

Environmentally Persistent Free Radicals stimulate CYP2E1-mediated generation of reactive  
oxygen species at the expense of substrate metabolism

George F. Cawley, J. Patrick Connick, Marilyn K. Eyer, and Wayne L. Backes

From the Department of Pharmacology and Experimental Therapeutics, and The Stanley S.  
Scott Cancer Center, Louisiana State University Health Science Center, New Orleans,  
Louisiana, United States.

**Running Title: EPFRs inhibit CYP2E1 by uncoupling of substrate metabolism**

**Corresponding Author:**

Wayne L. Backes, Ph.D.

Department of Pharmacology and Experimental Therapeutics

Louisiana State University Health Sciences Center

533 Bolivar St.

New Orleans, LA 70112

Tel: 504-568-6557

FAX: 504-568-2361

Email: wbacke@lsuhsc.edu

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**Nonstandard Abbreviations** – CYP or P450, cytochrome P450; CYP2E1, cytochrome P450 CYP2E1; POR, NADPH-cytochrome P450 reductase; ROS, reactive oxygen species; CuO-Si, the non-EPFR control particle; MCP230, the laboratory generated EPFR made by heating silica 5% copper oxide, and silica (<0.2  $\mu\text{m}$  in diameter) and 2-monochlorophenol at  $\geq 230^\circ\text{C}$ ; BRET, bioluminescence resonance energy transfer; 7BR, 7-benzyloxyresorufin; PD, protocatechuic acid dioxygenase; Rluc, Renilla luciferase; GFP, green fluorescent protein; DCFH, dichlorofluorescein; DCFH-DA, dichlorofluorescein diacetate; DCF, dichlorofluorescein.

## Abstract

Environmentally persistent free radicals (EPFRs) are a recently recognized component of particulate matter that cause respiratory and cardiovascular toxicity. The mechanism of EPFR toxicity appears to be related to their ability to generate reactive oxygen species (ROS), causing oxidative damage. EPFRs were shown to affect P450 function, inducing the expression of some forms through the Ah receptor. However, another characteristic of EPFRs lies in their ability to inhibit P450 activities. CYP2E1 is one of the P450s that is inhibited by EPFR (MCP230) exposure. As CYP2E1 is also known to generate ROS, it is important to understand the ability of EPFRs to influence the function of this enzyme and to identify the mechanisms involved. CYP2E1 was shown to be inhibited by EPFRs, and to a lesser extent by non-EPFR particles. As EPFR-mediated inhibition was more robust at subsaturating NADPH-cytochrome P450 reductase (POR) concentrations, disruption of POR•CYP2E1 complex formation and electron transfer were examined. Surprisingly, neither complex formation nor electron transfer between POR and CYP2E1 were inhibited by EPFRs. Examination of ROS production showed that MCP230 generated a greater amount of ROS than the non-EPFR CuO-Si. When a POR/CYP2E1-containing reconstituted system was added to the pollutant-particle systems there was a synergistic stimulation of ROS production. The results indicate that EPFRs cause inhibition of CYP2E1-mediated substrate metabolism, yet do not alter electron transfer and actually stimulate ROS generation. Taken together, the results are consistent with EPFRs affecting CYP2E1 function by inhibiting substrate metabolism and increasing the generation of ROS.

**Significance Statement** – Environmentally persistent free radicals affect CYP2E1 function by inhibition of monooxygenase activity. This inhibition is not due to disruption of the POR•CYP2E1 complex or inhibition of electron transfer, but due to uncoupling of NADPH and oxygen consumption from substrate metabolism to the generation of ROS. These results show that EPFRs block the metabolism of foreign compounds, and also synergistically stimulate the formation of reactive oxygen species that lead to oxidative damage within the organism.

## Introduction

Particulate matter is a leading environmental risk factor for toxicity, including respiratory (Fahmy et al., 2010; Balakrishna et al., 2011; Saravia et al., 2013; Jaligama et al., 2018), cardiovascular (Dugas et al., 2016; Feng et al., 2023), immune (Wang et al., 2011; Lee et al., 2014; Marchini, 2023), and endocrine systems (Saleem et al., 2024). Based on epidemiologic studies, the smaller particulates are capable of more deeply penetrating the lower airways and alveoli (Saravia et al., 2013; Marchini, 2023), and potentially entering the general circulation (Nemmar et al., 2002; Marchini, 2023). The mechanisms of toxicity include effects on redox mechanisms, and inflammation, in addition to more direct effects of the chemical pollutants, such as polycyclic hydrocarbons, associated with particulate matter. Environmentally persistent free radicals (EPFRs) are a recently identified component of particulate matter (Dellinger and Taylor, 1998; Dellinger et al., 2000; Cormier et al., 2006; dela Cruz et al., 2011). EPFRs are the result of incomplete combustion, leading to organic chemicals that combine with metals and particulates to produce an agent that is stable in the environment. However, when the EPFRs enter an organism, they are capable of redox cycling and ROS generation. In addition to their ability to cause oxidative damage, they also act through the aryl hydrocarbon receptor (AhR). EPFRs have been shown to activate AhR, leading to induction of CYP1A1 and CYP1B1, increase ROS generation, and stimulate the pulmonary immune response (Jaligama et al., 2018).

Cytochromes P450 are enzymes responsible for the oxidation of a wide variety of both exogenous and endogenous substrates, with several of the forms capable of uncoupling and leading to the formation of reactive oxygen species (ROS) (Hrycay and Bandiera, 2015; Albertolle and Guengerich, 2018; Harjumaki et al., 2021). CYP2E1 is an important and interesting P450 enzyme. It metabolizes small hydrophobic substrates that comprise many organic pollutant mixtures. It also is involved in the metabolism and bioactivation of many

carcinogenic chemicals such as benzene, nitrosamines, and chloroform (Harjumaki et al., 2021). Consequently, there are several reasons for interest in how CYP2E1 responds to EPFR exposure.

CYP2E1 is distinctive in that it is found in the high spin state, and readily able to accept electrons from NADPH-cytochrome P450 reductase (POR), even in the absence of substrate (Harjumaki et al., 2021). As a result, CYP2E1 is known to shift oxygen consumption from substrate metabolism to the generation of reactive oxygen species such as hydrogen peroxide and superoxide, which can lead to increased oxidative damage (Hrycay and Bandiera, 2015; Albertolle and Guengerich, 2018). This process is referred to as uncoupling of NADPH and oxygen consumption from monooxygenation to the generation of ROS.

In previous reports, EPFRs (Harmon et al., 2018; Jalgama et al., 2018; Aryal et al., 2023) and PM (Abbas et al., 2009; Hasan et al., 2020) have been shown to elevate the amounts of numerous P450 enzymes. Those of the CYP1 family have been shown to be the most sensitive to EPFR exposure (Harmon et al., 2018; Jalgama et al., 2018; Aryal et al., 2023) with induction mediated through the aryl hydrocarbon receptor (AhR). However, there also have been reports of CYP2E1 being induced by particulate matter (Abbas et al., 2009; Wu et al., 2023). In addition to the effects of EPFRs on P450 expression, EPFRs have been shown to inhibit the function of several P450s, including CYP1A2, CYP2B4, CYP2E1, CYP2D2, and CYP3A (Reed et al., 2014).

As CYP2E1 can be a source of ROS alone, and also can be inhibited by EPFRs (which can generate ROS), the goal of this study is to examine the mechanism by which EPFRs inhibit CYP2E1 function and to determine if ROS generation is affected. Our results confirm our previous report that CYP2E1 activity was dramatically inhibited by the presence of EPFRs (Reed et al., 2014), and despite its inhibition of CYP2E1 monooxygenation, ROS generation was stimulated by EPFR addition. The results showed that EPFRs inhibited CYP2E1-mediated

metabolism not by disrupting the interaction between POR and CYP2E1, but by uncoupling of NADPH and oxygen consumption from substrate metabolism to the production of ROS.

## Materials and Methods

*Materials* – Reagents were the highest quality available. Rabbit CYP2E1 was expressed and purified according to previously published methods (Cheng et al., 2004). The NADPH-cytochrome P450 reductase expression system was a gift from Dr. Lucy Waskell (University of Michigan) and was expressed and purified as previously described (Reed et al., 2010). The substrate 7-benzyloxyresorufin was purchased from Anaspec (Fremont, CA). Other reagents were obtained from Sigma (St. Louis, MO).

*Generation of EPFR and non-EPFR particles* – CAB-O-SIL EH-5 was purchased from Cabot corporation (Billerica, MA). This fumed silica was impregnated with 5% (w/w) copper oxide to generate the non-EPFR particle, CuO-Si as described previously (Reed et al., 2014; Reed et al., 2015a). To generate the EPFR, silica was impregnated with copper nitrate hemipentahydrate by incubation in a 0.1 M solution for 24 h at room temperature. Subsequently, the silica was dried at 120°C for 12 h, and then heated for 5 h in air at 450°C. To generate the EPFR, the particles were then placed in vacuum ( $<10^{-2}$  torr) and heated to 230 °C before being exposed to 2-chlorophenol vapors at 10 torr in a custom-made vacuum exposure chamber for 5 min. The samples were cooled to room temperature and evacuated for 1 h ( $10^{-2}$  torr). The radical contents of the EPFRs were analyzed by electron paramagnetic resonance (EPR) spectroscopy as described previously (Khachatryan et al., 2011), and had a spin content greater than  $1 \times 10^{17}$  spins/g. In summary, the EPFR particle, MCP230, is monochlorophenol that was heated to 230°C in the presence of silica particles and 5% copper oxide. CuO-Si was used as the non-radical containing control particle .

*Preparation of reconstituted systems* – For activity measurements, CYP2E1 and POR were reconstituted into dilauroylphosphatidylcholine (DLPC) vesicles, as previously described (Kelley et al., 2005; Kelley et al., 2006). Briefly, a stock suspension of DLPC (8 mM in 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.1 M NaCl, and 5 mM EDTA)



was prepared by sonication in a bath sonicator until clarification (~ 30 min). Proteins were mixed with DLPC at a 160:1 (DLPC:CYP2E1) ratio at 0.5:1, 1:1 or 2:1 POR:CYP2E1, and preincubated for 2 h at room temperature prior to addition of other assay components.

*7-Benzoyloxyresorufin metabolism* – The substrate 7-benzoyloxyresorufin was used to monitor CYP2E1 activities. The assay components and their final concentrations were, reconstituted system (160:1 [DLPC]:[CYP2E1] and [POR] as indicated), 7BR (32  $\mu$ M or as indicated in Results), in 50 mM HEPES, pH 7.5, containing 15 mM magnesium chloride and 0.1 mM EDTA (Cawley et al., 2001; Kelley et al., 2005; Kelley et al., 2006). Results are expressed as the mean  $\pm$  SD for at least 3 determinations.

*Measurement of physical complex formation using bioluminescence resonance energy transfer (BRET)* – To determine the effect of particles on CYP2E1/POR binding, we used bioluminescence resonance energy transfer (BRET). For BRET studies, HEK 293T/17 cells (ATCC) were co-transfected with a pair of vectors coding for NADPH-cytochrome P450 reductase (POR) and CYP2E1 with C-terminal protein tags: GFP for CYP2E1 (CYP2E1-GFP) and *Renilla* luciferase for POR (POR-Rluc). Transfections were performed to induce expression of the tagged proteins at a high (>10x) CYP2E1-GFP:POR-Rluc ratio to ensure that BRET signals generated were near BRET<sub>max</sub> and were not sensitive to small changes in expression ratio. Assays were performed as described previously with the addition of a 30-minute incubation step after the addition of MCP230 or CuO-Si particles at a range of concentrations (Connick et al., 2021). Untransfected cells, cells expressing a GFP-Rluc fusion protein, and cells expressing only POR-Rluc were used as controls to normalize the BRET signal at each MCP230 or CuO-Si concentration.

*First-electron transfer to CYP2E1* – These assays are modifications of methods described in previous reports (Backes and Reker-Backes, 1988; Backes and Eyer, 1989). CYP2E1 reduction was measured using an Olis stopped flow spectrophotometer (On-line Instrument

Systems). Reduction was measured by mixing the contents of two syringes, the first containing the reconstituted system (160  $\mu$ M dilauroylphosphatidylcholine (DLPC), 1  $\mu$ M POR and 2 $\mu$ M CYP2E1), in 50 mM potassium phosphate buffer, pH 7.4. The second syringe contained 375 nmol NADPH, in 50 mM phosphate buffer, pH 7.4. Prior to loading in the spectrophotometer, both samples were gently bubbled with carbon monoxide for 1 min, and protocatechuic acid, and protocatechuic acid dioxygenase (PD) were added. The samples were added to the stopped flow and after temperature equilibration (25°C), the formation of CO-reduced CYP2E1 was measured at 450 nm. After mixing, the final concentrations of assay components were: 0.5  $\mu$ M POR, 1  $\mu$ M CYP2E1, 160  $\mu$ M DLPC, 0.2 mM mM protocatechuic acid, 0.4 units/ml PD, and 250  $\mu$ M NADPH in 50 mM potassium phosphate buffer, pH 7.4. Where indicated, the concentration of both the EPFR and non-EPFR particles were 100  $\mu$ g/ml.

*Measurement of ROS generation (DCF metabolism)* – The generation of reactive oxygen species was monitored by measuring the metabolism of dichlorofluorescein (DCFH), using a modification of previous methods (Bass et al., 1983; LeBel et al., 1990; LeBel and Bondy, 1990; Bondy and Naderi, 1994a; Bondy and Naderi, 1994b; Serron et al., 2000). The reaction is based on the addition of the stable diacetate, dichlorofluorescein diacetate (DCFH-DA). In cellular systems DCFH-DA is readily taken into cells and rapidly converted to dichlorofluorescein (DCFH) by cellular esterases (Szejda et al., 1984; LeBel et al., 1990; LeBel and Bondy, 1990). Reactive oxygen species can readily convert DCFH to the fluorescent product dichlorofluorescein (DCF). As reconstituted systems are being used in the current study, there are no intracellular esterases to convert DCFH-DA to DCFH. Consequently, DCFH was generated by preincubation of DCFH-DA with porcine liver esterase (Sigma # 2884-1KU) prior to the addition of NADPH to the reconstituted system. In preliminary experiments, a 3 min preincubation of DCFH-DA with porcine esterase led to optimal fluorescence production (not shown).

The reaction mixture contained final concentrations of DCFH-DA (8 $\mu$ M), porcine esterase (40 units/ml), and either CuO-Si, or the EPFR MCP230 (as indicated in the Figures) in 40 mM Tris pH 7.4. Reconstituted systems where POR and CYP2E1 in DLPC were included, are indicated in the Figures. After preincubation at 37°C for 3 minutes, the reaction was initiated by the addition of NADPH to a final concentration of 500 $\mu$ M, and the linear rate of increase in fluorescence was determined using excitation and emission wavelengths of 488 nm and 535 nm, respectively.

*Statistical Analysis* – Data are expressed as the mean  $\pm$  SD in each of the Figures, with the number of determinations described in the figure legends. In Figure 6, the slopes of the lines and their 90% confidence intervals (dotted lines) were calculated using a linear regression analysis, and significance determined using an F test.

## Results

**Effect of EPFRs on CYP2E1 activity** – As shown with rat liver microsomes, EPFRs inhibited the function of several P450 enzymes, including CYP2E1 (Reed et al., 2014), which led us to examine the mechanism of this inhibition. As a first step, the effect of EPFRs on CYP2E1-mediated 7-benzoylresorufin activity was measured as a function of substrate concentration. At a 2:1 [POR]:[CYP2E1], 7-benzoyloxyresorufin (7BR) metabolism did not produce a hyperbolic response typical of Michaelis-Menten behavior, but was sigmoidal. Both the EPFR, MCP230, and the non-EPFR particle (CuO-Si) were effective inhibitors of this CYP2E1-mediated activity, with both having roughly similar responses (Fig. 1A). These titrations were repeated at subsaturating [POR] (Fig. 1B). There still was a tendency toward sigmoidicity in the absence of inhibitor. However, in contrast to the results from saturating POR, there was a difference in the behavior of the two particles. Whereas the non-EPFR particle CuO-Si produced modest inhibition of 7BR, MCP230 was a much more effective inhibitor. Because differential inhibition of CYP2E1 function was revealed at subsaturating POR concentrations, these results suggest that MCP230 appears to participate or modulate the inhibitory response by affecting the interaction between POR and CYP2E1.

Based on the increased inhibition by MCP230 found at subsaturating POR, the effects of these particulates were examined as a function of [POR] (Fig. 2). In the absence of inhibitor, 7BR metabolism could be readily described as following a Michaelis-Menten type mass action interaction between CYP2E1 and POR. A hyperbolic response also was observed by the non-EPFR particle, exhibiting an inhibitory response. However, the EPFR (MCP230) caused a much greater response as a function of the POR concentration, with the most dramatic inhibition being observed at low [POR]. Although the activities increased at higher [POR], they appeared to saturate at a lower activity than the uninhibited systems. Again, these results are consistent with EPFRs being more potent inhibitors than non-EPFR particulate matter (i.e. CuO-Si),

particularly at subsaturating POR, which is typical of the POR ratios found *in vivo* (Reed et al., 2011).

**Effect of EPFRs on POR•CYP2E1 complex formation** – The sigmoidal inhibition of 7ER when measured as a function of POR concentration in Figure 2, suggested that MCP230 may be inhibiting function by disrupting the formation of the POR•CYP2E1 complex. To address the potential for inhibition of CYP2E1 by MCP230 to be due to disruption of the POR•CYP2E1 complex, physical complex formation was examined using BRET at a range of particle concentrations. The results (Figure 3) showed that both the non-EPFR particle, CuO-Si, and the EPFR, MCP230, produced a similar decrease in the BRET response; however, this was only observed at particle concentrations well in excess of those capable of inhibiting activity (Figure 1 and 2), suggesting that the inhibition was not the result of disruption of the POR•CYP2E1 complex.

**Effect of EPFRs on first electron transfer.** Based on the predominant inhibition of 7BR dealkylation at subsaturating POR, we expected BRET to show disruption of the POR•CYP2E1 complex. As the POR•CYP2E1 complex was not disrupted, we further examined potential for EPFRs to inhibit electron transfer between POR and CYP2E1 without disrupting the functional complex. Therefore, we examined first-electron transfer between POR and CYP2E1 by measuring the rate of formation of the reduced-carbon monoxide CYP2E1 adduct at subsaturating POR. As shown in Fig. 4, in the absence of particles, an increase in CO-reduced CYP2E1 was observed – typical of anaerobic P450 reduction. Surprisingly, when either EPFR or non-EPFR particulates were included, we did not observe inhibition of the reaction, but actually saw an increase in the rate of first electron transfer. These results indicate that the POR•CYP2E1 complex is intact and transfers electrons to CYP2E1 more effectively in the presence of both EPFR and non-EPFR particulate.

**Effect of EPFRs on generation of reactive oxygen species (ROS)** – As CYP2E1 is known to generate significant amounts of ROS, we were interested in determining if CYP2E1-mediated ROS generation was inhibited in the presence of EPFRs. This was done by using a reconstituted system containing an equimolar ratio of CYP2E1 and POR and varying the EPFR or catalyst concentration. In these experiments, ROS generation was measured using the substrate DCFH-DA. This is a substrate that is used for measuring ROS generation in cellular systems and takes advantage of the presence of intracellular esterases to convert DCFH-DA to DCFH, which is then metabolized to the fluorescent DCF by ROS. As the reconstituted systems used in this study are not in a cellular system, we needed to generate DCFH in order to measure ROS production. This was accomplished by preincubation of DCFH-DA with porcine liver esterase. Based on preliminary experiments, preincubation for 3 min with 40 units/ml reaction of pig esterase produced a maximal effect on DCF production.

Using this method, we then examined the effect of CuO-Si and MCP230 on CYP2E1-mediated ROS generation. As shown in Figure 5, the **non-EPFR** CuO-Si produced a small amount of ROS, which increased as the particle concentration was increased (red). In contrast, MCP230 alone was able to generate much higher levels of ROS (blue). When these experiments were repeated in the presence of the CYP2E1/POR-containing reconstituted system, the results showed the reconstituted system alone was able to generate ROS (at zero particle concentration), and the increase with increasing CuO-Si (brown) roughly paralleled that seen with CuO-Si alone. As expected, ROS generation also was increased in the CYP2E1/POR system as the concentration of MCP230 was increased (green). There was a significant elevation of ROS at lower EPFR levels when both MCP230 and CYP2E1 were present, but the difference was not observed as the MCP230 levels exceeded 0.2 mg/ml of the particle as compared to MCP230 alone.

Based on the data in Figure 5, we observed a significant stimulation of DCF production by CYP2E1 at particle concentration of 0.1 mg/ml, with the EPFR being more effective. Therefore, we wanted to determine if greater amounts of CYP2E1 stimulated DCF production (Figure 6). In the absence of particles, increases in POR/CYP2E1 generated a linear increase in ROS, supporting the established evidence that functional CYP2E1 generates ROS. At 0.1 mg/ml particle concentration and in the absence of CYP2E1, both particles were able generate ROS, with MCP230 being more effective, which is consistent with that shown in Figure 5. An increase in the CYP2E1 concentration also caused a linear increase in ROS generation. Interestingly, the slopes of the lines in the presence of both the EPFR and non-EPFR particles were significantly greater than that found with CYP2E1 alone. These results indicate that that the particles synergistically stimulated CYP2E1-mediated ROS generation.

## Discussion

Environmentally Persistent Free Radicals (EPFRs) are an important component of particulate matter (PM) that cause both pulmonary and cardiovascular toxicity (Lord et al., 2011; Jaligama et al., 2018; Aryal et al., 2023). EPFRs are generated by the incomplete combustion of combinations of organic chemicals and metals, creating particulate matter where the metal and organic components are chemisorbed to create a pollutant-particle radical-containing system (Dugas et al., 2016). EPFRs are stable in the environment, with half-lives varying from days to months (Khachatryan et al., 2011; Kelley et al., 2013; Yuan et al., 2022); however, when present in an organism, EPFRs redox cycle to produce oxidative damage (Kelley et al., 2013; Khachatryan et al., 2014; Kiruri et al., 2014). This can lead to diminished pulmonary, immune, and cardiovascular function (Dellinger et al., 2001; Wang et al., 2008; Balakrishna et al., 2009; Balakrishna et al., 2011; Lord et al., 2011; Mahne et al., 2012; Kelley et al., 2013; Reed et al., 2014; Reed et al., 2015a; Stephenson et al., 2016; Jaligama et al., 2018).

Not only are EPFRs capable of modulating the expression of P450 enzymes, particularly those of the CYP1 family (Harmon et al., 2018; Jaligama et al., 2018; Harmon et al., 2021), but they also are capable of directly inhibiting P450 function (Reed et al., 2014; Reed et al., 2015a; Reed et al., 2015b). Using rat liver microsomal preparations, several P450 enzyme activities, including those catalyzed by CYP1A1, CYP1A2, CYP2B, CYP2E1, CYP2D2 and CYP3A were inhibited by EPFRs (Reed et al., 2014). In another study, CYP2B4 activities were non-competitively inhibited by MCP230 when measured as a function of substrate concentration, but competitively inhibited when measured as a function of POR concentration (Reed et al., 2015a). These results were consistent with MCP230 inhibiting CYP2B4 by disruption of the POR•CYP2B4 complex. With CYP2E1 being inhibited by MCP230 by more than 85% (Reed et al., 2014), there was an interest in determining whether the EPFR also disrupted the ability of CYP2E1 to complex with POR.



First, when examining the kinetic data, CYP2E1 exhibited a sigmoidal response as a function of substrate concentration (Fig. 1). This is potentially consistent with previous reports suggesting that CYP2E1 forms a homomeric complex that is inactive, even when POR is present (Jamakhandi et al., 2007). Sigmoidicity as a function of substrate concentration has previously been reported for other P450s, including CYP3A4 (Korzekwa et al., 1998; Davydov et al., 2013), CYP2C9 (Korzekwa et al., 1998), CYP2D6 (Davydov et al., 2017), and CYP3A5 (Davydov et al., 2015). In a report examining CYP2E1-mediated metabolism in a reconstituted system containing saturating POR, 7-methoxyfluorocoumarin-O-demethylation was hyperbolic when measured as a function of substrate (Davydov et al., 2017). There was a report of sigmoidicity as a function of substrate with CYP2E1 (Hartman, 2015) although these studies were done using microsomes containing multiple P450s and subsaturating POR. Both CuO-Si and MCP230 were equally effective inhibitors of CYP2E1-mediated 7BR activity. However, at subsaturating POR, MCP230 was a much more potent inhibitor, suggesting a difference in the way they inhibit the reaction. When 7BR activity was measured as a function of POR concentration, MCP230 was a much more potent inhibitor, particularly at subsaturating POR, suggesting that the EPFR may be inhibiting activity by influencing POR•CYP2E1 complex formation.

This was examined in more detail using BRET (Figure 3), where neither MCP230, nor CuO-Si disrupted complex formation at similar concentrations where activity was affected (Figures 1 & 2). In fact, when electron transfer to CYP2E1 was measured (Figure 4), an increase in the rate of electron transfer was observed in the presence of both the EPFR and non-EPFR particulate matter.

CYP2E1 is known to be capable of ROS generation (Harjumaki et al., 2021). With EPFRs and CYP2E1 both serving as a source of ROS, we were interested in determining if one of these components influenced the other. The results in Fig. 6 show that both MCP230 and

CYP2E1 produce ROS, and their combination synergistically stimulated ROS production as can be seen by the increased slope of the dependence when the EPFR was present. A similar stimulation also was observed with CuO-Si. Taken together, these data indicate that electron flow and POR•CYP2E1 complex formation were not affected by the particulates, yet monooxygenase activity was inhibited. The fact that ROS generation was stimulated by the presence of particles suggests that both particles lead to the uncoupling of substrate metabolism leading to the increased formation of reactive oxygen species. Interestingly, the EPFR MCP230 was a more potent inhibitor of monooxygenase activity than was the non-EPFR particle. The ability of EPFRs to lead to uncoupling of CYP2E1 is an important finding related to the P450 system and it will be interesting to determine if this response is common to other P450 enzymes that are inhibited by EPFRs.

In conclusion, the results showed that MCP230 was a more effective inhibitor of CYP2E1-mediated activities, particularly at subsaturating POR concentrations, initially suggesting that the POR•CYP2E1 complex formation was disrupted. However, BRET data showed that the POR•CYP2E1 complex was not disrupted, and that first electron transfer was actually stimulated. Despite the inhibition of CYP2E1-mediated substrate metabolism, its ability to generate ROS was not inhibited. In fact, there was a stimulation of ROS generation in the presence of CYP2E1. These results suggest that EPFRs (and to a lesser extent non-EPFR particulates) shift electron flow away from substrate metabolism and toward ROS generation: that is uncoupling of the monooxygenase reaction.

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**Data Availability** – *The data supporting the findings of this study are contained within the paper.*

**Author contributions:**

*Participated in research design:* Backes, Connick, Cawley

*Conducted experiments:* Cawley, Connick, Eyer, Backes

*Performed data analysis:* Backes, Connick, Cawley

*Wrote or contributed to the writing of the manuscript:* Backes, Connick, Cawley

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## Figure Legends

**Figure 1 – Effect of EPFR and non-EPFR particles on CYP2E1-mediated 7-benzyloxyresorufin dealkylation.** CYP2E1-mediated 7BR activity was measured in the absence and presence of 0.1 mg/ml of non-EPFR (CuO-SI), and EPFR (MCP230), using reconstituted systems containing CYP2E1 and POR. (A) The reconstituted systems consisted of CYP2E1 (0.025  $\mu$ M), POR (0.05  $\mu$ M), and DLPC (4  $\mu$ M). (B) CYP2E1 (0.025  $\mu$ M) was reconstituted with DLPC (4  $\mu$ M) and subsaturating POR (0.0125  $\mu$ M). The results represent the mean  $\pm$  SD for 4 determinations.

**Figure 2 – Effect of EPFR and non-EPFR particles on CYP2E1-mediated 7-benzyloxyresorufin metabolism as a function of POR concentration.** 7BR metabolism was examined in reconstituted systems containing 0.05  $\mu$ M CYP2E1, 8  $\mu$ M DLPC and POR (ranging from 0-0.5  $\mu$ M) and saturating 7BR (32  $\mu$ M) in the presence and absence of particles at 0.15 mg/ml. The data points were the mean  $\pm$  SD for at least 3 determinations.

**Figure 3 – Effect of particles on physical complex between POR and CYP2E1.** HEK 293T/17 cells were transfected with plasmids coding for POR-Rluc and CYP2E1-GFP. After 24 h, complex formation between POR and CYP2E1 was examined using BRET at a range of particle concentrations. Data points represent the mean  $\pm$  the SD for triplicate determinations from a single group of cells. A previous experiment was conducted with adjustments to transfection conditions for optimization of protein expression, generating similar results (not shown).



**Figure 4 – Effect of EPFR and non-EPFR particulates on first electron transfer to CYP2E1.**

Subsaturating POR and CYP2E1 (POR:CYP2E1 0.5:1) were reconstituted into DLPC vesicles, and first electron transfer was monitored by the formation of the reduced-CO complex of CYP2E1 in the absence and presence of the EPFR MCP230, and the non-EPFR CuO-Si. Each scan represents an average of at least quadruplicate stopped flow tracings for each condition. This was repeated on a subsequent day, obtaining similar results.

**Figure 5 – Effect of EPFRs on CYP2E1-mediated ROS formation.** ROS-mediated DCF formation was measured as a function of particle concentration for the EPFR-containing MCP230 and the control CuO-Si particle. When present, the POR:CYP2E1 ratio was 1:1. The results are the mean  $\pm$  SD for duplicate determinations.

**Figure 6 – Effect of CYP2E1 on EPFR-mediated ROS formation.** DCF formation was measured in the presence of 0.1 mg/ml of particles as a function of the POR/CYP2E1 reconstituted system. The POR:CYP2E1 ratio was 1:1. The results are the mean  $\pm$  SD for at least 4 determinations. The slopes of the lines were 20.6, 35.8 and 32.8 pmol DCF (min)<sup>-1</sup>( $\mu$ M CYP2E1)<sup>-1</sup>. The 90% confidence intervals are shown as dotted lines, and were determined to represent a significant difference between the “no particles” group and the two groups containing the particles (\*\*\*,  $p < 0.001$ ).

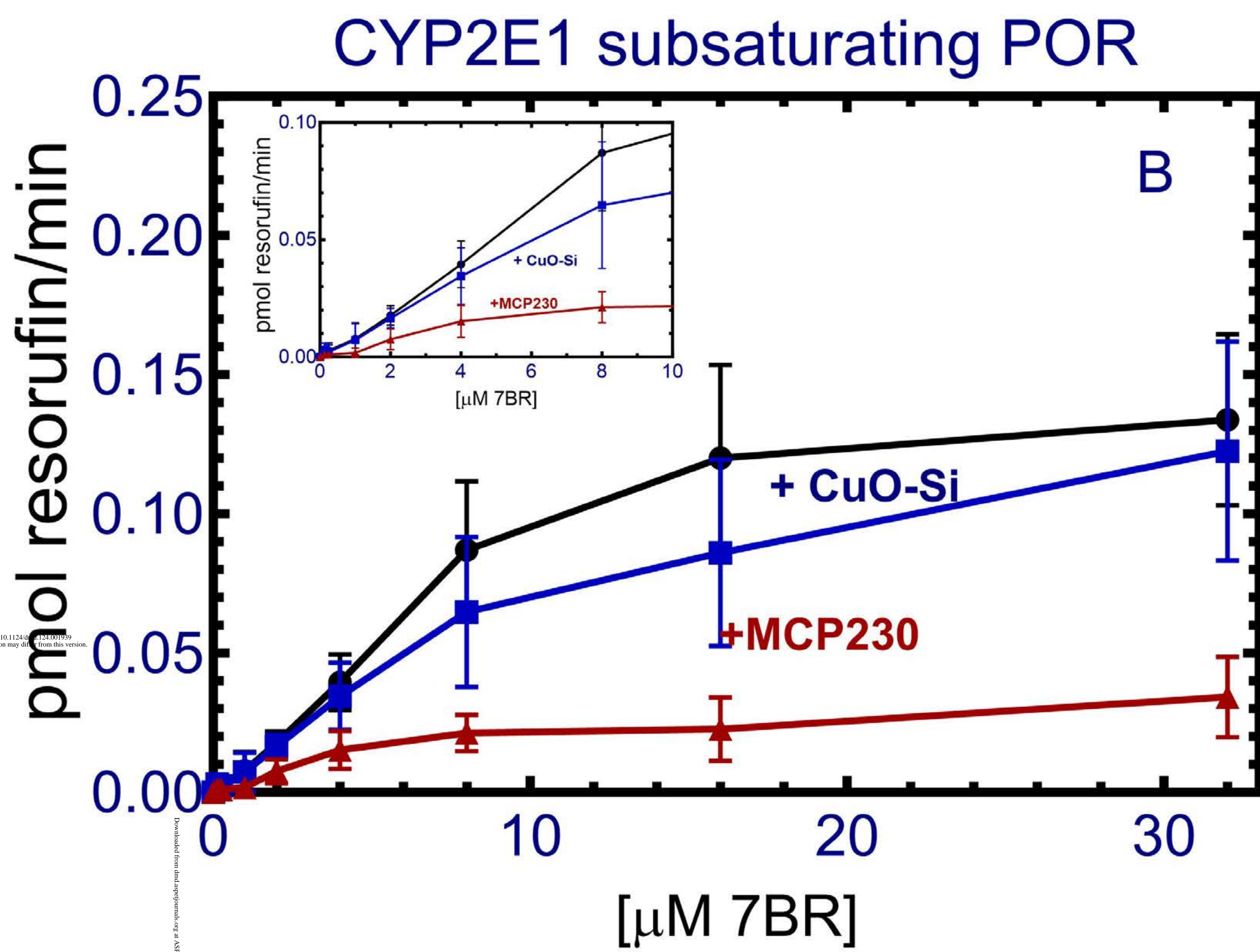
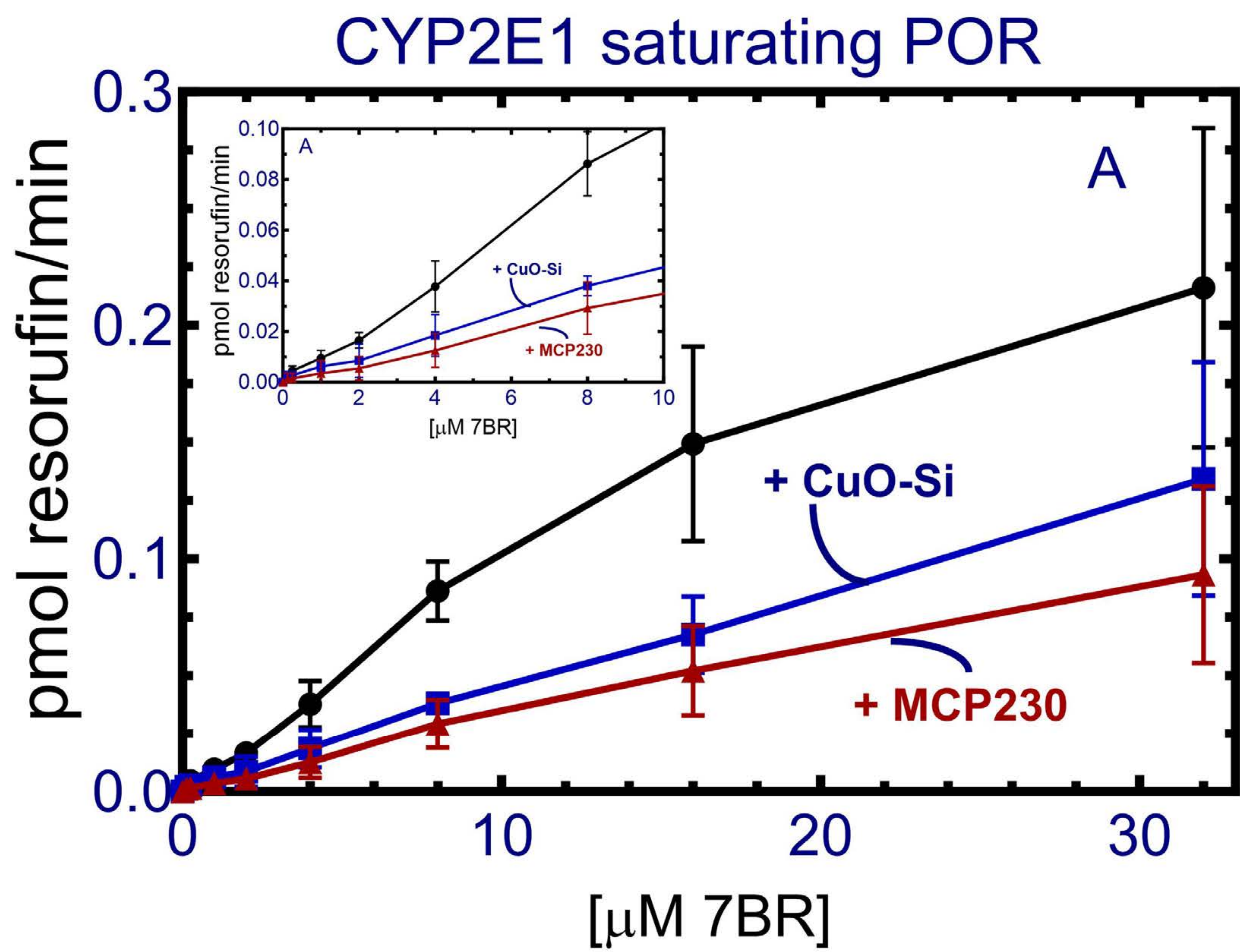


Figure 1

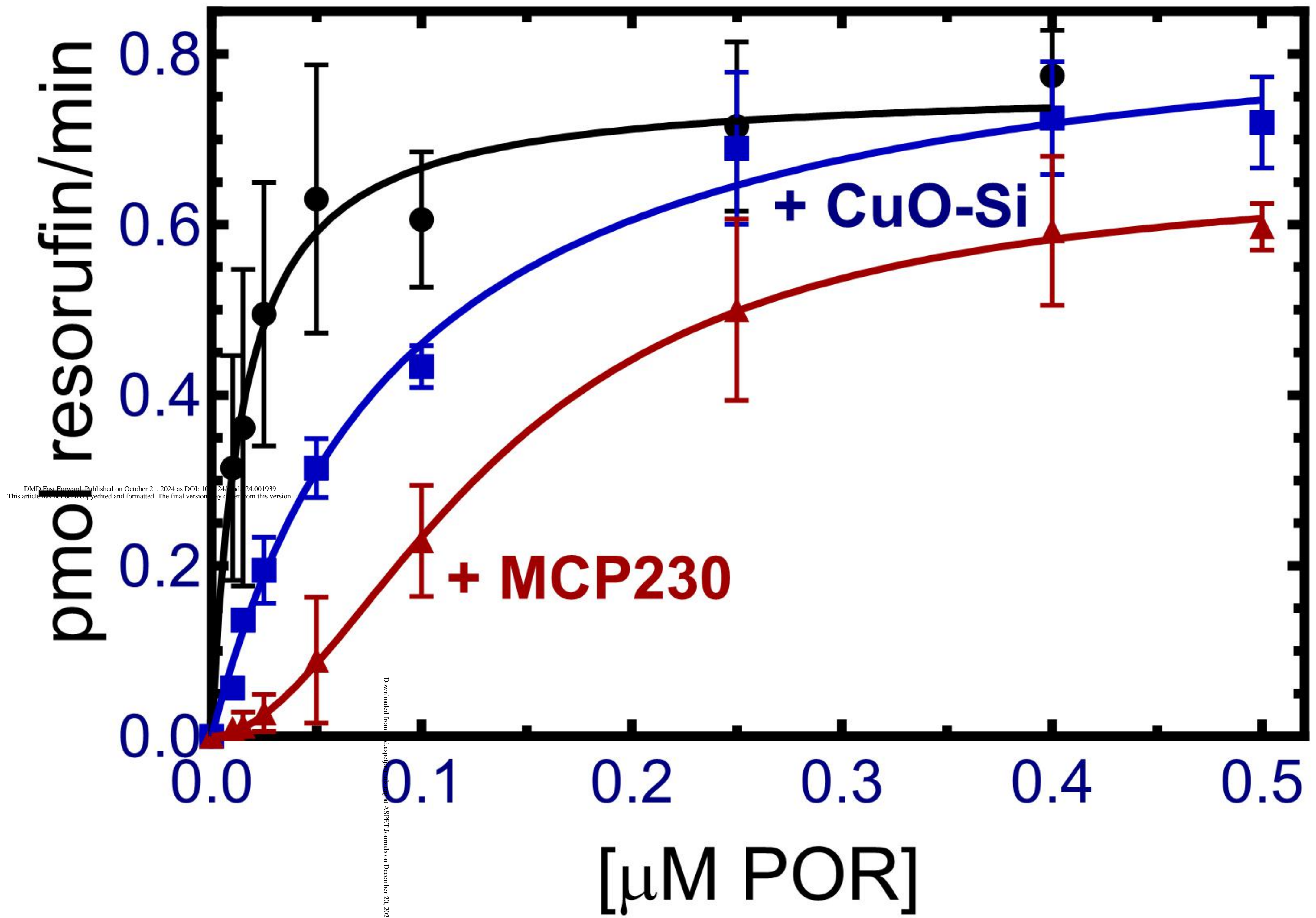


Fig. 2

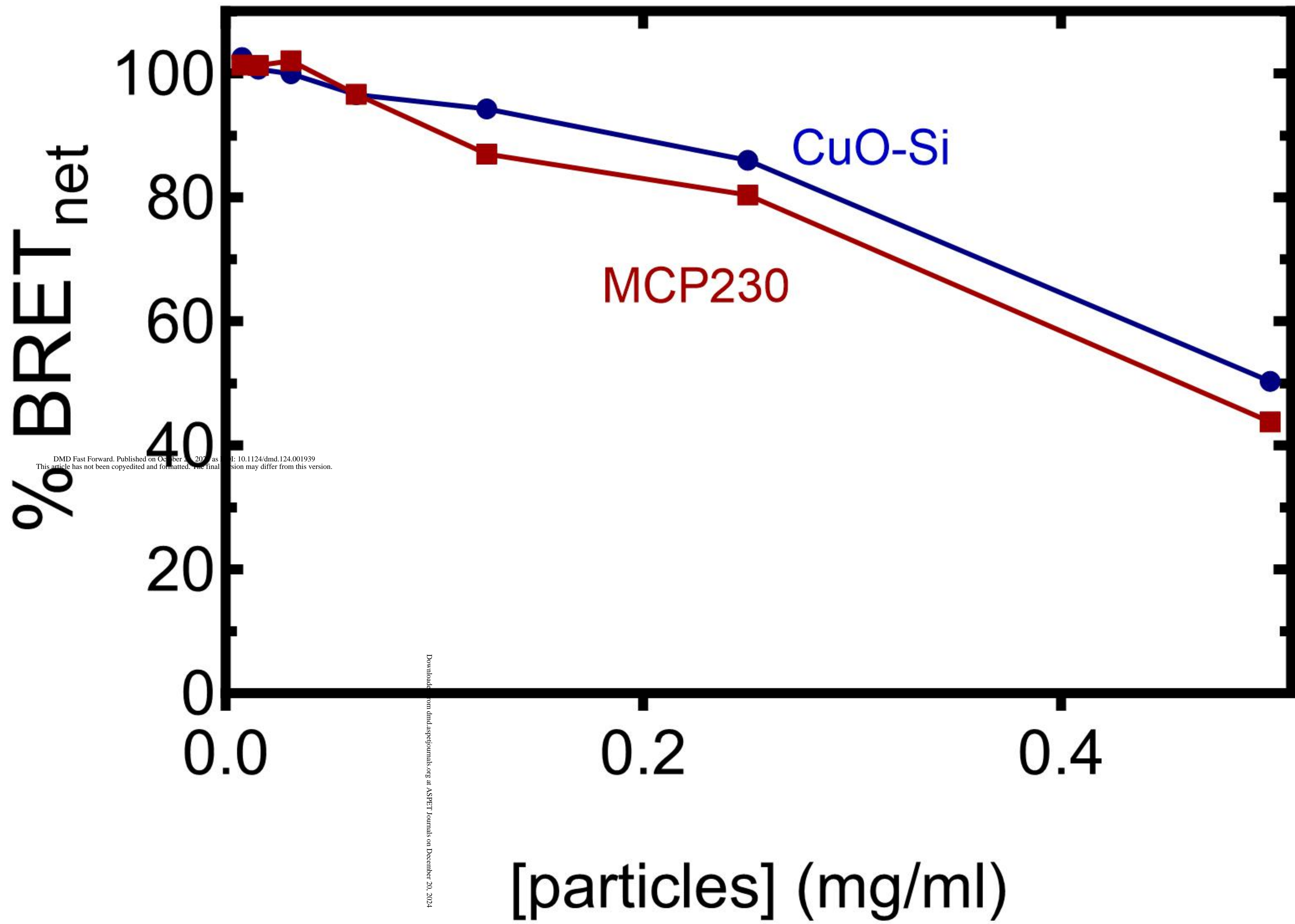


Fig. 3

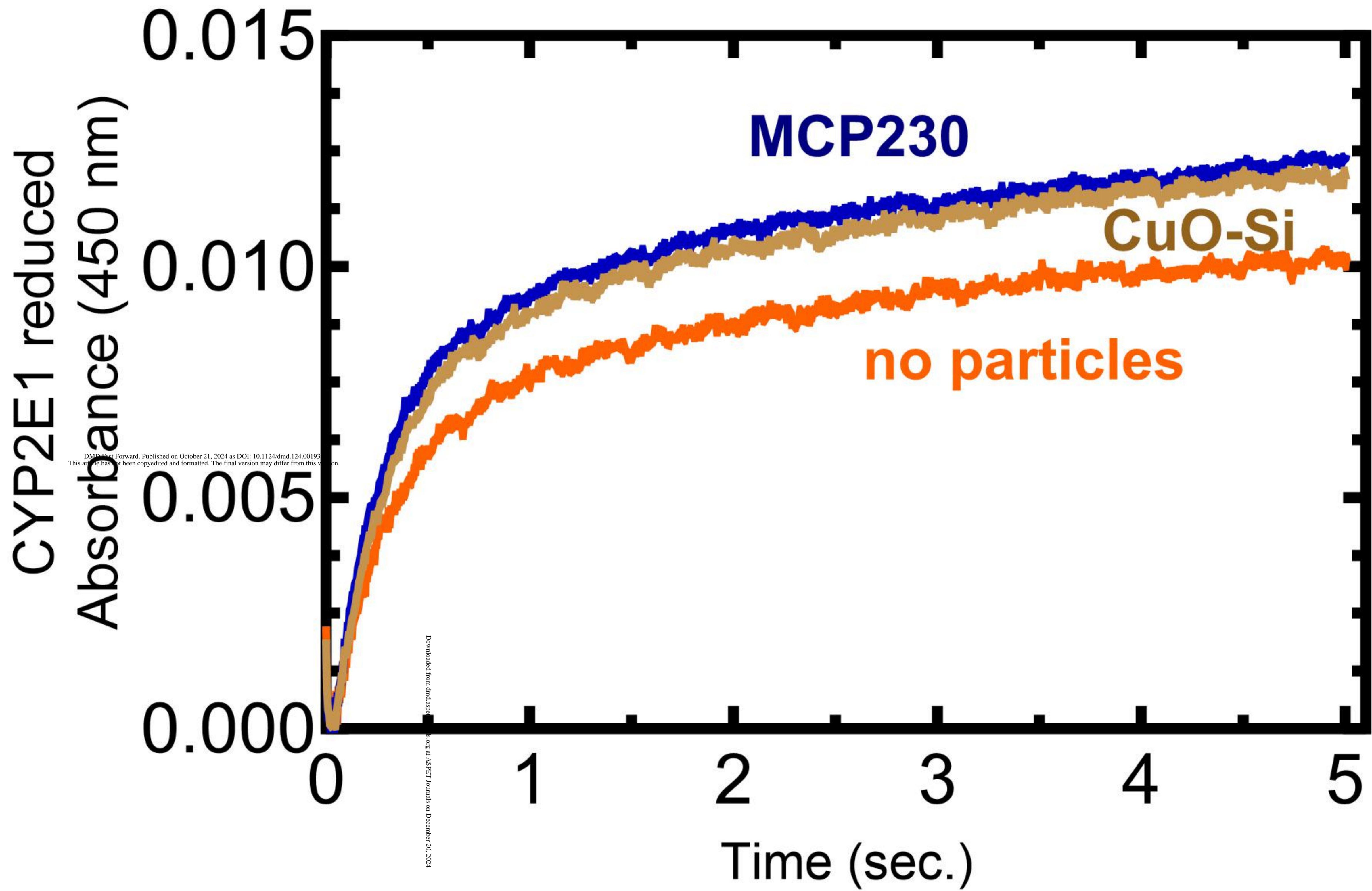


Fig. 4

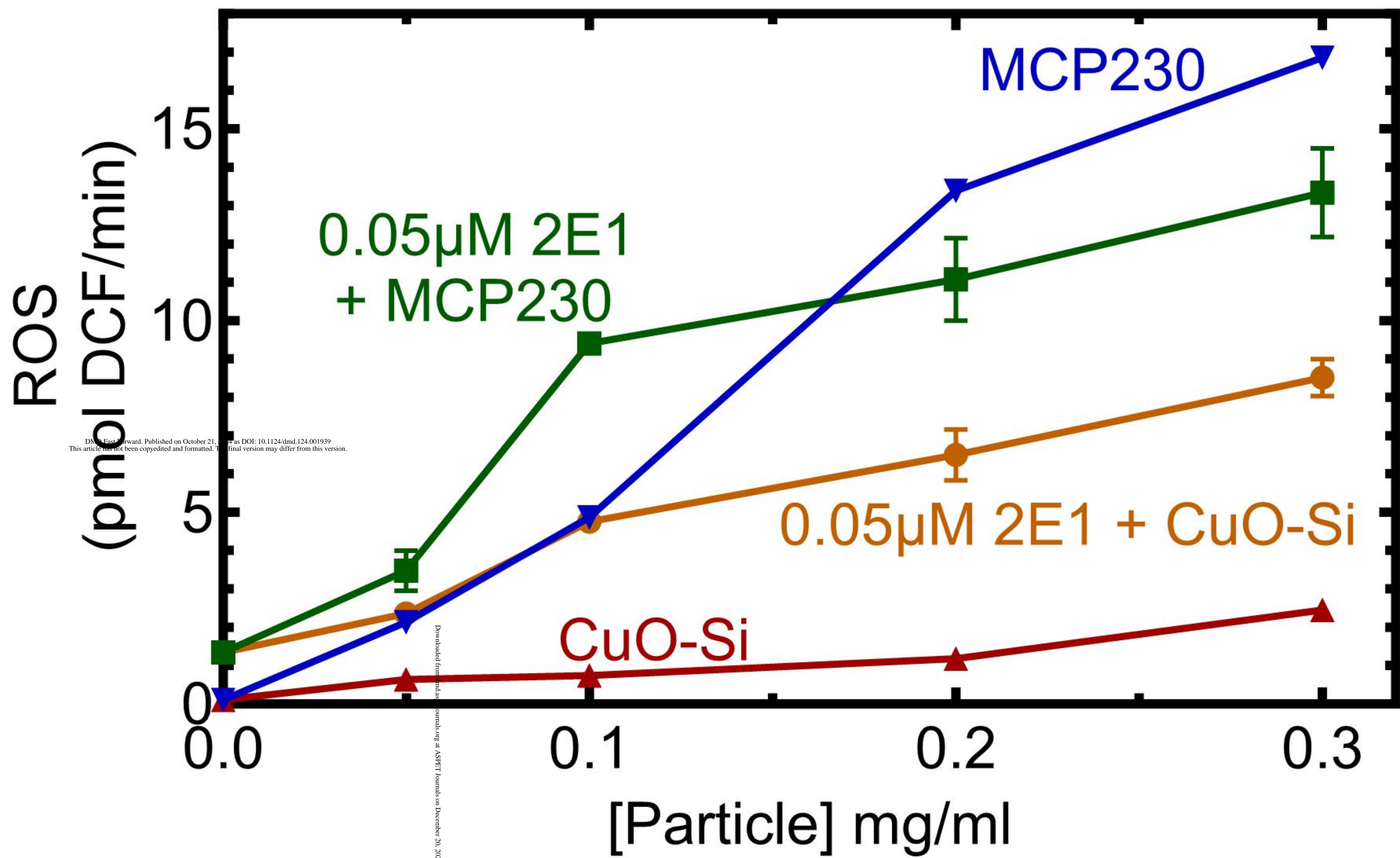


Fig. 5

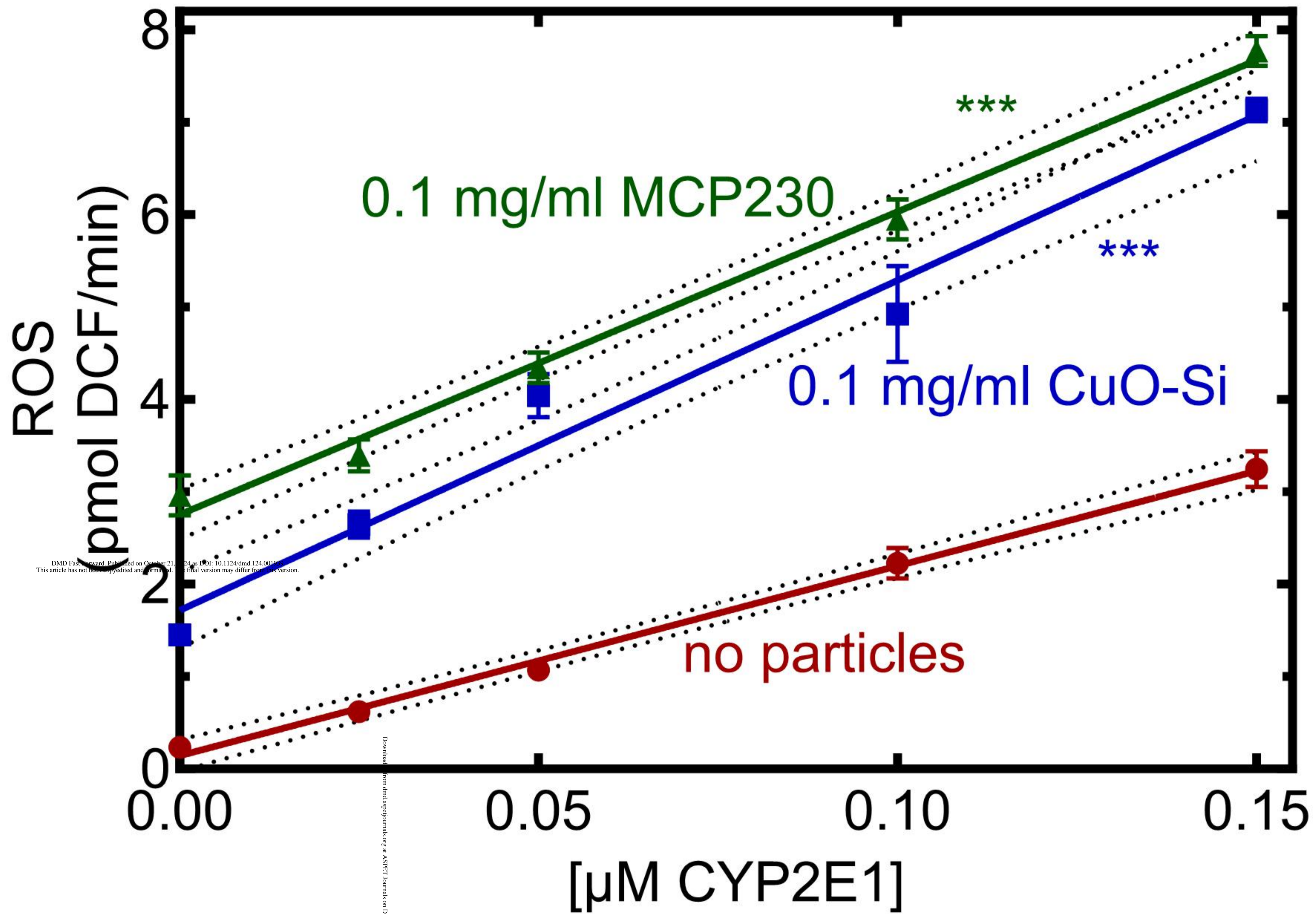


Fig. 6