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Structure of Pyrazole Derivatives Impact their Affinity, Stoichiometry, and Cooperative Interactions for CYP2E1 Complexes

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Abstract

CYP2E1 plays a critical role in detoxication and carcinogenic activation of drugs, pollutants, and dietary compounds; however, these metabolic processes can involve poorly characterized cooperative interactions that compromise the ability to understand and predict CYP2E1 metabolism. Herein, we employed an array of ten azoles with an emphasis on pyrazoles to establish the selectivity of catalytic and cooperative CYP2E1 sites through binding and catalytic studies. Spectral binding studies for monocyclic azoles suggested two binding events, while bicyclic azoles suggested one. Pyrazole had moderate affinity toward the CYP2E1 catalytic site that improved when a methyl group was introduced at either position 3 or 4. The presence of methyl groups simultaneously at positions 3 and 5 blocked binding, and a phenyl group at position 3 did not improve binding affinity. In contrast, pyrazole fusion to a benzene or cyclohexane ring greatly increased affinity. The consequences of these binding events on CYP2E1 catalysis were studied through inhibition studies with 4-nitrophenol, a substrate known to bind both sites. Most pyrazoles shared a common mixed cooperative inhibition mechanism in which pyrazole binding rescued CYP2E1 from substrate inhibition. Overall, inhibitor affinities toward the CYP2E1 catalytic site were similar to those reported in binding studies, and the same trend was observed for binding at the cooperative site. Taken together, these studies identified key structural determinants in the affinity and stoichiometry of azole interactions with CYP2E1 and consequences on catalysis that further advance an understanding of the relationship between structure and function for this enzyme.

Keywords

Cytochrome P450; CYP2E1; allostery; cooperativity; azole; structure-function

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1. Introduction

CYP2E1 plays an important role in the detoxication and carcinogenic activation of many environmental pollutants. CYP2E1 metabolizes a broad range of predominantly small, hydrophobic molecules (molecular weight < 100) comprised mainly of xenobiotic and endogenous compounds [1]. Substrates include monocyclic compounds, such as benzene, acetaminophen, isoniazid, and xylenes, as well as bicyclic compounds like coumarin, chlorzoxazone, and caffeine; nevertheless, there remain gaps in our knowledge of the molecular determinants for CYP2E1 specificity and metabolic efficiency toward these compounds. In some cases, the metabolism of these compounds improves their elimination from the body, and in other cases, they undergo activation into toxic compounds that increase the risk of several forms of cancer [2]. Consequently, an understanding of the mechanisms underlying the interactions between CYP2E1 and these molecules is critical for interpreting and predicting the biological significance of their metabolism.

The metabolism of many CYP2E1 substrates conforms to a simple one-site mechanism, such as that described by the Michaelis-Menten mechanism; however, there are several notable exceptions in which the stoichiometry of interactions between CYP2E1 and small molecules involves two binding sites and cooperative effects. Sigmoidal kinetic profiles of phenacetin, *m*-xylene, and 7-ethoxycoumarin by CYP2E1 indicate positive cooperativity [3–5], and in the latter two cases, Hill coefficients were reported to be 1.4 and 1.6, respectively [4, 6]. Moreover, we reported that CYP2E1 metabolism of styrene, a common pollutant, involves positive cooperativity [7]. Importantly, we proposed a mechanistic model to explain the findings in which two catalytic cycles display differing efficiency for styrene oxidation. The first cycle results from formation of a relatively inefficient catalytic binary complex with one substrate molecule bound to CYP2E1, and the second involves a more catalytically efficient ternary complex that forms when a second styrene molecule binds at higher concentrations. This mechanism for CYP2E1 oxidation of model substrates and pollutants is ultimately improved through cooperative binding of a second substrate molecule and could potentially impact the toxicological consequences of metabolism.

Conversely, 4-nitrophenol, a marker substrate for CYP2E1, undergoes oxidation through a substrate inhibition mechanism, whereby the binding of a second substrate molecule suppresses the reaction [8]. We were the first to provide a mechanistic model for the 4-nitrophenol kinetic profile through a negative cooperative mechanism for CYP2E1 [9]. In this mechanism, low 4-nitrophenol concentrations favor formation of an active binary complex. As 4-nitrophenol concentration increases, a second molecule binds to CYP2E1 to yield a catalytically inactive ternary complex leading to a decrease in overall activity. The interaction of 4-nitrophenol with two binding sites on CYP2E1 makes it an excellent reporter for cooperative interactions. Therefore, we employed binding data and inhibition kinetics to demonstrate heterotropic cooperative interactions in which 4-nitrophenol and 4-methylpyrazole bound to substrate and cooperative (effector) sites and modulated CYP2E1 activity. We postulated that these sites are likely proximal to one another and selectively bind specific monocyclic compounds, because bicyclic indazole bound to the catalytic and cooperative sites simultaneously and inhibited CYP2E1 activity.

The role of structure in mediating contacts with catalytic and cooperative sites has been explored through computational and catalytic studies. Early computational docking studies with models of CYP2E1 traditionally focused on single binding events consistent with the prevailing paradigm [10, 11]. Given growing evidence for multiple binding sites, we docked 4-methylpyrazole and indazole into the binding cavity of CYP2E1, which demonstrated the feasibility of binding more than one molecule and identified potentially important

hydrophobic contact residues for molecules [9]. These studies were followed up by work from other groups establishing a structural basis for CYP2E1 selectivity toward molecules [12, 13]. Moreover, we recently showed through catalytic studies with styrene and its metabolites that the structure of the molecules influenced their potency and mechanism of inhibition toward 4-nitrophenol metabolism [14]. Styrene and its primary oxidized metabolites, styrene oxide and 4-vinylphenol, differed little in hydrophobicity and shared similar affinities for both CYP2E1 binding sites. Interestingly, the presence of the hydroxyl group led to an inactive ternary complex as observed for 4-nitrophenol. The opening of the epoxide ring to form styrene glycol significantly increased the polarity of the molecule and led to almost a complete loss in binding to CYP2E1. Among all compounds, there was a positive correlation with binding and hydrophobicity. Taken together, these findings provide additional evidence for contributions of cooperative mechanisms by CYP2E1, yet the mechanisms underlying their prevalence and impact on the metabolism of drugs and pollutants remain largely unexplored.

To advance these discoveries, we demonstrate the selectivity of catalytic and cooperative effector sites for rabbit CYP2E1 through binding and inhibition studies for an array of seven commercially available pyrazole derivatives (Fig. 1). The compounds are well suited for exploring structure-function issues for CYP2E1, because they are known to induce spectral changes upon binding and inhibit CYP2E1 activity. A majority of the compounds were only available as the free base, rather than the salt, and thus, all studies involve the unprotonated (free base) forms of the compounds for consistency in terms of equilibrium between species and co-solvent (methanol) effects. This series includes 4-methylpyrazole, which we was previously characterized as a salt [9]. Spectral binding studies and catalytic inhibition studies using 4-nitrophenol as a reporter substrate were carried out and analyzed as reported elsewhere [14]. Through this approach, we were able to assess the impact of sterics and hydrophobicity on associations with catalytic and cooperative sites and their impacts on CYP2E1 catalysis. The corresponding dissociation and inhibition constants were then analyzed to determine the significance of hydrophobicity versus other factors, such as sterics and pi stacking, in forming CYP2E1 complexes. Based on initial findings, we expanded binding studies to analogs of pyrazole compounds, namely, imidazoles and triazoles (Fig. 2). These molecules also form spectral Type II complexes, but induce different orientations of the bound molecule and hence assess the steric constraints of the catalytic site. Taken together, these findings identified key structural determinants in the affinity and stoichiometry of their interactions with CYP2E1 and consequences on catalysis.

2. Methods

2.1 Materials

All chemicals were of at least reagent grade. HPLC-grade acetonitrile (CH_3CN) and trifluoroacetic acid, all pyrazole inhibitors, and other basic chemicals were purchased from Fisher Scientific. The following were obtained from Sigma-Aldrich: dilauroyl-L- α -phosphatidylcholine, 4-nitrophenol, 4-nitrocatechol, 2-nitroresorcinol, bovine erythrocyte superoxide dismutase, catalase, and sodium dithionite (hydrosulfite). In addition, components of the NADPH-regenerating system (NADP^+ , glucose 6-phosphate, torula yeast glucose-6-phosphate dehydrogenase) were purchased from Sigma-Aldrich. Rabbit CYP2E1 and CPR-K56Q were prepared from bacterial expression systems using previously published protocols [15, 16] with modifications [17]. Purified rabbit liver cytochrome b_5 was provided as a generous gift from Wayne L. Backes (Louisiana State University Health Science Center, New Orleans).

2.2 Spectral binding studies

We measured binding between CYP2E1 and azole molecules (Figs. 1 and 2) based on perturbations of heme absorbance in the Soret spectrum, as previously reported [9, 18]. Stock solutions of all unprotonated azoles were prepared in methanol due to solubility concerns. The titrations with these solutions were performed using tandem cuvettes to correct for any solvent effects on CYP2E1 absorbance and possible contributions from titrant to observed changes in absorbance. Specifically, we titrated 0.1 μM CYP2E1 in 50 mM potassium phosphate, pH 7.4, 20 μM dilauroyl-L- α -phosphatidylcholine with increasing amounts of ligand at 25 $^{\circ}\text{C}$. Spectral changes were recorded from 350 to 475 nm using a Jasco V-550 spectrophotometer. In the process, we generated difference spectra by subtracting the reference CYP2E1 sample with solvent from the absorbance of the sample with titrant present. Data from 4 to 6 experiments were compiled and averaged for analyses.

2.3 Statistical assessment of binding mechanisms

The source of the change in absorbance derives from the interaction between the CYP2E1 heme and one small molecule; nevertheless, the association of other molecules may further perturb the heme environment to alter the absorbance signal. Under those conditions, it may be possible to measure multiple binding events. As described in our previous publication [9], we determined the stoichiometry of CYP2E1 complexes with the pyrazoles using the software program DynaFit 3.28 (Biokin, Ltd.) [19]. Absorbance data were fit to mechanisms incorporating either one or two binding events (Fig. 3). When two molecules were bound to CYP2E1, we included the possibility that EL, EL₂, or both complexes yielded a spectroscopic signal as denoted by the asterisk. We then identified the most probable mechanism and corresponding binding parameters for these molecules using the advanced tools of numerical analysis and applied statistics, as implemented by DynaFit [19].

2.4 Inhibition of 4-nitrophenol oxidation

We measured initial velocities for rabbit CYP2E1 oxidation of 4-nitrophenol to 4-nitrocatechol using a high throughput HPLC method developed in our laboratory [17]. We employed a mutant form of the reductase, CPR-K56Q, due to its increased resistance to proteolysis [17]. For reactions, 25 nM CYP2E1 was reconstituted with 100 nM CPR-K56Q and 50 nM cytochrome *b*₅ in a 96-well assay block containing 50 mM potassium phosphate, pH 7.4, 20 μM dilauroyl-L- α -phosphatidylcholine, 4-nitrophenol (varied from 5 to 750 μM), 2 units μl^{-1} catalase, 0.04 $\mu\text{g} \mu\text{l}^{-1}$ superoxide dismutase, and an NADPH-regenerating system (2 $\mu\text{U} \mu\text{l}^{-1}$ glucose-6-phosphate dehydrogenase, 10 mM glucose 6-phosphate, 2 mM MgCl_2 , 500 μM NADP⁺). Prior to use, catalase was dialyzed against 20 mM potassium phosphate buffer, pH 7.4, 10% glycerol to remove the thymol preservative.

Specific reactions in the absence or presence of each pyrazole inhibitor (Fig. 1) were prepared in sets of eight, which facilitated sample manipulation with a multichannel pipette. All pyrazoles were prepared in methanol due to solubility concerns. Both control uninhibited and inhibited reactions contained a final 0.25% methanol to normalize potential solvent effects. Three concentrations of each inhibitor were used for these studies varying from 0.1 to 625 μM for high affinity (bicyclic pyrazoles) to low affinity (monocyclic pyrazoles) inhibitors. Following addition of all components except NADP⁺, reactions were incubated at 37 $^{\circ}\text{C}$ for 5 min. Upon addition of NADP⁺, a reaction aliquot was taken at four time points, transferred to a 96-well microplate, quenched with acetonitrile containing 2-nitroresorcinol (internal standard), and then centrifuged. The supernatant was transferred to a low volume HPLC vial held in a 96-vial rack that matched the 96-well format.

Quenched samples were then injected onto a Waters Symmetry C₁₈ 3.5 μm 4.6 $\times$ 75 mm column with a 75:25 0.1% trifluoroacetic acid/H₂O:CH₃CN mobile phase at a flow rate of

1.5 ml min⁻¹. All eluants were monitored at 360 nm. Product 4-nitrocatechol levels were normalized to the internal standard (4-nitroresorcinol) and quantitated relative to standards. The corresponding concentrations were plotted as a function of time, and the initial rate was determined by linear regression with the software program GraphPad Prism 4.0 (San Diego, CA). Reported initial rates reflect averages of data from 2 to 4 experiments.

2.5 Statistical assessment of catalytic inhibition mechanisms

The resulting kinetic profiles for pyrazole inhibitors were analyzed globally to identify the most probable inhibition mechanism toward 4-nitrophenol oxidation by CYP2E1 using DynaFit 3.28 (Biokin, Ltd) [19]. We limited the number of variable parameters during the analysis of the inhibition data by determining and setting constant the uninhibited steady-state parameters. Each inhibition data set included the uninhibited reaction as a negative control, and thus we compiled and analyzed all of those results to determine the kinetic parameters ($V_{\max}$, K_s , and K_{ss}) for uninhibited 4-nitrophenol reaction. The Dynafit script file used for analysis of the uninhibited data is included in Supplementary Data. As described previously [9], a substrate inhibition mechanism best described the metabolism of 4-nitrophenol by CYP2E1. A plot of the uninhibited kinetic profile as well as a table of parameters for the uninhibited reactions is also included in Supplementary Data, Fig. S.1 and Table S.1. These parameters were held constant and the inhibitory parameters ($K_{i,ap}$, $K_{si,ap}$, and $k_{cat2,ap}$) treated as variables.

For analysis of the inhibition experiments, as previously reported for styrene and metabolites [14], data were fit to one or two-site inhibition mechanisms for 4-nitrophenol metabolism, which involve multiple possible inhibitory complexes (Fig. 4). All proposed inhibition mechanisms included formation of an inactive ESS complex due to substrate inhibition during the uninhibited steady-state reaction. Consideration of a second binding site for substrate also opened the door to possible catalytically active ESI complexes. Moreover, possible mechanisms included the potential for cooperative effects of binding between substrate and inhibitors. Contributions of the EII and EIS complexes (shown in gray in Fig. 4) were not measurable using the catalytic marker assay. Therefore, $K_{si,ap}$ and $K_{i,ap}$ are reported as apparent parameters that may include contributions from multiple possible complexes. The binding events were stoichiometrically ordered but the two binding sites were indistinguishable. In total there were ten possible mechanisms involving inhibition at one or two sites and the possibility of cooperativity from the substrate on inhibitor binding (Table 1). The DynaFit script file used for the inhibition model discrimination analyses is included in the Supplementary Data.

2.6 Assessment of hydrophobicity in binding and catalysis

We analyzed the importance of hydrophobicity in mediating binding interactions between CYP2E1 and the pyrazoles in this study. We employed Marvin, ChemAxon (Budapest, Hungary) to determine the log of the standard octanol-water partition coefficient (logP) for all pyrazoles, as a measure of their respective hydrophobicity. We plotted the resulting logP values against the corresponding log of either the dissociation constants from the binding studies or inhibitor constants from the catalytic studies using GraphPad Prism (San Diego, CA). Data were fit to a simple linear regression.

3. Results

3.1 Binding of pyrazole derivatives to one or two sites on CYP2E1

The association between CYP2E1 and pyrazoles resulted in a traditional Type II difference spectrum [18]. Only 3,5-dimethylpyrazole did not induce a spectral “shift despite concentrations as high as 1000 μ M. For all other pyrazoles, there was an increase in

absorbance near 430 nm and a concomitant decrease in absorbance near 390 nm as a function of ligand concentration. During this process, the CYP2E1 heme shifted from the high spin to the low spin state indicating the heme iron ligated to nitrogen. The titration data were plotted and fit to multiple possible single and two binding site mechanisms. Statistical discrimination among these possible mechanisms demonstrated that monocyclic pyrazoles bound to two sites, while bicyclic pyrazoles bound to one. The fits of the data to these mechanisms are shown in Fig. 5, Panels A to E.

Structural differences among the pyrazole molecules significantly impacted binding interactions with CYP2E1 (Fig. 1, Table 2). For the catalytic site, the presence and size of substituents on the pyrazole ring played a role in the affinity toward the catalytic site. Of the monocyclic pyrazole derivatives, pyrazole had the lowest affinity toward CYP2E1. The introduction of a methyl group at the C3 or C4 positions led to a significant 2- or notably a 50-fold increase in affinity, respectively. Despite the introduction of a larger, hydrophobic phenyl group at the C3 position, there was no increase in affinity relative to pyrazole. This observation contrasts with the improvement in binding when a methyl group is at that position, i.e. 3-methylpyrazole. An improvement in binding affinity was also observed when comparing tetrahydroindazole to pyrazole. The corresponding K_d values for those molecules differed by 800-fold. Aromatization of the second ring further increased the affinity toward CYP2E1 about five-fold, when comparing tetrahydroindazole to indazole. The number of rings also impacted binding stoichiometry. Both bicyclic compounds bound to one site, while pyrazole and 3-methyl pyrazole bound to a second site, albeit more weakly compared to 4-methylpyrazole.

3.2 Binding of structural analogs through one or two sites

We further explored the steric limitations of the catalytic site through spectral binding studies using pyrazole analogs as titrants. Like the pyrazoles, the imidazoles and triazoles formed spectral Type II complexes but at different orientations (Fig. 2, Table 2). Plots of the binding data for the structural analogs can be found in Supplementary Data, Fig. S.2. For imidazole, a modest (nearly two-fold) improvement of binding was observed with the shift of the nitrogen to the N3 position compared to pyrazole. Unlike the trend observed with pyrazole and indazole, the bicyclic benzimidazole bound with 500-fold weaker affinity despite its relatively higher hydrophobicity (Fig. 2). 1,2,3-Triazole bound very weakly compared to pyrazole. Fusion of the triazole ring to a benzene ring caused an increase in affinity, albeit more modest than that observed between pyrazole and indazole.

3.3 Inhibition of CYP2E1 metabolism through one or two sites

All monocyclic pyrazoles inhibited CYP2E1 metabolism of 4-nitrophenol through a two-site mechanism with the exception of 3,5-dimethylpyrazole, (Table 3). Similar to the binding studies, 3,5-dimethylpyrazole did not seem to interact with CYP2E1. For the uninhibited reaction, the kinetic parameters and corresponding 95% confidence intervals were the following: $k_{cat} = 38$ (35 to 41) min^{-1} , $K_s = 23$ (19 to 27) μM , $K_{ss} = 210$ (180 to 240) μM . These parameters were held constant during the analysis of the inhibition mechanisms among tested compounds. Unlike the other pyrazole compounds, there was no inhibition of the 4-nitrophenol reaction at a 3,5-dimethylpyrazole concentration as high as 250 μM (data not shown).

The other monocyclic pyrazoles inhibited CYP2E1 through different two-site inhibition mechanisms. Pyrazole inhibition of the 4-nitrophenol reaction was best explained through two equally probable mechanisms. In both mechanisms, pyrazole interacted with two CYP2E1 sites, forming binary EI complexes as well as ternary heterotropic ESI complexes, which are catalytically inactive. In the first mechanism, pyrazole had equal affinity for each

site, while in the second mechanism, pyrazole had a slightly higher affinity for the ES complex than the free enzyme ($K_{si,ap} < K_{i,ap}$). Alternatively, the mechanism which best-described inhibition of the reaction by 3-methylpyrazole was a two-site mechanism where 3-methylpyrazole had a two-fold higher affinity for the free enzyme than the ES complex. Unlike inhibition by pyrazole, the heterotropic ESI complex was catalytically active at a rate nearly five-fold lower than the uninhibited 4-nitrophenol reaction. 4-Methylpyrazole inhibited the reaction with the same mechanism as 3-methylpyrazole but with 10-fold higher affinity for each site. The fit of these data to their respective mechanisms is shown in Fig. 6, Panels A through C. Overall, the associations of pyrazole and 3-methylpyrazole to both sites were much weaker than that observed for 4-methylpyrazole. This observation was similar for both catalytic and cooperative sites. The presence of the methyl group significantly improved binding to CYP2E1 relative to pyrazole.

The introduction of a six-member ring could significantly improve inhibitor potency, but the location of the ring determined this effect. The fused bicyclic pyrazoles shared a common inhibition mechanism toward CYP2E1 activity as shown in Fig. 6, Panels E and F, and Table 3. Both indazole and tetrahydroindazole blocked activity through two-site mixed cooperative mechanisms, whereby the ESI complex was active at rates three- to six-fold lower than the uninhibited rate, respectively. By contrast, there were inhibition mechanism for 3-phenylpyrazole that had essentially equally probability; however, simple competitive inhibition was the only mechanism which had closed 95% confidence intervals; therefore, the other models were excluded. The competitive mechanism inhibition constant $K_{i,ap}$ was similar to the dissociation constant K_d observed in the binding assays. The fusion of the second ring to pyrazole significantly improved the ability to inhibit CYP2E1 metabolism. Indazole was more than 200-fold more potent than pyrazole, while tetrahydroindazole was 100-fold more potent. The differences in inhibition between these fused bicyclic compounds presumably reflect the loss in aromaticity in the second ring.

As an alternative method of analysis, the binding parameters obtained in the spectral studies were used to represent binding at the catalytic site ($K_{i,ap}$) and were held constant, while the other parameters ($K_{si,ap}$ and $k_{cat2,ap}$) were allowed to vary. This approach would minimize the degrees of freedom in possible mechanisms and possibly improve model discrimination analyses. Nevertheless, the combination of binding and catalytic data led to poorer fits of the data and wider confidence intervals including the failure to fit parameters altogether (data not shown). These findings suggest that dissociation constants obtained in spectral binding assays may not reflect inhibition constants in catalytic assays.

3.4 Importance of hydrophobicity in binding and catalysis

Hydrophobicity and other factors presumably play a role in the association of the assorted azoles to CYP2E1. We first explored this premise by focusing on the structural determinants contributing to spectral binding constants. These experiments yielded values for interactions at the catalytic site based on Type II binding difference spectra. The plot of the hydrophobicity of the pyrazoles ($\log P$) versus the log of the dissociation constant validated the importance of those contacts based on a significant linear correlation (K_d , R^2 0.92), although 3-phenylpyrazole was treated as an outlier (shown as diamonds in Fig. 7, Panel A). There was not a sufficient amount of data from the other azoles to perform a similar analysis; we included the data in the figure for comparative purposes. Interestingly, 1,2,3-triazole fell on the line with pyrazoles, while the others did not. Moreover, the fused bicycles, benzimidazole and benztriazole, clustered together away from the line for the pyrazoles and the related monocyclic compounds. Collectively, these findings suggest that hydrophobicity is an important common driver for binding of pyrazoles at the catalytic site, but its role for other azoles may be more complex.

Inhibition constants from catalytic studies recapitulated trends observed for binding at the catalytic site based on spectral binding studies and provided the first insights on the driving forces in associations at the cooperative site (Fig. 7 Panels B and C). As observed in binding studies, 3-phenylpyrazole was an obvious outlier from the trend in the inhibition constants observed for the other pyrazole compounds. The analysis of the remaining data yielded similar linear correlations for $K_{i,ap}$, (R^2 0.71) and $K_{si,ap}$, (R^2 0.72) with respect to hydrophobicity. In both cases, the inhibition constants for tetrahydroindazole fell off the line, and so we repeated the linear regression analysis without those data. The exclusion of data from 3-phenylpyrazole and tetrahydroindazole resulted in a significant improved in the linearity of the fit for the inhibition constants ($K_{i,ap}$, R^2 0.91, $K_{si,ap}$, R^2 0.97), suggesting that sterics and aromaticity are important modulators of binding for those compounds. Taken together, these findings indicate that mainly favorable partitioning into a hydrophobic environment drove the formation of complexes between these pyrazoles and CYP2E1; however, other interactions can override its importance on binding.

4. Discussion

In this study, we utilized effectively an array of ten azole compounds to further establish the relevance of cooperative mechanisms for CYP2E1 and identify the importance of molecular substituents on mediating those interactions. Type II spectral binding titrations indicated that two monocyclic azoles bound to CYP2E1, while only one binding event was observed for bicyclic azoles. These binding experiments yielded a weak signal for the monocyclic pyrazoles at the second site and thus minimal information on the impact of their structure on binding. Interaction at a second site was confirmed by steady-state kinetic studies wherein both 4-nitrophenol and the pyrazoles competed for CYP2E1 catalytic and cooperative sites. Their respective mechanisms were similar yet the corresponding apparent inhibition constants were different due to alterations in the substituents on the pyrazole ring. Unlike the binding studies, these studies provided critical insights on the selectivity of binding at the less-characterized cooperative site and its role in modulating CYP2E1 activity through the heterotropic cooperative interactions.

Spectral binding studies demonstrated that the selectivity of the catalytic site toward pyrazole compounds depended upon the presence and location of substituents on the aromatic ring. A single methyl group at either position 3 or 4 improved binding, albeit more significantly at the latter position. These effects correlated with the increase in hydrophobicity of the molecule suggesting that substituents mediate contacts with the hydrophobic interior of the catalytic site. A limit for these favorable contacts was demonstrated by the complete loss in binding upon introduction of a second methyl group for 3,5-dimethylpyrazole. Higher hydrophobicity than 3-methylpyrazole also added steric bulk that presumably prevented formation of the critical iron-nitrogen bond with active site heme, as evidenced by the lack of a Type II difference spectrum. The introduction of a second ring to the molecule improved binding but also potentially caused steric clashes. Fusion of a ring to pyrazole significantly increased affinity for CYP2E1. These binding interactions were not significantly affected by the presence or lack of aromaticity in the second ring. Rather, the correlation of the dissociation constants with hydrophobicity suggested the location of the second ring facilitates highly favorable contacts with hydrophobic amino acid residues at the sterically restricted catalytic site. Like 3,5-dimethylpyrazole, introduction of another substituent, phenyl ring in this case, to the 3 position significantly increased hydrophobicity while inducing steric clashes such that binding affinity toward CYP2E1 was similar to that for the simple pyrazole molecule. Taken together, these findings are consistent with previous reports on the importance of hydrophobicity in CYP2E1 binding of molecules [9–13, 17, 20] and expand insights to the steric constraints of the catalytic site.

Another important contributor to binding is formation of the iron-nitrogen bond between the active site heme and the ligand, and thus we utilized this property to control orientation of the molecule in the active site and further explore steric clashes. Pyrazole bound heme iron through the nitrogen at position 2, such that fusion of a benzene ring (indazole) extended the length of the molecule perpendicular to the heme as suggested by our docking studies [9, 17] and demonstrated later by the crystal structure of this complex [21]. By contrast, imidazole interacted with the heme through the nitrogen at position 3, which determined the impact of ring substituents on binding. Specifically, introduction of the benzene ring to the imidazole moiety created an elongated molecule that must bind parallel to the heme to form the iron-nitrogen bond and observed Type II difference spectra. This restriction caused a significant 50-fold drop in affinity between imidazole and benzimidazole toward CYP2E1. Given the small active site for this enzyme (volume ~190 Å³ [21]), there are likely steric clashes between benzimidazole and neighboring residues. Studies with 1,2,3-triazole and benzotriazole provided further evidence for this interpretation of the data. The 1,2,3-triazoles were capable of forming bonds to heme iron through the nitrogens at positions 2 and 3, like pyrazole and imidazole, respectively. In following the fusion of the benzene ring to the triazole lead to favorable, albeit slight, improvement in binding that was intermediate between the observed effects for the other azole pairs. The imidazole and triazole compounds were not included in subsequent catalytic inhibition studies due to their overall poor binding interactions with CYP2E1 and the lack of structural diversity compared to the pyrazoles.

For catalytic studies, we statistically analyzed a wider array of possible inhibition mechanism than used previously [9] to more accurately explain effects of the pyrazoles on CYP2E1 activity. Specifically, we employed ten inhibition mechanisms that incorporated all possible cooperative or non-cooperative effects on binding of substrate and inhibitor as well as their effects on substrate turnover. Almost all of the compounds shared a common mixed cooperative inhibition mechanism in which the pyrazoles competed with 4-nitrophenol for both sites and impacted the overall rate of substrate turnover. There were a couple of exceptions. 3,5-Dimethylpyrazole did not inhibit CYP2E1 activity, and coupled with results from binding studies, it seems the presence of both methyl groups on the pyrazole ring precluded any significant binding to the enzyme. In addition, 3-phenylpyrazole behaved as a simple competitive inhibitor. The ability of the other bicyclic inhibitors, namely indazole and tetrahydroindazole, to bind both sites was surprising. Previous studies using simpler inhibition mechanisms suggested indazole inhibits CYP2E1 through a simple (single site) competitive mechanism [9]. These observations indicate that CYP2E1 binding sites are likely more flexible than assumed but still sterically restricted. In this case, the fused bicyclic compounds can associate with both catalytic and cooperative sites, while separation of the rings (3-phenylpyrazole) favors only one binding event likely due to steric clashes from a more elongated molecular structure.

The impacts of pyrazoles on CYP2E1 activity substantiated findings from spectral experiments on binding at the catalytic site indicated by $K_{i,ap}$. Overall, the trends and affinities in binding parameters to the catalytic site were similar between those obtained from spectral titrations and those from catalytic studies, but there were notable exceptions. Pyrazole was a much weaker inhibitor than anticipated by binding studies; this observation may reflect the importance of the stabilizing iron-nitrogen bond formed in the spectral binding studies, which is severed during the cycling of iron through ferric and ferrous states during catalysis as described for a peptide inhibitor toward CYP3A4 [22]. In addition, indazole and tetrahydroindazole demonstrated higher inhibition constants than dissociation constants by spectral titration. These differences are presumably an experimental artifact, because the protein concentration in titration experiments had to exceed the predicted dissociation constant to obtain an observable signal and thus rapid equilibrium conditions

were not met. Nevertheless, variations in inhibition constants among the pyrazoles demonstrated that the introduction of substituents on the pyrazole ring improved binding through more hydrophobic contacts at the catalytic site. As observed in binding studies, 3-phenylpyrazole was the only outlier presumably due to the additional contribution of steric clashes to form the binary CYP2E1 complex.

Importantly, these inhibition studies provided valuable information on interactions at the cooperative site toward CYP2E1 metabolic activity (Figure 8). At low concentrations, 4-nitrophenol associates with the CYP2E1 catalytic site to form catalytically competent complex leading to product formation (red arrow, Figure 8). Upon introduction of the pyrazoles, the inhibitors bound to the cooperative site (K_{si}) displaying mostly a slightly weaker affinity than that observed at the catalytic site. Apparently, presence of the 4-nitrophenol molecule in the catalytic site does not significantly impact pyrazole binding affinity, which is reasonable given the high hydrophobicity of the substrate. Hydrophobic interactions are then important in binding at both the cooperative and catalytic sites. When pyrazoles are bound at the cooperative site, there is a significant change in the metabolic flux of substrate to product by CYP2E1. Unlike the catalytically inactive ternary complex with two 4-nitrophenol molecules bound, the presence of a bound ring-substituted pyrazole resulted in a mixed ternary complex that was catalytically active (red arrow, Figure 8). This mixed substrate-inhibitor complex rescues enzyme activity by creating a new metabolic pathway, such that the inhibitor actually increased the metabolic flux of substrate to product. This mechanism is not possible in the absence of overly simplistic noncooperative mechanisms. These findings for pyrazole inhibitors further support the importance of CYP2E1 cooperative mechanisms in altering the trajectory of metabolism of styrene through positive cooperativity [7] and 4-nitrophenol through inhibition by styrene metabolites [14].

It is conceivable that the CYP2E1 binding cavity could accommodate multiple compounds based on recent X-ray crystal structures with imidazolyl fatty acids bound to CYP2E1 [23]. The binding cavity is rather small, $\sim 190 \text{ \AA}^3$ when a single 4-methylpyrazole or indazole molecule is bound to the enzyme [21]. However, in the presence of fatty acid analogs, the volume doubles in size ranging from 420 to $473 \text{ \AA}^3$ depending on the size of the respective compound [23]. This transformation of the binding cavity is not due to a gross change in structure; rather, Phe298 reorientates to connect a void in the structure to the catalytic site, and thus form the larger space necessary to accommodate the fatty acid analogs. If such a process occurs with mono and bi-cyclic pyrazoles as well as other compounds, then more than one may bind CYP2E1 and impact catalysis as reported in this study.

Taken together, our use of an array of azole compounds for binding and catalytic studies has advanced an understanding of mechanisms regulating CYP2E1 interactions with small molecules and their impact on metabolism. Binding events depended on competing roles for hydrophobicity and steric clashes. The importance of these effects on interactions at the catalytic and cooperative sites was similar but not the same, indicating subtle differences in specificity between the sites. More studies are needed to better delineate these differing characteristics. Once bound to the cooperative site, inhibitors generally rescued CYP2E1 activity from substrate inhibition and thus improved overall metabolic efficiency. Such nontraditional impacts of substrate competitors, such as those in this study, provide a potentially important paradigm when considering the effects of mixtures of compounds on CYP2E1 activity. The investigation of other combinations of compounds, including pollutants, through strategies discussed herein would help establish the biological relevance of these mechanisms on understanding and predicting the role of CYP2E1 metabolism in detoxication and carcinogenic activation of compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- ▶ Azole binding and catalytic studies showed selectivity of catalytic and cooperative CYP2E1 sites.
- ▶ Activity rescued from substrate inhibition through mixed cooperative mechanism for pyrazoles.
- ▶ Adding methyl groups or secondary rings improved affinity for both sites due to hydrophobicity.
- ▶ Substituent location sometimes led to loss in binding, possibly due to steric clashes.

Fig. 1.

Ligands used in this study to probe CYP2E1 binding and activity toward monocyclic and bicyclic compounds. Shown below each structure is the octanol/water partition coefficient (LogP), calculated using ChemAxon software.

pyrazole
LogP=0.28

imidazole
LogP=-0.15

1,2,3-triazole
LogP=-0.10

indazole
LogP=1.30

benzimidazole
LogP=1.26

benzotriazole
LogP=1.30

Fig. 2.
Structural analogs of pyrazole compounds used to further elucidate differences observed in CYP2E1 binding of monocyclic and bicyclic pyrazoles. Shown below each structure is the octanol/water partition coefficient (LogP), calculated using ChemAxon software.

Fig. 3.

Possible CYP2E1 binding modes for azoles. E = CYP2E1; L = Ligand

Model 1: Single-site inhibition

Model 2: Cooperative (two-site) inhibition

Fig. 4.

Possible mechanisms for inhibition of CYP2E1 4-nitrophenol activity by pyrazole compounds. Uninhibited reaction shown in bold; possible but undetectable complexes shown in gray. E = CYP2E1; S = 4-nitrophenol; P= product (4-nitrocatechol); I = pyrazole compounds.

Fig. 5. CYP2E1 binding data for pyrazole (Panel A), 3-methylpyrazole (Panel B), 4-methylpyrazole (Panel C), 3-phenylpyrazole (Panel D), and tetrahydroindazole Panel E). For these experiments, 0.1 μM CYP2E1 in 50 mM potassium phosphate, pH 7.4, 20 μM dilauroyl-L-phosphatidylcholine was titrated against increasing amounts of ligand to 1200 μM at 25 $^{\circ}\text{C}$ and the corresponding spectral changes recorded from 350 to 475 nm. The results from 4 to 6 experiments were compiled and averaged for analyses. Data were then fit to the most probable inhibition mechanism as identified by DynaFit software [19] and listed in Table 2.

Fig. 6. Inhibition of CYP2E1 metabolism of 4-nitrophenol by pyrazole (Panel A), 3-methylpyrazole (Panel B), 4-methylpyrazole (Panel C), 3-phenylpyrazole (Panel D), tetrahydroindazole (Panel E), and indazole (Panel F). For reactions, 25 nM CYP2E1 was reconstituted with 100 nM CPR-K56Q and 50 nM cytochrome b_5 in 50 mM potassium phosphate, pH 7.4, 20 μM dilauroyl-L- α -phosphatidylcholine, 4-nitrophenol (varied from 5 to 750 μM), 2 units μl^{-1} catalase, 0.04 $\mu\text{g} \mu\text{l}^{-1}$ superoxide dismutase, and an NADPH-regenerating system (2 $\mu\text{U} \mu\text{l}^{-1}$ glucose-6-phosphate dehydrogenase, 10 mM glucose 6-phosphate, 2 mM MgCl_2 , 500 μM NADP^+). Open circles and dashed lines represent the uninhibited reactions fit to a substrate inhibition mechanism, as reported previously [9,14]. Darker shading of data correlate with

higher concentrations of each inhibitor and the corresponding fit to the most probable inhibition mechanism, as identified by DynaFit software [19] and listed in Table 3. Reported initial rates reflect averages of data from 2 to 4 experiments.

Fig. 7. Contributions of hydrophobicity to pyrazole binding interactions with CYP2E1. (Panel A) Log of K_d from binding studies (Table 2) plotted against log of partition coefficients for pyrazoles (diamonds) and pyrazole analogs (circles). Data for pyrazole (PYR), 3-methylpyrazole (3MP), 4-methylpyrazole (4MP), tetrahydroindazole (THI) and indazole (IND) shown as black diamonds and fit to a linear regression. 3-Phenylpyrazole (3PP), open diamond, treated as an outlier. (Panel B) Log of K_i from catalytic studies (Table 3) plotted against log of partition coefficients for pyrazoles. (Panel C) Log of K_{si} from catalytic studies (Table 3) are plotted against the log of the partition coefficients for all pyrazoles. For panels B and C, data for pyrazole (PYR), 3-methylpyrazole (3MP), 4-methylpyrazole (4MP), and indazole (IND), shown in black diamonds, and fit to a linear regression. Tetrahydroindazole (THI) and 3-phenylpyrazole (3PP, Panel B only), shown as open diamonds, were treated as outliers, as discussed in Section 3.4 of Results.

Fig. 8.

Stylized depiction of the cooperative mechanism for inhibition of CYP2E1 metabolic reactions by pyrazoles. The reaction begins when 4-nitrophenol substrate binds to the CYP2E1 catalytic site as indicated by the red heme-iron group to form an active binary complex. Upon substrate oxidation (red arrow), product is released to regenerate free enzyme. At higher 4-nitrophenol concentrations, a second substrate molecule binds the enzyme-substrate complex, forming a catalytically inactive complex. Alternatively, a cooperative inhibitor, such as pyrazole compounds used in this study, can bind either free enzyme or the enzyme-substrate complex to form a binary or (possibly active, red arrow) ternary complex. This mechanism illustrates some of the different types of cooperativity observed in this study that can occur in the presence of mixtures of compounds.

Table 1

Possible Models for Inhibition of CYP2E1

Model	Complexes Formed	Cooperative Effects?	Possible Active ESI Complex? ^a	Parameters and Consequences ^a
Competitive	ES, ESS, EI	No	No	$K_s, K_{ss}, K_i, k_{cat}$
Uncompetitive	ES, ESS, ESI	No	Yes	$K_s, K_{ss}, K_{si}, k_{cat1}, k_{cat2}?$
Mixed	ES, ESS, EI, ESI	Yes	Yes	$K_s, K_{ss}, K_i, K_{si}, k_{cat1}, k_{cat2}?$
Noncompetitive	ES, ESS, EI, ESI	No	Yes	$K_s, K_{ss}, K_i=K_{si}, k_{cat1}, k_{cat2}?$

^aIn all models except competitive, ESI may be active or not, hence k_{cat2} . Both possibilities were included in mode I discrimination studies.

Table 2CYP2E1 binding parameters for azoles^a

Ligand	K _{d1} (μM)	K _{d2} (μM)
<i>Monocyclic pyrazoles</i>		
pyrazole	19 (15 to 24)	6000 (3700 to 12000)
3-methylpyrazole	11 (9.0 to 13)	1700 (1000 to 3800)
4-methylpyrazole ^b	0.36 (0.084 to 0.59)	0.67 (0.45 to 1.1)
3,5-dimethylpyrazole	-	-
<i>Bicyclic pyrazoles</i>		
3-phenylpyrazole	22.0 (19 to 24)	-
indazole ^c	0.005 (0.002)	-
tetrahydroindazole	0.023 (0.0072 to 0.047)	-
<i>Monocyclic pyrazole analogs</i>		
imidazole	11 (2.8 to 31)	120 (76 to 330)
1,2,3-triazole	330 (280 to 390)	-
<i>Bicyclic pyrazole analogs</i>		
benzimidazole	550 (430 to 720)	-
benzotriazole	150 (110 to 210)	-

^a99% confidence intervals shown in parentheses for binding constants^bStudies carried out using free form of compound instead of salt as reported previously [9]^cBinding constant with standard error reported previously[9]

Table 3Inhibition parameters for pyrazoles toward CYP2E1 oxidation of 4-nitrophenol^a

Inhibitor	Inhibition Mechanism ^b	K _{d1} (μM)	K _{d2} (μM)	k _{cat2} (min ⁻¹)
<i>Monocyclic pyrazoles</i>				
pyrazole	Mixed, coop. substrate	140 (72 to 520)	98 (77 to 131)	-
3-methylpyrazole	Mixed, coop. substrate	11 (8.3 to 16)	21 (13 to 35)	8.7 (7.0 to 10)
4-methylpyrazole	Mixed, coop. substrate	0.93 (0.72 to 1.2)	3.1 (2.1 to 5.1)	7.3 (6.0 to 8.7)
3,5-dimethylpyrazole ^c	-	-	-	-
<i>Bicyclic pyrazoles</i>				
3-phenylpyrazole	Competitive	19 (15 to 25)	-	-
indazole	Mixed, coop. substrate	0.13 (0.10 to 0.18)	0.35 (0.21 to 0.66)	11 (9.0 to 13)
tetrahydroindazole	Mixed, coop. substrate	1.0 (0.70 to 1.6)	2.7 (1.4 to 7.7)	6.6 (2.3 to 9.6)

^a95% confidence intervals shown in parentheses^bConsult Table 1 and Scheme 2^cNo inhibition was observed for 3,5-dimethylpyrazole