The Role of Mammalian Intestinal Bacteria in the Reductive Metabolism of Zonisamide

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Abstract

Zonisamide (1,2-benzisoxazole-3-methanesulphonamide), a new anticonvulsant, is mainly metabolized to 2-sulphamoylacetylphenol by reduction of the benzisoxazole ring. Recent studies have shown that mammalian liver enzymes are responsible for the reduction of zonisamide. Because intestinal bacteria can also mediate the reduction of xenobiotics, this study was designed to evaluate the role of intestinal bacteria in in-vivo reductive metabolism of zonisamide.

Treatment of rats with antibiotics significantly reduced the urinary and faecal excretion of 2-sulphamoylacetylphenol after oral administration of zonisamide. Re-contamination of the antibiotic-treated rats with microflora restored the excretion of the metabolite. The caecal contents of the control rats had significant zonisamide reductase activity, whereas little or no zonisamide reductase activity was observed with the caecal contents of the antibiotic-treated rats. Eight pure strains of intestinal bacteria were tested for zonisamide reductase activity and the highest was observed in *Clostridium sporogenes*.

We concluded that intestinal bacteria play a major role in the reductive metabolism of zonisamide to 2-sulphamoylacetylphenol in-vivo.

Zonisamide (1,2-benzisoxazole-3-methanesulphonamide), a new type of anticonvulsant, is clinically used for the treatment of epilepsy (Masuda et al 1980a, b; Sackellares et al 1985). Zonisamide is primarily metabolized to 2-sulphamoylacetyl-phenol by reduction of the benzisoxazole ring (Ito et al 1982; Stiff et al 1990). We recently showed that mammalian liver cytosols had significant zonisamide reductase activity originating from aldehyde oxidase (EC 1.2.3.1), a liver cytosolic enzyme, when supplemented with an electron donor of the enzyme (Sugihara et al 1996). Stiff et al (1992), on the other hand, reported that the reductive metabolism of zonisamide was mediated by rat-liver microsomal cytochrome P450, and Nakasa et al (1993a, b) suggested that cytochrome P450 belonging to the P450 3A subfamily might be predominantly responsible for the reductive metabolism of zonisamide in rat and human liver microsomes.

Thus, zonisamide reductase activity has been detected in mammalian liver preparations, but it is not clear whether enzymatic reduction observed in a mammalian tissue such as liver can account for the reduction of the drug in the whole animal. The reduction of certain xenobiotics is also mediated by mixed and pure cultures of intestinal bacteria (Scheline 1973). It therefore seemed worth investigating the role of intestinal bacteria in the reduction of zonisamide.

This study was designed to evaluate the role of the intestinal bacteria in zonisamide reduction in-vivo using conventional and antibiotic-treated rats.

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Materials and Methods

Chemicals

Zonisamide and 2-sulphamoylacetylphenol were generous gifts from Dainippon-Seiyaku. FMN, methyl viologen and neomycin sulphate were obtained from Sigma, NADPH and NADH from Oriental Yeast Co., streptomycin sulphate and bacitracin from Wako Pure Chemical Industries, 2-hydroxy-pyrimidine hydrochloride from Tokyo Chemical Industry, and β-glucuronidase (100 000 Fishman units mL^{-1})/aryl-sulphatase (800 000 Roy units mL^{-1}) from *Helix pomatia* was obtained from Boehringer Mannheim.

Bacterial strains

Clostridium sporogenes, *Bifidobacterium bifidum*, *Bacteroides vulgatus*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas fluorescens*, *Lactobacillus rhamnosus* and *Streptococcus faecalis* were obtained from the Institute for Fermentation (Osaka, Japan). Facultative anaerobic bacteria were grown in l-broth at 37°C and harvested by centrifugation at 9000 *g* for 10 min. Cells were washed twice with K,Na phosphate buffer (0·1 M, pH 7·4) and re-suspended in the phosphate buffer. Anaerobic bacteria were grown in GAM broth using a BBL Gas Pack anaerobic jar (Becton-Dickinson, Cockeysville, MD). Cells harvested by centrifugation were also suspended in the phosphate buffer.

Preparation of cell-free extract

Clostridium sporogenes cells (3·5 g) were suspended in K,Na phosphate buffer (0·01 M, pH 7·4; 15 mL), transferred to a glass pot placed in an ice-bath and sonicated for twenty cycles of 30 s on and 180 s off at maximum power using a Tomy Ultrasonic disruptor (UR-200p). Any undisrupted cells in the

suspension were removed by centrifugation at 17 000 g for 10 min. The supernatant was used as cell-free extract from *Clostridium sporogenes*.

Animals

Male Wistar rats, 140–220 g, ddY mice, 24–32 g, Syrian golden hamsters, 60–75 g, Hartley guinea-pigs, 215–260 g, and Japanese albino rabbits, 1.7–2·4 kg, were used. The first three and the last two species were fed standard MM-3 and RM-4 pellets (Funabashi Farm, Japan), respectively.

Treatment of rats with antibiotics

Rats were treated with antibiotics according to the method of Kinouchi et al (1993). A mixture of neomycin sulphate, streptomycin sulphate and bacitracin (200 mg kg^{-1} each) dissolved in sterilized water was given orally to rats twice daily for 6 days. Equivalent volumes of water were also administered orally twice daily for 6 days to conventional rats. In the in-vivo experiments antibiotic treatment was continued for three more days after administration of zonisamide. The antibiotic-treated rats were maintained in sterilized metabolic cages which enabled separation of urine and faeces and allowed free access to steam-sterilized diet and drinking water. Similarly, the control rats were kept in standard cages with free access to diet and water. In some experiments the antibiotic-treated rats were re-contaminated with the microflora of the animal house by keeping them for 3 weeks in cages containing faecal material from untreated rats.

Caecal contents

Caecal contents of animals were suspended and homogenized in two volumes of K, Na phosphate buffer (0·01 M, pH 7·4).

Tissue preparations

The control and antibiotic-treated rats were stunned by a blow on the head and exsanguinated. Livers were immediately perfused with 1·15% KCl and homogenized in KCl solution (4 vol) with a Potter-Elvehjem homogenizer. Microsomal and cytosolic fractions were obtained from the homogenate by successive centrifugation at 9000 g for 20 min and 105 000 g for 60 min. The microsomal fraction was resuspended in the KCl solution and re-centrifuged at 105 000 g.

Quantitative determination of in-vivo zonisamide metabolite (2-sulphamoylacetylphenol)

Zonisamide was suspended in 0·5% carboxymethyl cellulose and given orally to the antibiotic-treated or control rats at a single dose of 100 mg kg^{-1}. After administration, the urine and faeces were collected separately for 72 h. Urine (1 mL) was incubated with β-glucuronidase 3000 units/arylsulphatase 24 000 units in citrate-phosphate buffer (0·1 M, pH 6; 2 mL) at 37°C for 16 h. The faeces were dried in-vacuo over P$_2$O$_5$ and pulverized in a mortar. A sample (0·5 g) of the pulverized faeces was extracted twice with methanol (20 vol) by shaking. After centrifugation the supernatant was combined and evaporated to dryness under vacuum. The solid residues were further extracted by shaking once with citrate-phosphate buffer (10 vol). The buffer extract with β-glucuronidase 3000 units/ arylsulphatase 24 000 units was added to the residues from the supernatant and incubated as described above. For either urine or faeces the incubation mixture, after addition of 0·2 μmol

phenacetin, was extracted once with benzene (5 vol) and the benzene extract was washed by shaking with water (6 mL). The washed benzene extract was evaporated to dryness in-vacuo, and the residue was dissolved in a small amount of acetonitrile and analysed by HPLC. HPLC was performed with an Hitachi L-6000 chromatograph fitted with a 15 cm × 4·6 mm Inertsil ODS-2 column (GL Science, Tokyo, Japan). The mobile phase was CH$_3$CN–0·1 M KH$_2$PO$_4$ (3:7). The chromatograph was operated at a flow rate of 0·5 mL min^{-1} at ambient temperature and at a wavelength of 254 nm. The elution times of the metabolite (2-sulphamoylacetylphenol), zonisamide and phenacetin were 8·8, 9·7 and 11·7 min, respectively. The amount of the metabolite was determined from its peak area.

Assays of zonisamide reductase activity

Unless otherwise stated incubation was performed at 37°C for 30 or 60 min under an atmosphere of nitrogen in a Thunberg tube. A typical incubation mixture consisted of zonisamide (0·2 μmol) in methanol (10 μL), an electron-donor (0·5 μmol) and an enzyme source in a final volume of 2 mL K, Na phosphate buffer (0·1 M, pH 7·4). When caecal contents or bacteria were used the electron-donor was omitted. After incubation, the mixture, after addition of phenacetin (0·2 μmol) as internal standard, was extracted with ether (5 mL) and the ether extract was evaporated to dryness in vacuo. The residue was dissolved in methanol (0·1 mL) and then was analysed by HPLC. When liver microsomes or cytosol were used, the mixture, after addition of 10 μg phenacetin and acetonitrile (2 vol), was centrifuged and the supernatant was analysed by HPLC as before.

Determination of protein

Protein was determined by the method of Lowry et al (1951) with bovine serum albumin as the standard protein.

Results

The relative contribution of intestinal bacteria to the in-vivo metabolism of zonisamide to 2-sulphamoylacetylphenol was examined in rats. Treatment with antibiotics caused a significant decrease in the urinary and faecal excretion of 2-sulphamoylacetylphenol. Re-contamination of the antibiotic-treated rats with microflora restored the excretion of the metabolite (Table 1)..

Although the caecal contents of the control rats had significant zonisamide reductase activity under anaerobic condi-

Table 1. Urinary and faecal excretion of the zonisamide metabolite 2-sulphamoylacetylphenol by control, antibiotic-treated or re-contaminated rats after a single oral dose of zonisamide.

	Excretion of 2-sulphamoylacetyl-phenol (% of dose)	
	Urine	Faeces
Control	9·1 ± 1·9	3·1 ± 0·8
Antibiotic-treated	0·5 ± 0·7	ND
Re-contaminated	10·0 ± 3·2	4·0 ± 2·4

Results are means ± s.d. from five rats. Urine and faeces were collected separately for 72 h after administration of zonisamide (100 mg kg^{-1}). ND = not detected.

Table 2. Reduction of zonisamide to 2-sulphamoylacetylphenol by the caecal contents from control or antibiotic-treated rats.

	2-Sulphamoylacetylphenol formed (nmol h^{-1} (g caecal contents)$^{-1}$)	
	Anaerobic	Aerobic
Control	502 ± 177	3 ± 3
Antibiotic-treated	2 ± 2	ND
Re-contaminated	453 ± 87	3 ± 3

Results are the means ± s.d. from four 4 rats. ND = not detected.

Table 3. Influence of treatment with antibiotic on zonisamide reductase activity of rat liver microsomes and cytosol.

	2-Sulphamoylacetylphenol formed (nmol (mg protein^{-1} (30 min)$^{-1}$)	
	Microsomes*	Cytosol†
Control	16·2 ± 3·6	1·5 ± 0·6
Antibiotic-treated	16·3 ± 1·1	1·3 ± 0·5
Re-contaminated	19·8 ± 1·7	1·2 ± 0·1

Results are the means ± s.d. from four rats. *The assay was performed in the presence of NADPH and FAD. †The assay was performed in the presence of 2-hydroxypyrimidine.

Table 4. Reduction of zonisamide to 2-sulphamoylacetylphenol by the caecal contents from several mammalian species.

Species	2-Sulphamoylacetylphenol formed (nmol h^{-1} (g caecal contents)$^{-1}$)	
	Anaerobic	Aerobic
Mouse	1430 ± 9	28 ± 7
Hamster	294 ± 104	14 ± 11
Rabbit	91 ± 25	7 ± 8
Guinea-pig	76 ± 2	1 ± 0

Results are the means ± s.d. from four animals.

tions, little or no zonisamide reductase activity was observed with the caecal contents of the antibiotic-treated rats. When the antibiotic-treated rats were re-contaminated with microflora, the zonisamide reductase activity of the caecal contents was almost completely restored (Table 2).

The influence of antibiotic treatment and subsequent re-contamination with microflora on hepatic zonisamide reductase activity was examined. Little difference in liver microsomal or cytosolic reductase activity was detected among control, antibiotic-treated and re-contaminated rats (Table 3).

The caecal contents of mice, hamsters, rabbits and guinea-pigs, like those of rats, had the ability to catalyse the reduction of zonisamide to 2-sulphamoylacetylphenol under anaerobic conditions. Among the mammalian species examined, the highest activity was observed with mice and the lowest with guinea-pigs (Table 4).

The caecal contents contain a variety of bacteria. In this study, eight pure strains of intestinal bacteria were tested for

Table 5. Reduction of zonisamide to 2-sulphamoylacetylphenol by various bacteria.

Bacterium	2-Sulphamoylacetylphenol formed (nmol h^{-1} 10^{-10} cells)	
	Anaerobic	Aerobic
Clostridium sporogenes	4·9 ± 2·8	0·6 ± 0·4
Bifidobacterium bifidum	2·0 ± 0·5	0·4 ± 0·4
Bacteroides vulgatus	0·4 ± 0·1	0·08 ± 0·02
Escherichia coli	0·5 ± 0·3	ND
Salmonella typhimurium	0·4 ± 0·2	ND
Pseudomonas fluorescens	0·3 ± 0·2	0·2 ± 0·1
Lactobacillus rhamnosus	0·2 ± 0·1	0·08 ± 0·03
Streptococcus faecalis	0·07 ± 0·03	0·04 ± 0·01

Results are the means ± s.d. from five experiments. ND = not detected.

Table 6. Reduction of zonisamide to 2-sulphamoylacetylphenol by the cell-free extract from *Clostridium sporogenes*.

Addition	2-Sulphamoylacetylphenol formed (nmol (mg protein)$^{-1}$ (30 min)$^{-1}$)
None	0·3 ± 0·3
NADPH	0·5 ± 0·4
NADH	0·8 ± 0·3
NADPH, FMN	2·2 ± 1·2
NADH, FMN	1·6 ± 0·4
NADPH, methyl viologen	2·3 ± 0·3
NADH, methyl viologen	2·6 ± 1·0

Results are the means ± s.d. from three experiments.

zonisamide reductase activity. The activity was highest for *Clostridium sporogenes* followed the *Bifidobacterium bifidum*; the activity was lowest for *Streptococcus faecalis* under anaerobic conditions (Table 5).

The ability of the cell-free extract from *Clostridium sporogenes* to reduce zonisamide to 2-sulphamoylacetylphenol was examined. The zonisamide reductase activity of the cell-free extract was enhanced by addition of NADH or NADPH and FMN, or by addition of NADH or NADPH and methyl viologen (Table 6).

From these results, we concluded that intestinal bacteria play a major role in the reductive metabolism of zonisamide to 2-sulphamoylacetylphenol in-vivo.

Discussion

This study has provided the first evidence that intestinal bacteria play an important role in the in-vivo reduction of zonisamide to 2-sulphamoylacetylphenol. Similar results had been obtained from studies of the in-vivo reduction in rats of the azo linkage of prontosil and salicylazosulphapyridine (Gingell et al 1971; Peppercorn & Goldman 1972), and the nitro group of *p*-nitrobenzoic acid and nitrobenzene (Zachariah & Juchau 1974; Gardner & Renwick 1978; Levin & Dent 1982). The importance of intestinal bacteria in the metabolism of xenobiotics has been recognized increasingly, and the toxicological and pharmacological implications are of great concern (Scheline 1973).
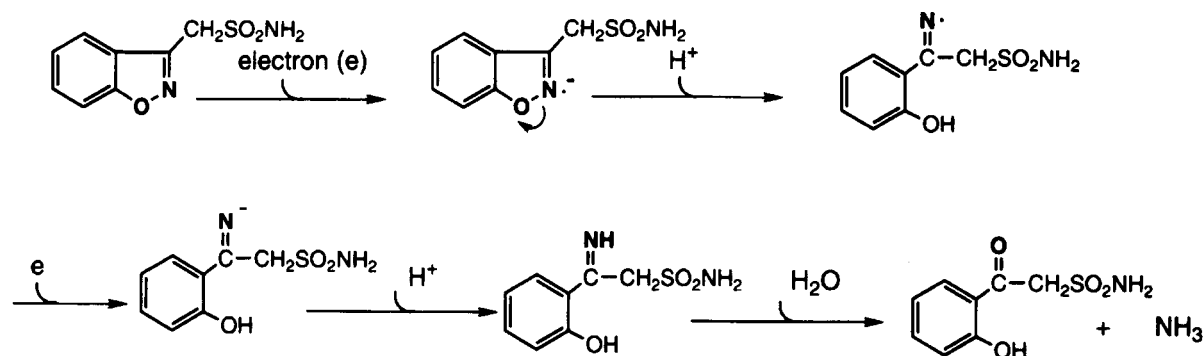
FIG. 1.   A postulated mechanism for the reduction of zonisamide to 2-sulphamoylacetylphenol by intestinal bacteria.

We recently proposed that 1,2-benzisoxazole and its derivatives such as zonisamide are metabolized reductively by aldehyde oxidase and cytochrome P450 to the corresponding oxo compounds, such as salicylaldehyde and 2-sulphamoylacetylphenol, via the corresponding ketimines that undergo nonenzymatic hydrolysis (Sugihara et al 1996). The proposed mechanism was supported by the stoichiometric study of the formation of 2-sulphamoylacetylphenol and ammonia from zonisamide. A similar mechanism is postulated for the reduction of zonisamide by intestinal bacteria; this is shown in Fig. 1.

The cell-free extract from *Clostridium sporogenes* had NADH or NADPH and FMN-dependent, or NADH or NADPH and methyl viologen-dependent reductase activities toward zonisamide. Studies on the bacterial enzymes responsible for the zonisamide reduction are under way in this laboratory.

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