Effect of Disease State on Human Carboxylesterase 1 Expression and Activity

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EFFECT OF DISEASE STATE ON HUMAN CARBOXYLESTERASE 1 EXPRESSION AND ACTIVITY

BY

ABDULLAH ALJUTAYLI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PHARMACEUTICAL SCIENCES

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OF
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2016
ABSTRACT

Background: Human carboxylesterase 1, CES1, is a phase I drug metabolizing enzyme that catalyzes the hydrolysis of about 20% of therapeutic agents. Factors, such as metabolic syndrome, ethnicity, gender and genetic polymorphism can influence the activity and/or the expression of drug metabolizing enzymes. Consequently, these factors can affect the pharmacokinetics of drugs leading to toxicity or therapeutic failure.

Objective: The objective of our study is to investigate the possible influence of ethnicity, gender, age, genetic polymorphism, metabolic syndrome or fatty liver on CES1 gene expression and activity.

Methods: Expression of CES1 hepatic mRNA was measured in 89 human livers using RT-PCR techniques. Enzymatic activity of CES1 was measured in S9 fractions and cytosols obtained from 40 and 96 human liver donors, respectively. The hydrolysis rates of two CES1 specific substrates clopidogrel and oseltamivir to their corresponding carboxylic acids were measured using LC-MS/MS methods. Statistical analyses were performed to compare between different groups.

Results and Conclusions: Our data showed that diabetic subjects had less CES1 hydrolytic efficiency although our data is limited by the small sample size. In addition, African American group had significantly higher CES1 activity as compared to Caucasian group. This result provides potential explanation to the clinical observation that African Americans subjects exhibit higher resistance to the CES1 substrates clopidogrel.
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CHAPTER 1

INTRODUCTION

Carboxylesterase1 (CES1) is a member of the mammalian carboxylesterases family of \(\alpha/\beta\)-hydrolase fold proteins (Redinbo et al., 2003). In general, mammalian carboxylesterases (CESs) are classified based on their amino acid homology into five different groups, CES1-CES5 (Holmes et al., 2010). Among the five, CES1 and CES2 are considered to be drug metabolizing enzymes and they share 40-50% amino acid sequence identity (Imai et al., 2006). CES1 has a critical function in catalyzing the hydrolysis of a large number of endogenous as well as exogenous compounds, including drugs and toxins. Many are commonly prescribed drugs that belong to diverse therapeutic classes, including antiplatelet agents, central nervous system (CNS) stimulants, antivirals, and anti-hyperlipidemics (Table 1) (Casey Laizure et al., 2013). The chemical classes of CES1 substrates are ester, carbamate, thioester and amides, but the majority are ester prodrugs. In contrast to cytochrome P450 (CYP) enzymes, our knowledge about CES1 is limited (Casey Laizure et al., 2013). Inter-individual variability of CES1 has been reported to be 430-fold for mRNA, 100-fold for protein and 127-fold for activity, causing unpredicted toxicity or non-responsiveness in pharmacological agents that are activated or cleared by this pathway (Yang et al., 2009). Inter-individual variability can be attributed to many factors including genetic polymorphism, race, drug-drug interaction, age, and/or different diseases (Sanghani et al., 2009) (Xu et al., 2002). The clinical implications of such
variability can be far-reaching. Therefore, more investigation on the role of CES1 as a mediator of drug metabolism and disposition is warranted (Casey Laizure et al., 2013).

**Table 1.** A number of important CES1 substrates among commonly used pharmacological agents (Casey Laizure et al., 2013).

<table>
<thead>
<tr>
<th>Therapeutic Class</th>
<th>Substrates</th>
<th>Product</th>
<th>Product activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiplatelet/Anticoagulant</td>
<td>Clopidogrel</td>
<td>Clopidogrel carboxylate</td>
<td>Inactive</td>
</tr>
<tr>
<td></td>
<td>2-oxo-clopidogrel</td>
<td>2-oxo-clopidogrel carboxylate</td>
<td>Inactive</td>
</tr>
<tr>
<td></td>
<td>Dabigatran etexilate</td>
<td>Dabigatran</td>
<td>Active</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitors</td>
<td>Enalapril</td>
<td>Enalaprilat</td>
<td>Active</td>
</tr>
<tr>
<td></td>
<td>Imidapril</td>
<td>Imidaprilat</td>
<td>Active</td>
</tr>
<tr>
<td>Anti-hyperlipidemia agents</td>
<td>Simvastatin</td>
<td>Dihydroxy acid metabolite</td>
<td>Active</td>
</tr>
<tr>
<td></td>
<td>Fenofibrate</td>
<td>Fenofibric acid</td>
<td>Active</td>
</tr>
<tr>
<td>CNS agents</td>
<td>Methylphenidate</td>
<td>Ritalinic acid</td>
<td>Inactive</td>
</tr>
<tr>
<td>Immunosuppressive agents</td>
<td>Mycophenolate mofetil</td>
<td>Mycophenolic acid</td>
<td>Active</td>
</tr>
<tr>
<td>Neuraminidase inhibitor</td>
<td>Oseltamivir</td>
<td>Oseltamivir carboxylic acid</td>
<td>Active</td>
</tr>
</tbody>
</table>
CHAPTER 2

REVIEW OF THE LITERATURE

2.1 First Pass Metabolism

Hydrolysis and transesterification are the two major reactions catalyzed by CES1 (Redinbo et al., 2003). Hydrolysis reactions serve as activation reactions for many prodrugs. Drug development scientists have taken advantage of the CES1 metabolism pathway to improve the oral bioavailability of many drugs by making ester prodrugs. Ester prodrugs are more lipophilic than both corresponding alcohol and carboxylic acid moieties that are part of the ester containing molecule (Merali et al., 2014). The high lipophilicity of prodrugs facilitates passive diffusion through cell membranes, resulting in higher oral bioavailability (Merali et al., 2014). For example, the active compound oseltamivir acid is a potent antiviral drug; however, due to its polarity, oseltamivir acid is not absorbed well through the gastrointestinal track (GIT). However, once synthetized as the corresponding ester (oseltamivir), the oral bioavailability reaches more than 80% (Taketani et al., 2007). CES1 mediated ester hydrolysis breaks down the ester bond forming two polar moieties, an alcohol moiety and an acyl moiety, facilitating their excretion (Fig. 1) (Merali et al., 2014). It should be noted that not every ester-containing drug is a prodrug. An ester functional group might also be a vital group in the structure of some drugs, such as aspirin and clopidogrel (Williams et al., 2008). For such drugs, CES1 mediated ester hydrolysis reaction is a de-activation reaction. In addition to ester hydrolysis, CES1 catalyzes
transesterification conjugation reaction, which occurs in the presence of ethyl alcohol. For example, clopidogrel in the presence of ethyl alcohol is transesterified into ethyl clopidogrel (Tang et al., 2006). Another example is the transesterification of cocaine into the ethylbenzoylecggonine metabolite that is 10 times more potent (Pennings et al., 2002).

![Figure 1](image)

**Figure 1.** Ester hydrolysis (1), and transesterification (2).

### 2.2 Tissue Distribution

All CES enzymes are located in the cytoplasmic and endoplasmic reticulum of the cells, and they are widely distributed in many tissues of different mammalians.

According to Protein Atlas, mRNA levels of CES1 are found in its highest abundance in the liver, approximately 50% of its total distribution ([www.proteinatlas.org](http://www.proteinatlas.org), Last accessed June 30, 2016). The next highest is the gallbladder, making about 20% of the total mRNA tissue distribution. It should be noted that CES1 is not the only abundant carboxylesterase in the liver. Human carboxylesterase 2 (CES2) is abundant in the liver as well. However, CES2 is the predominantly found in the gastrointestinal tract, GIT, making about 60% of CES2 mRNA total expression in the whole body (Taketani et al., 2007). In contrast to CYPs, the distribution of CES2 along the entire small intestine is relatively constant (Satoh and Hosokawa, 2006). **Figure 2** shows the pattern of mRNA tissue expression of both CES1 and CES2 in various tissues.
2.3 Substrate Specificity

Metabolism mediated by CESs usually lacks binding specificity (Holmes et al., 2010) (Hosokawa, 2008). However, specific preferences have been observed between CES1 and CES2 enzymes. It has been reported that CES1 usually prefers substrates with a large acyl group and a small alcohol group. In contrast, CES2 prefers substrates with a large bulky alcohol group and a small acyl group. As shown in Table 2, clopidogrel is preferably hydrolyzed by CES1 due to its relatively large acyl group, whereas irinotecan is preferably hydrolyzed by CES2 because of its relatively large alcohol group. Furthermore, CESs display stereo-selectivity as well. For example, DL-threo-methylphenidate (MPH), a central nervous system stimulant, is mainly metabolized to ritalinic acid by CES1. In addition, hydrolysis efficiency for L-isomer is 6- to 7-fold higher than the corresponding D-isomer (Sun et al., 2004). Another example is permethrin. The trans isoform is equally preferred by both CES1 and CES2, but its cis isoform is hydrolyzed only by CES2, Figure 3 (Zhu et al., 2000).
Table 2. Differences in substrate preferences for CES1 and CES2.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CES1</th>
<th>CES2</th>
</tr>
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<tbody>
<tr>
<td>Clotidogrel</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Irinotecan</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 3. Stereoselectivity for CES1 and CES2, adapted from (Zhu et al., 2000)

2.4 Species Differences

Animal models are utilized pre-clinically for prediction of pharmacology, toxicology, and pharmacokinetics properties of drugs. The accuracy of the prediction of drug metabolism/elimination is highly dependent on the resemblance between the enzyme in the human and the animal model. Generally, drug biotransformation between different species may differ in metabolic pathways and/or reaction velocity (Taketani et al., 2007). Among many species including humans, mice, rats, rabbits, dogs, and
monkeys, CESs are the most abundant hydrolases in the small intestine and the liver. Although all previously mentioned species share CES1 and CES2 families in their livers, protein levels of both enzymes vary widely (Taketani et al., 2007). In addition, protein level of CES1 family was reported to be abundant in the intestines of some species, such as monkey and rabbit, in contrast to being absent in human (Taketani et al., 2007).

Human plasma contains four esterases, three abundant and one rare, which are paraxonase, butrylcholinesterase, albumin, and acetylcholinesterase, respectively. In contrast to other animal species, such as rodents and rabbits, none of the CESs are expressed in human plasma (Schopfer et al., 2005). A single molecule of albumin has a weak hydrolytic activity; however, albumin’s significant contribution to hydrolysis is due to its high plasma concentration. The hydrolytic activity of albumin is believed to be due to Tyr411 amino acid in human albumin (Hosokawa et al., 1995).

Another important consideration is the level of protein homology between animal and human isoenzymes. Monkey CES1 showed 92.9% homology with human CES1. On the other hand, rabbit CES2 shows only 46.9 homology with human CES2. In fact, due to the differences in selectivity and activity of different species isoforms, pharmacological activity and toxicity of CES substrates are difficult to predict based on animal models and, as a result, experimental animals typically are not adequate pre-clinical models for carboxylesterases (Wood et al., 2003).
2.5 Effect of Gender

Gender can influence the activity and expression of drug metabolizing enzymes (DMEs). For example, CYP3A4 activity is reported to be two-times higher in female subjects as compared with male subjects (Patrick et al., 2007). Although the CES1 gender differences are not well established, some evidence has been reported supporting the effect of gender on metabolism of some drugs. For example, one study reported that the CES1 substrates lovastatin and simvastatin, are hydrolyzed more efficiently in women than in men (Patrick et al., 2007). Similarly, another clinical study showed differences in response and area under the concentration-time curve (AUC) for a dose of 0.3 mg/kg of methylphenidate administered to men and women subjects. The value of AUC (SD), in average, was much higher in men as compared with women [93.4 (25.3) ng h/ml vs. 73.5 (12.8) ng h/ml respectively] (Patrick et al., 2007; Casey Laizure et al., 2013).

2.6 Age-dependent Expression and Activity

Age can potentially influence many drug metabolizing enzymes including cytochrome P450 (CYP) enzymes (Koukouritaki et al., 2004). The activity of some CYPs (i.e. CYP2D6, CYP2C9, and CYP2E1) increase gradually with age (Stevens et al., 2003). In contrast, CYP3A7 protein level showed a drastic decrease by more than 60-fold by the end of their first birthday (Mehrotra et al., 2016). The influence of age on CES1 have been reported by Yang et al. and Zhu et al. (Yang et al., 2009)(Zhu et al, 2009). The average expression of CES1 in subjects younger than one year was significantly lower in comparison with pooled samples. For example, only 20.3% and 11.1 % of the
activity of the pooled samples were observed in 13-day and 30-day old subjects. Studies conducted using human liver microsome showed 4-fold and 10-fold higher adult CES1 activity compare to children and fetal microsomes, respectively (Yang et al., 2009). Furthermore, the correlation between growth hormone (GH) and CESs age-related expression in mice models was studied (Zhu et al., 2009). However, no correlation was observed neither after a short-term treatment with GH (1 day) nor after a longer term treatment of 9 days, indicating no contribution of GH to reduced CESs expression in young mice.

The results of Yang et al. were consistent with these findings (Yang et al., 2009). In their study, they grouped livers from 104 subjects into three groups, fetal, child, and adults. The expression and the activity were age related in such that it was higher in adults than in children, which was, also, higher than the expression in the fetal group.

2.7 Lipid Metabolism

The role of CES1 in lipid metabolism is very significant. For example, CES1 metabolizes endogenous substrates, such as cholesteryl esterase, triacylglycerol, and 2-arachidonylglycerol through a mechanism of forming a fatty acyl-enzyme intermediate and releasing an alcohol substituent (Merali et al., 2014). The intermediate acyl-enzyme, then, reacts with water releasing an acyl containing-substrate. In addition, CES1 possesses cholesterol transferase activity that helps with de-esterification and trans-esterification of lipids and forms cholesteryl esters in the presence of free cholesterol (Friedrichsen et al., 2013). Friedrichsen et al. reported a correlation between CES1 expression in adipose tissue and obesity, in which CES1
expression was higher in obese subjects (Friedrichsen et al., 2013). Furthermore, diet induced weight loss decreased CES1 expression in obese subjects. In fact, some speculate that CES1 expression in adipose tissue is connected to measures of metabolic function and obesity, such as plasma insulin level, triglycerides level, waist circumference, and homeostasis model assessment-insulin resistance, and, therefore, it may contribute to obesity and type-2 diabetes mellitus (Merali et al., 2014).

2.8 Effect of Pathological Conditions

Disease conditions are another factor that can alter CES1 expression or activity. Severe health conditions are accompanied by an elevation in the concentration of interleukin 6 (IL-6), an inflammatory biomarker, which has a suppression effect on the CES1 expression leading to decreases in CES1 activity (Yang et al., 2007). Hepatic dysfunction, such as cirrhosis and hepatitis, reduces the hydrolysis activity of CES1 (Thiollet et al., 1992; Eriksson et al., 2003). However, CES1 expression in mice infected with hepatitis C virus was up-regulated, which is believed to help the formation of lipid droplets that may be necessary for viral proliferation (Zhu et al., 2000).

Type 2 diabetes mellitus was reported to decrease CES1 protein level (Valle et al., 2012). Diabetes is characterized by many metabolism disorders including hyperglycemia, hyperinsulinemia, and abnormalities in fat, carbohydrates and protein metabolism (Valle et al., 2012). Moreover, steatosis and NASH are associated with diabetes and obesity (Fabbrini et al., 2010; Leite et al., 2011). Non-alcoholic fatty liver disease (NAFLD) is characterized by steatosis, a condition that is defined as an
abnormal retention of lipids in hepatocytes (Merali et al., 2014). Steatosis per se is an independent and important marker for multi-organ insulin resistance (Vega et al., 2007; Korenblat et al., 2008). NAFLD can progress to severe stages such as non-alcoholic steatohepatitis (NASH), cirrhosis or hepatocellular carcinoma (8,9).

2.9 Enzyme Inhibition

Competitive inhibition is likely to occur between CES1 substrates; especially some of CES1 substrates that are commonly prescribed together (Zhu et al., 2000). For example, it is reported that clopidogrel inhibits the conversion of oseltamivir to its active metabolite by 90% (Goel et al., 2007). A clinical trial reported that severe mania episode occurred as a result of concurrent administration of aripiprazole and the CES1 selective substrate DL-threo-methylphenidate (MPH) (Holmes et al., 2010). This effect might be as a result of the aripiprazole potential inhibitory effect on CES1 metabolism reported in vitro (Zhu et al., 2010).

2.10 Pharmacogenomics and Genetic Polymorphism of CESs

The highly polymorphic CES1 gene is located on chromosome 16q12.1, and it consists of 14 exons (Merali et al., 2014). Six CES1 single nucleotide polymorphisms (SNP) have been identified to be associated with alterations in drug disposition (Hines et al., 2016). The CES1 alleles rs3785161 and rs2241409 are associated with increase promoter activity and decreased transcript levels (Marsh et al., 2004; Geshi et al., 2005). CES1 rs71647871 results in a total loss-of-activity due to a substitution in amino acid number 143 from glycine to glutamic acid in the protein product. This
substitution is a result of a nonsynonymous transition of G to A at cDNA position 428 of CES1 in exon 4 (Hines et al., 2016). The frequency of this variant is reported to be around 4.3% in African Americans, 3.7% in Caucasians, and 2% in Hispanics, and 1% in Asians (Casey Laizure et al., 2013).

2.11 Oseltamivir and Clopidogrel

In our study, we were interested on the role of CES1 in oseltamivir and clopidogrel metabolism. Therefore, both pharmacological agents are reviewed below.

2.11.1 Oseltamivir

The World Health Organization (WHO) recommends use of oseltamivir during influenza pandemics as a treatment and prevention for influenza A and B virus infections (Merali et al., 2014). Oseltamivir carboxylate, a CES1 metabolism product, is the active form of the prodrug oseltamivir. Oseltamivir carboxylate is a neuraminidase inhibitor that prevents viral reproduction. *In vivo* and *in vitro* response variability has been reported. Rs71647871 mutant cell lines showed 25% lower activity than the corresponding wild type (Merali et al., 2014). The average increase in the AUC of oseltamivir was 360% and 18% for heterozygotes (428AA) and homozygote (428GA) alleles, respectively, compare to the wild type (428GG) allele (Taketani et al., 2007). In healthy volunteers, heterozygous rs121912777 SNP carriers showed 18% increase in oseltamivir AUC, and 23% decrease in metabolite-to-drug AUC compare to non-carriers (Merali et al., 2014).

2.11.2 Clopidogrel
Clopidogrel is a prodrug that undergoes a cascade of biotransformation by several CYP enzymes including CYP1A2, CYP3A4, CYP2B6, CYP2C9, and CYP2C19 (Fig. 4) (Zhu et al., 2013) (Rehmel et al., 2006; Kazui et al., 2010). However, only 15% of the oral dose is metabolized to 2-oxo-clopidogrel and the remaining 85%, undergoes hydrolysis by CES1 to the corresponding inactive metabolite (Hagihara et al., 2009). The metabolite, 2-oxo-clopidogrel undergoes further metabolism to the pharmacologically active 2-thiol clopidogrel (clopidogrel-AM), which is a bio reactive compound that irreversibly blocks P2Y\textsubscript{12} adenosine diphosphate (ADP) receptor on platelet membrane by forming a disulfide bridge (Bouman et al., 2011). CES1 dominate the metabolism of both 2-oxo- and 2-thiol-clopidogrel to the inactive corresponding carboxylic acids (Bouman et al., 2011).

Prolonged dual antiplatelet therapy with aspirin and clopidogrel, an antiplatelet thienopyridine inhibitor, is recommended to reduce ischemic events in atherothrombotic patients. Dual antiplatelet therapy is pivotal in conjunction with the use of drug-eluting stents (DES) to prevent stent thrombosis, ST, which is associated with high rates of morbidity and mortality (Anderson et al., 2007; Antman et al., 2008)(Yusuf et al., 2001; Sabatine et al., 2005). DES is a widely used technique making about 75% of the total percutaneous coronary intervention, PCI. Although the dual therapy with clopidogrel and aspirin decreases the occurrence of ST, several studies reported rare yet life threatening STs in some patients (Vaknin-Assa et al., 2007).
A significant inter-individual variability in the response to clopidogrel has been reported (Yusuf et al., 2001; Steinhubl et al., 2002; Sabatine et al., 2005). This variability has an impact in a large proportion of the population and increases the risk of cardiovascular events in case of hypo-responsiveness (Wang et al., 2006; Angiolillo and Alfonso, 2007; Angiolillo et al., 2007; Gurbel et al., 2007). Many studies have associated CYP2C19 reduced-function alleles (*2, *3, *4, *5) with lower levels of clopidogrel-AM and consequently lower platelet inhibition and higher rate of adverse cardiovascular events, such as ST (Mega et al., 2009; Simon et al., 2009). However, it has been suggested that black race is an independent predictor of definite drug-eluting stent thrombosis, and its potential mechanism of influence on ST has been provoked for further investigation (Collins et al., 2010). CES1 is believed to play a major role in clopidogrel inter-individual variability and it might explain the mechanism of higher cardiovascular events among African American (AA) patients.
Figure 4. Metabolic pathways of clopidogrel (Hall et al., 2011).
CHAPTER 3

METHODOLOGY

3.1 Chemicals and Supplies

Oseltamivir, oseltamivir acid, and oseltamivir-d3-acid were obtained from TRC (Toronto, ON, Canada). Clopidogrel and clopidogrel carboxylic acid were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Naproxen was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sections of human livers were obtained from a commercial source (Xenotech LLC, Lenexa, KS). Unless otherwise indicated, all other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

3.2 Tissue Preparation

Human liver samples and their demographic data, i.e. gender, age, height, weight, smoking and drinking habits, were obtained from XenoTech LLC. Thawed liver samples, 0.5 g each, were homogenized using automated Omni Bead Rutor 24 bead mill homogenizer (Omni International, Kennesaw, GA). The homogenates were further centrifuged at 10,000 g for 20 min at 4°C using Eppendorf centrifuge (Hamburg, Germany). The supernatant, S9 fractions, were aliquoted and stored for further experiments at -80°C. For microsome and cytosols preparations, the supernatants were subjected to ultracentrifugation at 100,000 g for 60 min at 4°C. The pallets were washed and re-suspended in glycerol buffer (20% glycrol, 20 uM BHT, 0.5 mM EDTA, 50mM Tris-HCL, and 0.25 M sucrose) and stored in -80 °C.
3.3 Total Protein Quantification

Total protein concentration in S9, microsomes, and cytosols samples were quantified using Bicinchoninic Acid Protein Assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer’s protocol.

3.4 LC-MS/MS Analyses

3.4.1 Oseltamivir Assay

A tandem mass spectrometry technique was used. Aquiety Xevo TQ MS mass spectrometry (Waters Corp.) equipped with an electrospray ionization source (ESI). Analytes detection was performed in the positive ion mode, and the mass transitions used were \( m/z \) 313.11 to 120 with retention time 0.83 minutes for oseltamivir, and \( m/z \) 285.13 to 197 with retention time of 0.75 minutes for oseltamivir acid. The desolvation gas flow rate was 650 L/h at 350 °C. Cone gas flow rate was set at 2 L/h and the source temperature was 150°C. The column was Acquity UPLC BEH C18 column, 2.1 x 50 mm, 1.7 µm (Waters Corp., Milford, MA) and was maintained at 45°C. Mobile phases were (A) 0.05% formic acid and 5% acetonitrile in deionized water, and (B) acetonitrile sustained at a flow rate 0.5 ml/min during the entire run of 2 minutes. The elution mode was 95:5 (A:B) for 0.1 min. From 0.1 to 1.0 minutes, there was continuous gradient change to A: B 5:95 (v/v). The composition was kept constant for 1.5 minutes. The mobile phase composition was reversed back with a fast gradient to A:B 95:5 (v/v), and the column was re-equilibrated for 2 minutes.
The linearity was established from 5 ng/ml to 1000 ng/ml for both oseltamivir and its metabolite. All standards and quality control points were prepared in Tris-HCl buffer (50 mM, pH 7.4). Then, they were mixed with 1:2 acetonitrile containing the internal standard, oseltamivir-d3-acid, before injecting in the system.

3.4.2 Clopidogrel Assay

For clopidogrel LC-MS/MS analysis, the mass transitions used were m/z 322.0 to 211.85 with retention time 1.6 minutes for clopidogrel, m/z 307.7 to 197 with retention time 1.2 minutes clopidogrel carboxylate, and 228.89 to 168.95 for the IS naproxen. Mobile phases were (A) 0.05% formic acid, and 5% acetonitrile in deionized water, and (B) acetonitrile sustained at a flow rate 0.5 ml/min during the entire run of 3 minutes. The elution mode was 95:5 (A:B) for 0.2 min. From 0.1 to 1.0 minutes, there was continues gradient change to A: B 5:95 (v/v). The composition of mobile phase was kept constant to 2.0 minutes. It retained back with a fast gradient.

Figure 5. Chromatogram obtained from oseltamivir LC-MS/MS assay.
to A:B 95:5 (v/v), and re-equilibrated until 3 minutes. The instrument used and its parameters were the same as described earlier using the same analytical column. The linearity was established from 0.4 ng/ml to 300 ng/ml clopidogrel carboxylic acid. All standards and quality control points were prepared as described above with oseltamivir.

![Figure 6. Chromatogram obtained from clopidogrel LC-MS/MS assay.](image)

### 3.5 Determination of Enzymatic Activity

Human carboxylesterase 1, CES1, activity was assessed by the measuring the rate of CES1 hydrolysis of the specific substrate, oseltamivir to its carboxylic acid metabolite. Oseltamivir hydrolysis was carried out by incubation with human liver cytosols. With 30 ul of the reaction buffer, Tris-HCl (50 mM, pH 7.4), containing 0.2 mg/ml protein were pipetted into 1 mL Waters 96/well microplates. This was mixed with an equal volume of the substrate at concentrations ranged from 9.15 uM to 12.55 mM for S9 fraction, 10 and 40 uM for cytosols. After 10 minutes of incubation at 37°C, the
reaction was terminated using 120 ul of ice-cold acetonitrile containing the internal standard, oseltamivir-d3-acid. Then, the mixture was centrifuged at 4000 g and 4°C for 15 minutes. Reactions that were performed without protein, served as controls. The concentrations of metabolites were measured using the LC-MS/MS method described above.

Clopidogrel incubations with S9 fractions followed the same protocol. However, incubation time was 20 min and [S] ranged from 2.24 uM to 605 uM for enzyme kinetics and 8 uM for determination of incubation time curves.

For all enzyme kinetics experiments, samples were diluted according to dilution factors shown in Table 3 to fit in the range of analysis calibration curve.

<table>
<thead>
<tr>
<th>#</th>
<th>[Oseltamivir] uM</th>
<th>Dilution Factor</th>
<th>[Clopidogrel] uM</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12556</td>
<td>1371.7</td>
<td>605.9</td>
<td>4285</td>
</tr>
<tr>
<td>2</td>
<td>3766.5</td>
<td>411.5</td>
<td>393.8</td>
<td>2785</td>
</tr>
<tr>
<td>3</td>
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<td>256</td>
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<td>6</td>
<td>30.51</td>
<td>3.3</td>
<td>24.9</td>
<td>176.5</td>
</tr>
<tr>
<td>7</td>
<td>9.153</td>
<td>1</td>
<td>7.48</td>
<td>53</td>
</tr>
<tr>
<td>8</td>
<td>2.2</td>
<td>1.1</td>
<td>2.2</td>
<td>15.8</td>
</tr>
</tbody>
</table>

Ahead of all experiments, pilot studies were performed using pooled S9 fractions and cytosols from 6 livers of healthy donors to optimize incubation conditions and to maintain the reactions in the linear range. Incubation period (0 to 60 min) and protein concentration (0.035 to 1 mg/ml) experiments were performed as described earlier; however, using 1.5 ml Eppendorf vials.
3.6 Real-Time PCR

Isolation of total RNA was performed with High Pure RNA Paraffin Kit (Roche Diagnostics, Indianapolis, IN, USA). For cDNA reparation we used QIAamp DNA Mini Kit (QIAGEN Sciences, Maryland). Messenger RNA (mRNA) levels were measured using TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) on a Vii7 real-time PCR machine from Applied Biosystems (Foster City, CA). The results were based on Ct values, which were normalized to B-actin mRNA level, and corrected based on 16s rRNA.

3.7 Statistical Analysis

The experimental results were analyzed using IBM SPSS Statistics software (Version 23, Chicago, USA). Results are expressed as the mean ± standard deviation (SD) or geometric mean ± SEM. Distribution normality of the variables were confirmed using Kolmogorov-Smirnov’s test. Differences between groups were determined using either independent samples t-test or nonparametric Kruskal-Wallis test was used as specified in each case. P values of 0.05 or less was considered statistically significant.
CHAPTER 4

RESULTS

4.1 Differences in CES1 Expression and Intrinsic Clearances between Groups of Subjects Donating Livers

Differences in CES1 expression and intrinsic clearances between groups of subjects donating livers are presented in Table 4 and Table 5, respectively. Data for the factors including fibrosis, NASH or alcohol consumption in Table 5 was not available for all subjects. Subjects with diabetes showed significantly higher intrinsic clearance (Clint) compare to non-diabetic subjects, $P=0.007$. Despite the limited sample size of 6 subjects with probable NASH, it seems that NASH might affect as compare to subjects with no NASH, $P=0.099$.

Table 4. Differences in CES1 expression levels between groups of subjects donating livers.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Variables (N)</th>
<th>Mean (SD) ΔΔ CT</th>
<th>$P$ Value (Kruskal-Wallis test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Female (43) Male (46)</td>
<td>1.23 (1.04) 1.82 (2.6)</td>
<td>0.521</td>
</tr>
<tr>
<td>Race</td>
<td>Caucasian (81) African American (8)</td>
<td>1.60 (2.1) 0.88 (0.52)</td>
<td>0.521</td>
</tr>
<tr>
<td>Alcohol Consumption</td>
<td>Yes (45) No (44)</td>
<td>1.66 (1.73) 1.40 (2.28)</td>
<td>0.030</td>
</tr>
<tr>
<td>Smoker</td>
<td>Yes (47) No (42)</td>
<td>1.55 (1.79) 1.51 (2.22)</td>
<td>0.475</td>
</tr>
<tr>
<td>Diabetes</td>
<td>No (37) Yes (52)</td>
<td>1.44 (1.82) 1.59 (2.15)</td>
<td>0.603</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>No (77) Yes (12)</td>
<td>1.58 (2.14) 1.20 (0.7)</td>
<td>0.867</td>
</tr>
<tr>
<td>NASH according to Kleiner et al. classification system (Kleiner et al., 2005)</td>
<td>No NASH (50) Borderline NASH (26) NASH (13)</td>
<td>1.505 (1.76) 1.58 (2.7) 1.5 (1.19)</td>
<td>0.513</td>
</tr>
</tbody>
</table>
Table 5. Differences in intrinsic clearances between groups calculated based on full enzyme kinetics obtained from oseltamivir incubations with S9 fraction as determined in Fig. 17.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Variables (N)</th>
<th>Mean (SD) ul/min*mg</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Female (18) Male (21)</td>
<td>160.9 (125.1) 189.9 (129)</td>
<td>0.338³</td>
</tr>
<tr>
<td>Race</td>
<td>Caucasians (19) African Americans (20)</td>
<td>135.33 (98.5) 215.5 (193.4)</td>
<td>0.047⁷⁴</td>
</tr>
<tr>
<td>Alcohol Consumption</td>
<td>Yes (13) No (16)</td>
<td>130.2 (65.7) 150.2 (104.1)</td>
<td>0.772⁴</td>
</tr>
<tr>
<td>Smoker</td>
<td>Yes (27) No (12)</td>
<td>180.2 (133) 168.2 (115.3)</td>
<td>0.903⁵</td>
</tr>
<tr>
<td>Diabetes</td>
<td>No (27) Yes (12)</td>
<td>204.5 (183.1) 113.5 (62)</td>
<td>0.007⁷⁴</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>No (26) Yes (3)</td>
<td>144.9 (91.9) 109.4 (35.7)</td>
<td>0.523⁵</td>
</tr>
<tr>
<td>NASH according to Kleiner et al. classification system (Kleiner et al., 2005)</td>
<td>Not NASH (22) Probable NASH (6)</td>
<td>158.2 (90.2) 94.7 (60.2)</td>
<td>0.099⁵</td>
</tr>
</tbody>
</table>

*a Independent samples t-test
*b Kruskal-Wallis test

4.2 Experiment Conditions

4.2.1 Time-dependent carboxylesterase 1 activity

Oseltamivir experiments conditions were determined to be linear in the range of experiments as shown in (Fig. 7) for S9 and (Fig. 8) for cytosols pooled from 6 different subjects.
4.2.2 Establishment of the linearity between protein concentration and hydrolysis activity

Oseltamivir experiments conditions were determined to be linear (Fig. 9) for S9 and (Fig. 10) for cytosols in the range of the experiments. Cytosol and S9 fractions were pooled from 6 different subjects.
Figure 9. Scatter plot showing protein concentration vs. oseltamivir carboxylic acid formation rate in S9 fractions.

Figure 10. Scatter plot showing protein concentration vs. oseltamivir carboxylic acid formation rate in cytosol.

4.3 Clopidogrel Enzyme Kinetics in S9 fraction

Pooled liver S9 fractions of 20 AA and 20 Caucasian subjects were used to compare CES1 activity in metabolizing clopidogrel to clopidogrel carboxylic acid.
The pooled AA group had significantly higher CLint, $P = 0.036$ (Fig. 11). Substrate inhibition was observed and the data were fitted using equation 1. At high substrate concentrations, clopidogrel inhibited its own metabolism to clopidogrel carboxylic acid \textit{in vitro}. Clopidogrel was reported to inhibit oseltamivir hydrolysis \textit{in vitro} (Tang et al., 2006). Organic Solvents used to dissolve clopidogrel did not exceed 1%.

**Equation 1:** $Y = V_{max} \frac{x}{K_m + x \left(1 + \frac{x}{K_i}\right)}$

**Figure 11.** Clopidogrel incubations in pooled S9 fractions obtained from 20 African American subjects and 20 Caucasian subjects showed inhibition effect.

### 4.4 Clopidogrel Hydrolysis Activity as a Function of Time

Incubations with 8 uM clopidogrel (Fig. 12) shows that although both groups were able to form 550 nM clopidogrel carboxylic acid, pooled S9 fractions from Caucasians had slower reaction time as compared with AA. This confirms that CES1 activity in AA is more efficient even at low substrate concentrations.
4.5 Higher CES1 activity in African Americans is Highly Influenced by CES1 Activity in Five Subjects

Among the 20 African American subjects, 5 subjects showed relatively and constantly higher enzyme activity in metabolizing both clopidogrel and oseltamivir. When removing these subjects, African American group losses the significant level as shown in plot with Clint, P<0.001 (one way ANOVA comparing the three groups) (Fig. 13).
4.6 African Americans had Significantly Higher Oseltamivir Hydrolysis Rates Compare to Caucasians in Human Liver Cytosol

The efficiency of hydrolysis 10 uM and 40 uM oseltamivir to oseltamivir carboxylic acid expressed as formation rates (pmol/min/mg protein) in human liver cytosols of 8 African Americans were significantly higher than the rates in cytosols of 88 Caucasian subjects indicating higher CES1 enzymatic activity in African Americans, P=0.006, P=0.002 (Kruskal-Wallis test) for incubations with 10 uM and 40 uM, respectively. Data plotted in (Fig. 14) compares the hydrolysis rates of 10 uM oseltamivir in African Americans and Caucasians, while (Fig. 15) shows the frequency of distribution for incubations with 40 uM.

![Boxplot showing oseltamivir carboxylic acid rates of formation of 88 Caucasian and 8 African American subjects incubation in cytosol at 10 uM.](image)

**Figure 14.** Boxplot showing oseltamivir carboxylic acid rates of formation of 88 Caucasian and 8 African American subjects incubation in cytosol at 10 uM.
4.7 Oseltamivir Enzyme Kinetics Using S9 fractions showed Higher Clint in African American Livers

Averaged and Individual (Fig. 16 A and B) enzyme kinetic results obtained from individual incubations in S9 fractions of 20 African Americans showed significantly higher internist clearance (Clint) in AA compare to averaged 19 Caucasians, \( P=0.047 \). Intrinsic clearance was calculated for each subject according to the equation: Clint = \( \frac{V_{\text{max}}}{K_m} \). Parameters \( K_m \) (\( p=0.05 \)) and \( V_{\text{max}} \) (\( p=0.87 \)) were calculated using Prism (Table 6).
Table 6. Individual Km, Vmax, and Clint values.

<table>
<thead>
<tr>
<th>ID</th>
<th>Km (uM)</th>
<th>Vmax (nmol/min/mg)</th>
<th>Clint (ul/min*mg)</th>
<th>ID</th>
<th>Km (uM)</th>
<th>Vmax (nmol/min/mg)</th>
<th>Clint (ul/min*mg)</th>
</tr>
</thead>
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<td>8707</td>
<td>641.48</td>
<td>73.67</td>
</tr>
</tbody>
</table>

4.10 Inter Individual Variability in Hydrolysis of Oseltamivir

Human Carboxylesterase shows high inter-individual variability in both cytosol (CV= 66%) and S9 fractions (CV= 73%) as shown in Figures 16 (a) and (b).

4.8 Establishment of the correlation between CES1 activities measured by oseltamivir and clopidogrel
Positive correlation was observed between CES1 activities measured using oseltamivir and clopidogrel hydrolysis in S9 fractions (Fig. 17 (a) and (b)). The data used in these correlations are oseltamivir enzyme kinetics data above and clopidogrel enzyme kinetics data mentioned in the appendix, which defined the metabolism phase by seven [S] points in contrast to 3 [S] points in Figure 11.

**Figure 17 (A).** Positive correlation between CES1 activities measured by oseltamivir and clopidogrel in African American subjects.

**Figure 17 (B).** Positive correlation between CES1 activities measured by oseltamivir and clopidogrel in Caucasian subjects.
CHAPTER 5

CONCLUSION

The goal of this study was to investigate variables influencing CES1 expression and activity, and subsequently the PK/PD of drugs undergo CES1 hydrolysis. Metabolism efficiency of oseltamivir mediated by CES1 appeared to be lower in diabetic subjects compare to non-diabetic subjects. Despite the limited sample size of 6 subjects with probable NASH, it is likely that NASH might reduce the CES1 hydrolysis efficiency. However, the effect of both diabetes and NASH should be studied with a larger sample size. Also, among many variables, subjects who consume alcohol had higher level of CES1 gene expression although it was not translated into higher enzymatic activity.

Another important and clinically relevant finding is that African Americans had a significantly higher CES1 activity compare to non-African Americans. This finding can provide potential mechanism explaining the clinical observations of African Americans developing nearly three times stent thrombosis compare to non-blacks after implantation with drug-eluting stent (DES) (Collins et al., 2010). Our data support that black race per se is an independent strong predictor for developing ST after DES (Collins et al., 2010). Our results indicate that CES1 activity in human liver S9 fractions and Cytosols were significantly higher in Africans American group compare to Caucasians using two different CES1 probes, oseltamivir and clopidogrel.
In case of clopidogrel metabolism, a two-step metabolism process by CYP1A2 and CYP2B6 to clopidogrel thiolacton, and then by CYP2B6, CYP2C9, and CYP2C19 are necessary for forming clopidogrel AM. However, clopidogrel and its metabolites are subject to a parallel competitive inactivation pathway. The inactivation pathway is predominant since more than 85% of clopidogrel and 49% of clopidogrel thiolacton is inactivated by CES1 to the corresponding carboxylic acid metabolite. Hydrolysis pathway by CES1 is a very efficient pathway compare to CYP450 pathways involved in clopidogrel metabolism. For example, Hagihara et al. reported the mean (activity pmol/mg/min) in 20 human liver microsome of CYP1A2 to be (39.0), CYP2B6 (2.0), CYP2C9 (54.8), CYP2C19 (10.1), CYP2D6 (27.3), CYP2E1 (1522.4), CYP3A4 (2084.8), and CES1 to be (20,299.7) (Hagihara et al., 2009). In addition to CES1 efficiency over other CYPs, CES1 has a combined effect on clopidogrel metabolism such that CES1 is involved in inactivation of clopidogrel and all its metabolites. Therefore, due to CES1 efficiency and combined effect, it is very likely that CES1 activity correlate inversely with clopidogrel AM formed and any variability in CES1 activity might lead to variation in clopidogrel AM, and consequently its plate inhibition activity. However, clopidogrel resistant appears to be multifactorial, and some factors, such as CYP2C19 polymorphisms have been reported as contributors.

Clopidogrel inhibited its own metabolism in vitro (Fig. 11). It was reported that clopidogrel inhibited oseltamivir hydrolysis (Shi et al., 2006). Substrate inhibition
was reached at a relatively high concentration and does not seem to have a clinical significant.

In contrast to clopidogrel, variability in CES1 activity might not be of a clinical implication for drugs that follow sequential non-competitive CES1 metabolism pathways, such as oseltamivir. Assuming enough presentation time to CES1 in the liver, CES1 will eventually metabolize all available substrate owning to its high efficiency and low \textit{in vivo} concentration of most drugs. Such variability in CES1 activity is likely to be counterbalanced although it might influence some pharmacokinetic parameters such as Tmax.

In conclusion, this study gives a potential explanation of the clinical observation and strongly supports the conclusion of \textit{Collines et al.} that black populations are exposed to more risk incidence of stent thrombosis (Collins et al., 2010). Our data support that black race \textit{per se} is a strong predictor of stent thrombosis.
APPENDIX

I. Results of clopidogrel Incubation, Experiment # 2.

![Graph showing the rate of clopidogrel incubation over different concentrations. The graph has a y-axis labeled "Rate (pmol/mg/min)" and an x-axis labeled "[Clopidogrel] uM". Two curves are depicted, one for AA (blue) and one for C (red).]


Hines RN, Simpson PM, and McCarver DG (2016) Age-Dependent Human Hepatic Carboxylesterase 1 (CES1) and Carboxylesterase 2 (CES2) Postnatal Ontogeny. Drug Metabolism and Disposition: dmd. 115.068957.


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