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Letter

In vitro metabolism of 4-methyl- and 5-methyl-2-mercaptobenzimidazole, thyrotoxic and hepatotoxic rubber antioxidants, in rat liver microsomes

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ABSTRACT — The metabolism of 4-methyl-2-mercaptobenzimidazole (4-MeMBI), 5-methyl-2-mercaptobenzimidazole (5-MeMBI), and 2-mercaptobenzimidazole (MBI) was examined *in vitro* in rat liver microsomes. The test chemicals were incubated in the presence of liver microsomes from male Sprague-Dawley rats, and their metabolism was analyzed by HPLC. The metabolism amount increased in an incubation time-dependent manner, and was similar among the test chemicals. SKF-525A, a non-selective inhibitor of cytochrome P450 (CYP) enzymes, decreased the metabolic rate of all the test chemicals, indicating the involvement of liver microsomal CYP enzymes. When liver microsomes from rats treated with CYP-inducers (β-naphthoflavone, phenobarbital, and isoniazid) were used, 4-MeMBI was more decreased than 5-MeMBI, particularly in the phenobarbital-treated group. These results, together with the reported inducibility of the drug-metabolizing activity by the test chemicals, partly explained the counteraction in the toxic effects between 4-MeMBI and 5-MeMBI in the *in vivo* study.

Key words: Methyl-2-mercaptobenzimidazole, Liver microsomes, Rat, *In vitro* metabolism, Cytochrome P450, SKF-525A

INTRODUCTION

4-Methyl-2-mercaptobenzimidazole (4-MeMBI) and 5-methyl-2-mercaptobenzimidazole (5-MeMBI) constitute 4(or5)-methyl-2-mercaptobenzimidazole (4(5)-MeMBI) as the 1:1 mixture that has been widely used as an industrial material, including a rubber antioxidant, corrosion inhibitor, and copper-plating brightener, similar to its unmethylated chemical form, 2-mercaptobenzimidazole (MBI) (Teng *et al.*, 2014). In 28-day repeated oral administration studies, both 4(5)-MeMBI and MBI were thyrotoxic owing to their thioureylene structure, but only the latter was considered hepatotoxic (Kawasaki *et al.*, 1998; Saitoh *et al.*, 1999). However, we previously showed that 4(5)-MeMBI was also hepatotoxic in terms of inhibitory effects on the drug-metabolizing activity in rat liver microsomes (Miyajima *et al.*, 2017). We also showed that the toxicity of 4-MeMBI and 5-MeMBI counteract-

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ed each other; the hepatotoxicity of 4(5)-MeMBI were the same or weaker than either of its constituents alone. As a possible mechanism of this counteraction, it was supposed that the drug-metabolizing activity induced by 4-MeMBI detoxified efficiently 5-MeMBI, or *vice versa*. In the present study, we therefore examined the *in vitro* metabolism of 4-MeMBI, 5-MeMBI, and MBI in rat liver microsomes. Liver microsomes from rats treated with inducers of cytochrome P450s (CYPs) also were used to investigate the CYP species involved in the metabolism of the test chemicals.

MATERIALS AND METHODS

Chemicals

The structures of the test chemicals, 4-MeMBI, 5-MeMBI, and MBI, are shown in Fig. 1A. 4-MeMBI (CAS No. 27231-33-0) was isolated from 4(5)-MeMBI

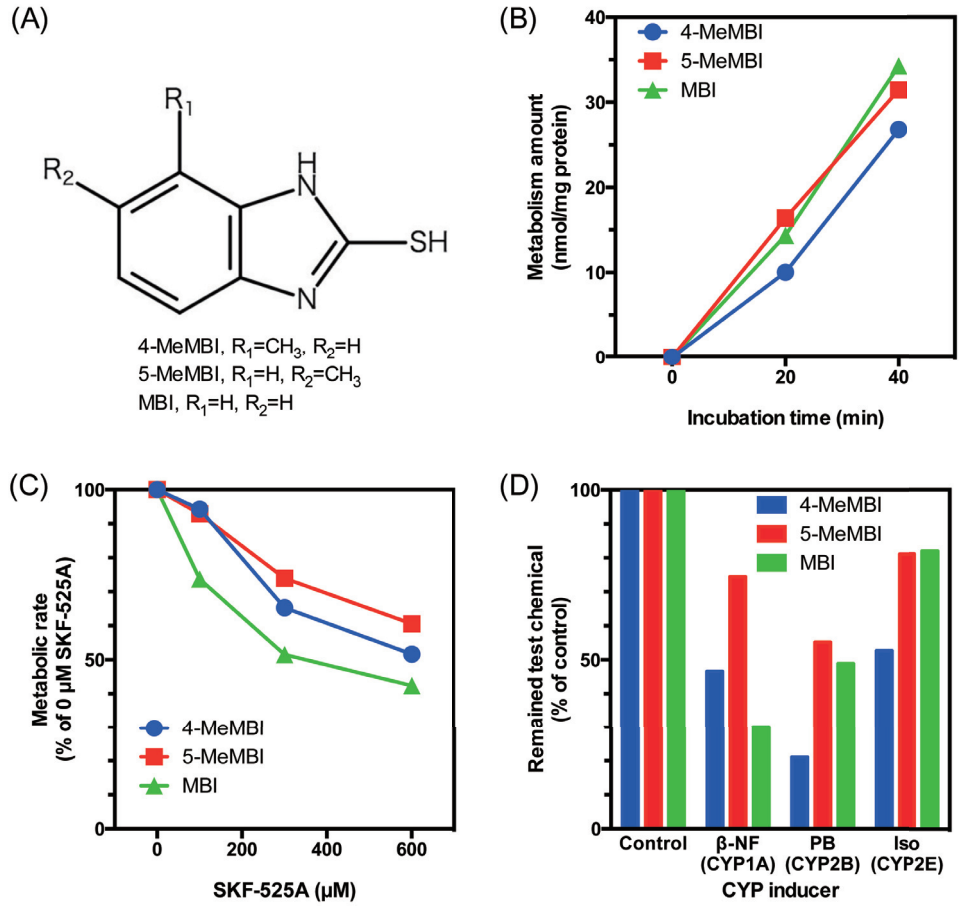

Fig. 1. Test chemicals and their *in vitro* metabolism in rat liver microsomes. 4-MeMBI, 4-Methyl-2-mercaptobenzimidazole; 5-MeMBI, 5-methyl-2-mercaptobenzimidazole; MBI, 2-mercaptobenzimidazole. (A) Structures of the test chemicals. Marvin was used for drawing, Marvin JS (17.15.0), 2017, ChemAxon (http://www.chemaxon.com). (B) Time-course analysis of the metabolism. Metabolism amount was calculated from the decrease in the peak area of the test chemicals as determined by HPLC, and was corrected for the amount of microsomal protein. The reaction mixtures were incubated for 40 min. Averages of two determinations are shown. (C) Effects of SKF-525A on the metabolism. Metabolic rate was calculated from the decrease in the peak area of the test chemicals as determined by HPLC, and was expressed as percentage of the values at 0 μM of SKF-525A. Averages of two determinations are shown. (D) Metabolism of the test chemicals by liver microsomes from rats treated with CYP-inducers: β-naphthoflavone (β-NF) for CYP1A, phenobarbital (PB) for CYP2B, and isoniazid (Iso) for CYP2E. Remained test chemical was calculated from the peak area of the test chemicals as determined by HPLC, and was expressed as percentage of the values by liver microsomes from untreated rats in the control groups. The reaction mixtures were incubated for 20 min. Averages of two determinations are shown.

by repeated fractional recrystallization of 4(5)-MeMBI (CAS No. 53988-10-6) supplied from Ohuchi Shinko Chemical Ind., Ltd (Tokyo, Japan). 5-MeMBI (CAS No. 27231-36-3) was purchased from Aldrich Japan Inc. (Tokyo, Japan). MBI (CAS No. 583-39-1) and thiabendazole (CAS No. 148-79-8) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). SKF-525A (CAS No. 62-68-0) was purchased from Salford Ultrafine Chemicals and Research Ltd (Manchester, UK). β-NADPH was purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). The test chemicals (10 mM) and thiabendazole (1 mM) were dissolved in methanol before use.

Rat liver microsomes

Liver microsomes prepared from male Sprague-Dawley rats were purchased from XenoTech, LLC (Kansas City, KS, USA). For the control groups, pooled liver microsomes from rats treated with saline (product no. R1073) or corn oil (R1098) were used. For the CYP-induced groups, pooled liver microsomes from rats treated with β-naphthoflavone for CYP1A (R1083), phenobarbital for CYP2B (R1078), and isoniazid for CYP2E (R1088) were used.

In vitro metabolism experiments

The test chemicals (10 μM) were incubated in a reaction mixture (250 μL) composed of 100 mM potassium phosphate (pH 7.4), 0.33 mM β-NADPH, and liver microsomes (200 μg protein/mL). The reaction was started by the addition of the liver microsomes (50 μL) to the pre-incubation mixture (200 μL) containing the other components at 37°C. After the incubation time (20-40 min), the reaction was stopped by the addition of methanol (200 μL), and 50 μM thiabendazole solution (50 μL) was added as an internal standard for the HPLC analysis of test chemicals.

HPLC analysis

The amount of the test chemicals in the reaction mixture was determined in duplicate by HPLC using thiabendazole as an internal standard (Sakemi *et al.*, 1999). The retention times of the peaks, measured at 300 nm, were 14.9 min for 4-MeMBI, 15.8 min for 5-MeMBI, 8.17 min for MBI, and 21.7 min for thiabendazole.

RESULTS AND DISCUSSION

The metabolism amount of the test chemicals in the reaction mixture increased similarly in an incubation time-dependent manner, indicating that their metabolism can be evaluated quantitatively under these experimental conditions (Fig. 1B). The similar metabolism of the test chemicals suggests that the major metabolic reaction involves their common structure, possibly thioureylene that can be desulfurized. This is supported by the findings that the desulfurized metabolites of the test chemicals appeared rapidly in the urine after the single oral administration to rats (Sakemi *et al.*, 1999; Sakemi *et al.*, 2002).

The addition of SKF-525A, a non-selective inhibitor of CYP enzymes, to the reaction mixtures decreased the metabolic rate of all the test chemicals in a concentration-dependent manner, indicating the involvement of CYP enzymes in their metabolism (Fig. 1C). The order of inhibition of metabolic rate, MBI > 4-MeMBI ≥ 5-MeMBI, suggested that the metabolism of 4-MeMBI had slightly greater CYP-dependence than that of 5-MeMBI. The involvement of CYP enzymes is consistent with the desulfurization of the test chemicals described above since some CYPs have this activity (Kyle *et al.*, 2013).

When liver microsomes from rats treated with CYP-inducers were used, 4-MeMBI was more decreased than 5-MeMBI, particularly in the phenobarbital (PB) group, which contained more CYP2B (Fig. 1D). These results explain, at least partly, the counteraction in the toxic effects between 4-MeMBI and 5-MeMBI because the latter markedly induce CYP2B1/2 (Miyajima *et al.*, 2017), which can result in more efficient detoxification of the former.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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