Letter

***In vitro* cytotoxicity of the thyrotoxic and hepatotoxic rubber antioxidant 2-mercaptobenzimidazole and its 4- or 5-methyl derivatives in rabbit corneal cells**

**Yukie Kuroda^{1,*}, Atsuko Miyajima^{2,*}, Kazue Sakemi-Hoshikawa¹, Makoto Usami^{1,3},
Katsuyoshi Mitsunaga⁴, Yasuo Ohno^{1,5} and Momoko Sunouchi¹**

¹Division of Pharmacology, National Institute of Health Sciences,
3-25-26 Tonomachi, Kawasaki, Kanagawa 210-9501, Japan

²Division of Medical Devices, National Institute of Health Sciences,
3-25-26 Tonomachi, Kawasaki, Kanagawa 210-9501, Japan

³Graduate School of Veterinary Medicine, Azabu University,
1-17-1 Fuchinobe, Chuo-ku, Sagami-hara 252-5201, Japan

⁴School of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan

⁵Kihara Memorial Yokohama Foundation for the Advancement of Life Sciences,
1-6 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

(Received August 4, 2020; Accepted August 13, 2020)

ABSTRACT — 2-Mercaptobenzimidazole (MBI) and its methyl derivatives 4-methyl-MBI (4-MeMBI), 5-methyl-MBI (5-MeMBI), and 4(or 5)-methyl-MBI (4(5)-MeMBI) are widely applied industrial agents with substantial thyrotoxicity and hepatotoxicity detected in rats *in vivo*. Here, we examined the *in vitro* cytotoxicity of MBI and its derivatives in cultured SIRC rabbit corneal cells. SIRC cells were cultured in the presence of the test chemicals for 72 hr, and cell viability was determined by estimating the number of cells using a crystal violet staining assay. The median lethal concentration (LC₅₀) was calculated for each of the chemicals. The methyl derivatives showed higher cytotoxicity than MBI, which is in contrast to previous *in vivo* findings demonstrating higher thyrotoxicity and hepatotoxicity of MBI compared to its derivatives. According to the LC₅₀ values, the ranking of the tested agents in terms of cytotoxicity was 5-MeMBI (761.5 μ M) $\geq$ 4-MeMBI (796.3 μ M) $\geq$ 4(5)-MeMBI (822.9 μ M) $>$ MBI (1002.9 μ M). The present results suggest that the lower thyrotoxicity and hepatotoxicity of methyl derivatives of MBI is related to their faster detoxification *in vivo*, because SIRC cells are considered to have lower drug-metabolizing activity than hepatic cells.

Key words: Methyl-2-mercaptobenzimidazole, Cytotoxicity, SIRC cells

INTRODUCTION

4-Methyl-2-mercaptobenzimidazole (4-MeMBI) and 5-methyl-2-mercaptobenzimidazole (5-MeMBI) are methyl derivatives of 2-mercaptobenzimidazole (MBI), which have a thioureyne structure that is considered to

be responsible for their substantial thyrotoxicity and hepatotoxicity (Heidari *et al.*, 2015). 4-MeMBI and 5-MeMBI are typically prepared in a 1:1 mixture, resulting in 4(or 5)-methyl-2-mercaptobenzimidazole (4(5)-MeMBI, hereafter collectively referred to as methyl-MBIs) for broad industrial applications, including as rubber antioxi-

Correspondence: Makoto Usami (E-mail: makoto_usami@mac.com)

*These authors equally contributed to this work.

dants, corrosion inhibitors, and copper-plating brighteners, along with MBI (Teng *et al.*, 2014).

Both MBI and methyl-MBIs have been shown to cause thyrotoxicity and hepatotoxicity after repeated oral administration in rats (Saitoh *et al.*, 1999; Kawasaki *et al.*, 1998; Sakemi *et al.*, 2002). However, differences in their hepatic drug-metabolizing activity have been detected. For example, methyl-MBIs could induce the production of cytochrome P450 proteins (CYPs), including CYP1A1/2, in rats both *in vivo* and *in vitro*, whereas MBI did not (Miyajima *et al.*, 2017; Miyajima *et al.*, 2020). One proposed mechanism to explain this difference is that 4-MeMBI and 5-MeMBI are metabolized by CYP1A and CYP2B that are induced in rat liver microsomes to their desulfurized forms, allowing for their detoxification (Miyajima *et al.*, 2018; Sakemi *et al.*, 2002).

To help confirm this possibility, in the present study, we examined the *in vitro* cytotoxicity of MBI and methyl-MBIs to explore the intrinsic toxic effects under a condition that avoids detoxification activity. Cultured Statens Seruminstitut Rabbit Cornea (SIRC) rabbit corneal cells were used for the *in vitro* cytotoxicity assay because they are considered to have much lower drug-metabolizing activity than hepatic cells.

MATERIALS AND METHODS

Chemicals

The structures of 4-MeMBI, 5-MeMBI, and MBI are shown in Fig. 1. MBI (CAS No. 583-39-1, PubChem CID: 707035) was purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-MeMBI (CAS No. 27231-33-0, PubChem CID: 3034478) was isolated from 4(5)-MeMBI by repeated fractional recrystallization as described previously (Isama *et al.*, 1998). 5-MeMBI (CAS No. 27231-36-3, PubChem CID: 712373) was purchased from Sigma-Aldrich Co. LLC (Merck KGaA, Darmstadt, Germany) and 4(5)-MeMBI (CAS No. 53988-10-6) was supplied from Ohuchi Shinko Chemical Industries, Ltd. (Tokyo, Japan). All chemicals were dissolved in dimethyl sulfoxide (DMSO; final concentration 0.1%) before addition to the culture medium.

Cytotoxicity assay

The general cytotoxicity of the chemicals was determined by measuring the viability of SIRC cells using the crystal violet staining assay as described previously (Feoktistova *et al.*, 2016). In brief, SIRC cells (ATCC No. CCL 60; American Type Culture Collection, Manassas, VA, USA) were cultured in minimum essential medium (Thermo Fisher Scientific, Waltham, MA, USA) contain-

Fig. 1. Structures of 2-mercaptobenzimidazole (MBI), 4-methyl-2-mercaptobenzimidazole (4-MeMBI), and 5-methyl-2-mercaptobenzimidazole (5-MeMBI). The structure of 4(or 5)-methyl-MBI (4(5)-MeMBI) is not shown because it is a 1:1 mixture of 4-MeMBI and 5-MeMBI. Substituted methyl groups on the benzene ring are depicted in bold. Marvin JS (19.11.0, 2019) [ChemAxon (<http://www.chemaxon.com>)] was partly used for drawing the chemical structures.

ing 10% fetal bovine serum (Thermo Fisher Scientific) at 37°C with 5% CO₂. The SIRC cells were subcultured every 3 or 4 days using a 0.25% trypsin-ethylenediaminetetraacetic acid, phenol red solution (Thermo Fisher Scientific) for the detachment of cells prior to the cytotoxicity assay. All chemicals were dissolved in DMSO (final concentration $\leq 0.75\%$) and diluted with the culture medium. The cell suspensions were dispensed to 96-well culture plates (Clear Flat Bottom TC-treated Culture Microplate, Corning) at 100 μ L/well (2.0×10^5 cells/mL or 6.25×10^4 cells/cm²). After a 30-min period of standing on a clean bench, the cells were cultured for 72 hr at 37°C with 5% CO₂. The culture wells were washed with 200 μ L of phosphate-buffered saline (-) twice, and stained with 200 μ L of 0.4% crystal violet (Wako Pure Chemical) dissolved in methanol for 30 min. After washing with tap water and air-drying, the absorbance at 595 nm was determined for each of the wells with a microplate reader (Model 450, Bio-Rad Laboratories, Hercules, CA, USA). The median lethal concentration (LC₅₀) was calculated according to the decrease in absorbance due to each chemical using log-logistic curve fitting with the R software (R Core Team, 2017) extension package *drc* (Ritz *et al.*, 2015).

RESULTS AND DISCUSSION

The crystal violet staining assay showed that MBI decreased the viability of SIRC cells at concentrations

Cytotoxicity of methyl-2-mercaptobenzimidazoles

Fig. 2. Cytotoxicity of 2-mercaptobenzimidazole (MBI), 4-methyl-2-mercaptobenzimidazole (4-MeMBI), 5-methyl-2-mercaptobenzimidazole (5-MeMBI), and 4(or 5)-methyl-MBI (4(5)-MeMBI) in SIRC cells. SIRC cells were cultured for 72 hr in the presence of each chemical. Cell viability was determined by the crystal violet staining assay. Data from three experiments were combined after normalization. Each data point is the mean of three or four determinations from three experiments, which are distinguished by different colors. The 50% lethal concentration (LC_{50}) and its 95% confidence limits calculated by the log-logistic curve fitting are shown.

greater than 200 μM with an LC_{50} value of approximately 1000 μM (Fig. 2A). In addition, 4-MeMBI, 5-MeMBI, and 4(5)-MeMBI decreased cell viability at concentrations greater than 200 μM with similar concentration-response curves to each other, but showed lower LC_{50} values than that of MBI of around 800 μM (Fig. 2B-D). According to the LC_{50} values, the rank order of cytotoxicity in SIRC cells was 5-MeMBI $\geq$ 4-MeMBI $\geq$ 4(5)-MeMBI $>$ MBI.

The present results indicate that methyl-MBIs have similar cytotoxicity to each other, but are more cytotoxic than MBI in SIRC cells. This suggests that presence of a methyl group on the benzene ring intensifies the cytotoxicity of MBI derivatives. This effect is similar to the bioactivity of benzimidazole derivatives used as medical drugs, which is potentiated by substituents at position 5 on the benzene ring (Naaz *et al.*, 2018; Shimomura *et al.*, 2019; Arshad *et al.*, 2017).

The precise mechanisms of cytotoxicity of MBI and methyl-MBIs remain elusive. However, the benzimidazole structure was identified as the active center of various derivatives with several cytotoxic mechanisms suggested, including inhibition of the ubiquitin-proteasome pathway (Dogra and Mukhopadhyay, 2012), inhibition of angiogenesis (Ranganatha *et al.*, 2013), inhibition of protein kinases (Singla *et al.*, 2014), destabilization of microtubules (Lai *et al.*, 2017), and generation of reactive oxygen species (Prosser *et al.*, 2017). Therefore, it is possible that the cytotoxicity of MBI and methyl-MBIs involves similar mechanisms.

Notably, the rank order of the cytotoxicity of MBI and methyl-MBIs determined in SIRC cells in this study is in direct contrast with the ranking of their *in vivo* thyrotoxicity and hepatotoxicity based on studies in rats. That is, methyl-MBIs showed less thyrotoxicity based on an

increase in thyroid weight and inhibition of thyroid hormone production (Sakemi *et al.*, 2002), and showed reduced hepatotoxicity in terms of an increase in liver weight and inhibition of CYP activity (Miyajima *et al.*, 2017) compared with the effects of MBI. It has been considered that the lower *in vivo* thyrotoxicity and hepatotoxicity of methyl-MBIs is due to their rapid detoxification by induced drug-metabolizing enzymes (Miyajima *et al.*, 2017; Sakemi *et al.*, 2002). Because SIRC cells are considered to have lower drug-metabolizing activity than hepatic cells, the present results support this hypothesis.

ACKNOWLEDGMENTS

This work was partly supported by a Health and Labor Science Research Grant from the Ministry of Health, Labor and Welfare of Japan. We greatly appreciate Ohuchi Shinko Chemical Industries Co. for kindly providing 4(5)-MeMBI (Nocrac MMB).

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

REFERENCES

- Arshad, T., Khan, K.M., Rasool, N., Salar, U., Hussain, S., Asghar, H., Ashraf, M., Wadood, A., Riaz, M., Perveen, S., Taha, M. and Ismail, N.H. (2017): 5-Bromo-2-aryl benzimidazole derivatives as non-cytotoxic potential dual inhibitors of α -glucosidase and urease enzymes. *Bioorg. Chem.*, **72**, 21-31.
- Dogra, N. and Mukhopadhyay, T. (2012): Impairment of the ubiquitin-proteasome pathway by methyl N-(6-phenylsulfanyl-1H-benzimidazol-2-yl)carbamate leads to a potent cytotoxic effect in tumor cells: A novel antiproliferative agent with a potential therapeutic implication. *J. Biol. Chem.*, **287**, 30625-30640.
- Feoktistova, M., Geserick, P. and Leverkus, M. (2016): Crystal Violet Assay for Determining Viability of Cultured Cells. *Cold Spring Harb. Protoc.* **2016**, pdb.prot087379.
- Heidari, R., Niknahad, H., Jamshidzadeh, A., Eghbal, M.A. and Abdoli, N. (2015): An overview on the proposed mechanisms of antithyroid drugs-induced liver injury. *Adv. Pharm. Bull.*, **5**, 1-11.
- Isama, K., Momma, J. and Kaniwa, M. (1998): Structure-activity relationships for skin sensitization potential of 2-mercaptobenzimidazole and its methyl derivatives. *Environ. Dermatology*, **5**, 26-33.
- Kawasaki, Y., Umemura, T., Saito, M., Momma, J., Matsushima, Y., Sekiguchi, H., Matsumoto, M., Sakemi, K., Isama, K., Inoue, T., Kurokawa, Y. and Tsuda, M. (1998): Toxicity study of a rubber antioxidant, 2-mercaptobenzimidazole, by repeated oral administration to rats. *J. Toxicol. Sci.*, **23**, 53-68.
- Lai, S.R., Castello, S.A., Robinson, A.C. and Koehler, J.W. (2017): *In vitro* anti-tubulin effects of mebendazole and fenbendazole on canine glioma cells. *Vet. Comp. Oncol.*, **15**, 1445-1454.
- Miyajima, A., Kuroda, Y., Sakemi-Hoshikawa, K., Usami, M., Mitsunaga, K., Irie, T., Ohno, Y. and Sunouchi, M. (2018): *In vitro* metabolism of 4-methyl- and 5-methyl-2-mercaptobenzimidazole, thyrotoxic and hepatotoxic rubber antioxidants, in rat liver microsomes. *Fundam. Toxicol. Sci.*, **5**, 113-116.
- Miyajima, A., Kuroda, Y., Sakemi-Hoshikawa, K., Usami, M., Mitsunaga, K., Irie, T., Ohno, Y. and Sunouchi, M. (2020): Inhibitory and inductive effects of 4- or 5-methyl-2-mercaptobenzimidazole, thyrotoxic and hepatotoxic rubber antioxidants, on several forms of cytochrome P450 in primary cultured rat and human hepatocytes. *Toxicol. Rep.*, **7**, 979-985.
- Miyajima, A., Sakemi-Hoshikawa, K., Usami, M., Mitsunaga, K., Irie, T., Ohno, Y. and Sunouchi, M. (2017): Thyrotoxic rubber antioxidants, 2-mercaptobenzimidazole and its methyl derivatives, cause both inhibition and induction of drug-metabolizing activity in rat liver microsomes after repeated oral administration. *Biochem. Biophys. Res. Commun.*, **492**, 116-120.
- Naaz, F., Srivastava, R., Singh, A., Singh, N., Verma, R., Singh, V.K. and Singh, R.K. (2018): Molecular modeling, synthesis, antibacterial and cytotoxicity evaluation of sulfonamide derivatives of benzimidazole, indazole, benzothiazole and thiazole. *Bioorg. Med. Chem.*, **26**, 3414-3428.
- Prosser, K.E., Chang, S.W., Saraci, F., Le, P.H. and Walsby, C.J. (2017): Anticancer copper pyridine benzimidazole complexes: ROS generation, biomolecule interactions, and cytotoxicity. *J. Inorg. Biochem.*, **167**, 89-99.
- R Core Team. (2017): R: A Language and Environment for Statistical Computing. Vienna, Austria.
- Ranganatha, V.L., Vijay Avin, B.R., Thirusangu, P., Prashanth, T., Prabhakar, B.T. and Khanum, S.A. (2013): Synthesis, angiopreventive activity, and *in vivo* tumor inhibition of novel benzophenone-benzimidazole analogs. *Life Sci.*, **93**, 904-911.
- Ritz, C., Baty, F., Streibig, J.C. and Gerhard, D. (2015): Dose-Response Analysis Using R. *PLoS One*, **10**, e0146021.
- Saitoh, M., Umemura, T., Kawasaki, Y., Momma, J., Matsushima, Y., Sakemi, K., Isama, K., Kitajima, S., Ogawa, Y., Hasegawa, R., Suzuki, T., Hayashi, M., Inoue, T., Ohno, Y., Sofuni, T., Kurokawa, Y. and Tsuda, M. (1999): Toxicity study of a rubber antioxidant, mixture of 2-mercaptomethylbenzimidazoles, by repeated oral administration to rats. *Food Chem. Toxicol.*, **37**, 777-787.
- Sakemi, K., Ito, R., Umemura, T., Ohno, Y. and Tsuda, M. (2002): Comparative toxicokinetic/toxicodynamic study of rubber antioxidants, 2-mercaptobenzimidazole and its methyl substituted derivatives, by repeated oral administration in rats. *Arch. Toxicol.*, **76**, 682-691.
- Shimomura, I., Yokoi, A., Kohama, I., Kumazaki, M., Tada, Y., Tatsumi, K., Ochiya, T. and Yamamoto, Y. (2019): Drug library screen reveals benzimidazole derivatives as selective cytotoxic agents for KRAS-mutant lung cancer. *Cancer Lett.*, **451**, 11-22.
- Singla, P., Luxami, V. and Paul, K. (2014): Benzimidazole-biologically attractive scaffold for protein kinase inhibitors. *RSC Advances*, **4**, 12422-12440.
- Teng, Y., Zou, L., Huang, M. and Chen, Y. (2014): Molecular interaction mechanism between 2-mercaptobenzimidazole and copper-zinc superoxide dismutase. *PLoS One*, **9**, e106003.