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Differences in Butadiene Adduct Formation between Rats and Mice Not Due to Selective Inhibition of CYP2E1 by Butadiene Metabolites

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Abstract

CYP2E1 metabolizes 1,3-butadiene (BD) into genotoxic and possibly carcinogenic 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxybutane (DEB), and 1,2-epoxy-3,4-butanediol (EB-diol). The dose response of DNA and protein adducts derived from BD metabolites increase linearly at low BD exposures and then saturate at higher exposures in rats, but not mice. It was hypothesized that differences in adduct formation between rodents reflect more efficient BD oxidation in mice than rats. Herein, we assessed whether BD-derived metabolites selectively inhibit rat but not mouse CYP2E1 activity using B6C3F1 mouse and Fisher 344 rat liver microsomes. Basal CYP2E1 activities toward 4-nitrophenol were similar between rodents. Through IC₅₀ studies, EB was the strongest inhibitor (IC₅₀ 54 μM, mouse; 98 μM, rat), BD-diol considerably weaker (IC₅₀ 1200 μM, mouse; 1000 μM, rat), and DEB inhibition nonexistent (IC₅₀ >25 mM). Kinetic studies showed that in both species EB and BD-diol inhibited 4-nitrophenol oxidation through two-site mechanisms in which inhibition constants reflected trends observed in IC₅₀ studies. None of the reactive epoxide metabolites inactivated CYP2E1 irreversibly. Thus, there was no selective inhibition or inactivation of rat CYP2E1 by BD metabolites relative to mouse Cyp2e1, and it can be inferred that CYP2E1 activity toward BD between rodent species would similarly not be impacted by the presence of BD metabolites. Inhibition of CYP2E1 by BD metabolites is then not responsible for the reported species difference in BD metabolism, formation of BD-derived DNA and protein adducts, mutagenicity and tumorigenesis.

Keywords

CYP2E1; butadiene; cooperativity; inactivation; mouse; rat

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Conflicts of Interest Statement:

The authors declare that there are no conflicts of interest.

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1. Introduction

1,3-Butadiene (BD) is an important industrial chemical and byproduct of incomplete combustion found in cigarette smoke, auto exhaust, and fossil fuels (Himmelstein et al., 1997; IARC, 2008). BD has been rated the cigarette constituent with highest cancer risk index (Fowles and Dybing, 2003). Federal and international agencies have classified BD as a human carcinogen based on evidence from epidemiology and animal studies (IARC, 2008; Program, 1984, 2011). Our most recent published data in humans clearly shows that environmental BD exposure is sufficiently high enough to lead to formation of pro-mutagenic metabolites in subjects lacking any evidence of BD exposure (Boysen et al., 2012). These findings suggest that BD is an ubiquitous environmental carcinogen with a high potential to adversely affect public health.

The carcinogenic potential of BD depends on the balance between bioactivation to genotoxic epoxide metabolites and detoxification through cleavage of epoxides and subsequent conjugative processing (Figure 1). During metabolism, BD is oxidized to 3,4-epoxy-1-butene (EB), and then further metabolized to other epoxide metabolites, 1,2:3,4-diepoxybutane (DEB) and 1,2-dihydroxy-3,4-epoxybutane (EBD), as well as 3-butene-1,2-diol (BD-diol). The epoxide metabolites vary in mutagenicity up to 200-fold, such that DEB is the most mutagenic (Meng et al., 2007; Walker, 2009). In fact, BD-derived genotoxicity is attributed mainly to DEB and to a lesser extent, EB and EB-diol (Fred et al., 2008; Swenberg et al., 2011). CYP2E1 has been shown to be primarily responsible for oxidation of BD to reactive epoxide metabolites (Csanády et al., 1992; Seaton et al., 1995), and thus an understanding of the factors impacting CYP2E1-mediated metabolism BD is necessary for appropriately estimating risk associated with exposure to this pollutant.

An important strategy for identifying the link between toxicity and P450-mediated bioactivation of pollutants involves the use of rodent models; nevertheless, mice and rats demonstrate distinctly different sensitivities toward carcinogenesis when exposed to BD (Himmelstein et al., 1997; IARC, 2008). At similar BD exposure levels, mice form significantly more genotoxic DEB than rats and the corresponding DEB-specific hemoglobin adducts correlate with observed species-dependent mutagenesis (Georgieva et al., 2010). In mice, dose-responses for DEB and EB-diol are supralinear based on formation of DNA (Boysen et al., 2009; Goggin et al., 2007) and hemoglobin adducts (Boysen et al., 2007; Georgieva et al., 2010). To date, there is no clear consensus mechanism to explain differences in dose response of DNA and protein adducts and tumorigenesis between rodent species.

At high BD exposures, it has been suggested that BD metabolites inhibit overall BD-metabolism leading to saturation of metabolism by CYP2E1 (Filser et al., 2007). In rodents, we hypothesize that butadiene metabolites inhibit CYP2E1 activity in rats to a greater extent than in mice. If true, a selective inhibitory effect in rats would suppress CYP2E1 activity at higher BD levels and potentially cause the observed saturation in oxidation of BD. Mechanistically, the accumulation of metabolites would favor reversible binding to the CYP2E1 active site and block metabolism of substrates including BD. Direct evidence for inhibition of CYP2E1 has only been reported for BD-diol using mouse liver microsomes (Krause et al., 2001). Another plausible mechanism involves covalent modification of CYP2E1 by reactive epoxide metabolites that either block substrate access to the enzyme or compromise the electron transfer steps necessary for substrate oxidation to occur (Blobaum et al., 2002; Blobaum et al., 2004; Boysen et al., 2007; Guengerich, 2002; Hollenberg et al., 2007). EB has been shown to modify human CYP2E1 protein at sites theoretically important for enzyme activity (Boysen et al., 2007). Despite these seminal studies, the possibility of

BD metabolite inhibition of CYP2E1 activity has not been adequately explored between mice and rats.

Herein, we tested our hypothesis by comparing the inhibitory effects of BD metabolites and their reversibility on microsomal CYP2E1 activity between mice and rats. Specifically, we report the inhibitory potency and mechanisms of three BD metabolites, i.e. EB, DEB, and BD-diol, toward microsomal CYP2E1 activity derived from B6C3F1 mice and Fisher 344 rats. These species were chosen based on their common use in BD toxicity studies (Csanády et al., 1992; Goggin et al., 2007; Meng et al., 2007; Program, 2011; Sabourin et al., 1992). As a reporter for CYP2E1 activity, we measured oxidation of 4-nitrophenol and initially screened the inhibitory potency of BD metabolites through IC₅₀ studies. Based on those findings, we designed and carried out subsequent mechanistic steady state inhibition kinetic studies. The resulting inhibition profiles were fit globally to 10 different mechanisms involving catalytic and/or cooperative sites to identify the most probable one. We then determined whether the epoxide metabolites were capable of inactivating mouse and/or rat CYP2E1 to provide mechanistic insights toward observed dose-responses between mice and rats in formation of pro-mutagenic oxides.

2. Experimental Procedures

2.1 Materials

All chemicals in this study were ACS grade or higher. All butadiene metabolites were purchased from Sigma-Aldrich (St. Louis, MO). Fisher Scientific (Pittsburgh, PA) was the source of HPLC grade methanol, acetic acid, and NADPH. B6C3F1 mouse liver and Fisher 344 rat liver microsomes were purchased from BD Biosciences (San Jose, CA).

2.2 Steady State Kinetics Assay

We used the hydroxylation of 4-nitrophenol to 4-nitrocatechol as a marker reaction for the inhibition of CYP2E1 by compounds in this study. As a baseline for CYP2E1 activity, we determined steady-state parameters for the 4-nitrophenol reaction catalyzed by rat and mouse liver microsomes (Koop, 1986). Specifically, 0.25 mg/mL protein from either mouse or rat liver microsomes was incubated with 0 to 2 mM 4-nitrophenol in 50 mM potassium phosphate, pH 7.4 at 37°C and reactions initiated with 1 mM NADPH. After 30 min, reaction was quenched with equal volume of 0.4 N perchloric acid containing 25 µM 4-nitroanisole, the internal standard. The quenched reaction was centrifuged, and supernatant analyzed by HPLC to quantify product formed by CYP2E1 as described (Hartman et al., 2013). Six independent replicates were performed each with mouse and rat liver microsomes. The resulting kinetic profiles were plotted and fit to a substrate inhibition mechanism using DynaFit software ver. 3.28 (Biokin Ltd.) to determine the kinetic parameters as described previously (Collom et al., 2008).

2.3 Inhibition of microsomal CYP2E1 activity by BD metabolites through IC₅₀ studies

4-Nitrophenol hydroxylation was used as a marker reaction for these studies. Inhibition experiments contained 50 µM 4-nitrophenol and seven concentrations of inhibitor (EB, BD-diol, DEB, or structural analogue 1,4-butanediol) plus a negative control (inhibitor buffer solution). Reactions were initiated, quenched, and prepared for HPLC analysis as described in the previous section on steady-state experiments. At least three replicates were performed each with mouse and rat liver microsomes. After normalizing the rates to the negative control, relative activity was plotted against the log of 4-nitrophenol concentration, and the data fit to the standard IC₅₀ equation in GraphPad Prism 4.0 to determine the corresponding inhibition constants.

2.4 Inhibition Kinetics for BD Metabolites

Further inhibition kinetics studies were performed to more quantitatively assess the mechanism of CYP2E1 inhibition by BD metabolites. Substrate and inhibitor stocks were prepared in 50 mM potassium phosphate, pH 7.4. In these reactions, 0.25 mg/mL protein in mouse and rat liver microsomes was incubated with eight 4-nitrophenol concentrations ranging from 0 to 2 mM and three different inhibitor concentrations along with a negative control (inhibitor buffer solution). Based on previous experiments, inhibitor concentrations were set threefold below the IC_{50} value, at the IC_{50} value, and threefold above the IC_{50} value. The reaction was initiated, quenched, and prepared for HPLC analysis as described in the previous section on steady-state experiments. At least three replicates were performed each with mouse and rat liver microsomes.

The resulting kinetic inhibition profiles were analyzed globally to identify the most probable inhibition mechanism toward 4-nitrophenol oxidation by CYP2E1 using DynaFit 3.28 (Biokin Ltd, Watertown, MA) (Kuzmic, 1996). As described previously for rabbit (Collom et al., 2008) and human (Hartman et al., 2013) CYP2E1, a substrate inhibition mechanism involving an inactive ESS complex best describes metabolism of 4-nitrophenol by CYP2E1 (Figure 2, reaction pathway in bold). The Dynafit script file used for determining the corresponding V_{max} , K_s , and K_{ss} for the uninhibited data is included in Supplementary Data.

Data were then fit to one- or two-site inhibition mechanisms for 4-nitrophenol metabolism involving multiple possible inhibitory complexes (Figure 2) as reported previously (Hartman et al., 2013). 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. The DynaFit script file used for the inhibition model discrimination analyses is included in the Supplementary Data.

2.5 Inactivation Studies

BD metabolites may reversibly bind or covalently modify CYP2E1 to alter activity. Therefore, we tested these possibilities by measuring residual CYP2E1 activity after pre-incubation of microsomes with each metabolite. For these studies, 6.7 mg/mL microsomal protein was incubated with 1.3 mM NADPH along with inhibitor and a negative control (inhibitor dilution buffer). For mouse EB concentrations were 0, 18, 54, and 162 μ M, and for rat were 0, 32.7, 98, and 294 μ M. DEB concentrations were 0 and 25000 μ M for both mouse and rat experiments. After 15 min incubation, reactions were diluted 30-fold in 50 mM potassium phosphate, pH 7.4. A subsequent enzymatic reaction was then carried out and analyzed with diluted mixtures containing 0.22 mg/mL protein liver microsomes and 50 μ M 4-nitrophenol as described for IC_{50} experiments. Each experiment was performed in three independent replicates.

2.6 Statistical Analyses

For inhibition kinetic studies, the most probable mechanism and corresponding parameters for inhibition by these molecules were identified using the advanced tools of numerical analysis and applied statistics, as implemented in the software DynaFit. After globally fitting reactions to these mechanisms, we generated a corresponding Akaike information criterion (AICc) to identify statistically the most plausible based on the quality of the fits (Kuzmic, 1996).

3. Results

3.1 Steady State Kinetics

The metabolism of 4-nitrophenol by B6C3F1 mouse and F344 rat liver microsomes was consistent with substrate inhibition (Figure 4). The reaction rates initially increased as a function of substrate concentration and then decreased at higher values. As we reported previously for rabbit (Collom et al., 2008) and human (Hartman et al., 2013) CYP2E1, the data were fit to a two-site model involving negative cooperativity (bold reaction pathway shown in Figure 2), so that we could obtain kinetic parameters for the reaction (Table 1). The overall rate of substrate turnover was similar between rodent microsomal fractions, while mouse CYP2E1 bound 4-nitrophenol with slightly higher affinity. Similarly, the association of 4-nitrophenol with the cooperative site was also more favorable for mouse CYP2E1 than that for the rat enzyme (Table 1).

3.2 IC₅₀ values for BD metabolites

BD metabolites varied widely in inhibitory potency toward 4-nitrophenol metabolism by CYP2E1 (Table 2, Figure 3). For B6C3F1 mouse and Fisher 344 rat liver microsomes, EB was the most effective inhibitor with IC₅₀ values approximate two-fold greater for rat than mouse microsomes. BD-diol yielded weak inhibitory potency toward mouse and rat CYP2E1 activity, specifically, a 20- and 10-fold decrease in inhibitor potency relative to EB for mouse and rat, respectively. Residual CYP2E1 activity varied from 15 and 24% at concentrations of 25 mM EB and BD-diol. Surprisingly, DEB showed little to no inhibition toward mouse or rat CYP2E1 activity even up to a final concentration of 25 mM DEB and therefore was excluded from further kinetics studies. Nevertheless, we further investigated whether DEB polarity played a role in these findings by determining the IC₅₀ value for 1,4-butanediol as an analog of the metabolite. Consistent with previous studies (Hartman et al., 2013), this compound bound very poorly to CYP2E1 such that the IC₅₀ value was greater than that observed for BD-diol, but much lower than DEB.

3.3 Inhibition Kinetics for B6C3F1 mouse liver microsomes

For mouse liver microsomes, we determined the mechanisms of CYP2E1 inhibition by EB and BD-diol toward the oxidation of 4-nitrophenol. Data for the uninhibited reaction were plotted, and the kinetic parameters were determined. The inhibition data for both EB and BD-diol were fit to ten possible inhibition mechanisms for the oxidation of 4-nitrophenol (Figure 2). Both BD metabolites inhibited 4-nitrophenol metabolism through similar mechanisms. For EB, the preferred model was a mixed cooperative substrate mechanism in which the ESI complex was active at a k_{cat2} approximately one half of the uninhibited rate (Figure 4, Panel A, Table 3). The $K_{si,ap}$ value was determined to be twice the $K_{i,ap}$ value. Both parameters were within the confidence intervals of the IC₅₀ value determined from prior experiments. For BD-diol, there were two similar mechanisms with essentially the same probability. The most probable mechanisms, both mixed cooperative substrate models, differed in that, in one model, k_{cat2} was approximately equal to k_{cat1} and, in the other model, k_{cat2} was greater than k_{cat1} . However, the latter mechanism had open confidence intervals and was therefore excluded, leaving only the $k_{cat1}=k_{cat2}$ mechanism (Table 3). $K_{si,ap}$ was between two- and threefold higher than $K_{i,ap}$, suggesting a negative cooperative effect of 4-nitrophenol on inhibitor binding.

3.4 Inhibition Kinetics for Fisher 344 rat liver microsomes

The inhibition mechanisms for the rat liver microsomes were determined in the same manner as for the mouse liver microsomes. Two mechanisms had equal statistical probability for EB as an inhibitor for the 4-nitrophenol reaction (Figure 4, Panel C, Table 3).

Both mechanisms were mixed cooperative substrate models. However, in one model, k_{cat2} is the same as k_{cat1} and, in the other, k_{cat2} is slightly higher than k_{cat1} . Preference is given to the former mechanism due to consistency with the mechanism observed for mouse liver microsomes and similar cooperative studies performed with human CYP2E1 (Hartman et al., 2013). The $K_{si,ap}$ was between two- and threefold greater than the $K_{i,ap}$. Statistical analysis of the BD-diol inhibition data for rat liver microsomes determined it to also follow a mixed cooperative substrate model. The preferred model had k_{cat1} greater than k_{cat2} , but there were open confidence intervals. The next highest preferred model was the same, but with k_{cat1} equal to k_{cat2} . The $K_{si,ap}$ was nearly twofold greater than the $K_{i,ap}$ for the reaction, which is consistent with the cooperative effect observed in mouse liver microsomes.

3.5 Reversibility of CYP2E1 inhibition by BD metabolites

For the previous inhibition studies, we assumed inhibition was reversible and in these experiments validated that possibility. Specifically, we tested the reversibility of EB and DEB inhibition of recombinant CYP2E1 activity using 4-nitrophenol oxidation as a marker reaction. After incubation for 15 min with both EB and DEB, no inactivation of CYP2E1 activity by either mouse or rat liver microsomes was observed at levels threefold higher than the measured IC_{50} for both metabolites (Figure 5).

4. Discussion

In this study, we demonstrated that the hypothesized inhibitory potency of BD metabolites toward CYP2E1 activity cannot explain differences in BD metabolic activation between B6C3F1 mice and Fisher 344 rats. For the first time, we show that these compounds, including reactive epoxides, perturb CYP2E1 activity through reversible mechanisms and not irreversible modification and inactivation of the enzyme. CYP2E1 from both rodent species shared common inhibition mechanisms and affinities for BD metabolites, although EB was the only promising inhibitor identified in this study. Consequently, the differences between B6C3F1 mice and Fisher 344 rats in response to BD exposure are not due to their affinity or reactivity toward CYP2E1.

BD metabolites possess the capacity to inhibit CYP2E1 activity through reversible and irreversible mechanisms. Irreversible covalent bond formation is a possibility for electrophilic epoxide metabolites EB and DEB. These molecules are susceptible to nucleophilic attack by amino acid side chains to form irreversible covalent modifications that could affect CYP2E1 activity. In fact, four histidine and two tyrosine residues present in human CYP2E1 react with EB to form adducts (Boysen et al., 2007), although their impact on activity was not investigated. As a bifunctional electrophile, DEB can modify individual residues and form intra- and inter-protein crosslinks (Cho and Guengerich, 2012), as well as protein-DNA crosslinks (Loecken et al., 2009). In this study, we did not specifically investigate the covalent modification of rodent CYP2E1 enzymes by BD metabolites; however, we did not observe any irreversible inhibition/inactivation of CYP2E1 by EB and DEB that would suggest covalent binding. Consequently, the differences in BD metabolism between these rodents, especially in the dose-response curves of some pathways, are not likely due to selective inactivation of rat CYP2E1. Any effects of EB and DEB on CYP2E1 activity would then manifest in a simple reversible inhibition mechanism.

Studies assessing inhibition of 4-nitrophenol metabolism by BD metabolites demonstrated that all compounds interacted with catalytic and cooperative CYP2E1 sites. As reported previously (Koop, 1986), CYP2E1 metabolism of 4-nitrophenol involves substrate inhibition. We have shown that this kinetic profile for rabbit (Collom et al., 2008) and human (Hartman et al., 2013) enzymes can be explained through a two-site mechanism in which binding of the second substrate suppresses the reaction through an inactive complex.

Similarly, this mechanism was suitable for investigating interactions during 4-nitrophenol metabolism by mouse and rat CYP2E1 in this study. Like substrate, EB and BD-diol bound to both catalytic and cooperative sites modulating CYP2E1 activity, while the presence of DEB did not significantly impact CYP2E1 activity at all. Importantly, inhibitors induced a significant change in the metabolic flux of substrate to product by CYP2E1. Occupancy of the cooperative site by either EB or BD-diol resulted in a mixed ternary complex with 4-nitrophenol that was catalytically active, as opposed to the dead end complex with substrate in the cooperative site. Consequently, the “inhibitors” rescued enzyme activity by creating a new metabolic pathway as reported for styrene, styrene metabolites, and several pyrazoles (Hartman et al., 2013). CYP2E1 from both mice and rats shared this cooperative mechanism for binary mixtures of BD metabolites with 4-nitrophenol such that any differences between the metabolic systems must reflect CYP2E1 affinities for the metabolites and/or their levels in rodents.

The most potent inhibitor in this study was EB, whereas BD-diol and DEB had negligible effects on CYP2E1 activity present in B6C3F1 mouse and Fisher 344 rat liver microsomes. The stronger affinity of EB for CYP2E1 compared to the other metabolites may reflect its higher hydrophobicity based on logP values. The weak binding of 1,4-butanediol to CYP2E1 supported this possibility. These findings are consistent with other studies investigating the molecular basis of CYP2E1 selectivity for compounds (Hartman et al., 2013; Wang, 1995). We excluded EBD from our study due to a lack of stability (Figure 1). Based on our results herein, it is estimated that presence of polar groups at both ends of the EB-diol is likely to abolish significant interactions with CYP2E1 as observed for DEB and 1,4-butanediol, making it a poor inhibitor. In following, the ultimate question is whether these values for the BD metabolites are sufficient to impact BD metabolism by CYP2E1. While CYP2E1 affinity for EB is similar to that for BD (120 to 140 μM), there is no significant difference in K_m values for BD metabolism by B6C3F1 mouse and Sprague-Dawley rat CYP2E1 (Duescher and Elfarra, 1994), and thus differences in BD toxicity in rodents cannot be due to CYP2E1 affinities for substrate or products of metabolism.

Only differences in EB levels could then drive selective inhibition of rat over mouse CYP2E1 to suppress BD activation and impact adduct formation. After exposures from low to high BD doses, EB levels in blood from B6C3F1 mice were shown to range from two- to eightfold higher than those reported in Sprague-Dawley rats (Bechtold et al., 1995; Himmelstein et al., 1994). Similar values were also reported for metabolite levels in various rodent tissues (Csanady et al., 2011; Thornton-Manning JR, 1997). This difference in EB levels between rodent species is consistent with a higher rate for metabolic elimination of BD in mice than for rats (Kreiling et al., 1986). Collectively, these findings would suggest that BD metabolite levels are more likely to inhibit mouse CYP2E1 metabolic activity under in vivo conditions rather than that for rat, which undermines the hypothesis tested in this study.

In conclusion, these findings eliminate the selective inhibition of rat CYP2E1 as a possible explanation for the differences in dose-responses to BD exposure between mice and rats. Following BD exposure, mice and rats achieve similar saturation levels of BD (Bechtold et al., 1995; Himmelstein et al., 1994). The intrinsic capacity of CYP2E1 to metabolize BD in both rodent species are essentially equivalent (Duescher and Elfarra, 1994); however, mice metabolize BD more efficiently (Kreiling et al., 1986) suggesting the existence of differences in processes upstream or downstream of CYP2E1 metabolism of BD between rats and mice. We report therein that downstream BD metabolites do not selectively inhibit reversibly or irreversibly rat CYP2E1 activity. In following, it can be inferred that CYP2E1 activity toward BD between rodent species would similarly not be impacted by the presence of BD metabolites. Rather, the metabolic flux of BD to genotoxic metabolites EB and DEB

in rats and mice differs in the upstream regulation of CYP2E1 and other P450s metabolizing BD and/or the downstream processing of BD metabolites by CYP2E1 and other P450s, epoxide hydrolase, and other enzymes. Taken together, these possible mechanisms require further study to determine if one or more impact relative CYP2E1 activity among rodents and thus mitigate its role in adduct formation related to BD toxicity and tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BD	1,3-butadiene
EB	1,2-epoxy-3-butene
EB-diol	1,2-epoxy-3,4-butanediol
BD-diol	3-butene-1,2-diol
LM	liver microsomes
HPLC	high-performance liquid chromatography

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Appendix A. Supplemental Data

Supplemental Data can be found in on-line version of this article.

HIGHLIGHTS

- Toxic response to butadiene saturates at high levels in rats but not mice
- Difference attributed to selective CYP2E1 inhibition by butadiene metabolites
- Butadiene metabolites reversibly, but not irreversibly, inhibit CYP2E1 metabolic activity
- No difference in butadiene metabolite inhibition of rat and mouse CYP2E1 activity

Figure 1. Metabolic pathways for butadiene

CYP2E1 activates butadiene (BD) and its metabolites to carcinogenic epoxides. Epoxide hydrolase (EH) deactivates them into less harmful diols that can undergo subsequent conjugation and elimination. As indicated by red blocking arrow, BD metabolites hypothesized to selectively inhibit rat CYP2E1 but not the mouse enzyme to effectively suppress toxicity related to BD metabolism.

Model 1: Single-site inhibition

Model 2: Cooperative (two-site) inhibition

Possible Models for Inhibition of CYP2E1

Model	Complexes Formed	Cooperative Effects?	Possible Active ESI Complex? ^a	Parameters and Consequences ^b
Competitive	ES, ESS, EI	No	No	$K_i, K_{ss}, K_s, k_{\text{cat}}$
Uncompetitive	ES, ESS, ESI	No	Yes	$K_i, K_{ss}, K_{si}, k_{\text{cat}1}, k_{\text{cat}2}?$
Mixed	ES, ESS, EI, ESI	Yes	Yes	$K_i, K_{ss}, K_i, K_{si}, k_{\text{cat}1}, k_{\text{cat}2}?$
Noncompetitive	ES, ESS, EI, ESI	No	Yes	$K_{ii}, K_{ss}, K_i=K_{si}, k_{\text{cat}1}, k_{\text{cat}2}?$

^aIn all models except competitive, ESI may be active or not, hence $k_{\text{cat}2}$. Both possibilities were included in model discrimination studies.

Figure 2. Possible inhibition mechanisms for single-site (Model 1) versus cooperative (two-site) inhibition (Model 2) of CYP2E1 4-nitrophenol activity by BD metabolites

The uninhibited reaction is shown in bold; possible, but undetectable, complexes are shown in gray. E = CYP2E1; S = 4-nitrophenol; P = product (4-nitrocatechol); I = BD metabolites.

Figure 3. IC₅₀ for BD metabolites on CYP2E1 activity with mouse liver microsomes (Panel A) and rat liver microsomes (panel B)

Plots represent nonlinear regression analyses of normalized microsomal activity versus log of inhibitor concentration in micromolar. Individual tracings represent different metabolites, with EB as inverted triangles and BD-diol as squares, respectively. The reported values reflect the average from 4 experiments, including the mean $\pm$ S.D.

Figure 4. Inhibition kinetics with BD metabolites as inhibitors toward oxidation of 4-nitrophenol
 Data presented as rate versus concentration of substrate, 4-nitrophenol. Dashed line with open circles represent uninhibited reaction fit to substrate inhibition model (Collom et al., 2008; Hartman et al., 2013). Increasing inhibitor concentration is represented by darker shading of symbols and lines. Specific inhibitor-microsome combinations are as follows: (A) EB inhibition of B6C3F1 mouse liver microsomes; (B) BD-diol inhibition of B6C3F1 mouse liver microsomes; (C) EB inhibition of F344 rat liver microsomes; and (D) BD-diol inhibition of F344 rat liver microsomes. Experimental details are given in the Experimental Procedures. Data were fit to the most probable of 10 mechanisms (Table 1); mechanisms and parameters are listed in Tables 4 and 5. The reported values reflect the average from 4 experiments, including the mean $\pm$ S.D.

Figure 5. Reversibility of CYP2E1 inhibition by DEB and EB

Data shows residual activity after 15 min preincubation of mouse liver microsomes or rat liver microsomes with a BD metabolite. Following a 30-fold dilution of the preincubated mixture, the residual microsomal activity was assayed as described in the Experimental Procedures. Individual tracings represent different microsomes, with mouse liver microsomes as squares and rat liver microsomes as circles, respectively. The reported values reflect the average from 3 experiments, including the mean $\pm$ S.D.

Table 1Parameters for 4-nitrophenol steady-state metabolism by microsomal CYP2E1.^a

Enzyme System	Parameters for Cooperative Model ^b		
	$V_{\max}^c$	K_s (μM)	K_{ss} (μM)
B6C3F1 mouse liver microsomes	1.4 (1.2 to 1.6)	46 (31 to 69)	1900 (1200 to 3000)
F344 rat liver microsomes	1.2 (1.1 to 1.3)	61 (43 to 86)	3200 (2100 to 5300)

^aThe nonsymmetrical 95% confidence intervals for parameters are shown in parentheses.^bSee Figure 2.^cUnits are nmol/min/mg protein.

Table 2

IC₅₀ values for BD metabolites toward 4-nitrophenol metabolism by microsomal CYP2E1.^a

Inhibitor	logP	B6C3F1 Mouse LM		F344 Rat LM	
		IC ₅₀ (μM)	Residual Activity (%)	IC ₅₀ (μM)	Residual Activity (%)
EB	0.74	54 (18 to 89)	18 (11 to 25)	98 (58 to 140)	24 (19 to 28)
BD-diol	-0.42	1200 (670 to 1600)	15 (7.7 to 23)	1000 (620 to 1400)	18 (12 to 25)
DEB	-0.15	> 25000	—	>25000	—
1,4-butane diol ^b	-0.63	3200 (170 to 6200)	46 (31 to 60)	5800 (2300 to 9400)	17 (0.31 to 33)

^aLM, liver microsomes

^bIncluded in study as BD metabolite analog to impact of polarity on CYP2E1 inhibition

Table 3

Apparent inhibition parameters for butadiene metabolites toward CYP2E1 activity present rodent liver microsomes^a

Inhibitor	Enzyme	Mechanism	k_{cat}^b	$K_{i,app}$ (μ M)	$K_{sl,app}$ (μ M)
EB	B6C3F1 Mouse LM	Mixed Coop. Substrate	0.77 (0.68 to 0.86)	29 (19 to 42)	59 (30 to 140)
	F344 Rat LM	Mixed Coop. Substrate	1.2 (1.1 to 1.3)	24 (13 to 45)	42 (22 to 81)
BD-diol	B6C3F1 Mouse LM	Mixed Coop. Substrate	1.4 (1.2 to 1.6)	830 (520 to 1400)	2100 (1300 to 3700)
	F344 Rat LM	Mixed Coop. Substrate	1.2 (1.1 to 1.3)	640 (440 to 940)	1200 (790 to 1800)

^aFigure 2 depicts reaction mechanism used to determine kinetic parameters. Nonsymmetrical 95% confidence intervals for parameters are shown in parentheses.

^bUnits are nmol/min/mg protein