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**Inhibitory effect of oxethazaine on midazolam metabolism in rats**

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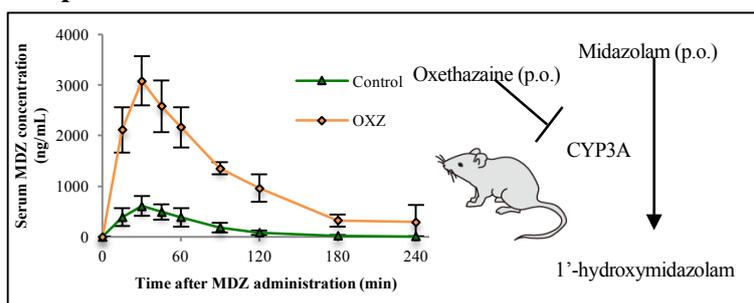
## Summary

There have been few reports concerning to the drug-drug interactions (DDIs) with over-the-counter (OTC) drugs although an increase in the use of OTC drugs in recent years. This current study was conducted to clarify the DDIs through cytochrome P450 3A (CYP3A) inhibition by oxethazaine (OXZ), an antacid available as an OTC drug. Midazolam (MDZ) was used as a probe drug for CYP3A activity. In an *in vivo* study, a single oral dose of OXZ (50 mg/kg) was administered to rats 30, 60, or 120 min before oral MDZ administration (15 mg/kg). Serum concentrations of MDZ were analyzed by high-performance liquid chromatography (HPLC), and its pharmacokinetic parameters were compared with a water-treated control group. The inhibitory effect of OXZ on MDZ 1'-hydroxylation (MDZ 1'-OH) activity was investigated *in vitro* using rat liver and intestinal microsomes. Pretreatment of OXZ 120 min before MDZ administration significantly increased the  $AUC_{0-\infty}$  of MDZ six-fold compared to the control group without a change in  $t_{1/2}$ . In contrast, OXZ pretreatment 30 or 60 min before MDZ administration did not show any remarkable change in MDZ pharmacokinetic parameters. The *in vitro* study showed that OXZ inhibited MDZ 1'-OH activity in a concentration-dependent manner both in liver and intestinal microsomes. These results suggested that OXZ increases serum MDZ concentration presumably by the inhibition of liver and/or intestinal CYP3A activity. OXZ was predicted to cause the DDIs mediated by CYP3A inhibition, although this effect depended on the dose interval.

## Keywords

Over-the-counter drugs, oxethazaine, drug-drug interactions, cytochrome P450 3A

## Graphical abstracts



## INTRODUCTION

Over-the-counter (OTC) drugs can be easily purchased at the drugstore and on the internet without a doctor's prescription. In Japan, with the increase in the elderly population and the incidence of lifestyle-related diseases, self-medication is expected to become more widespread. The number of nonprescription OTC drugs that have been approved as prescription drugs has also increased.<sup>1,2)</sup> For this reason, the use of OTC drugs is more likely to increase in the future and attract more attention in the clinics.

OTC drugs have been considered relatively safe for use. However, in recent years, diphenhydramine, an antihistamine that has been available on prescription drug and OTC for many years, was reported to inhibit cytochrome P450 (CYP) 2D6-mediated metoprolol metabolism in humans.<sup>3)</sup> Thus, the possibility of drug-drug interactions (DDIs) between diphenhydramine and CYP2D6 substrates with narrow therapeutic indices was suggested. Although most of OTC drugs have been available for a long time, information regarding their metabolic pathways and DDIs with other drugs is inadequate. The safety use of OTC drugs with respect to DDIs is therefore important.

Many gastrointestinal drugs can be easily obtained as OTC, and their high-use frequency increases the likelihood of combined use with other drugs. In a previous study, we examined the inhibitory effects of five OTC gastrointestinal drugs on CYP activities in human liver microsomes. The results showed that oxethazaine (OXZ) competitively inhibited CYP3A activity with a  $K_i$  value of  $3.08 \mu\text{M}$  (personal data). OXZ exerts a local

anesthetic effect on gastric mucosa (Fig. 1). This drug inhibits gastric acid secretion secondarily by suppressing of gastrin secretion.<sup>4)</sup> In fact, OXZ is used to alleviate pain associated with peptic ulcer disease, heartburn, and anorexia and is available OTC and on prescription in Asian countries such as Japan, Korea, and India.<sup>5,6)</sup> Because OXZ is used to improve of the common gastrointestinal symptoms, there may be multiple opportunities to take it in combination with various drugs: those used regularly for the maintenance treatment of comorbidities, or those taken temporarily for acute conditions such as the common cold. However, it has not been clarified whether DDIs through CYP3A inhibition by occurs with OXZ *in vivo* as well as *in vitro*.

OXZ is usually taken after symptom onset. The timing of administration is often entrusted to the patient, so various inter-dose intervals are possible between concomitant drugs. Meanwhile, the pharmacologic effects of OXZ that raise gastric pH and inhibit gastrointestinal motility<sup>7,8)</sup> may influence the pharmacokinetics of the concomitant drugs depending on the inter-dose interval. For this reason, it is necessary to investigate the DDI by OXZ at various inter-dose intervals rather than on co-administration with other drugs.

The current study was conducted to clarify the DDI through CYP3A inhibition by OXZ and investigate the inhibitory effect of OXZ on CYP3A-mediated midazolam (MDZ) metabolism in rats. The inter-dose intervals of both drugs were settled at 30, 60, 120 min considering the actual situation to take them.

## MATERIALS AND METHODS

**Chemicals** MDZ maleate was a kind gift from Nippon Roche Co. Ltd. (Tokyo, Japan). OXZ and diazepam were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 1'-hydroxymidazolam was purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan). All other chemicals were of analytical grade.

**Animals** Male Sprague-Dawley (SD) rats, weighing 220-240 g, were obtained from Japan Laboratory Animals Inc. (Tokyo, Japan). Rats were bred at an animal laboratory for one week as an adaptation period and starved overnight before *in vivo* studies.

The study procedure was reviewed and approved by the Showa University Ethics Committee for Animal Care and Use.

**Treatment and sampling** Considering its pharmacological action, OXZ was predicted to combine with CYP3A substrates at various dosage intervals in different clinical situations. For this reason, we investigated the effect of OXZ treatment on serum MDZ concentrations at three different dose intervals. The dose of OXZ was determined based on the previous report that investigated the inhibition of gastric acid secretion in rats.<sup>7)</sup>

Rats were divided into four groups of 3-4 each. MDZ was orally administered to rats at a dose of 15 mg/kg after 30, 60, or 120 min of single oral treatment with OXZ 50 mg/kg. Control rats were given water 30 min before MDZ administration.

Blood samples (0.2 mL) were collected from jugular vein before and after 15, 30, 45, 60,

90, 120, 180, and 240 min of MDZ administration. The samples were centrifuged at  $15,000 \times g$  for 10 min at 4 °C, and collected serum samples were stored at -80 °C until analysis.

**Determination of serum midazolam concentrations** The serum concentrations of MDZ were determined using previously described HPLC methods with slight modifications.<sup>9-11)</sup> Briefly, 100  $\mu\text{L}$  of each serum was diluted with 500  $\mu\text{L}$  of 0.1 N NaOH, and 5 ng of diazepam was added as an internal standard. The mixture was extracted with 8 mL of dichloromethane : pentane (1:1) for 1 min and centrifuged at  $1,600 \times g$  for 10 min. The upper organic solvent phase was transferred into a clean glass tube and evaporated to dryness under the nitrogen stream. The dried residue was dissolved in 60  $\mu\text{L}$  of mobile phase, and 15  $\mu\text{L}$  was injected onto an HPLC equipped with a CAPCELLPAK C18 MG II column (2.0 mm  $\times$  250 mm, and Shiseido, Tokyo, Japan). As the mobile phase 50/50 mixture (v/v) of 10 mM phosphate buffer (pH 5.0)  $\text{CH}_3\text{CN}$  was used with the flow rate of 0.2 mL/min. UV detector was set at 220 nm.

**Pharmacokinetic analysis** The pharmacokinetic parameters of MDZ were assessed by non-compartment analysis using MOMENT (Excel<sup>®</sup>) based on the moment analytic method.<sup>12,13)</sup> The area under the serum concentration-time curve ( $\text{AUC}_{0-\infty}$ ) was calculated according to the trapezoidal rule. The maximum serum concentrations ( $C_{\text{max}}$ ) was obtained from the actual data. The elimination half-life ( $t_{1/2}$ ) was calculated, dividing  $\ln 2$  by  $\lambda$ , which is the terminal elimination rate constant calculated by a linear regression analysis of

four or more data points from the terminal portion.

**Assay of CYP3A activity** Pooled liver and intestinal microsomes were prepared from male SD rats according to the conventional methods<sup>14-16)</sup> to investigate the inhibitory effect of OXZ on MDZ 1'-hydroxylation (MDZ 1'-OH) activity *in vitro*. All microsomes were frozen by liquid nitrogen and kept at  $-80^{\circ}\text{C}$  until used. Protein concentrations were measured by the method of Lowry *et al.*<sup>17)</sup>

The reaction mixtures consisted of pooled rat liver (100  $\mu\text{g}$ ) or intestinal (200  $\mu\text{g}$ ) microsomes, 30  $\mu\text{M}$  MDZ, and the nicotinamide adenine dinucleotide phosphate (NADPH) generating system in 80 mM phosphate buffer (pH 7.4) were incubated at  $37^{\circ}\text{C}$  for 15 min in the presence of OXZ at the final concentration of 1-300  $\mu\text{M}$ . The reactions were quenched by the addition of cold methanol:acetonitrile (35:21), and then nitrazepam (200 ng) was added as an internal standard. 1'-hydroxymidazolam was analyzed by HPLC according to a previous report.<sup>18)</sup> Control activity was determined in the same conditions but with the addition of water instead of OXZ. The analytic results were shown as the percentage of control activity.

In addition, the preincubation study was conducted to determine the possibility of the metabolite or metabolic intermediate production of OXZ that inhibit or inactivate CYP3A. OXZ (3  $\mu\text{M}$ ) was preincubated with pooled rat liver microsomes at  $37^{\circ}\text{C}$  for 10, 20, and 30 min in the presence or absence of NADPH. After the preincubation step, MDZ was added and MDZ 1'-OH activity was analyzed as described above.

**Statistical analysis** *In vivo* data were presented as the mean  $\pm$  standard deviation (S.D.). The differences between the four groups were analyzed by non-repeated measures analysis of variance (ANOVA) followed by the Bonferroni's method. A difference of  $p < 0.05$  was considered statistically significant.

## RESULTS

**Pharmacokinetics of MDZ** The serum concentration-time profiles and the pharmacokinetic parameters of MDZ administered orally after 30, 60, or 120 min of oral OXZ treatment are shown in Fig. 2 and Table 1.

In the group treated with OXZ 120 min before MDZ administration, the mean  $AUC_{0-\infty}$  and  $C_{max}$  of MDZ increased from  $710 \pm 255$  to  $4562 \pm 690$  ng · h/mL and from  $615 \pm 192$  to  $3083 \pm 486$  ng/mL, respectively ( $p < 0.01$  versus controls), without a change in  $t_{1/2}$ . Administration of OXZ 30 or 60 min before MDZ administration showed a tendency toward an increase in MDZ  $AUC_{0-\infty}$  and  $C_{max}$  but the differences were not statistically significant.

**MDZ 1'-hydroxylation activity *in vitro*** OXZ inhibited CYP3A-mediated MDZ 1'-OH activity in a concentration-dependent manner both in rat liver and intestinal microsomes with  $IC_{50}$  values of 8.6 and 65  $\mu$ M, respectively (Fig. 3).

To identify the possible contribution of CYP-mediated metabolites or active intermediates on the inhibitory effect of OXZ against CYP3A activity, another inhibition study was conducted including a preincubation step. As a result, the increase of inhibition potency of OXZ on MDZ 1'-OH activity was not observed even after 30 min of preincubation (Fig. 4).

## DISCUSSION

The current study was conducted to clarify the DDIs between the OTC antacid OXZ and MDZ through CYP3A inhibition. Our results revealed that OXZ inhibited MDZ metabolism in rats.

Our previous *in vitro* study has shown that OXZ inhibited MDZ 1'-OH activity in human liver microsomes (personal data). In the current study, the concentration-related inhibition of MDZ 1'-OH activity by OXZ was also observed in intestinal microsomes as well as liver microsomes in rats. Furthermore, this *in vivo* study showed that pretreatment with OXZ changed MDZ pharmacokinetic parameters, and the degree of this effect differed according to the dose interval between the two drugs. When OXZ was given 120 min before MDZ administration, MDZ  $AUC_{0-\infty}$  and  $C_{max}$  showed a remarkable five-to-six-fold increase compared to the control group, without a change in  $t_{1/2}$ . These results suggested that OXZ significantly increased serum MDZ concentrations presumably by the inhibition of liver and/or intestinal CYP3A activity. Generally, in addition to CYPs, drug transporters such as p-glycoprotein (P-gp) have an important role in the pharmacokinetics of orally administered drugs.<sup>19)</sup> Therefore, if the intestinal absorption by P-gp was inhibited by OXZ, blood concentrations of the concomitant drug increase. However, because MDZ is not a substrate of P-gp,<sup>19)</sup> this possibility can be excluded.

At the same time, it is known that OXZ is mainly metabolized in liver with a short  $t_{1/2}$

of 1 h, and less than 0.1 % of the unchanged form is excreted in the urine within 24 h.<sup>20)</sup> Nevertheless, the pharmacokinetics of OXZ has not been clarified enough and the amount of OXZ remaining in liver or small intestine 120 min after its oral administration is unknown. For the DDI study mediated by intestinal CYP3A, as the maximal concentration of inhibitors in the intestinal lumen after oral administration, it is recommended to use dose/250 mL.<sup>21)</sup> According to this, the predicted value of maximal intestinal concentration of OXZ is estimated to reach higher enough compared to IC<sub>50</sub> value of 65  $\mu$ M obtained by an *in vitro* experiment. Thus, OXZ is thought to have likelihood to inhibit intestinal CYP3A activity even 120 min after oral administration.

Meanwhile, little information is available concerning OXZ metabolism, only that mephentermine and phentermine are known to be its major metabolites.<sup>5)</sup> Particularly, the involvement of CYPs on OXZ metabolism has not been reported. However, because it is known that phentermine does not inhibit CYP3A4 activity<sup>22)</sup> and also mephentermine is further metabolized to phentermine, these metabolites are not likely to inhibit CYP3A activity. In addition, as one of the mechanism of CYP inhibition, a mechanism-based inhibition (MBI) has been demonstrated. In this case, the inhibitory effect persists because the reactive intermediate that is catalytically activated by CYP binds to a target CYP irreversibly. Therefore, we conducted an *in vitro* that included a preincubation step to clarify the possibility of OXZ as a mechanism-based inhibitor. As a result, no additional inhibition of MDZ 1'-OH was observed when OXZ was preincubated with rat liver

microsomes and NADPH before the addition of MDZ. Therefore, the inhibition mechanism of OXZ on CYP3A activity did not seem to exert a MBI, which the intermediate metabolite of OXZ inhibited this enzyme irreversibly. These results suggested that the inhibitory effect of OXZ itself on CYP3A was involved in the marked elevation of serum MDZ level.

In contrast to the remarkable effect of OXZ on serum MDZ concentration with a dose interval of 120 min, OXZ administration 30 or 60 min before MDZ treatment showed no significant change in the pharmacokinetic parameters of MDZ. One possible explanation for the different influence of OXZ treatment on MDZ metabolism at different dose intervals is the pharmacological effect of OXZ on MDZ. Because OXZ has the ability to restrain gastrointestinal motility that reaches the maximum from 30 to 60 min after dosage.<sup>23)</sup> Therefore, MDZ might have been transferred to the small intestine gradually when OXZ was given 30 or 60 min before MDZ administration. For this reason, it was suggested that rapid increase of serum MDZ concentration did not occur even if intestinal CYP3A was inhibited by OXZ treatment. In fact, the serum MDZ concentration-time profiles with a broad apex were observed in the 30 or 60 min dose interval group as shown in Fig. 2.

In general, because OXZ is used for the improvement of gastrointestinal symptoms, patients do not take this drug before symptom onset. Accordingly, it is speculated that patients might take OXZ at various dose intervals, including 120 min after taking other

drugs. Therefore, the investigation of DDIs, including the consideration of inter-dose intervals, can provide more practical information for clinical situations.

OXZ is as available on prescription and OTC. Despite their easy availability, there has been little information about DDIs between OTC drugs: therefore, the doctors and pharmacists are unable to give the appropriate safety warnings to patients. As shown in the current study, it is notable that OXZ which is used as OTC drug increased the  $AUC_{0-\infty}$  of the concomitant drug six-fold. The cause of this DDI was not proved directly because we could not measure the concentration of OXZ in the liver and small intestine and did not examine the inhibitory effect of OXZ treatment on intravenously administered MDZ concentration. Nevertheless, MDZ is known to be a typical probe drug to estimate CYP3A-mediated metabolism and many drugs are metabolized via CYP3A. Because gastrointestinal medications such as OXZ are more likely to be used together with other drugs, careful attention is necessary to avoid severe DDIs. The DDIs observed in this study on rats suggests the need for a clinical study on humans. Furthermore, more studies investigating DDIs between other OTC drugs are warranted.

In the current study, we have shown that OXZ markedly increased serum MDZ concentration in rats. This DDI was probably caused by the mechanism including liver and/or intestinal CYP3A inhibition. The effect of OXZ treatment on MDZ metabolism differed according to dose interval, as shown by the significant six-fold increase in the  $AUC_{0-\infty}$  of MDZ when OXZ was administered 120 min before MDZ treatment.

**Conflict of Interest** The authors declare no conflict of interest.

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## FIGURE LEGENDS

Fig. 1. Chemical structure of oxethazaine.

Fig. 2. Serum concentration-time profiles of midazolam (MDZ) after a single treatment of oxethazaine (OXZ) in rats. OXZ (50 mg/kg) was orally administered to rats, followed by oral administration of MDZ (15 mg/kg) after 30, 60, or 120 min. Control rats were treated with water 30 min before MDZ administration. Each point represents the mean  $\pm$  S.D. of three to four rats.

Fig. 3. Inhibitory effect of oxethazaine (OXZ) on midazolam 1'-hydroxylation (MDZ 1'-OH) activity in rat liver and intestinal microsomes. OXZ (1-300  $\mu$ M) was incubated in the incubation mixture including rat liver or intestinal microsomes, MDZ (30  $\mu$ M) and nicotinamide adenine dinucleotide phosphate (NADPH). Data are shown as the mean of duplicate determinations.

Fig. 4. Preincubation effect of oxethazaine (OXZ) on midazolam 1'-hydroxylation (MDZ 1'-OH) activity in rat liver microsomes. OXZ (30  $\mu$ M) was preincubated with rat liver microsomes for 10, 20, and 30 min in the presence or absence of nicotinamide adenine dinucleotide phosphate (NADPH) before the addition of MDZ. Data are shown as the mean of duplicate determinations.



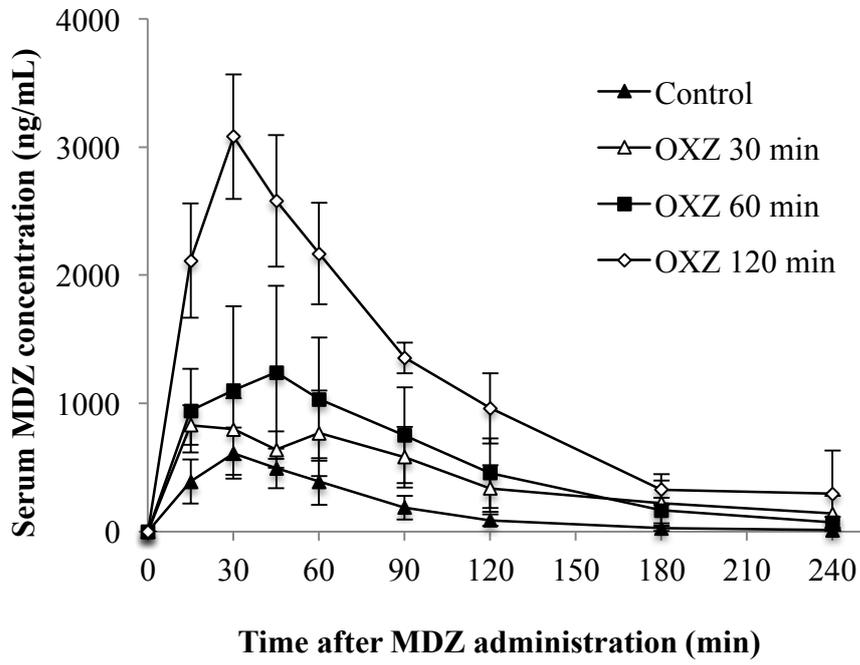
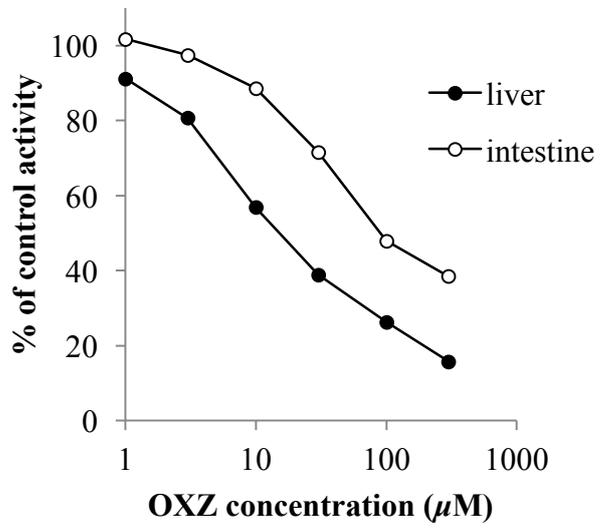
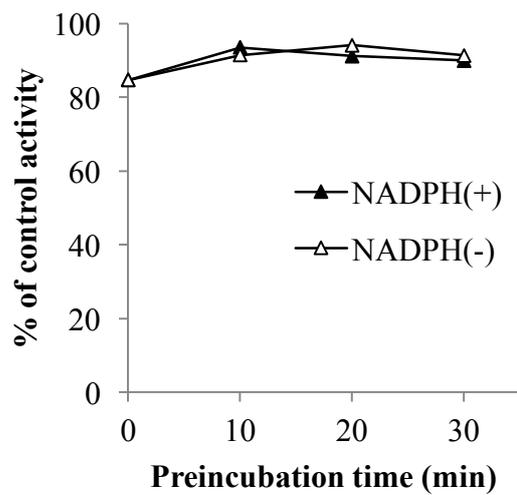


Fig. 2.



**Fig. 3.**



**Fig. 4.**

**Table 1.** Pharmacokinetic parameters of midazolam (MDZ) after a single treatment of oxethazaine (OXZ) in rats.

Dose interval (min)		AUC <sub>0-∞</sub> (ng.h/mL)	C <sub>max</sub> (ng/mL)	t <sub>1/2</sub> (min)
30	Control	710 ± 255	615 ± 192	33.11 ± 4.21
	OXZ	2090 ± 1062	1083 ± 162	76.84 ± 43.98
60	OXZ	2191 ± 1066	1445 ± 683	44.23 ± 10.77
120	OXZ	4562 ± 690 *	3083 ± 486 *	31.10 ± 6.80

Data are shown as the mean ± S.D. of 3-4 rats. \* P < 0.01 versus control.