Ozagrel Hydrochloride Monohydrate, a Thromboxane Synthase Inhibitor, and Its Metabolites as Inhibitors of Hepatic Microsomal Drug Metabolism

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The change in the hepatic oxidative drug-metabolizing capacity in humans treated with ozagrel hydrochloride monohydrate (OZA), an imidazole derivative and a new thromboxane A_2 synthase inhibitor, was studied and the inhibitory potencies of the metabolites of OZA (M-1 and M-2) on the mouse hepatic microsomal monooxygenase system *in vitro* were compared with that of OZA.

***In vitro*, M-1 and M-2, which are the β-oxidized form and the reduced form of OZA, respectively, inhibited aminopyrine N-demethylation, aniline hydroxylation and testosterone hydroxylations in mouse hepatic microsomes and produced type II difference spectra in the same manner as OZA. The kinetic data indicated that the inhibitory potencies and the affinities of these compounds for cytochrome P-450 were decreased in the order of M-2 > OZA > M-1.**

The ratio of 6β-hydroxycortisol (6β-OHF) to cortisol (F) in urine, used as an indicator of oxidative drug-metabolizing capacity in humans, did not change significantly during oral treatment with 400 mg/d of OZA, while the ratio decreased to 80—85% of the original level during treatment with 800 mg/d of OZA. Although the participation of the metabolites of OZA in the reduction of drug-metabolizing capacity *in vivo* is not yet clear, the results suggest that hepatic oxidative drug-metabolizing enzyme activities in humans are inhibited by treatment with a relatively high dose of OZA.

Keywords ozagrel hydrochloride monohydrate; thromboxane synthase inhibitor; imidazole derivative; cytochrome P-450; drug-metabolism; enzyme inhibition; 6β-hydroxycortisol

Introduction

Ozagrel hydrochloride monohydrate (OZA) is an imidazole derivative and a new thromboxane synthase inhibitor that has become available for the treatment and prevention of various thrombotic diseases.[1-3] It has been shown that the pharmacological actions of OZA occur *via* the specific inhibition of thromboxane A_2 biosynthesis, which is catalyzed by thromboxane synthase, a cytochrome P-450 enzyme.[1,4]

In general, several groups of imidazole derivatives are well-known to be potent inhibitors of the cytochrome P-450-mediated drug-metabolizing enzyme system in hepatic microsomes.[5-7] We have demonstrated the inhibitory or inducing potencies of some imidazole-containing drugs, such as cimetidine,[8-10] ketoconazole and miconazole.[11,12] If OZA as an imidazole derivative inhibits the cytochrome P-450-containing enzyme system in hepatic microsomes in addition to its known inhibitory action on thromboxane synthase as a cytochrome P-450 enzyme, it will be necessary to monitor for change in the hepatic oxidative drug-metabolizing capacity in patients during the clinical use of OZA. Our previous paper reported that OZA was an inhibitor of some cytochrome P-450-mediated drug-metabolizing activities in mouse hepatic microsomes *in vitro* and *in vivo*, being comparable in potency to cimetidine.[13] However, the effect of treatment with OZA on the oxidative drug-metabolizing capacity in humans remains to be examined.

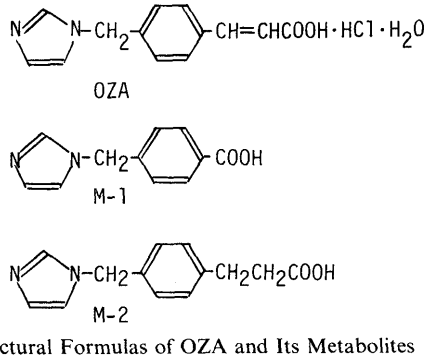

Fig. 1. Structural Formulas of OZA and Its Metabolites

On the other hand, it has been reported that the main metabolites of OZA in animals and humans are its β-oxidized form (M-1) and reduced form (M-2), which also have an imidazole ring structure[14,15] as shown in Fig. 1. Although it has been demonstrated that the inhibitory potencies of M-1 and M-2 on thromboxane A_2 synthase activity in rabbit platelets *in vitro* are only about 1/400 and 1/4 of that of OZA, respectively,[16] no other detailed study has been reported on the inhibitory effects of these metabolites on hepatic microsomal drug-metabolizing enzyme activities.

The aims of the present study were: to ascertain the relative inhibitory potencies of OZA and its metabolites on the mouse hepatic microsomal monooxygenase system *in vitro*; and to examine the effect of the treatment with OZA on the oxidative drug-metabolizing capacity in humans.

Materials and Methods

Materials OZA, M-1, M-2 and OZA capsules were kindly supplied by Kissei Pharmaceutical Co., Ltd., Matsumoto, Japan and Ono Pharmaceutical Co., Ltd., Osaka, Japan. Aminopyrine, aniline and testosterone were purchased from Nacalai Tesque Inc., Kyoto, Japan. All other chemicals and solvents were of analytical grade.

Preparation of Mouse Hepatic Microsomes Hepatic microsomes from male ddY mice (4—6 weeks old) were prepared according to the method previously described.[13] Microsomal protein concentration was determined by the method of Lowry *et al.*[17]

Biochemical Analysis The enzyme assay mixture was the same as that described in a previous paper.[13] Aminopyrine N-demethylation was determined by measurement of formaldehyde according to the procedure of Nash.[18] Aniline hydroxylation was determined by the method of Imai *et al.*[19] Testosterone hydroxylations were determined as described in a previous paper.[8] The inhibition by OZA and its metabolites was tested by the addition of 0.01—10 mM OZA, M-1 or M-2. Spectrophotometric

determination of the binding of OZA, M-1 and M-2 to cytochrome P-450 was carried out according to the method previously described.[13]

Measurement of the Ratio of 6β-Hydroxycortisol (6β-OHF) to Cortisol (F) in Urine as an Indicator of Hepatic Oxidative Drug-Metabolizing Capacity in Humans Five normal volunteers, male hospital pharmacists between 26 and 37 years old, participated in the study. All subjects had normal hepatic and renal functions, and they were taking no medication. All subjects received, on separate occasions, an oral dose of OZA (OZA capsule, 400 mg q.i.d. or 800 mg q.i.d.) for 3 d. Successive treatments were given two weeks after the previous drug treatment. On day 0 (just before treatment) and days 1, 2, 3, 4 and 5, random urine samples were taken for the evaluation of oxidative drug-metabolizing capacity. All urine samples were collected during the time period from 12:00 to 16:00 h. Urine samples were stored frozen at −80 °C until assay. Oxidative drug-metabolizing capacity of each subject was evaluated in terms of the ratio of 6β-OHF to F in urine. Urinary 6β-OHF was determined by the method described previously,[20] and urinary F was determined by the fluorescence polarization immunoassay technique (TDX® system, Dainabot kit).

Results and Discussion

Comparison of the Inhibitory Potencies and the Affinities of M-1 or M-2 for Mouse Hepatic Microsomal Cytochrome P-450 *in Vitro* with Those of OZA Aminopyrine N-demethylase, aniline hydroxylase and testosterone 6β-, 7α- and 16α-hydroxylase activities in mouse hepatic microsomes were inhibited significantly by OZA, M-1 and M-2 *in vitro*. Figure 2 shows the double-reciprocal plots of these enzyme activities in the presence of OZA, M-1 or M-2 at the same concentration (0.2 mM or 1 mM). The modes of inhibition of aminopyrine N-demethylation and aniline hydroxylation by these compounds were of mixed type while the modes for testosterone hydroxylations were of competitive type. The inhibition constants (K_i) of OZA, M-1 and M-2 for these enzyme activities are shown in Table I. The K_i values for each enzyme activity generally decreased in the order of M-1 > OZA > M-2, that is, the inhibitory

TABLE I. Inhibitory and Spectral Dissociation Constants of OZA and Its Metabolites Toward Cytochrome P-450-Mediated Monooxygenase System in Mouse Hepatic Microsomes

	OZA	M-1	M-2
Inhibition constants (K_i) (mM)			
Aminopyrine N-demethylase	0.21	0.74	0.05
Aniline hydroxylase	0.24	0.67	0.06
Testosterone 6β-hydroxylase	0.52	0.83	0.25
Testosterone 7α-hydroxylase	3.67	2.65	3.23
Testosterone 16α-hydroxylase	ND	ND	1.24
Spectral dissociation constants (K_s) (μM)			
K_{s1}	24.9	33.3	11.8
K_{s2}	163.0	196.1	104.1

ND, not detected.

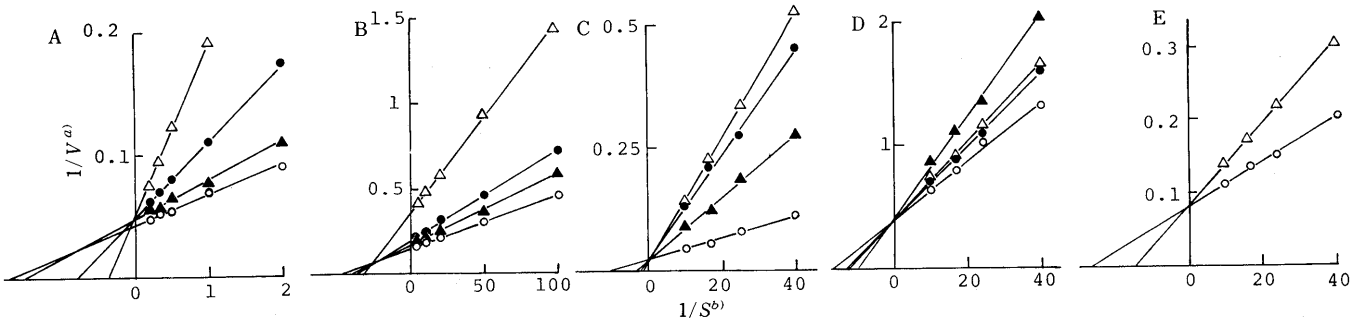

Fig. 2. Double-Reciprocal Plots of Aminopyrine N-Demethylation (A), Aniline Hydroxylation (B), Testosterone 6β-Hydroxylation (C), 7α-Hydroxylation (D) and 16α-Hydroxylation (E) Obtained with Mouse Hepatic Microsomes in the Absence and Presence of OZA, M-1 and M-2

The concentrations of OZA (●), M-1 (▲) and M-2 (△) added were 0 mM (○) and 0.2 mM for aminopyrine N-demethylase and aniline hydroxylase assays, and 1 mM for testosterone hydroxylase assays. Each enzymatic reaction was started by adding the microsomal suspension and was carried out at 37 °C with shaking for 5 min for aminopyrine N-demethylation, 10 min for testosterone hydroxylations and 20 min for aniline hydroxylation. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid, except for the assay of testosterone hydroxylations where 20 ml of dichloromethane was added. Each value is the mean of experiments with three different mouse hepatic microsomal preparations. *a*) V = nmol/mg protein/min. *b*) S = concentration of substrate (mM).

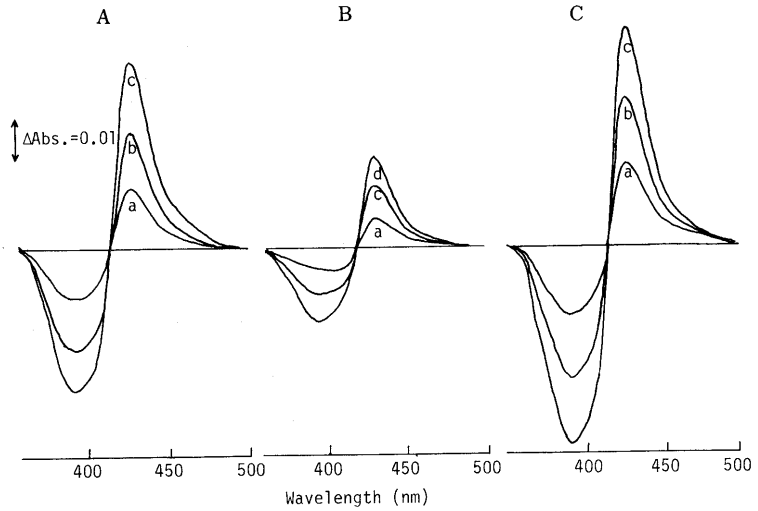

Fig. 3. Spectral Changes of Mouse Hepatic Microsomal Cytochrome P-450 Caused by the Additions of OZA (A), M-1 (B) and M-2 (C)

Difference spectra were recorded after the successive additions of 10 μM (a), 30 μM (b), 70 μM (c) and 200 μM (d) drug.

potencies of M-2 for hepatic microsomal monooxygenase activities were much stronger and the potencies of M-1 were much weaker than those of OZA. With regard to the inhibitory effects of OZA, M-1 and M-2 on the three hydroxylations of testosterone, 6β-hydroxylation was inhibited more potently than the 7α- and 16α-hydroxylations. Whereas individual microsomal xenobiotic oxidation pathways are often catalyzed by more than a single cytochrome P-450, the same is not true of steroid hydroxylations. It has been reported that different cytochrome P-450 isozymes hydroxylate testosterone at the 6β-, 7α- and 16α-positions in mouse hepatic microsomes.[21] These findings are strongly suggestive of the inhibitory effects of M-1 and M-2 on the monooxygenase activities being relatively selective for some cytochrome P-450 isozymes, in the same manner as in the case of OZA itself.

Figure 3 shows the difference spectra induced by the addition of OZA, M-1 and M-2 to mouse hepatic microsomes. All had an absorption maximum (peak) at 428 nm and an absorption minimum (trough) at 392 nm, characteristic of a type II spectral change.[22] Type II spectral change is strongly suggestive of the formation of a nitrogenous ligand to cytochrome P-450,[23] *i.e.*, interaction of the imidazole group in the drug with the heme moiety. Therefore, these results indicate that M-1 and M-2 also have a direct interaction of their imidazole groups with cytochrome P-450 in a similar manner to OZA. These spectral changes were converted to double-reciprocal plots in order to obtain the dissociation constants (K_s). As shown in Fig. 4, the plots for these compounds were biphasic and two K_s values (K_{s1} for high-affinity sites and K_{s2} for low-affinity sites) were obtained. The K_s values obtained are also summarized in Table I. The K_s values for high and low affinity sites on cytochrome P-450 decreased in the order of M-1 > OZA > M-2. In agreement with the order of their inhibitory potencies, the results show that the affinity of M-2 for cytochrome P-450 was much higher and the affinity of M-1 was much lower than that of OZA. It is particularly interesting that although the difference in chemical structure between OZA and M-2 is only slight (the presence or absence of the double bond in the carbon side chain) (Fig. 1), the inhibitory potency and the affinity of M-2 for cytochrome P-450 in hepatic microsomes *in vitro* are 2- to 4-fold greater than those of OZA (Table I). It has been reported that the interaction between cytochrome P-450 and 1- or 4(5)-substituted imidazole is dominated by its hydrophobic character and the spatial orientation of the nitrogen atom at position 3 of the imidazole ring.[7] Therefore, the reasons for the more potent inhibitory activity of M-2 may be as follows, that is, the hydrophobicity of M-2 may be higher than that of OZA, or the greater degree of rotational freedom of the drug molecule owing to the lack of the double bond in the carbon side chain may facilitate the approach of the nitrogen atom at position 3 of the imidazole ring to the heme moiety in cytochrome P-450. On the other hand, contrary to the order of the inhibitory potencies of OZA and M-2 on hepatic microsomal cytochrome P-450 described above, it has been demonstrated that the inhibitory potency of M-2 on the activity of thromboxane A_2 synthase in rabbit platelets *in vitro* is only about one-fourth of that of OZA itself.[16] These findings suggest that the properties of the active sites of cytochrome P-450 as the drug-metabolizing enzyme in hepatic microsomes and cytochrome P-450 as thromboxane synthase in platelets may differ significantly.

Change in the Drug-Metabolizing Capacity in Humans by the Administration of OZA Measurement of the ratio of 6β-OHF to F or 17-hydroxycorticosteroids in urine is useful as a noninvasive method for evaluating changes in human hepatic monooxygenase activity such as enzyme inhibition[9,10,24-26] and induction,[12,27-31] because 6β-OHF is a polar metabolite formed by the oxidative metabolism of F by hepatic microsomes. Furthermore, there is no circadian change in 6β-OHF/F ratios, that is, the ratio in randomly obtained urine specimens from subjects may be used to detect the changes in drug-metabolizing capacity in the same manner as in 24-h urine.[26] By the use of this method, we examined whether OZA affects the hepatic oxidative drug-metabolizing capacity in humans.

The change in the ratio of 6β-OHF to F in urine from 5 healthy volunteers, before, during and after 3 d of OZA treatment is shown in Fig. 5. No significant change in the ratio was found during treatment with a low dose (400 mg/d) of OZA, while the ratio decreased to 80—85% of the original level during the treatment with a high dose (800 mg/d) of OZA. The ratio returned to its original level

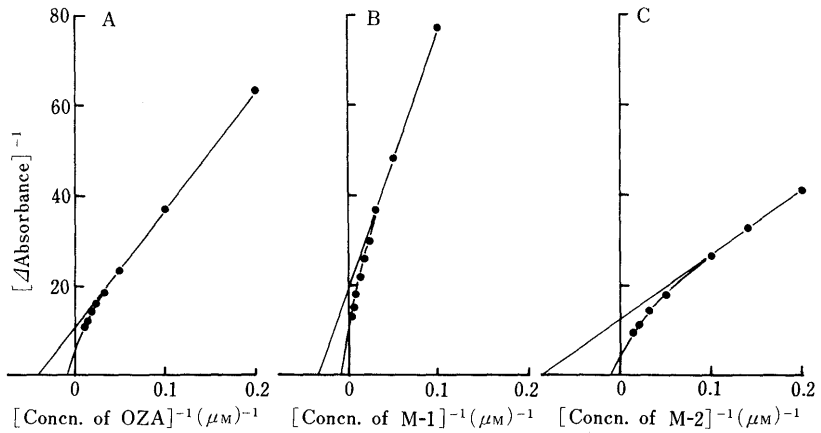

Fig. 4. Double-Reciprocal Plots of the Changes in Absorbance Produced by the Addition of Various Concentrations of OZA (A), M-1 (B) and M-2 (C)

Bindings of drugs to cytochrome P-450 were estimated from the absorbance difference between the peak (428 nm) and trough (392 nm). Each value is the mean of experiments with three different mouse hepatic microsomal preparations.

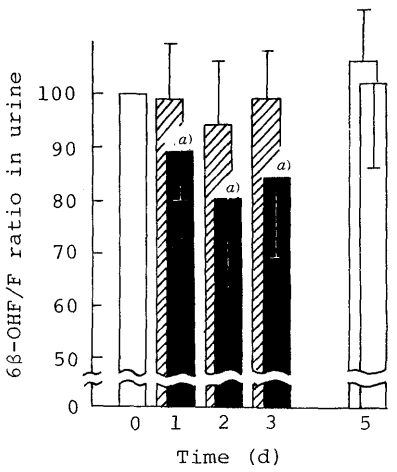

Fig. 5. Change in the Ratio of 6β-OHF to F in Urine Obtained from 5 Healthy Volunteers, before, during and after 3 d of OZA Treatment

The dosage of OZA was 400 mg/d (▨) or 800 mg/d (■). The value of the ratio on day 0 (mean ± S.D.) was 1.51 ± 0.30. The ordinate shows the percentage to the ratio on day 0 in each subject. Each column represents the mean ± S.D. *a*) Significantly different from day 0 ($p<0.05$).

within 2 d after the cessation of treatment with OZA. The mode of the change in the urinary excretion of 6β-OHF after treatment with 800 mg/d of OZA was similar to that of cimetidine.[9,10] In the previous paper, we reported that the potencies and the modes of OZA inhibitions of monooxygenase activities in mouse hepatic microsomes *in vitro* and *in vivo* are similar to those of cimetidine.[13] The results obtained in this study suggest strongly that the oxidative drug-metabolizing enzyme activity responsible for the formation of 6β-OHF from F in human liver is inhibited by treatment with a relatively high dose of OZA.

On the other hand, whether the reduction of oxidative drug-metabolizing capacity in humans given a high dose of OZA (Fig. 5) is due to the inhibitory activity of OZA alone or that of OZA and its metabolites is not clear. Although we found that the inhibitory potencies of M-2 on some enzyme activities in mouse hepatic microsomes *in vitro* are about 4-fold greater than those of OZA (Table I), the ratio of the area under the plasma concentration–time curves (*AUC*) of OZA, M-1 and M-2 after oral dosing in clinical use is 46 : 45 : 9,[15] that is, the concentration of M-2 in the systemic circulation is only about one-fifth of that of OZA. Further detailed study is needed to examine the participation of M-2 in the inhibition of drug-metabolizing enzyme activities *in vivo*, particularly to establish the concentration ratio of OZA, M-1 and M-2 in the liver and the differences among mouse and humans.

In conclusion, the *in vitro* study indicated that both M-1 and M-2 interact with mouse hepatic microsomal cytochrome P-450 in the same manner as OZA, and in particular, the inhibitory potency of M-2 on the enzyme system is greater than that of OZA itself, while the *in vivo* study indicated that the possibility of interaction between OZA and some coadministered drugs in high-dose therapy must be considered in the clinical context.

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