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Time-Dependent Inhibition of CYP1A2 by Stiripentol and Structurally Related Methylenedioxyphenyl Compounds via Metabolic Intermediate Complex Formation

Yasuhiro Masubuchi*, Chieko Takahashi, and Rina Gendo

Laboratory of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Chiba Institute of Science, 15-8 Shiomi-cho, Choshi, Chiba, Japan (Y.M., C.T., R.G.)

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b) Corresponding author: Yasuhiro Masubuchi, Laboratory of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Chiba Institute of Science, 15-8 Shiomi-cho, Choshi, Chiba 288-0025, Japan. E-mail: ymasubuchi@cis.ac.jp

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d) Abbreviations: STP, stiripentol; P450, cytochrome P450; MDP, methylenedioxyphenyl; MIC, metabolic intermediate complex; MDB, methylenedioxybenzene; SF, safrole; PBO, piperonyl butoxide; ISF, isosafrole; DSF, dihydrosafrole; G-6-P, glucose 6-phosphate; G-6-PDH, glucose 6-phosphate dehydrogenase

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ABSTRACT

Stiripentol (STP), an antiepileptic agent, causes drug-drug interactions by inhibiting cytochrome P450 (P450) enzymes. STP contains a methylenedioxyphenyl (MDP) group, which could form inhibitory metabolic intermediate complexes (MICs) with P450. The present study examined the possible time-dependent inhibition of CYP1A2 via MIC formation by STP and structurally related MDP compounds such as isosafrole. Time-dependent inhibition was observed in human liver microsomes for CYP1A2, but not CYP3A4. Spectral analysis of the liver microsomes from CYP1A-induced rats incubated with STP and NADPH revealed a Soret peak at approximately 455 nm, which was largely eliminated by potassium ferricyanide. Similar spectra were obtained for all the other MDP compounds, albeit in varying amounts. Thus, the extent of time-dependent CYP1A2 inhibition and MIC formation were in good agreement. In addition, the dissociation of MIC by potassium ferricyanide partially attenuated the impairment of CYP1A2 activity, suggesting that MIC is involved in the time-dependent inhibition of CYP1A2 by STP. In conclusion, STP, like other MDP compounds, caused time-dependent CYP1A2 inhibition via MIC formation, and this may be involved in drug-drug interactions associated with the clinical use of STP.

SIGNIFICANCE STATEMENT

The present study found that stiripentol, an antiepileptic agent, caused a time-dependent inhibition of CYP1A2. Stiripentol like isosafrole has a methylenedioxyphenyl group and generated MI complexes with CYP1A2. This is a new case of the time-dependent CYP inhibition by a methylenedioxyphenyl containing drug via MI complex formation.

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Introduction

Stiripentol (STP) is an antiepileptic agent used to treat Dravet syndrome in combination with clobazam and valproic acid (Chiron et al., 2000). In addition to enhancing GABA signaling, STP is assumed to increase the blood levels during concomitant-antiepileptic-drug medication and enhance their anticonvulsant effects by inhibiting cytochrome P450 (P450) enzymes (Giraud et al., 2006). However, the mechanisms underlying STP-mediated P450 inhibition has not been fully elucidated. STP possesses a methylenedioxyphenyl (MDP) group in its structure that can be converted into a carbene metabolite to form an inhibitory complex (metabolic intermediate complex, MIC) with reduced forms of P450 (Murray, 2000; Masubuchi and Horie, 2007; Taxak et al., 2013) (Fig.1). MDP compounds contained in natural oil components, such as safrole, inhibit P450 enzymes (Hodgson and Philpot, 1974).

Regarding STP, a spectroscopic study using rat liver microsomes in the 1990s revealed the generation of MIC with a metabolite of STP; however, P450 inhibition associated with MIC formation was not examined in that study (Zhang et al., 1990). Another in vitro study indicated that STP inhibited multiple P450s, including CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4; however, that study found no time-dependent inhibition of these P450s, implying that the metabolites were not involved in P450 inhibition (Tran et al., 1997). In contrast, clinical studies in the same report suggested the in vivo inhibition of CYP3A4 and CYP1A2, but not CYP2D6 (Tran et al., 1997). After that, reports on the mechanism of inhibition of P450 enzymes by STP have been lacking.

In recent years, reports on the inhibition of several P450 enzymes by MDP compounds in drugs and foods have emerged (Bertelsen et al., 2003; Yasuda et al., 2015; Cui et al., 2020). Therefore, it is necessary to characterize the mechanisms underlying STP-mediated inhibition of P450 enzymes. In this study, we examined the

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time-dependent inhibition of CYP3A4 and CYP1A2 in human liver microsomes. Next, focusing on CYP1A2, which showed time-dependent inhibition, we investigated the relationship between time-dependent inhibition and MIC formation caused by STP and structurally related MDP compounds (Fig.2) using liver microsomes from CYP1A-induced rats.

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Materials and methods

Chemicals. STP, methylenedioxybenzene (MDB), safrole (SF), and piperonyl butoxide (PBO) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Isosafrole (ISF), phenacetin, and potassium ferricyanide were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). Dihydrosafrole (DSF) was purchased from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). Testosterone and acetaminophen were purchased from Sigma-Aldrich (St. Louis, MO, USA). 6β-Hydroxytestosterone was purchased from Ultrafine Chemicals Ltd. (Manchester, UK). Glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH) and NADPH were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Liver microsomes. Eight-week-old male Wistar rats were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were housed in a climate-controlled chamber under a 12/12 h light/dark cycle and were fed a standard chow diet and water *ad libitum* for 1 w before testing. All animal experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee of the Chiba Institute of Science. The rats were intraperitoneally pretreated with 80 mg/kg/d of β-naphthoflavone (BNF) in corn oil for 4 d and killed by decapitation 24 h after the final dose. Microsomal fractions were isolated from the livers according to the method described by Omura and Sato (Omura and Sato, 1964), and their protein concentrations were assayed using the method described by Lowry et al. (Lowry et al., 1951). Liver microsomes as pooled fractions from 50 donors were purchased from XenoTech LLC. (Lenexa, KS).

Preincubation of microsomes with MDP compounds. Human liver microsomes were preincubated with STP in the presence of NADPH to determine the metabolism-dependent inhibition of CYP1A2 and CYP3A4. An incubation mixture

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volume of 0.5 ml contained 0.25 mg microsomal protein, 10 mM G-6-P, 1 unit G-6-PDH, 10 mM $MgCl_2$, 0.1 mM EDTA, and some concentrations of STP in 0.15 M potassium phosphate buffer (pH 7.4). After setting the temperature to 37 °C, microsomes were preincubated with STP by initiating with NADPH at a final concentration of 0.5 mM and it was carried out for 20 min. Subsequent incubation was initiated by adding phenacetin and testosterone to the CYP1A2 and CYP3A4 assays, respectively. The structural requirements for the metabolite-dependent inhibition of CYP1A2 by MDP compounds, including drugs other than STP, were examined using liver microsomes from BNF-treated rats. In this experiment with rat liver microsomes, preincubation was performed for 10 min to determine metabolism-dependent CYP1A2 inhibition.

Assay of CYP1A2 and CYP3A4 activities. Phenacetin O-deethylation and testosterone 6β-hydroxylation activities were assessed as CYP1A2 and CYP3A4 indices, respectively. Probe substrates were added to the reaction mixture after preincubation for the metabolism of STP and other MDP compounds, as described above. The substrate concentrations of phenacetin and testosterone were 50 and 200 μM, respectively. Incubation was performed for 2 min and terminated by the addition of ethyl acetate. Phenacetin O-deethylation and testosterone 6β-hydroxylation activities were assayed using HPLC as previously described (Masubuchi and Horie, 2003; Masubuchi and Kawaguchi, 2013).

Assessment of MIC formation. MIC formation was assessed by measuring the difference in spectroscopic features using a Hitachi U3310 spectrophotometer (Tokyo, Japan) over a wavelength range of 400–500 nm. A 1.0 mL incubation mixture containing 1.0 mg of microsomal protein from BNF-treated rats, 10 mM G-6-P, 1 unit/mL G-6-PDH, 10 mM MgCl2 and 0.5 mM NADPH in 0.15 M potassium phosphate buffer (pH 7.4) were divided into 0.5 mL portions for sample and reference cuvettes. After setting the temperature to 37 °C, STP or other MDP compound at a final

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concentration of 100 μM was added into the sample cuvette and incubated for 40 min. The spectrophotometer was set to scan for the difference spectra from 500 to 400 nm every 5 or 10 min. ΔAbsorbance maximum was obtained from the difference between maximum absorption and absorption at 490 nm.

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Results

Time-dependent inhibition of CYP1A2 by STP in human liver microsomes. As a result of examining the effects of STP on P450 isozymes in human liver microsomes, as previously reported (Tran et al., 1997), an inhibitory effect on CYP3A4 (IC_{50} = 18.9 μM) was observed, and a similar or slightly weaker inhibitory effect on CYP1A2 (IC_{50} = 28.7 μM) was obtained (Fig.3). Of these, preincubation of microsomes with STP in the presence of NADPH did not affect the inhibitory effect on CYP3A4 (IC_{50} = 13.6 μM) (Fig.3A), but that on CYP1A2 was enhanced by preincubation, resulting in a shift of the inhibition curve to the low concentration side (IC_{50} = 2.6 μM) (Fig.3B). This time-dependent CYP1A2 inhibition indicated that the metabolites produced by CYP1A2 are involved in the inhibitory effects of STP on CYP1A2.

MIC formation by STP and other MDP compounds. Based on the above considerations, structural characterization of time-dependent P450 inhibition by STP was performed using CYP1A2. In a preliminary study, spectra associated with STP metabolism, suggesting MIC formation, were detected in human liver microsomes. However, these amounts were too small to be suitable for quantitative analysis (data not shown). Therefore, spectroscopic analysis was performed using liver microsomes from CYP1A2-induced rats. Liver microsomes were incubated with the MDP compounds in the presence of NADPH to obtain difference spectra. All MDP compounds used in this study gave spectra with a Soret peak at approximately 455 nm (Supplemental Fig.1), which is characteristic of MIC formation, and there was no major difference between the drugs at the maximum wavelength (Table 1). The MIC formation was comparable and the largest for STP and ISF (Fig.4) with common groups, similar to that for DSF, where the side chain of ISF was substituted with a propyl group (Fig.2). This followed SF, an ISF isomer. PBO, which is a classical CYP inhibitor, was smaller than these, and MIC formation by MDB, which is the backbone of MDP without a side chain, was the

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smallest. When potassium ferricyanide was added 20 min after the start of the reaction with STP, the absorbance rapidly decreased, as clearly shown by the spectral change (Supplemental Fig.2). Similar results were obtained for other MDP compounds (Fig.5), suggesting that the complexes were quickly dissociated by ferricyanide.

Time-dependent inhibition rat CYP1A2 by STP and structurally related MDP compounds. The inhibition of CYP1A2 activity in liver microsomes by various MDP compounds was evaluated with or without preincubation with the test drug in the presence of NADPH. Preincubation with any compound shifted the inhibition curve to the left (Fig.6), indicating potentiation of inhibition due to the formation of metabolites. As indicated by the IC_{50} values (Table 2), without preincubation, inhibition was the strongest with ISF, followed by STP. However, after preincubation to exert metabolism-dependent inhibition, inhibition was the strongest with STP, followed by ISF. The inhibitory potency acquired by preincubation was evaluated using the ratio of IC_{50} value at 0 min preincubation to that at 20 min preincubation (Table 2). The inhibitory potency in DHS was the strongest, followed by STP.

Association of STP- and other MDP compound-induced CYP1A2 inhibition with MIC formation. A strong correlation existed between the absorbance at the peak value of the MIC spectrum and the degree of CYP1A2 inhibition by the MDP compounds, when preincubated; however, PBO tended to deviate from this trend slightly (Fig.7). Since the MIC of CYP1A2 with metabolites of MDP compounds can be dissociated by ferricyanide, the effect of ferricyanide on the time-dependent inhibition was examined. The addition of potassium ferricyanide partially but significantly attenuated STP-induced CYP1A2 inhibition after preincubation (Fig.8). These results suggest that MIC contributes to the time-dependent CYP1A2 inhibition by STP and other MDP compounds.

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Discussion

A previous *in vitro* study indicated that STP inhibited multiple P450s, including CYP1A2 and CYP3A4; however, that study observed no time-dependent inhibition of P450s (Tran et al., 1997). In the present study, we found, for the first time, that STP caused a time-dependent inhibition of CYP1A2 (Fig.3). It is unclear why the previous study did not observe time-dependent inhibition. However, that study used only R-warfarin as the index substrate for activity measurement, which may be one reason why time-dependent inhibition could not be detected. Moreover, clinical studies in the same report (Tran et al., 1997) suggested that STP inhibits *in vivo* CYP1A2 as well as CYP3A4. Therefore, time-dependent inhibition should be implicated in the *in vivo* inhibition of CYP1A2, which is an important clinical target for STP-mediated drug-drug interactions.

Since STP has an MDP group, carbene-mediated MIC formation with the heme iron of the P450 enzyme (Fig.1) was presumed to be the mechanism for the time-dependent CYP1A2 inhibition by STP found in the present study. The spectroscopic studies yielded spectra indicative of MIC formation (Supplemental Fig.1). MDP compounds (structurally similar to STP) also showed similar spectra, which caused time-dependent CYP1A2 inhibition (Fig.6). Correlation analysis showed that the ability of the six MDP compounds to inhibit CYP1A2 and generate MIC were significantly correlated (Fig.7). Interestingly, a better correlation was observed when PBO was excluded from this analysis, suggesting that PBO, a classical inhibitor of P450 enzymes, can also inhibit CYP1A2 via mechanisms other than MIC formation. Furthermore, the addition of potassium ferricyanide, an oxidizing agent that dissociates the MIC (Fig.5), partially but significantly attenuated the time-dependent inhibition by STP (Fig.8). A similar attenuation of CYP3A4 inactivation by ferricyanide has been reported for the MDP compound, piperine (Cui et al., 2020). These results suggest that

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STP-mediated time-dependent CYP1A2 inhibition is caused by MIC formation of STP-derived carbenes with the heme iron of CYP1A2.

All MDP compounds used in this study could be converted into the corresponding carbene species, which generate a complex with CYP1A2. The observed difference in the potencies of MDPs to generate MICs indicates that the structures of small alkyl groups determine their favorability to generate the postulated inhibitory carbenes. While examining the MICs formed with MDP compounds, including STP (Fig.4), STP showed the same potency for MIC formation as ISF, which showed the highest value among the six MDP compounds. Although STP has an unsaturated bond similar to ISF, it is not considered essential for MIC formation because DSF, which has a propyl group, also showed a similar extent of MIC formation. Moreover, SF, which is an isomer of ISF, actively formed MIC next to these MDPs. In contrast, MDB without a side chain and PBO with a long side chain formed MICs to a lower extent than the other compounds. STP and ISF are well-matched compounds that generate carbenes via oxidative metabolism by CYP1A2.

ISF was widely used in the earlier studies on MIC formation by MDP compounds (Hodgson and Philpot, 1974; Murray, 2000). Although structure-activity relationship has not been determined for the shortening or lengthening of ISF side chains, studies using a series of synthetic 4-n-alkoxymethylenedioxybenzene derivatives (Murray et al., 1985) indicated that the alkoxy chain length for optimal MIC formation with P450 was approximately five or six carbon atoms. The side chains of ISF and STP contained three and seven carbon atoms, respectively, which are close to these conditions. Hydrophobicity and steric properties of MDP compounds, which are determined by the size of the side chain, are important factors, in addition to the facilitation of carbene formation and stability in the mechanism of P450 inhibition. As compounds that exist naturally or as pharmaceuticals, STP and ISF are likely to be the most potent inhibitors; however, stronger inhibitors may be found in the future.

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Previous studies using liver microsomes of phenobarbital-induced and BNF-induced rats suggested that the P450 species had low specificity in complex formation with ISF and were relatively isoform-nonspecific; however, different isoforms were reported to generate complexes with different stabilities, i.e., different abilities to inhibit P450 function (Murray, 2000). On the other hand, there is little information on MDP compound-mediated inhibition of human P450s, but one report showed a high selectivity of ISF function, which inhibited CYP1A2 but not CYP1A1 (Pastrakuljic et al., 1997). In the present study, since time-dependent inhibition by STP was observed for human CYP1A2 but not for CYP3A4, we focused on CYP1A2 in subsequent mechanistic MIC studies. In the future, more precise elucidation of isoform specificity is desirable in STP-mediated MIC formation with P450.

Several decades have passed since the series of ISF studies, and little progress has been made in promoting the importance of MIC for MDP; however, recently, inhibition of P450 isoforms by MDP compounds for pharmaceutical and supplement use has been actively reported. These include CYP2D6 inhibition by paroxetine, a selective serotonin reuptake inhibitor (Bertelsen et al., 2003); CYP2C9 inhibition by sesamin, a major lignan in sesame (Yasuda et al., 2015); and CYP3A4 inhibition by piperine, a component of black pepper (Cui et al., 2020). Interestingly, each MDP compound (including STP) preferentially inhibited different P450 isoforms; however, their selectivity requires further investigation. Since the site where an MDP molecule binds to the heme iron of P450 is specific, it is assumed that a side chain of the opposite site determines isoform specificity; therefore, a series of MDP compounds are expected to be useful for further analysis of substrate-binding sites of different P450 isoforms.

In conclusion, the present study demonstrates for the first time that the antiepileptic drug STP causes time-dependent CYP1A2 inhibition via MIC formation, which may lead to drug-drug interactions. This can lead to the continuous inhibition of the metabolism of concomitant antiepileptic agents, which is beneficial for maintaining

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blood concentrations. Moreover, the chemical structure of STP may favor the formation of stable carbenes, leading to a potent inhibition of CYP1A2. The present study not only provides a new example of CYP inhibition by MDP compounds but also suggests the universality of time-dependent CYP inhibition by this group of compounds.

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Data Availability.

The authors declare that all the data supporting the findings of this study are contained within the paper and its Supplemental Materials.

Authorship Contributions

Participated in research design: Masubuchi

Conducted experiments: Takahashi and Gendo

Performed data analysis: Takahashi and Gendo

Wrote or contributed to the writing of the manuscript: Masubuchi

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Footnotes

The authors declare that there are no conflicts of interest.

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Send reprint requests to: Dr. Yasuhiro Masubuchi, Laboratory of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Chiba Institute of Science, 15-8 Shiomi-cho, Choshi, Chiba 288-0025, Japan. E-mail: ymasubuchi@cis.ac.jp

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TABLE 1

Maximum absorption wavelength and Δabsorption maximum of MDP compound

MDPs	Maximum absorption wavelength (nm)	⊿Amax
STP	455.8	0.0916 ± 0.0029
MDB	455.3	0.0363 ± 0.0022
PBO	456.5	0.0432 ± 0.0036
SF	455.7	0.0544 ± 0.0018
ISF	455.3	0.0884 ± 0.0019
DSF	455.5	0.0806 ± 0.0016

The data shown in Fig.S1 and Fig.4 are obtained for quantitative evaluation. Results for ⊿Amax are presented as the mean ± SE of 3 determinations.

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TABLE 2

Shift in IC_{50} values of MDP compounds for CYP1A2 inhibition after preincubation with NADPH

MDPs	IC_{50} (μM)		Ratio
	Preincubation time		
	0 min	10 min	
STP	129.0 ± 26.0	6.0 ± 26.0	23.7
MDB	> 1000	290.9 ± 77.8	> 4.3
PBO	294.0 ± 36.6	39.2 ± 0.3	7.5
SF	467.3 ± 68.9	45.1 ± 1.8	10.2
ISF	48.2 ± 4.7	8.1 ± 0.8	6.1
DSF	285.9 ± 59.4	11.0 ± 0.6	26.4

The data in Fig.6 are obtained for quantitative evaluation. Results are presented as the mean ± SE of 3 determinations.

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Legends of figures

Fig.1. Proposed metabolic pathway of MDP compounds leading to MIC formation with heme-iron of P450 via generation of reactive carbene.

Fig.2. Chemical structure of MDP compounds used in this study.

Fig.3. Inhibition of CYP1A2 and CYP3A4 by STP in human liver microsomes. CYP3A4 (A) and CYP1A2 (B) activities in human liver microsomes were determined using various concentrations of STP, which represent the percentage of control activity without STP. Open and closed circles represent direct inhibition and time-dependent inhibition after preincubation with STP in the presence of NADPH, respectively. Results are presented as the mean ± SE of 3 determinations. The mean IC_{50} values obtained for each experimental condition are described in the Result section.

Fig.4. Time course of Δabsorption revealing MIC of MDP compounds with P450. The spectral data in Fig.S1 were subjected to calculation for determining the absorbance differences between the maximum absorption at the wavelength listed in Table 1 and the basal absorption at 490 nm. The absorbance differences thus obtained at each sampling time point are plotted against time. Results are presented as the mean ± SE of 3 determinations.

Fig.5. Dissociation of MIC by ferricyanide presented as Δabsorption. Incubation was performed in the same manner as shown in Fig.4, except that potassium ferricyanide was added to both cuvettes after 20 min. The difference in spectra for the incubates were recorded at 5, 10, 20, 30, and 40 min after the addition of NADPH. The absorbance differences thus obtained at each sampling time point are plotted against

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time. Results are presented as the mean of 3 determinations.

Fig.6. Inhibition of CYP1A2 by STP and other MDP compounds in liver microsomes of BNF-pretreated rats. CYP1A2 activity in liver microsomes collected from BNF-pretreated rats was determined using various concentrations of STP and other MDP compounds, which represents the percentage of control activity without MDPs. Open and closed circles represent direct inhibition and time-dependent inhibition after preincubation with MDPs in the presence of NADPH, respectively. Results are presented as the mean ± SE of 3 determinations. The mean IC_{50} values thus obtained for each experimental condition are listed in Table 2.

Fig.7. Correlation between MIC formation and time-dependent CYP1A2 inhibition by MDPs. The correlation between the abilities of MDP compounds to generate MIC and inhibit CYP1A2 in a time-dependent manner was analyzed. The horizontal axis indicates ΔAmax listed in Table 1 as MIC. On the vertical axis, the time-dependent CYP1A2 inhibition by each MDP compounds was calculated from the residual activity after preincubation of microsomes with 100 μM MDP plotted in Fig.6. The dotted line and correlation coefficients in parentheses indicate the correlation obtained without PBO. Results are presented as the mean ± SE of 3 determinations.

Fig.8. Partial recovery of CYP1A2 activity by adding potassium ferricyanide in the microsomes preincubated with STP and NADPH. Microsomes were preincubated for 0 or 10 min in the presence of STP and NADPH, and potassium ferricyanide was added to some cuvettes to dissociate the MIC, which is shown as a plus or minus on the horizontal axis. Results are shown as relative activities. STP(-)/STP(+), and are presented as the mean ± SE of 3 determinations. *, $p<0.05$ vs preincubation(-); †, $p<0.05$ vs without potassium ferricyanide.

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Fig.1

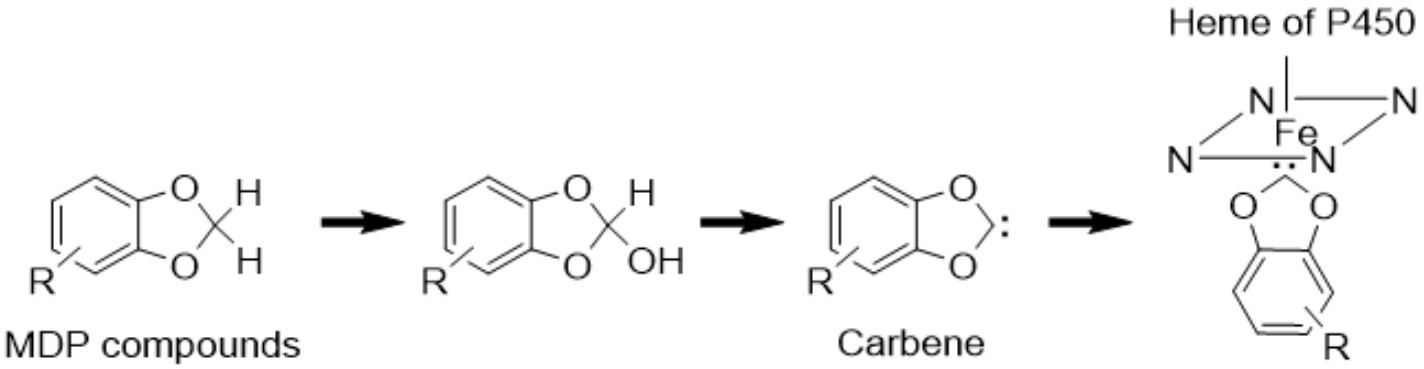

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Fig.2

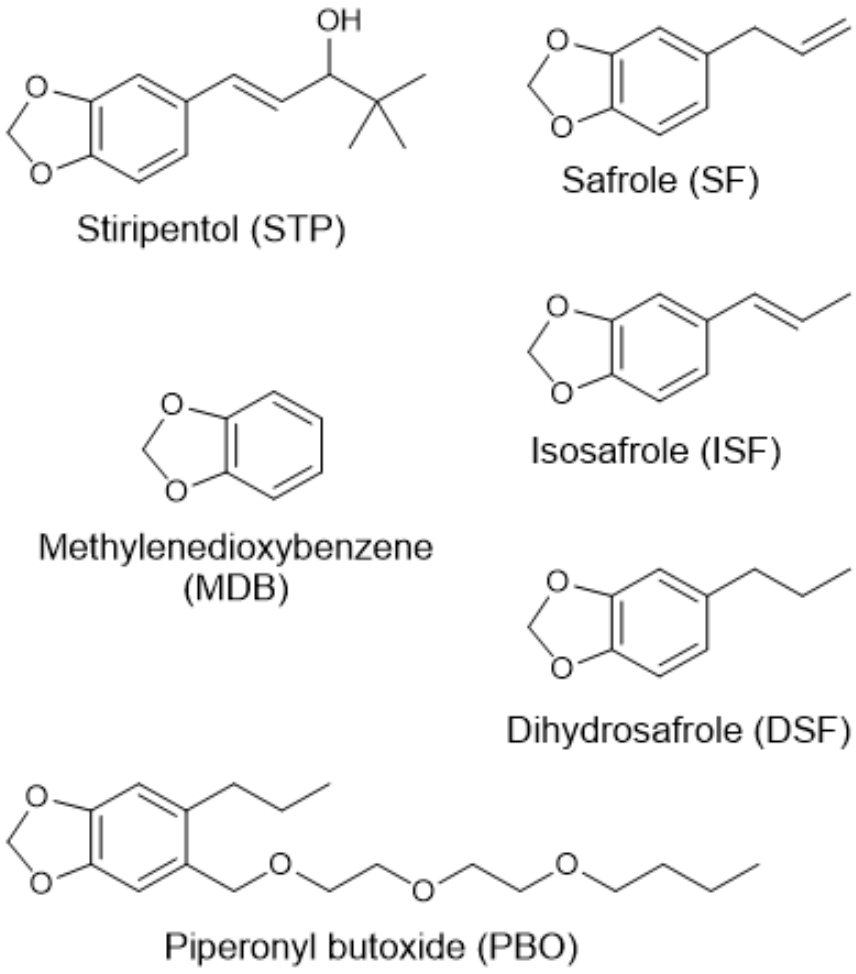

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Fig.3

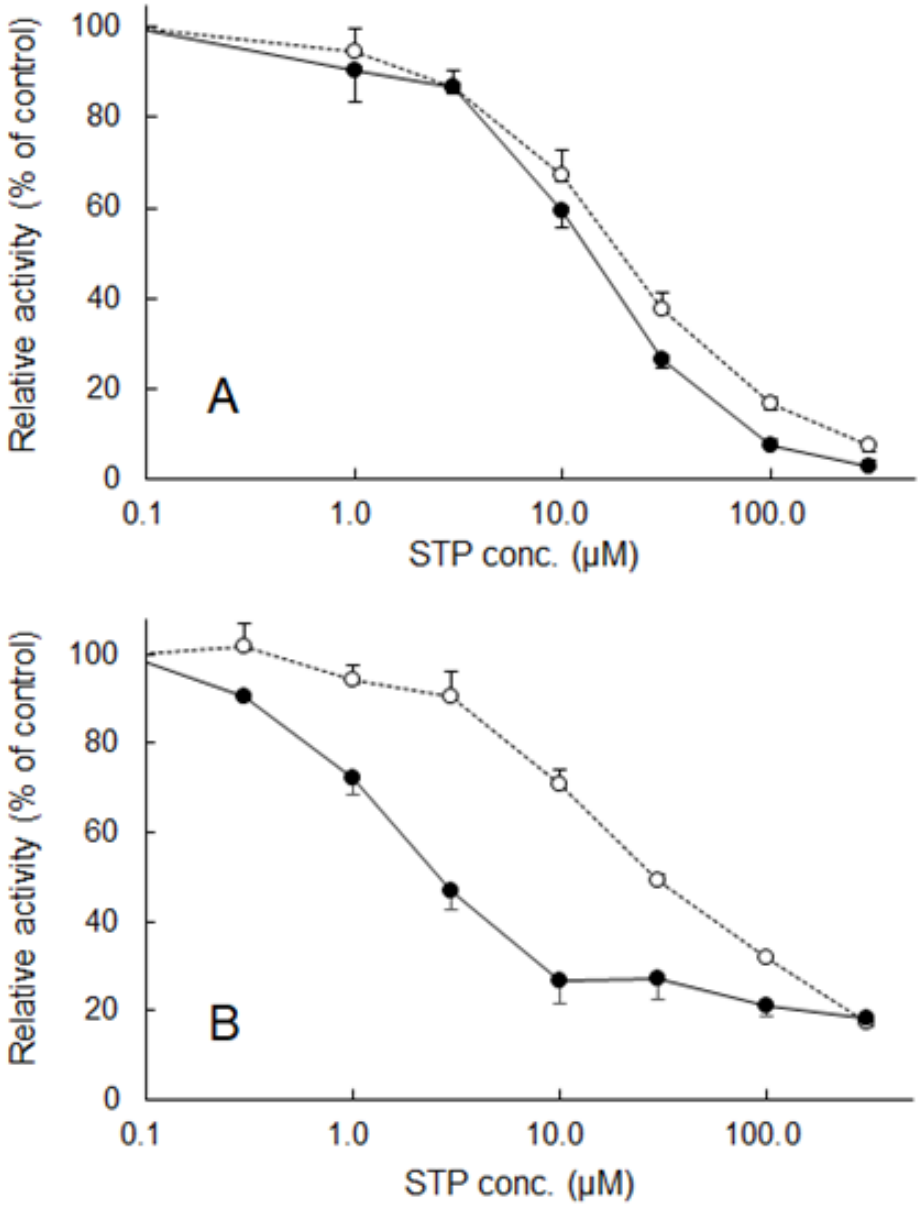

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Fig.4

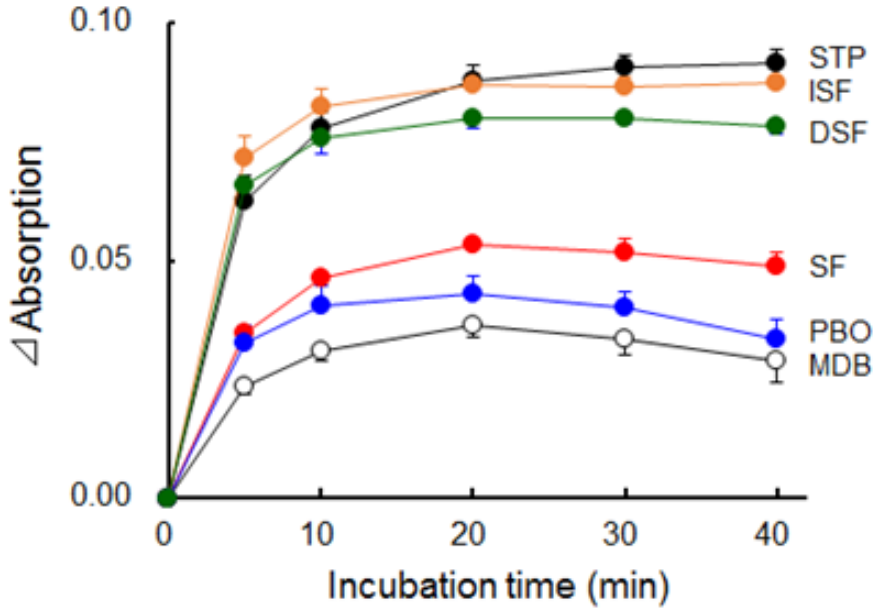

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Fig.5

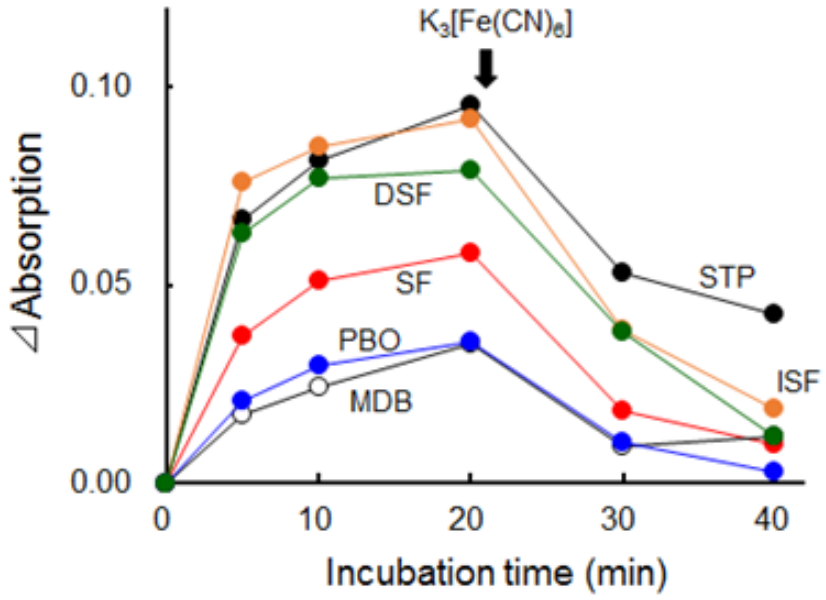

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Fig.6

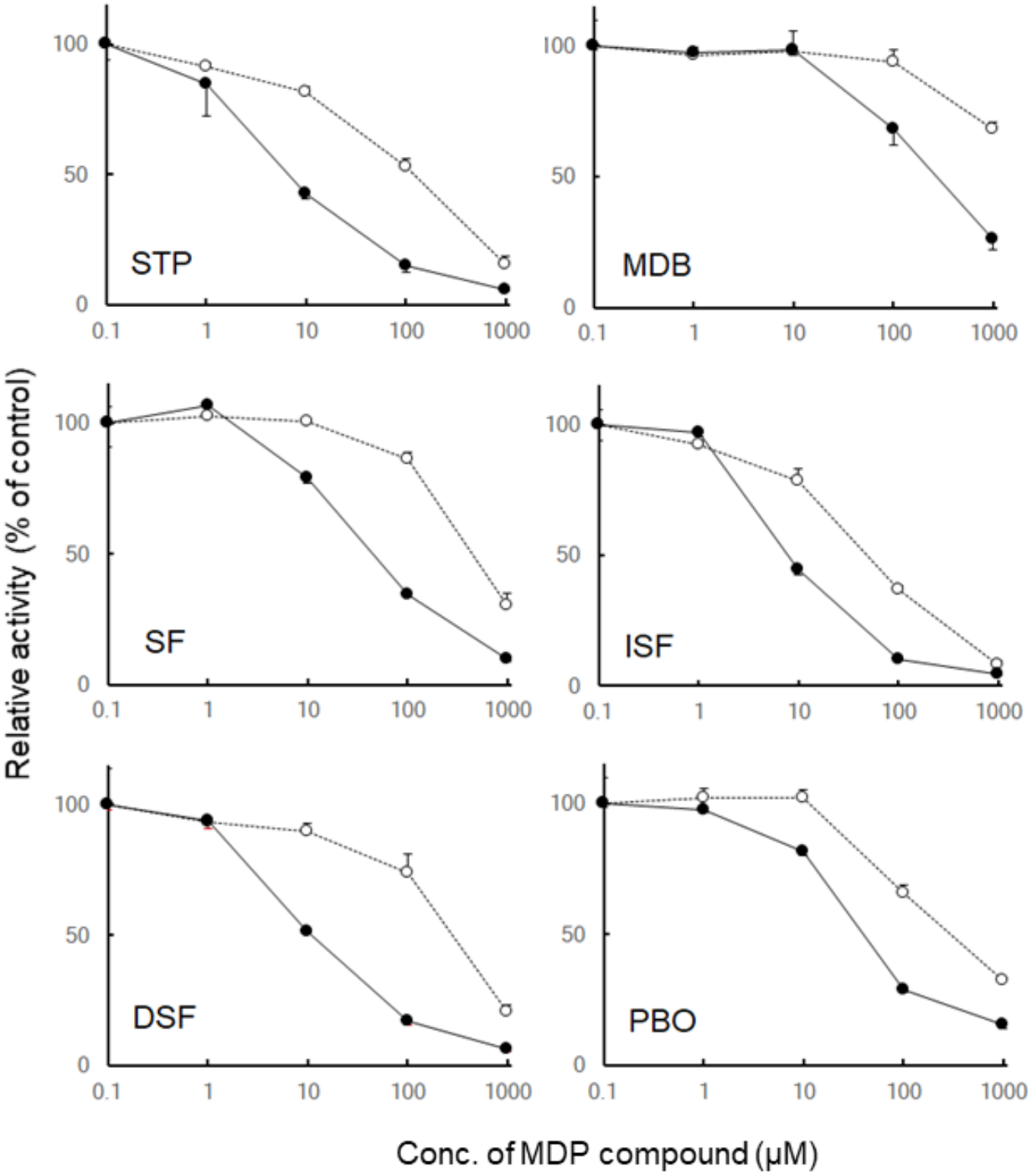

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Fig.7

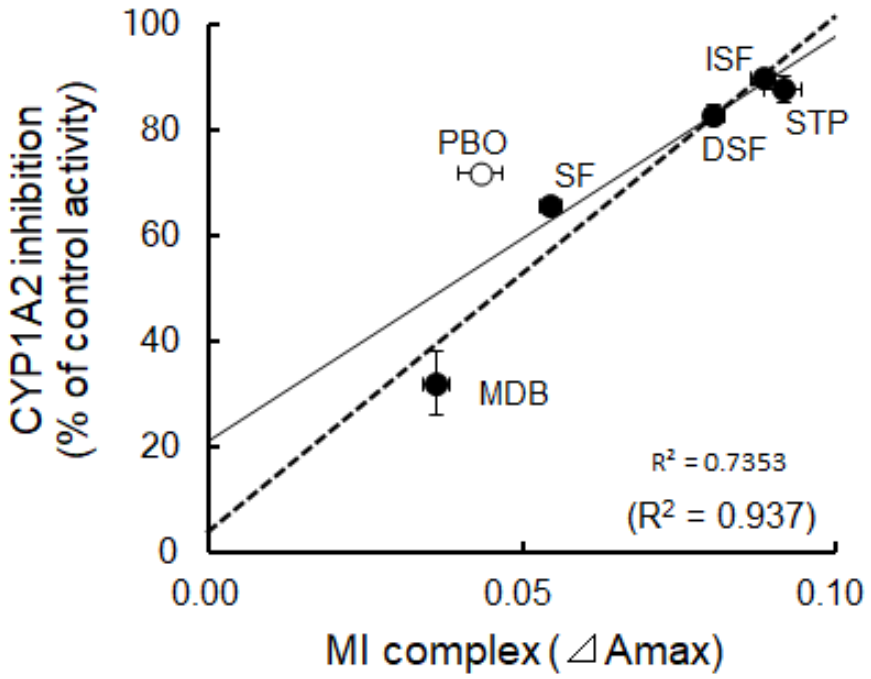

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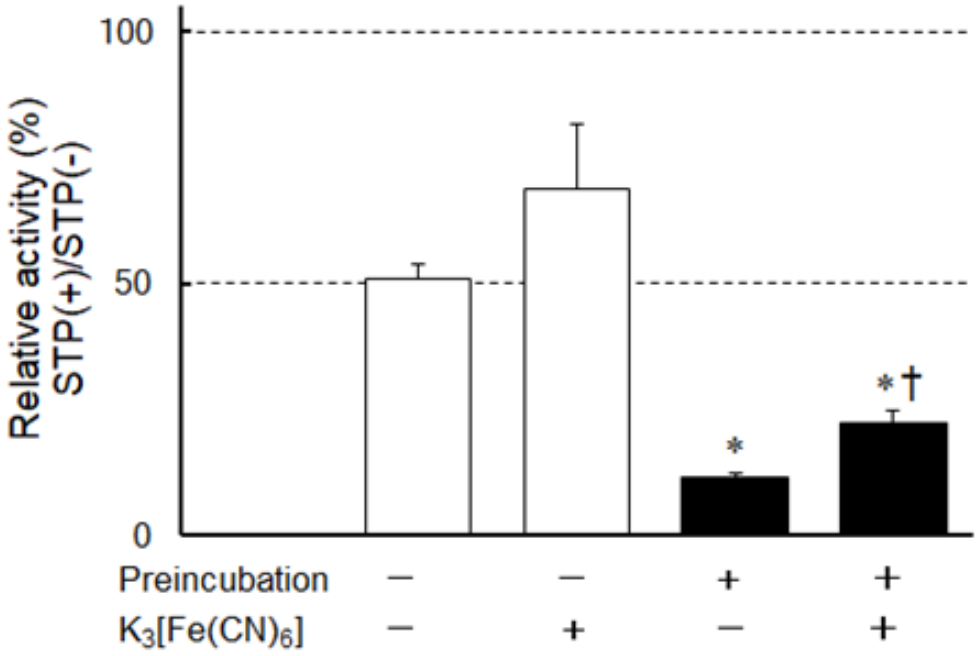
Fig.8



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