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Environmentally persistent free radical-containing particulate matter competitively inhibits metabolism by cytochrome P450 1A2

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Abstract

Combustion processes generate different types of particulate matter (PM) that can have deleterious effects on the pulmonary and cardiovascular systems. Environmentally persistent free radicals (EPFRs) represent a type of particulate matter that is generated after combustion of environmental wastes in the presence of redox-active metals and aromatic hydrocarbons. Cytochromes P450 (P450/CYP) are membrane-bound enzymes that are essential for the phase I metabolism of most lipophilic xenobiotics. The EPFR formed by chemisorption of 2-monochlorophenol to silica containing 5% copper oxide (MCP230) has been shown to generally inhibit the activities of different forms of P450s without affecting those of cytochrome P450 reductase and heme oxygenase-1. The mechanism of inhibition of rat liver microsomal CYP2D2 and purified rabbit CYP2B4 by MCP230 has been shown previously to be noncompetitive with respect to substrate. In this study, MCP230 was shown to competitively inhibit metabolism of 7-benzyl-4-trifluoromethylcoumarin and 7-ethoxyresorufin by the purified, reconstituted rabbit CYP1A2. MCP230 is at least 5- and 50-fold more potent as an inhibitor of CYP1A2 than silica containing 5% copper oxide and silica, respectively. Thus, even though PM generally inhibit multiple forms of P450, PM interacts differently with the forms of P450 resulting in different mechanisms of inhibition. P450s function as oligomeric complexes within the membrane. We also determined the mechanism by which PM inhibited metabolism by the mixed CYP1A2-CYP2B4 complex and found that the mechanism was purely competitive suggesting that the CYP2B4 is dramatically inhibited when bound to CYP1A2.

Keywords

cytochrome P450 1A2; inhibition; particulate matter; environmentally persistent free radicals

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1. Introduction

Combustion processes generate large amounts of particulate matter (PM) in the environment [1–3]. Exposure to fine (< 2.5 μM) and ultra-fine (< 0.1 μM) PM can penetrate the lower airways of the lungs from which they can enter the systemic circulation [4–6] and have been associated with oxidative stress [7], deleterious immune responses [8], pulmonary infirmities [9], and cardiac morbidities [10;11].

Environmentally persistent free radicals (EPFRs) represent a type of PM that is generated after combustion of a mixture of redox-active metal, aromatic hydrocarbon, and inert PM. After combustion and cooling below 600°C, the aromatic hydrocarbons chemisorb to the PM through transition metal oxides and form resonance-stabilized, semiquinone-type free radicals that have half-lives as long as several weeks in ambient air [12;13]. EPFRs have been shown to be capable of redox cycling with molecular oxygen [14] and act as a source of reactive oxygen species in biological systems [15;16]. As with other types of PM, EPFRs have been shown to trigger inflammation [17;18] and abnormal immune responses [19]. They also have been shown to cause cardiac damage in rats [20;21]. Thus, EPFRs may be an environmental contaminant that can have prolonged, deleterious effects in living systems by acting as a source of reactive oxygen species.

Cytochromes P450 (P450/CYP) represent a ubiquitous superfamily of enzymes that catalyze the mixed-function oxygenation of both endogenous and foreign compounds [22]. To carry out these reactions, P450s use electrons provided by redox protein partners [23;24]. For the P450s in mammalian liver, these electrons are delivered primarily by interactions with the NADPH cytochrome P450 reductase (CPR). P450s are widely expressed across species and are variably expressed in tissues throughout the body [22]. The forms representing the gene families 1–3 are mostly involved in the metabolism of xenobiotics and are essential for the elimination and disposition of most hydrophobic compounds [25].

Recently, we showed that EPFRs are potent inhibitors of cytochromes P450-mediated metabolism relative to other types of PM [26;27]. Using fine (≈0.2 μm in diameter) EPFRs of defined composition prepared by the combustion of silica containing 5% (w/w) copper oxide and vapors of either 1,2-dichlorobenzene (DCB230) or 2-monochlorophenol (MCP230), it was shown that EPFRs inhibited metabolism by six different P450s in rat liver microsomes but did not inhibit the ability of CPR to reduce cytochrome c or metabolism mediated by heme oxygenase 1 [26]. As P450 inhibitors, EPFRs were at least 10 times more potent than other types of PM such as free silica, silica containing 5% copper oxide, and silica exposed to aromatic hydrocarbons at lower temperatures (DCB50 and MCP50) that did not result in chemisorption of the organics to the silica. When metabolism by microsomal rat CYP2D2 [26] and purified rabbit CYP2B4 [27] were measured as a function of substrate concentration, the EPFRs and other types of PM were shown to inhibit the reactions noncompetitively. Furthermore, when metabolism by CYP2B4 was measured as a function of the CPR concentration in the presence of a saturating concentration of substrate, the EPFRs were shown to inhibit the P450-mediated metabolism competitively [27], demonstrating that the EPFRs inhibited CYP2B4 metabolism by physically interfering with

the binding of CPR to the P450, and in turn, preventing the flow of electrons needed for P450-mediated catalysis. Because P450s are needed for the metabolism and elimination of most hydrophobic xenobiotics, individuals exposed to EPFRs may be at altered risk from the harmful effects and toxicity of environmental pollutants.

The P450s from the CYP1 family are induced in various tissues following activation of the aryl hydrocarbon receptor. Not coincidentally, the majority of substrates of these enzymes are aryl hydrocarbons. CYP1A2 is highly expressed in liver but has also been identified in tissues affected by exposure to PM. Although its expression in human pulmonary tissues has been controversial {Raunio, 1998 3003 /id}{Wei, 2002 3000 /id}{Wei, 2001 3001 /id}, a published method to immunochemically distinguish CYP1A2 from the closely related CYP1A1 clearly indicated expression of the former in all of the lung samples examined, whereas CYP1A1 was expressed in some but not all of the tested lung samples {Bernauer, 2006 3002 /id}. Furthermore, this study showed that only 70% of 7-ethoxyresorufin metabolism (a probe reaction for CYP1A activity) could be inhibited by an antibody specific for CYP1A1. CYP1A2 has also been shown to be expressed in human endothelial tissue from the endocardium and coronary vessels {Minamiyama, 1999 3004 /id}. Furthermore, its importance in heart function has been implicated by a study showing that a polymorphism of CYP1A2 is associated with increased risk of myocardial infarction {Cornelis, 2004 3006 /id}, and its expression is inversely related with the incidence of heart failure {Frye, 2002 3007 /id}.

Rabbit CYP1A2 is also interesting because it has a tendency to form complexes with itself {Reed, 2012 2662 /id} and other P450s {Backes, 1998 1482 /id}{Reed, 2010 2295 /id}{Kelley, 2006 2032 /id}, and these P450-P450 interactions modify the catalytic activities of the enzymes. Thus, the interaction of CYP1A2 with other P450s may have important ramifications on xenobiotic metabolism and disposition. The most studied P450-P450 interaction is that of CYP1A2 and CYP2B4 which causes stimulation of CYP1A2 activity and inhibition of that by CYP2B4.

Because of the presence of CYP1A2 in cardiovascular and pulmonary tissue and the ability of the P450 to form mixed complexes with CYP2B4, the PM-related inhibition of metabolism by purified, rabbit CYP1A2 and the mixed CYP1A2-CYP2B4 complex was measured in this study. Unlike the other P450 reactions, the EPFR (MCP230) and other types of PM inhibited CYP1A2-mediated metabolism competitively. Metabolism by a mixed CYP1A2-CYP2B4 complex also was competitively inhibited by PM supporting the existing hypothesis that CYP1A2 and CYP2B4 form a heteromeric complex, and that CPR primarily interacts with the CYP1A2 moiety of this complex at subsaturating CPR. As seen in our previous studies examining the interaction of P450 with PM, EPFRs were much more potent than other PM in inhibiting the catalytic activity of CYP1A2. Our study shows that although EPFRs (and other PM) generally inhibit numerous P450s, PM inhibits CYP2B4 and CYP1A2 by different mechanisms.

2. Methods

2.1 Chemicals

The reagents used were of the highest commercial quality available. TWEEN 80, HEPES sodium salt, EDTA, potassium phosphate, NADPH, sodium chloride, glycerol, dimethyl sulfoxide, resorufin, and dilauroylphosphatidylcholine (DLPC) were purchased from Sigma (St. Louis, MO). 7-hydroxy-4-trifluoromethylcoumarin was purchased from Molecular Probes (Eugene, OR). 7-ethoxyresorufin (7ERF) and 7-ethoxy-4-trifluoromethylcoumarin (7EFC) were purchased from Anaspec (Fremont, CA). 7-benzyloxy-4-trifluoromethylcoumarin (7BFC) was purchased from BD Gentest (Woburn, MA).

2.2. Particle Preparation

Fumed Silica (CAB-O-SIL EH-5) was purchased from Cabot Corporation (Billerica, MA) and was used to make silica impregnated with 5% copper oxide (w/w) (CuO-Si); MCP230; and silica with physisorbed MCP (MCP50) as described previously [13]. Briefly, silica was first impregnated with copper nitrate hemipentahydrate by incubation in a 0.1M solution for 24 hours at room temperature. The impregnated silica was then dried at 120°C for 12 hours and subsequently heated for 5 hours in air at 450°C to complete the calcination process. The prepared particles were placed in vacuum ($<10^{-2}$ torr) and heated to 230°C before being dosed with vapors of the organic constituents at 10 torr in a custom-made vacuum exposure chamber for 5 min. The samples were cooled to room temperature and evacuated for 1 hr (10^{-2} torr). The radical contents of the EPFRs were analyzed by electron paramagnetic resonance spectroscopy as described previously [14], and the samples were then weighed in 15 mg portions and sealed in ampoules under vacuum. Only EPFRs that contained greater than 1×10^{17} spins/g were used in the experiments described herein and were used within one week after synthesis. The PM suspensions were previously characterized by flow cytometry and transmission electron microscopy and were shown to consist of disaggregated particles approximately 200 nm in diameter [15]. The zeta potentials of the CuO-Si and MCP230 used in this study were measured as −9.32 mV and −3.83 mV, respectively.

2.3 Enzymes

CYP1A2 was purified from the livers of rabbits treated with β-naphthoflavone as described previously [28]. Rabbit CYP2B4 and CPR were expressed in *E.* coli and purified as described previously [29;30].

2.4 Enzymatic assays

Before assaying for activities, the CYP1A2 and CPR were pre-incubated at a P450 concentration greater than 5 μM with an amount of DLPC that was equal to 160 times the amount of P450. Before adding to the enzymes, the DLPC was bath-sonicated to clarity in an 8 mM solution containing 0.05 M HEPES (pH 7.5), 100 mM NaCl, 20% glycerol, and 0.1 mM EDTA. The enzymes and lipid were incubated for 2 hours at room temperature before adding other reaction components and performing the assays. Unless otherwise indicated, the assays were performed at 0.1 μM CYP1A2, 0.2 μM CPR, and 16 μM DLPC.

When the CYP2B4- and CYP1A2-mediated metabolism of 7EFC was assayed in mixed reconstituted systems, the CYP2B4 was tested at a concentration of 0.1 μM, whereas the concentration of CYP1A2 was 0.3 μM. The CPR concentration was 0.05 μM in all of these reactions. The DLPC concentration was present at a 160-fold excess to the total P450 present in the assay. Simple systems containing only one of the P450s were tested at the same enzyme concentrations as those used in the assays with the mixed systems in order to directly compare the rates of metabolism by the simple and mixed systems. 7EFC was added at the indicated concentrations by diluting 100-fold from stocks prepared in methanol (7EFC).

Each type of PM was added to the assay mixtures from a suspension in 0.9% NaCl containing 0.02% TWEEN 80 (v/v). The pH of the saline/TWEEN 80 solution was adjusted to > pH 7.0 with 1% sodium bicarbonate before adding to the PM. The nanoparticle suspensions were prepared at a concentration of 2 mg/ml by vortexing vigorously for one minute followed by probe sonication (15 watts) on ice for four × 30 sec cycles with 30 sec intervals between each sonication. The suspension was then added to assay mixtures at the desired concentrations (indicated in Results). The PM suspensions were vortexed intermittently to keep the particles from settling before they were added to the reaction mixtures. The effects of the PM were determined by comparison to assays without PM that contained equal concentrations of the saline/TWEEN 80 solution used to suspend the particles.

The *O*-dealkylation reactions (0.1 ml volume) of 7BFC, 7EFC, and 7ERF mediated by the P450 reconstituted systems were measured at 37°C in a buffer containing 0.05 M HEPES (pH 7.5) and 15 mM $MgCl_2$ and were initiated by the addition of 0.01 ml of 0.4 mM NADPH. The reactions were monitored in real-time by measuring the fluorescence associated with the formation of 7-hydroxy-4-trifluoromethylcoumarin (7HFC) and resorufin, respectively (7HFC: 410 nm - excitation; 510 nm – emission; resorufin: 535 nm - excitation; 585 nm - emission). The rates of product formation for the 7BFC (and 7EFC) and 7ERF reactions were quantified by reference to standard curves for 7HFC and resorufin, respectively. Rates of metabolism were observed to be linear over the course of the reactions. Furthermore, the mechanism of inhibition by the EPFR, MCP230, was identical to that by other types of PM, including the relatively inert PM, silica. These facts indicate that the different types of PM do not inhibit by mechanism-based inactivation.

Enzyme kinetics were performed by fitting the data obtained by measuring the rates of metabolism as a function of substrate concentrations to best-fit curves using nonlinear regression using Prism v 5.02 from GraphPad Software (San Diego, CA). The data were analyzed using a nonlinear regression of kinetic data, which were used for calculation of the K_m and V_{max} values. The kinetic data were by transformed by plotting 1/rate of metabolism as a function of 1/substrate concentration. This transformation was done for illustrative purposes. Using this transformation, the mechanisms of inhibition by PM were readily visualized using the the point of intersection of the family of lines derived from the kinetic data generated at different PM concentrations.

3. Results

3.1 Inhibition of CYP1A2 activity by different types of PM

Each type of PM was tested over a range of concentrations for the ability to inhibit the rate of 7BFC *O*-dealkylation mediated by CYP1A2 (Fig 1). In these assays, the CPR concentration was half that of CYP1A2 in order to compare PM effects under conditions that simulate the limiting concentrations of CPR observed *in vivo* [31;32]. The substrate was tested at the approximate K_m for CYP1A2 (2.5 μM). Similar to the findings with CYP2B4-mediated metabolism [27], MCP230 (IC50 = 0.008 mg/ml) was about 10-fold more potent as an inhibitor than CuO-Si (IC50 = 0.06 mg/ml). The reaction was completely inhibited by MCP230 at a concentration of 0.03 mg/ml, whereas CuO-Si completely inhibited the reaction at a concentration of 0.2 mg/ml. Silica and MCP50 (IC50s = 0.48 and 0.41 mg/ml, respectively) seemed equally potent in inhibiting the reaction, and the two types of PM may only be partial inhibitors of the reaction (see below). Compared to its ability to inhibit CYP2B4-mediated metabolism (IC50 = 0.018 mg/ml), MCP230 was more potent in inhibiting metabolism by CYP1A2 (IC50 = 0.008 mg/ml) [27].

3.2 PM inhibits CYP1A2 competitively as a function of substrate

The metabolism of 7BFC mediated by CYP1A2 was measured over a range of substrate concentrations in the presence of different concentrations of MCP230 (Fig 2), CuO-Si (Fig 3), and MCP50 (Fig 4) in order to determine the mechanism of inhibition by each type of PM. For these experiments, the CPR concentration was in excess to that of P450 (2:1), so that the inhibition by PM was not obfuscated by limiting CPR. The B panels of each figure show the double reciprocal plots derived from the corresponding, untransformed data from panel A. The double reciprocal plots in Figs 2–4 show that the best-fit regressions pass through a common point on the ordinate of the graphs indicating that each type of PM is a pure, competitive inhibitor of CYP1A2 with respect to substrate. These effects are in stark contrast to those observed with CYP2B4-mediated metabolism as each type of PM was characterized as a pure, noncompetitive inhibitor [27]. The physisorbed MCP50 (Fig 4) (and presumably silica) was a partial inhibitor of the reaction, similar to our previous findings regarding the inhibition of CYP2B4 by these PM [27]. However, the inhibition of CYP1A2 by these particles seems to be even more limited than that by CYP2B4 as the inhibition of CYP1A2-mediated activity by MCP50 did not exceed 35% (when the CPR concentration was saturating) even at a particle concentration of 0.5 mg/ml. From the data in Figs 2–4, the K_i constants for MCP230, CuO-Si, and MCP50 were calculated to be 2.7 μg/ml, 19 μg/ml, and 166 μg/ml, respectively. These data also indicate that MCP230 is a much more potent inhibitor of CYP1A2-mediated metabolism than the other PM.

To ensure that the results were not attributable solely to the substrate used, the inhibition of CYP1A2-mediated metabolism of 7ERF by MCP230 was also characterized (Fig 5). Consistent with the findings using 7BFC as a substrate, MCP230 also competitively inhibited metabolism of 7ERF by CYP1A2, and the K_i for this reaction was 1.3 μg/ml. Thus, the data indicate that CYP1A2 interacts with PM in a manner that leads to competitive inhibition of its metabolism.

3.3 Effect of bovine serum albumin on inhibition of P450s by EPFRs

Our previous study examining the effects of PM on CYP2B4-mediated activity showed that PM noncompetitively inhibited metabolism by this P450 with respect to the substrate [27]. Furthermore, we provided evidence that the noncompetitive inhibition stemmed from direct competition between the PM and the CPR for binding to the CPR-binding site of CYP2B4. In combination with our data from this study showing that PM competitively inhibit substrate binding to CYP1A2, it appears that PM specifically interact with different parts of P450s in a form-dependent manner. In order to assess the specificity of PM interactions with P450, we compared the inhibition of CYP1A2- and CYP2B4-mediated activities by 0.025 mg/ml MCP230 in the absence and presence of 0.2 mg/ml bovine serum albumin (BSA). It might be expected that the high concentration of BSA would also bind the EPFR if the PM binds non-specifically, generally adsorbing to the protein surface. If this were the case, BSA would be expected to attenuate the PM-mediated inhibition of the P450-mediated activities. Figure 6 shows that MCP230 remained an effective inhibitor both in the absence and presence of 0.2 mg/ml BSA. Although BSA had no significant effect on the uninhibited rates for either P450, BSA did affect the degree of inhibition by MCP230. Inhibition of CYP2B4-mediated 7EFC metabolism was attenuated by BSA which may be attributable to non-specific adsorption of EPFR to BSA; however, inhibition of CYP1A2 by the EPFR was actually exacerbated by the presence of BSA.

3.4 PM competitively inhibit metabolism by the mixed CYP1A2-CYP2B4 complex

P450 enzymes have been shown to function as oligomeric complexes in membranes [33–35] and in reconstituted systems (reviewed in [36;37]). From the extensive study of the specific interaction between CYP1A2 and CYP2B4 [38–40], it has been determined that CYP1A2-mediated activity is stimulated whereas that by CYP2B4 is inhibited when the two P450s interact in the presence of a limiting concentration of CPR in the reactions. Typically, when assays are performed with equal concentrations of CYP1A2 and CYP2B4 and a CPR concentration equal to half of the total P450 concentration, the CYP2B4-mediated activity is inhibited 20% to 50%, depending on the substrate, whereas the CYP1A2-mediated activity is stimulated more than 60% relative to the P450 activities measured in the absence of the alternate P450 [40]. These results were shown to be consistent with a model where CYP1A2 and CYP2B4 form a heteromeric complex, with CPR binding selectively to the CYP1A2 moiety of CYP1A2•CYP2B4 complex [39].

The different mechanisms by which PM inhibits CYP1A2- and CYP2B4-mediated activities afford a unique opportunity to elucidate the catalytic behavior of the mixed CYP1A2-CYP2B4 complex. Our previous studies could not discern whether the decrease in CYP2B4-mediated activity in the presence of CYP1A2 reflected limited or abolished catalytic activity by the CYP2B4 when it was bound in a mixed complex with CYP1A2. It is possible that the remaining 50 to 80% of CYP2B4 activity observed in the presence of CYP1A2 (discussed above) may have represented that by homomeric CYP2B4 complexes and/or monomeric CYP2B4 that were present in a physical equilibrium with the CYP1A2:CYP2B4 heterocomplex when metabolism was measured at a 1:1 CYP2B4:CYP1A2 ratio.

The effects of PM on P450-mediated activities in this study and in our previous study were assessed in the presence of an excess of CPR in order to simplify the kinetic analysis when substrate concentrations were varied. The alterations in metabolism by CYP1A2 and CYP2B4 that are caused by the physical interaction of the enzymes are only manifest when the CPR concentration is limiting. In order to confirm that PM inhibit CYP1A2 and CYP2B4 by different mechanisms when there is a rate-limiting concentration of CPR, we tested the effects of CuO-Si on both the CYP2B4-mediated metabolism of 7-ethoxy-4-trifluoromethylcoumarin (7EFC) and the CYP1A2-mediated metabolism of 7-benzyloxy-4-trifluoromethylcoumarin (7BFC) at a subsaturating 1:2 CPR:P450 ratio. We chose CuO-Si for these experiments because the PM is more easily generated and is more stable than the EPFR, MCP230, allowing for comparisons of different conditions to be made more easily. The data in Figure 7 show that the CuO-Si also inhibited CYP1A2-mediated metabolism of 7BFC competitively (Panel A) and CYP2B4-mediated metabolism of 7EFC noncompetitively (panel B) when the CPR concentration was limiting [27].

Using a CYP2B4-selective substrate, 7EFC, we were able to assess the relative contribution of each P450 to overall metabolism by the mixed P450 system by determining the mechanism of inhibition by PM. Figure 8 shows the effects of CuO-Si on metabolism of 7EFC mediated by the mixed CYP2B4/CYP1A2 reconstituted system. Panel 8A also shows the rates of metabolism by simple reconstituted systems containing either CYP1A2 or CYP2B4 for comparison to those by the mixed system. For these experiments, CYP1A2, when present, was assayed at 3-fold greater concentration than CYP2B4, so that any metabolism by CYP2B4 would likely represent that by the mixed CYP1A2-CYP2B4 complex because equilibrium would favor the formation of the mixed P450 complex over monomeric CYP2B4 and homomeric CYP2B4 complexes. In addition, the CPR concentration was kept constant for all of the conditions, and was present at half the concentration of CYP2B4 to favor conditions that have been shown to cause alterations in the activities of the P450s in the mixed complex [39].

The results show that the rates of metabolism of this substrate by CYP1A2 alone are much less than those by CYP2B4 even though the former was present at three-fold greater concentration. The rates of metabolism of the CYP2B4-selective substrate by the mixed system were approximately $1/10^{th}$ the rates observed with the system containing CYP2B4 alone. Although the rates are greater than those observed for the system containing only CYP1A2, the catalytic activity of this P450 is increased in the presence of CYP2B4 {Backes, 1998 1482 /id}{Reed, 2010 2295 /id}, so some of the increase in activity over that by the simple CYP1A2 system is likely attributable to CYP1A2-mediated metabolism. As would be expected for a CYP1A2-mediated reaction, the mechanism by which CuO-Si inhibits metabolism of the CYP2B4-selective substrate by the mixed system is competitive (Figure 8B).

4. Discussion

The identification of the adverse health effects related to the exposure to combustion-derived PM is an area of intensive research. It has been established that exposure to EPFRs and to a lesser extent other PM can lead to cardiovascular and pulmonary maladies caused

by inflammation and oxidative stress [15;20;21] Recent studies have also demonstrated that different types of PM can inhibit P450-mediated activities. The PM-related inhibition of a number of forms of P450 have been studied using ultra-fine (≈10 nm in diameter) gold and silver nanoparticles [41], ultra-fine to fine (20 nm to 200 nm) carboxyl polystyrene PM [42], fine (≈ 200 nm) silica, CuO-Si, MCP50, MCP230, DCB50, and DCB230 [26;27].

Various hypotheses have been postulated to explain the effects of PM on P450-mediated activities. In the study with gold and silver nanoparticles, it was suggested that the nanoparticles disrupted the membrane environment of the P450s and disrupted its interaction with CPR [41]. The study using carboxyl polystyrene PM hypothesized that the disruption of the membrane by PM may impair the interaction of hydrophobic substrates with P450 [42]. Although plausible, the outcomes suggested by these studies were speculative. Our studies have been the first to characterize the mechanism of P450 inhibition by PM using enzyme kinetics [26;27]. In previous studies, we determined that the activities of both rat liver, microsomal CYP2D2 [26] and purified rabbit CYP2B4 [27] were inhibited noncompetitively by EPFRS. In the more detailed study using purified CYP2B4, we showed that other types of PM also inhibited this enzyme noncompetitively as a function of substrate concentration. However, when EPFR inhibition of CYP2B4-mediated activities was measured as a function of CPR concentration, the inhibition was competitive, demonstrating the inhibitory effect of the PM was to disrupt the interaction between CPR and CYP2B4 [27].

Interestingly, the PM-related inhibition of CYP1A2-mediated activity examined in this study was purely competitive with respect to substrate indicating that the major effect of PM was to prevent substrate binding to the active site of the P450. As observed in the previous studies from our lab, the EPFR, MCP230, was a much more potent inhibitor than other types of PM (silica, MCP50, and CuO-Si). Clearly, EPFRs interact with P450s with greater affinity than other types of PM. We also showed that CuO-Si was a more potent CYP1A2 inhibitor than silica and MCP50. The chemical properties of the EPFRs that are responsible for its ability to potently inhibit P450-mediated metabolism, in comparison to other types of PM, are not understood.

It is intriguing that PM inhibits CYP2B4 and CYP1A2 by different mechanisms. If the suggestion [41;42] that a mechanism of P450 inhibition involving membrane disruption by PM is valid, it seems that both CYP1A2 and CYP2B4 would be inhibited by similar processes; however, this does not appear to be the case as both activities are clearly inhibited by distinct mechanisms. The hydrophobicity of the substrates used for CYP1A2 and CYP2B4 also cannot explain the different mechanisms of inhibition because 7ERF used as a substrate for CYP1A2 in this study is less hydrophobic than the 7-benzyloxyresorufin used as a CYP2B4 substrate in our previous study. Thus, the CYP1A2 substrates are not necessarily more hydrophobic and their binding to P450 would not be more likely to be impaired by membrane disruption. The effects might reflect the general disruption of the membrane by PM if the CYP1A2-mediated metabolism was limited by substrate binding and that by CYP2B4 was limited by electron transfer. However, this does not appear to be the case for CYP1A2-mediated metabolism [43]. Our finding that 0.2 mg/ml BSA only moderately attenuated the inhibition of CYP2B4-mediated metabolism and had no effect on

the inhibition of metabolism by CYP1A2 also suggests that PM selectively bind to P450s relative to other types of proteins. Other evidence for this assumption is the fact that comparable concentrations of PM did not inhibit the activity of heme oxygenase 1 [26]. Thus, it seems likely that the PM inhibits by directly and specifically interacting with the P450s. Our data imply that this specificity varies with respect to the part of the P450 bound by PM for different forms of P450. If PM interact directly with P450s, they would appear to selectively bind to the CPR-binding regions of rabbit CYP2B4 and rat CYP2D2 and over the substrate access channel of rabbit CYP1A2.

Using the different mechanisms of CYP1A2 and CYP2B4 inhibition by PM, it was possible to determine the relative contributions of CYP1A2- and CYP2B4-mediated activities to overall metabolism of a CYP2B4-selective substrate by the mixed P450 reconstituted system. More specifically, it can be assumed that the mechanism of inhibition of the mixed system activity by each type of PM would be competitive if CYP1A2 is mainly responsible for metabolism but noncompetitive if metabolism is largely mediated by CYP2B4. If both P450s significantly contribute to metabolism by the mixed P450 complex, the mechanism of inhibition by PM would be expected to be mixed. By showing that the PM competitively inhibited metabolism of a CYP2B4-selective substrate, 7EFC, by the mixed P450 system, we have demonstrated that the catalytic activity by CYP2B4 is dramatically inhibited (≈ 90%) when it is in a complex with CYP1A2. Thus, our findings provide novel insight into the catalytic properties of the mixed CYP1A2-CYP2B4 complex. Our previous studies had shown that CYP2B4-mediated activity was only moderately decreased (20% to 50%) in the presence of equimolar concentrations of CYP1A2 [39;40]. From our results in this study, we can conclude that a large proportion of the CYP2B4-mediated activity observed in the previous studies represented metabolism by monomeric CYP2B4 and/or homomeric CYP2B4 complexes that were also present in equilibrium with homomeric CYP1A2 and heteromeric CYP1A2-CYP2B4 complexes in the mixed reconstituted systems.

It is worth considering our results with respect to the size of PM tested. All of the PM used in our experiments were in the fine range (≈ 200 nm in diameter). It is conceivable that higher levels of P450 inhibition would have been attained using smaller particles as the previous study with carboxyl polystyrene nanoparticles showed that the smallest particles tested (20 nm in diameter) were the most inhibitory when its effects were tested on the activities of human CYP1A2, CYP2C9, CYP2D6, and CYP3A4 {Frohlich, 2010 2738 /id}. Interestingly, the carboxyl polystyrene nanoparticles that were 200 nm in diameter (the size used in this study) had no effect on metabolism mediated by human CYP1A2 even when tested at concentrations as high as 0.2 mg/ml. In addition to the possibility that smaller particles would be more inhibitory, ultrafine nanoparticles (<100 nm) have been shown to penetrate deeper into respiratory airways and reach higher concentrations in the circulatory system after inhalation {Chen, 2006 1912 /id}. This suggests that finer particles also would be more likely to have an impact on P4540s *in vivo* at the same levels of exposure.

The question of toxicological relevance with regards to the concentrations of PM tested in *in vitro* studies is always an important consideration when assessing the implications of the findings. Although precise determinations of the concentrations of PM in the circulation after inhalation are inscrutable, they are likely to be much lower than 0.1 mg/ml

{Oberdorster, 2005 2743 /id}. Significant inhibition of CYP1A2 was measured at a MCP230 concentration of 0.005 mg/ml. Thus, if the PM can be concentrated over time in cells, it is possible that MCP230 might influence drug metabolism following inhalation and passage into the systemic circulation. However, it is unlikely that the other types of PM would reach the levels necessary to inhibit CYP1A2 under normal conditions of exposure. Despite this limitation to the findings, the conditions used in our study are consistent with most in vitro studies examining the effects of PM {Frohlich, 2010 2738 /id}{Sereemaspun, 2008 2737 /id}{Watterson, 2009 3008 /id}{Veronesi, 2002 3009 /id}{Diabate, 2011 3010 /id}{Balakrishna, 2009 2747 /id}.

The inhibition of P450s by PM is a relatively new consideration with regards to the adverse health effects of PM. PM are generated by combustion processes and are prevalent in industrial areas. A study that collected ambient air samples in proximity to a highway area and four different Superfund waste sites found that significant levels of particulates, including EPFRs, were collected near all of the sites over a 24 hour interval {Dellinger, 2001 1902 /id}. Because P450-mediated metabolism is often a requisite step in the elimination of many xenobiotics, the concurrent exposure to PM and pollutants would make exposed individuals more susceptible to the harmful effects of the pollutants. More studies are required to elucidate the health consequences of P450 inhibition by PM.

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Abbreviations

DLPC	dilauroylphosphatidylcholine
EPFR	environmentally persistent free radical
CPR	cytochrome P450 reductase
CYP/P450	cytochrome P450
MCP230	EPFR derived from 2-monochlorophenol
MCP50	2-monochlorophenol physisorbed to silica
CuO-Si	silica containing 5% copper oxide (w:w)
PM	particulate matter
7EFC	7-ethoxy-4-trifluoromethylcoumarin
7BFC	7-benzyloxy-4-trifluoromethylcoumarin
7-ERF	7-ethoxyresorufin

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Highlights

- Combustion of organic pollutants generates long-lived particulate radicals (EPFRs).
- Particulate matter (PM) competitively inhibited CYP1A2 activity.
- EPFRs were much more potent CYP1A2 inhibitors than other types of PM.
- PM interact differently with different forms of P450.
- PM competitively inhibited metabolism by the mixed CYP1A2-CYP2B4 complex.

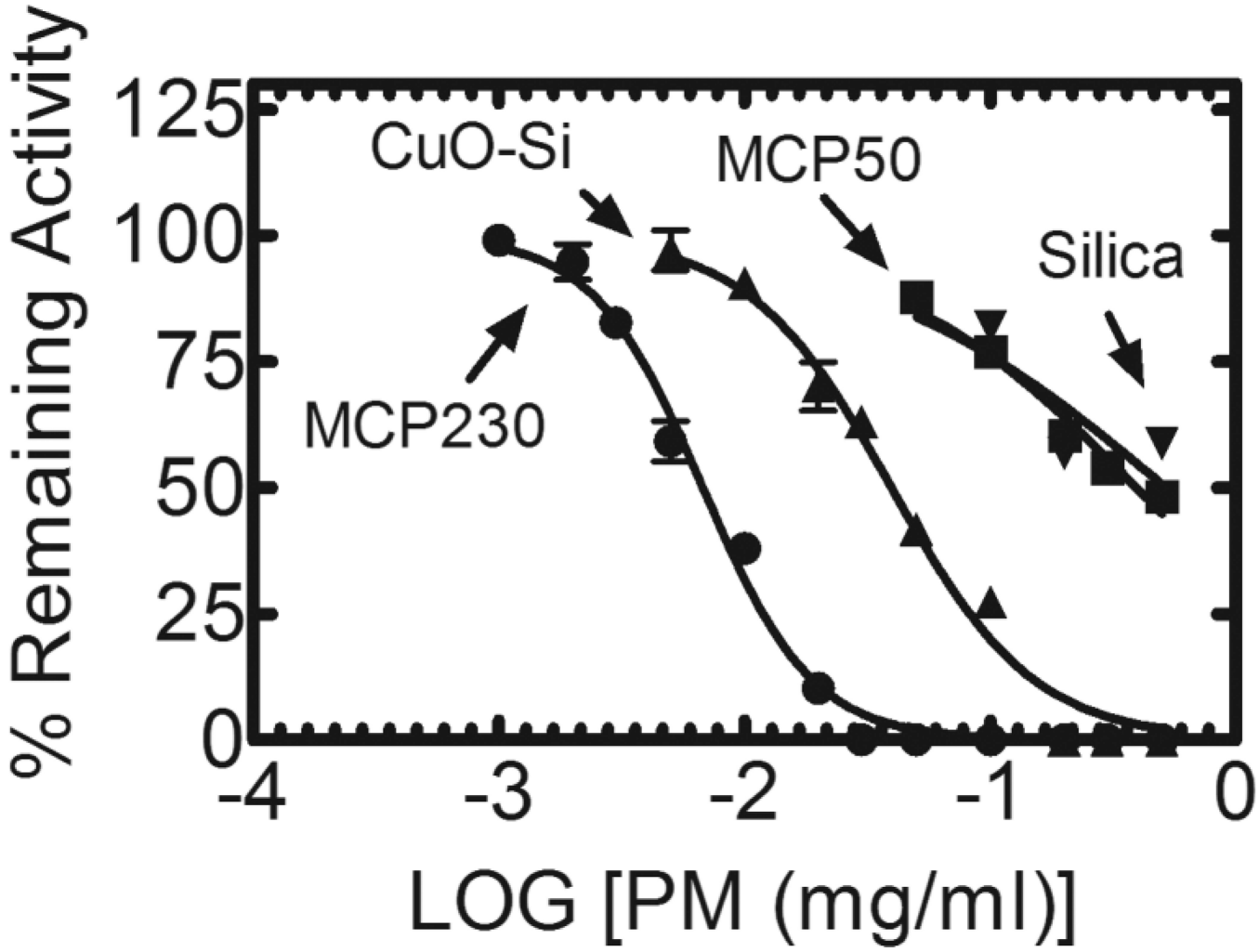

Figure 1. Effects of PM on metabolism of 7BFC by CYP1A2

Reconstituted systems containing 0.1 μM CYP1A2, 0.05 μM CPR, and 16 μM DLPC were prepared and assayed to measure the rate of 7BFC metabolism as described in Materials and Methods. The reactions were performed at 2.5 μM 7BFC which is approximately the K_m for metabolism by CYP1A2 (see figure 2). The relative rates of activity as a function of nanoparticle concentration were expressed as the percentages of the rates measured in the absence of particles. These reactions were performed in the presence of 0.23% saline and 0.005% TWEEN 80. The data points represent the average ± the standard error of three separate determinations. IC50 values were calculated from nonlinear fits through the data using the LOG of the PM concentration versus the normalized response with variable slopes model in Prism v. 5.02 (GraphPad, La Jolla, CA).

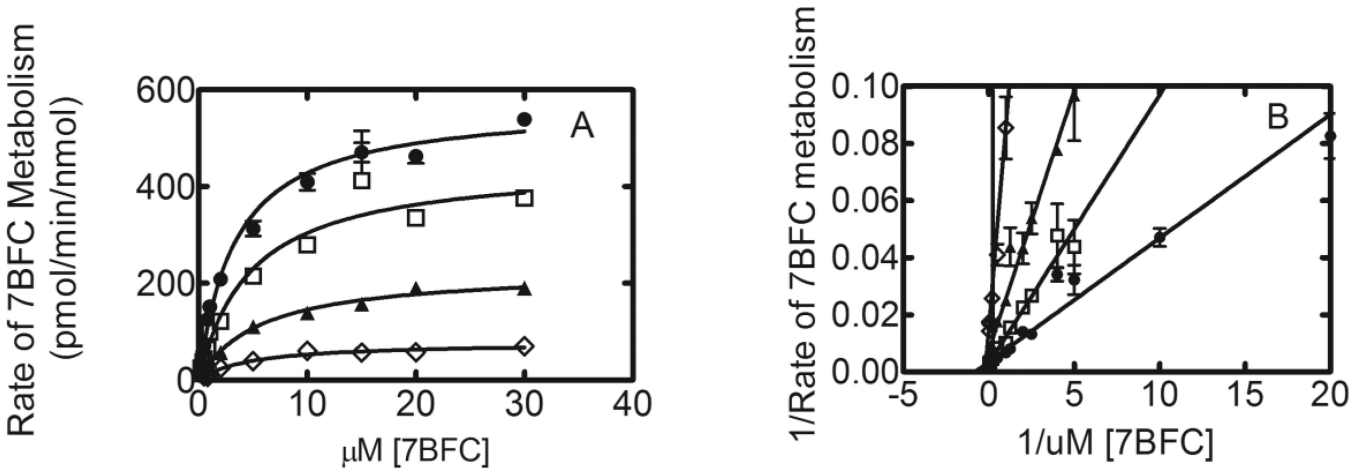

Figure 2. Rates of CYP1A2-mediated metabolism as a function of 7BFC concentration in the presence of different concentrations of MCP230

The rates of 7BFC metabolism by CYP1A2 were determined as described in Materials and Methods. These reactions were performed in the presence of 0.7% saline and 0.015% TWEEN 80. The data points represent the average ± the standard error of at least three separate determinations. The curves in panel A were generated by nonlinear regression as described in Materials and Methods. Panel B shows the double reciprocal plots of the experimental data in addition to the lines derived from taking the reciprocals of the nonlinear regressions shown in panel A. Legend: Saline/TWEEN 80 control, circles; 0.005 mg/ml MCP230, open squares; 0.01 mg/ml MCP230, triangles; and 0.015 mg/ml MCP230, open diamonds.

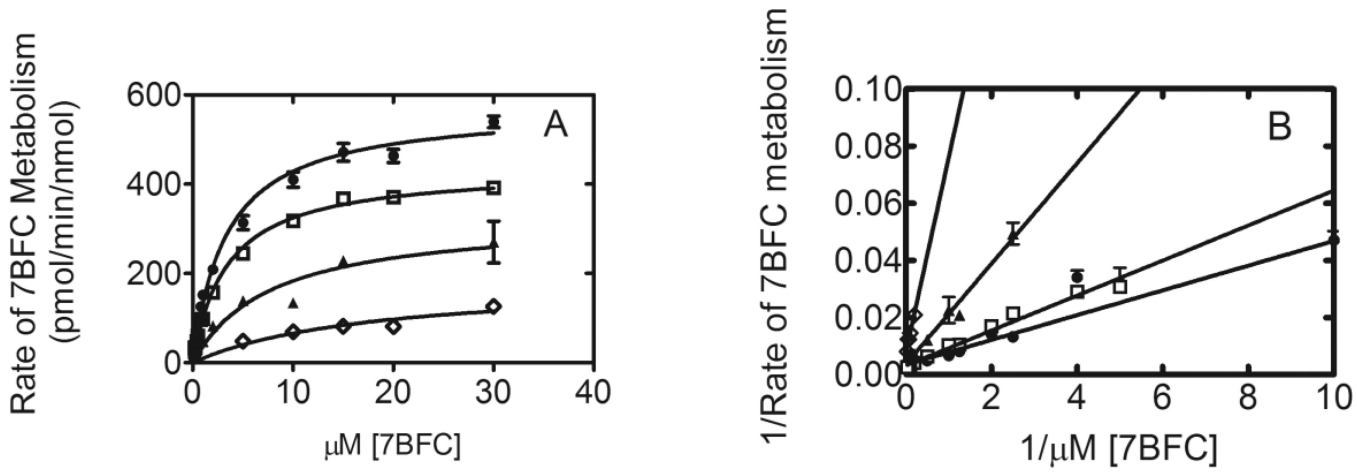

Figure 3. Rates of CYP1A2-mediated metabolism as a function of 7BFC concentration in the presence of different concentrations of CuO-Si

The rates of 7BFC metabolism by CYP1A2 were determined as described in Materials and Methods. These reactions were performed in the presence of 0.7% saline and 0.015% TWEEN 80. The data points represent the average ± the standard error of at least three separate determinations. Panel B shows the double reciprocal plots of the experimental data and the regressions shown in panel A. Legend: Saline/TWEEN 80 control, circles; 0.125 mg/ml CuO-Si, open squares; 0.2 mg/ml CuO-Si, triangles; and 0.3 mg/ml CuO-Si, open diamonds.

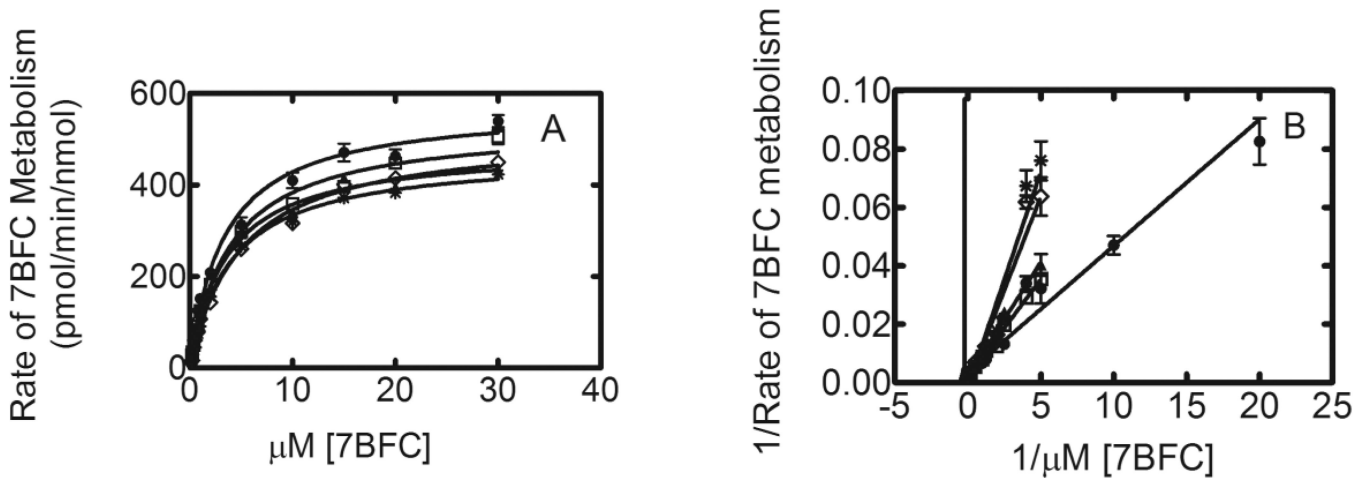

Figure 4. Rates of CYP1A2-mediated metabolism as a function of 7BFC concentration in the presence of different concentrations of physisorbed MCP50

The rates of 7BFC metabolism by CYP1A2 were determined as described in Materials and Methods. These reactions were performed in the presence of 0.7% saline and 0.015% TWEEN 80. The data points represent the average ± the standard error of at least three separate determinations. Panel B shows the double reciprocal plots of the experimental data and the regressions shown in panel A. Legend: Saline/TWEEN 80 control, circles; 0.3 mg/ml MCP50, open squares; 0.4 mg/ml MCP50, triangles; 0.45 mg/ml MCP50, open diamonds; and 0.5 mg/ml MCP50, stars.

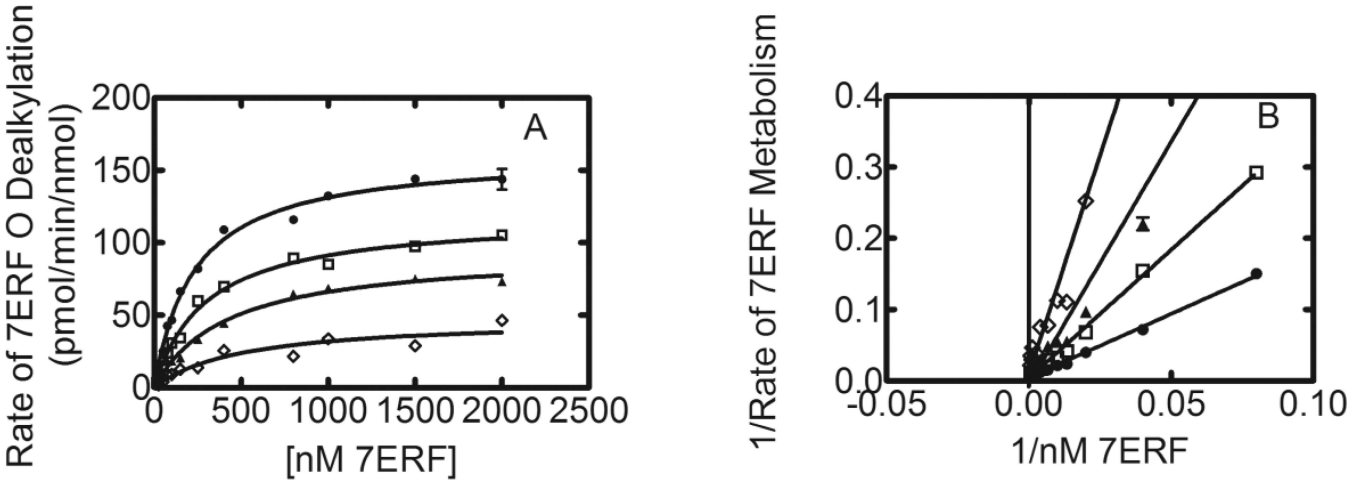

Figure 5. Rates of CYP1A2-mediated metabolism as a function of 7ERF concentration in the presence of different concentrations of MCP230

The rates of 7ERF metabolism by CYP1A2 were determined as described in Materials and Methods. These reactions were performed in the presence of 0.7% saline and 0.015% TWEEN 80. The data points represent the average ± the standard error of at least three separate determinations. Panel B shows the double reciprocal plots of the experimental data and the regressions shown in panel A. Legend: Saline/TWEEN 80 control, circles; 0.005 mg/ml MCP230, open squares; 0.01 mg/ml MCP230, triangles; and 0.015 mg/ml MCP230, open diamonds.

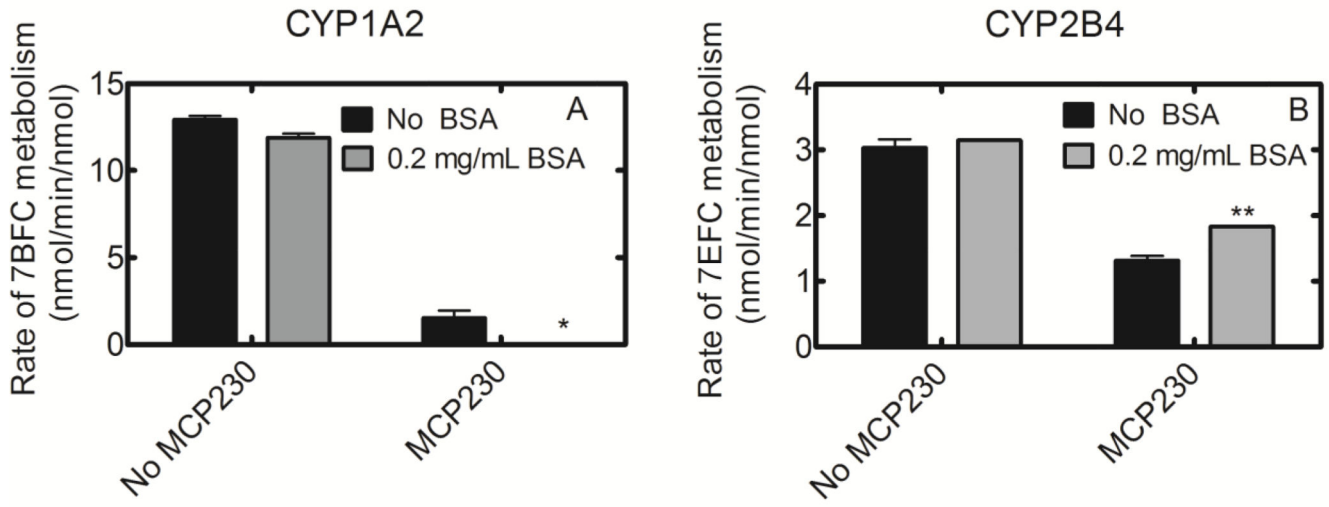

Figure 6. Effect of BSA on the inhibition of P450-mediated metabolism by MCP230

Assays to measure the CYP1A2 mediated metabolism of 7BFC (Panel A) and the CYP2B4-mediated metabolism of 7EFC (Panel B) were performed as described in Materials and Methods. The MCP230 and BSA concentrations (when present) were 0.025 mg/ml and 0.2 mg/ml, respectively. The substrates, 7BFC and 7EFC, were incubated at the K_m values for the two enzymes (2.5 μM and 25 μM, respectively). The data represent the average and the standard error of measurement (error bars) of triplicate determinations.

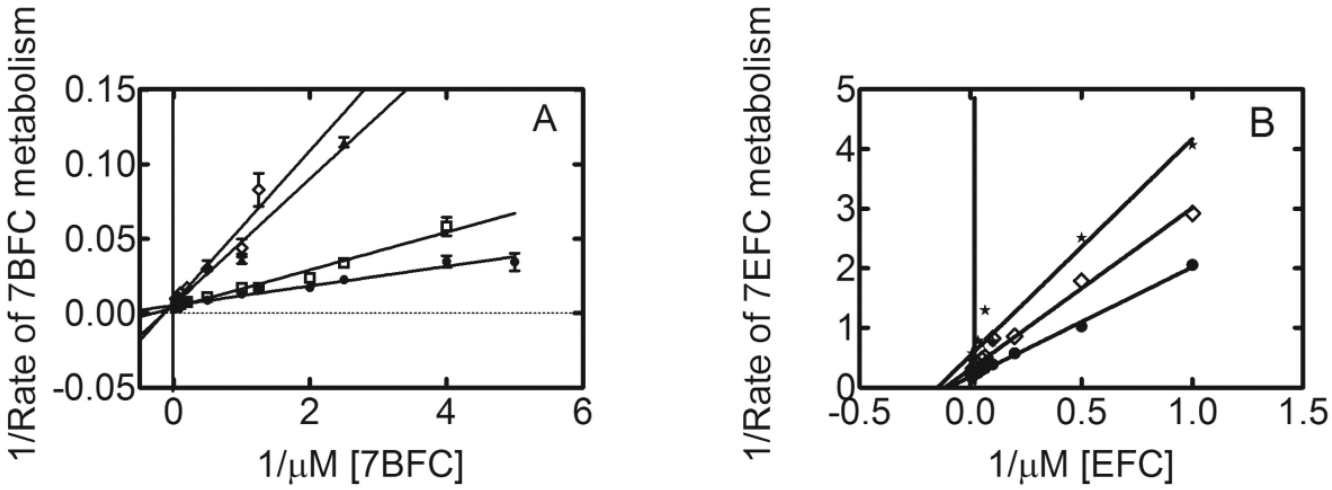

Figure 7. Double reciprocal plots derived from the rates of CYP1A2- (7BFC) and CYP2B4-mediated (7EFC) metabolism in the presence of different concentrations of CuO-Si

Assays to measure the CYP1A2 mediated metabolism of 7BFC (Panel A) and the CYP2B4-mediated metabolism of 7EFC (Panel B) were performed as described in Materials and Methods except the CPR:P450 ratio was 1:2. The data points represent the average ± the standard error of three separate determinations. The double reciprocal plots were generated as described in Materials and Methods and in the caption of figure 2. Legend: Saline/TWEEN 80 control, circles; 0.04 mg/mL CuO-Si, open squares; 0.075 mg/mL CuO-Si, triangles; 0.1 mg/mL CuO-Si, open diamonds; 0.25 mg/mL CuO-Si, closed diamonds.

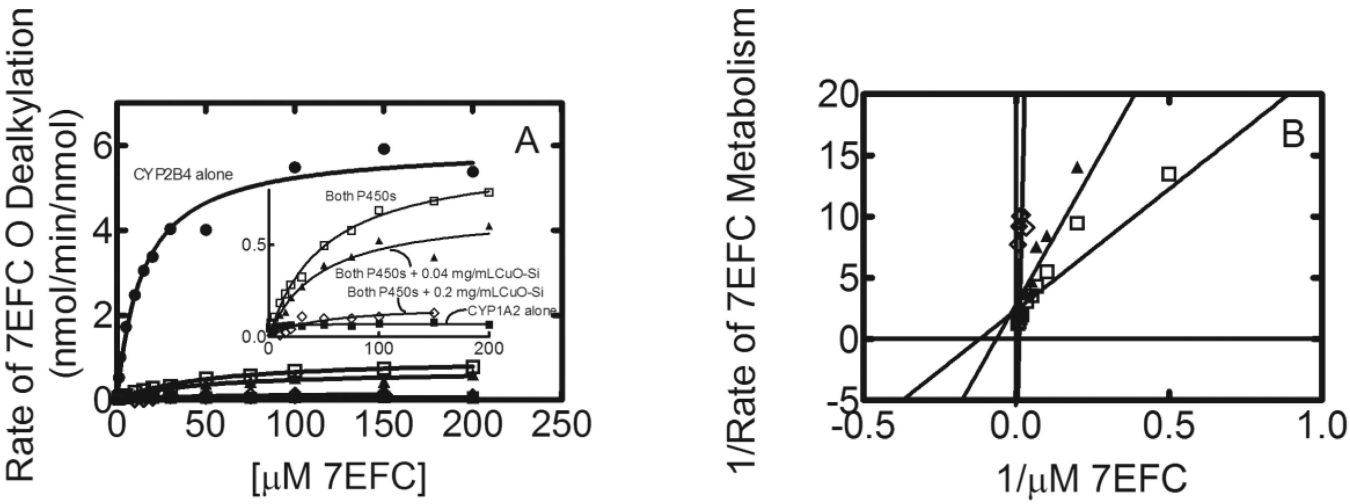

Figure 8. Rates of P450-mediated metabolism by simple and mixed CYP1A2 and CYP2B4 systems as a function of 7EFC concentration in the presence of different concentrations of CuO-Si

Assays to measure the P450-mediated metabolism of 7EFC were performed as described in Materials and Methods. These reactions were performed in the presence of 0.7% saline and 0.015% TWEEN 80. The data points represent the average ± the standard error of at least three separate determinations. The insert in panel A is a magnification of the scale that encompasses the rates of the simple CYP1A2 system and the mixed CYP1A2-CYP2B4 systems in the presence and absence of copper oxide. Panel B shows the double reciprocal plots of the experimental data and the nonlinear regressions for the three conditions using the mixed P450 systems (± CuO-Si) shown in panel A. The data represent the activities associated with assays using reconstituted systems that contained 0.05 μM CPR and a DLPC:P450 ratio of 160:1. The conditions that differed for each set of data are as follows: CYP2B4 alone (tested at 0.1 μM), circles; CYP1A2 alone (tested at 0.3 μM), closed squares; both P450s (tested at the same concentrations as those used in the respective simple systems), open squares; both P450s in the presence of 0.04 mg/mL copper oxide, triangles; and both P450s in the presence of 0.2 mg/mL CuO-Si, open diamonds.