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Characterization of Human Liver Microsomal Cytochrome P450 Involved in the Reductive Metabolism of Zonisamide

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SUMMARY

Zonisamide (1,2-benzisoxazole-3-methanesulfonamide) was metabolized to 2-sulfamoylacetylphenol (SMAP) in human liver microsomes under anaerobic conditions. The formation of SMAP was remarkably inhibited by cimetidine, *n*-octylamine, ketoconazole, and carbon monoxide, indicating that a cytochrome P450 is involved in the metabolism of zonisamide to SMAP in human liver microsomes. The SMAP-producing activity did not correlate with the spectrally determined amount of cytochrome P450. In contrast, the SMAP-producing activity from zonisamide correlated closely with the activity of testosterone 6β-hydroxylase ($r^2 = 0.96$) and correlated slightly but significantly with the activity of imipramine 2-hydroxylase ($r^2 = 0.28$), but not with those of aniline hydroxylase ($r^2 = 0.09$) or benzphetamine *N*-demethylase ($r^2 = 0.20$). In addition, immunoquantitation of cytochrome P450 enzymes in 21 human liver microsomal samples revealed that SMAP formation correlated closely with the amount of P450 3A enzyme and correlated moderately well with that of P450 2D6 but not with that of P450 2C enzyme in human liver microsomes. P450 3A4 exhibited SMAP-producing activity in a reconstituted monooxygenase system. The metabolism of zonisamide to SMAP was almost completely inhibited by anti-P450 3A4 antibody but not by anti-P450 2C9 or anti-P450 2D6 antibodies, suggesting that the amount of P450 3A enzyme may be a major factor influencing the level of metabolism of zonisamide to SMAP in human liver microsomes.

The P450s are a family of hemoproteins, located predominantly in the endoplasmic reticulum of the liver, that catalyze not only the oxidative metabolism but also the reductive metabolism of a wide variety of xenobiotics (1). There is conclusive evidence that the P450 enzymes consist of multiple forms of structurally distinct isoenzymes (2). An important characteristic of some of these cytochromes is that they can exhibit overlapping substrate specificities, that is, numerous structurally diverse substrates are metabolized by the same form of P450. It is becoming increasingly evident that drug metabolism in liver microsomes is directly involved in a number of drug interactions. Therefore, identification of the form of P450 responsible for the metabolism of drugs in question is important for predicting and/or avoiding drug interactions.

Zonisamide is a recently developed sulfonamide anticonvulsant that is used for the treatment of epileptic disorders (3–6). From the analysis of the metabolites excreted in urine, SMAP has been proposed as one of the major reductive metabolites of zonisamide in rats and human (7, 8). However, the enzymes involved in the reductive metabolism of zonisamide were unclear. More recently, it has been demonstrated that zonisamide is metabolized to SMAP in rat liver microsomes under anaerobic conditions and that P450 contributes predominantly to the formation of SMAP from zonisamide (9, 10). The aim of this study was to investigate the enzyme(s) responsible for the reductive metabolism of zonisamide in human liver microsomes.

We show herein that zonisamide is metabolized to SMAP in human liver microsomes and that P450 3A enzyme is a major enzyme responsible for the metabolism of zonisamide to SMAP in human liver microsomes.

Experimental Procedures

Materials. NADPH, NADH, NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). Sepharose 4B, 2′,5′-ADP-Sepharose 4B, and Protein A-Sepharose (Fast Flow) were obtained from Pharmacia (Uppsala, Sweden). Testosterone metabolites and Emulgen 913 were kindly provided by Dr. Kirk, Queen Mary College, University of London, and Kao-Atlas (Tokyo, Japan), respectively. Zonisamide and SMAP were generous gifts from Dainippon-Seiyaku Co. Ltd. (Osaka, Japan). Other chemicals used were of the highest grade commercially available. Pre-

ABBREVIATIONS: P450, cytochrome P450; zonisamide, 1,2-benzisoxazole-3-methanesulfonamide; SMAP, 2-sulfamoylacetylphenol; DLPC, dilauroyl-L-3-phosphatidylcholine.

parative DEAE-5PW and hydroxylapatite columns were obtained from Toso (Tokyo, Japan) and Koken (Tokyo, Japan), respectively.

Preparation of liver microsomes. Liver autopsy samples were obtained within 20 hr after death. The livers were excised and homogenized, and the liver homogenate was centrifuged at 9000 × *g* for 20 min. The supernatant was ultracentrifuged at 105,000 × *g* for 60 min, and the resulting microsomal pellets were resuspended with 1.15% KCl solution and were ultracentrifuged at 105,000 × *g* for 30 min. The pellets were homogenized with 50 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA, at a protein concentration of about 20 mg/ml, and were stored at −80° until use.

Assay for SMAP-producing activity. A typical reaction mixture consisted of 100 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, an NADPH-generating system (0.33 mM NADP, 8 mM glucose-6-phosphate, 0.1 unit of glucose-6-phosphate dehydrogenase, and 6 mM $MgCl_2$), an oxygen-consuming system (10 mM glucose, 5 units of glucose oxidase, and 30 units of catalase), microsomal protein (approximately 0.5 mg), and substrate, in a final volume of 1.0 ml. The concentration of zonisamide was 0.4 mM. Various agents were also added, if necessary. The concentrations of the agents used were as specified in the figures. Zonisamide was dissolved in acetone at a concentration of 40 mM. After the construction of a reaction mixture in Thunburg cuvettes, the oxygen-consuming system was added to remove oxygen from the reaction mixture. The gas phase was then exchanged with argon gas by the vacuum-flush method. The addition of an oxygen-consuming system was necessary to obtain stable and reproducible formation of SMAP from zonisamide. The reaction was started by the addition of the NADPH-generating system, which had been preincubated at 37° for 5 min, and the incubations were carried out at 37° for 20 min. To stop the reaction, 0.5 ml of reaction mixture was mixed with 4 ml of dichloromethane containing 100 μl of bromvalerylurea (0.4 mg/ml in methanol) as the internal standard. After the dichloromethane extract had been evaporated to dryness under reduced pressure, the residue was dissolved in 50 μl of methanol. An aliquot of the methanol solution was applied to a high performance liquid chromatograph equipped with a dual-delivery pump (Waters model 6000A) and a UV absorbance detector (Soma model S-310A), and reverse phase chromatography was performed on an Inertsil ODS-2 column (particle size, 5 μm; 4.6 × 250 mm; GL Science). The SMAP was separated at a flow rate of 0.8 ml/min with isocratic elution (0.1 M potassium phosphate, pH 4.0/acetonitrile/2-propanol, 15:5:1), and the effluent was monitored at 260 nm. Standard peak heights were linear over the concentration range to 8000 ng/ml for SMAP, and the limit of detection (defined as the concentration that would provide a signal equivalent to 3 times the noise level) was 2 ng/ml. Other monooxygenase activities were measured under aerobic conditions.

Assays for monooxygenase activities. The reaction mixture was as described above except that potassium phosphate, pH 7.4, was used in place of potassium phosphate, pH 7.0, and the oxygen-consuming system was omitted. The concentrations of aniline, benzphetamine, testosterone, and imipramine were 2 mM, 1 mM, 0.2 mM, and 0.1 mM, respectively. Testosterone was dissolved in acetone at a concentration of 20 mM. The activities of aniline hydroxylase and benzphetamine *N*-demethylase were measured by the methods of Imai *et al.* (11) and Nash (12), respectively. The activities of testosterone 6β-hydroxylase and imipramine 2-hydroxylase were measured by high performance liquid chromatography according to the methods of Hayashi *et al.* (13) and Ohmori *et al.* (14), respectively.

Reconstitution of reductive metabolizing activity of zonisamide. A reconstituted system contained 100 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, the NADPH-generating system, 20 pmol of P450 3A4, 1 unit of rat liver microsomal NADPH-P450 reductase, 25 μg of DLPC, zonisamide, and the oxygen-consuming system, in a final volume of 1.0 ml. Cytochrome b_5 (20 pmol) purified from rat livers was added to the system if necessary. Other methods were as described above.

Immunoinhibition studies. Microsomes were preincubated with varying amounts of antibodies at 37° for 10 min before the reaction was started. For the titration experiments, 1.0, 2.0, 5.0, and 10 mg of anti-P450 2C9, anti-P450 2D1, and anti-P450 3A4 antibodies per nmol of P450 were used in 1.0 ml of reaction mixture. Each antibody was diluted with preimmune IgG to make a final protein concentration of 10 mg in the reaction mixture.

Other methods. The content of P450 was estimated by the method of Omura and Sato (15), except that the difference spectrum of the carbon monoxide-reduced form minus the reduced form was measured in the presence of 20% glycerol and 0.2% Emulgen 913. P450 2C9, P450 2D6, and P450 3A4 were purified from human liver microsomes according to methods reported elsewhere (16, 17). Purification of NADPH-P450 reductase from phenobarbital-treated rats was carried out according to the method described by Yasukochi and Masters (18), with minor modifications (19). Cytochrome b_5 was purified from rat liver microsomes by the method described previously (20). Antibodies to the purified P450 2C9 and 3A4 were raised in rabbits as reported previously (21). Anti-P450 2D1 antibody, which was able to cross-react with human P450 2D6 (22), was kindly provided by Dr. F. P. Guengerich, Vanderbilt University. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot peroxidase/antiperoxidase staining were carried out essentially as described by Laemmli (23) and Guengerich *et al.* (24), respectively. Protein was measured according to the method of Lowry *et al.* (25), using bovine serum albumin as the standard.

Results

SMAP, zonisamide, and bromvalerylurea (internal standard) were well separated on a high performance liquid chromatogram (Fig. 1). The formation of SMAP was sensitive to oxygen and was about 3-fold lower at an oxygen concentration of 50 μM, compared with that observed under anaerobic conditions (data not shown). SMAP formation from zonisamide in human liver microsomes required NADPH. The rate of formation was linear between 5 and 30 min and was directly dependent on microsomal protein between 0.25 and 1.0 mg/ml of reaction mixture (data not shown).

As shown in Fig. 2, various agents affected the formation of

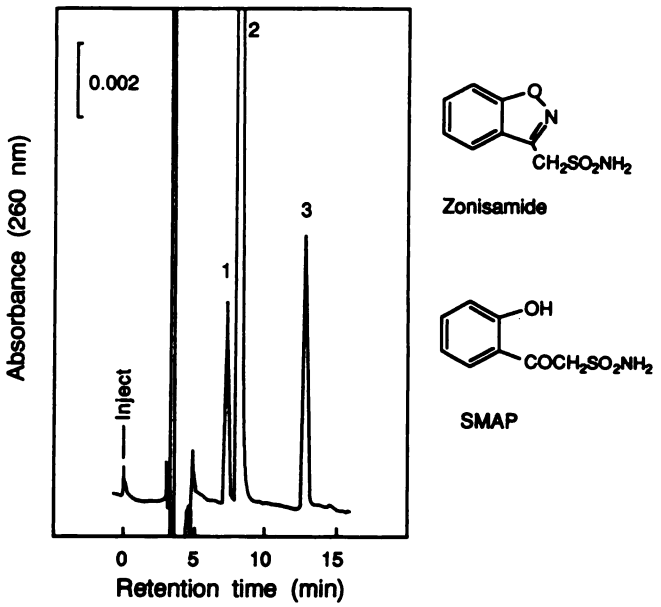

Fig. 1. High performance liquid chromatogram of zonisamide metabolites formed by human liver microsomes. Incubation was carried out as described in Experimental Procedures. *1*, SMAP; *2*, zonisamide; *3*, bromvalerylurea (internal standard).

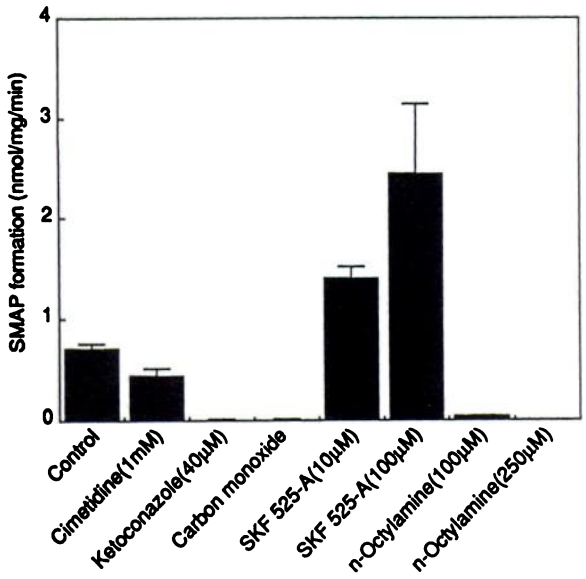

Fig. 2. Effects of various agents on the formation of SMAP from zonisamide in human liver microsomes. The formation of SMAP from zonisamide was measured as described in Experimental Procedures, in the presence or absence of cimetidine, *n*-octylamine, ketoconazole, and SKF 525-A, under anaerobic conditions. When the effect of carbon monoxide on the reaction was studied, carbon monoxide was used in place of argon as the gas phase. Each value represents the mean ± standard deviation of duplicate determinations.

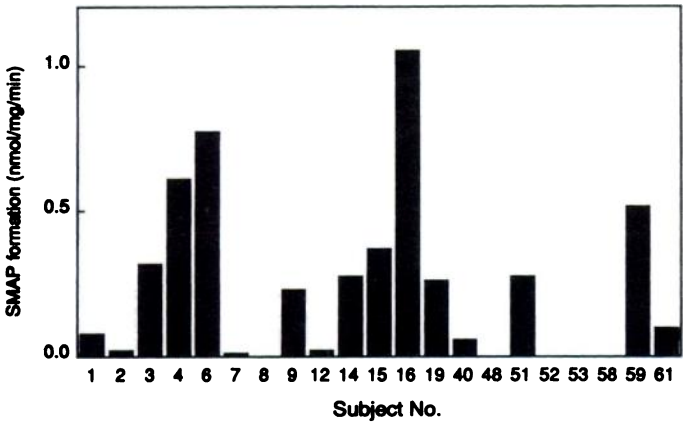

Fig. 3. Reductive metabolism of zonisamide to SMAP in human liver microsomes. The SMAP-producing activity was determined as described in Experimental Procedures, in different human liver microsomal samples.

SMAP from zonisamide in human liver microsomes under anaerobic conditions. Cimetidine inhibited about 40% of the formation of SMAP in human liver microsomes at a concentration of 1 mM. In addition, carbon monoxide, *n*-octylamine, and ketoconazole almost completely inhibited the SMAP-producing activity of human liver microsomes, indicating that the P450 may catalyze the reductive metabolism of zonisamide to SMAP in human liver microsomes. In contrast, SMAP formation from zonisamide was activated rather than inhibited by the addition of SKF 525-A, in a dose-dependent manner.

When the SMAP-producing activity was measured in different human liver microsomal samples, the activities ranged from not detectable to 1.05 nmol/mg/min (Fig. 3). However, as shown in Fig. 4, no good correlation between the SMAP-producing activities and the spectrally determined content of total P450 was observed, suggesting that a specific form of P450

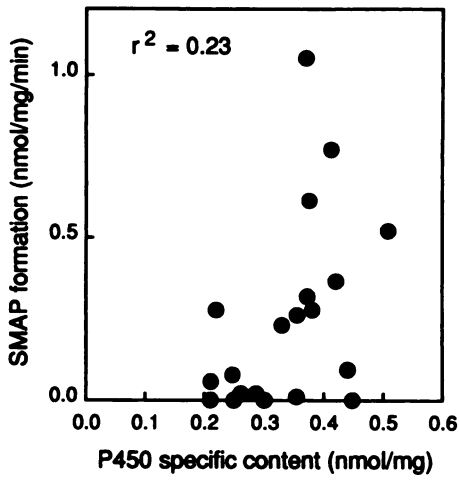

Fig. 4. Correlation of SMAP formation and spectrally determined P450 content. SMAP-producing activity and P450 content were measured with different human liver samples, as described in Experimental Procedures.

is involved in the reductive metabolism of zonisamide to SMAP in human liver microsomes. The correlation of SMAP formation with the activities of aniline hydroxylase, benzphetamine *N*-demethylase, testosterone 6β-hydroxylase, and imipramine 2-hydroxylase in 21 human liver microsomal samples is shown in Fig. 5. The SMAP-producing activity from zonisamide correlated closely with the activity of testosterone 6β-hydroxylase and correlated weakly but significantly with that of imipramine 2-hydroxylase. In contrast, neither aniline hydroxylase activity nor benzphetamine *N*-demethylase activity correlated with SMAP formation in human liver microsomes. To obtain additional evidence to support the idea that a specific form of P450 is responsible for SMAP formation in human liver microsomes, immunoquantitation of P450 enzymes was also carried out in 21 human liver samples. As expected from the results shown in Fig. 6, the formation of SMAP from zonisamide was found to correlate closely with the amount of P450 3A enzyme immunochemically determined. Furthermore, the SMAP-producing activity correlated weakly with the amount of P450 2D6 but not with that of P450 2C enzymes. These results indicate that P450 3A enzyme, at least, is one of the forms of P450 responsible for the reductive metabolism of zonisamide to SMAP in human liver microsomes. As shown in Table 1, P450 3A4 was capable of metabolizing zonisamide to SMAP in a reconstituted system containing NADPH-P450 reductase and DLPC, under anaerobic conditions. The formation of SMAP from zonisamide was dependent on P450 and was not activated by the addition of cytochrome b_5 to the reconstituted system. The effects of anti-P450 antibodies on the formation of SMAP from zonisamide in human liver microsomes are shown in Fig. 7. Neither anti-P450 2C9 antibody nor anti-P450 2D1 antibody inhibited the metabolism of zonisamide to SMAP in human liver microsomes. In contrast, anti-P450 3A4 antibody inhibited SMAP formation in a dose-dependent manner, and the SMAP-producing activity was inhibited by >90% at a concentration of 10 mg of IgG/nmol of P450, indicating that P450 3A enzyme is a major form of P450 involved in the reductive metabolism of zonisamide.

Discussion

It has been shown that an oral dose of zonisamide, a new antiepileptic compound containing a 1,2-benzisoxazole hetero-

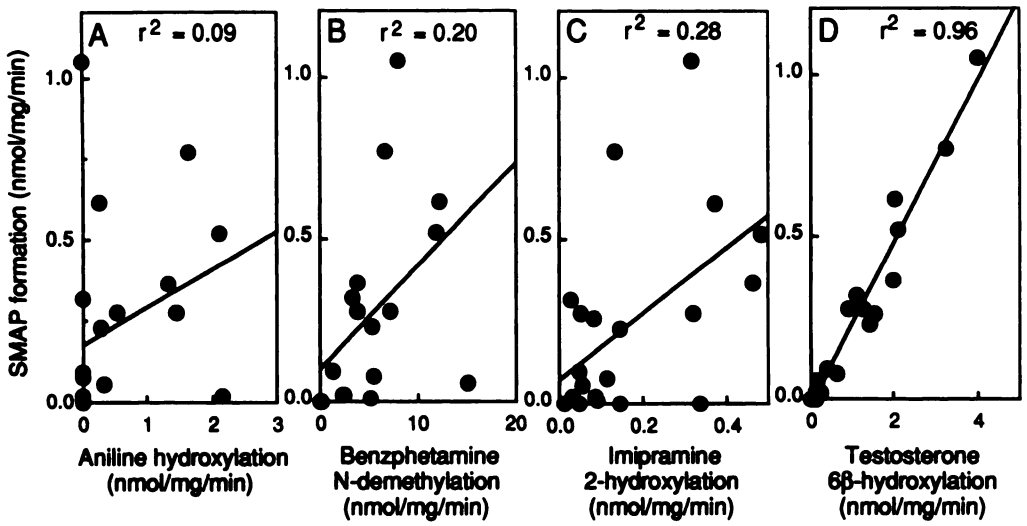

Fig. 5. Correlations of SMAP formation with aniline hydroxylation (A), benzphetamine *N*-demethylation (B), testosterone 6β-hydroxylation (C), and imipramine 2-hydroxylation (D) in different human liver microsomal samples. SMAP-producing activity and monooxygenase activities were measured as described in Experimental Procedures.

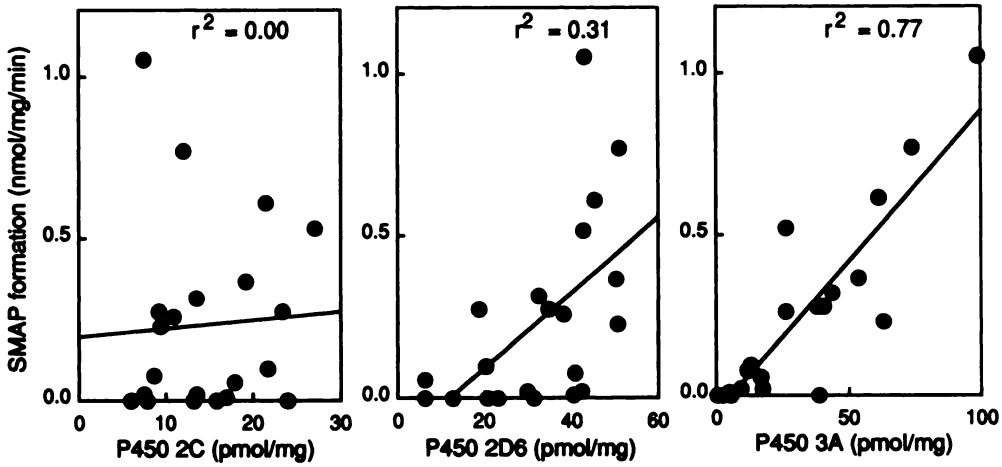

Fig. 6. Correlation of SMAP formation with the amounts of P450 2C, P450 2D6, and P450 3A in different human liver microsomes. The amounts of P450 2C, P450 2D6, and P450 3A were immunochemically quantitated as described in Experimental Procedures.

TABLE 1
SMAP formation by purified P450 3A4 in a reconstituted system

	SMAP formation	
	Aerobic	Anaerobic
	nmol/nmol of P450/min	
DLPC + P450 reductase	<0.02	<0.02
DLPC + P450 reductase + P450	<0.02	0.47
DLPC + P450 reductase + P450 + cytochrome b_5	<0.02	0.42

cycle in its structure, is predominantly excreted in urine as unchanged zonisamide and SMAP glucuronide. However, the mechanism by which the 1,2-benzisoxazole heterocycle N-O bond is reductively cleaved to form SMAP and the enzymes involved in the reductive metabolism of zonisamide remained unclear. More recently, the reductive metabolism of zonisamide to SMAP has been demonstrated to be catalyzed by P450 in rat liver microsomes under anaerobic conditions. Therefore, in the present study, we investigated whether P450 is also responsible for SMAP formation in human liver microsomes.

The reductive metabolism of zonisamide to SMAP in human liver microsomes required NADPH and was inhibited by the addition of various inhibitors of P450 except for SKF 525-A, indicating that P450 may be responsible for SMAP formation in human liver microsomes, as in the case of rat liver microsomes. SKF 525-A enhanced the reductive metabolism of zonisamide in human liver microsomes. At this time, we do not have a completely satisfying explanation for the stimulation by SKF 525-A. However, because SKF 525-A is well known to stimulate the rate of reduction of P450, due to conformational changes in the cytochrome (26), the stimulation of the rate of reduction of P450 might account for the enhancement by SKF 525-A of the reductive metabolism of zonisamide. In fact, it has been also reported that SKF 525-A stimulated the CO-sensitive pathway of reduction of azo compounds (27). However, the possibility that an allosteric effect of SKF 525-A is responsible for the activation should also be considered.

SMAP formation in 21 human liver microsomes did not correlate with the total content of P450. Because multiple forms of P450 are present in human liver microsomes, it is likely that a specific form of P450 may contribute to the reductive metabolism of zonisamide to SMAP in human liver microsomes.

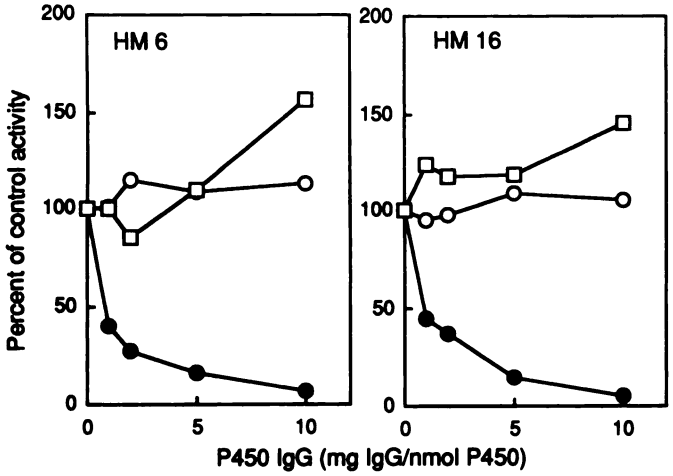

Fig. 7. Effects of anti-P450 antibodies on the formation of SMAP from zonisamide in human liver microsomes. SMAP formation was measured in the presence of anti-P450 2C9 antibody (○), anti-P450 2D1 antibody (□), or anti-P450 3A4 (●) antibody, as described in Experimental Procedures. The concentrations of antibodies added to the reaction mixture are specified in the figure. SMAP-producing activities of HM6 and HM16 in the absence of antibodies were 0.36 ± 0.11 and 0.55 ± 0.12 nmol of product/mg/min, respectively.

Therefore, the correlation of SMAP formation with the activities of monooxygenase was investigated in different human liver samples. The SMAP-producing activity from zonisamide was found to correlate closely with the activity of testosterone 6β-hydroxylation but not with those of other monooxygenases studied, except that the activity of imipramine 2-hydroxylation catalyzed by P450 2D6 in human liver microsomes (28) correlated weakly with SMAP formation. In addition, a good correlation between the amount of P450 3A enzyme immunochemically determined and the SMAP-producing activity was observed in different human liver microsomal samples. These results suggested that P450 3A enzyme may be one of the major forms of P450 involved in SMAP formation in human liver microsomes. Furthermore, the results showing that P450 3A4 exhibited catalytic activity for reductive metabolism of zonisamide in a reconstituted system and that anti-P450 3A4 antibody almost completely inhibited SMAP formation in liver microsomes indicated that P450 3A enzyme contributes predominantly to the metabolism of zonisamide to SMAP in human liver microsomes.

It has been demonstrated that one of the characteristics of P450 enzymes classified into the 3A subfamily is that the oxidative catalytic activity of the cytochrome is stimulated by cytochrome b_5 (29). However, cytochrome b_5 did not stimulate SMAP formation in a reconstituted system containing P450 3A4. Because cytochrome b_5 has been shown to supply the second electron for monooxygenation by P450, it is suggested that the metabolism of zonisamide to SMAP may be a one-electron reductive reaction. However, the possibility that the lipid used here is not appropriate for the effect of cytochrome b_5 cannot be excluded. More precise kinetic measurements using different lipid mixtures are required before the mechanism of the reaction can be firmly established.

In summary, we demonstrated in this communication that zonisamide is metabolized to SMAP under anaerobic conditions and that P450 classified into the 3A subfamily is a major form of P450 responsible for the formation of SMAP from zonisamide in human liver microsomes.

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