PUCKETT, SARA PARKER, M.S. Cytochrome P450's and the Effects upon the Addition of Undecylenic Aldehyde. (2016)
Directed by Dr. Gregory M. Raner 50 pp.

Cytochrome P450's are vital enzymes for the metabolism of chemicals that are foreign to the human body. By altering the activity of these enzymes through enzyme inhibition, it is possible to alter rates of metabolism of these so-called xenobiotics many of which belong to the pharmaceutical class, resulting in what is termed a "Drug Interaction". There are many well-known drug interactions known, though most involve inhibition of the human cytochrome P4503A4 enzyme. A classic example is the case of statins drugs and furanocoumarins found in grapefruit juice. Research aimed at determining the effects of foreign chemicals on human cytochrome P450's is an important area of pharmacology and toxicology, as it has the potential to identify toxic or dangerous drug interactions before a drug reaches clinical trials. Toward this end, prior studies with aldehydes and terminal olefins have shown that both general classes of molecules can potentially destroy certain mammalian cytochrome P450 enzymes. The compound, undecylenic aldehyde, which is an additive in a variety of consumer products, contains both functional groups, and was therefore a target for evaluation in the current study. The effects of adding various concentrations of undecylenic aldehyde to different human P450 isoforms were monitored using HPLC-based enzyme assays, and the results showed a significant decrease in the activity of isoforms 2E1 and 3A4 in the presence of this compound. Modes of inhibition were analyzed through Michaelis-Menten kinetics and appeared to be reversible and non-competitive (mixed) in nature.

CYTOCHROME P450'S AND THE EFFECTS UPON THE ADDITION OF UNDECYLENIC ALDEHYDE

by

Sara Parker Puckett

A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2016

Approved by

_____
Committee Chair

To my family

Your continuous love and endless patience is what made all of this possible. I would not be the person I am today without your continuous encouragement.

To my friends

Thank you for your unwavering support. You all have helped me with writing, presentations, but most of all boosting my morale and I am forever grateful.

APPROVAL PAGE

This thesis, written by Sara Parker Puckett, has been approved be the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

_____

_____

Date of Acceptance by Committee

_____

Date of Final Oral Examination

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Gregory Raner as a source of continual guidance throughout my time at UNCG. I would also like to thank Dr. Jason Reddick for aiding in my admission as well as serving on my committee. Lastly, I would like to thank Dr. Alice Haddy for helping me during your office hours as well as the time you invested as being a member of my committee.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SCHEMES

Page

CHAPTER I

INTRODUCTION

1.0.0. Xenobiotics

Foreign substances can enter the body through a variety of pathways. One of the main ways chemicals can enter is through inhalation, such as for pollen. We can also come into contact with exogenous compounds through skin absorption, which can lead to direct entry into the blood. Another common way for foreign substances to enter the body is through ingestion. Whether it is food or pharmaceuticals, what people consume has the potential to affect other systems in the body. Compounds that are not naturally found in the body or a compound that may be naturally found but at the present has an abnormally high concentration may be referred to as xenobiotics[1]. To rid the body of these compounds, they are usually metabolized, thus changing their physical properties, in order to then be excreted. Certain enzymes play a role in metabolizing these foreign compounds, and a major class of xenobiotic metabolizing enzymes are the Cytochrome P450's.

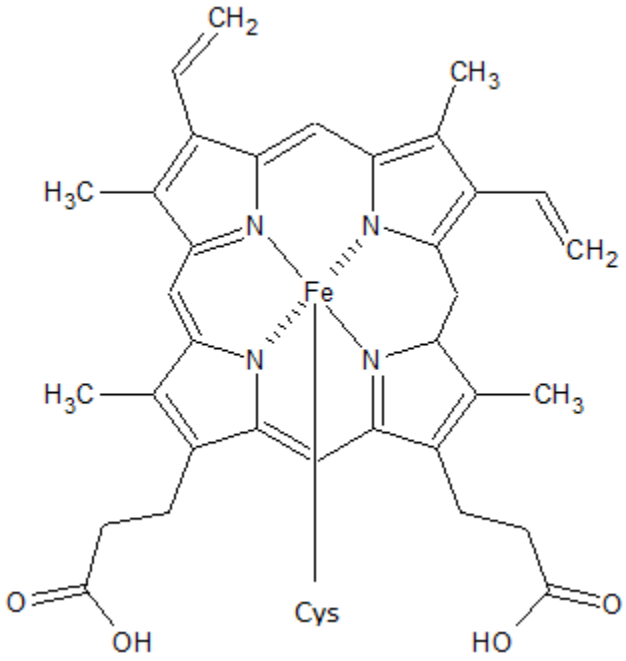
1.1.0. Background on Cytochrome P450's

Cytochrome P450's are monooxgyenase enzymes whose primary function in humans and other mammals is to make xenobiotics more soluble to facilitate their excretion. P450 enzymes are found in most forms of life ranging from plants to animals

to prokaryotes, with equally diverse functions. Their role in plants contributes to pigment formation in flowering species as well as to toxic compounds produced as a defense mechanism[2].

At the heart of cytochrome P450 catalysis is a heme cofactor that is essential for the oxidative function of these enzymes. Figure 1 below shows the central heme molecule tethered by a cysteine residue in the active site. The center is responsible for catalysis and typically has a 3+ charge in the resting form of the enzyme. The iron center is surrounded by four pyrrole rings where the respective nitrogen is attached to the iron center.

Figure 1. Structure of Heme Component in Cytochrome P450's

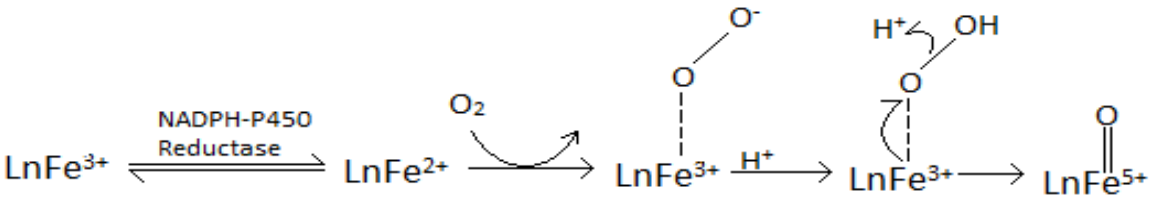


In human cells, the enzymes that are involved in xenobiotic metabolism are bound to the endoplasmic reticulum (ER) by the N-terminus. A redox partner, P450 reductase (CPR), is needed to transfer the electrons from an electron donating compound, NADPH, to the heme cofactor of P450 in order to initiate catalysis[3].

1.1.1. Catalytic Cycle

Upon the binding of the substrate into the active site of a cytochrome P450, a monooxygenase reaction occurs in which an oxygen atom is incorporated into the substrate where the source of the oxygen atom is molecular oxygen. As shown in Scheme 1[4], the stable resting form of the enzyme is in the Fe^{3+} state, where Ln is the abbreviation for ligand. This ferric center is oxidized by NADPH P450 reductase. Along with the incorporation of molecular oxygen, a ferrous oxygen species emerges[5]. Another electron then enters the cycle but can enter through another oxidation of NADPH reductase or through the oxidation of cytochrome b5[4]. An important step in this cycle is the heterolytic bond cleavage and releasing water as a byproduct[5]. The remaining iron-oxo compound sets the stage for the substrate to get oxidized. A water molecule replaces the newly oxidized product in the active state, thus taking the ferric center to the high-spin state.

Scheme 1. General P450 Catalytic Cycle.



1.2.0. Properties of Cytochrome P450's

There are thousands of different cytochrome P450 isoforms. The one thing they have in common is that they carry out a monooxygenase reaction and contain a heme group connected to the enzyme via a cysteine thiol. These isoforms are named based on genetic similarities. The first number denotes the family the isoform belongs to. A letter used to categorize between subfamilies may be present if there are two or more subfamilies known to exist for that family. An additional number at the end is used to represent the individual gene[6]. Just by analyzing the abbreviated name of a P450, it can help illuminate similar characteristics and functionalities amongst different isoforms.

Cytochrome P450 enzymes are essential, metabolic tools for the human body. Nearly 80% of all pharmaceuticals are metabolized by P450's[7]. According to "The Seattle Times," 4.3 billion prescriptions were filled in the United States in 2014, costing United States citizens 347 billion dollars. That would mean approximately 3 billion of these drugs consumed are processed by P450's. The percent of pharmaceuticals metabolized by P450's is overwhelming which is why the understanding of their individual compositions, possible substrates, and mechanisms of action is crucial not only to researchers but the general population.

P450's are present in many tissues throughout the body. Different types of P450's are present in the liver, small intestinal mucosa, lungs, kidneys, brain, olfactory mucosa, and skin[8]. The specific group of human P450's of interest for monitoring drug metabolism are human liver and intestinal enzymes since they are the primary organs of metabolism in the human body or site of first exposure to oral pharmaceuticals. Of the total 80% of pharmaceuticals metabolized by P450's, 90% are metabolized by five different isoforms: 1A2, 2C9, 2C19, 2D6, and 3A4.

1.2.1. Types of Reactions by Cytochrome P450's

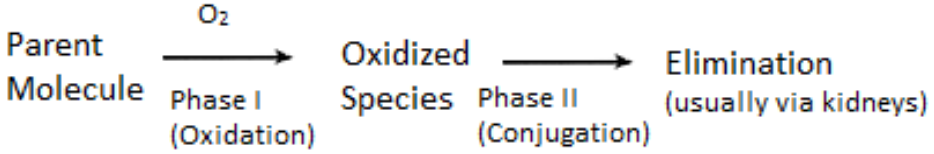
The science behind cytochrome P450's is crucial to understanding drug interactions, and learning how they function is essential. This single type of enzyme can catalyze many different types of reactions: alkane oxidation, aromatic oxidation, olefin epoxidation, N- or O-dealkylations, dehalogenations, and aromatization, to name a few[9]. These types of reactions play a vital role in the processing of endogenous chemicals consistent with the function of their class of enzymes, or can also be hazardous in metabolizing foreign compounds. A unique and interesting reaction catalyzed by a specific mammalian P450 involves an aromatization reaction which is responsible for converting testosterone to estrogen in the human body[10]. Alkane hydroxylation is a necessary reaction for increasing the polarity of xenobiotics to facilitate their excretion. This type of metabolism is an important aspect of Phase I metabolism, or the first step that is taken before a compound is able to be excreted. Aromatic compounds may also pose a threat to mammals, however it is often the oxidation of such compounds that lead to their toxicity. An example of the type of harmful reaction is when benzopyrene is

metabolized by P450's to benzopyrene oxides. This reaction is harmful because the carbocation which is formed cannot be stabilized through delocalization of the charge without destroying the aromaticity of the adjacent benzene. This high degree of instability causes the epoxide to not open until it is attacked by a nucleophile which is often water thus forming the diol. This diol can then form a diol epoxide which can be excreted through two different reactions. If the diol epoxide could undergo rearrangement, it could be excreted without injury. The other pathway forces the epoxide to open and a carbocation to form which is delocalized by the two hydroxyl groups. Other nucleophiles can then attack and cause the formation of carcinogenic products. Not only do benzopyrene metabolites have genotoxic (tumor-initiating) effects but also nongenotoxic (tumor-promoting) effects[11].

1.2.2. Structure, Mechanism, and Metabolic Processes

There are two main stages of metabolism in the human body, Phase I and Phase II, which differ in the mode of action but aid in the ultimate goal of excretion as seen in Scheme 2. Phase I primes the xenobiotic, typically through oxidation. Conjugation of the xenobiotic is a primary function of Phase II. This phase adds particular groups to the compound to make it more water soluble to ease the excretion[12]. Phase I usually precedes Phase II due to the addition of the hydroxyl which can aid in renal excretion.

Scheme 2. Phase I and Phase II Metabolism.



1.2.3. Drug Interactions with Cytochrome P450's

With P450's able to metabolize a vast majority of pharmaceuticals, it is imperative for researchers to know whether these substances interact with common, everyday chemicals. That is to say other chemicals have the ability to influence the metabolism of drugs by the P450 enzymes. Such is the case with cytochrome P450 3A4. This particular isoform has been studied in interactions with grapefruit juice to monitor for changes in overall activity. A compound in the juice inactivates this enzyme, preventing it from metabolizing a wide variety of pharmaceuticals known to be substrates for this isoform. A class of chemicals found in grapefruit juice are the furanocoumarins, which contain a three-ring system with an aliphatic tail. Furanocoumarins, which are present in grapefruit juice, were studied in the presence of drugs such as calcium channel blockers, benzodiazepines, and statins which are commonly prescribed in an effort to lower cholesterol levels. Upon ingestion of grapefruit juice while taking one of the previously mentioned drugs, the furanocoumarin binds to all of those in the CYP 3A family including CYP 3A4. Once the furanocoumarin is metabolized, it creates a furanoepoxide that irreversibly binds to CYP apoproteins to halt all enzymatic activity[13]. With this isoform (3A4) having known effects on pharmaceuticals upon metabolizing certain xenobiotics, it was a launching point for further investigation of other xenobiotics. A significant need in this area is to examine xenobiotics that we may come in contact with on a regular basis that may not have been previously tested for the effect on P450's.

1.2.4. Cytochrome P450 Genes and Selectivity

There are 57 genes that encode for P450's and 58 distinct monooxygenase enzymes regulating metabolism of either xenobiotics or endogenous compounds in the human body. Those that metabolize xenobiotics have a much lower selectivity for their substrates as compared to those that selectively metabolize compounds such as sterols, vitamins, or fatty acids[7]. For this reason, xenobiotic metabolizers have a wider array of possible substrates than those that purely metabolize endogenous compounds. While this class of P450's has more possible substrates, they have a great specificity for what they do metabolize such as pharmaceuticals which ultimately affects the amount of activity generated in the reaction.

1.2.5. Description of Specific Isoforms

1.2.5.1. 1A1

Each isoform has its own unique physical and chemical properties. Isoforms of the same family may have overlapping characteristics. Such is the case for 1A1 and 1A2. The isoform 1A1 is found in several tissues throughout the body including the lungs, gastrointestinal tract, skin, and in low quantities in the liver. This isoform is used as an aryl hydrocarbon hydroxylase. It is used in the metabolic pathways of procarcinogens such as polycyclic aromatic hydrocarbons (PAHs) and polyhalogenated aromatics hydrocarbons (PHAHs). These types of molecules are often found in environmental pollutants[14]. This isoform is of high interest in oncology studies due to its role in the

activation of polycyclic hydrocarbons. Modulation of the 1A1 isoform activity is being evaluated for cancer chemoprevention[15].

1.2.5.2. 1A2

Unlike 1A1, isoform 1A2 is one of the predominant P450's in the human liver at approximately 13%[16]. This isoform's primary role is the metabolism of select arylamines and heterocyclic arylamines. Among the compounds metabolized are mainstream pharmaceuticals such as phenacetin, lidocaine, tacrine, and theophylline[14]. Isoform 1A2 is similar to 1A1 in that bioactivated compounds upon metabolism are procarcinogens. Inhibition of this isoform can potentially be studied for cancer prevention[12].

1.2.5.3. 2A6

Isoform 2A6 is present at approximately 6% of the total P450 volume in the liver[13]. Unlike most of the other isoforms mentioned, 2A6 metabolizes very few pharmaceuticals. The primary role of 2A6 in the liver is to detoxify the body of nicotine. Much like the other "A" family counterparts, this isoform impacts the generation of procarcinogens[16].

1.2.5.4. 3A4

Of all the hepatic cytochrome P450's, the 3A family is by far the most prominent at nearly 40% of the total P450's present[17]. This isoform is even more prevalent in the intestines where it is present in 82% of the total P450's present[17]. As mentioned previously, this isoform is responsible for the metabolism of statins and

benzodiazepines[5]. Since this isoform metabolizes nearly two-thirds of all drugs, it is considered one of the most important hepatic isoforms[5]. Among these substrates are prominent drugs are the numerous drug classes and specific drugs below in Table 1. Besides grapefruit juice, other inhibitors are indinavir, clarithromycin, and diltiazem. Known inducers of isoform 3A4 are carbamazepine and St. John's Wort[18].

Table 1. List of Common Substrates for 3A4.

List of Common Substrates for Isoform 3A4[18]		
Macrolide Antibiotics -clarythromycin -erythromycin	Benzodiazepines -diazepem -triazolam	HIV Antivirals -ritonavir -nevirapine
Antihistamines -astemizole -chlorpheniramine	HMG CoA Reductase Inhibitor -atorvastatin -lovastatin	PDE-5 Inhibitors -vardenafil -sildenafil
Immune Modulators -cyclosporine -tacrolimus	Calcium Channel Blockers -amlodipine -nifedipine	Other -quinine -trazodone

1.2.5.5. 2C8

Common substrates for this isoform include but are not exclusive to amodiaquine, paclitaxel, torsemide, cerivastatin, and repaglinide[18]. Along with 2C9 and 2J2, the isoform 2C8 has been found to be present in prostate carcinoma cells[19]. A recent study shows that genetic polymorphisms in 2C8 decrease the disease-free survival in breast cancer patients[20]. This may be attributed to the fact 2C8 metabolizes cancer therapeutic drugs such as paclitaxel, and that a shift in the metabolism of such drugs may cause an increase in the recidivism of certain cancers like breast cancer[18].

1.2.5.6. 2D6

The isoform 2D6 is an important liver enzyme that comprises only a small fraction at 2% of the total liver cytochrome P450's[16]. Although it comprises a small total percentage, this isoform metabolizes various types of drugs such as beta blockers, antidepressants, antipsychotics, and pain relievers. Examples of the drugs include: carvedilol, S-metroprolol, amitriptyline, haloperidol, codeine, dextromethorphan, oxycodone, tramadol, and risperidone[18].

1.2.5.7. 2E1

Another prominent isoform in the liver found at 9% of the total P450 volume is isoform 2E1[16]. Possible substrates include methoxyfurane, acetaminophen, aniline, benzene, ethanol, N,N-dimethylformamide, and theophylline[18]. A known inhibitor is disulfiram and possible inducers are ethanol and isoniazid[14]. Research analyzing rats who have been exposed to alcohol and smoke were seen having elevated levels of 2E1, which may be a contributor to alcoholic liver failure[21].

1.3.0. Inhibition of Cytochrome P450's

An inhibitor is generally found to be a small molecule that upon binding with the enzyme will cause a decrease in activity. There are two main types of inhibition. Reversible inhibition occurs when a molecule binds reversibly that can either slow down product formation or either prevent enzyme turnover[22]. This effect of the inhibitor only lasts as long as the molecule is in contact with the active or allosteric sites. Reversible inhibitors of cytochrome P450's belong to one of three different classes. Competitive

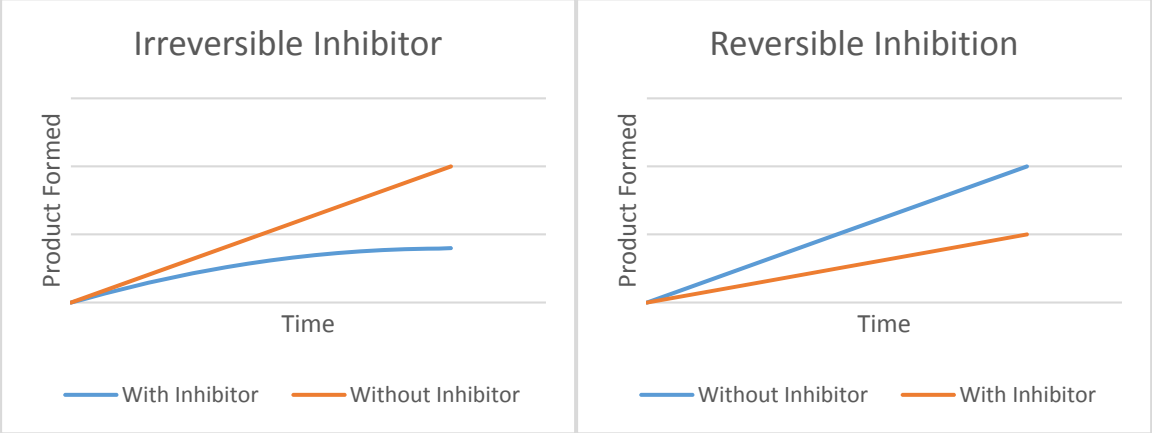
inhibition is when the substrate and the inhibitor compete for the active site. A competitive inhibitor will bind only to the enzyme itself. An uncompetitive inhibitor is an inhibitor that will bind only to the enzyme/substrate complex. Noncompetitive inhibition occurs when the substrate aims to bind to the active site but it unable to function due to the binding of the inhibitor at an allosteric site or binding to the active site causing the enzyme to be inactive. This type of inhibitor will bind to either the enzyme or to the enzyme/substrate complex[22].

The other possible type of inhibition is irreversible inhibition. This type of inhibition is reflective of the inhibitor binding to the enzyme that permanently inactivates the enzyme, thus muting all functionality of the enzyme[23]. There are two main types of irreversible inhibitors: reactive substrate analogs (affinity labels) and mechanism based inhibitors (suicide inactivators). Affinity labels are small molecules that are similar in structure to the substrate but are able to bind to active site residues to inhibit activity[24]. Tosyl-L-phenylalanine dimethyl ketone (TPCK) is a substrate analog to the substrate for chymotrypsin[25]. The other main type of irreversible inhibitor is a suicide inhibitor. The type of inhibitor is similar to that of affinity labels but has a greater degree of similarity to the natural substrate. This substrate goes through the traditional catalytic pathway and is initially metabolized. After this initial metabolism, the substrate is converted into an unstable product that halts the enzyme permanently. Since the enzyme is able to halt its functions through "suicide," this suggests that the mechanism based inhibitor is important to a feedback mechanism[26]. This feedback is able to prevent the amount of the natural substrate turning over to desired product from becoming excessive.

1.4.0. Measuring Activity of Cytochrome P450's (Kinetics)

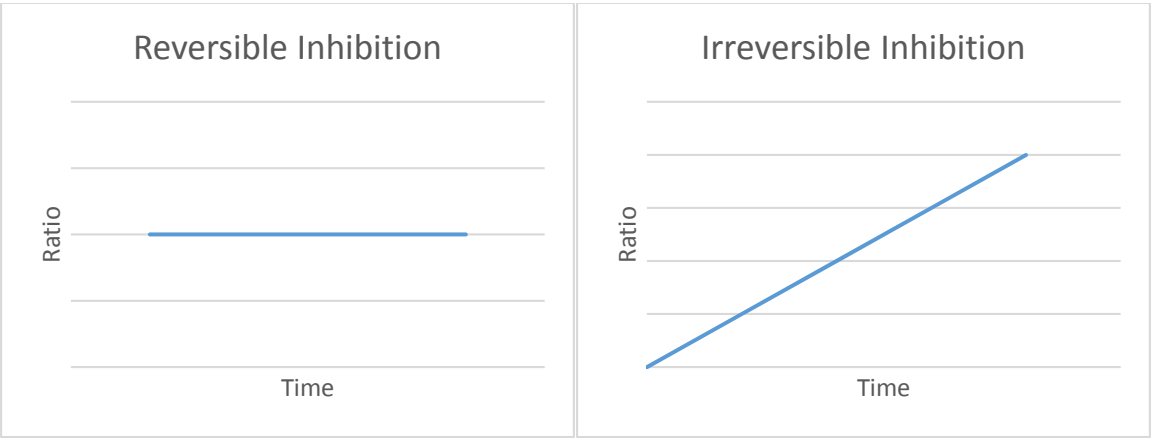
The Michaelis-Menten equation has served as the quintessential measurement of the kinetics of an enzymatic reaction. Fundamentally this equation relates reaction rates to substrate concentration. The V_{max} of the reaction is the maximum velocity of the reaction and is only possible to measure with an infinite substrate concentration. However, it can be determined via regression analysis. The K_m of the reaction is the concentration of the substrate needed to yield a rate of one half of the V_{max}. It is used to measure the strength of the interaction between the enzyme and the substrate. The smaller the K_m the stronger the interaction between the substrate and the enzyme. The k_{cat} of the equation is the rate constant for the turnover of substrate to product from the binding of the substrate and enzyme. It can also be thought of as the enzymatic rate of catalysis of the reaction. Using the Michaelis-Menten equation, it can be determined what type of inhibition is being exhibited. As seen in Figure 2 the changes in V_{max} and K_m between different types of inhibition are visible. The data from the Michaelis-Menten plot can be used to create a Lineweaver-Burke plot which can help to differentiate between the varying classes of inhibition. The type of inhibition can also be found through correlating activity over time as seen in Figure 2.

Figure 2. Product Formation over Time in Comparing Reversible and Irreversible Inhibitors.



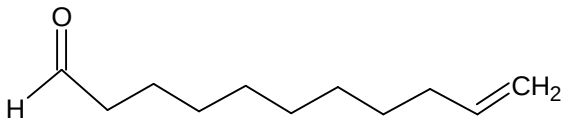
If there is reversible inhibition, the ratio of activity over time will be constant. In the presence of an irreversible inhibitor, the ratio of activity of time will have a positive trend as seen in Figure 3.

Figure 3. Ratio of Activity over Time in Comparing Reversible and Irreversible Inhibitors.



As foreign substances enter the body, they are able to alter the efficiency of certain enzymes. An example of one such foreign chemical that could possibly alter the activity of P450's is undecylenic aldehyde. This specific aldehyde is interesting since it not only contains an aldehyde end, but also a terminal olefin that contributes to its unique qualities as seen in Figure 4. In previous studies conducted by Dr. Kandagatla et al., the addition of an aldehyde containing an olefin was shown to decrease the overall activity as a competitive inhibitor of 2E1 and 2A6[27].

Figure 4. Structure of Undecylenic Aldehyde.



1.5.0. Interactions with Xenobiotics

In previous studies by Ortiz de Montellano, upon the addition of an olefin to a cytochrome P450 there was a destruction of the central heme. The addition of the undecylenic aldehyde which contains an olefin end is predicted to affect the selected isoforms' central heme component in a similar way[28]. Alternatively, aldehydes have been shown to inactivate microsomal P450 enzymes via a mechanism based reaction, thus the aldehyde portion of this molecule may lead to destruction.

Both aldehydes and olefins are known to irreversibly destroy cytochrome P450 enzymes via suicide inactivation. We can come in contact with undecylenic aldehyde through of means. It can be inhaled since it is highly used for scent longevity in

perfumes, candles, and deodorants (US Patent US US5380707 A). In the 1970's, it was used as an herbicide (US Patent US2626862 A), while today, undecylenic aldehyde could also be ingested through its use in anti-cavity chewing gum (US Patent US4048299 A). Little was known then and now about the effects of undecylenic aldehyde once it enters the body, and specifically how it could have the ability to alter the metabolic efficiency of P450's.

1.6.0. Objectives

The primary objectives of this project are to determine the effects of undecylenic aldehyde on human drug metabolizing P450's, and to identify precise chemical mechanisms involved. If the assays prove that low concentrations of inhibitor cause a significant amount of inhibition, this could possibly have adverse effects on the metabolism by cytochrome P450's and require a great deal of universal testing. A patient prescribed a drug could witness possible effects of the interaction between a drug and a xenobiotic, much like the case of statins and grapefruit juice. The number of possible compounds encountered daily that could alter the metabolism of these enzymes has just been grazed. For the benefit of patients alike, the safety behind pharmaceutical testing is an important safety measure of pharmaceuticals everywhere.

1.6.1. Screening with Varying Isoforms of Liver Xenobiotic-Metabolizing CYP's

The whole familial umbrella of cytochrome P450's can be subdivided according to what substrates they are able to metabolize such as: sterols, fatty acids, eiconasoids, vitamins, and xenobiotics. While there are always exceptions and ones that a substrate is

not known for yet, these five main groupings show similarities between what they metabolize[29]. The current study focuses on the microsomal/ drug metabolizing P450's with the goal of identifying possible inhibition of certain members. The approach used will be to add undecylenic aldehyde and monitor changes in overall activity of P450's. Inhibition of specific human enzymes may suggest potential drug interactions with this compound.

The following group of xenobiotic metabolizing Cytochrome P450's are in the highest quantities in the liver: 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5. Of this grouping, 3A4 would be most significant since it is in the most prominent human isoform, being responsible for the oxidation of approximately two-thirds of all known drugs, including the drugs as previously mentioned, statins and benzodiazepines[5]. Since 3A4 metabolizes a large percentage of all drugs, that makes it a natural candidate to consider for the monitoring of interactions with other xenobiotics. Although 3A4 would be affected the most, the following also have a high likelihood of being affected since in conjunction with 3A4 they collectively metabolize 80% of clinically used drugs: 2D6, 2C, 1A2, and 2E1[1].

1.6.2. Mechanistic Studies

In previous studies by Ortiz de Montellano, upon the addition of an olefin to a cytochrome P450 there has been a destruction of the central heme. The addition of the undecylenic aldehyde which contains an olefin end is predicted to affect the selected isoforms' central heme component in a similar way[28]. Alternatively, aldehydes have been

shown to inactivate microsomal P450 enzymes via a mechanism based reaction, thus the aldehyde portion of this molecule may lead to destruction. By monitoring heme structure following inhibition, it should be possible to assess the mode of inactivation.

CHAPTER II

MATERIALS AND METHODS

2.1.0. Preparation of Reagents and Stock Solutions

2.1.1. Substrate Preparations

2.1.1.1. Naphthalene

This substrate was used as the substrate in the 3A4, 1A1, 1A2, and 2D6. Naphthalene was purchased from Arcos Organics. A stock solution of 200 μM was prepared by adding 2.6 mg to 100 mL of nanopure water.

2.1.1.2. Coumarin

The coumarin used in the 2A6 assay was purchased from Arcos Organics. A stock concentration of 100 μM was achieved by adding 1.5 mg of coumrain to 100 mL of nanopure water.

2.1.1.3. Amodiaquine

Amodiaquine was purchased from Arcos Organics. A stock concentration of amodiaquine was prepared by adding 35.6 mg to a solution of 90 mL of nanopure water and 10 mL of methanol, for a total of 100 mL. Before adding to the 2C8 experiments, the stock solution was diluted by adding 1 mL of the stock solution to 99 mL of nanopure water to ensure that the methanol was less than one percent.

2.1.1.4. P-nitrophenol

This substrate was used in the 2E1 experiments. P-nitrophenol was purchased from Crescent Chemical Company Incorporated. A stock solution was prepared by adding 13.9 mg to 100 mL of nanopure water, creating a 1mM stock.

2.1.1.5. Fluorophenol

This substrate was purchased from Arcos Organics. A stock solution of 60 mM 4-fluorophenol was made by adding 336.3 mg to 50 mL of nanopure water.

2.1.2. Preparation of Phosphate Buffer Solution

A 1 M solution of phosphate buffer was made using monobasic and dibasic potassium phosphate. These compounds were purchased from Carolina Biological Supply Inc. The pH of the solution was adjusted to 7.4 through combining both phosphate solutions. This buffer solution was used for all of the following assays below unless otherwise stated.

2.1.3. Preparation of NADPH

Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Research Products International (RPI). The NADPH was diluted with nano-pure water and partitioned into 10 mM aliquots. The aliquots were then stored in a -80°C freezer until they were used in an assay.

2.1.4. Preparation of Glutathione

The glutathione used in the assays was purchased from Sigma-Aldrich. A stock solution of glutathione was prepared by adding 1.5366 g to 50 mL of nanopure water. The concentration of the stock solution was 100 mM.

2.1.5. Preparation of Rabbit Liver Microsomes

The rabbit liver microsomes that were used were prepared in the lab. The rabbit liver was initially washed in 0.05 M potassium phosphate buffer with 0.1 mM EDTA. The liver and buffer solution was then blended using a hand-held homogenizer. Once this mixture was thoroughly blended, the mixture was centrifuged at 5,000 rpm for 10 minutes and the pellet was discarded. The supernatant was further centrifuged at 37,000 x g for one hour. The microsomes were then homogenized in potassium phosphate buffer and stored in a -80°C freezer in 75 µL aliquots until they were further used.

2.1.6. Human S9 Liver Microsomes

The human S9 liver microsomes used to determine the mode of inhibition of undecylenic aldehyde were purchased from Molecular Toxicology Inc. in Asheville, North Carolina. The solution was partitioned into 75 µL aliquots and stored in a -80°C freezer until they were used in an assay.

2.2.0. Cytochrome P450 Assays

2.2.1. Time Trial of 2E1 Using Rabbit Microsomes

Rabbit microsomes were used to conduct a time trial experiment to monitor inhibition over time. To begin, incubation tubes were prepared by mixing phosphate buffer (0.1M, pH 7.4), water, 5 $x10^{-5}$ M of the substrate p-nitrophenol, and 5 $x10^{-4}$ M NADPH. Phosphate buffer, microsomes, and water were added to all the preincubation tubes. Half of the preincubation tubes had undecylenic aldehyde (1.25 $x10^{-5}$ M) added and the other half received that same amount of water. Then via a "rolling method" in 15 second intervals, 5 $x10^{-4}$ M NADPH was added, vortexed, and placed in the heat block at 37 ^{o}C. One twenty-fifth of the total contents (20 µL) of the preincubation tube was removed in intervals of 0, 2, 5, 10, and 15 minutes of preincubation and placed into an incubation tube to make the total volume 200 µL. Once the contents were delivered to the incubation tube, it was vortexed and all samples incubated for 30 minutes. After each tube's incubation time, the sample was quenched with 20 µL of 60% perchloric acid, vortexed, and placed on ice for at least 10 minutes. The samples were then centrifuged at 14,000 rpm for 10 minutes after which the supernatant was removed and analyzed by HPLC. The HPLC conditions included a C-18 column and a mobile phase with 40 % acetonitrile, 59% nanopure water, and 1% trifluoroacetic acid.

2.2.2. Human Liver S9 Microsomes

Human liver S9 fractions were used to determine the mode of inhibition of undecylenic aldehyde on CYP 2E1 isoform. A time trial using samples with and without

added aldehyde were monitored for activity over time. All of the reactions received 0.1 M pH 7.4 phosphate buffter, nanopure water, 50 μM paranitrophenol, 10 μL of microsomes, and 0.5 mM glutathione. Half of the samples had 250 μM undecylenic aldehyde added while the other half received water added to make the total volume 800 μL. Then via a "rolling method" in 15 second intervals, 1 mM NADPH was added, vortexed, and left to incubate for 15 minutes. At 15 minutes one fourth of the contents or 200 μL was removed, quenched, and placed on ice. The remaining 600 μL continued to incubate for an additional 15. After the total 30 minutes elapsed, 200 μL was removed, quenched, and placed on ice. The remaining 400 μL continued to incubate for an additional 15. After the total 45 minutes elapsed, 200 μL was removed, quenched, and placed on ice. The remaining 200 μL continued to incubate for an additional 15 in which after the total 60 minutes elapsed, the remaining contents were removed, quenched, and placed on ice. After all sample had been placed on ice for at least 10 minutes, the samples were then centrifuged at 14,000 rpm for 10 minutes. The supernatant was then analyzed by HPLC. A C18 column was used with a mobile phase of 45% acetonitrile, 54% nanopure water, and 1% trifluoroacetic acid.

All expressed P450 enzymes were purchased from Xentotech.

2.2.3. Individually Expressed Isoforms, Supersomes

2.2.3.1. 1A1 and 1A2

For each sample, 0.1 M pH 7.4 phosphate buffer, 60 μM naphthalene, 5 mM catalase, 5 mM ascorbate, 5 μL of the individually expressed 1A1 or 1A2 isoform, and

water were added. Undecylenic aldehyde was then added in 0 mM, 1.25 mM, 3.75 mM, and 12.5 mM concentrations to the individual samples respectively. Via a "rolling method" in 15 second intervals, a final concentration of 1 mM NADPH was added to the reaction, vortexed, and incubated at 37 ºC for 10 minutes. The final volume was 200 µL. The samples were then quenched via the "rolling method" with 300 µL of 6% perchloric acid, vortexed, and placed on ice for 10 minutes. The samples were then centrifuged at 14,000 rpm for 10 minutes. A HPLC analysis was then performed on the supernatant and the pellet was discarded. The mobile phase conditions consisted of 60% acetonitrile, 39% nanopure water, and 1% trifluoroacetic acid.

2.2.3.2. 2A6

Phosphate buffer (0.1M) with a pH of 7.4, nanpure water, 125 µM glutathione, 5 µM coumarin, and 5 µL of the individually expressed 2A6 isoform were added to each vial. In separate samples, varying amounts of undecylenic aldehyde were added: 0 mM, 1.25 mM, 3.75 mM, and 12.5 mM. Then via a "rolling method" in 15 second intervals, 1 mM NADPH was added, vortexed, and incubated at 37 ºC for 30 minutes. The final reaction volume was 200 µL. After the incubation period, via the "rolling method," in 15 second intervals ice-cold 2M HCl was added to each reaction, vortexed, and placed on ice for 10 minutes. The samples were then centrifuged at 14,000 rpm and the supernatant was analyzed by HPLC with a C18 column at 328 nm. The mobile phase conditions were 59% nanopure water, 40% methanol, and 1% trifluoroacetic acid.

2.2.3.3. 3A4

In separate vials, 0.1 M pH 7.4 phosphate buffer, 60 µM naphthalene, 5 mM catalase, 5 mM ascorbate, 3 µL of the individually expressed 3A4 isoform, and water were added. Then 0 mM, 1.25 mM, 3.75 mM, and 12.5 mM concentrations of undecylenic aldehyde were added to the individual samples respectively. Via a "rolling method" in 15 second intervals, a final concentration of 1 mM NADPH was added to the reaction, vortexed, and incubated at 37° C for 30 minutes. The final volume was 200 µL. After this incubation period, the samples were quenched via the "rolling method" with 70% perchloric acid and placed on ice for 10 minutes. The samples were then centrifuged at 14,000 rpm for 10 minutes. HPLC analysis was then performed on the supernatant and the pellet was discarded. Mobile phase conditions consisted of 50% acetonitrile, 49% nanopure water, 1% isopropanol, and 0.1% trifluoroacetic acid, and a C18 column was used.

2.2.3.4. 2C8

The supersomes were diluted eight-fold with 0.1 M phosphate buffere pH 7.4. The substrate amodiaquine was diluted 100-fold by adding 0.01 mM amodiquine in 10% methanol to nanopure water. Phosphate buffere pH 7.4, nanopure water, 10 µL of the individually expressed isoform 2C8, and 20 µL of the substrate were added. Then 0 mM, 1.25 mM, 3.75 mM, and 12.5 mM concentrations of undecylenic aldehyde were added to the individual samples respectively. Via a "rolling method" in 15 second intervals, a final concentration of 1 mM NADPH was added to the reaction, vortexed, and incubated at 37° C for 4 minutes. The reactions were quenched in the same "rolling method" with 7.5%

perchloric acid and placed on ice for 10 mintues. The samples were then centrifuged at 14,000 rpm for 10 minutes. The supernatant was then analyzed by HPLC with a C18 column at 347 nm. A time program of the mobile phase conditions is listed below in Table 2. Two different mobile phases were used, and both included 0.1% trifluoroacetic acid. Pump A is nanopure water. When pump A is less than 100%, the other mobile phase incorporated is acetonitrile.

Table 2. 2C8 HPLC Conditions.

2C8 HPLC Conditions	
Time (minutes)	% A
0	100
2	80
8	70
8.1	0
10.1	0
10.2	100
13.2	100

2.2.3.5. 2D6

Each trial contained 0.1 M pH 7.4 phosphate buffer, 100 μM naphthalene, 5 mM catalase, 5 mM ascorbate, 5 μL of the individually expressed 2D6 isoform, and water were added. Undecylenic aldehyde was then added in 0 mM, 1.25 mM, 3.75 mM, and 12.5 mM concentrations to the individual samples respectively. Via a "rolling method" in 15 second intervals, a final concentration of 1 mM NADPH was added to the reaction, vortexed, and incubated at 37 ^{o}C for 10 minutes. The samples were then quenched via the "rolling method" with 300 μL of 6% perchloric acid, vortexed, and placed on ice for 10 minutes. The samples were then centrifuged at 14,000 rpm for 10 minutes. HPLC analysis with a C18 column was then performed on the supernatant and the pellet was discarded. The mobile phase conditions consisted of 60% acetonitrile, 39% nanopure water, and 1% trifluoroacetic acid. The final volume of the reactions was 200 μL.

2.2.3.6. 2E1

With a final volume of 200 μL, 2 μL of individually expressed isoform 2E1, 0.1 M pH 7.4 phosphate buffer, 50 μM paranitrophenol, 5 mM catalase, 5 mM ascorbate, and water were added collectively. Then 0mM, 1.25 mM, 3.75 mM, and 12.5 mM concentrations of undecylenic aldehyde were added to individual samples respectively. Then via a "rolling method" in 15 second intervals, 1 mM NADPH was added to the reaction, vortexed, and incubated at 37^{o} C for 30 minutes. After this incubation period, the samples were quenched via the "rolling method" with 70% perchloric acid and placed on ice for 10 minutes. The samples were then centrifuged at 14,000 rpm for 10 minutes.

The supernatant was removed and analyzed by HPLC at 340 nm. Mobile phase conditions consisted of 60% acetonitrile, 39% nanopure water, and 1% trifluoroacetic acid.

2.2.4. Michaelis-Menten Analyses

2.2.4.1. Study with 2E1 and p-Nitrophenol

A Michaelis-Menten study was performed to monitor the mode of inhibition. All of samples received 0.1 M potassium phosphate buffer with a pH of 7.4 and 15 µL of rabbit liver microsomes. A series of five concentrations of substrate were utilized: 50 µM, 100 µM, 200 µM, 300 µM, and 400 µM. For each of these varying substrate concentrations, varying concentrations of the inhibitor, undecylenic aldehyde, were added to the samples: 0 µM, 12.5 µM, and 62.5 µM. The remaining volume was filled with nanopure water and a final volume of 200 µL was achieved after taking into the future account of volume of NADPH. Then via a "rolling method," 1 mM NADPH was added, vortexed, and incubated for incubated for 30 minutes. After the incubation period in a "rolling method" format, the samples were then quenched with 60% perchloric acid, vortexed, and placed on ice for at least 10 minutes. The samples were then centrifuged at 14,000 rpm for 10 minutes. The pellet was discarded and the supernatant was analyzed by HPLC. The mobile phase conditions included 60% acetonitrile, 39% nanopure water, and 1% trifluoroacetic acid.

2.2.5. BM3

BM3 trials were used as a mechanistic probe and serve no biological relevance. These trials contained 0.1 M pH 7.4 phosphate buffer, 15 mM fluorophenol, nanopure water, and enzyme. Undecylenic aldehyde was then added in 0 mM, 1.25 mM, 3.75 mM, and 12.5 mM concentrations to the individual samples respectively. The samples were then pre-incubated for two minutes each. The samples individually had NADPH, with a final concentration of 1mM, added to the reaction, vortexed, and incubated at 37 ºC for 20 seconds. After this 20 second incubation, the reaction was then rapidly quenched with 10 µL of 60% perchloric acid and placed on ice for 10 minutes. After being placed on ice, the samples were then centrifuged at 14,000 rpm for 10 minutes. The pellet was discarded and the supernatant was analyzed by HPLC through use of a C18 column. The mobile phase used contained 55% nanopure water, 44% acetonitrile, and 1% trifluoroacetic acid.

CHAPTER III

RESULTS AND DISCUSSION

3.1.0. Isoform Screening Assays

A wide spectrum of liver Cytochrome P450's were individually monitored for changes in overall activity upon the addition of undecylenic aldehyde. The following P450 isoforms were tested to see if there was a change in relative activity in response to varying concentrations of undecylenic aldehyde: 1A1, 1A2, 2A6, 3A4, 2B6, 2C8, 2C19, 2D6, and 2E1. A good substrate had to be used for each specific isoform, meaning it was able to be metabolized by that specific isoform and also generate a high yield of its respective product after being oxidized. The substrates shown in Table 3 were used to probe the activity of their respective isoforms and products, which are also shown in the table.

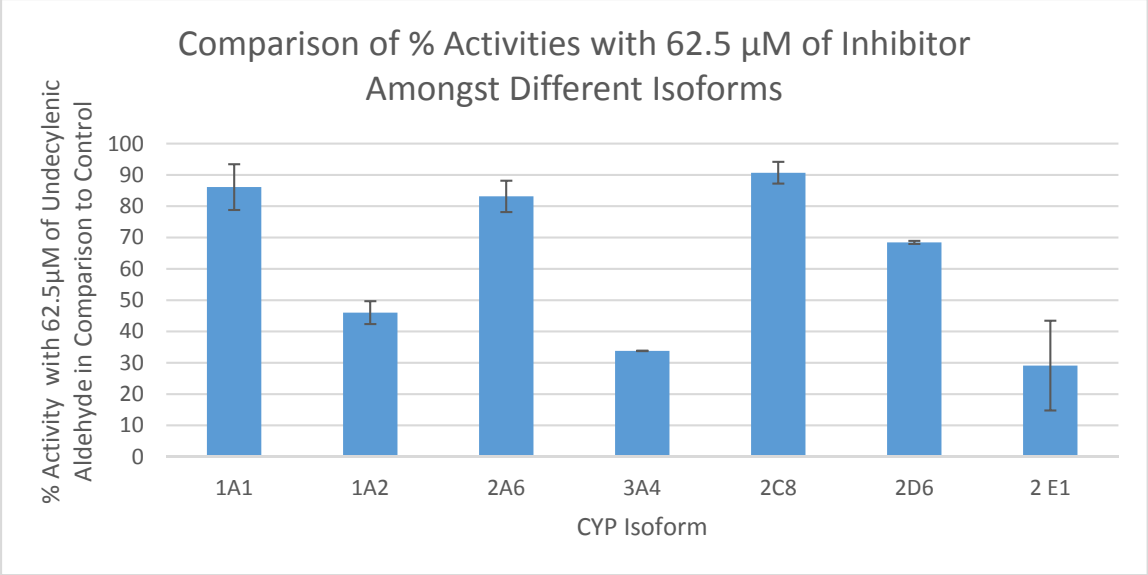
Table 3. List of Isoforms and Respective Substrates and Products.

Isoform	Respective Substrate	Oxidized Product
1A1	Naphthalene	1-naphthol
1A2	Naphthalene	1-naphthol
2A6	Coumarin	7-hydroxy coumarin
3A4	Naphthalene	1-naphthol
2C8	Amodiaquine	Oxidized Amodiaquine
2D6	Naphthalene	1-naphthol
2E1	4-nitrophenol	4-nitrocatechol

Once the samples were analyzed via their respective HPLC conditions, a measure of total activity for each sample was obtained as a control for subsequent inhibition studies. All activities with inhibitor are reported as a percent of these controls. Some of the isoforms presented good overall activity in their respective assays, while others did not. Isoforms 2E1, 2A6, and 2C8 had high overall activity. Isoform 3A4 and 2D6 had moderate overall activity. Isoforms 1A1 and 1A2 had a low overall activity. The activity of the reactions was then judged after the addition of undecylenic aldehyde (12.5 μM, 31.25 μM, and 62.5 μM) and placed into one of three categories: low, moderate, and high inhibition. Low inhibition was defined as less than 30% of overall activity. Moderate inhibition was defined as less than 50% of overall activity. High inhibition was characterized by inhibition of 60% or greater.

A comparative graph at the highest concentration of undecylenic aldehyde, or 62.5 µM, were used to determine which isoforms showed the highest percent inhibition. Those with the highest degree were further analyzed by different methods. In Figure 5, the graph shows the comparisons of all the isoforms tested. Isoforms 3A4 and 2E1 had a percent inhibition of greater than 60% and had high overall activity. The other isoforms were either eliminated from further consideration due to low overall activity or low inhibition. Isoform 1A1 and 1A2 had a low overall activity. The remaining isoforms (2A6, 2C8, and 2D6) all had moderate to high activity but exhibited low inhibition upon the addition of undecylenic aldehyde. Since all the different isoforms displayed some inhibition under these conditions, a dose response for each isoform was carried out.

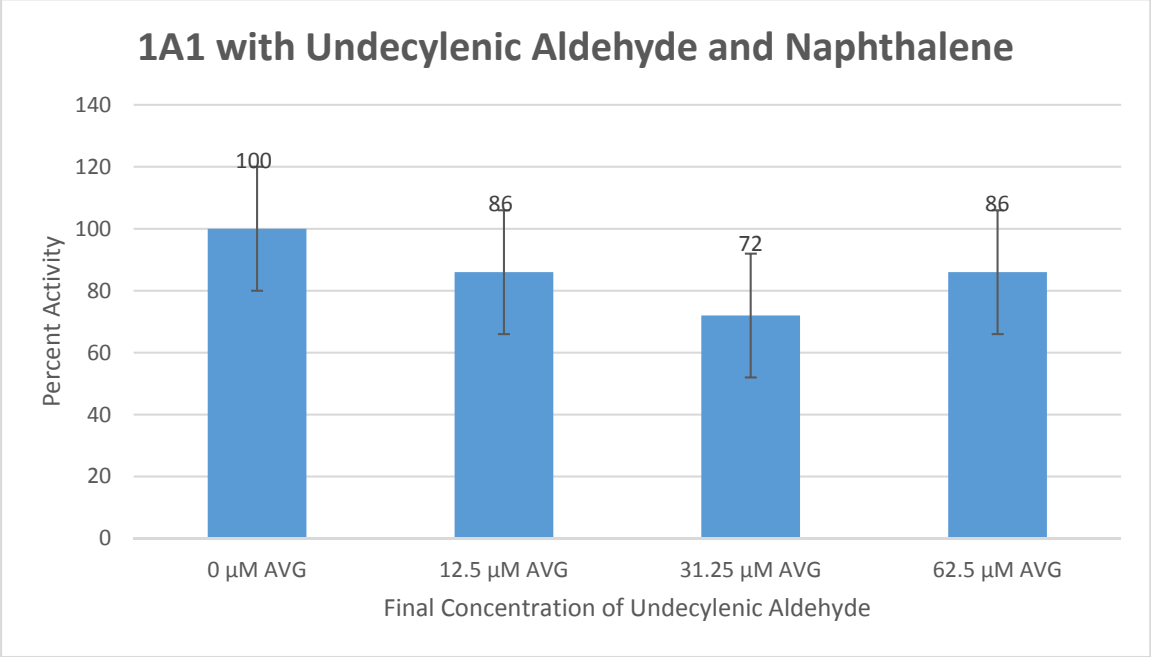
Figure 5. Comparative Graph between Isoforms with Highest Concentration of Undecylenic Aldehyde, 62.5 µM.



3.1.1. 1A1

In Figure 6a, results from the dose response study involving inhibition of 1A1 by undecylenic aldehyde are displayed. Isoform 1A1 exhibited low overall activity with naphthol as a substrate however, was sufficient for evaluating effects of undecylenic aldehyde. The error bars were created using the standard deviation. Here, a low percent decrease in activity with the addition of aldehyde was observed indicating very little inhibition of 1A1. To resolve this issue, a new stock of enzyme could be used or a better substrate could be used, resulting in higher percent activities.

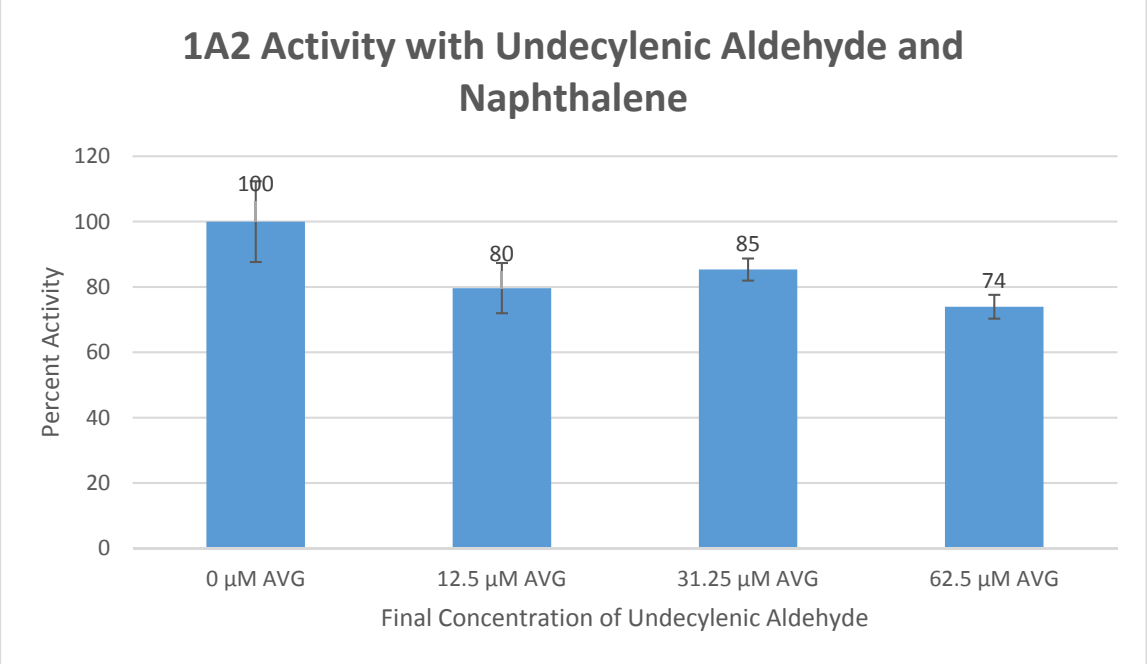
Figure 6a. Isoform 1A1 Activity with Addition of Varying Amounts of Aldehyde.



3.1.2. 1A2

Results from the reactions with 1A2 are shown in Figure 6b. Again low activity was observed with naphthalene, but unlike with 1A1, moderate decrease in activity after the addition of undecylenic aldehyde was seen. The error bars were created using the standard deviation. Though the data from the 1A2 screening is more reliable, the activity was moderate at best. Like the 1A1 suggested modification, a better substrate could be utilized in hopes of increasing the overall activity.

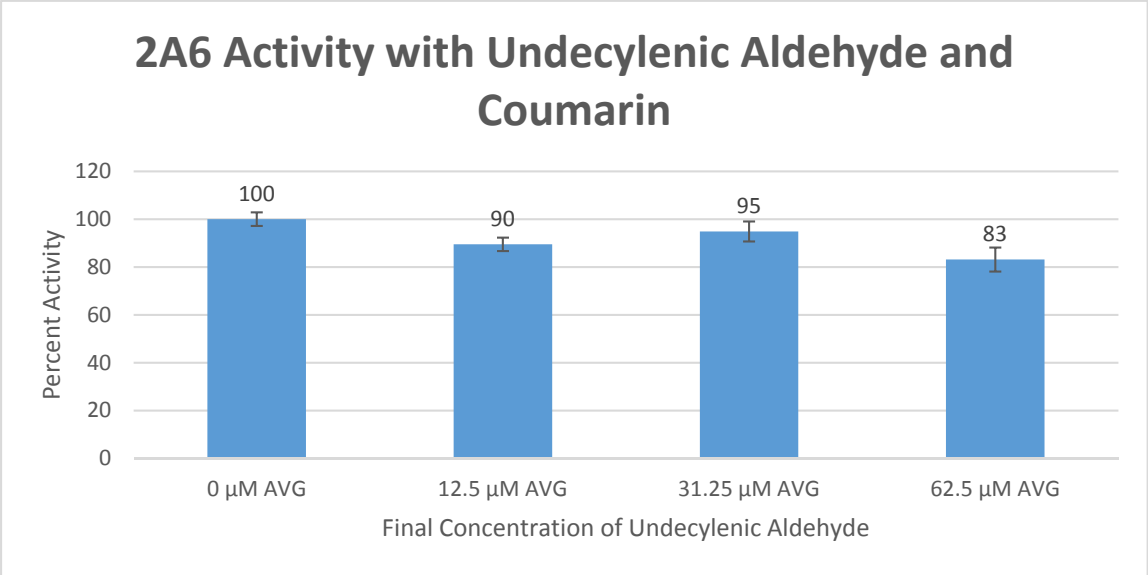
Figure 6b. Isoform 1A2 Activity with Addition of Varying Amounts of Aldehyde.



3.1.3. 2A6

The data for isoform 2A6 is displayed in Figure 6c; this enzyme was assayed with coumarin as a substrate, however a low degree of inhibition was observed. The error bars were created using the standard deviation. The addition of 12.5 µM of undecylenic aldehyde resulted in approximately a 10% decrease in activity. Addition of 31.25 µM showed a decrease in the percent inhibition, but addition of 62.5 µM undecylenic aldehyde showed a slight decrease in percent activity.

Figure 6c. Isoform 2A6 Activity with Addition of Varying Amounts of Aldehyde.

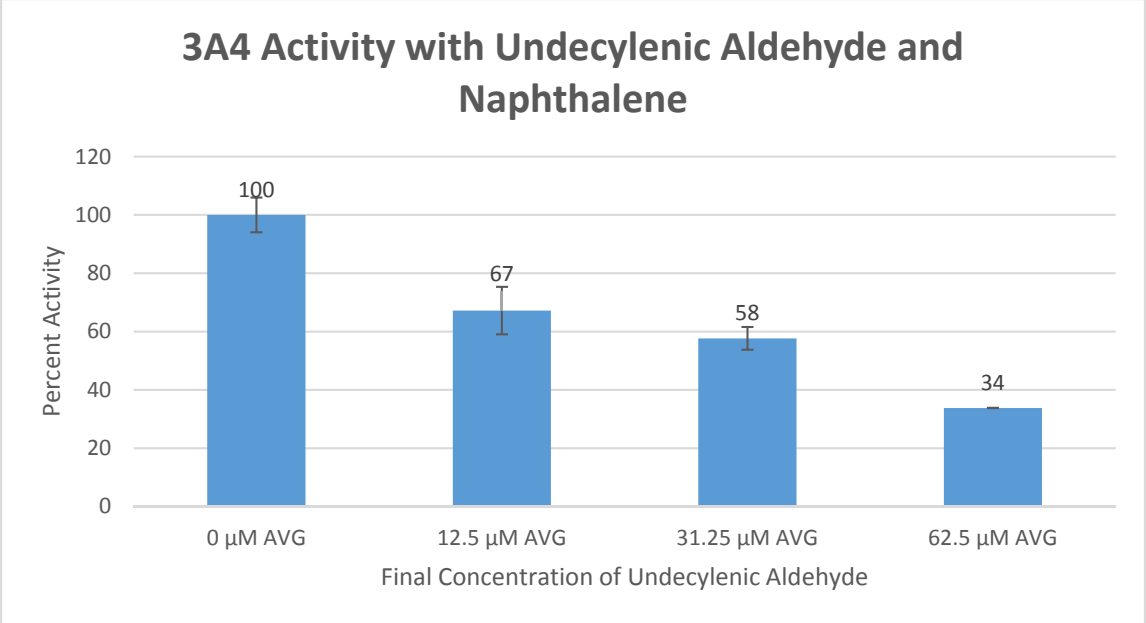


3.1.4. 3A4

Another isoform with high activity, 3A4, had a high degree of overall inhibition as seen in Figure 6d using naphthalene as a substrate. The error bars were created using the standard deviation. Here, the addition of 31.25 µM produced an inhibitory effect of

close to 50%. The addition of the highest concentration of undecylenic aldehyde, 62.5 µM, approximately 34% of the initial activity was seen. Of the isoforms examined, this was the second most prominent level of inhibition observed.

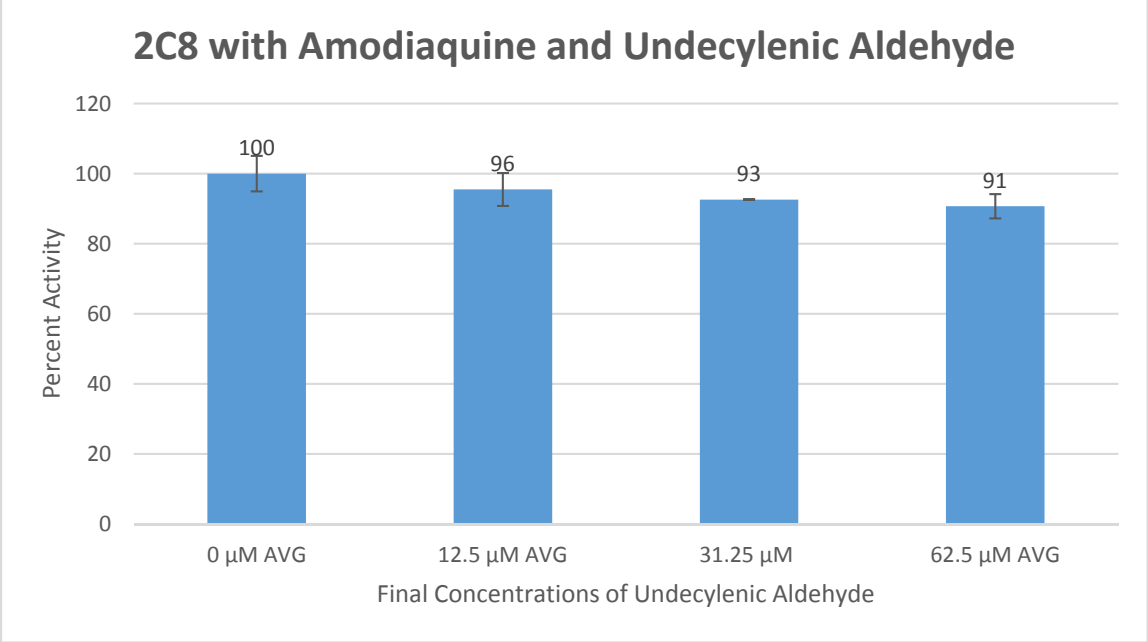
Figure 6d. Isoform 3A4 Activity with Addition of Varying Amounts of Aldehyde.



3.1.5. 2C8

Isoform 2C8 as seen in Figure 6e was also an isoform with a high activity using amodiaquin as a substrate, but in contrast to 3A4 had a low degree of inhibition. After the addition of the highest quantity of inhibitor, 62.5 µM, only a 9% decrease in activity was seen. This marginal decrease in activity with the highest concentration of inhibitor was not significant enough to perform further studies. The error bars were created using the standard deviation.

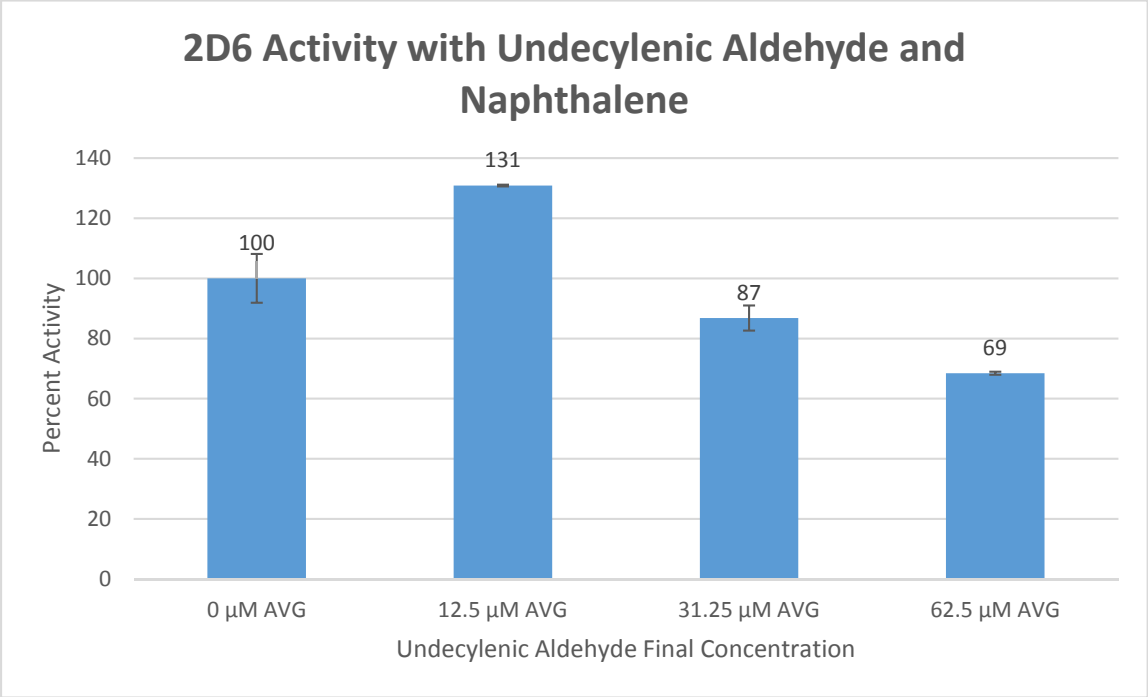
Figure 6e. Isoform 2C8 Activity with Addition of Varying Amounts of Aldehyde. For the 31.25 µM Concentration There Was Only One Data Point Due to Error, Thus the Standard Deviation is 0.



3.1.6. 2D6

For 2D6 which is shown in Figure 6f, the overall activity was low and there was modest inhibition. Naphthalene as a substrate did not produce a high turnover to product. This low activity could be a contributing factor to the increase in activity seen in the reactions with 12.5 µM undecylenic aldehyde. The error bars were created using the standard deviation.

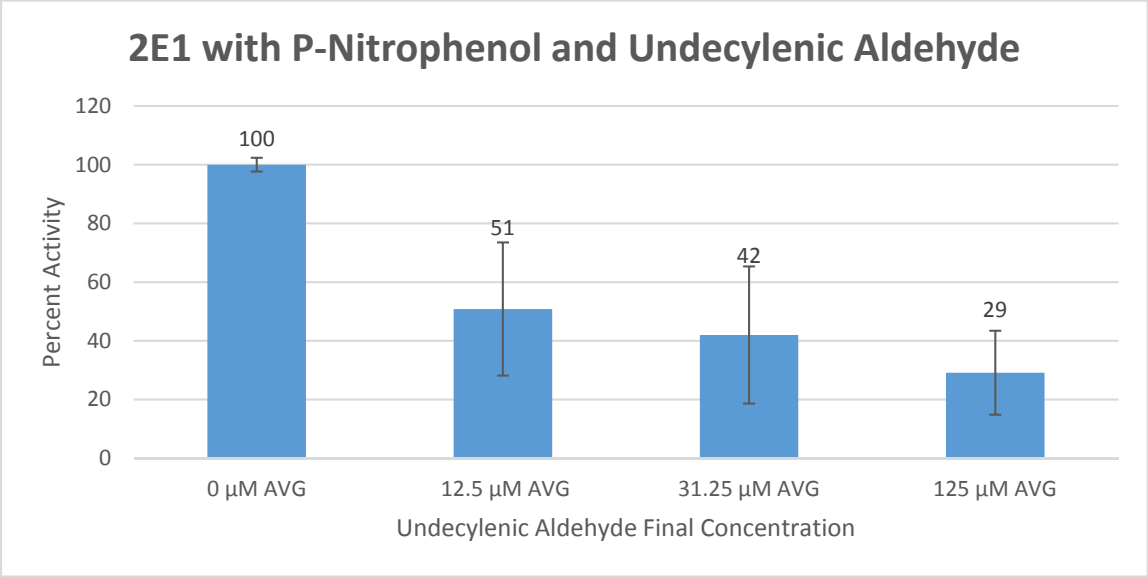
Figure 6f. Isoform 2D6 Activity with Addition of Varying Amounts of Aldehyde.



3.1.7. 2E1

Isoform 2E1 exhibited a high degree of activity as well as a high degree of inhibition as shown in Figure 6g. This isoform was the most susceptible to undecylenic aldehyde inhibition. For example, a concentration of 12.5 µM undecylenic aldehyde inhibited the activity by 50%. After the highest concentration of inhibitor was added, less than 30% of the wildtype activity was seen. The error bars were created using the standard deviation.

Figure 6g. Isoform 2E1 Activity with Addition of Varying Amounts of Aldehyde.



3.1.8. Overall Results

For these seven isoforms tested, screening studies were carried out based on the observed level of inhibition seen with the addition of undecylenic aldehyde at varying concentrations. Isoforms 1A1, 2A6, 2C8, and 2D6 showed marginal inhibition and were not considered for further analysis. A slightly higher inhibition was seen in isoform at approximately 30% for 1A2 but experimentation was not continued due to low overall activity. Isoforms 3A4 and 2E1 both showed high degrees of activity and inhibition.

This data suggest that very low concentrations of undecylenic aldehyde can contribute to a significant decrease in the activity of 3A4 and 2E1. Isoform 2E1 had the largest decrease in activity upon the addition of the highest concentration of undecylenic aldehyde. This data is important for the study of drug reactions. If it takes a minute amount of undecylenic aldehyde to greatly affect the activity of specific isoforms, this

could possibly cause a threat to the efficacy of drugs being prescribed. From the screening of isoforms, 3A4 and 2E1 were good candidates to continue kinetic analysis due to their significant decrease in activity even at the lower concentrations.

3.2.0. Determining the Mode of Inhibition for 2E1

Initially, rabbit liver microsomes were used to monitor the mode of inhibition for the 2E1 isoform. P-nitrophenol was used since it is a selective substrate of 2E1. Over the course of one hour, four reactions were carried out, two with undecylenic aldehyde and two without. The four reactions were then compared to monitor differences due to the addition of undecylenic aldehyde. In Figure 7, the results of this time course experiment is shown. The activity is representative of irreversible binding. For example, when irreversible binding occurs, it blocks enzymatic activities most likely due to covalent binding to the enzyme. As shown in the graph, this should then give a time dependent decrease in activity that correlates with the kinetics of the covalent attachment. As more and more enzyme gets inactivated over time, the ratio (slope) of the time course plot drops to zero eventually. To assess this, one can compare the ratio of activities in the absence and presence of inhibitor as seen in Figure 8. If the ratio remains constant, it suggests a simple reversible mode of inhibition. However, irreversible inhibition should result in an increasing ratio of without inhibitor to with inhibitor. Irreversible binding is marked by a time-dependent reduction in activity as seen in Figure 7. In other words, the activity is lost over time when undecylenic aldehyde is present. Irreversible binding is indicative of complete binding of the aldehyde to the active site of the enzyme without the possibility of the two separating.

Figure 7. Comparison of Activity between Samples without Inhibitor to Those with Inhibitor Using the Substrate for 2E1, p-Nitrophenol.

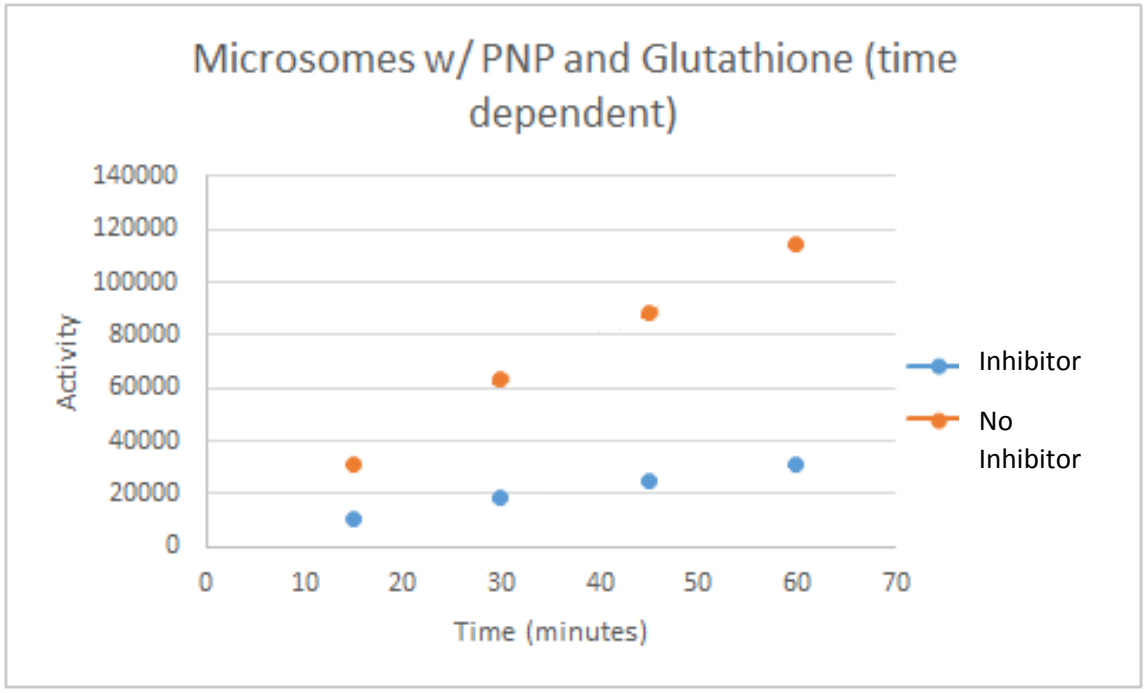
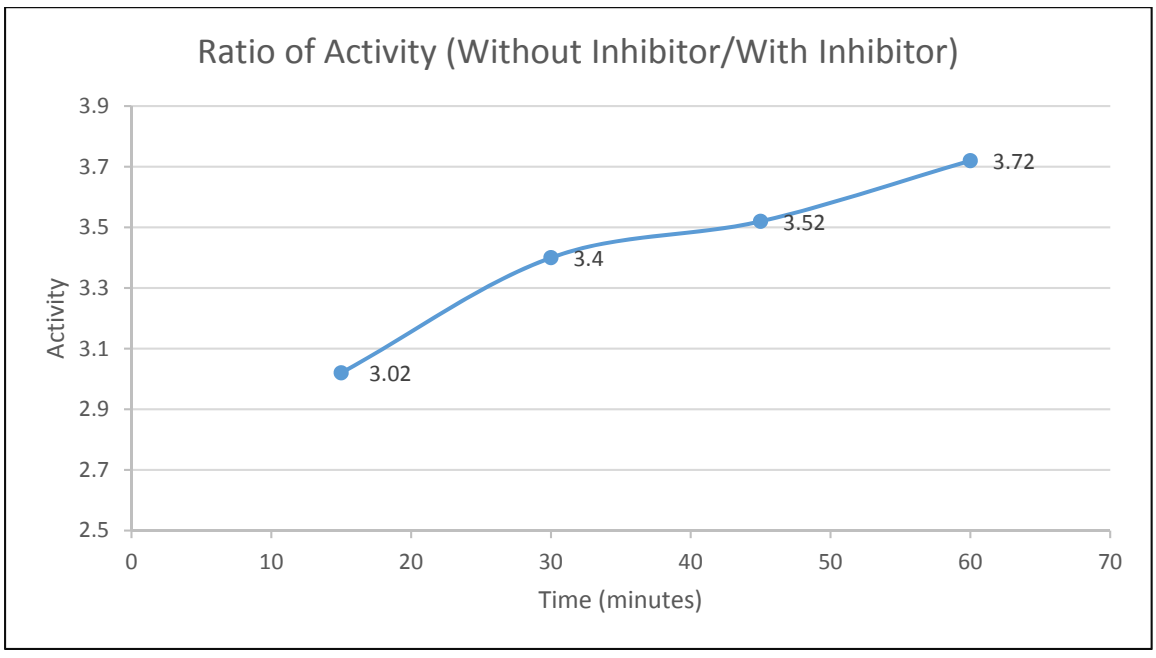


Figure 8. Ratios from Figure 7 between Samples without Inhibitor Compared to Those with Added Undecylenic Aldehyde.



3.2.1. Michaelis-Menten Studies of 2E1 with Lineweaver-Burk Plot

To monitor the mode of inhibition, a Michaelis-Menten study was performed. This study was conducted to monitor if a reversible type of inhibition was present. The concentrations of the substrate, or p-nitrophenol, were tested in 50 μM, 100 μM, 200 μM, 300 μM, and 400 μM. These concentrations were tested with 0 μM, 12.5 μM, and 62.5 μM concentrations of undecylenic aldehyde. In Figure 10, the concentrations were plotted and show a decrease in the K_m and the V_{max}. A competitive mode of inhibition would show a change in the Km. A change in the V_{max} would be reflective of a noncompetitive inhibitor. Since there is a change in both the K_m and the $V_{max,}$ this is suggestive of mixed inhibition. Mixed inhibition has certain characteristics from both competitive and non-competitive inhibition. In order to better determine which type of inhibition it is most associated, a Lineweaver-Burk plot or a double reciprocal plot was made to better analyze the data from Figure 9.

The results from the Michaelis-Menten study were then used to create a Lineweaver-Burk plot. This double reciprocal plot is also used to distinguish between different modes of inhibition. A competitive inhibitor would share the same y-intercept as the sample without the inhibitor present. A non-competitive inhibitor would share the same x-intercept as the sample without inhibitor. In Figure 10, the data points along the y-axis are close to the same value, whereas the data points crossing the x-axis differ in value. This type of inhibition suggests primarily mixed inhibition but most closely suggests competitive inhibition.

Figure 9. Michael-Menten of 2E1 with p-Nitrophenol in the Presence of Undecylenic Aldehyde.

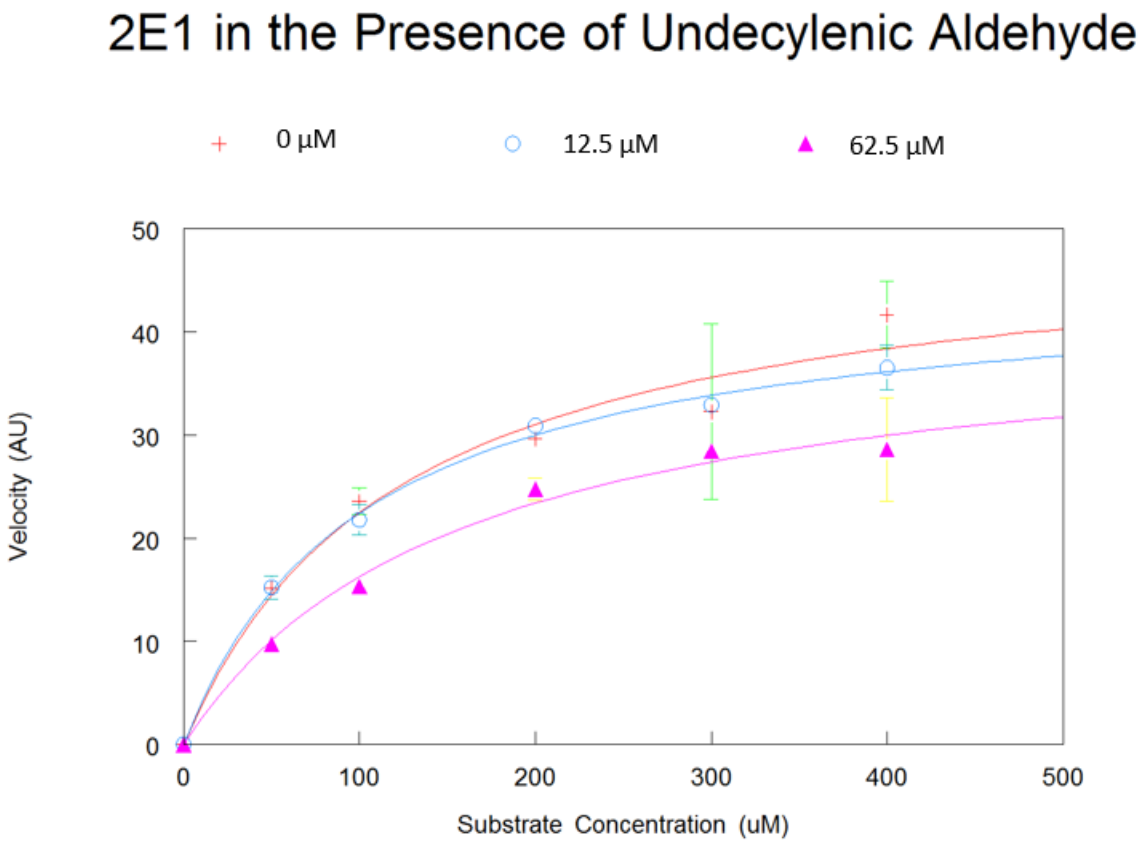
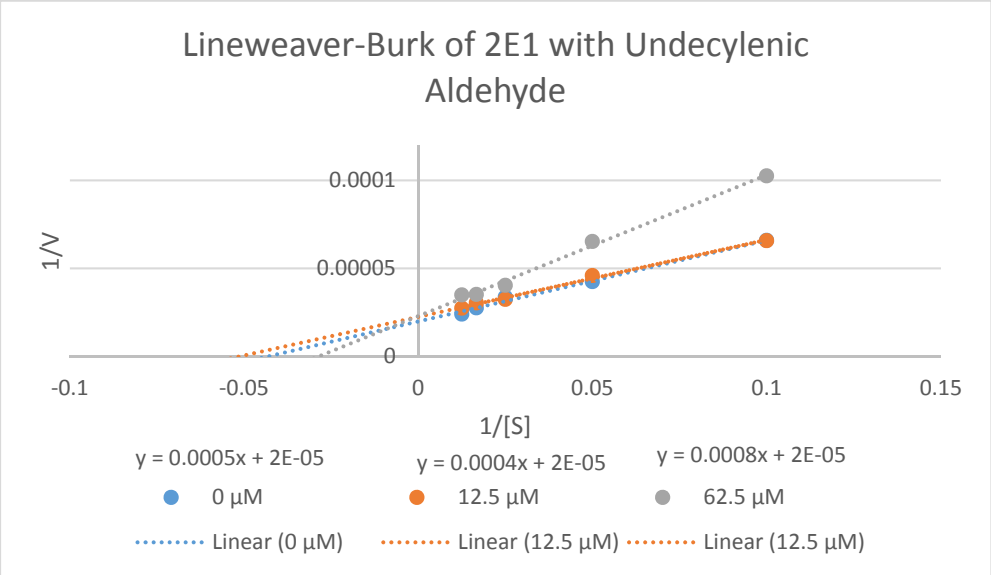


Figure 10. A Lineweaver-Burk Plot of Isoform 2E1 at Three Varying Concentrations of Undecylenic Aldehyde. The Blue is Used for Reactions with 0 µM of Undecylenic Aldehyde, the Orange Lines is Used for Reactions with 12.5 µM of Undecylenic Aldehyde, and the Gray Line is Used for Reactions with 62.5 µM of Undecylenic Aldehyde.



3.3.0. BM3 Used as a Mechanistic Probe

To address mechanistic aspects of the reaction, the bacterial P450 BM3 was used as a model because we could acquire high concentrations of purified enzyme. This enzyme is found in bacteria, *Bacillus megaterium,* and is useful in an industrial setting. Since it is derived from bacteria, it can be harvested more abundantly since it is easier to control their growth and harvest the enzyme in a controlled setting. Cytochrome P450's taken from cadavers are costly and harder to harvest and purify. Another reason BM3 is used in place of human P450's is that BM3 is highly versatile and can be a very effective model for human P450 enzymes. At the University of Torino, researchers tested a small group of a certain class of drugs and of the group, nine of these prodrugs were able to be

turned over by BM3[3]. The efficiency of BM3 was seen in the preceding reaction's coupling efficiency which ranged from 55% to 100%.

The studying of BM3 treated with undecylenic aldehyde can reveal what happens to the central heme component of cytochrome P450's during the reaction. For example, Raner et al. demonstrated with certain aldehydes that during turnover, the heme is alkylated by reactive intermediates produced by the reaction[29]. Fluorophenol was used as the substrate for BM3 to test for effects on catalytic activity. Initial studies using NADPH as the source of electrons in the presence of aldehyde resulted in increased activity consistent with prior studies involving aldehyde stimulation of BM3. No evidence of inactivation was observed, however, suggesting that although the aldehyde function of undecylenic aldehyde was probably oriented toward the active site, its proximity to activated oxygen is not optimal for heme alkylation.

CHAPTER IV

CONCLUSIONS

Cytochrome P450's play a vital role in the metabolism of xenobiotics. Performing a screening of the different liver Cytochrome P450' showed that only two of the proposed seven human enzymes examined had a decrease in activity. In the case of isoforms 3A4 and 2E1, these two isoforms had a significant decrease in overall activity upon the addition of undecylenic aldehyde.

A kinetic study was performed to analyze the type of binding present. Initially, isoform 2E1 data displayed signs of irreversible binding. This was concluded through the continual increase in activity over time. The ratio over time also increased suggesting irreversible inhibition.

Using a spectrophotometer, there was no significant change in the absorption upon the addition of undecylenic aldehyde. There was no evidence of inactivation observed, suggesting the aldehyde function of undecylenic aldehyde was bound at the active site.

The Michaelis-Menten study suggests that the type of inhibition experienced in the 2E1 isoform is the result of mixed inhibition, as seen in the changes in the K_m and V_{max}. A Lineweaver Burk plot was created and the results showed mixed inhibition.

However, the data more closely resembled that of competitive inhibition. This research is valuable for analyzing how xenobiotics affect the activity of specific enzymes. In the case of cytochrome P450's, isoforms 2E1 and 3A4 saw an overall reduction of activity. As seen in the case of furanocoumarins and P450's, these compounds alter the metabolism of a class of drugs referred to as statins. Studying other compounds and monitoring for activity change is crucial to how drugs are dosed and prescribed. Other similar experiments should be performed on drugs before they are available to consumers. This area of research is essential for drug safety, and will help reduce the amount of potentially harmful drug/xenobiotic interactions.

REFERENCES

1. Guengerich FP, Cheng Q. Orphans in the Human Cytochrome P450 Superfamily: Approaches to Discovering Functions and Relevance in Pharmacology. *Pharmacol Rev*. **2011**; 63(3): 684–699.
2. Bak S, Beisson F, Bishop G, Hamberger B, Höfer R, Paquette S, Werck-Reichhart D. Cytochromes P450. *The Arabidopsis Book / American Society of Plant Biologists*. **2011**, 9: e0144.
3. Di Nardo G, Gilardi G. Optimization of the Bacterial Cytochrome P450 BM3 System for the Production of Human Drug Metabolites. *Int J Mol Sci*. **2012**, 13(12): 15901–15924.
4. Guengerich, FP, Johnson WW. "Kinetics of Ferric Cytochrome P450 Reduction by Nadph-Cytochrome P450 Reductase: Rapid Reduction in the Absence of Substrate and Variations Among Cytochrome P450 Systems." *Biochemistry*. **1997**, 36(48): 14741-50.
5. Ortiz de Montellano PR. Cytochrome P450-activated prodrugs. *Future Med Chem*. **2013**, 5(2): 213–228.
6. Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR, Waxman DJ. The P450 Gene Superfamily: Update Mapping on New Sequences, and Recommended Nomenclature. *DNA and Cell Biology*. **1991**, 10(1): 1-14.
7. Johnson EF, Connick JP, Reed JR., Backes WL, Desai MC, Xu L, Estrada DF, Laurence JS, Scott EE. Correlating Structure and Function of Drug-Metabolizing Enzymes: Progress and Ongoing Challenges. *Drug Metab Dispos*. **2014**, 42(1): 9–22.
8. Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC. The human intestinal cytochrome P450 "pie". Drug Metab Dispos. **2006**, 34 (5): 880-6.
9. Guengerich FP. Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity. *Chemical Research in Toxicology*. **2001**, 14 (6): 611-650.
10. Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshel Wood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, Mendelson CR, Bulun SE. Aromatase Cytochrome P450, The Enzyme Responsible for Estrogen Biosynthesis. *Endocrine Reviews*. **1994**,15(3): 342-355.

11. Barhoumi R, Catania JM, Parrish AR, Awooda I, Tiffany-Castiglioni E, Safe S, Burghardt RC. Multiphoton spectral analysis of benzo[a]pyrene uptake and metabolism in breast epithelial cell lines. *The Journal of Toxicological Sciences*. **2009**, *34*(1): 13–25.
12. Xu C, Li C, Kong A. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Archives of Pharmacal Research.* **2005**, 28 (3): 249-268.
13. Hanley, M.J.; Cancalon, P.; Widmer, W.W.; Greenblatt, D.J., The effect of grapefruit juice on drug disposition. *Expert Opin Drug Metab Toxicol*. **2012**, 7(3): 267–286.
14. Androutsopoulos VP, Tsatsakis AM, Spandidos DA. Cytochrome P450 CYP1A1: wider roles in cancer progression and prevention. BMC Cancer. 2009;9:187.
15. Zhou SF, Chan E, Zhou ZW, Xue CC, Lai X, Duan W. Insights into the structure, function, and regulation of human cytochrome P450 1A2. Curr Drug Metab. 2009; 10(7):713–729.
16. Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC. The human intestinal cytochrome P450 "pie". *Drug Metab Dispos*. **2006**, 34 (5): 880-886
17. Kim D, Guengerich FP. Cytochrome P450 activation of arylamines and heterocyclic amines. *Annu Rev Pharmacol Toxicol*. **2005**, 45:27–49.
18. Flockhart D. Drug Interactions: Cytochrome P450 Drug Interaction Table. Indiana University. **2015**.
19. Nithipatikom K, Brody DM, Tang AT, Manthati VL, Falck, JR., Williams CL, Campbell WB. Inhibition of carcinoma cell motility by epoxyeicosatrienoic acid (EET) antagonists. *Cancer Sci*. **2010**, 101(12): 2629-2636.
20. Jernstrom H, Bageman E, Rose C, Jonsson PE, Ingvar C. CYP2C8 and CYP2C9 polymorphisms in relation to tumour characteristics and early breast cancer related events among 652 breast cancer patients. *British Journal of Cancer*. **2009**, 101: 1817–1823.
21. Howard LA, Miksys S, Hoffmann E, Mash D. Brain CYP2E1 Is Induced by Nicotine and Ethanol in Rat and Is Higher in Smokers and Alcoholics. *Britsih Journal of Pharmacology.* **2003**.
22. Wrighton SA, Maurel P, Schuetz EG, Watkins PB, Young B, Guzelian PS. Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450p. *Biochemistry*. **1985**, 24 (9): 2171-2178.
23. Ortiz de Montellano PR., Stearns RA, Langry KC. *Molecular Pharmacology*. **1984**, 25: 310.
24. McCray JW, Weil R. Inactivation of interferons: halomethyl ketone derivatives of phenylalanine as affinity labels. *Proceedings of the National Academy of Sciences of the United States of America*. **1982**; *79*(16): 4829–4833.
25. Jitkaew S, Trebinska A, Grzybowska E, Carlsson G, Nordström A, Lehtiö J, Fröjmark A, Dahl N. Fadeel B. N^{α}-Tosyl-l-phenylalanine Chloromethyl Ketone Induces Caspase-dependent Apoptosis in Transformed Human B Cell Lines with

Transcriptional Down-regulation of Anti-apoptotic HS1-associated Protein X-1. *The Journal of Biological Chemistry*, **2009**; 284(41): 27827–27837.
26. Correia MA, Sinclair PR, De Matteis F. Cytochrome P450 Regulation: The Interplay Between its Heme and Apoprotein Moieties in Synthesis, Assembly, Repair, and Disposal. *Drug Metabolism Reviews*. **2011**; 43(1): 1–26.
27. Kandagatla SK, Mack T, Simpson S, Sollenberger J, Helton E, Raner GM. Inhibition of human Cytochrome P450 2E1 and 2A6 by aldehydes: Structure and activity relationships. *Chemico-Biological Interactions*. **2014**, 219: 195–202.
28. Ortiz de Montellano PR, Beilan HS, Kunze K, Mico BA. Destruction of Cytochrome P450 by Ethylene. *J Biol Chem.* **1981**, 256: 4395-4399.
29. Montellano GM, Hatchell JA, Dixon MU, Loy TL, Haddy AE, Johnston ER. Regioselective Peroxo-Dependent Heme Alkylation in $P450_{BM3}$-F87G by Aromatic Aldehydes: Effects of Alkylation on Catalysis. *Biochemistry*. **2002**, 41: 9601-9610.