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RESEARCH ARTICLE

Cooperative effects for CYP2E1 differ between styrene and its metabolites

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Abstract

1. Cooperative interactions are frequently observed in the metabolism of drugs and pollutants by cytochrome P450s; nevertheless, the molecular determinants for cooperativity remain elusive. Previously, we demonstrated that steady-state styrene metabolism by CYP2E1 exhibits positive cooperativity.
2. We hypothesized that styrene metabolites have lower affinity than styrene toward CYP2E1 and limited ability to induce cooperative effects during metabolism. To test the hypothesis, we determined the potency and mechanism of inhibition for styrene and its metabolites toward oxidation of 4-nitrophenol using CYP2E1 Supersomes® and human liver microsomes.
3. Styrene inhibited the reaction through a mixed cooperative mechanism with high affinity for the catalytic site (67 μM) and lower affinity for the cooperative site (1100 μM), while increasing substrate turnover at high concentrations. Styrene oxide and 4-vinylphenol possessed similar affinity for CYP2E1. Styrene oxide behaved cooperatively like styrene, but 4-vinylphenol decreased turnover at high concentrations. Styrene glycol was a very poor competitive inhibitor. Among all compounds, there was a positive correlation with binding and hydrophobicity.
4. Taken together, these findings for CYP2E1 further validate contributions of cooperative mechanisms to metabolic processes, demonstrate the role of molecular structure on those mechanisms and underscore the potential for heterotropic cooperative effects between different compounds.

Keywords

Allostery, cooperativity, CYP2E1, inhibition, P450 2E1, styrene

History

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Introduction

Oxidation by cytochrome P450 enzymes is the primary metabolic pathway for many xenobiotic compounds including drugs and pollutants and thus, identifying suitable models for interpreting and predicting those metabolic reactions is critical to advancing pharmacological and toxicological research (Guengerich, 2007). In many cases, the Michaelis–Menten mechanism explains reported kinetic profiles and provides parameters for assessing the metabolic clearance of compounds. Nevertheless, it has become increasingly clear that P450 metabolism of compounds can be more complex involving homo- and heterotropic cooperative (allosteric) interactions. In fact, these non-Michaelis–Menten mechanisms may be more of the rule than the exception for many P450s especially CYP1A2 (Isin et al., 2008; Miller & Guengerich, 2001), CYP2C9 (Liu et al., 2005; Řemínek & Glatz, 2010) and CYP3A4 (Kapelyukh et al., 2008; Roberts et al., 2011; Woods et al., 2011), as extensively reviewed elsewhere (Atkins, 2006; Denisov et al., 2009; Niwa 2008). Consequently, identification of the underlying mechanisms for these processes is necessary for establishing a biochemical explanation for their biological impacts on drug and pollutant metabolism.

Although less characterized than other P450s, CYP2E1 is similarly capable of metabolizing small drugs and pollutants through cooperative mechanisms. Aromatic and heterocyclic compounds inhibit CYP2E1 activity through uncompetitive and mixed inhibition mechanisms, which require two binding sites for substrate and inhibitor (Collom et al., 2008; Hargreaves et al., 1994). The kinetic profiles for several CYP2E1 substrates demonstrate positive cooperativity such as styrene, *m*-xylene and 7-ethoxycoumarin (Hill coefficients 1.7, 1.4 and 1.6, respectively) (Harrelson et al., 2008; Hartman et al., 2012; Spatzenegger et al., 2003). Similarly, the kinetic profile for CYP2E1 *O*-deethylation of phenacetin deviates from linearity at low substrate concentrations,

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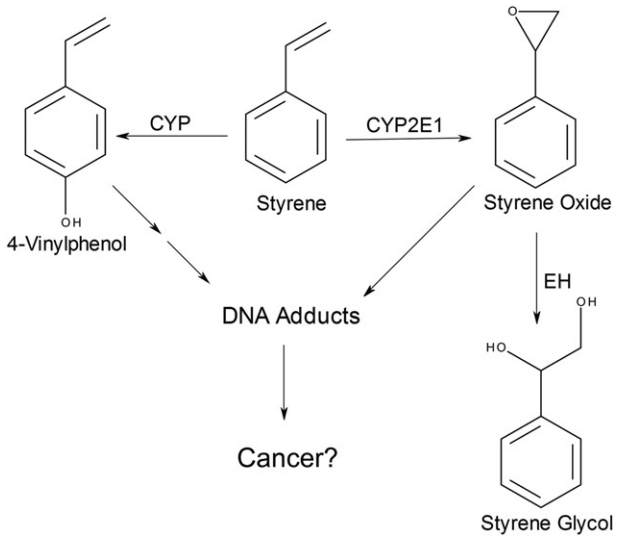

Figure 1. Metabolic pathways for styrene.

although the authors did not investigate the possibility of cooperativity (Venkatakrishnan et al., 1998). Unlike those substrates, the metabolism of 4-nitrophenol is not sigmoidal, and instead displays substrate inhibition (negative cooperativity) for this commonly used marker substrate for CYP2E1 (Koop, 1986). We were the first to provide a mechanistic explanation for the 4-nitrophenol kinetic profile through a cooperative mechanism for CYP2E1. Low concentrations favor 4-nitrophenol binding to a catalytic CYP2E1 site and undergoing oxidation to 4-nitrocatechol. At higher concentrations, 4-nitrophenol binds to a second, cooperative effector site, which leads to a catalytically inactive CYP2E1 complex (Collom et al., 2008). Similarly, homotypic negative cooperative effects for aniline were investigated through molecular dynamics, whereby binding of a second substrate molecule to a cooperative effector site led to reorientation of crucial active site residues resulting in negative cooperativity (Li et al., 2011). Despite insight into some cooperative interactions, the molecular determinants that favor Michaelis–Menten or cooperative metabolism of compounds by CYP2E1 still remain unknown.

An investigation of styrene and its metabolites provides an excellent opportunity to probe the role of structure in favoring occupancy of CYP2E1 catalytic and cooperative sites. Styrene is a common industrial pollutant and potential carcinogen (Barale, 1991; Bond & Bolt, 1989; National Toxicology Program, 2008). Styrene is oxidized primarily by CYP2E1 to the genotoxin styrene oxide and, to a lesser extent, 4-vinylphenol (Figure 1; Bond & Bolt, 1989). Styrene oxide then undergoes hydrolysis to the less toxic metabolite 1,2-phenylethanediol (styrene glycol) through action catalyzed by epoxide hydrolase. Our studies with CYP2E1 Supersomes and human liver microsomes demonstrated that steady-state metabolism of styrene by CYP2E1 exhibits positive cooperativity (Hartman et al., 2012). At low styrene concentrations, weak styrene binding to CYP2E1 leads to inefficient oxidation of the pollutant. As the styrene concentration increases, a second molecule binds to CYP2E1 causing an increase in affinity for substrate and subsequent increase in metabolic efficiency. The transition between the catalytic cycles results in the observed positive cooperativity in the kinetic profile and occurs within reported styrene concentrations in the blood of workers exposed to this pollutant, and thus this CYP2E1 mechanism may be relevant *in vivo*. During oxidative metabolism of styrene, the molecule becomes more polar and increases in steric bulk. These changes in structure conflict with the known preference for CYP2E1 to bind hydrophobic compounds without steric hindrance (Backes et al., 1993; Miller, 2008; Porubsky et al., 2008). Therefore, we hypothesize that oxidative products of styrene metabolism possess decreased affinity toward CYP2E1 and are unable to induce cooperative effects during metabolism.

We tested this hypothesis by identifying the inhibitory potency and mechanism for styrene metabolites using CYP2E1 Supersomes and human liver microsomes pooled from 150 donors as a model for liver metabolism (Crespi, 2009). We chose oxidation of 4-nitrophenol as a reporter reaction for these inhibition studies to avoid interference between styrene metabolites as inhibitors and those generated during styrene metabolism. Moreover, 4-nitrophenol binds to catalytic and cooperative sites and thus its metabolism is sensitive to inhibitory styrene metabolites binding to both sites. We initially screened the inhibitory potential of styrene metabolites through IC_{50} studies. Those results were used to design and carry out subsequent steady-state kinetic inhibition studies with the styrene metabolites. Inhibition kinetic profiles were fit globally to 10 different possible mechanisms to identify the most probable one involving binding at catalytic and/or cooperative sites. The role of hydrophobicity in driving these interactions was determined through correlative analysis between the partition coefficient ($\log P$) of the compounds and the corresponding apparent binding constants. Taken together, these approaches demonstrate oxidative metabolites of styrene possess altered affinity and mechanism of interaction with CYP2E1 and thus, provide insights toward the significance of molecular structure in cooperativity during CYP2E1 catalysis of metabolic reactions.

Materials and methods

Materials

All chemicals used in this study were ACS grade or higher. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): styrene (substrate), styrene glycol (product), benzyl alcohol (internal standard), 4-nitrophenol (substrate), 4-nitrocatechol (product), 4-nitroanisole (internal standard) and most inhibitors (8,9-styrene oxide, (R)-1,2-phenylethanediol and (S)-1,2-phenylethanediol. The inhibitors (R/S)-1,2-phenylethanediol and 4-vinylphenol were purchased from Fisher Scientific (Wilmington, MA). Methanol (100%) was purchased from AAPER (Shelbyville, KY). Pooled human liver microsomes (HLM150s) and human CYP2E1 (Supersomes®) were purchased from BD Biosciences (San Jose, CA). Supersomes are recombinant cDNA-expressed human cytochrome P450 enzyme prepared from the baculovirus-infected insect cell system. These insect cell microsomes contain supplemental cDNA-expressed human reductase and cytochrome b5.

IC_{50} studies to determine inhibitory potential of compounds

We assessed the specific interactions between styrene and its metabolites and CYP2E1 through IC_{50} studies using the oxidation of 4-nitrophenol to 4-nitrocatechol as a marker reaction for CYP2E1 (Koop, 1986). Either 25 nM CYP2E1 Supersomes or 0.25 mg/mL protein for HLM150s was incubated with 50 μM 4-nitrophenol and at least seven concentrations of each inhibitor (plus a negative control with solvent only) in 50 mM potassium phosphate pH 7.4, at 37 °C. Because of solubility, all inhibitor stocks were prepared in methanol. All reactions contained a final concentration of 1% methanol. Reactions were initiated upon addition of 1 mM NADPH and after 30 min, quenched with an equal volume of 0.4 N perchloric acid containing the internal standard (25 μM 4-nitroanisole). Quenched reactions were centrifuged and the resulting supernatant analyzed by HPLC to quantify formation of the 4-nitrocatechol product formed. Each experiment was performed in at least three independent experimental replicates. Under these experimental conditions, product formation was linear with respect to time.

HPLC analysis of 4-nitrophenol oxidation by CYP2E1

We improved a previously published in-house HPLC method to analyze 4-nitrophenol reactions (Collom, 2007). For this study, we employed a different column to maintain resolution of compounds, while eliminating the use of acetonitrile and decreasing run times. Samples were injected onto a Waters Breeze HPLC system and resolved using a 4.6×150 mm Zorbax Eclipse 5 μm XDB-C18 column (Agilent, Santa Clara, CA) heated to 45 °C under isocratic conditions (40:60 0.1% acetic acid/H_2O:methanol) at a flow rate of 1.2 mL/min. The elution of 4-nitrocatechol (product), 4-nitrophenol (substrate) and 4-nitroanisole (internal standard) were monitored at 360 nm. Final 4-nitrophenol concentrations were calculated from the ratio of product area to internal standard area in the sample and compared to calibration curves as determined with authentic standards.

Analysis of IC_{50} data

For each set of inhibition reactions, the corresponding rates of product formation were normalized relative to the control rate in the absence of inhibitor and presence of 1% methanol. These data were plotted as a function of the log of the inhibitor concentration and fit to the standard IC_{50} equation using GraphPad Prism 4.0 (San Diego, CA). The corresponding IC_{50} value was used to select metabolite concentrations for subsequent inhibition kinetic studies.

Assessing CYP2E1 inactivation by styrene oxide

Styrene oxide may reversibly interact with CYP2E1 binding sites and/or react with the enzyme to alter activity. Consequently, we assessed the significance of styrene oxide-mediated inactivation of CYP2E1 by measuring residual activity following pre-incubation of the enzyme with the metabolite. For these studies, 667 nM CYP2E1 Supersomes was incubated with 1.3 mM NADPH and 0, 18.3, 55 and 165 μM styrene oxide in 50 mM potassium phosphate pH 7.4, at 37 °C. Due to low solubility in water, all styrene, styrene oxide and 4-vinylphenol stocks were prepared in methanol. As in the inhibition studies, all reactions contained a final concentration of 1% methanol. After 15 min of incubation, the reactions were diluted 30-fold in 50 mM potassium phosphate, pH 7.4. A second reaction was then carried out with the diluted reaction mixtures containing 22 nM CYP2E1 and 50 μM 4-nitrophenol. Reactions were initiated upon addition of 1.3 mM NADPH and after 30 min, quenched with an equal volume of 0.4 N perchloric acid containing internal standard (25 μM 4-nitroanisole). Quenched reactions were centrifuged and the resulting supernatant analyzed by HPLC to quantify formation of the 4-nitrocatechol product. Each experiment was performed in at least three independent experimental replicates. Under these experimental conditions, product formation was linear with respect to time.

Inhibition kinetics for styrene and its metabolites toward CYP2E1 activity

We determined the inhibition mechanisms for styrene and its metabolites toward CYP2E1 to elucidate their mode of interaction with the enzyme and subsequent effect on activity. For these reactions, either 25 nM CYP2E1 Supersomes or 0.25 mg/mL protein for HLM150s was incubated with 25, 50, 100, 500, 1000 and 2000 μM 4-nitrophenol in 50 mM potassium phosphate pH 7.4, at 37 °C. Each set of reactions for a particular inhibitor was carried out in the presence of at least three different inhibitor concentrations, along with a set without inhibitor as a negative control. Reactions were performed, quenched, and analyzed by HPLC, as described for the IC_{50} studies. Each experiment was performed in at least four independent experimental replicates.

The resulting kinetic profiles for styrene and its metabolites were analyzed globally to identify the most probable inhibition mechanism toward 4-nitrophenol oxidation by CYP2E1 using DynaFit software version 3.28 (Biokin Ltd, Watertown, MA) (Kuzmic, 1996). In order to limit the number of variable parameters during the analysis of the inhibition data, the uninhibited steady-state mechanism and parameters were first obtained. 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 the uninhibited 4-nitrophenol reaction. The DynaFit script file used for analysis of the uninhibited data is included in ''Supplementary material'', with the model discriminations shown in Table S2a and b. As described previously for the reconstituted rabbit CYP2E1 system (Collom et al., 2008), a substrate inhibition mechanism best described the metabolism of 4-nitrophenol by CYP2E1 Supersomes and HLM150. For each enzyme system, a plot of the uninhibited kinetic profile as well as a table of parameters for the uninhibited reactions is also included in ''Supplementary material'' (Figure S1 and Table S1). These parameters were held constant and the inhibitory parameters (K_i, K_{si} and k_{cat2}) treated as variables.

For analysis of the inhibition experiments, data were fit to one or two-site inhibition mechanisms for 4-nitrophenol

metabolism, which involves multiple possible inhibitory complexes (Figure 2). Although the models varied in complexity (and number of corresponding parameters), simpler models were favored over more complex mechanisms in the Dynafit statistical analysis. The proposed inhibition mechanisms are based on traditional Michaelis–Menten mechanisms (competitive, uncompetitive, noncompetitive and mixed), but necessarily included formation of an inactive ESS complex due to known substrate inhibition during the uninhibited steady-state reaction. Consideration of a second substrate-binding site also opened the door to formation of ESI complexes, which may be catalytically active. In addition to those possibilities, other mechanisms included the potential for cooperative effects on binding between substrate and inhibitors. Contributions of the EII and EIS complexes (shown in gray in Figure 2) were not measurable using the catalytic marker assay. Therefore, $K_{si,ap}$ and $K_{i,ap}$ are designated as apparent parameters that may include contributions from multiple possible complexes. 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 material''.

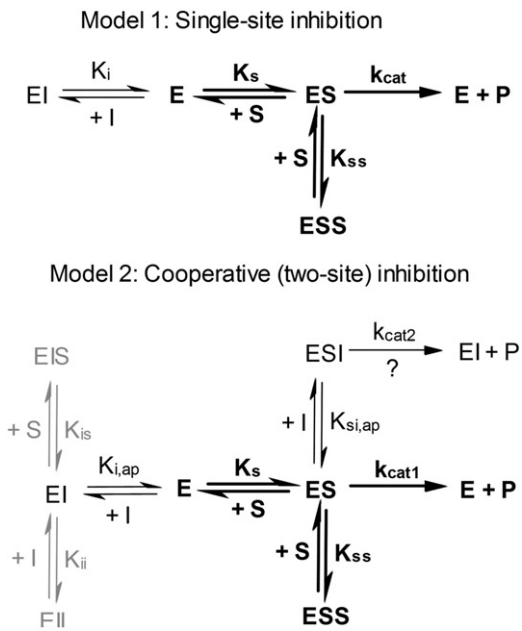

Figure 2. Possible inhibition mechanisms for CYP2E1 4-nitrophenol activity by styrene and metabolites. Uninhibited reaction shown in bold; possible but undetectable complexes shown in gray. E = CYP2E1; S = 4-nitrophenol; P = product (4-nitrocatechol) and I = styrene and metabolites.

Assessment of hydrophobicity in binding to the CYP2E1 catalytic site

We analyzed the importance of hydrophobicity in mediating binding interactions between CYP2E1 and the styrene derivatives as well as 4-nitrophenol. We used parameters obtained from the preferred model describing binding of either substrate or inhibitor to the enzyme catalytic site. We employed Marvin software, ChemAxon (Budapest, Hungary) to determine the log of the standard octanol-water partition coefficient ($\log P$) for all compounds, as a measure of their respective hydrophobicity. We plotted the resulting $\log P$ values against the corresponding log of either the inhibition constants (K_i) or substrate binding constants (K_s) from the catalytic studies using GraphPad Prism (San Diego, CA). Data were fit to a simple linear regression.

Results

IC_{50} values for styrene and its metabolites reveal inhibitory potential

We screened the inhibitory potential of styrene and its metabolites through IC_{50} studies using 4-nitrophenol as the marker reaction (Table 2 and Figure 3). For CYP2E1 Supersomes and HLM150, styrene, 4-vinylphenol and styrene oxide had similar and potent inhibitory properties. Styrene glycol was a very poor inhibitor, approximately 300-fold above styrene and the other two metabolites. There was almost no residual activity remaining at high concentrations of these compounds with CYP2E1 Supersomes. With HLM150, somewhat greater residual activity remained at high concentrations.

Styrene oxide inhibition of CYP2E1 is reversible

We tested the reversibility of styrene oxide inhibition of recombinant CYP2E1 activity by using 4-nitrophenol oxidation as a reporter reaction following incubation with the epoxide metabolite. Even at levels three-fold higher than the measured IC_{50} for styrene oxide, the enzyme retained all of its initial activity (Figure 4). The negative control (without any styrene oxide present) had slightly less activity than the incubations with styrene oxide, suggesting a slight increase in the stability of CYP2E1 when a ligand was available to occupy the catalytic site as reported for other compounds (Roberts et al., 1995; Song et al., 1989).

Styrene and its metabolites interact with two CYP2E1 sites during 4-nitrophenol metabolism by recombinant enzyme.

Through kinetic studies with CYP2E1 Supersomes and HLM150s, we determined the mechanism of CYP2E1

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}?

[a]In all models except competitive, ESI may be active or not, hence k_{cat2}. Both possibilities were included in model discrimination studies.

Table 2. IC_{50} values for styrene and its metabolites.[a]

Metabolite	Recombinant CYP2E1		HLM150	
	IC_{50} (μM)	Residual activity (%)	IC_{50} (μM)	Residual activity (%)
Styrene	75 (54–95)	11 (5.2–16)	45 (33–58)	20 (16–24)
Styrene oxide	55 (35–75)	13 (5.1–20)	150 (24–280)	28 (12–44)
4-Vinylphenol	24 (15–24)	16 (8.7–23)	38 (26–50)	19 (12–26)
R-Styrene glycol	11 000 (6200–15 000)	9.7 (0.0–21)	12 000 (7500–16 000)	26 (19–32)
S-Styrene glycol	15 000 (9200–22 000)	5.6 (0.0–17)	15 000 (10 000–20 000)	11 (2.7–18)
R/S-Styrene glycol	13 000 (2800–23 000)	9.2 (0.0–24)	14 000 (6600–21 000)	18 (6.5–29)

[a]The nonsymmetrical 95% confidence intervals for parameters are shown in parentheses.

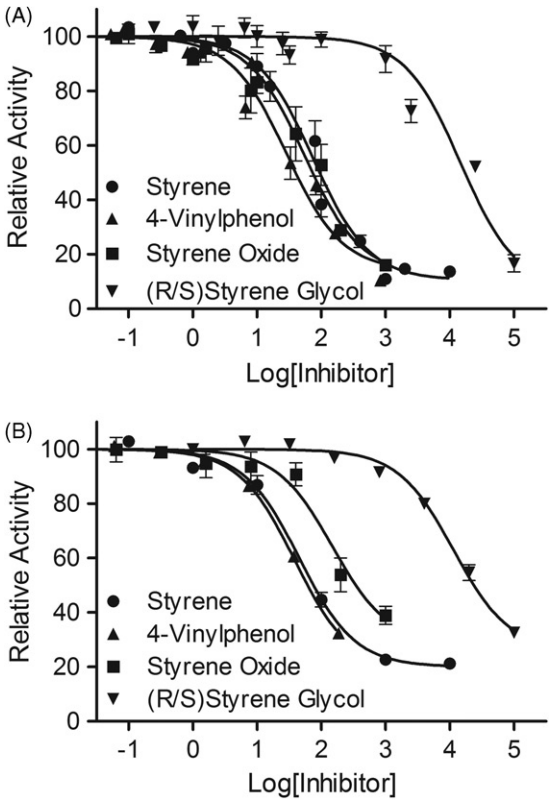

Figure 3. IC_{50} plots for styrene metabolites with CYP2E1 Supersomes (Panel A) and HLM150 (Panel B). Nonlinear plots represent the IC_{50} nonlinear regression for the normalized activity versus log of inhibitor concentration in micromolar. Individual tracings represent different metabolites. Reactions were performed at least four times at 37 °C and pH 7.4. Further reaction conditions are described in Experimental Procedures.

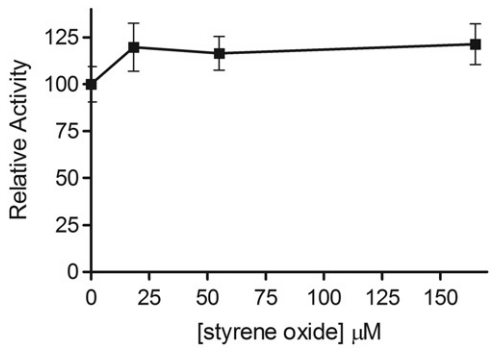

Figure 4. Residual CYP2E1 activity after preincubation with styrene oxide. For reactions, 670 nM CYP2E1 and 1.3 mM NADPH were incubated with 0, 18.3, 55 and 165 μM styrene oxide at 37 °C and pH 7.4 for 15 min. Following a 30-fold dilution, the residual activity was assayed at a final concentration of 22 nM CYP2E1 with 50 μM 4-nitrophenol and 1.3 mM NADPH. The reported values represent the average of three experimental replicates, including the mean ± standard deviation. Further reaction conditions are described in Experimental Procedures.

inhibition by styrene and its metabolites toward oxidation of 4-nitrophenol. As a foundation for these studies, data for the uninhibited reaction were plotted for CYP2E1 Supersomes and HLM150 and the corresponding parameters determined (Figure S1 and Table S1). The inhibition data for each compound were fit to ten possible mechanisms for inhibition of the oxidation of 4-nitrophenol, generalized in Figure 2 and detailed in Table 1. In these mechanisms, binding of inhibitor can either lead to formation of an EI complex, an ESI complex, or both. If both complexes form, heterotropic cooperative interactions may occur in the form of bound substrate affecting binding of the inhibitor molecule. Alternatively, cooperative effects on turnover can be observed; the substrate 4-nitrophenol is known to form an inactive ESS complex, so ESI complexes may be inactive or active. If active, the ESI complex may turn over product at the same or differing rates from the uninhibited reaction. A statistical analysis was performed to determine the most probable mechanism according to the AIC (Akaike Information Criteron). The resulting statistical outputs are included in Tables S3(a)–(d) and S4(a)–(d).

In the studies with CYP2E1 Supersomes (Table 3), the inhibitory potency was strongest for styrene and 4-vinylphenol, slightly weaker for styrene oxide, and very weak for styrene glycol, although the compounds interacted with CYP2E1 through differing mechanisms (Figure 5). Styrene inhibition of the reaction was best explained through two mixed cooperative mechanisms (Table S3a) that were essentially equally probable. In these mechanisms, inhibitor can bind to either free enzyme or the enzyme-substrate complex (Figure 2). Similar to the inhibitory mechanism for 4-methylpyrazole (Hartman et al., 2012), substrate was a cooperative effector on inhibitor binding. However, unlike that interaction, bound 4-nitrophenol had a negative cooperative effect on styrene binding, as reflected by a 10-fold higher $K_{si,ap}$ relative to $K_{i,ap}$. In both preferred mechanisms, the ESI complex was active; in one case, the k_{cat} of turnover from the ESI complex was greater than that of the uninhibited reaction ($k_{cat2} > k_{cat1}$). In the other case, the two reaction rates were equal. However, in the first case,

Table 3. Apparent inhibition parameters for styrene metabolites toward recombinant CYP2E1 activity.[a,b]

Inhibitor	LogP	Mechanism	k_{cat2}[c]	$K_{i,ap}$ (μM)	$K_{si,ap}$ (μM)
Styrene	2.71	Mixed cooperative substrate	19 (17–21)	67 (56–80)	1100 (700–2000)
Styrene oxide	1.74	Mixed cooperative substrate	19 (17–21)	110 (76–170)	1000 (450–10 000)
4-Vinylphenol	2.41	Mixed cooperative substrate	–	47 (33–72)	230 (120–1500)
R/S-Styrene glycol	0.58	Competitive	–	29 000 (19 000–52 000)	–

[a]Figure 2 depicts reaction mechanism used to determine kinetic parameters. Nonsymmetrical 95% confidence intervals for parameters are shown in parentheses.
[b]Parameters for uninhibited 4-nitrophenol oxidation by recombinant CYP2E1 were K_s 290 μM, K_{ss} 1700 μM and k_{cat1} 19 nmol/min/nmol CYP2E1.
[c]Units are nmol/min/nmol CYP2E1.

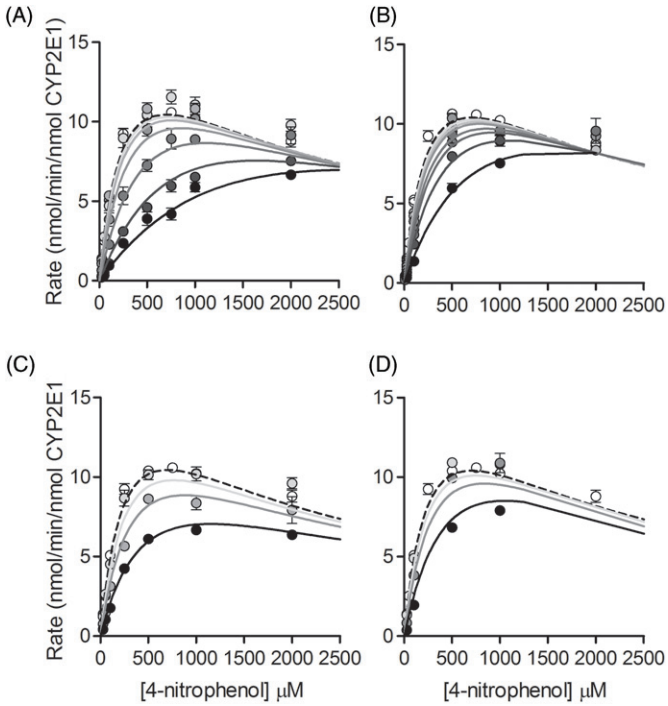

Figure 5. CYP2E1 Supersomes inhibition kinetic profiles for styrene and its metabolites toward oxidation of 4-nitrophenol. The dashed line and open circles represent the uninhibited reaction; increasing inhibitor concentration is indicated by darker lines and markers. Specific inhibitors include (A) 0, 3.7, 11, 33, 99, 300 and 890 μM styrene; (B) 0, 18, 55, 170 and 500 μM styrene oxide; (C) 0, 8.7, 26 and 78 μM 4-vinylphenol or (D) 0, 4000, 12 000 and 36 000 μM styrene glycol. For reactions, 25 nM CYP2E1, varying 4-nitrophenol concentrations, and 1 mM NADPH were incubated at 37 °C and pH 7.4 (Experimental Procedures). The reported values reflect the average of four experimental replicates, including the mean ± standard deviation. Data were fit to the most probable mechanism listed in Table 3, which were identified among 10 possibilities (Table 1) using DynaFit software version 3.28 (Biokin Ltd) (Kuzmic, 1996).

the confidence intervals were open and thus, we could not properly fit the parameters. Thus, there is essentially one preferred mechanism for styrene inhibition of 4-nitrophenol oxidation by CYP2E1.

Inhibition induced by styrene oxide was best explained through a mechanism similar to that observed for styrene. There was an equal probability of two different mixed inhibition mechanism in which 4-nitrophenol (substrate) acted as a negative cooperative effector on styrene oxide binding ($K_{si,ap} >> K_{i,ap}$); however, they differed on the rates of catalysis (Table S3b). One mechanism had different product formation rates for the uninhibited reaction and the ESI complex, but the parameters had open confidence intervals. The other mechanism had an identical k_{cat} for each, and closed nonsymmetrical intervals at 95% confidence, as shown in Table 3.

Like styrene and styrene oxide, the preferred inhibition mechanism for 4-vinylphenol involved binding at the catalytic and cooperative sites in a mixed inhibition model in which substrate acted cooperatively (Table S3d). As with the other compounds, 4-nitrophenol had a negative cooperative effect on inhibitor binding, such that $K_{si,ap} > K_{i,ap}$. However, unlike those compounds, the preferred mechanism for 4-vinylphenol inhibition involved an ESI complex that was not catalytically active. The favored mechanism for styrene glycol inhibition was its role as a simple competitive inhibitor only for the catalytic site (Table S3c).

Styrene and its metabolites interact with two CYP2E1 sites during 4-nitrophenol metabolism by human liver microsomes

For human liver microsomes, the observed substrate inhibition is less pronounced due to contribution from other P450s at high 4-nitrophenol concentrations (Figure 6). Because of this, there was ambiguity in the discriminations between mechanisms and interactions at the cooperative site were difficult to observe. However, the preferred inhibition mechanism for styrene was the mixed cooperative substrate model, in which substrate acted cooperatively on the reaction as observed for the studies with recombinant enzyme (Table S4a). The model that had the greatest statistical support was that with ESI active and $k_{cat1} \neq k_{cat2}$. However, the corresponding parameters for this model had open confidence intervals. The model with the next highest probability was the same model but with the $k_{cat1} = k_{cat2}$, and closed confidence intervals. The parameters associated with this model are shown in Table 4.

The discriminations for styrene oxide, styrene glycol, and 4-vinylphenol (Tables S4b, d and c, respectively) showed a preference for competitive models and mixed cooperative substrate models; however, the mixed cooperative models had kinetic parameters with open confidence intervals. The parameters associated with a competitive inhibition mechanism are reported in Table 4.

Taken together, the corresponding inhibition constants for the catalytic site (K_i) from CYP2E1 Supersomes and HLM150 reactions in Tables 3 and 4 were similar to the IC_{50} values in Table 2.

Hydrophobicity plays dominant role in binding interactions to CYP2E1 catalytic site

For CYP2E1 Supersomes and human liver microsomes, the apparent $K_{d,ap}$ ($K_{i,ap}$ for styrene and metabolites and $K_{s,ap}$ for 4-nitrophenol) were significantly correlated with the log of the partition coefficient ($\log P$). The correlation had a coefficient of determination (r^2) of 0.85 for CYP2E1 Supersomes and 0.93 for human liver microsomes (Figure 7). The same trends were observed for each system with approximately the same magnitude for each. There were no apparent outliers from this analysis. These correlations suggest that for the compounds included in this study hydrophobicity is likely important for protein binding rather than steric hindrance or other factors.

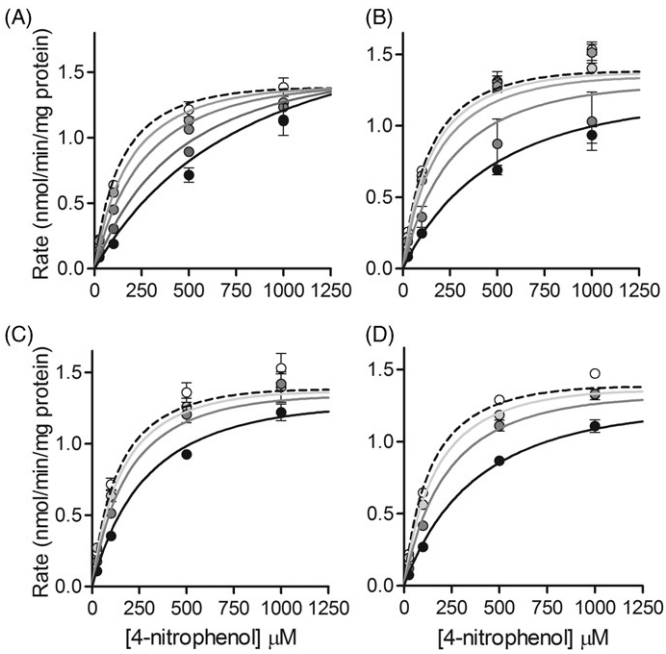

Figure 6. Inhibition kinetic profiles for styrene and its metabolites toward oxidation of 4-nitrophenol with human liver microsomes (HLM150). The dashed line and open circles represent the uninhibited reaction; increasing inhibitor concentration is indicated by darker lines and markers. Specific inhibitors include (A) 0, 3.7, 11, 33, 99 and 300 μM styrene; (B) 0, 18, 55, 170 and 500 μM styrene oxide; (C) 0, 8.7, 26 and 78 μM 4-vinylphenol or (D) 0, 4000, 12 000, and 36 000 μM styrene glycol. For reactions, 0.25 mg/mL HLM150, varying 4-nitrophenol concentrations, and 1 mM NADPH were incubated at 37 °C and pH 7.4 in the presence of inhibitor (Experimental Procedures). The reported values reflect the average of four experimental replicates, including the mean ± standard deviation. Data were fit to the most probable mechanism listed in Table 4, which were identified among 10 possibilities (Table 1) using DynaFit software version 3.28 (Biokin Ltd) (Kuzmic, 1996).

Discussion

Herein, we demonstrated the impact of styrene metabolism on the ability of the molecule to modulate CYP2E1 activity through a cooperative mechanism. Specifically, we investigated the mode of interaction between CYP2E1 and an array of molecules, i.e. styrene, its oxidized metabolites (styrene oxide and 4-vinylphenol), and a secondary metabolite, styrene glycol. Initial IC_{50} studies using 4-nitrophenol as a reporter provided valuable insights on the inhibitory potential of the compounds. Nevertheless, the simplicity of those experiments obviated the ability to truly investigate the mechanism of interaction as revealed by kinetic inhibition studies. Styrene and its metabolites preferably interacted with CYP2E1

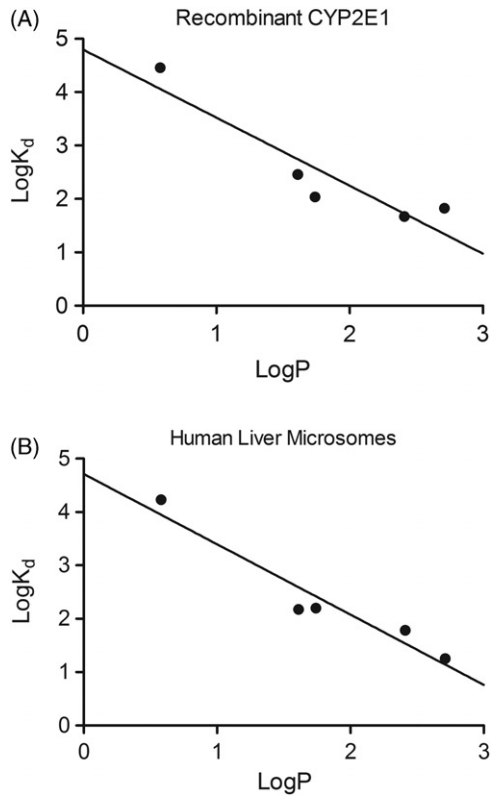

Figure 7. Importance of hydrophobicity in binding interactions with the CYP2E1 catalytic site. log*P* values were calculated using the Marvin software, ChemAxon (Table 3) were plotted against K_d representing the inhibition constant K_i for styrene and metabolites or equilibrium constant K_s for 4-nitrophenol. Data were then fit to a linear regression.

Table 4. Apparent inhibition parameters for styrene metabolites toward CYP2E1 activity with HLM150.[a,b]

Inhibitor	Mechanism	k_{cat2}[c]	$K_{i,ap}$ (μM)	$K_{si,ap}$ (μM)
Styrene	Mixed cooperative substrate	1.7 (1.5–1.9)	18 (7.6–42)	58 (21–190)
Styrene oxide	Competitive (mixed cooperative substrate)[d]	–	160 (140–190)	–
4-Vinylphenol	Competitive (mixed cooperative substrate)	–	61 (47–83)	–
R/S-Styrene glycol	Competitive	–	17 000 (15 000–19 000)	–

[a]Figure 2 depicts reaction mechanism used to determine kinetic parameters. Nonsymmetrical 95% confidence intervals for parameters are shown in parentheses.
[b]Parameters for uninhibited 4-nitrophenol oxidation by HLM150 were $K_s = 150$ μM, $K_{ss} = 11\,000$ μM and $k_{cat1} = 1.7$ nmol/min/mg protein.
[c]Units are nmol/min/mg protein.
[d]Probability for both models was statistically equivalent, but the parameters for the model in parentheses had open confidence intervals.

through cooperative mechanisms dependent on the respective structure of the molecules. The strongest evidence came from studies employing CYP2E1 Supersomes, which were devoid of competing P450s and other possible reactions. Human liver microsomes recapitulated the findings, although it was not always possible to define confidence intervals for parameters. There was some unavoidable ambiguity in the models, which did not allow true equilibrium constants to be determined; however, the mechanisms reported herein provide crucial evidence for multiple CYP2E1 binding sites for styrene and its metabolites. Collectively, these CYP2E1 studies expanded the array of known compounds to interact with its cooperative site and revealed the importance of structure on determining the impacts on metabolism (Harrelson et al., 2008; Hartman et al., 2012; Spatzenegger et al., 2003).

Styrene inhibition of the oxidation of 4-nitrophenol shared many mechanistic similarities with our findings for styrene metabolism, in particular, the association with catalytic and cooperative effector sites (Hartman et al., 2012). In both cases, styrene bound to CYP2E1 through two sites – one high affinity site and another low affinity one. Styrene metabolism involved an initial weak binding event between styrene and CYP2E1 followed by a much stronger second one. Presumably, the first binding event created a more hydrophobic environment in the catalytic site to favor binding of a second styrene molecule. In the current studies, both of these binding events for styrene collectively blocked 4-nitrophenol binding to the catalytic site to inhibit its metabolism. Consequently, the apparent inhibition constant for styrene ($K_{i,ap}$ 67 μM for CYP2E1 Supersomes, Table 3) is comparable to the high affinity apparent binding constant observed during styrene metabolism (K_s 110 μM for CYP2E1 Supersomes) (Hartman et al., 2012). By contrast, when 4-nitrophenol bound to the catalytic site, the association of styrene with the cooperative site was rather poor ($K_{si,ap}$ 1100 μM for CYP2E1 Supersomes, Table 3). The lack of productive binding contacts to styrene may be due to a decrease in steric space and an increase in polarity in the catalytic site because of the presence of 4-nitrophenol. These findings indicate that the properties of the compound bound at the catalytic site can influence binding to the cooperative site.

Similarly, occupancy of the cooperative effector site impacted substrate binding and catalysis although specific effects depended on the structure of the respective compounds. Styrene bound to the cooperative effector site improved the binding of another styrene molecule at the catalytic site (Hartman et al. 2012). The formation of favorable hydrophobic and/or pi stacking interactions may account for the increased affinity between styrene and CYP2E1. A consequence of this mode of binding for styrene created a new more efficient catalytic cycle for CYP2E1, even though styrene did not act cooperatively on k_{cat} for the reaction (Hartman et al., 2012). Likewise, styrene bound at the cooperative site produced no cooperativity on k_{cat} for 4-nitrophenol oxidation; rather, the effects manifested in changes in the reaction pathway for 4-nitrophenol and an overall increase in catalytic efficiency as shown in Figure 8. In the absence of styrene, 4-nitrophenol bound to the cooperative site to create an inactive complex and subsequent substrate inhibition. When styrene occupied the cooperative site, 4-nitrophenol could not associate with the site to inactivate CYP2E1. Consequently, CYP2E1 remained active toward 4-nitrophenol and metabolized it through a new catalytic cycle involving a mixed complex with both 4-nitrophenol and styrene bound to the enzyme.

Oxidation of styrene significantly altered its inhibition mechanism toward the 4-nitrophenol reaction and not its affinity toward CYP2E1 sites. Styrene epoxide and 4-vinylphenol inhibited 4-nitrophenol metabolism through a mixed cooperative mechanism. Similar affinities of these molecules for the catalytic site to those observed for styrene (Table 3) indicated epoxidation of the vinyl group or hydroxylation of the phenyl ring did not alter the ability to form effective binding contacts. Both alterations in the structure introduce minimal steric bulk, although they do introduce some polarity, especially the epoxide moiety. When 4-nitrophenol bound to the catalytic site, there were much less productive binding interactions with these metabolites at the cooperative site as suggested for styrene. Nevertheless, the specific binding modes for styrene oxide and 4-vinylphenol were different given their impacts on catalysis. Like styrene, styrene oxide changed the metabolic pathway for 4-nitrophenol by creating a mixed complex that was catalytically active (Figure 8). The analogous mixed complex for 4-vinylphenol was catalytically inactive. In other words, 4-vinylphenol binding at the cooperative site impacted catalysis more like 4-nitrophenol rather than styrene. It is possible that the introduction of the hydroxyl group to styrene may create new binding interactions that are not possible for styrene or its epoxide. For example, 4-vinylphenol and 4-nitrophenol are both hydrogen bond donors and acceptors. In following, phenolic compounds may favor the formation of higher order but catalytically inactive CYP2E1 complexes, although further studies are necessary to better establish this relationship.

An alternate but not exclusive inhibition mechanism for styrene oxide would involve adduct formation between the metabolite and CYP2E1. Epoxides are electrophilic and are prone to attack by nucleophilic sites on proteins. We have shown that treatment of CYP2E1 with 1,2-epoxy-butadiene resulted in the modification of four residues (Boysen et al., 2007). Although no adducts between styrene oxide and CYP2E1 have been reported, exposure to styrene leads to formation of styrene oxide adducts to albumin and hemoglobin (Christakopoulos, 1993; Rappaport et al., 1996). If an adduct formed between styrene oxide and CYP2E1, the modification of its structure could have blocked 4-nitrophenol access to the catalytic site and/or compromised interactions with cytochrome P450 reductase, which are necessary for activating oxygen during catalysis (Guengerich, 2002). Pre-incubation of styrene oxide with CYP2E1 did not alter activity (Figure 4), indicating that there are no functional consequences even if adducts formed between styrene oxide and CYP2E1. In the absence of covalent modifications, styrene oxide inhibition of CYP2E1 activity is solely a reversible process.

Cleavage of styrene oxide significantly altered the inhibition mechanism and affinity of the compound toward CYP2E1. Styrene glycol binding was extremely poor and behaved as competitive binder interacting solely though the catalytic site. However, this observation does not rule out possible binding to the cooperative site. Styrene and its oxidized metabolites bound more poorly to the cooperative

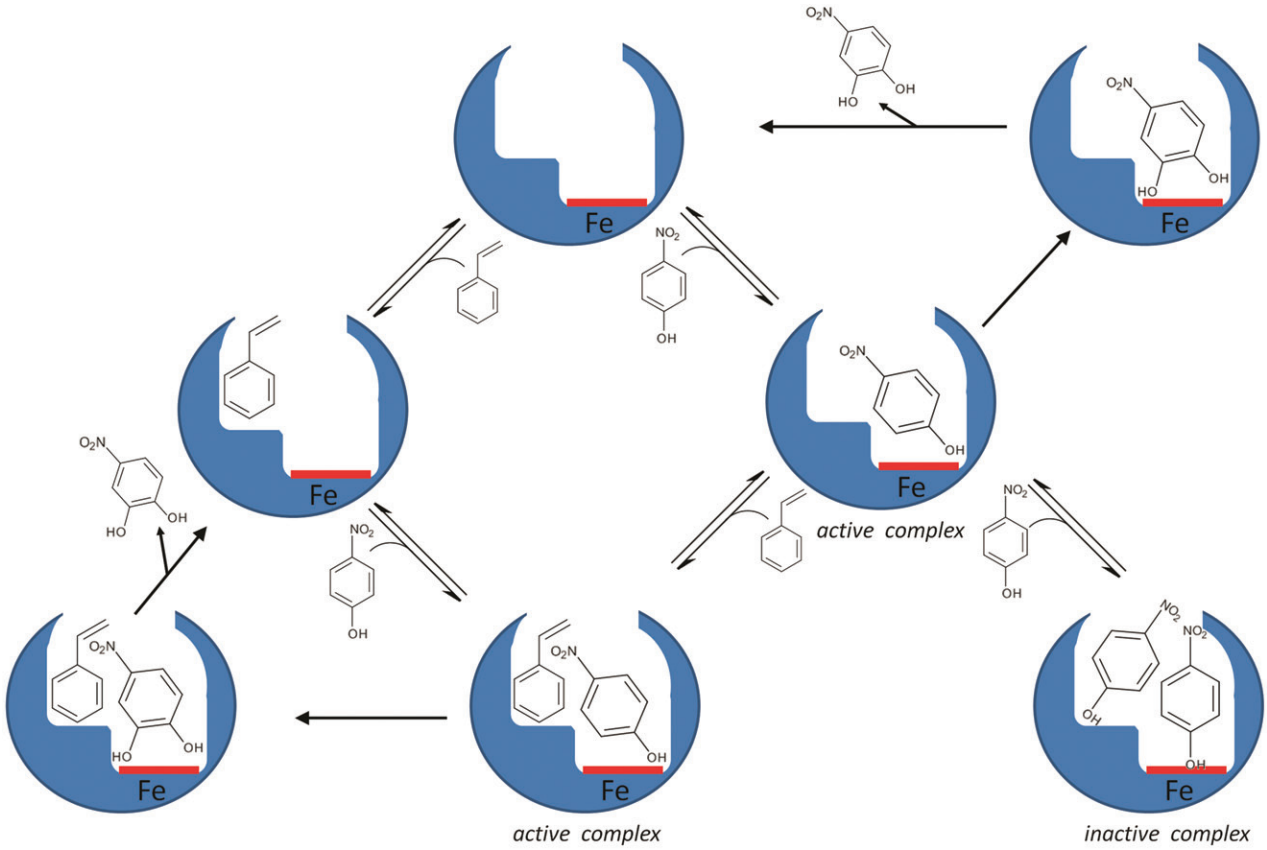

Figure 8. Stylized depiction of the cooperative mechanism for inhibition of CYP2E1 metabolic reactions. In the case of 4-nitrophenol, substrate binds to the CYP2E1 catalytic site indicated by the red heme-iron group and forms an active binary complex, which releases product and regenerates the free enzyme. At higher 4-nitrophenol concentrations, a second substrate molecule binds the enzyme-substrate complex at a distal cooperative site and forms a dead-end complex. When a cooperative inhibitor such as styrene is also present, it can bind either the free enzyme or the enzyme-substrate complex to form a binary or (active) ternary complex. This illustrates some of the different types of cooperativity that can occur when a mixture of compounds is present in the system.

site than the catalytic site. For the glycol metabolite, the weakness of the initial binding event may preclude observation of binding at the cooperative site. The opening of the epoxide ring to form the glycol introduced more steric bulk and more importantly, increased the polarity of the molecule leading to the significant decrease in CYP2E1 binding.

The studies performed with human liver microsomes allowed us to interrogate the potential inhibition mechanisms in a system that better approximated in vivo conditions. While 4-nitrophenol is more effectively metabolized by CYP2E1, other P450s can contribute at higher concentrations. Those data points also played an important role in discriminating between possible inhibition mechanisms, and thus contribution from multiple enzymes to observed rates likely account for difficulties identifying a single preferred inhibition mechanism. Nevertheless, the preferred mechanisms with human liver microsomes did recapitulate the findings with CYP2E1 Supersomes: styrene and its metabolites likely interact at two sites on CYP2E1 and modulate its activity.

Concluding remarks

Styrene and its metabolites interacted with CYP2E1 through cooperative mechanisms; however, the consequences of those interactions depended on differences in the structure of the molecules. Specifically, these inhibition studies demonstrated that the occupancy of the cooperative site by an array of molecules led to alternate modes of interactions between mixtures of molecules and CYP2E1 than simple competition for a single catalytic site. For these compounds, the driving force for binding events including at the cooperative site was formation of favorable hydrophobic interactions and not steric clashes. The corresponding evidence was clearer for binding at the catalytic site than the cooperative site indicating further studies are necessary to better characterize its specificity. Once occupied, the cooperative site impacted CYP2E1 metabolism of molecules overall through two significant, and not mutually exclusive, ways. Firstly, occupancy of the cooperative site modulated binding and the rate of catalysis, and thus steps along the metabolic pathways to product. Secondly, the binding of molecules to cooperative site created new, mixed complexes that altered the trajectory of metabolic pathways and impacted the overall efficiency of metabolism. There were ambiguities with proposed models due to the inability to define parameters, such as inhibition constants at either the catalytic or cooperative sites rather than treatment as a single constant. Other biophysical methods including binding studies would provide a powerful complement to these efforts. Nevertheless, these findings for CYP2E1 further validate the contributions

of cooperative mechanisms to metabolic processes, demonstrate the role of molecular structure on those mechanisms, and underscore the potential for heterotropic cooperative effects on binding and catalysis between different compounds.

Supplementary material

An analysis of the uninhibited 4-nitrophenol (substrate) reaction including statistical analysis of the mechanism, a plot of the kinetic profile and a table of parameters is included. Also included are the DynaFit script files and statistical outputs from the model discrimination analysis of the inhibition kinetic data.

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Declaration of interest

The authors report no declarations of interest.

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