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Assessing Specificity of Indazole Derivatives as Inhibitors to CYP2E1

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This Honors thesis entitled

"Assessing Specificity of Indazole Derivatives as Inhibitors to CYP2E1"

written by

Hannah Hart

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meets the criteria for acceptance
and has been approved by the undersigned readers.

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Assessing Specificity of Indazole Derivatives as Inhibitors to CYP2E1

A Research Paper

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Abstract

Metabolism of drugs in the human body can yield both desirable and undesirable results. Some of these side-effects are due to drug-drug interactions, drug-diet interactions, or lack of drug specificity. The enzymes that drugs attack are structurally selective and complex; therefore, many drugs are very general in structure and could possibly react with a variety of enzymes. Indazole has been used as a backbone for several drugs as it has the general structure that may react with most P450s. To determine a derivative, or a group of derivatives, of indazole that may be reactive only with cytochrome P450 2E1 (CYP2E1), several indazole derivatives were employed as inhibitors of various P450s in enzyme-substrate reactions for IC_{50} assays. We hypothesize that the polarity and steric bulk of substituents to indazole will control the affinity and selectivity of this class of molecules toward P450s, especially CYP2E1. We developed IC_{50} assays that indicated the effectiveness of the inhibitors in relation to indazole itself. These findings will provide insight into (1) the effects of substituents to the indazole ring on affinity toward CYP2E1, and (2) the structural specificity of CYP2E1.

Assessing Specificity of Indazole Derivatives as Inhibitors to CYP2E1

Enzymes and Their Importance within the Body

Enzymes have been known to exist for over a hundred years. The name itself, meaning "in yeast", explains where they were first discovered. Dr. Benjamin Horowitz wrote in 1918 of the recent discoveries made in the area of enzymatic research which suggested enzymes to be primarily catalysts produced within and by a cell itself (p. 253). The widely known textbook definition of "catalyst" is a substance that increases the rate of a reaction but itself remains unaltered. In layman's terms, then, an enzyme is a substance produced within a cell to speed up reactions and not be used up or changed in the process. It is now known that these enzymes catalyze reactions by lowering the energy required for activation of a process. They do this by optimizing either the environment in which the reaction takes place or the orientation and/or proximity of reactants. The vitality of these enzymes may only be fully understood when one realizes that life is sustained by cellular activity. This cellular activity occurs largely due to intracellular chemical reactions regulated by catalytic substances such as these enzymes, which can be produced only by the cell itself. Therefore, life cannot be sustained without cellular production of enzymes. They are present not only within human cells but all living cells. Enzymes were first discovered in everyday processes, such as the spoiling of milk, alcoholic fermentation, and putrefaction. Various experiments showed that these processes were due not to the living cells within the substances but rather due to something within the cells—later found to be enzymes. They aid in a vast majority of intracellular reactions, their primary function being increasing the rate at which the reactions take place. While Horowitz (1918) acknowledged that society had "far from a comprehensive knowledge on the chemical configuration of an enzyme", he was on the right path to learning more about these vital cellular components (p. 259).

Cytochrome P450 Enzymes

Enzymes do not come in one set shape or form. As a whole species, enzymes are not mass-produced by assembly line to be exact copies of one another and serve exactly

one function; rather, there are categories of enzymes, designating to each a more specified purpose. Within these categories or families, the enzymes may be produced in multitude. You may think of the general term "enzyme" as similar to the general term "jacket". There are many different kinds of jackets that have a generally similar function, though they may be different colors, sizes, styles, materials, etc. Many of those jackets, with their different specifics that make them unique, are mass-produced, though they still differ from another mass-produced jacket. Similarly, enzymes serve the same general purpose as described earlier, but they also show slight differences that allow them to complete their function in different ways. However, these enzymes may also not be so specific that they are ill-prepared for the unpredictable multitude of compounds and reactions they may encounter. As F. P. Guengerich (1993) puts it, "It would be impractical for the body to produce elaborate and specific enzymatic machinery to deal with every new molecule that comes its way" (p. 440). In terms of fighting off potential toxins, a function largely reliant upon enzymes, the body produces multiple generalized lines of defense. The first of these is most obviously the immune system. Beyond it, however, stands a class of molecules now referred to as the cytochrome P450 (CYP) enzymes.

The study of such compounds began in late 1950s, when a single enzyme believed to be found exclusively in the liver was detected by spectrophotometry at a wavelength of 450 nanometers (Guengerich, 1993, p. 440). At the time, these compounds were believed to function solely in xenobiotic, that is 'foreign chemical compound', metabolism. Since then, discoveries in this field have made it clear that there are multiple cytochrome P450 enzymes with differing functions and cell-type localizations.

Upon experimental observation of aerobic processes within cells, researchers determined that a heme-bearing protein was responsible for the liver-microsomal control of oxidation reactions, which led to the aforementioned conclusions. Further experimentation revealed that this protein was the component at the end of the electron-transport chain responsible for catalysis of oxidation, which led to its name, "cytochrome" (Guengerich, 1993, p. 442). By definition, as *cytochromes*, these enzymes are 'iron-containing proteins that participate in cell respiration as catalysts of oxidation-reduction reactions' (Nebert et al. 2013, p. 1). (However, it must be noted that these enzymes are

unrelated to the mitochondrial cytochromes a, b and c. Guengerich (1993) says, "Cytochrome P450 enzymes transfer electrons to oxygen, whereas most respiratory cytochromes transfer electrons to other proteins" (p. 443).) As experimentation continued, the cytochrome P450 enzymes were discovered in eukaryotic mitochondria, requiring iron-sulfur proteins that were not needed by the liver-microsomal proteins. After findings were published by various labs, the existence of multiple types of cytochrome P450 molecules was confirmed. It is now known that these enzymes are found in every type of cell within the body, except in red blood cells and skeletal muscle cells, with most of them residing in the liver (Guengerich, 1993, p.442).

The compounds that these enzymes metabolize cannot be categorized solely as things we ingest deliberately, although they have proven to be instrumental in understanding the science behind drug metabolism. Many of the xenobiotics, substances foreign to the body, processed by these molecules are introduced to the body unknowingly as environmental pollutants. In addition to metabolizing foreign substances, the cytochrome P450 enzymes also play important roles in the metabolism of endogenous substances, 'those produced within the body' (Guengerich, 1993, p.440). Since multiple types of cytochrome P450 enzymes exist, their function is usually designated exclusively either to the processing of foreign chemicals or to the metabolism of endogenous substances.

Relating to the processing of foreign chemicals, more applicably, important functions of cytochrome P450 enzymes include drug metabolism and carcinogenic response. Drug metabolism, being a large factor in this study, will be discussed in more depth later in this paper. Carcinogenic responses activated by the presence of specific cytochrome P450 enzymes may be positive or negative, depending upon the enzyme. Some P450 enzymes may activate the harmful effects of substances that would otherwise be essentially harmless, whereas some other cytochrome P450 enzymes may be responsible for the removal of such compounds. This difference in function applies not only to the effects of carcinogenic substances but also to the toxic activation of other compounds within the body. That is to say that the toxicity of a substance within an individual's biological system may be greatly affected by the type and quantity of P450 enzymes

present in the system (Guengerich, 1993, p.445). Nelson (2013) says, "Human P450s are the basis for sometimes fatal drug interactions and the beneficial conversion of codeine into morphine" (p.4). Therefore, these enzymes may be either beneficial or detrimental in mode of action.

In regard to the modification of endogenous substances, P450 enzymes are crucial to the hydroxylation of important molecules within the body. The lack of such hydroxylation reactions results in a number of genetic diseases known today, "salt-wasting syndrome" for example (Guengerich, 1993, p. 445). Several compounds vital to survival are created by reactions directly dependent upon these enzymes. The production of sterols, compounds which stabilize the cell membrane structure, depends upon the P450 CYP51. Lignin, the molecule responsible for the rigid support in woody plants and the making of water conduction vessels within them, is a product of the reactions of CYP73, CYP98 and CYP84. Components of pollen are also produced by P450 enzymes, as are the pigments for color in many flowers (Nelson, 2013, p.1-2). They are vital to the synthesis of eicosanoids, 20-carbon compounds produced from the modification of arachidonic acid. This group of compounds includes vital signaling molecules and molecule transport modifiers. It has also been shown that these enzymes are involved in the production of nitric oxide (NO), a neurotransmitter, blood pressure mediator, and natural defender against invading pathogens (Guengerich, 1993, p. 446). Nebert, Wikvall, and Miller (2013) outline the importance of P450 enzymes in endogenous, or internally originating, functions, stating:

> It was proposed that CYP enzymes are important upstream in the synthesis and degradation of virtually all non-protein ligands that bind to receptors or activate second-messenger pathways regulating growth, differentiation, apoptosis, homeostasis and neuroendocrine functions. (p.2)

While the cytochrome P450 enzymes have been found to exist in every biological kingdom, the human genome is of most interest to this study. The P450 enzymes are arranged into families and subfamilies, based on percent amino acid sequence identity. Studies have found that "the human genome contains 18 CYP families, divided into 41 protein-coding subfamilies encoding 57 genes." The CYP2, CYP3, and CYP4 families contain a much

larger number of genes than the other families. These three, along with the CYP1 family, are inducible by a variety of stimuli, whereas the other 14 families function mainly in critical life functions, and are thus more likely to be associated with genetic diseases when mutated, as mentioned earlier (Nebert et al. 2013, p. 2). The members of these 14 families are more unique, having well-defined substrates and functions (Nelson, 2013, p.3). Meanwhile, the first 4 families participate primarily in the general metabolism of eicosanoids, drugs, and other foreign chemicals. There are now more than 150 known eicosanoids, and therefore the enzymes binding them have low specificity. In addition, there are a number of sequential redundancies amongst the genes of these P450 families; thus, allelic mutations within these enzymes rarely result in serious disease. However, single-nucleotide polymorphisms (SNPs) in these genes may result in the inter-individual differences affecting parameters such as drug dosage (Nebert et al. 2013, p. 5).

A better understanding of the cytochrome P450 enzymes as a molecular species allows us to be able to put this study into full perspective in regard to human biological systems and to the world in which we live.

CYP2E1

The cytochrome P450 enzyme of interest in this study was CYP2E1. It is an enzyme that resides in the liver and is involved in its own metabolism and that of several other protoxicants, meaning that it regulates the production or degradation of each to maintain them in certain amounts. This metabolism is inducible, meaning that the production of CYP2E1 may be affected by the presence or absence of certain stimuli. This enzyme is responsible for the activation of many procarcinogens, which is the first step in development of "chemically mediated cancers" (Mendoza-Cantu et al. 2006, p.494). Growing evidence supports a possible role of CYP2E1 in gluconeogenesis (Collom et al. 2007, p.1). CYP2E1 is distinguished from other P450s by its rapid turnover rate, making it the most unstable of the liver-microsomal CYPs. Its short half-life leads to quick degradation of the enzyme, however its half-life is increased when bound by substrates or heme-iron ligands, meaning that the binding of this enzyme, and therefore its induction, is its primary means of survival (Zhukov et al. 1999, p.453).

One of the better-known metabolites of CYP2E1 is ethanol, connecting high CYP2E1 expression in the liver to chronic alcohol users. A common result of persistent alcohol use is liver toxicity caused by oxidative stress. Oxidative stress is an imbalance within a cell in which a reactive oxygen species (ROS), a molecule containing an oxygen free radical, pulls electrons from other sources for its own stabilization while destabilizing the molecules from which it takes the electrons. Alcohol metabolism-mediated liver toxicity occurs when such an ROS and the reactive metabolite acetaldehyde cause damage within the cell. In occasional drinkers, CYP2E1 is only mildly expressed because the metabolism of ethanol in these cases can be handled by alcohol dehydrogenase (ADH). ADH is present at very low levels in liver cells. Therefore, as alcohol levels increase, the role of CYP2E1 is also greater. Its induction by ethanol may occur translationally, post-translationally, and transcriptionally. CYP2E1 contributes to the formation of the ROS and therefore oxidative stress, which ultimately results in apoptosis (or systematic cell death). These processes occur not only in the liver, but also in the brain. CYP2E1 appears to play a dominant role in ethanol-mediated neurodegeneration and neurotoxicity (Jin et al. 2013, p.1-8).

CYP2E1 is also inducible by compounds such as toluene, a systemic toxicant that targets primarily the central nervous system and is present in organic solvents to which print workers are often exposed (Mendoza-Cantu et al. 2006, p.494).

Although CYP2E1 metabolizes many important chemical compounds, which will be discussed later, its role in protoxicant and carcinogenic activation suggest the relevance of finding better inhibitors for this enzyme.

Experimental findings suggest the presence of a restrictive active site. This site is the oxidizing component of many CYP2E1-catalyzed reactions. From previous studies, it is known that this oxidation is caused by a heme moiety located at the active site. While some active site amino acid residues have been identified, their effect on substrate binding, and therefore active site selectivity, remains uncertain. Due to the limited knowledge of the CYP2E1 structure and conformation, and therefore its mechanisms of interaction, most structural substrate predictions have been made based on a model constructed by knowledge of similar CYPs such as CYP2C5. "[This] model predicts CYP2E1 possesses

a hydrophobic channel from the solvent leading to small binding pocket bordered by three structural elements" (Collom et al. 2007, p.1, 9-13). (Fig. 1).

Because CYP2E1 metabolizes such a broad range of compounds, its "metabolic processes can involve poorly characterized cooperative interactions" (Hartman et al. 2013a, p.755). As such, it is difficult to gain much insight into the conformation of the enzyme. However, observation of common interactions between CYP2E1 and its substrates has allowed for better characterization of conformation.

Common Substrates and Inhibitors to CYP2E1

Because the structure of CYP2E1 is highly complex and not yet completely known, substrate affinity is difficult to predict and has been partially determined experimentally. Through such experimentation it has been found that this enzyme metabolizes a broad range of more than 70 chemicals—small, hydrophobic molecules, including monocyclic and bicyclic compounds, such as benzene, acetaminophen, coumarin, caffeine, and chorzoxazone, etc. (Collom et al. 2007, p.1) Although many of these substrates bind via a one-site mechanism, following Michaelis-Menten kinetics, there are several exceptions which bind cooperatively at two binding sites (Hartman et al. 2013a, p.756). These insights apply to inhibitory binding as well.

Aromatic and heterocyclic compounds act on CYP2E1 through uncompetitive and mixed inhibition mechanisms, requiring two binding sites—one for the substrate and another for the inhibitor (Hartman et al. 2013b, p.13). The research of Hartman et al. (2013a) suggested that monocyclic azoles show two binding events, while bicyclic azoles show only one (p.755). The experiments conducted in this study used a compound called pyrazole as a standard substrate compared to various derivatives of the compound. Pyrazole is a monocyclic compound containing two nitrogen atoms within its ring (Fig. 2). Analysis of results showed that the addition of a methyl group to the third or fourth carbons on the pyrazole ring would increase affinity toward CYP2E1, however there was no increase in affinity when a phenyl group was placed at the third carbon (p.761). The addition of a second ring showed an even greater increase in affinity for the enzyme and inhibitor potency depending on the location of the ring. The aromatization of the second ring also causes an increase in affinity and potency (p.762). Steric clashes caused by

adding bulky substituents to the rings decreases affinity for CYP2E1 due to the conformation and environment of the binding site. Past docking studies determined that the active site heme was located at the end of an access channel that became suddenly narrower just past the cooperative site (Levy et al. 2014, p.49-50). (Fig. 1).
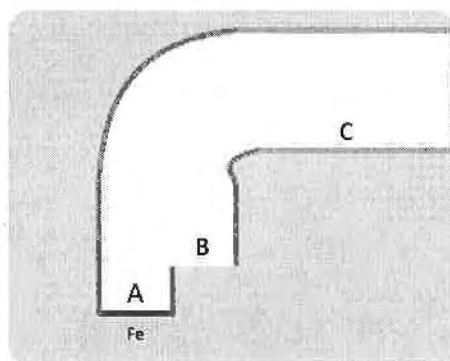

Figure 1   Stylized depiction of the CYP2E1 active site (A), cooperative site (B), and access channel (C).

The broad group of P450-binding substances, including those we chose as the inhibitors of our study, namely the azoles, bind to the enzymes by the formation of an iron-nitrogen bond. An azole is any five-membered aromatic ring structure containing two hetero-atoms, one of which must be a nitrogen atom. The chemically favorable formation of the iron-nitrogen bond determines the ideal orientation of the compound, which would also have some bearing on selectivity of the enzyme (Levy et al. 2014, p.50). Upon forming the iron-nitrogen bond, compounds rotate to form more favorable interactions with residues along the access channel (Fig. 1) while avoiding steric clashes. For this reason, both structure and identity play a vital role in the enzyme active site selectivity and affinity of substances for the enzyme.

As stated earlier, some compounds bind cooperatively to a second site on the enzyme, rightly named the "cooperative site" as seen in Fig. 1(B). Monocyclic azoles fall into this category. In the CYP2E1 enzyme, the cooperative site is located as a platform in the channel just above the active site. When a compound binds to the cooperative site, it overlaps the compound bound to the active site and a variety of interactions (favorable or unfavorable) may occur depending upon the identity and characteristics of each molecule and its interactions with the enzyme environment (Levy et al. 2014, p.53). Although

previous studies suggested that bicyclic compounds inhibit CYP2E1 by a single-site mechanism, later experimentation found that compounds such as indazole and tetrahydroindazole may act cooperatively, indicating greater flexibility of CYP2E1 binding sites (Hartman et al. 2013a, p.764).

Larger molecules, such as the bicyclic indazole derivatives used in our experimentation, have trouble rotating once reaching the narrowed active site due to their steric bulk. Their final orientation may therefore be determined by the interactions that occur with residues along the access channel as they travel toward the active site. Factors such as these have a great bearing on everyday life. The structure of this enzyme's access channel and the residues of which it is composed determine the general rules and regulations for any compound seeking to bind to it. This is an example of the **selectivity** of the enzyme. The identity of the compound determines if and how it will be able to enter and bind to the enzyme. This is an example of the enzyme's **affinity** for the compound. The interaction of a compound with an enzyme is not quite so simple as the lock and key idea so often presented. These interactions, depending upon a variety of factors, may produce many different results regarding the compound's effects on the enzyme. This area of research is important because it allows one to discover new possibilities for drug specificity and to enhance those innovations already discovered.

Why Indazole?

Previous experiments revealed the high affinity of CYP2E1 for the fused bicyclic compounds indazole (IND) and tetrahydroindazole (THI). (Fig. 2). These compounds were found to interact at both the active site and the cooperative site. Experiments showed IND and THI to be 200-fold and 100-fold more potent than pyrazole, respectively, in CYP2E1 inhibition (Hartman et al. 2013a, p.762-764).

Because IND is ideal for CYP2E1 binding, it is rapidly becoming a base unit for drug design. However, the molecule occurs rarely in nature. It is possible that a more potent and naturally occurring derivative could be found.

The inhibitory success of IND and THI combined with the previously existing information about substituent addition to other compounds for increased affinity brought us to the experiment described in this paper.

Chosen Compounds

Eight inhibitors were chosen for this study. Indazole (IND) and its derivative tetrahydroindazole (THI) were used as references for relative inhibition by other compounds. The lack of aromaticity in the second ring of tetrahydroindazole was expected to cause a decrease in inhibition. 5-methyl-1H-indazole and 6-methyl-1H-indazole were chosen to compare the effects of having a group on carbon-5 as opposed to carbon-6. 5-bromo-1H-indazole was chosen to compare the bromine substituent to the methyl group as they are comparable in size but differ significantly in properties. 5-hydroxymethyl-1H-indazole was chosen for its polar hydroxyl group, which was predicted to show low affinity toward the hydrophobic binding site of CYP2E1. 5-phenyl-1H-indazole was used due to
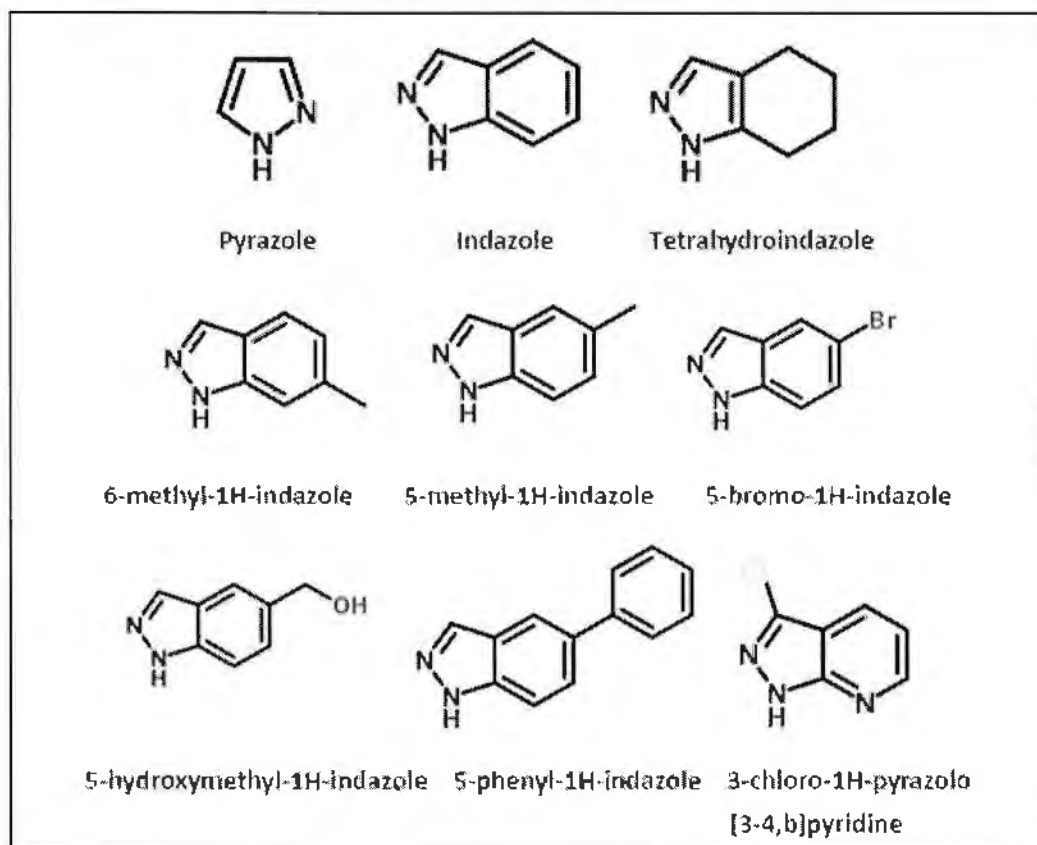

Figure 2   Indazoles used as inhibitors in IC50 assays with CYP2E1.

its bulky substituent phenyl ring, which was predicted to be hindered in its inhibition due to steric hindrance. Finally, 3-chloro-1H-pyrazolo[3,4-b]pyridine was chosen based on its structural similarity to chlorzoxazone (CHZ), a substrate of CYP2E1 with which reaction has been shown to occur at a carbon adjacent to a large chlorine atom. CHZ was first used as a well-known skeletal muscle relaxant in drugs such as Paraflex and was known to act by binding to CYP2E1 ("Nurses' Drug Alert," 1986, p.1049-1050). This final compound was expected to cause a significant increase in inhibition, yielding more insight into the structure-specificity of CYP2E1.
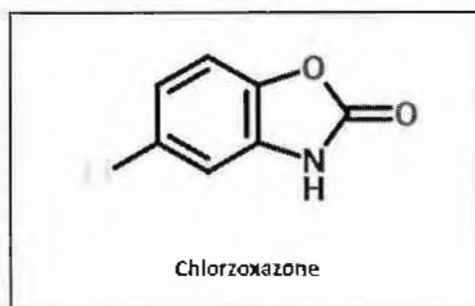

Figure 3   Chlorzoxazone (CHZ), a substrate of CYP2E1 known to bind at the carbon adjacent to the chlorine atom.

Goal of the Study

The goal of this study was to determine the structure-function relationships of indazoles as inhibitors for CYP2E1, and perhaps other P450s. The initial step was to identify suitable high throughput assay conditions for recombinant P450s, then apply them to experimental design and carry out subsequent IC_{50} studies. Knowledge of the inhibition of P450s by indazoles will identify how different types of substituents determine affinity and selectivity toward each of the enzymes and, in doing so, expand understanding of structure-function relationships governing the enzymes' interactions with small molecules.

Materials

All chemicals in this study were of at least reagent grade. HPLC-grade acetonitrile (CH_3CN) and other basic chemicals were purchased from Fisher Scientific. Human CYP2E1 (Supersomes®) were purchased from BD Biosciences (San Jose, CA). Indazole, tetrahydroindazole, and components of the NADPH-regenerating system were purchased

from Sigma-Aldrich (St. Louis, MO). All remaining inhibitors were purchased from Chem-Impex International (Wood Dale, Illinois).

Methods

The steady state kinetics (K_m in particular) for the investigated enzymes were determined with 7-methoxy-4-trifluoromethyl-coumarin (7-MFC) and 7-ethoxy-4-trifluoromethyl-coumarin (7-EFC) as substrates, using the fluorescence of their common product, 7-hydroxy-trifluoromethylcoumarin. The results were then analyzed using the Michaelis-Menten equation to yield a value for the substrate concentration at which the enzyme works most efficiently. The K_m value was then used to set the substrate concentration for IC_{50} experiments with indazole derivatives. Finally, IC_{50} values could then be used to estimate K_i values, inhibitor concentration of greatest efficiency, using the Cheng-Prusoff equation.

K_m assays

For this experiment, substrates of varying concentrations in methanol were diluted to 4% methanol in 50 mM potassium phosphate pH 7.4 (KPi 7.4). The wells were prepared with 17 µL of deionized water, 2 µL of 50 mM KPi 7.4, 1 µL of enzyme, and 10 µL of diluted substrate (7MFC). NADPH solution at a concentration of 4 mM was prepared and incubated along with the well plate in a shaking incubator for 5 min at 37°C with shaking at 350 rotations per minute. Of the incubated NADPH, 10 µL was added to each of the wells. The reaction plate was incubated for 30 min at 37°C at 350 rotations per minute. Reactions were quenched by adding 30 µL of reaction mixture to 30 µL acetonitrile that had been chilled on ice for 10 min in a black well plate. Fluorescence of the product solutions was measured on a plate reader. Fluorescence values were then analyzed using Excel—values were blank adjusted, and product concentrations were calculated based on the standard curve. Rates of reaction were then calculated by taking the time of reaction (30 min) into account. All data was then analyzed in GraphPad Prism 5 (Fig. 3), to give a K_m value.
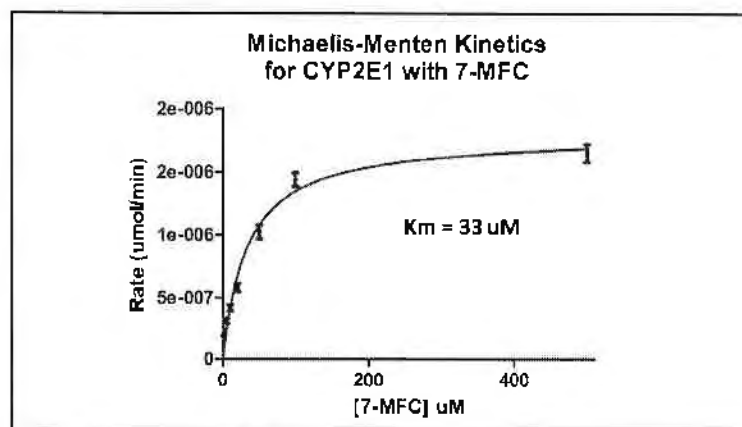
Figure 3 Michaelis-Menten curve of reaction of 7-MFC and CYP2E1 expressed as a function of 7-MFC concentration.

IC_{50} assays

For this experiment, inhibitors of varying concentrations in methanol were diluted to 2% methanol in 50 mM KPi 7.4. Substrate at a concentration of 50 mM was diluted 10-fold in 50 mM KPi 7.4. The wells were prepared with 1 μL of enzyme, 0.4 μL of diluted substrate, 10 μL of diluted inhibitor, and 18.6 μL of 50 mM KPi 7.4. NADPH solution at a concentration of 4 mM was prepared and incubated along with the well plate in a shaking incubator for 5 min at 37°C at 350 rotations per minute. Of the incubated NADPH, 10 μL was added to the wells. The reaction plate was incubated for 60 min at 37°C with shaking at 350 rotations per minute. Reactions were quenched by adding 30 μL of reaction mixture to 30 μL acetonitrile that had been chilled on ice for 10 min in a black well plate. Fluorescence of the product solutions was measured on a plate reader. Fluorescence values were then analyzed using Excel—the values were blank adjusted, then percent relative activity was calculated assuming 100% at 0μM inhibitor. All data was then analyzed in GraphPad Prism 5 (Fig. 4), to give an IC_{50} value.

Results and Discussion

We investigated the structure-function relationships for a number of indazoles to assess the impact of substituents on affinity for and inhibition of CYP2E1 as a foundation for their potential to be high-affinity and selective inhibitors of P450s. We were only able to explore reactions of the inhibitors with one P450, CYP2E1. The results of this study were able to give significant insight into the structural preferences of CYP2E1. We were

able to determine affinity effects of the given substituents; however selectivity information was not established.

The K_m of 7-MFC with CYP2E1 was found to be approximately 33 µM. This value and the concentration of substrate being held constant in all experimental runs allows us to directly compare IC_{50} values, as seen in Figure 4.

Loss of aromaticity with THI led to a slight decrease in affinity, as expected, although significantly less than reported previously with more in-depth spectral binding and inhibition kinetic studies (Collom et al. 2007, p.9). The current observation may reflect differences in experimental design, but would require further study for verification.

Substituent addition at position 5 altered binding to CYP2E1. A methyl group slightly increased affinity, whereas the presence of a phenyl group had a more pronounced effect indicating that bulkier, hydrophohic groups improve affinity at position 5. A bromine at position 5 had a similar effect as a methyl group. Not surprisingly, the presence of a polar hydroxyl group on the 5-hydroxymethyl group led to a three-fold decrease in binding.

By contrast, the addition of a methyl group at position 6 had an effect on affinity quantifiably similar to that of indazole, suggesting a lack of significant interaction of the methyl group with the enzyme.

In the case of the 3-chloro derivative, the large halide close to the site binding with the heme led to a significant six-fold increase in affinity. The increase in affinity may improve selectivity for CYP2E1 given a similar effect for chlorzoxazone even though the drug binds relatively poorly to the enzyme. As seen in Fig. 4, the maximum inhibition caused by this compound is only 50%, while the others reach approximately 80-90% inhibition. Further study would be required to determine possible reasons that the compound binds tightly to the enzyme but does not cause a great decrease in enzyme activity. These results could reflect a conformational change of the binding site because of the inhibitor, which could still allow some enzyme-substrate interaction despite the inhibitor being bound to the enzyme. These possibilities should be further studied as they could also lead to new insights on enzyme function within the body.

This study established, as expected, that affinity of indazole for CYP2E1 is greater than that of tetrahydroindazole, due to the aromaticity of the indazole rings. It was determined that placing a hydrophobic group on the 5th carbon significantly improves affinity, while a polar group at this position decreases affinity and the presence of smaller non-polar groups at this position show slight improvement. Addition to the sixth carbon showed little interaction and change in activity from the standard indazole. Finally, the presence of a large halide close to the binding site showed significant improvement in affinity. Overall, these trends provide important insights on CYP2E1 binding of indazole and will be bolstered by in-depth kinetic inhibition studies to confirm these trends and provide mechanisms of inhibition. With these interactions in mind, further discoveries may be made as to the possible structural changes and the binding characteristics of the enzyme that were previously unknown.

Future Studies

Because experimental replicates are needed to decrease noise in the data and more clearly define trends, more assays should be run under the same conditions using different inhibitor concentrations. More steric groups may further enhance binding. A study of the 3-chloro derivative may provide insights on possible partial binding of the enzyme. Lastly, indazoles will be used as inhibitors against other P450s to assess affinity and selectivity among them. It is hoped that the results of more studies will help to identify the structure-specificity relationship of CYP2E1 and provide more insight into the structure effects on the selectivity of indazoles toward P450s.
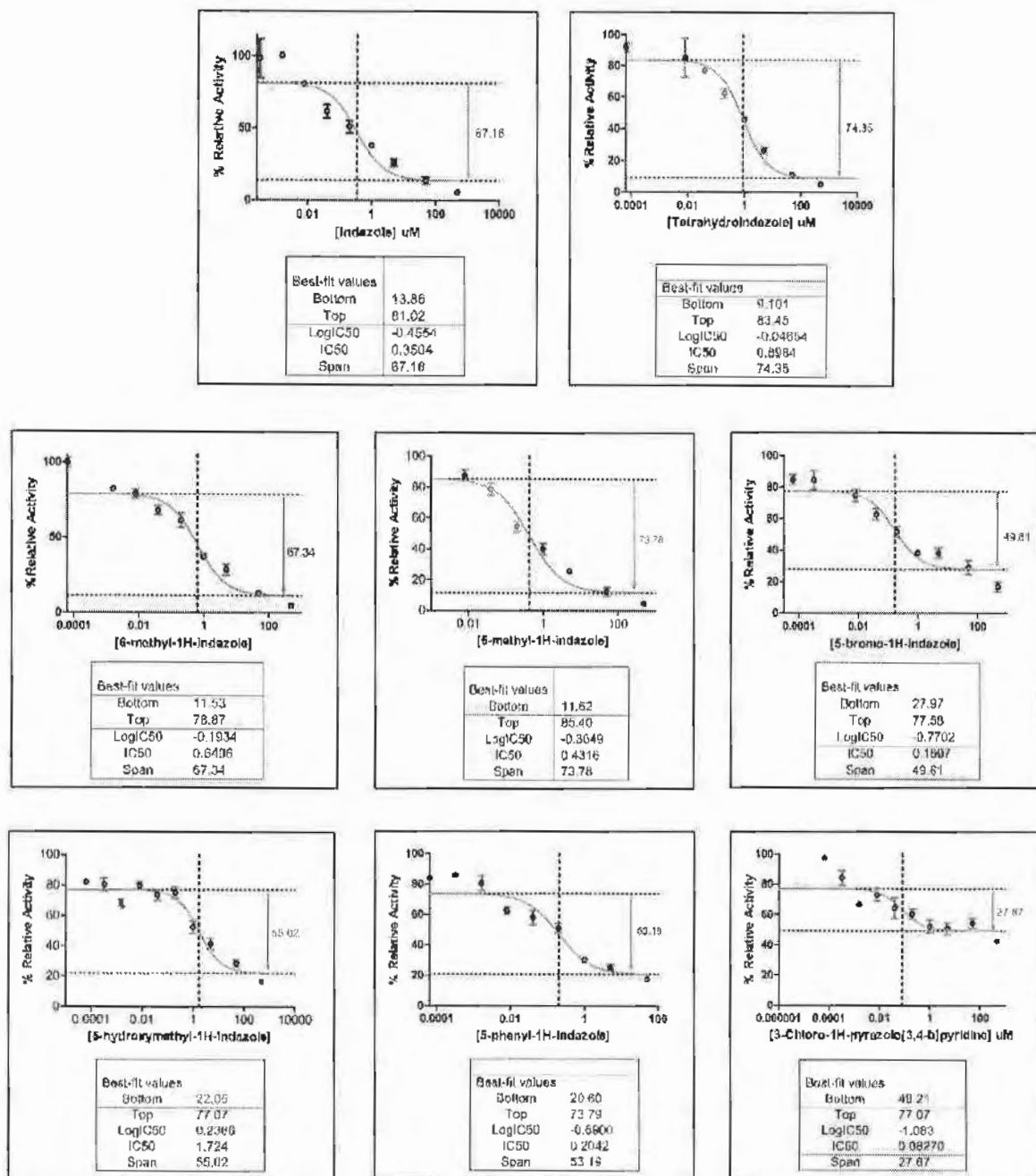
Figure 4   Inhibition of CYP2E1 activity expressed as % relative activity as a function of inhibitor concentration.   Experiments were performed three times with technical duplicates of each inhibitor concentration in each of the experimental replicates (n=6). IC_{50} values and the corresponding 95% confidence intervals.

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