Carboxylesterase 1 as a determinant of clopidogrel metabolism and activation

Hao-Jie Zhu, Xinwen Wang, Brian E Gawronski, Bryan J. Brinda, Dominick J. Angiolillo, and

John S. Markowitz

Department of Pharmacotherapy and Translational Research, University of Florida, Gainesville, Florida (H.J.Z, X. W., B.E.G., B.J.B, D.J.A., J.S.M.)

Center for Pharmacogenomics, University of Florida, Gainesville, Florida (H.J.Z, J.S.M.)

The Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, P.R. China (X.W.)

Division of Cardiology, University of Florida, Jacksonville, Florida (D.J.A.)

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Running title: CES1 affects clopidogrel metabolism and activation

Corresponding Author and Reprints:

John S. Markowitz, Pharm.D.

Department of Pharmacotherapy and Translational Research

University of Florida College of Pharmacy

1600 SW Archer Road, RM PG-23

Gainesville, FL 32610-0486

Tel: 352-273-6262; Fax: 352-273-6121

E-mail: jmarkowitz@cop.ufl.edu

Number of text pages: 30

Number of tables: 1

Number of figures: 4

Number of references: 43

Number of words in Abstract: 247

Number of words in Introduction: 626

Number of words in Discussion: 1938

List of non-standard abbreviations: CES1, carboxylesterase 1; BNPP, bis(4-nitrophenyl)

phosphate; MPB, 2-bromo-3'-methoxy acetophenone; AM, active metabolite

Sections: Metabolism, Transport, and Pharmacogenomics

Abstract

Clopidogrel pharmacotherapy is associated with substantial interindividual variability in clinical response which can translate into an increased risk of adverse outcomes. Clopidogrel is a recognized substrate of hepatic carboxylesterase 1 (CES1), and undergoes extensive hydrolytic metabolism in the liver ultimately forming an active metabolite. Significant interindividual variability in the expression and activity of CES1 exists, which is attributed to both genetic and environmental factors. The aim of the present study was to determine whether CES1 inhibition and *CES1* genetic polymorphisms would significantly influence the biotransformation of clopidogrel and alter formation of the active metabolite. Co-incubation of clopidogrel with the CES1 inhibitor bis(4-nitrophenyl) phosphate in human liver s9 fractions significantly increased the concentrations of clopidogrel, 2-oxo-clopidogrel, and clopidogrel active metabolite, while the concentrations of all formed carboxylate metabolites were significantly decreased. As anticipated, clopidogrel and 2-oxo-clopidogrel were efficiently hydrolyzed by the cell s9 fractions prepared from wild type *CES1* transfected cells. The enzymatic activity of the CES1 variants G143E and D260fs were completely impaired in terms of catalyzing the hydrolysis of clopidogrel and 2-oxo-clopidogrel. However, the natural variants G18V, S82L, and A269S failed to produce any significant effect on CES1-mediated hydrolysis of clopidogrel or 2-oxo-clopidogrel. In summary, deficient CES1 catalytic activity resulting from CES1 inhibition or *CES1* genetic variation may be associated with higher plasma concentrations of clopidogrel-active metabolite and hence, enhance antiplatelet activity. Additionally, *CES1* genetic variants have the potential to serve as a biomarker to predict clopidogrel response, and individualize clopidogrel dosing regimens in clinical practice.

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Introduction

Clopidogrel is a second-generation thienopyridine derivative used extensively as an orally administered antiplatelet agent. The use of clopidogrel has become standard treatment in patients with acute coronary syndromes and undergoing percutaneous coronary interventions. Nevertheless, it has been consistently documented that a significant percentage (5-40%) of individuals treated with clopidogrel do not receive the anticipated therapeutic benefit, which in turn has been associated with an increased risk of adverse outcomes (Angiolillo et al., 2007; Karazniewicz-Lada et al., 2012).

Clopidogrel is a prodrug which undergoes a complex metabolic scheme. It is initially absorbed in its inactive prodrug (parent) form, and after a multi-step biotransformation sequence in the liver, is ultimately metabolized to its active 5-thiol metabolite (Figure 1). However, the majority of administered clopidogrel never enters this bioactivation cascade since ~ 85% of the absorbed prodrug is rapidly hydrolyzed to the inactive metabolite, clopidogrel carboxylic acid (Hagihara et al., 2009). This reaction is catalyzed by hepatic carboxylesterase 1 (CES1) (Tang et al., 2006). Accordingly, only ~15% of a clopidogrel dose is available to undergo further oxidative metabolism catalyzed by cytochrome P450 (CYP) 1A2, 2B6, and 2C19, resulting in the formation of the thiolactone derivative, 2-oxo-clopidogrel. A portion 2-oxo-clopidogrel is then hydrolyzed by CES1 to form 2-oxo-clopidogrel carboxylate, an inactive metabolite, while the balance of 2-oxo-clopidogrel is further hydrolyzed to the unstable but active 5-thiol metabolite. This final activation step is mediated by CYP2B6, CYP2C9, CYP2C19 and CYP3A4 (Kazui et al., 2010). The 5-thiol clopidogrel active metabolite (clopidogrel-AM) is a labile bioreactive compound that forms a disulfide bridge that binds irreversibly to $P2Y_{12}$ receptors located on a

platelet membrane causing irreversible blockade. Finally, CES1 again plays a role in the further hydrolysis of the clopidogrel-AM, ultimately forming the 5-thiol carboxylic acid metabolite (Bouman et al., 2011).

With regard to formation of the clopidogrel-AM and its dependence on CYP enzymes, numerous studies have focused upon CYP2C19 and its functional variants such as the loss of or reduced function alleles *2, *3, *4,*5, *6, *7, *8 and the gain-of-function allele *17. Of these, *CYP2C19*2* may have particular significance with regard to its influence on clopidogrel metabolism and ensuing pharmacokinetic and pharmacodynamic response (Mega et al., 2009; Shuldiner et al., 2009). However, *CYP2C19*2* explains only 5-12% of the variability in clopidogrel response (Karazniewicz-Lada et al., 2012), and the majority of therapeutic variability in clopidogrel treatment remains unknown.

In humans, CES1 is the most predominant hydrolytic enzyme, catalyzing the hydrolysis of numerous ester- and amide-containing endogenous compounds, toxins, and medications to their respective free acids (Imai et al., 2006; Ross and Crow, 2007). CES1 contributes to 80%- 95% of total hydrolytic activity in the human liver. Significant interindividual variability in the expression and activity of CES1 has been consistently observed and reported in the biomedical literature. This variability is likely to be to the result of both genetic and environmental factors (Hosokawa et al., 1995; Fukami et al., 2008; Yoshimura et al., 2008; Yang et al., 2009; Zhu et al., 2009a; Shi et al., 2011; Ross et al., 2012). We have reported that the *CES1* single nucleotide polymorphisms (SNPs) G143E (rs71647871) and D260fs (rs71647872) exhibit markedly decreased enzymatic activity towards the hydrolysis of the CES1 substrates methylphenidate, oseltamivir, and trandolapril (Zhu et al., 2008; Zhu et al., 2009b; Zhu and Markowitz, 2009). Furthermore, we

and others demonstrated that some therapeutic agents can significantly inhibit CES1 activity (Shi et al., 2006; Zhu et al., 2010; Rhoades et al., 2012). Thus, we hypothesized that variable CES1 function is an important contributing factor to interindividual variability of clopidogrel activation and antiplatelet activity. In the present study, we investigated the influence of CES1 inhibition on the activation of clopidogrel, and the effect of several *CES1* nonsynonymous variants on the hydrolysis of clopidogrel and its intermediate metabolite 2-oxo-clopidogrel *in vitro*.

Materials and Methods

Materials

S-(+)-clopidogrel, 2-oxo-clopidogrel, 2-bromo-3'-methoxy acetophenone (MPB) derivatized clopidogrel active metabolite (*cis*-clopidogrel thiol metabolite, clopidogrel-AM), clopidogrel carboxylate, and the internal standard d4-clopidogrel were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). The hydrolytic metabolites of 2-oxo-clopidogrel and clopidogrel-AM were obtained via incubation of the parent compounds (100 µM) with the cell s9 fractions (1 mg protein/ml) prepared from the transfected cells stably expressing wild type CES1. The hydrolytic reaction was completed in 90 min at 37 ˚C. The completion of the bioconversion was confirmed by LC-MS/MS analysis. The derivatizing agent MPB and the CES inhibitor bis(4-nitrophenyl) phosphate (BNPP) were products from Sigma-Aldrich (St. Louis, MO). Flp-In™-293 cells, pcDNA5/FRT/V5-His TOPO®TA Expression Kit, hygromycin B, and Lipofectamine 2000™ were obtained from Invitrogen (Carlsbad, CA). *Taq* polymerase was purchased from Takara (*Takara EX Taq*™ HS, Shiga, Japan). Human *CES1A1* cDNA cloned into a pCMV-SPORT6 vector was from American Type Culture Collection (ATCC, Manassas, VA). Pooled human liver s9 fraction was obtained from BD Biosciences (Woburn, MA). All other chemicals and agents were of the highest analytical grade commercially available.

CES1 inhibition study in human liver s9 fractions

Pooled human liver s9 fractions were pre-incubated with an NADPH generating system (0.1 mg/ml yeast glucose-6-phosphate dehydrogenase, 3 mg/ml NADP+, and 0.07 M glucose-6-phosphate) in the presence and absence of the CES1 inhibitor BNPP at 37 ˚C for 5 min. The

reaction was initiated by adding clopidogrel. The final concentrations of the liver s9 fractions,

BNPP, and clopidogrel in the reaction system were 4 mg/ml, 10 µM, and 20 µM, respectively,

and the total volume was 200 µl. Samples (20 µl) were collected at 0.25, 0.5, 1, 2, 4, 6, 8, 22 h

after initiation of the reaction. The reaction was terminated by adding a 5-fold volume of

acetonitrile containing the internal standard d4-clopidogrel (25 ng/ml) and the derivatizing

reagent MPB (5 mM). The concentrations of clopidogrel and its five metabolites including 2-

oxo-clopidogrel, clopidogrel-AM, clopidogrel carboxylate, 2-oxo-clopidogrel carboxylate,

clopidogrel-AM carboxylate were determined by a validated LC-MS/MS assay. The area under

the time-concentration curve (AUC) of each compound was calculated using linear-trapezoidal

rule by the software WinNonlin 5.2.1 (Pharsight, Mountain View, California). The differences

between BNPP treated and untreated groups were compared using independent student t-test, and

were considered statistically significant when p value was less than 0.05.

Establishment of cell lines stably expressing CES1 variants G18V, S82L, and A269S

The Flp-In™-293 cell lines stably expressing wild type *CES1* and its variants G143E and D260fs

have been developed previously and applied to our ensuing *CES1* pharmacogenetic studies (Zhu

et al., 2008; Zhu et al., 2009b; Zhu and Markowitz, 2009). The minor allele frequency (MAF) of

G143E is estimated to be 3.7%, 4.3%, 2.0%, and 0% in Caucasian, Blacks, Hispanic, and Asian

populations, respectively, whereas D260fs appears to be a rare mutation in all racial and ethnic

groups studied to date (Zhu et al., 2008). In the present study, we developed three additional cell

lines expressing the *CES1* variants G18V (rs3826190), S82L (rs62028647), and A269S

(rs115629050) to study the effect of these nonsynonymous variants on CES1 enzymatic activity

and clopidogrel hydrolysis. Briefly, the mutant *CES1A1* plasmids were obtained via site-directed

mutagenesis assay using specific primers and probes. All plasmids were bidirectionally

sequenced to confirm that the desired constructs were generated. The plasmids were co-

transfected with the pOG44 plasmid at a ratio of 1:10 into Flp-In[TM]-293 cells using the

transfection reagent Lipofectamine 2000[TM] in serum free RPMI Medium 1640. The cell lines

were established after hygromycin B selection (100 µg/ml) for 3 weeks. The choice of these three

CES1 variants was based on their relatively high MAFs, and all of these variants were predicted

by the *in silico* programs SIFT (https://sift.jcvi.org) and Polyphen2

(https://genetics.bwh.harvard.edu/pph2/) to be possibly detrimental to enzyme function. The

MAFs of G18V, S82L, and A269S range from 0.072 to 0.340, 0.026 to 0.368, and 0.014 to

0.057, respectively, among different populations (detailed information about the MAFs of these

SNPs in different populations can be found in the Supplemental Table 1). Cells were cultured in

Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. To prepare cell

supernatant 9000 (s9) fractions, the cells were rinsed and suspended in PBS buffer solution (pH

7.4). The cells were then sonicated and the s9 fractions were collected after centrifugation at

9000g for 30 min at 4°C. The protein concentration was determined using a Pierce BCA assay kit

(Rockford, IL).

Hydrolysis of clopidogrel and 2-oxo-clopidogrel by wild type and mutant CES1

An *in vitro* incubation study was conducted to determine the catalytic activity of CES1 and its

variants G18V, S82L, G143E, D260fs, A269S on the hydrolysis of clopidogrel and 2-oxo-

clopidogrel. Selected concentrations of clopidogrel (0.5, 1.5, 5.0, 15, 50, 150 µM) and 2-oxo-

clopidogrel (0.05, 0.15, 0.5, 1.5, 5 µM) were incubated with cell s9 fractions at 37 °C for 10 and

20 min, respectively. The final concentrations of the s9 fractions were 0.05 mg/ml and 0.2 mg/ml

for clopidogrel and 2-oxo-clopidogrel, respectively. The reaction was terminated by the addition of a 5-fold volume of acetonitrile containing the internal standard d4-clopidogrel (25 ng/ml). After centrifugation at 16,000 $\times g$ at 4 °C for 10 min, the supernatant was collected and analyzed for concentrations of the hydrolytic products (i.e. clopidogrel carboxylate and 2-oxo-clopidogrel carboxylate) utilizing established validated LC-MS/MS assay. Data were fit to the Michaelis-Menten equation, and kinetic parameters K_m and V_{max} were calculated using nonlinear regression analysis (Graphpad Prism software Version 4.0 for Windows, Graphpad Software Inc., San Diego, CA). Additionally, in an effort to capture any substrate specificity exhibited by any variant, a well characterized CES1 selective substrate, methylphenidate was included in the incubation study at a single concentration of 100 µM. The experiment procedures were identical to those previously published (Zhu et al., 2008). The catalytic activity of CES1 and its variants towards methylphenidate was evaluated by measuring the concentrations of the formed hydrolytic metabolite ritalinic acid.

HPLC-MS/MS assay

An HPLC-MS/MS assay was developed for the simultaneous quantification of clopidogrel and its five metabolites including the intermediate metabolite 2-oxo-clodipgrel, the active metabolite clopidogrel-AM, and three hydrolytic metabolites (i.e. clopidogrel carboxylate, 2-oxo-clopidogrel carboxylate, and clopidogrel-AM carboxylate). This assay was a modification of a previously published method (Tuffal et al., 2011). For the human liver s9 inhibition study, the samples were prepared by mixing 20 µl of reaction mixtures with 100 µl of acetonitrile containing the internal standard d4-clopidogrel (25 ng/ml) and the derivatizing agent MPB (5 mM). Clopidogrel-AM is very unstable, and thus was carefully derivatized with MPB to form the

stable derivative clopidogrel-AM-MPB for analysis (Takahashi et al., 2008; Tuffal et al., 2011).

The mixtures were left standing at room temperature for 10 min to allow the derivatization

reaction to proceed to completion. No derivatizing agent was used for the samples from the

clopidogrel and 2-oxo-clopidogrel cell s9 hydrolysis studies. All samples were centrifuged at

16,000 $\times g$ at 4ºC for 10 min to remove proteins. The resulting supernatant was then collected for

HPLC-MS/MS analysis.

A Shimadzu HPLC system coupled to an Applied Biosystems API 3000 triple quadrupole mass

spectrometer was employed. Ionization was achieved via ESI in the positive mode and ions were

monitored by multiple reaction monitoring. Clopidogrel, 2-oxo-clopidogrel, clopidogrel-AM,

clopidogrel carboxylate, 2-oxo-clopidogrel carboxylate, clopidogrel-AM carboxylate, and the

internal standard d4-clopidogrel were monitored via the m/z transition 322.1>194.0,

338.0>183.0, 504.0>354.0, 308.0>197.9, 324.0>169.0, 490.0>340.0, and 326.2>188.1,

respectively. The compounds were separated on a C18 reverse phase column with the mobile

phase consisting of 48% of acetonitrile, 2 mM ammonium acetate, and 0.2% formic acid, and

delivered at a flow rate of 0.3 ml/min. The assay is highly sensitive, selective, and reliable. The

lower limit of quantification (LLOQ) of all analytes was estimated to be 1 nM. Accuracy and

precision were within FDA guidelines (FDA, 2001). A representative chromatogram obtained

following the incubation of 20 µM of clopidogrel with human liver s9 fractions for 30 min in the

presence of a NADPH-generating system can be found in the associated Supplemental Materials.

As anticipated, two isomers of the thiol metabolites were observed (panel #4 from the top),

which are Z compounds with reference to the C3-C16 double bond of 7S clopidogrel (Pereillo et

al., 2002; Tuffal et al., 2011). These two isomers differ in terms of whether the C4 configuration

is R or S. After being separated by a conventional reverse-phase column (e.g. C18), the first and

second peaks are noted to correspond to H3 and H4 metabolites, respectively (Tuffal et al.,

2011). It is essential that the analytical assay is enantioselective as both the H3 and H4

metabolites can be formed after hepatic metabolism, but only the H4 isomer is believed to be

pharmacologically active. The identity of the active H4 metabolite was confirmed by performing

comparisons with pure analytical reference standards.

Results

BNPP affected clopidogrel metabolism and activation

Co-incubation of BNPP with human liver s9 fractions significantly *increased* the concentrations

of clopidogrel, 2-oxo-clopidogrel, and clopidogrel-AM while the concentrations of all formed

carboxylate metabolites were significantly *decreased* (Figure 2). Notably, the AUC_{0-22h} of

clopidogrel-AM increased by more than 100% in the presence of the CES1 inhibitor BNPP

(Table 1). The ratios of clopidogrel to 2-oxo-clopidogrel and 2-oxo-clopidogrel to clopidogrel-

AM were not increased following co-administration of BNPP, indicating the increased formation

of clopidogrel-AM was not due to the interaction between BNPP and CYP isoenzymes. The data

suggest that CES1 inhibition led to enhanced formation of clopidogrel-AM as well as the

increased concentrations of the intermediate metabolite, 2-oxo-clopidogrel. .

**The CES1 variants G143E and D260fs are loss-of-function variants for clopidogrel and 2-
oxo-clopidogrel hydrolysis**

Consistent with previous reports, both clopidogrel and 2-oxo-clopidogrel were efficiently

hydrolyzed by wild type CES1 (Tang et al., 2006; Bouman et al., 2011). The V_{max} and K_m values

for clopidogrel were determined to be 3558 ± 371 pmol/min/mg protein and 62.7 ± 15.4 μM,

respectively, while the V_{max} and K_m values for 2-oxo-clopidogrel were 158.1 ± 16.2 pmol/min/mg

protein and 2.4 ± 0.6 μM, respectively. The enzymatic activity of the CES1 variants G143E and

D260fs were completely impaired in terms of catalyzing the hydrolysis of clopidogrel and 2-oxo-

clopidogrel (Figure 3. The D260fs data overlapped with G143E, and are not shown in the

Figure). However, the variants G18V, S82L, and A269S produced no significant effect on CES1-

mediated hydrolysis of clopidogrel or 2-oxo-clopidogrel. Similar to clopidogrel and 2-oxo-

clopidogrel, no catalytic activity of G143E or D260fs was observed with regard to

methylphenidate metabolism, while the activity of G18V, S82L, and A269S appeared to remain

intact and was comparable to that of wild type enzyme (Figure 4). Thus, the study demonstrated

that both 143E and D260fs are loss-of-function alleles and the G18V, S82L, and A269S are

nonfunctional variants for all three tested CES1 substrates. The results also suggest that the

commonly used *in silico* programs SIFT and Polyphen2 may not be useful approaches for the

prediction of the function of *CES1* nonsynonymous variants.

Discussion

CES1 is encoded in humans by the *CES1* gene, which consists of three isoforms, i.e. *CES1A1*, *CES1A2*, and *CES1A3* (Hosokawa et al., 2007; Fukami et al., 2008). *CES1A1* and *CES1A3* are inversely located on chromosome 16 q13-q22.1 while *CES1A2* is a variant of the *CES1A3* gene (Fukami et al., 2008). Both *CES1A1* and *CES1A2* are functional whereas *CES1A3* is a pseudogene due to a premature stop codon located in exon 3 (Fukami et al., 2008; Hosokawa et al., 2008; Zhu and Markowitz, 2012). *CES1A1* and *CES1A2* are identical with the exception of the exon 1 and promoter regions. In the liver, the majority of CES1 is the product of the *CES1A1* gene since the transcription efficiency of the *CES1A2* gene is only ~ 2% of that of *CES1A1*, probably due to additional Sp1 and C/EBP binding sites in the promoter region of *CES1A1* (Fukami et al., 2008; Hosokawa et al., 2008). Thus, only *CES1A1* genetic variants are likely to produce a significant impact on CES1 expression and activity.

CES1 expression and activity vary markedly among individuals. Consolidated evidence supports the presence of both genetic and non-genetic factors as significant contributors to observed variability (Hosokawa et al., 1995; Fukami et al., 2008; Yoshimura et al., 2008; Yang et al., 2009; Zhu et al., 2009a; Ross et al., 2012). We have identified and characterized two novel CES1 nonsynonymous variants G143E and D260fs within the *CES1A1* and *CES1A2* genes, respectively, in a human subject participating in a normal volunteer pharmacokinetic study of *dl*-methylphenidate (Patrick et al., 2007; Zhu et al., 2008). Systemic blood concentrations of both *d*- and *l*-methylphenidate were grossly elevated following a single modest dose (0.3 mg/kg) of *dl*-methylphenidate relative to typical values found in the published literature as well as his 19 study peers. Additionally, all hemodynamic measures (i.e. systolic blood pressure, diastolic

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blood pressure, mean arterial blood pressure, heart rate) in this poor metabolizer differed significantly from his study peers (Zhu et al., 2008). Our *in vitro* functional studies have demonstrated that catalytic function of both G143E and D260fs are profoundly impaired in terms of hydrolyzing *dl*-methylphenidate and other CES1 substrates including the prodrugs trandolapril and oseltamivir (Zhu et al., 2008; Zhu et al., 2009b; Zhu and Markowitz, 2009). Subsequently, a clinical study was conducted in patients with attention deficit-hyperactivity disorder (ADHD) treated with methylphenidate. The results showed that patients carrying the 143E allele required significantly lower doses of methylphenidate for symptom reduction relative to non-carriers (Nemoda et al., 2009). These findings are consistent with the expectation that impaired hydrolysis of methylphenidate would result in higher systemic and CNS concentrations, and accordingly, a decreased need for upward dosage titration. Recently, and consistent with our published *in vitro* findings (Zhu and Markowitz, 2009), a healthy volunteer pharmacokinetic study demonstrated that the G143E variant significantly impaired the activation of the prodrug oseltamivir (Tarkiainen et al., 2012). Notably, the $AUC_{0-\infty}$ of oseltamivir in a homozygous variant type (143EE) was found to be 360% greater than the non-carrier peers.

Beyond the nonsynonymous SNPs, a number of SNPs within the promoter and 5'-untranslated region (5'-UTR) of *CES1A1* and *CES1A2/CES1A3* genes have been reported (Geshi et al., 2005; Yoshimura et al., 2008; Sai et al., 2010; Yamada et al., 2010). Among them, -816A>C was reported to be significantly associated with the efficacy of the ACE inhibitor prodrug imidapril (Geshi et al., 2005). However, a later study demonstrated that this SNP resides in the nonfunctional pseudogene *CES1A3* (Sai et al., 2010). Furthermore, it was not found to be associated with the activation of the partial CES1 substrate irinotecan, leaving its clinical significance unresolved (Sai et al., 2010). Additionally, the variant -75T>G within the 5'-

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untranslated region (5'-UTR) of *CES1A1* gene was recently found to be associated with isoniazid-induced hepatotoxicity in patients with latent tuberculosis (Yamada et al., 2010). A subsequent clinical study revealed that the AUC ratios of irinotecan metabolites (SN-38+SN-38G) to irinotecan, an indicator of in vivo CES1 and CES2 activity, were significantly lower in cancer patients carrying the minor G allele relative to wild type patients (Sai et al., 2010). Most recently, the -75G allele was associated with the worsening of appetite deduction in ADHD patients treated with methylphenidate (Bruxel et al., 2012). In addition to the promoter, 5'-UTR, and nonsynonymous variants, more than 900 other *CES1* variants, such as synonymous and intronic variants, have been documented in several SNP databases (e.g. NCBI dbSNP). However, the function of essentially none of these variants has been systematically evaluated to date.

Beyond the presence of functional *CES1* variants, patient age and potential drug-drug interactions may contribute to interindividual variability in CES1 function as well. Data recently generated in our laboratory and by others demonstrate that the expression of CES1 is markedly lower in neonates and infants, gradually increases in a developmental manner, and full maturation in expression and function is observed by age 6 to 9 years (Yang et al., 2009; Zhu et al., 2009a; Shi et al., 2011). Additionally, a number of commonly used medications have been recently identified as CES1 inhibitors or inducers (Shi et al., 2006; Fukami et al., 2010; Zhu et al., 2010; Hatfield and Potter, 2011; Rhoades et al., 2012). However, the magnitude of effect and clinical significance of CES1 inhibitors/inducers and developmental age need to be validated by *in vivo* assessments, and both areas of study are in their relative infancy.

CES1 is responsible for the initial hydrolysis of ~85% of clopidogrel, converting it to its inactive carboxylate metabolite, leaving a balance of ~15% of clopidogrel for further hepatic metabolism

(Figure 1). Furthermore, 2-oxo-clopidogrel and clopidogrel-AM are also hydrolyzed by CES1 forming their respective inactive metabolites. In the present study, we have demonstrated that co-incubation of clopidogrel with 10 μM BNPP resulted in significant inhibition of the hydrolysis of clopidogrel, 2-oxo-clopidogrel, and clopidogrel-AM. Based upon the AUCs generated for the three carboxylate metabolites assessed in the inhibition study (Table 1), the inhibitory rates of BNPP (10 μM) on the hydrolysis of clopidogrel, 2-oxo-clopidogrel, and clopidogrel-AM were determined to be 81.6%, 60.6%, and 44.0%, respectively. Several CYP enzymes including CYP1A2, 2B6, 2C9, 2C19, and 3A4 are involved in the two-step activation process of clopidogrel. In order to fully appreciate the effect of CES1 inhibition on the metabolism and activation of clopidogrel, it is critical that the CES1 inhibitor should not significantly interact with these CYP enzymes at the concentration(s) utilized in the study. In the present study, 10 μM BNPP significantly inhibited CES1 activity resulting in decreased hydrolytic metabolism and increased formation of clopidogrel-AM. However, at this concentration, the inhibitor did not significantly alter the ratios of clopidogrel-to-2-oxo-clopidogel and 2-oxo-clopidogrel-to-clopidogrel-AM, indicating that 10 μM BNPP had no significantly effect on the activity of those CYP enzymes under the utilized experimental conditions. Thus, the concentration (10 μM) of BNPP appeared to inhibit CES1 as intended, without perturbing native CYP function.

Clopidogrel undergoes a fairly complex metabolism, and two sequential metabolic reactions lead to the formation of clopidogrel-AM. Accordingly, we employed a relatively lengthy incubation (22 h) approach with multiple time points sample collections.. Under our experimental conditions, the formation of clopidogrel-AM and clopidogrel-AM carboxylate peaked at 2.5 and 4.5 hours, respectively, after the initiation of the incubation period. If only a brief incubation period were employed (i.e. ≤ 60 min), the study would have failed to detect the influence of

CES1 inhibition on the production of clopidogrel-AM. The prolonged incubation time permitted a more thorough characterization of the effect of CES1 inhibition on clopidogrel metabolism and activation. The results showed CES1 inhibition can lead to significant increases in the metabolic production of both active and intermediate metabolites of clopidogrel. This is speculated to be the result of the accumulation of the parent compound coupled with the impairment of hydrolytic metabolism.

Beyond the demonstrated effects of chemical inhibition of CES1 and the ensuing influence on clopidogrel biotransformation, we further assessed the influence of CES1 genetic variants on clopidogrel metabolism utilizing transfected cell lines. Functional *CES1* SNPs such as G143E and D260fs exhibited null activity for the hydrolysis of both clopidogrel and 2-oxo-clopidogrel. However, catalytic activity of the variants G18V, S82L, and A269S on the hydrolysis of clopidogrel or 2-oxo-clopidogrel was comparable to the wild type enzyme, indicating that these variants are nonfunctional SNPs for clopidogrel and 2-oxo-clopidogrel metabolism though all of these three variants were predicted to be potentially damaging to enzyme function by the *in silico* programs SIFT and Polyphen2. Clopidogrel-AM is very unstable, and is currently not commercially available. Thus, we were not able to evaluate whether these variants exert similar effects on clopidogrel-AM hydrolysis. However, given the fact that clopidogrel-AM shares a high degree of similarity in its chemical structure with clopidogrel and 2-oxo-clopidogrel, we anticipate that G143E and D260fs are loss-of-function variants for clopidogrel-AM as well. Native expression of major drug metabolizing enzymes including CYP enzymes and CES1 are undetectable in the parent HEK293 cells which were used to create the transfected cell lines (Bouman et al., 2011). Therefore, the cells serve as an excellent model to study the effect of genetic variation on the function of CES1. However, the oxidative intermediate and active

metabolites of clopidogrel cannot be observed in the transfected HEK293 cells due to the lack of expression of CYP enzymes in the cells.

The clinical benefits of clopidogrel therapy have been demonstrated in numerous large-scale trials, and have established this agent as one of the mainstay treatments in patients with acute coronary syndromes and those undergoing percutaneous coronary intervention, as also reflected in clinical practice guidelines (Levine et al., 2011; Wright et al., 2011). However, numerous investigations have revealed a broad interindividual variability in response profiles to clopidogrel therapy. Importantly, this has shown to have prognostic implications (Angiolillo et al., 2007). In particular, patients with reduced clopidogrel mediated antiplatelet effects who persist with high on-treatment platelet reactivity (also known as "poor responders") have an increased risk of recurrent ischemic events, including stent thrombosis, while patients with enhanced clopidogrel mediated antiplatelet effects who have low on-treatment platelet reactivity (also known as "hyper responders") have an increased risk of bleeding complications (Ferreiro et al., 2010). Enormous efforts have been made to determine the causes of this marked variability of outcomes. Genetic polymorphisms of drug metabolizing enzymes, such as CYP2C19, are considered to be an important contributing factor to varied clopidogrel treatment outcomes. However, all biomarkers currently used for the prediction of clopidogrel response including CYP2C19 genotypes can only explain a very small portion of the observed variability. Given the very broad application in clinical practice of this pivotal antiplatelet agent, defining determinants of variability in clopidogrel response is critical and may have important therapeutic implications. Our present study suggests that CES1 can markedly affect clopidogrel metabolism and activation, indicating functional CES1 SNPs may be associated with higher plasma concentrations of clopidogrel-AM and enhanced antiplatelet activity. In fact, while this manuscript was under preparation, Lewis et

al reported that the *CES1A1* variant G143E discovered in our laboratory (Zhu et al., 2008) was associated with significantly increased plasma concentrations of clopidogrel-AM and greater clopidogrel response in participants of the Pharmacogenomics of Anti-Platelet Intervention (PAPI) study (n=566) and in 350 patients with coronary heart disease (Lewis et al., 2012). This clinical observation is fully in agreement with our *in vitro* data of the present study.

Beyond *CES1* genetic polymorphisms, CES1 mediated drug-drug interactions have the potential to influence clopidogrel activation and the ensuring pharmacological effect as well. Additional clinical investigations are needed to evaluate the effect of CES1-mediated drug-drug interactions on therapeutic outcomes of clopidogrel, and whether *CES1* genetic variants can be utilized as a biomarker to predict clopidogrel response, and individualize clopidogrel doses in clinical practice.

Authorship Contributions

Participated in research design: Zhu, Wang, Gawronski, and Markowitz.

Conducted experiments: Zhu, Wang, Gawronski, Brinda.

Performed data analysis: Zhu, Wang.

Wrote or contributed to the writing of the manuscript: Zhu, Angiollilo, and Markowitz.

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Footnotes

Legends for Figures

Figure 1. Metabolic pathways of clopidogrel.

Figure 2. Effect of CES1 inhibition on the metabolism and activation of clopidogrel. The concentrations of clopidogrel and its five metabolites were determined utilizing an established LC-MS/MS assay after incubation of clopidogrel (20 µM) in pooled human liver s9 fractions (4 mg/ml) in the presence and absence of the CES1 inhibitor BNPP (10 µM). Data are the averages of three independent experiments with error bars representing SD.

Figure 3. Hydrolysis of clopidogrel (A) and 2-oxo-clopidogrel (B) by CES1 and its variants. Catalytic activity of CES1 and its nonsynonymous variants G18V, S82L, G143E, and A269S on the hydrolysis of clopidogrel and 2-oxo-clopidogrel was determined by measuring the formation of the respective carboxylate metabolites utilizing LC-MS/MS assay. Data were expressed as the means from three independent experiments.

Figure 4. Hydrolysis of methylphenidate by CES1 and its variants. The established CES1 selective substrate methylphenidate (100 µM) was incubated with the s9 fractions prepared from the WT CES1 cells and the cells transfected with the variants G18V, S82L, G143E, D260fs, and A269S. Enzymatic activity was determined by measuring the formation of the hydrolytic metabolite ritalinic acid. Data were expressed as mean ± SD (n=3).

Table 1. The area under the concentration-time curve (AUC, µM·hr) of clopidogrel and its metabolites from 0 to 22 hours after incubation of clopidogrel (20 µM) with pooled human liver s9 fractions in the presence or absence of the CES1 inhibitor BNPP (10 µM). Data are expressed as mean ± SD from three independent experiments. ** P<0.001 compared to controls.

	Clopidogrel	2-oxo-clopidogrel	Clopidogrel-AM	Clopidogrel carboxylate	2-oxo-clopidogrel carboxylate	Clopidogrel-AM carboxylate
Control	1.76 ± 0.29	0.57 ± 0.04	0.45 ± 0.04	45.38 ± 2.46	0.66 ± 0.07	0.25 ± 0.02
BNPP	9.02 ± 0.50**	1.93 ± 0.11**	0.99 ± 0.04**	8.35 ± 0.55**	0.26 ± 0.03**	0.14 ± 0.01**

Figure 1
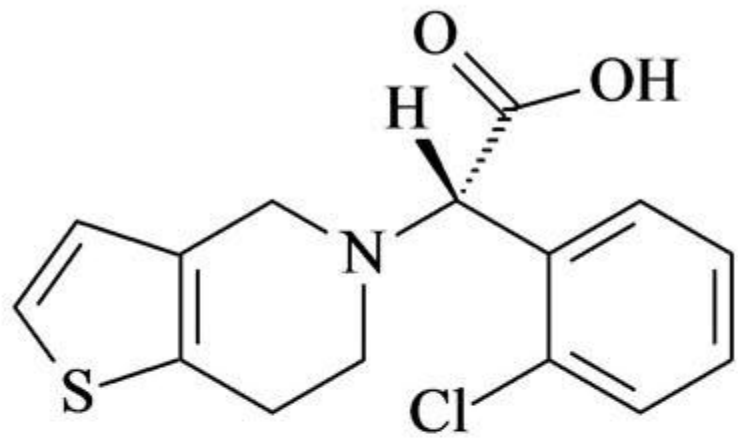
clopidogrel-carboxylic acid
(SR26334, inactive)

85%

clopidogrel
(inactive)

15%

CYP2C19
CYP1A2
CYP2B6
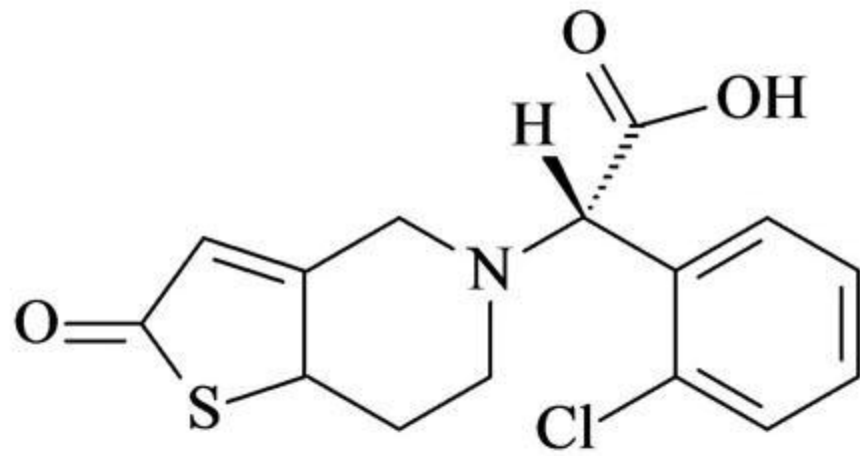
2- oxo-clopidogrel-carboxylic acid
(inactive)

hydrolysis
CES1
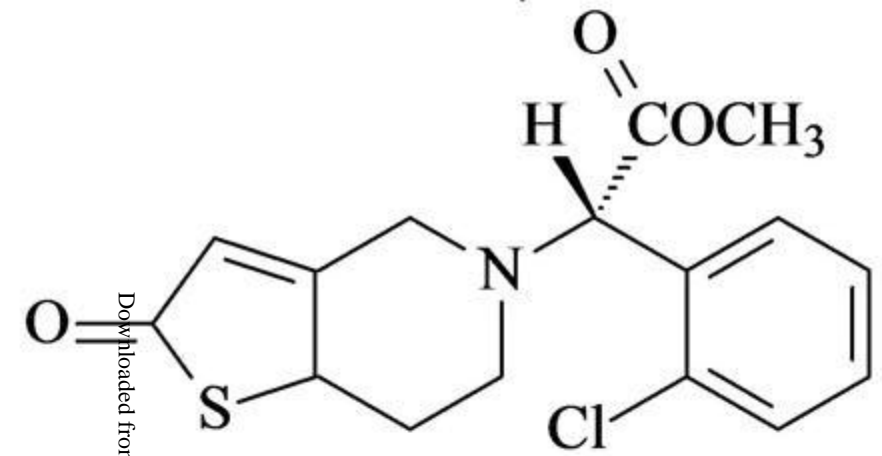
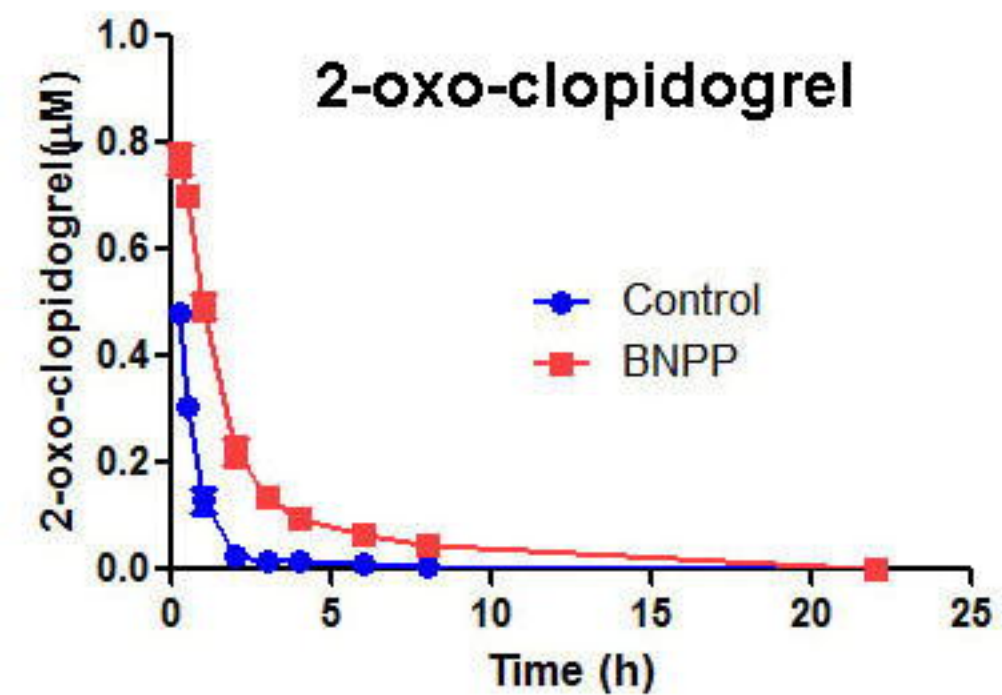**2- oxo-clopidogrel**
(inactive)

CYP3A4
CYP2B6
CYP2C9
CYP2C19

5-thiol carboxylic acid metabolite
(inactive)

hydrolysis
CES1
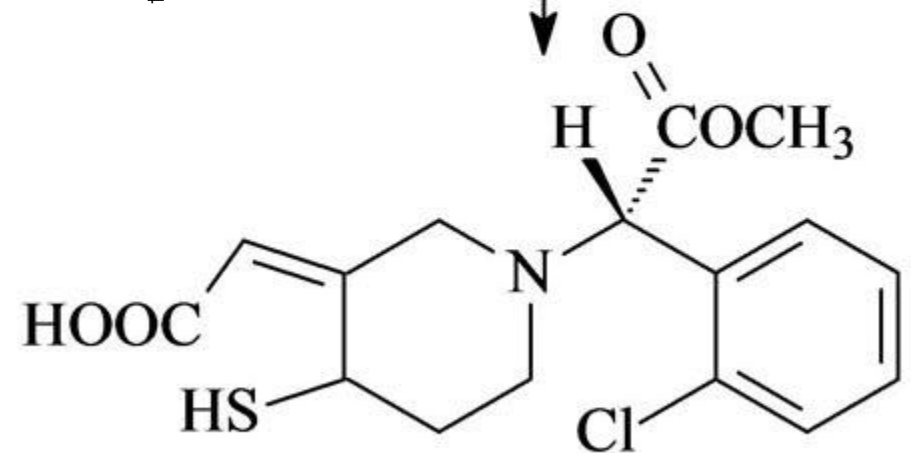
5-thiol metabolite
(R-130964, *active*)
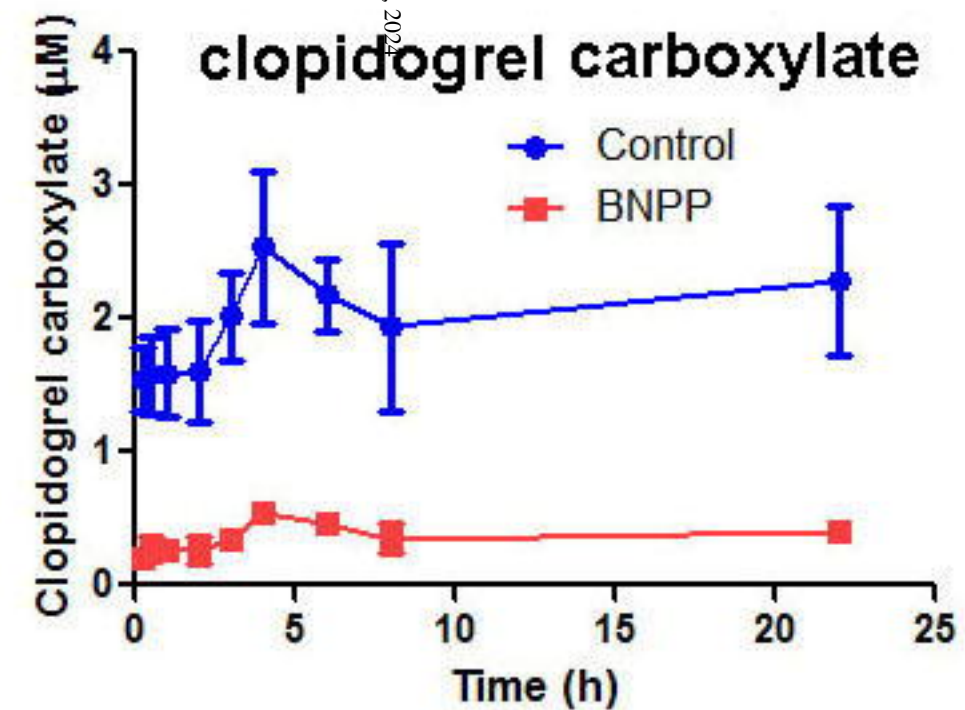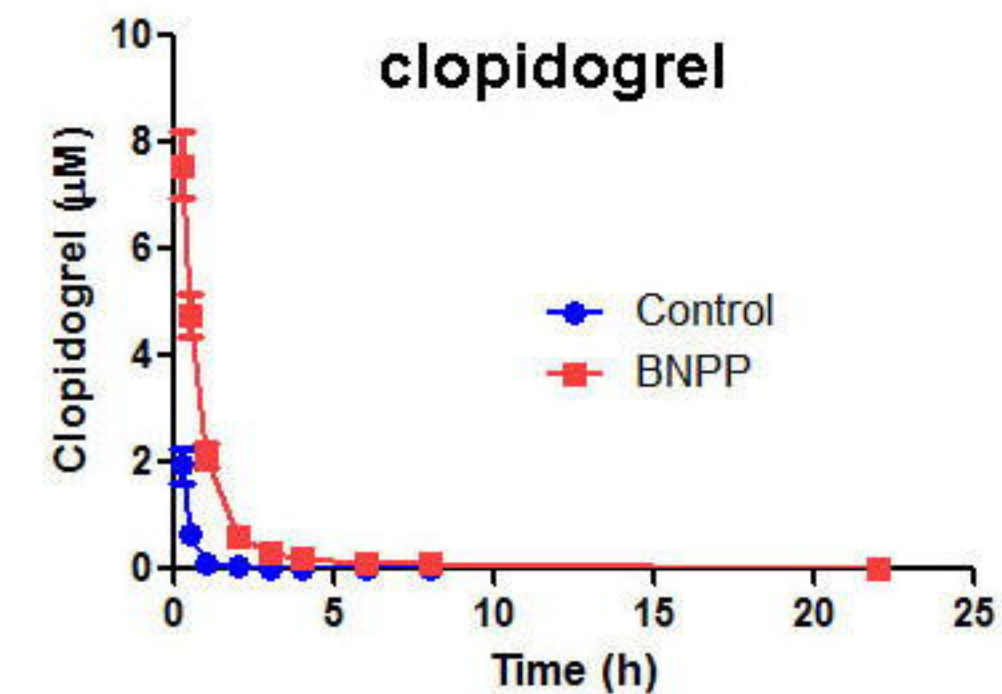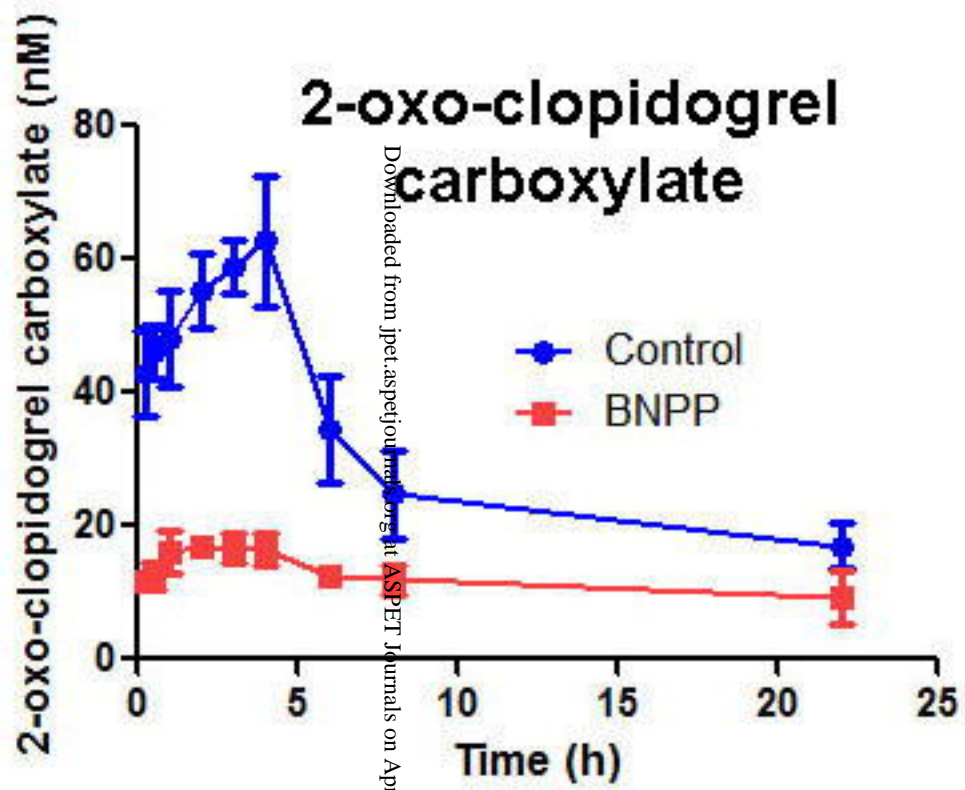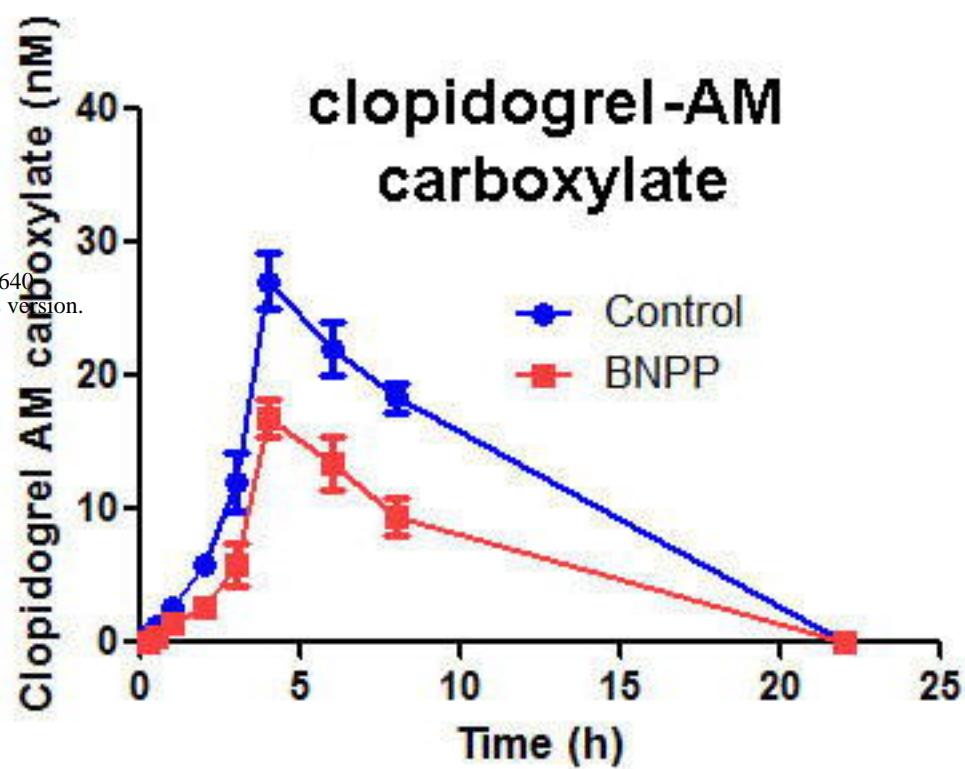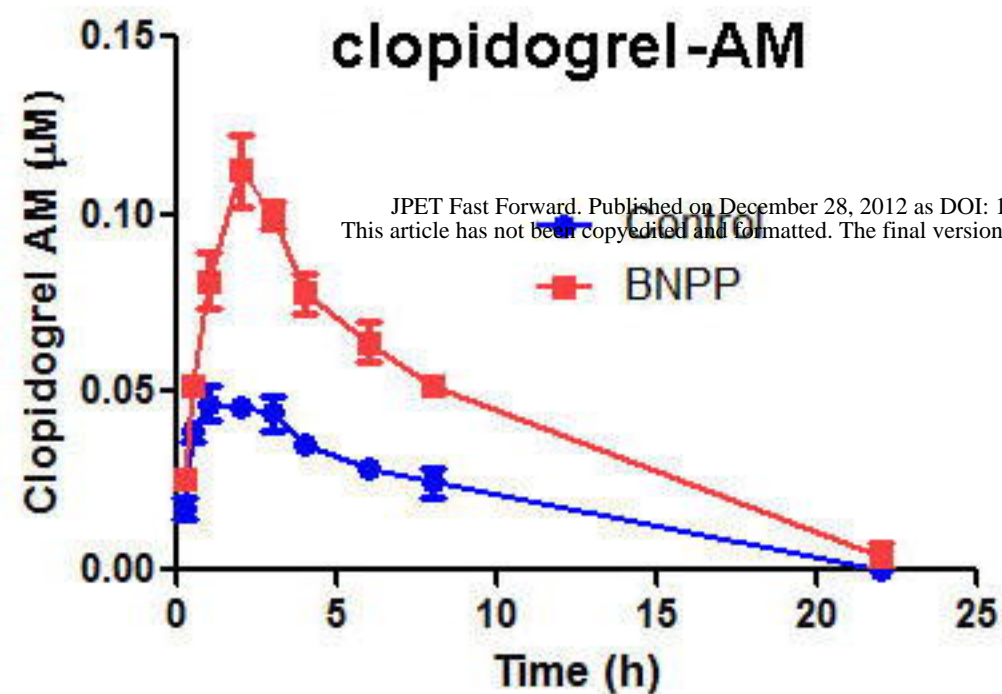
Figure 2

Figure 3

A clopidogrel hydrolysis

- WT
- G18V
- S82L
- A269S
- G143E

B 2-oxo-clopidogrel hydrolysis

- WT
- G18V
- S82L
- A269S
- G143E

Figure 4