 10,000 times as much as oxidized clopidogrel (Heestermans et al., 2006). Therefore, the prolonged presence of clopidogrel by ethyl alcohol likely allows the oxidation to be proceeded longer, thus increasing the pharmacological activity (Heestermans et al., 2006), particularly when ethyl alcohol is moderately consumed.

In summary, our work points to several important conclusions. First, clopidogrel is hydrolyzed by HCE1, whereas aspirin is hydrolyzed predominately by HCE2. Therefore, clopidogrel and aspirin lack hydrolytic interactions. Second, several natural variants of HCE1 and HCE2 show altered activity toward clopidogrel or aspirin, thus genetic polymorphism on each carboxylesterase may alter the pharmacokinetics and ultimately the efficacy. Third, clopidogrel is converted to ethyl clopidogrel in the

presence of ethyl alcohol, therefore, consumption of alcohol may alter the hydrolytic elimination of clopidogrel. Given the fact that hydrolytic biotransformation represents pharmacological inactivation (clopidogrel) or reduction (aspirin), identification of the primary enzyme for the hydrolysis of each drug provides a molecular basis for individual variation, drug-drug interaction and toxicity regarding the use of these two anti-thrombogenic agents.

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FOOTNOTES

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Legends for Figures

Fig. 1. Hydrolysis of aspirin and clopidogrel by plasma, intestine and liver microsomes (A) *representative chromatograms containing aspirin, clopidogrel, metabolites and internal standard.* The traces were generated by injecting a mixture (10 μ l) of aspirin (8 ng/ml), salicylic acid (31 ng/ml) and IS acetaminophen (38 ng/ml), or a mixture (20 μ l) of clopidogrel (8 ng/ml), clopidogrel carboxylate (31 ng/ml) and IS naproxen (0.5 μ g/ml). (B) *Hydrolysis of aspirin, clopidogrel and para-nitrophenylacetate by plasma, intestine and liver microsomes* For hydrolysis of aspirin, plasma (100 μ g protein) or microsomes (25 μ g) were prepared in 50 μ l phosphate buffer (0.1 M, pH 7.4) and then mixed with an equal volume of aspirin (usually 2 mM) in the same buffer. The incubations lasted for 20 min depending on the samples and the reactions were terminated with 150 μ l of acetonitrile containing acetaminophen (38 ng/ml). The reaction mixtures were subjected to centrifugation for 15 min at 4°C (12,000 g). For hydrolysis of clopidogrel, samples (10-25 μ g protein) were prepared in 50 μ l reaction buffer Tris-HCl (50 mM, pH 7.4), mixed with an equal volume of clopidogrel (200 μ M) in the same buffer, and incubated at 37°C for 15-20 min. The reactions were terminated and centrifuged as described for the hydrolysis of aspirin, however, naproxen (0.5 μ g/ml) was used to replace acetaminophen. The metabolism of both aspirin and clopidogrel was monitored by HPLC. Hydrolysis of *para*-nitrophenylacetate was spectrophotometrically determined as described previously (Xie et al., 2002). All assays were performed in triplicate with three transfection experiments. Hydrolytic rates were expressed as the mean \pm SD (nmol or μ mol/mg/min).

Fig. 2. Hydrolysis of aspirin and clopidogrel by recombinant carboxylesterases and determination of enzyme kinetics (A) *Hydrolytic rate* Lysates (100 μ g) from cells transfected with the empty vector or a cDNA construct encoding HCE1, HCE2, hydrolase A or B were assayed for their activity to hydrolyze aspirin (1 mM) and clopidogrel (100 μ M). The hydrolytic activity toward clopidogrel was monitored by HPLC. (B) *Enzyme kinetics* Lineweaver-Burk plot of oseltamivir hydrolysis by recombinant HCE1

(clopidogrel and aspirin) or HCE2 (aspirin). The hydrolytic rate was determined as a function of aspirin (0.025-5 mM) or clopidogrel (0.02-300 μ M). The K_m and V_{max} were calculated by VisualEnzymics.

Fig. 3. Hydrolysis of aspirin and clopidogrel by individual liver samples and correlation analysis of the hydrolysis with the abundance of HCE1 (A) *Clopidogrel hydrolysis by liver microsomes* Individual liver samples (25 μ g) were incubated with clopidogrel with a final concentration of 100 μ M, and the formation of clopidogrel carboxylate was detected by HPLC. For Western blots, samples (1 μ g) were subjected to SDS-PAGE and transferred electrophoretically to a Trans-Blot nitrocellulose membrane. The immunoblots were blocked in 5% non-fat dry milk, incubated with the antibody (10 μ g/ml) against HCE1 and detected by alkaline phosphatase-conjugated anti-rabbit IgG. The immunostaining intensity was quantified by densitometry and plotted with the hydrolytic rate of each sample toward clopidogrel. (B) *Aspirin hydrolysis by liver microsomes* Hydrolysis and correlation analysis were performed as described for clopidogrel. However, aspirin (1 mM) was used to replace clopidogrel, and the correlation analysis was conducted with the level of HCE2 (3 μ g proteins were used for Western blot).

Fig. 4. Hydrolysis of aspirin and clopidogrel by HCE1 or HCE2 variants (A) *Hydrolysis by HCE1 variants* Lysates (15 μ g) from cells transfected with a cDNA construct encoding HCE1 or a variant were assayed for the activity to hydrolyze clopidogrel (100 μ M) or aspirin (1 mM). The hydrolysis was monitored by HPLC. The level of HCE1 or a variant was determined by Western blots with 1 μ g of lysates. (B) *Hydrolysis by HCE2 variants* Lysates (100 μ g) from cells transfected with HCE2 construct were assayed for the hydrolysis of aspirin (1 mM) or para-nitrophenylacetate (1 mM). *Significantly different between cells transfected with an HCE2 variant and those transfected with wild type HCE2 ($P < 0.05$).

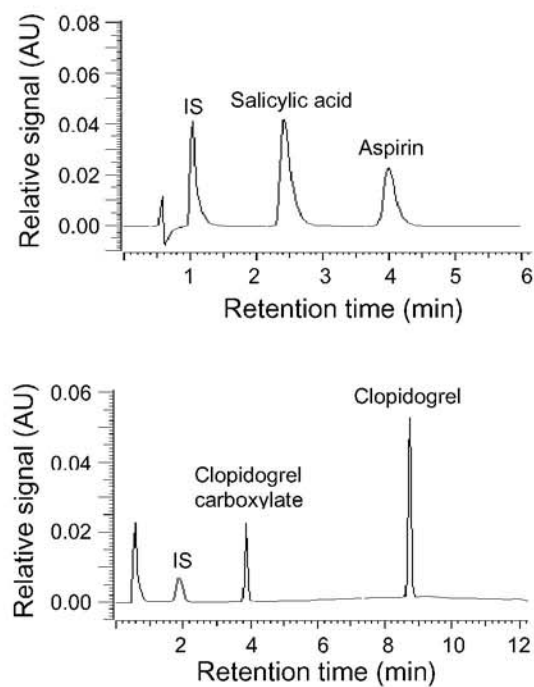
Fig. 5. Effect of clopidogrel hydrolysis on cell viability (A) *Cell viability* Cells (293T) were cultured in 6-well plates and transfected with the vector or a construct encoding HCE1 or HCE2. After a 12-h

incubation, the cells were collected and seeded into 96-well plates at a density of 10,000/well. After additional 12-h incubation, cells were treated with clopidogrel at various concentrations (0-50 μ M) for 36 h. Cell viability was determined with MTT assay as described in Materials and Method. The cell viability was expressed as relative O.D. values. All concentration points are statistically significant between clopidogrel treatment and vehicle control in HCE2 or vector transfected cells, and between HCE1 and HCE2 transfected cells. (B) *Morphological analysis* The images were taken under bright field from cells treated with 50 μ M clopidogrel (250 X). (C) The media collected from clopidogrel-treated cells (vector, HCE1 or HCE2 transfected) and analyzed for the level of clopidogrel and clopidogrel carboxylate by HPLC. The expression of HCE1 and HCE2 in respectively transfected cells was monitored by Western blots.

Fig. 6. Transesterification of clopidogrel (A) *representative chromatograms generated from incubation with clopidogrel and ethyl alcohol* Lysates (60 μ g) from HCE1-transfected cells were incubated with clopidogrel (0.2 mM) and ethyl alcohol (200 mM) at 37°C for 20 min. The reaction was terminated by acetonitrile and analyzed by HLPC (Top) or LC-MS (Bottom). (B) *Formation of ethyl alcohol as a function of ethyl alcohol* Incubations were conducted with clopidogrel (0.2 mM) and ethyl alcohol (0-200 mM). The reactions were terminated by acetonitrile and analyzed by HPLC. The results are expressed as percentages (a peak area of clopidogrel or a metabolite over the area totaled from clopidogrel, clopidogrel carboxylate and ethyl clopidogrel). (C) *Formation of ethyl alcohol by individual liver microsomes* Human microsomes (80 μ g) were incubated with clopidogrel (0.1 mM) and ethyl alcohol (200 mM) at 37°C for 20 min. The reactions were terminated by acetonitrile and analyzed by HLPC. Again, the results are expressed as percentages.

Fig. 7. Biotransformation pathway of aspirin and clopidogrel by carboxylesterases

A



B

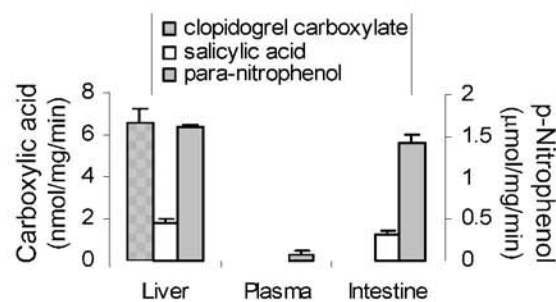
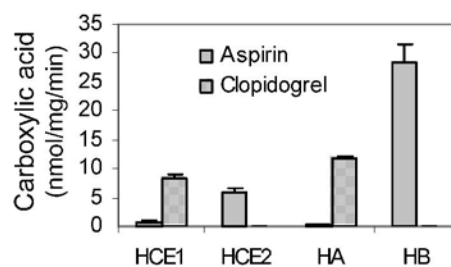
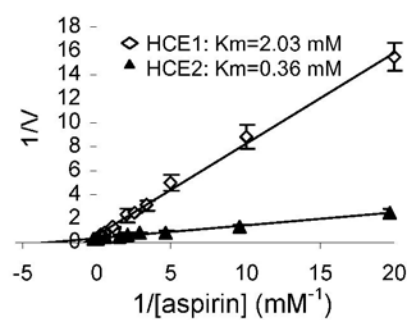


Figure 1

A



B



C

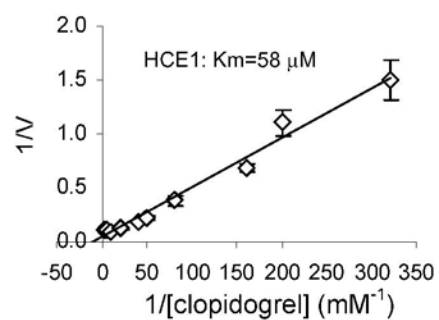
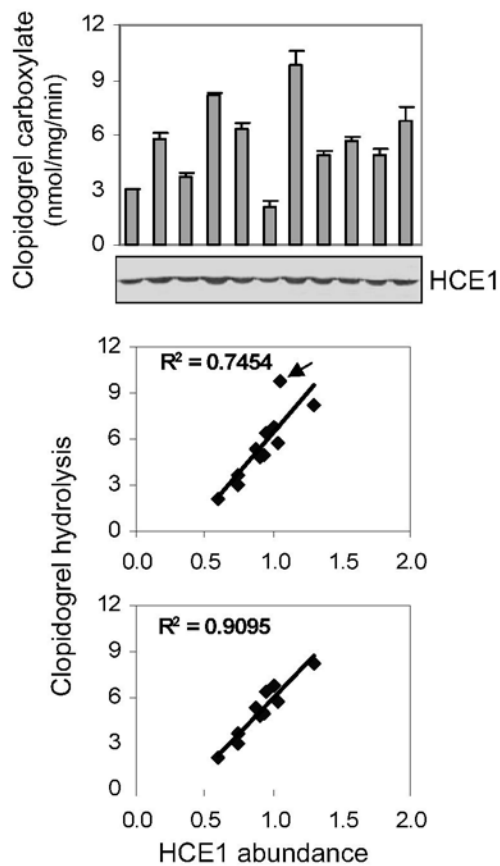


Figure 2

A



B

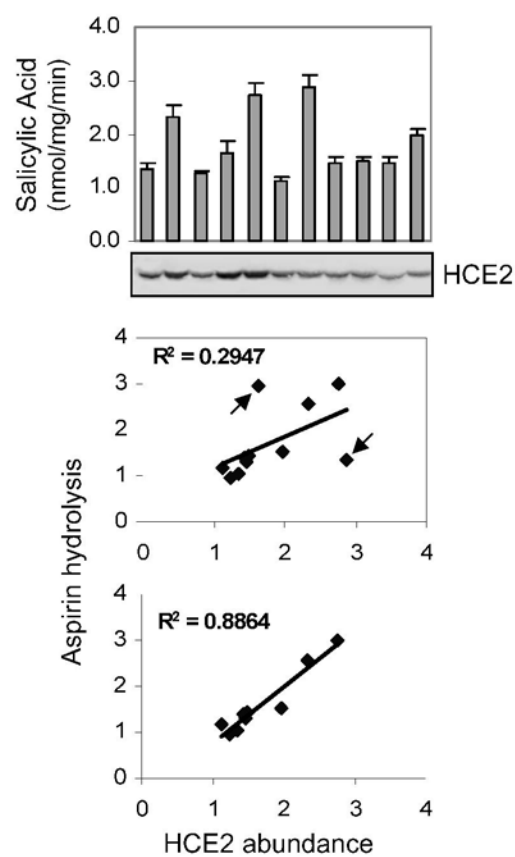
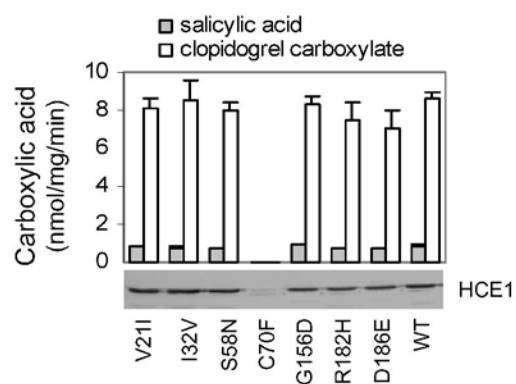


Figure 3

A



B

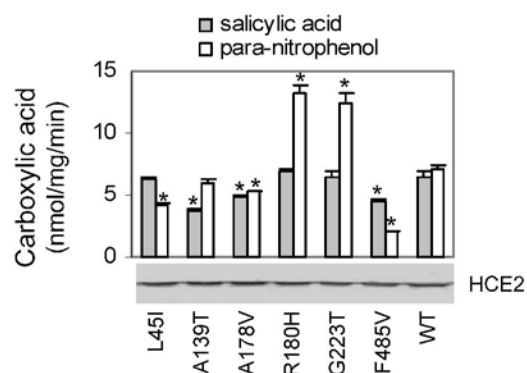
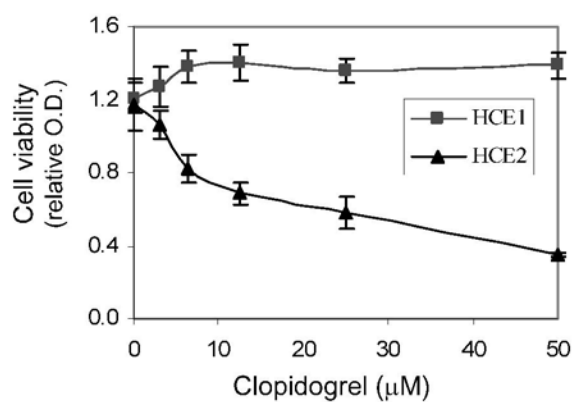
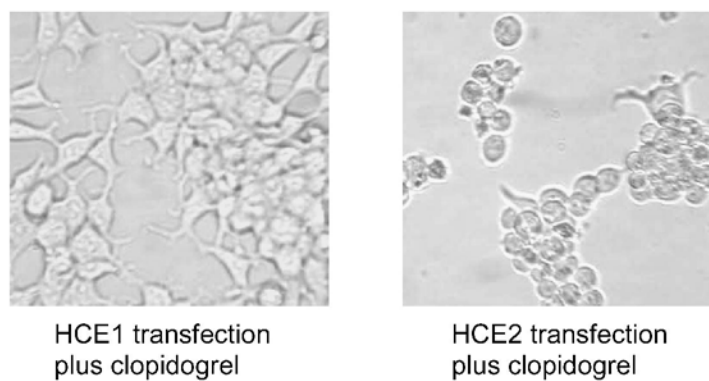


Figure 4

A



B



C

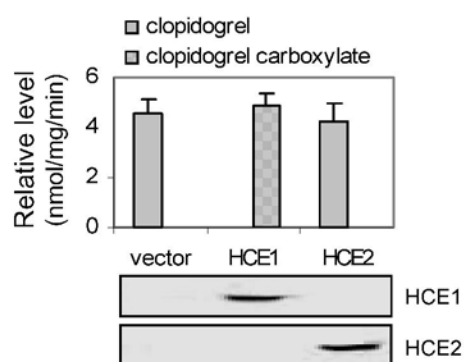
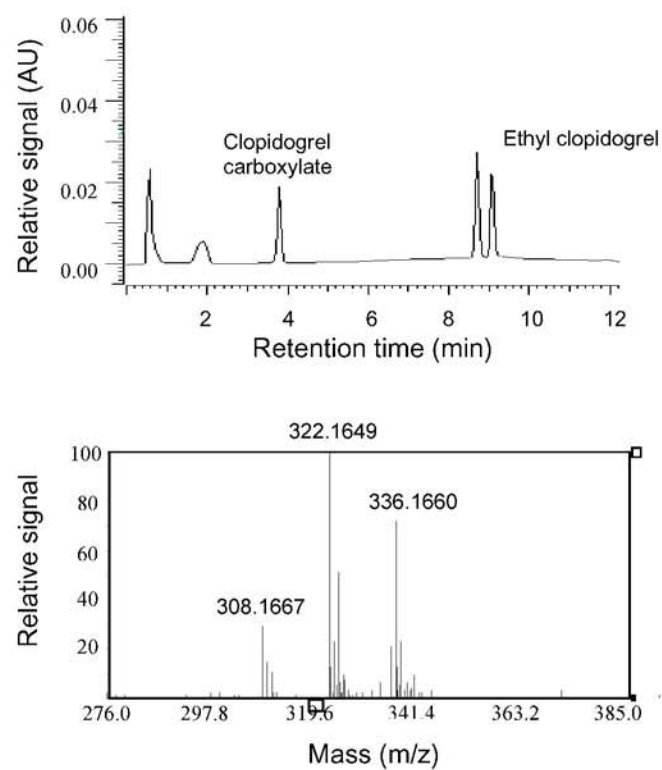
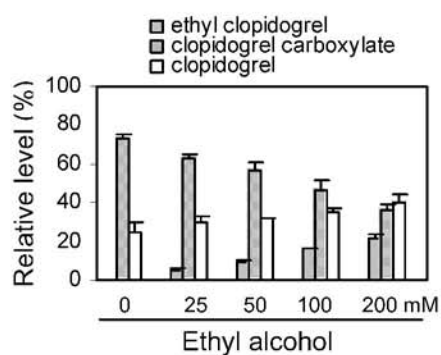


Figure 5

A



B



C

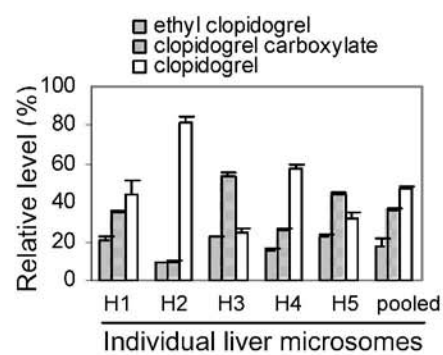


Figure 6

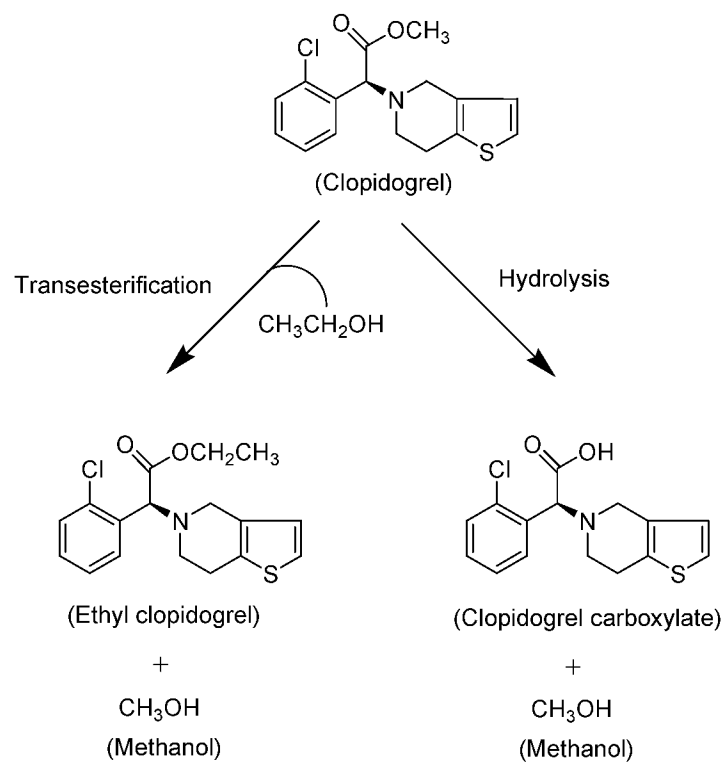
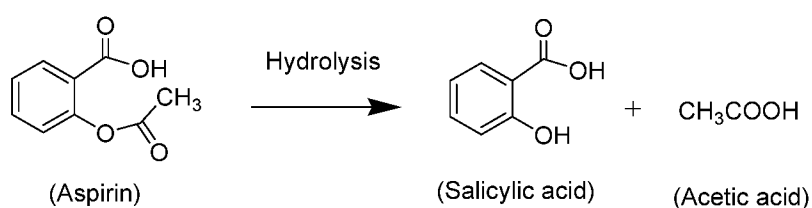


Figure 7