The current study demonstrated that TMP at 10^{-3} *M* acts on both apical and basolateral membranes by different mechanisms, however, the question arises as to which action contributes to the TMP-induced hyperkalemia. When 800 mg of SMX is given with 160 mg of TMP twice daily, the peak concentration of TMP in plasma is approximately 6.8×10^{-6} *M* [19]. Peak plasma concentration of TMP is 1.1×10^{-5} *M* after intravenous infusion of 800 mg of SMX and 160 mg of TMP over a period of 1 h [20]. These reports indicate that plasma concentrations of TMP do not reach as high as 10^{-3} *M*. Also, the effects of TMP at 10^{-3} *M* in the bath on V_T and V_B were trivial (fig. 7; table 5). Therefore, the basolateral action is unlikely to contribute to the TMP-induced hyperkalemia. On the other hand, patients receiving 320 mg of oral TMP per day in two doses have mean urinary TMP concentrations of 2×10^{-4} *M* [20]. It has been shown that the urinary concentrations of TMP may reach 1.1×10^{-3} *M* after a single oral dose of 200 mg TMP in humans [8]. In humans, TMP has a half-life of 8 to 12 h and 80% is excreted unchanged into the urine [21]. Therefore, the concentrations of TMP indeed can reach the level of 10^{-5} to 10^{-3} *M* in the lumen of the CCD, exerting natriuresis and antikaliuresis. Eiam-Ong et al. [11] also reported that acute and chronic administration of TMP into rats decreased Na^+-K^+-ATPase activity in the CCD. This inhibitory action of TMP on Na^+-K^+-ATPase in the CCD can be secondary to its direct effect on apical Na^+ conductance, because apical Na^+ entry is known to modulate basolateral Na^+-K^+ pump activity in the CCD [14, 22].

In conclusion, TMP mainly acts on the apical membrane of the rabbit CD cell, and inhibits macroscopic Na^+ conductance. This effect causes a decrease in the net driving force for K^+ exit across the apical membrane, leading to an inhibition of K^+ secretion.

Acknowledgments

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PHARMACOKINETICS AND DISPOSITION

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Interaction between grapefruit juice and hypnotic drugs: comparison of triazolam and quazepam

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Abstract *Objective:* Grapefruit juice (GFJ) inhibits cytochrome P450 (CYP) 3A4 in the gut wall and increases blood concentrations of CYP3A4 substrates by the enhancement of oral bioavailability. The effects of GFJ on two benzodiazepine hypnotics, triazolam (metabolized by CYP3A4) and quazepam (metabolized by CYP3A4 and CYP2C9), were determined in this study. *Methods:* Nine healthy subjects were administered 0.25 mg triazolam or 15 mg quazepam, with or without GFJ. Each trial was performed using an open, randomized, cross-over design with an interval of more than 2 weeks between trials. Blood samples were obtained during the 24-h period immediately following the administration of each dose. Pharmacodynamic effects were determined by the digit symbol substitution test (DSST) and utilizing a visual analog scale. *Results:* GFJ increased the plasma concentrations of both triazolam and quazepam and of the active metabolite of quazepam, 2-oxoquazepam. The area under the curve (AUC)(0–24) of triazolam significantly increased by 96% ($p<0.05$). The AUC(0–24) of quazepam (+38%) and 2-oxoquazepam (+28%) also increased; however, these increases were not significantly different from those of triazolam. GFJ deteriorated the performance of the subjects in the DSST after the triazolam dose (−11 digits at 2 h after the dose, $p<0.05$), but not after the quazepam dose. Triazolam and quazepam produced similar sedative-like effects, none of which were enhanced by GFJ. *Conclusion:* These results suggest that the effects of GFJ on the pharmacodynamics of triazolam are greater than those on quazepam. These GFJ-related different effects are partly explained by the fact that triazolam is presystemically metabolized by CYP3A4, while quazepam is presystemically metabolized by CYP3A4 and CYP2C9.

Keywords Grapefruit juice · CYP3A4 · CYP2C9 · Triazolam · Quazepam

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Introduction

The ingestion of grapefruit juice (GFJ) increases the oral bioavailability of a number of drugs, including felodipine, terfenadine, cyclosporine and simvastatin [1–4]. The mechanism by which GFJ substantially reduces the oral clearance of these cytochrome P450 (CYP) 3A4 substrate drugs is by increasing their oral bioavailability through a post-transcriptional mechanism that decreases CYP3A4 protein content in the small intestine [5]; hepatic CYP3A4 activity is not blunted by GFJ [5]. Furanocoumarin-induced inhibition of CYP3A4 is thought to be involved in the drug-food interaction [6, 7].

Triazolam and quazepam are benzodiazepine derivatives which are frequently prescribed for the treatment of sleep disorders. In an in vitro study, von Moltke et al. demonstrated that triazolam is metabolized by CYP3A4 to α-hydroxytriazolam and 4-hydroxytriazolam [8]. As expected, GFJ has been found to increase plasma triazolam concentration and enhance its pharmacodynamic effects in human subjects [9, 10]. However, because the metabolism of quazepam to 2-oxoquazepam is mediated by CYP3A4 and CYP2C9 [11], it is anticipated that the effects of GFJ on the pharmacokinetics and pharmacodynamics of triazolam may be greater than those on quazepam. This study was undertaken to examine this hypothesis. The effects of GFJ on the pharmacokinetics and pharmacodynamics of triazolam and quazepam were determined by an open, randomized, cross-over design in healthy subjects.

Methods

Study design

The study was performed in an open, randomized, crossover design with four phases. The interval between each phase (study day) was more than 2 weeks. The protocol was approved by the Ethics Committee of Jichi Medical School (Tochigi, Japan).

Nine healthy Japanese men (age: 23–44 years old; weight: 56–80 kg) participated in this study after giving written informed consent. Genetic analysis [12] showed that all subjects were extensive metabolizers of CYP2C9 substrates. Four of the participants were smokers. The subjects were instructed not to take any medications, herbal dietary supplements and herbal tea throughout the study period. The consumption of GFJ was not allowed during the entire study period, except for when a trial was being conducted, while the consumption of caffeine-containing beverages, including coffee and green tea, and alcohol and smoking were prohibited from one night before the trial until a final blood sampling on the next morning. The subjects were given 250 ml of normal-strength GFJ (Tropicana, Kirin Beverage, Tokyo, Japan) three times daily for 3 days immediately prior to the trial according to a randomized schedule. The recommended dose for the treatment of sleep disorders in Japan is 0.25–0.5 mg triazolam and 15–30 mg quazepam. However, because 0.25 mg triazolam and 15 mg quazepam are most frequently used as initial doses, we chose these doses for the trials. On the day of the trial, subjects took a single oral dose of triazolam (0.25 mg; Halcion, Pharmacia & Upjohn, Tokyo, Japan) or quazepam (15 mg; Doral; Mitubishi Pharma, Tokyo, Japan) together with 250 ml of GFJ or water at 8:00 A.M. after an overnight fast. In the trials with GFJ, the subjects also took 250 ml of GFJ 4 and 12 h after the drug had been taken. The subjects had a light meal 4 h after taking either the triazolam or quazepam.

Blood sampling

Blood samples (5 ml in each) for the analyses of triazolam and quazepam were collected in heparinized tubes immediately before and at 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after the participants had taken the doses of triazolam or quazepam. Plasma samples were stored at −80°C until the assay was carried out.

Pharmacodynamic measurements

The pharmacodynamic effects of triazolam and quazepam were determined using the digit symbol substitution test (DSST) [13] and a visual analog scale (VAS) immediately before each blood sampling and from 0–12 h following the administration of the drug. In the DSST, the participants in the study substituted simple digit symbols using a pencil and paper. The number of digits correctly substituted in 2 min was recorded. Subjective assessments were made on 100-mm long horizontal ungraded visual analogue scales. Sedative-like effects observed were the pairs of adjectives (in Japanese) such as drowsy/alert, calm/nervous, clumsy/skilled, mentally slow/quick-witted and discontented/contented. The subjects had been trained to perform the tests before the initiation of the study: prior to the trials they performed DSST several times after being given oral instructions; thereafter, they completed 12 h of baseline study (DSST and VAS) without medication on a different day.

Assay of plasma triazolam

Plasma concentration of triazolam was measured by a high-performance liquid chromatography (HPLC) method developed in our laboratory. A 2-ml plasma sample was added to 4 ml of methanol, and the mixture was then shaken vigorously for 10 min, followed by centrifugation at 1,500 *g* for 10 min. The procedure was repeated three times, and the supernatant was evaporated to dryness under nitrogen stream. The residue was reconstituted with 100 μl ethanol, and the whole amount was subjected by analysis using a two-step drug extraction process by HPLC.

The HPLC system consisting of a chromatography pump (PU-880; Jasco, Tokyo, Japan), an ultraviolet detector (Uvidec 875; Jasco) and a column (Fine Pak SilC18T5, 5 μm, 250×4.6 mm; Jasco) was used to separate triazolam. The column temperature was set at 40°C with a column oven module. In the first process, the mobile phase consisted of methanol/H_2O (70:30, v/v). The fraction containing triazolam was separated from the interfering substances existing in the residue by collecting the effluent between 6.0 and 7.5 min after the injection. The collected effluent was evaporated to dryness and reconstituted with 100 μl of a mobile phase consisting of methanol/H_2O (54.5:45.5, v/v), which was then injected into the identical HPLC system with the mobile phase. To reduce sample loss, the tube containing the residue obtained in the first process was again washed with the mobile phase, and this reconstituent (100 μl) was also applied to the second separation process. Triazolam was separated out by collecting effluent between 12.0 and 13.5 min. The mobile phase was pumped at a flow rate of 1.0 ml/min for the separation of triazolam. The absorbance of the effluent was monitored at 220 nm.

The effluent containing triazolam was evaporated to dryness and reconstituted with 50 μl of internal standard (1 μg/ml butyl *p*-hydroxybenzoate in methanol), and 20-μl aliquots were applied to the HPLC system. The HPLC system was identical to that described above except for the mobile phase [methanol/H_2O (52.4:47.6, v/v)]. The recovery of 2.5 ng/ml triazolam was 88.7% (n=6). The method was validated for the concentration range from 0.5 to 2.5 ng/ml. The intra-assay coefficient and accuracy were less than 8.6% and 85.1–108.4% (n=6), respectively. The inter-assay coefficient and accuracy were less than 18.8% and 73.4–137.4% (n=6), respectively. The lower detection limit was 0.15 ng/ml.

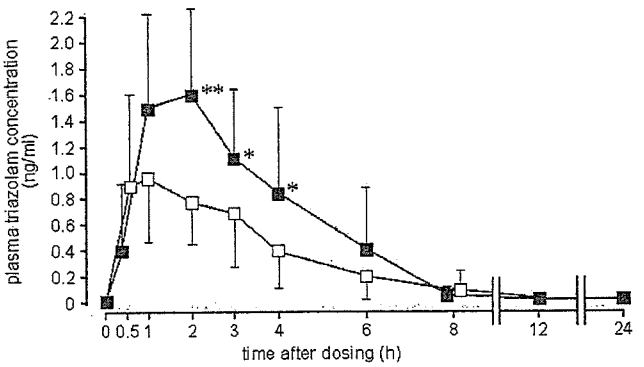

Fig. 1 Plasma concentrations of triazolam after a single oral dose of 0.25 mg triazolamwith grapefruit juice (*black squares*) or water (*open squares*). Points represent the mean (n=9) ± SD. *p<0.05, **p<0.01 versus the control (water) values

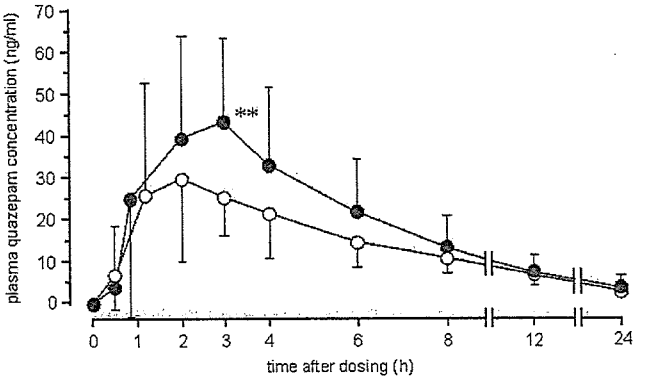

Fig. 2 Plasma concentrations of quazepam after a single oral dose of 15 mg quazepam with grapefruit juice (*black circles*) or water (*open circles*). Points represent the mean (n=9) ± SD. **p<0.01 versus the control (water) values

Assay of plasma quazepam and its metabolites, 2-oxoquazepam and *N*-desmethyl-2-oxoquazepam

Plasma concentrations of quazepam and its metabolites were measured by a column-switching HPLC analysis. An aliquot of each plasma sample (1.5 ml), to which 0.1 ml of cisapride (800 ng/ml) was added as an internal standard, was first alkalinized by adding 500 µl 0.5 *M* NaOH followed by the addition of 0.4 ml H_20 and 5 ml of toluene/chloroform (85:15, v/v). The mixture was shaken vigorously for 15 min and then centrifuged at 2,000 *g* for 10 min. A 4.5-ml portion of the organic layer was evaporated to dryness in vacuo at 45°C. The residue was reconstituted with 0.8 ml of eluent A (see below) and used as an extract.

A 0.5-ml aliquot of the extract was injected onto the column-switching HPLC system which consisted of a chromatography pump (LC-10A; Shimadzu, Tokyo, Japan), an autoinjector (AS-8020; Tosoh, Tokyo, Japan) and an ultraviolet detector (SPD-10A; Shimadzu). Column I (TSK-BSA-C8, 5 µm, 10×4.6 mm; Tosoh) was used for pretreatment and column II (STR-ODS II, 5 µm, 150×4.6 mm; Shimadzu) for the separation of quazepam and 2-oxoquazepam. The column temperature was set at 30°C with a column oven module. Between 0 and 13.0 min after the injection of a sample, cisapride was separated from the interfering substances present in the extract on column I by means of a mobile phase solvent (eluent A) consisting of acetnitrile/0.02 mol/l KH_2PO_4 (13:87, v/v). Between 13.0 and 20.0 min after the injection, quazepam and its metabolites, which had been retained on column I were eluted with a mobile phase (eluent B) consisting of acetnitrile/perchloric acid/0.02 mol/l KH_2PO_4 (41.00:0.05:58.95, v/v/v), and the effluent from column I was switched to column II. Then *N*-desmethyl-2-oxoquazepam was separated on column II by eluting with eluent B (between 20.0 and 32.0 min). Quazepam and 2-oxoquazepam were separated on column II by eluting with a mobile phase solvent (eluent C) consisting of acetnitrile/0.02 mol/l KH_2PO_4 (62.5:37.5, v/v) between 32.0 and 46.5 min. The mobile phase was pumped at a flow rate of 0.6 ml/min. The absorbance of the effluent from column II was monitored at 254 nm for the metabolites and 286 nm for quazepam.

The lower detection limits were 0.8 ng/ml, with a linear calibration range from 1 to 100 ng/ml of quazepam and its metabolites. The recoveries of quazepam and its metabolites was more than 93.2% at 10 ng/ml of the concentrations (n=6). The inter-and intra-assay coefficient of variations were less than 4.6% at 10 ng/ml of these compounds.

Pharmacokinetic calculations

Pharmacokinetics were characterized by maximum plasma concentration C_{max}), time to maximum plasma concentra-

Table 1 Pharmacokinetic parameters[a] of triazolam, quazepam and 2-oxoquazepam after a single oral dose of the drug (0.25 mg triazolam or 15 mg quazepam) with grapefruit juice (GFJ) or water in nine healthy Japanese male volunteers

	C_{max} (ng/ml)			t_{max} (h)		AUC(0–24) (ng h/ml)		
	Water	GFJ	Ratio	Water	GFJ	Water	GFJ	Ratio
Triazolam	1.3±0.4[b]	1.9±0.6*	1.55±0.46 (1.27; 1.84)	1.4±1.0	1.6±0.5	6.3±2.7	10.9±4.0*	1.96±1.02 (1.34; 2.59)
Quazepam	36.9±21.7	52.0±20.8*	1.62±0.59 (1.25; 2.00)	2.2±1.0	2.6±0.7	247.5±110.2	324.2±150.1	1.38±0.43 (1.12; 1.64)
2-Oxoquazepam	6.1±1.4	9.7±3.5*	1.57±0.36 (1.35; 1.79)	2.4±1.0	2.4±0.9	53.5±14.8	67.6±24.5	1.28±0.34 (1.07; 1.50)

*p<0.05 versus water

[a]C_{max}, Maximum plasma concentration; t_{max}, time to maximum concentration; AUC(0–24), area under the plasma concentration time curve from 0 to 24 h after administration of the dose

[b]Values are the mean ± SD (90% confidence interval)

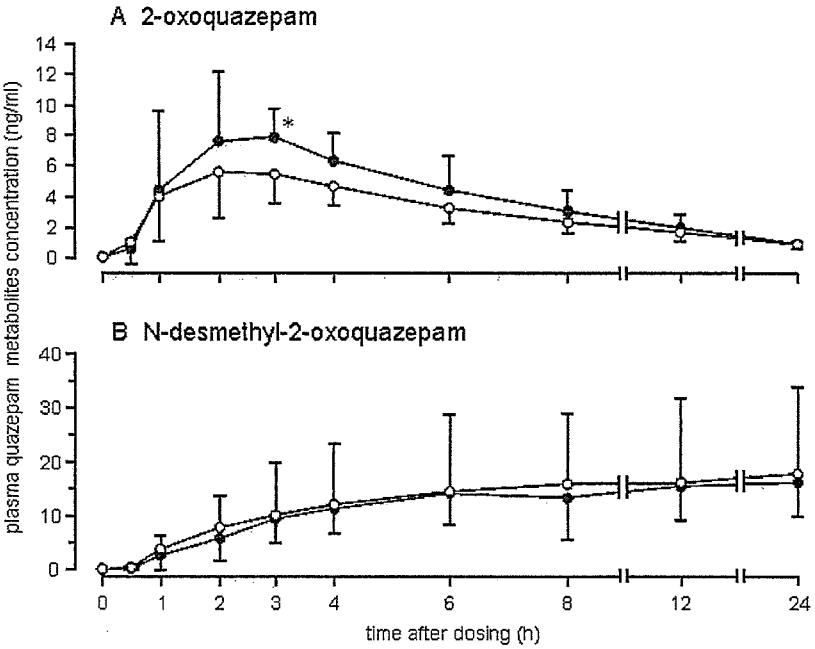

Fig. 3 Plasma concentrations of quazepam metabolites, 2-oxo-quazepam (**a**) and *N*-desmethyl-2-oxoquazepam (**b**), after a single oral dose of 15 mg quazepam with grapefruit juice (*black circles*) or water (*open circles*). Points represent the mean (n=9) ± SD. *p<0.05 versus the control (water) values

tion (t_{max}), elimination half-life ($t_{½}$) and area under the plasma concentration-time curve from 0–24 h post-administration of the drug [AUC(0–24)]. The elimination rate constant (K_e) was determined using last three points of a linear regression analysis of a log-linear phase of the plasma drug concentration-time curve. Elimination half-life ($t_{½}$) was calculated as follows:

$$t_{1/2} = \ln 2/K_e$$

The AUC(0–24) was calculated by the trapezoidal rule.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Data were analyzed by paired Student's *t*-test or analysis of variance (ANOVA) using the statistical program *Statview* for Windows, version 5.0 (SAS Institute, Cary, N.C.). A correlation between plasma drug concentrations and the decrease in the number of digit substitutions was analyzed by the Pearson's correlation coefficient. Differences were regarded as statistically significant when the p value was less than 0.05.

Results

Plasma concentration of triazolam

The plasma concentration of triazolam increased after the 0.25 dose had been given with GFJ relative to the same dose given with water (Fig. 1). The C_{max} and AUC(0–24) of the agent were significantly greater with GFJ than with water (Table 1). No significant difference was observed in the $t_{½}$ (in hours) between the two groups (water: 3.6, GFJ: 3.4).

Plasma concentrations of quazepam and 2-oxoquazepam

Plasma concentrations of quazepam and 2-oxoquazepam increased after a dose of quazepam was given with GFJ (Figs. 2 and 3a). The values of C_{max} of these agents in the trial with GFJ were significantly greater than those in the trial with water (Table 1). Their AUC(0–24) were also greater in the trial with GFJ (Table 1), but they did not

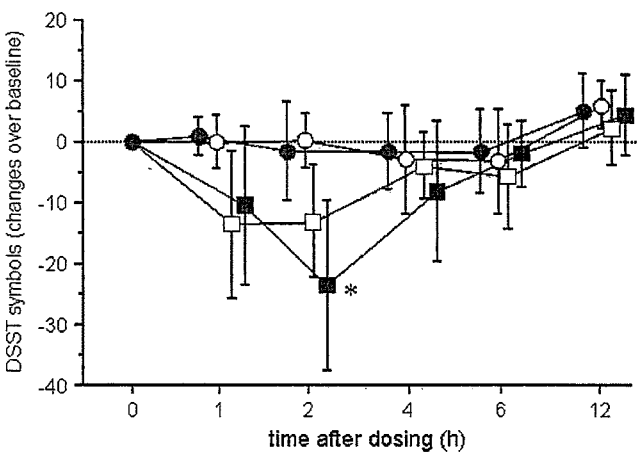

Fig. 4 Digit symbol substitution test (DSST, expressed as changes over the predose baseline) after a single oral dose of 0.25 mg triazolam or 15 mg quazepam with grapefruit juice or water. *Open circle* Quazepam with water, *black circle* quazepam with grapefruit juice, *open square* triazolam with water, *closed square* triazolam with grapefruit juice. Points represent the mean (n=9) ± SD. *p<0.05 versus the control (water) values

Table 2 Pharmacodynamic parameters[a] of quazepam following the administration of a single oral administration with grape fruit juice (GFJ) or water

	DSST (digits/h)		Drowsiness (VAS mm/h)		Mental slowness (VAS mm/h)	
	AUC(0–6)	AUC(0–12)	AUC(0–6)	AUC(0–12)	AUC(0–6)	AUC(0–12)
Triazolam + water	−49 (−68, −26)	−57 (−100, −15)	160 (42, 277)	200 (−13, 412)	126 (59, 192)	212 (64, 360)
Triazolam + GFJ	−64 (−98, −31)	−58 (−102, −14)	147 (33, 261)	136 (−88, 360)	126 (55, 198)	146 (−12, 305)
Quazepam + water	−9 (−30, 12)a,b[b]	0 (−40, 39)	187 (81, 292)	343 (116, 569)	100 (25, 175)	191 (19, 363)
Quazepam + GFJ	−6 (−22, 11)a,b	5 (−28, 37)a,b	162 (114, 210)	283 (196, 370)	88 (33, 143)	188 (85, 292)

[a]DSST, Digit substitution symbol test; VAS, visual analog scale; AUC(0-*n*), area under the symbol number or analog scale-time curve between time zero and *n* hours after administration

[b]Data are mean values (90% confidence interval); values followed by 'a', $p<0.05$ versus triazolam + water; values followed by 'b', $p<0.05$ versus triazolam + GFJ

reach statistical significance. The concentration of *N*-desmethyl-2-oxoquazepam in the plasma rose up to 24 h following the administration of quazepam; consequently, the C_{max} of this metabolite could not be determined in this study. However, GFJ did not affect the plasma concentrations of this metabolite during the period we examined (Fig. 3b). No significant difference in the GFJ-related increase in AUC(0–24) was observed between triazolam and quazepam (difference: 58%). There was also no significant difference in the increase in C_{max} between triazolam (+55%) and quazepam (+62%). The increase in the AUC(0–24) or C_{max} of 2-oxoquazepam did not significantly differ from that of triazolam. The ratio of 2-oxoquazepam to quazepam in the C_{max} or AUC(0–24) did not differ between two trials C_{max}: water, 0.21; C_{max}: GFJ, 0.19; AUC(0–24): water, 0.24; AUC(0–24): GFJ, 0.22). The $t_{½}$ (in hours) of these agents did not significantly differ between the water and GFJ trials (quazepam: water, 5.9; quazepam: GFJ, 4.5; 2-oxoquazepam: water, 6.3; 2-oxoquazepam: GFJ, 5.8).

Pharmacodynamics

Triazolam remarkably reduced the number of digit substitutions (Fig. 4, Table 2). On the other hand, the effect of quazepam on the objective performance in the DSST was small. Triazolam with GFJ caused an addition decrease in digit substitution, especially at 2 h following administration of the dose (Fig. 4). However, GFJ did not deteriorate the performance in the DSST after the quazepam dose.

Triazolam caused significant ($p<0.0001$) drowsiness and mental slowness, with a peak at 2 h after administration of the dose (Fig. 5). Time courses of other subjective effects were similar (data not shown). Quazepam also produced similar sedative-like drug effects but its peak was slightly later – at 4–6 h after administration of the dose (Fig. 5). GFJ did not significantly enhance these triazolam- or quazepam-induced subjective effects (Table 2).

A significant negative correlation was noted between triazolam concentrations in the plasma and the decrease in

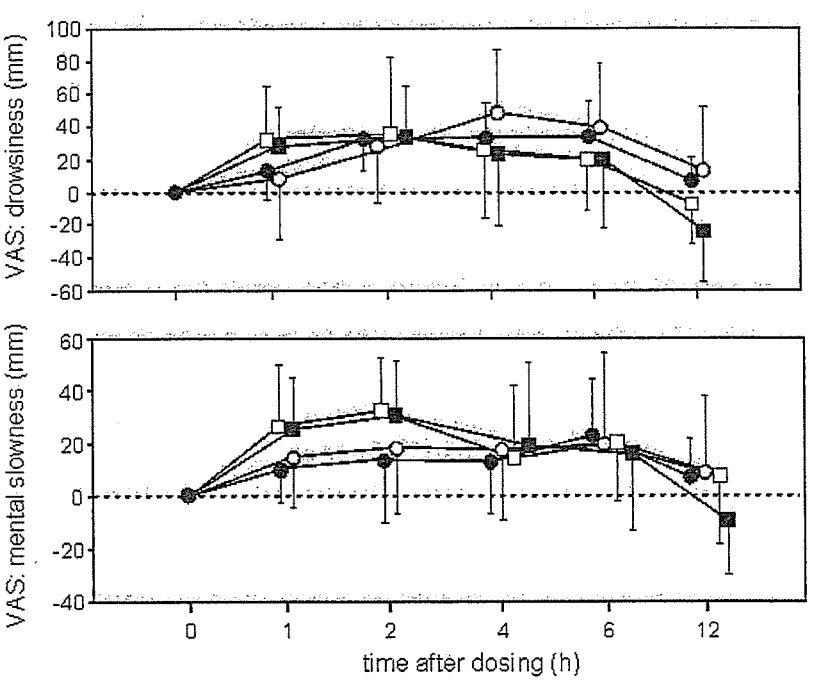

Fig. 5 Sedative-like self-rated drug effects [based on scores from the 100-mm visual analog scale (VAS), expressed as changes over the predose baseline] after a single oral dose of 0.25 mg triazolam or 15 mg quazepam with grapefruit juice or water. *Open circle* Quazepam with water, *black circle* quazepam with grapefruit juice, *open square* triazolam with water, *black square* triazolam with grapefruit juice. Points represent the mean (n=9) ± SD

the number of digit substitutions at 2 h after the dose had been given (R=−0.539, p<0.05, n=18). There were no significant correlations between the plasma concentrations of quazepam or 2-oxoquazepam and any pharmacodynamic parameters.

Discussion

In this study, the C_{max} of triazolam, quazepam and 2-oxoquazepam significantly increased during the trials with GFJ. The oral bioavailability of triazolam was about 60% due to presystemic elimination. Triazolam is mainly metabolized by CYP3A4 [8], and the elimination $t_{½}$ is 2–4 h. The bioavailability of quazepam is assumed to be 29–35% based on a calculation of the ratio of the dose-normalized cumulative amount excreted in Japanese subjects (personal communication, Mitsubishi Pharma, Tokyo, Japan). Quazepam is metabolized by CYP3A4 and CYP2C9 [11], and it has been reported that in plasma the elimination $t_{½}$ of quazepam and an active metabolite, 2-oxoquazepam, are 25–40 h and that of *N*-desmethyl-2-oxoquazepam is 70–75 h [14, 15]. The calculated elimination $t_{½}$ of quazepam and 2-oxoquazepam in the present study were shorter than those reported in the previous studies because the length of sampling period was not enough to estimate terminal half-lives of these compounds. In the present study, the effect of GFJ on the pharmacokinetics of quazepam and its metabolites were examined for 24 hours after administration of the dose; however, a longer sampling period post-dose administration and an analysis system with a high sensitivity are required to correctly estimate the half-lives of quazepam, 2-oxoquazepam and in particular *N*-desmethyl-2-oxoquazepam. In fact, we were unable to determine even the C_{max} for *N*-desmethyl-2-oxoquazepam.

The metabolism of quazepam and 2-oxoquazepam is mediated by CYP3A4 and CYP2C9, and the contribution of CYPs to the metabolic process is presumed to be 50:50 based on an inhibition study using the cDNA-expressed human CYPs and antiserum for CYP3A4 and CYP2C [11]. These findings led us to speculate that the inhibition of CYP3A4 activity by GFJ has a greater influence on the pharmacokinetics of triazolam than on those of quazepam. The AUC(0–24) of triazolam was significantly increased by GFJ, while the elevation in this parameter for quazepam or 2-oxoquazepam by GFJ did not reach statistical significance. However, the GFJ-related changes of AUC (0–24) or C_{max} for triazolam did not significantly differ from those for quazepam or 2-oxoquazepam. It therefore appears that the inhibition of intestinal CYP3A4 activities by GFJ enhances the oral bioavailability of both quazepam and triazolam. The ratio of 2-oxoquazepam to quazepam in the C_{max} or AUC(0–24) did not differ significantly between the trials with and without GFJ. GFJ elevated the plasma concentrations of quazepam and 2-oxoquazepam, but not of *N*-desmethyl-2-oxoquazepam. The quazepam metabolite 2-oxoquazepam is further metabolized to *N*-desmethyl-2-oxoquazepam by CYP3A4 and CYP2C9 and to another metabolite by CYP3A4 [11]. Consequently, it is likely that in the present study the effect of GFJ diminished with the formation of *N*-desmethyl-2-oxoquazepam. In addition, GFJ did not prolong the $t_{½}$ of triazolam, quazepam and 2-oxoquazepam. These pharmacokinetic alterations may be caused by the GFJ-induced inhibition of CYP3A4 activity in the intestine, but not in the liver [5]. Lilja et al. [10] been reported that repeated consumption of GFJ prolonged the $t_{½}$ of triazolam, suggesting the inhibition of hepatic CYP3A4 by GFJ. In their study, subjects were given 200 ml of double-strength GFJ three times a day for 3 days, while we gave 250 ml of normal-strength GFJ three times a day for 4 days to our participants. Not only the length of the treatment period but the strength of GFJ may be a determinant of the GFJ-induced inhibition of hepatic CYP3A4, if this inhibition is truly caused by GFJ.

GFJ increases plasma triazolam concentration [9, 10], which is consistent with the present results. A significant increase in the pharmacodynamic effects of triazolam has been observed following multiple doses of GFJ [10], but not following a single dose [9, 16]. Hukkinen et al. [9] found that psychomotor function, as assessed by DSST, was not further impaired by a single dose of 250 ml GFJ despite an increase in plasma triazolam concentration [9]. In their study, triazolam C_{max} increased by only 30% following a single dose of GFJ. Similarly, Vanakoski et al. [16] observed that a single, concomitant ingestion of 300 ml GFJ did not influence the triazolam-induced objective effects, including DSST. In the present study in which multiple-doses of GFJ were given, C_{max} increased by 55% on average; furthermore, the maximum reduction in DSST performance showed a weak – but significant – correlation with the plasma concentration of triazolam. The present results suggest that pharmacodynamic alteration by the GFJ-triazolam interaction may be caused by the increase in plasma triazolam concentration. Taken together, a single dose of GFJ may not enough to cause the interaction with triazolam on psychomotor function, probably because of an insufficient increase in plasma triazolam concentration.

GFJ increased plasma quazepam and 2-oxoquazepam concentrations, but it did not alter the pharmacodynamic effects. The major metabolites of quazepam, 2-oxoquazepam and *N*-desmethyl-2-oxoquazepam are reported to be pharmacologically active, although the involvement of these metabolites has not been proven to date. Roth et al. [17] suggested that the hypnotic effect of quazepam is caused by the metabolite *N*-desmethyl-2-oxoquazepam [17]. In their study, a repeated dose of quazepam, however, did not increase the incidence or the duration of daytime napping; similarly, the hypnotic and sedative effect of quazepam disappeared 12 h after the dose despite a high plasma concentration of *N*-desmethyl-2-oxoquazepam. If the effect of quazepam is solely caused by *N*-desmethyl-2-oxoquazepam, the sedative-like subject-rated effects quazepam should have been evident even 12 h after administration of the dose of quazepam. However, we observed the maximum sedative-like subject-rated effects by quazepam 4–6 h after the dose. It therefore appears that the sedative effect observed in the present study was caused

by quazepam itself. A contribution by 2-oxoquazepam to the effects of quazepam is possible, but it is not obvious based on our results, and the contribution of *N*-desmethyl-2-oxoquazepam was small, if any. The effect of quazepam on psychomotor function as assessed by DSST was negligible, a result similar to that found by Nikaido et al. [18]. Our results suggest that the detrimental effects of 15 mg quazepam on psychomotor functions may be small even if the drug is taken with GFJ. However, 0.25 mg triazolam and 15 mg quazepam produced significant sedative-like, subject-rated effects in this study. The effect of GFJ on plasma quazepam or 2-oxoquazepam concentration was diminished at 4–6 h after the dose had been given, which is the time span when the sedative effect was at its maximum; consequently, the subject-rated effect of quazepam may not be enhanced by GFJ.

CYP2C9 metabolizes many clinically important drugs including the S-enantiomer of warfarin and tolbutamide [19]. The enzyme is also involved in the metabolism of quazepam [11]. Two common polymorphisms of CYP2C9 have been reported; the *2 allele (Arg144Cys) and the *3 allele (Ile359Leu) [20]. CYP2C9*2 and CYP2C9*3 diminish the clearance of S-warfarin and tolbutamide, respectively [19]. The frequencies of these alleles are as follows: CYP2C9*2, 8% in Caucasians and 0% in Japanese; CYP2C9*3: 6% in Caucasians and 2% in Japanese [12, 21]. Therefore, when quazepam is given to patients with one or these CYP2C9 polymorphisms, the effects of GFJ on the pharmacokinetics and pharmacodynamics of the drug may be exaggerated. Further study is needed to address the issue.

In summary, the results of this study demonstrate that the effects of GFJ on the pharmacodynamics of triazolam are greater than on those of quazepam. These GFJ-induced different effects are partly explained by the fact that triazolam is mainly metabolized by CYP3A4 while quazepam is metabolized by CYP3A4 and CYP2C9.

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Indoxyl sulfate stimulates proliferation of rat vascular smooth muscle cells

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Vascular smooth muscle cell (VSMC) proliferation is a key event in the progression of arteriosclerosis. Clinical studies show that uremic toxins deteriorate the arteriosclerosis in renal failure patients. Indoxyl sulfate (IS) is a strong protein-bound uremic toxin, but the effect of IS on VSMC proliferation has not been studied. We examined the effect of IS on rat VSMC proliferation, assessed by a cell counting kit (4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2*H*-5-tetrazolio-1, 3-benzene disulfonate] assay) and by [^{3}H]thymidine incorporation *in vitro*. We further evaluated a contribution of mitogen-activated protein kinase (MAPK; p44/42 MAPK) to VSMC proliferation by IS. Immunohistochemical staining was performed for VSMCs using antirat organic anion transporter (OAT)3 antibody. The mRNA expressions of platelet-derived growth factor (PDGF)-A and -C chains, and PDGF-β receptor were evaluated by real-time PCR. IS stimulated the proliferation of VSMCs in a concentration-dependent manner and activated p44/42 MAPK. Concentration of IS needed to stimulate the proliferation of rat VSMC was about 250 μM, which is compatible with that in the serum of end-stage renal failure patients. PD98059 (10 μM), a selective inhibitor of MAPK/extracellular signal-regulated kinase, inhibited the IS-induced (250 μM) VSMC proliferation and phosphorylation of MAPK. Probenecid (0.5 mM), an inhibitor and substrate of OAT, inhibited the IS-induced (250 μM) VSMC proliferation. Rat OAT3 was detected in VSMCs. The mRNA expressions of PDGF-C chain and PDGF-β receptor were significantly increased by IS. We conclude that IS directly stimulates rat VSMC proliferation and activates MAPK *in vitro*. This might be one of the mechanisms underlying the progression of atherosclerotic lesions in end-stage renal disease patients.

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KEYWORDS: indoxyl sulfate; uremic toxin; vascular smooth muscle cells; MAP kinase cascade; organic anion transporter

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Improvements in dialysis treatment have resulted in prolongation of the survival period of hemodialysis patients, but their prognosis is still poor compared to that in the general population.[1] The major cause of death in patients undergoing dialysis is cardiovascular disease.[2] The pathophysiology of cardiovascular disease in end-stage renal disease patients is not completely understood; however, accumulation of uremic toxins that are difficult to be removed from the body by current dialysis procedures is partly involved in the condition.[3,4] Indoxyl sulfate (IS) is one of the organic anions metabolized in the liver from indole, which is produced by the intestinal bacteria as a metabolite of tryptophan.[5] Although the molecular weight of IS is small, the rate of binding to albumin is high in the blood,[6] which, in turn, leads to its large secretion from the proximal tubule cells in urine.[7] Various organic anion transporters (OATs) exist in proximal tubules, and it has recently been revealed that IS is secreted in urine mainly by OAT3, one of the members of the OAT family.[8] Therefore, IS accumulates in the body of patients with reduced renal function.[5]

Removal of IS by hemodialysis is difficult because the size of the IS–albumin complex molecule in blood is larger than the pore size of a dialysis membrane.[5] Thus, IS is considered to be one of the main uremic toxins that are difficult to be removed from the body by current dialysis procedures.[5] It has been reported that IS accelerates the progression of renal failure,[9] but only one datum is available about the effects of IS on the cardiovascular system.[10]

In the progression of atherosclerotic lesions, the proliferation of vascular smooth muscle cells (VSMCs) is of particular importance. The mitogen-activated protein kinase (MAPK) cascades are a well-documented family of serine/threonine kinases that include p44/42 MAPK (also called extracellular signal-regulated kinases (ERK1/2)), p38 MAPK, and c-Jun N-terminal kinase.[11] The p44/42 MAPK cascade is the most well characterized and is shown to mediate proliferative responses in various cells, including mesangial cells[12] and VSMCs.[13] Growth factors, such as platelet-derived growth factor (PDGF),[14] angiotensin II,[15] erythropoietin,[16] and uremic toxins such as homocysteine,[17] and uric acid[18] can activate intracellular signaling cascades, leading to the proliferation of VSMCs. However, the effects of IS on VSMC proliferation and activation of MAPK have not been studied.

In this study, we therefore directly investigated the effects of IS *in vitro* on VSMC proliferation and MAPK activation using cultured rat VSMCs.

RESULTS

IS increases rat VSMC proliferation and DNA synthesis

First, we examined the effect of IS on the number of VSMCs. IS significantly increased the number of VSMCs in a concentration-dependent manner (Figure 1). PDGF (10 ng/ml) also increased the number of VSMCs.

We next evaluated whether the increase in cell proliferation was accompanied with DNA synthesis. Figure 2a shows that [^{3}H]thymidine incorporation was also significantly increased by IS (100–500 μM). Because IS is mainly bound to albumin in the serum, we also evaluated the effect of IS in the presence of 4 g/dl albumin in the medium. Although basal value was significantly lower with albumin, IS at 250 and 500 μM significantly increased the uptake (Figure 2b). This result indicates that IS stimulated VSMC proliferation even in the presence of 4 g/dl albumin. IS in the body is made from tryptophan in the intestine[5] and the medium used in this study contained tryptophan (78 μM), which may affect the results. Thus, we further examined the effect of the removal of tryptophan from the medium. As shown in Figure 3, 250 μM IS increased the [^{3}H]thymidine incorporation by VSMCs in both the presence and absence of tryptophan (78 μM). Therefore, IS, but not its precursor, directly stimulated the proliferation of VSMC *in vitro*.

As IS is reported to be the substrate of OAT3 in the proximal tubule cell membrane,[8] we next examined whether the proliferation of VSMCs is mediated by cellular transport of IS via the OAT. As shown in Figure 4, the increase of [^{3}H]thymidine incorporation in VSMCs by 250 μM IS was partly prevented by co-administration of 0.5 mM probenecid, an inhibitor and substrate of OAT,[8] whereas probenecid alone did not affect it. Next, to determine whether OAT3 is present in rat VSMCs, we performed immunostaining of rat VSMCs by rat OAT3 antibody. As shown in Figure 5, the rat VSMCs had strong signals of rat OAT3, mainly in the cell membrane.

IS activates the p44/42MAPK pathway in rat VSMCs

To evaluate further intracellular signaling event in the effects of IS on the rat VSMC proliferation, we investigated the

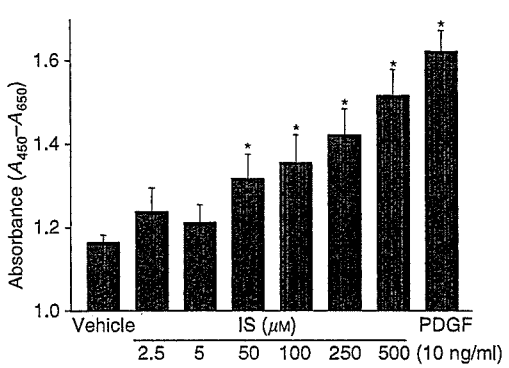

Figure 1 | Effect of IS on the proliferation of rat VSMCs by 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] **proliferation assay.** Growth-arrested VSMCs were stimulated by IS and PDGF for 24 h. 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] was added for the last 4 h. PDGF (10 ng/ml) was used as a positive control. Absorbance was measured by an enzyme-linked immunosorbent assay reader. Values are mean ± s.e.m. ($n = 8$). *$P < 0.05$ compared with the vehicle.

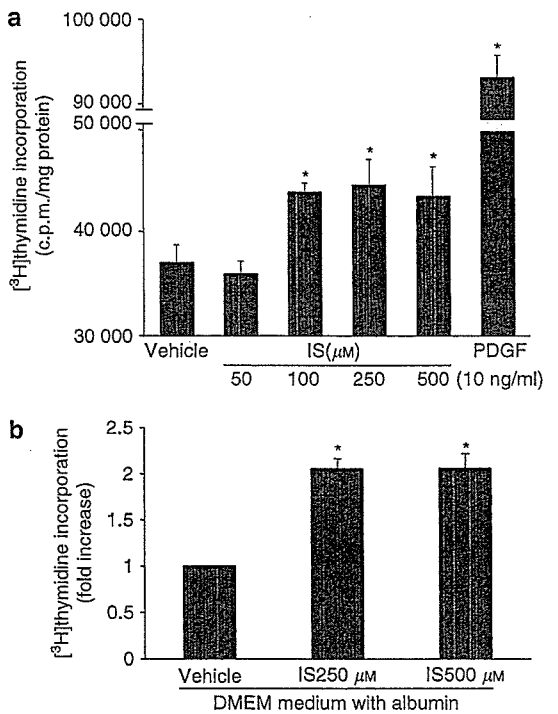

Figure 2 | Effect of IS on the proliferation of rat VSMCs assessed by DNA synthesis. (**a**) Growth-arrested VSMCs were stimulated by IS and PDGF for 24 h. [^{3}H]Thymidine was added for the last 6 h. PDGF (10 ng/ml) was used as a positive control. (**b**) Effect of IS on the proliferation of rat VSMCs in the presence of 4 g/dl albumin in the medium assessed by DNA synthesis. Values are mean ± s.e.m. ($n = 8$). *$P < 0.05$ compared with the vehicle.

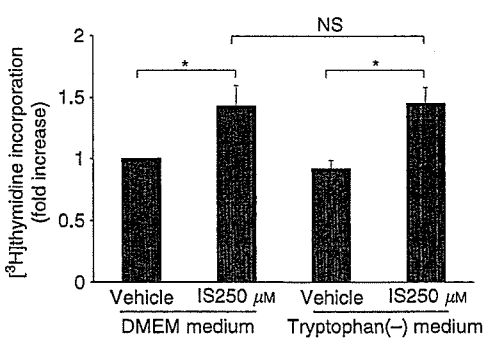

Figure 3 | Effect of the removal of tryptophan on IS-induced increase of DNA synthesis in rat VSMCs. Growth-arrested VSMCs were incubated for 24 h in DMEM or tryptophan (–) medium with or without IS (250 μM). [^{3}H]Thymidine was added for the last 6 h. Values are mean ± s.e.m. ($n = 8$). *$P < 0.05$ compared with vehicle.

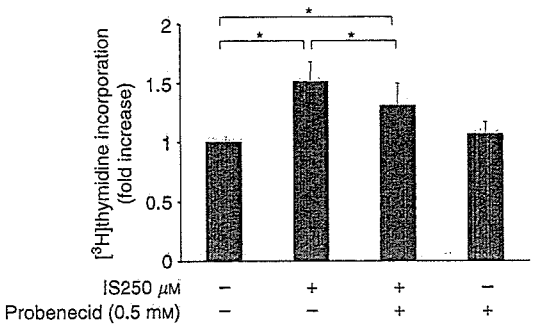

Figure 4 | Effect of probenecid on IS-induced increase of DNA synthesis in rat VSMC. Growth-arrested VSMCs were pretreated for 1 h with probenecid (0.5 mM) and then stimulated by IS (250 μM) for 24 h. Values are mean ± s.e.m. ($n = 8$). *$P < 0.05$.

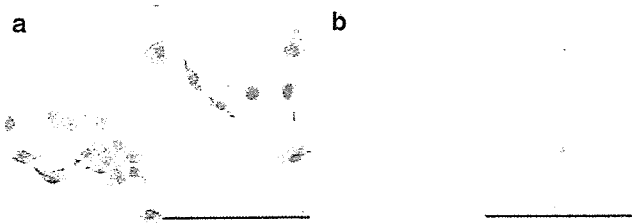

Figure 5 | Immunohistochemistry of rat OAT3 in rat VSMCs. (**a**) VSMCs were stained with polyclonal antibodies against rat OAT3 (**b**) There was no staining in the negative control with rabbit immunoglobulin fraction. Bar = 200 μm.

activation of p44/42 MAPK in VSMCs. As plasma concentration of IS in hemodialysis patients is reported to be about 250 μM,[5] we selected this dose for the following experiments. IS at 250 μM induced p44/42 MAPK phosphorylation in the cells, with a maximal intensity at 5 min (Figure 6). To confirm that the p44/42 MAPK pathway was actually involved in the IS-induced mitogenesis, we pretreated VSMCs with 10 μM PD98059, an MAPK/ERK kinase inhibitor,[14] to inhibit the pathway. The IS-induced phosphorylation was significantly inhibited by PD98059 (Figure 7). We further evaluated the effect of pretreatment with PD98059 at the same dose on the increase in IS-induced [^{3}H]thymidine incorporation. PD98059 inhibited the increase in DNA synthesis by IS, whereas PD98059 alone had no effect on DNA synthesis (Figure 8).

IS increases mRNA expressions of PDGF-C chain and PDGF-β receptor

To discuss the mechanisms of IS-induced proliferation of VSMC, we evaluated the mRNA expressions of PDGF-A and -C chains, and PDGF-β receptor by real-time PCR. We found that mRNA expressions of PDGF-C chain and PDGF-β receptor were significantly increased by the addition of IS (Figure 9). Therefore, the increased expressions by IS are partly involved in the mechanism of IS-induced VSMC proliferation.

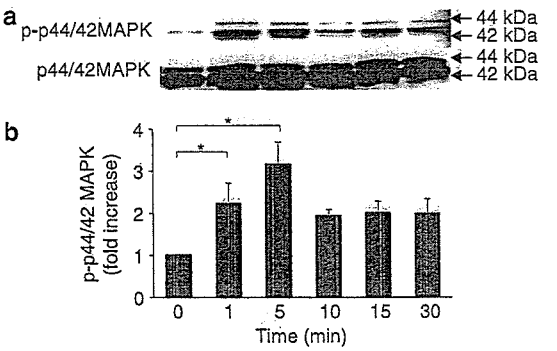

Figure 6 | Effect of IS on p44/42 MAPK phosphorylation. Growth-arrested VSMCs were stimulated by IS (250 μM) for the indicated times. (**a**) Representative immunoblots are shown with antibodies that recognize phosphorylated p44/42 MAPK and total p44/42 MAPK. (**b**) Densitometric analysis of phosphorylated p44/42 MAPK. Values are mean ± s.e.m. ($n = 5$). *$P < 0.05$.

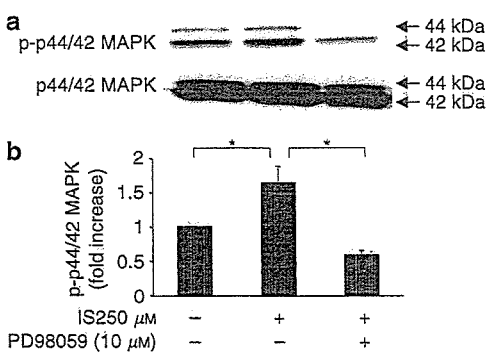

Figure 7 | Effect of PD98059 on IS-induced phosphorylation of p44/42 MAPK. Growth-arrested VSMCs were pretreated with PD98059 (10 μM) for 1 h and then stimulated by IS (250 μM) for 5 min. (**a**) Representative immunoblots are shown with antibodies that recognize phosphorylated p44/42 MAPK and total p44/42 MAPK. (**b**) Densitometric analysis of phosphorylated p44/42 MAPK. Values are mean ± s.e.m. ($n = 5$). *$P < 0.05$.

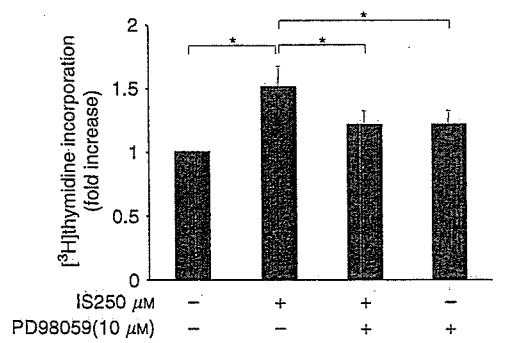

Figure 8 | Effect of PD98059 on IS-induced proliferation of rat VSMCs assessed by DNA synthesis. Growth-arrested VSMCs were pretreated for 1 h with PD98059 (10 μM) and then stimulated with IS (250 μM) for 24 h. Values are mean ± s.e.m. ($n = 8$). *$P < 0.05$.

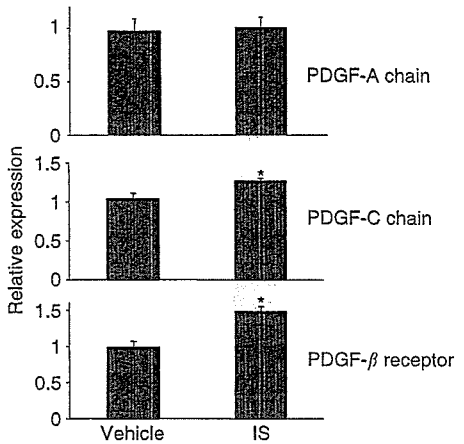

Figure 9 | Effect of IS on mRNA expressions of PDGF-A and -C chains and PDGF-β receptor. Growth-arrested VSMCs were stimulated with IS (250 μM) for 6 h and then total RNA was isolated. Real-time quantitative PCR was performed as described in Materials and Methods. Values are mean ± s.e.m. of two identical samples performed in at least triplicate ($n = 4$ in each). *$P < 0.05$ compared with the vehicle.

DISCUSSION

It is known that IS is a protein-bound uremic toxin that accelerates the progression of renal failure,[5] but data are limited about its actions on other organs. A recent *in vitro* study has shown that IS has a suppressive effect on the repair of damage in human umbilical vein endothelial cells, suggesting that the agent is involved in endothelium dysfunction in renal failure.[10] The present study shows for the first time that IS directly promotes VSMC proliferation. This is the most important finding in the present study. Serum concentration of IS in patients with end-stage renal failure is about 250 μM,[5] which is compatible with that needed to stimulate the proliferation of rat VSMCs in this study. As the enhanced proliferation of VSMCs in vascular walls is believed to promote hypertrophy of arteries, it is possible that VSMC proliferation induced by IS is one of the causes of cardiovascular complications that occur in dialysis patients with a high serum IS.

As IS is known to be transported by OATs on tubular cell membranes, the effect of probenecid, an inhibitor and substrate of OATs, on VSMC proliferation was investigated. In this experiment, we found that probenecid had an inhibitory effect on the proliferation of VSMCs. We also confirmed the presence of OAT3 in VSMCs by immunostaining. These findings suggest that IS is at least in part taken into VSMCs via OAT. Proliferation of VSMCs plays some role in the development of hypertension and arteriosclerosis,[19] and the p44/42 MAPK pathway is thought to be the intracellular signaling pathway involved in the proliferation of VSMCs.[13] When we evaluated the involvement of MAPK in the effect of IS on VSMCs, we found that pretreatment of VSMCs with PD98059, an inhibitor of MAPK, caused inhibition of VSMC proliferation. We also found that IS induced p44/42 MAPK phosphorylation, which was inhibited by PD98059. These findings indicate that the p44/42 MAPK pathway is involved in the induction of VSMC proliferation by IS.

In the present study, serum was not used in experiments examining the effects of IS stimulation, although no significant difference was found between the degrees of VSMC proliferation in serum with and without IS (data not shown). However, serum contains various growth factors, some of which might have a strong proliferative effect in addition to IS on VSMCs. Indeed, we found that the mRNA expressions of PDGF-C chain and PDGF-β receptor were increased by IS. Therefore, the increased expressions by IS are partly involved in the mechanism of IS-induced VSMC proliferation. The culture medium used in the present study contained tryptophan, a precursor of IS, but the possibility that the presence of tryptophan in the medium affected the results was ruled out by comparison of results of experiments using a medium containing tryptophan and a medium from which tryptophan had been removed.

The mechanisms by which IS induces proliferation of VSMC and activation of MAPK are not known. Uric acid has also been reported to be a uremic toxin that induces proliferation of VSMCs. Rao *et al.*[18] reported that uric acid induces proliferation of VSMCs by promoting the production of PDGF-A chain, and Johnson *et al.*[20,21] reported that uric acid is taken up by VSMCs via an OAT. As a potential mechanism for VSMC proliferation, uric acid, after being taken into VSMCs, activates MAPK and induces the production of cyclooxygenase-2 and the expression of PDGF-A chain, -C chain, and PDGF-α receptor mRNA.[20] We found that the induction of VSMC proliferation by IS was inhibited by probenecid and that MAPK was involved in the induction of VSMC proliferation by IS. Therefore, the mechanism of IS-induced VSMC proliferation might be similar to that of uric acid. To our knowledge, other protein-bound uremic toxins that have been shown to induce VSMC proliferation *in vitro* are homocysteine[22] and leptin.[23] Further study is needed to determine whether MAPK activation by IS is mediated by various growth factor receptors on the cell membrane surface or by an as yet unidentified receptor. To understand the pathophysiology of cardiovascular disease in end-stage renal disease patients, the effect of IS on other cell types of blood vessels and their crosstalk must be evaluated in the future.

In summary, the present *in vitro* study shows for the first time that IS directly induces cell proliferation of rat VSMC. Concentration of IS required to induce VSMC proliferation is similar to its blood concentration in patients on maintenance dialysis. Part of the action of IS on VSMC is mediated by OAT3, and the induction of VSMC proliferation by MAPK activation caused by IS. Increases of mRNA expressions of PDGF-C chain and PDGF-β receptor are also involved in the phenomenon. This might be one of the mechanisms underlying the progression of atherosclerotic lesions in end-stage renal disease patients.

MATERIALS AND METHODS

Materials

A p44/42 MAPK assay kit, including anti-p44/42 MAP kinase and anti-phospho-p44/42 MAP kinase (Thr202/Try204) antibodies, was purchased from Cell Signaling Technology (Beverly, MA, USA). A Cell Counting Kit was purchased from DOJINDO Laboratories (Kumamoto, Japan). [^{3}H]Thymidine was purchased from Perkin Elmer Life Science (Boston, MA, USA). Anti-rat OAT3 polyclonal antibody was purchased from TransGenic Inc. (Kumamoto, Japan). All other materials including IS were purchased from Sigma (St Louis, MO, USA). IS was dissolved with distilled water as vehicle in all experiments.

Cell culture

VSMCs were isolated from the aortas of male Sprague–Dawley rats (150–200 g) as previously described.[13] In brief, VSMCs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (ICN Biomedicals, Osaka, Japan), 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technology Inc., Rockville, MD, USA) in a 5% CO_2 incubator at 37°C. VSMCs at 70–80% confluence were growth-arrested by incubation in DMEM with 0.5% FBS for 48 h. The cells were used between passages 3 and 8. VSMCs were identified by their typical hill and valley morphology and by indirect immunofluorescent staining for α-smooth muscle actin (R&D Systems Inc., Minneapolis, MN, USA). We measured endotoxin concentration in the medium by limulus amebocyte lysate method (Wako Pure Chemical Industries, Osaka, Japan) and found that it was under the detection limit, which strongly indicated the absence of lipopolysaccharide in the medium.

Cell proliferation assay

VSMC proliferation was assessed using the Cell Counting Kit.[24] The cells were seeded at a density of 5×10^3 cells/well on 96-well culture plates in DMEM with 10% FBS for 72 h. After serum starvation for 48 h in DMEM with 0.5% FBS, the cells were stimulated by IS or PDGF as a positive control for 24 h. For the final 4 h of incubation, 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] was added to each well and the absorbance was measured at 450 nm with a reference wavelength of 650 nm using a microplate spectrophotometer system (SOFTmax PRO, Molecular Devices Corporation, Sunnyvale, CA, USA). The difference of absorbance between 450 and 650 nm was regarded as the cell proliferation.

[^{3}H]Thymidine incorporation

The cells were seeded on 24-well culture plates, allowed to grow to 70–80% confluence, and then growth-arrested by incubation in DMEM with 0.5% FBS for 48 h. The cells were incubated for 24 h, and [^{3}H]thymidine (1 μCi/ml; specific activity, 79 Ci/mmol) was added to for the last 6 h of the incubation period. The cells were then washed three times with phosphate-buffered saline, treated with ice-cold 10% trichloroacetic acid at 4°C for 15 min, and washed with phosphate-buffered saline. The acid-insoluble material was dissolved in 0.5 ml of 0.3 N NaOH. The protein content was measured by a DC Protein Assay (Bio-Rad, Hercules, CA, USA), and radioactivity was determined by using a liquid scintillation counter (Aloka, Japan).

Western blot analysis

Growth-arrested cells cultured in 100-mm dishes were stimulated by 250 μM IS for the indicated times. For the inhibitor studies, cells were pretreated for 1 h with PD98059 (10 μM), an MAPK/ERK kinase inhibitor.[14] The cells were washed with ice-cold phosphate-buffered saline and lysed in 400 μl of lysis buffer (1% Triton, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM ethyleneglycol tetraacetate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml L-leupeptin, 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. The cell lysates were centrifuged for 15 min at 15 000 *g* and the supernatants were collected. Equal amounts of protein (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrophoretically transferred to polyvinylidine difluoride membranes (Invitrogen Corp., Carlsbad, CA, USA). The membranes were blocked for 1 h at room temperature with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% bovine serum albumin. After washing with TBS-T, the membranes were incubated overnight with phospho-p44/42 MAPK (Thr202/Tyr204) antibody (1:1000) or p44/42 MAPK antibody (1:1000) (Cell Signaling Technology, Beverly, MA, USA) at 4°C with gentle shaking. The primary antibodies were detected using horseradish peroxidase-conjugated goat antirabbit IgG and visualized by enhanced chemiluminescence Western blotting reagents (Amersham Biosciences, Buckinghamshire, UK). Band intensity was analyzed using KODAK 1D Image Analysis Software (KODAK, Rochester, NY, USA).

Immunohistochemical staining

Immunostaining of OAT3 in rat VSMCs was performed by the labeled streptavidin biotin (LSAB) method using a DAKO LSAB Kit (Dako, Carpinteria, CA, USA). Briefly, VSMCs were cultured on eight-well Lab-Tek chamber slides (Nalge Nunc International) and fixed with paraformaldehyde lysine periodate solution (containing 0.01 M $NaIO_4$, 0.075 M lysine, 0.0375 M phosphate buffer with 2% paraformaldehyde; pH 6.2) for 1 h at 4°C. After fixation, the cells were permeabilized in 0.1% Triton X-100 in phosphate-buffered saline for 5 min and incubated with 3% hydrogen peroxide for 30 min to suppress endogenous peroxidase activity and then with blocking solution for 10 min. The cells were incubated with a polyclonal antibody against rat OAT3[8] (1:20) at 4°C overnight. The negative controls were treated with the rabbit immunoglobulin fraction (Dako, Carpinteria, CA, USA). After rinsing with TBS-T, the cells were incubated with a biotinylated link antibody against rabbit immunoglobulin for 30 min. The cells were washed with TBS-T and incubated with horseradish peroxidase-conjugated streptavidine solution for 30 min. Horseradish peroxidase labeling was detected using a peroxidase substrate diaminobenzidine, and then counterstaining was performed with hematoxylin.

RNA extraction and real-time quantitative PCR

The isolation of total RNA was achieved using the RNeasy Mini Kit (Quiagen, Valencia, CA, USA). Reverse transcription was performed with 1.2 μg of total RNA, random hexamer primers, and RevertAid M-MuLV reverse transcriptase (Fermentas, Hanover, MD, USA). The resulting cDNA equivalent to 60 ng of RNA was used for real-time quantitative PCR in the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) as previously described.[25] All of the specific sets of primers and TaqMan probes in the present study were obtained from Applied Biosystems (Assays-on-Demand Gene Expression Products and TaqMan Rodent GAPDH Control Reagants). All primer sets but that of TaqMan Rodent GAPDH Control Reagents were designed to be located in two exons to avoid amplification of potentially contaminating

genomic DNA. To control the variation in the amount of DNA available for PCR in the different samples, gene expressions of the target sequence were normalized in relation to the expression of an endogenous control, glyceraldehyde-3-phosphate dehydrogenase. As the efficiency of the target amplification was approximately equal to that of the glyceraldehyde-3-phosphate dehydrogenase amplification, data were analyzed using the comparative threshold cycle method.[26] Because the intra- and interassay coefficients of variation of the relative expression values were $<20\%$, we considered the mean relative values of less than 0.8 or more than 1.2 to be significant in this study.

Statistics

The results are expressed as the mean $\pm$ s.e.m. Data were analyzed by the unpaired Student's *t*-test or by one-way analysis of variance combined with Fisher's protected least significant difference using personal computer with StatView version 5.0 (SAS Institute, Cary, NC, USA). Differences with $P<0.05$ were considered to be significant.

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PHARMACOEPIDEMIOLOGY AND PRESCRIPTION

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Favorable effect on postgraduate clinical practice of a drug-interaction exercise for undergraduate students

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Abstract *Aim:* Undergraduate students in Jichi Medical School participated in a laboratory exercise investigating the furosemide–probenecid interaction at the end of their clinical pharmacology (CP) course. The aim of this study was to determine whether they learned to recognize drug interactions better than students who did not take such a course. *Methods:* We conducted a postal survey of physicians who had graduated from Jichi Medical School or from other medical schools without a CP course including the exercise. Questions were asked concerning: (1) the recognition of furosemide–probenecid and nine other drug interactions, and (2) the need to anticipate drug interactions and their adverse effects before writing prescriptions. *Results:* The degree of the recognition of all drug interactions, and the percentage of physicians who responded that knowledge of drug interactions and adverse effects were essential before writing prescriptions, were significantly greater in physicians who had taken an undergraduate CP course than in those who had not. *Conclusions:* CP courses with specific laboratory exercises on drug interactions lead future physicians to recognize drug interactions and their adverse effects.

Keywords Drug-interaction exercise · Postgraduate clinical practice · Undergraduate course

Introduction

During the last decade, dramatic changes in medical education have occurred worldwide because of dissatisfaction with the way in which doctors were being trained. A traditional passive approach has been replaced by a more active approach. Educational reform has also taken place in the fields of basic and clinical pharmacology [1–7].

The clinical pharmacology (CP) course in the Jichi Medical School is a compulsory course and consists of 16 lectures concerning the core knowledge of CP, such as clinical pharmacokinetics, adverse drug reactions, and drug interactions. At the end of the course, undergraduate students participate in a laboratory exercise investigating the furosemide–probenecid interaction, as described previously [8]. In brief, students are randomly assigned to one of three groups in a double-blind fashion: (1) placebo plus 20 mg of furosemide; (2) 250 mg of probenecid plus 20 mg of furosemide; and (3) 1,000 mg of probenecid plus 20 mg of furosemide. They take probenecid or its placebo 1 h before furosemide. Urine volume and urinary sodium excretion are measured for 3 h after they take furosemide.

We analyzed data obtained for 5 years (1995–1999) and found that the furosemide–probenecid interaction exercise was easy to perform, reproducible, and safe [8]. In addition, more than 80% of the students considered the exercise to be useful. Based on these observations, we hypothesized that the CP course with the furosemide–probenecid laboratory exercise would motivate students to recognize drug interactions and their adverse effects. To evaluate this hypothesis, we conducted a postal survey of physicians who had graduated from Jichi Medical School or other medical schools without a CP course.

Methods

Physicians who had been in clinical practice for 3–7 years participated in this study. A questionnaire was sent by mail to 527 graduates of Jichi Medical School whose mailing addresses were available on file. Each was asked to provide copies of the questionnaire to colleagues who had graduated from other medical schools and worked at the same hospital. Their colleagues were asked whether they took a CP course during their undergraduate education.

As shown in Table 1, questions were asked concerning the recognition of the furosemide–probenecid interaction

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t1.1 **Table 1** List of questions

t1.2 No.	Drug interaction	Recognition
t1.3 1	Digoxin–verapamil	A B C
t1.4 2	Tetracycline–aluminum	A B C
t1.5 3	Warfarin–aspirin	A B C
t1.6 4	Cisapride–clarithromycin	A B C
t1.7 5	Nifedipine–rifampicin	A B C
t1.8 6	Aspirin–sodium bicarbonate	A B C
t1.9 7	Furosemide–probenecid	A B C
t1.10 8	Ciclosporine–grapefruit juice	A B C
t1.11 9	Quinolones–NSAIDs	A B C
t1.12 10	HMG CoA reductase inhibitors–clofibrates	A B C

I. Do you recognize the following drug interactions?
A: yes, and understand its mechanism; B: yes, but do not understand its mechanism; C: no
II. Is the knowledge of drug interactions and adverse effects essential for prescribing drugs?
1. Drug interactions: yes no
2. Drug-related adverse effects: yes no

and nine clinically important drug interactions documented in National drug formulary by the Ministry of Health, Labour and Welfare, Japan [9]. The possible answers were: (A) knew it and understood its mechanism; (B) knew it but did not understand its mechanism; or (C) did not know it. Respondents were also asked whether understanding drug interactions and their adverse effects was essential for prescribing drugs.

Because physicians who had graduated from Jichi Medical School participated in the furosemide–probenecid laboratory exercise, these respondents were divided into three groups: (I) knew the furosemide–probenecid interaction and understood its mechanism; (II) knew the drug interaction but did not understand its mechanism; (III) did not know the drug interaction. Respondents who had graduated from other medical schools and did not participate in CP courses or laboratory exercises during their undergraduate education belonged to group IV.

Data were analyzed by chi-square or Kruskal-Wallis tests as appropriate. The level of significance was set at $P<0.05$.

Results

Of the 527 survey recipients, 357 (68%) responded (group I=139, group II=118, group III=100). In addition, 126 physicians who graduated from other medical schools responded; because 23 of them took a CP course with laboratory exercises (other than drug-interaction exercises) during their undergraduate education, they were not included (group IV=103). Thirty-five (34%) doctors in group IV recognized the furosemide–probenecid interaction. The various medical specialties of respondents are shown in Table 2; specialty did not differ significantly between the groups who took a CP course (I + II + III) and the group that did not (IV; $P>0.05$) or among groups I, II, and III ($P>0.40$).

There was a significant difference between groups I, II, and III and group IV with regard to recognition of all drug interactions (Fig. 1). Among groups I, II, and III, significant differences were also observed in the degree of the recognition of eight drug interactions (except quinolones–NSAIDs). In these eight drug interactions, the degree of recognition was greater in group I than in groups II and III. Physicians in group II recognized more drug interactions than did those in group III, except for the tetracycline–aluminium interaction.

The percentages of physicians who responded that understanding drug interactions and their adverse effects was essential for prescribing drugs were significantly greater in the group with the CP course (I + II + III) than in the group without (IV; Table 3).

t2.1 **Table 2** Medical specialty in each group

	Number (and %) in each group			
t2.2 Specialty	I	II	III	IV
t2.3 Medicine	81 (58%)	65 (55%)	50 (50%)	43 (42%)
t2.4 Surgery	24 (17%)	14 (12%)	20 (20%)	18 (18%)
t2.5 Pediatrics	10 (7%)	7 (6%)	4 (4%)	3 (3%)
t2.6 Gynecology	4 (3%)	7 (6%)	4 (4%)	6 (6%)
t2.7 Other	20 (15%)	25 (21%)	22 (22%)	32 (31%)
t2.8 No answer	0 (0%)	0 (0%)	0 (0%)	1 (0%)
t2.9 Total (male/female)	139 (112/27)	118 (95/23)	100 (79/21)	103 (85/18)
t2.10 Age (mean±SE)	30.2±2.2	29.8±2.5	30.9±2.0	31.0±2.2

(I) Doctors who knew the furosemideprobenecid interaction, understood its mechanism and participated in CP courses or laboratory exercise in Jichi Medical School; (II) octors who knew the drug interaction but did not understand its mechanism and participated in CP courses or laboratory exercise in Jichi Medical School; (III) doctors who did not know the drug interaction and participated in CP courses or laboratory exercise in Jichi Medical School; (IV) doctors who graduated from other medical schools and did not participate in CP courses or laboratory exercises during their undergraduate education

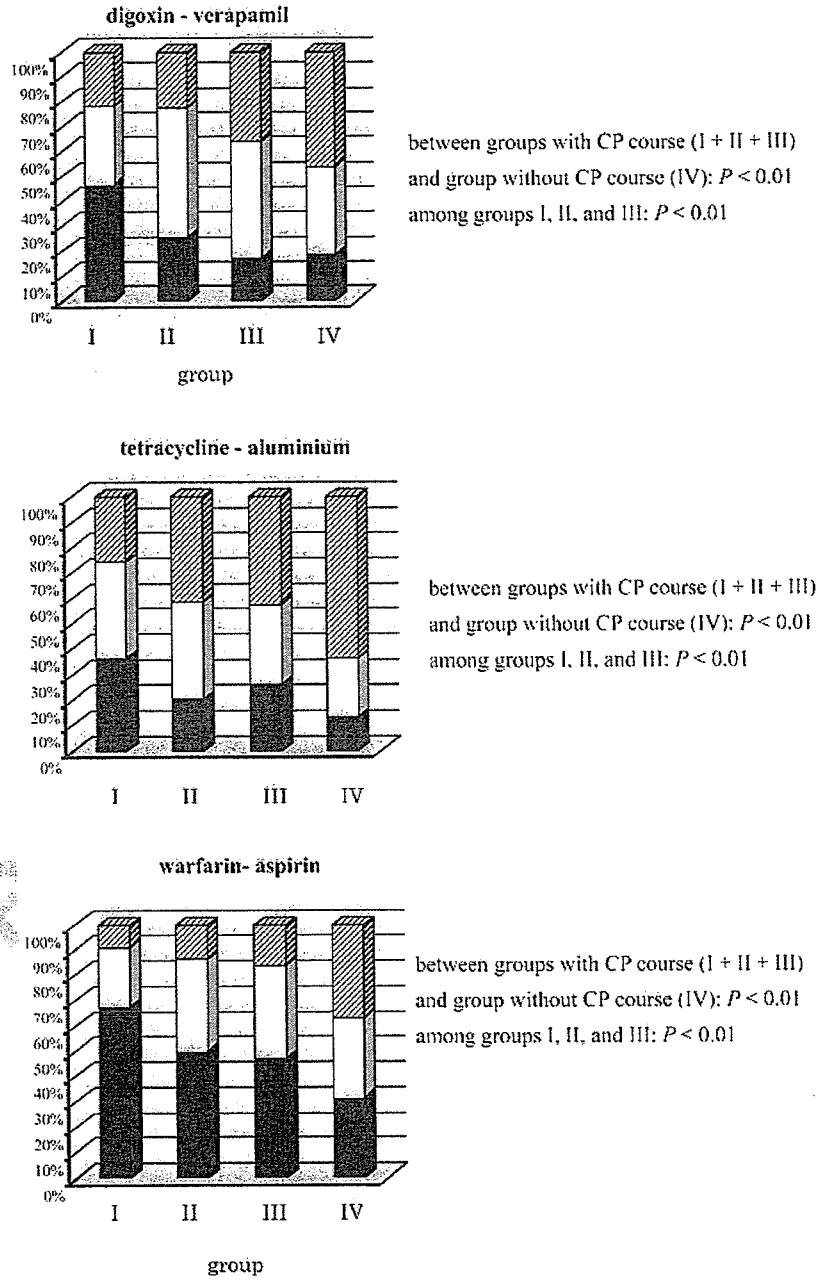


Fig. 1 Recognition of clinically important drug interactions that were documented by the Ministry of Health, Labour and Welfare, Japan. Sample sizes of groups I, II, III, and IV were 139, 118, 100, and 103, respectively. (I) Doctors who knew the furosemide–probenecid interaction, understood its mechanism and participated in CP courses or laboratory exercise in Jichi Medical School; (II) doctors who knew the drug interaction but did not understand its mechanism and participated in CP courses or laboratory exercise in Jichi Medical School; (III) doctors who did not know the drug interaction and participated in CP courses or laboratory exercise in Jichi Medical School; (IV) doctors who graduated from other medical schools and did not participate in CP courses or laboratory exercises during their undergraduate education

a: knew it and understood its mechanism

b: knew it but did not understand its mechanism

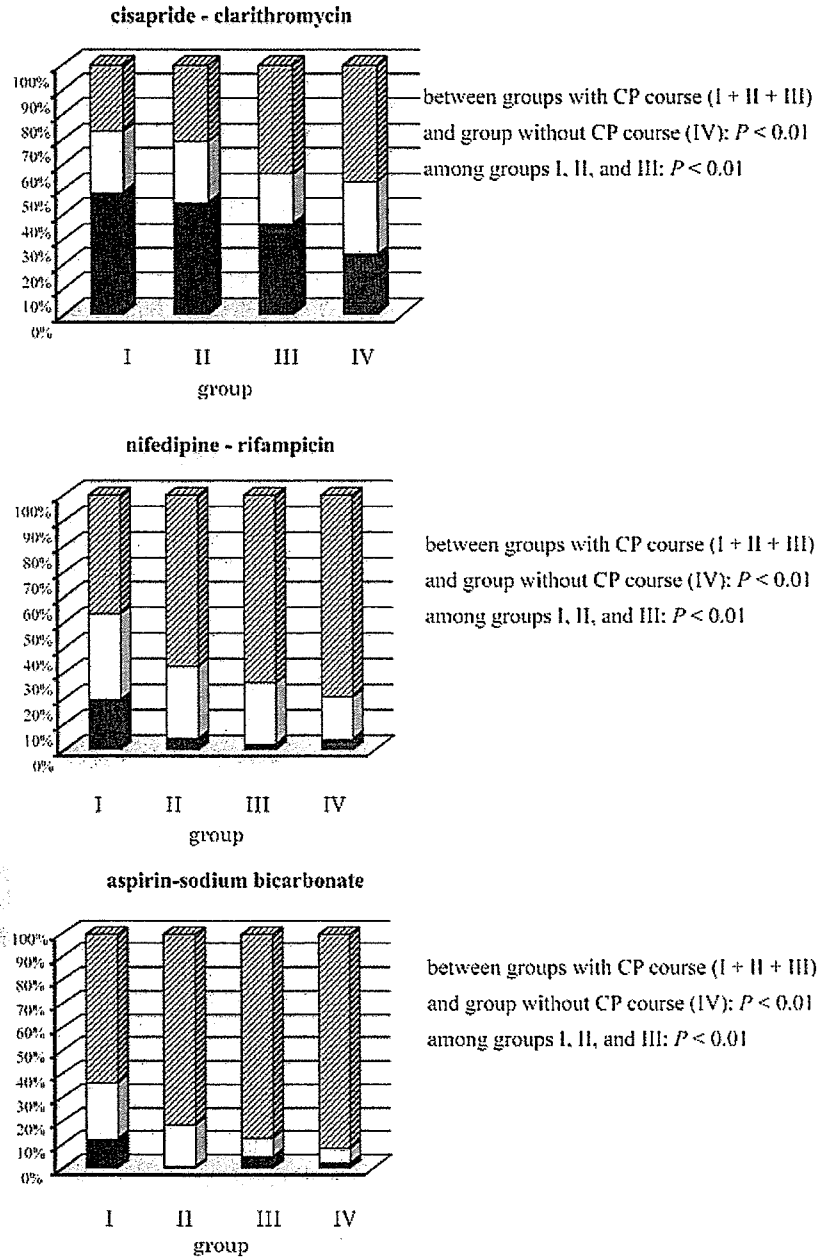
c: did not know it

Discussion

At the beginning of the 1990s, it was recognized that the inhibition of terfenadine metabolism by erythromycin and ketoconazole led to serious adverse effects [8], thus highlighting the importance of physicians recognizing drug interactions. Subsequently, the U.S. Food and Drug Administration has published guidelines for studies of in vitro and in vivo drug interactions [10, 11]. To avoid serious drug-related adverse effects in patients, it is important to improve the system of transmitting information about drug interactions to physicians [12]. It is also necessary that undergraduate students acquire the knowledge and understanding of drug interactions during CP courses. However, for medical students to develop an attitude that will lead them to assess and estimate potential drug interactions before prescribing drugs, we think it is important that the students themselves experience a drug interaction.

The drug interaction between furosemide and probenecid is well known. Both furosemide and probenecid are secreted in urine by the renal organic anion transport system [13, 14]. Therefore, when these agents are coadministered, probenecid reduces the renal secretion of furosemide and subsequent diuretic effects. For more than 15 years at Jichi Medical School, we have performed a laboratory exercise of the furosemide–probenecid interaction at the end of the CP course for undergraduate students.

Fig. 1 (continued)

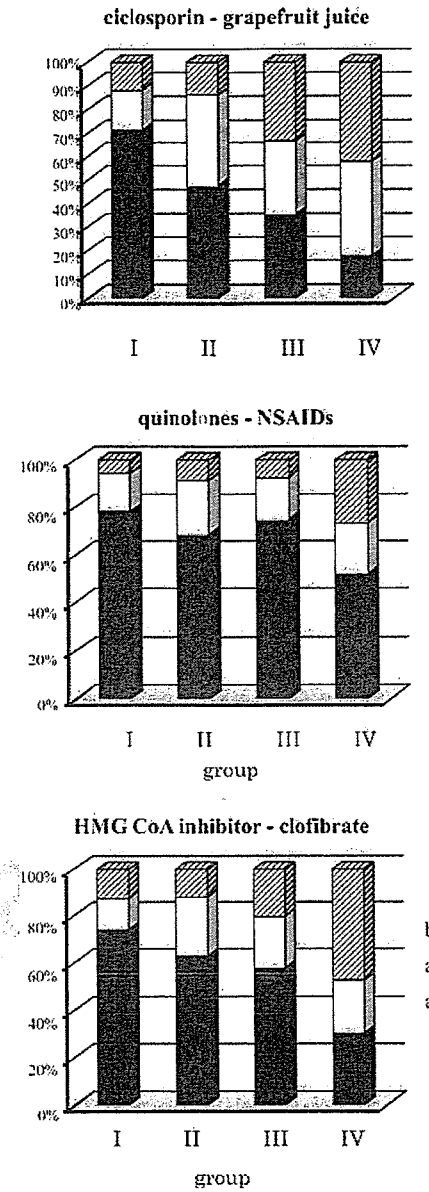


Recently, we analyzed data obtained from 1995 to 1999 and found that the exercise was easy to perform, reproducible, and safe [8].

In this study, we surveyed physicians who had been prescribing drugs for 3–7 years, and we evaluated the effect of the CP course including the furosemide–probenecid exercise on their recognition of clinically important drug interactions. The percentage of physicians who responded that knowledge of drug interactions was essential for prescribing drugs and the degree of recognition of ten drug interactions was significantly greater in the group which had taken an undergraduate CP course than in those who had not. In addition, the percentage of doctors who thought that the knowledge of drug-related adverse effects was essential before writing a prescription was also significantly greater in the group which had taken the CP course. Based on these results, we think that a CP course including the furosemide–probenecid laboratory exercise provides a good opportunity for active learning of drug interactions and adverse reactions in patients.

In general, among physicians who had taken the CP course, the degree of the recognition of drug interactions was group I (knew the furosemide–probenecid interaction and understood its mechanism) > group II (knew the drug interaction but did not understand its mechanism) > group III (did not know the drug interaction). These findings suggest that both the recognition of potential drug interactions as well as an understanding of the underlying

Fig. 1 (continued)



mechanisms are needed to prevent serious adverse effects in future clinical practice.

Because this study is based on a questionnaire survey, it is difficult to estimate the honesty of the responses, which is a limitation of the study. However, because we can assume that the incidence of dishonesty was evenly distributed in all groups, the results still indicated the effectiveness of the laboratory exercise in the undergraduate CP course. Another possible interpretation of the present results could be that the graduates of Jichi Medical School had greater knowledge about drug interaction whether they had done the laboratory exercise or not. However, we think that such a possibility is small because the degree of recognition in group I is greater than group II

Table 3 Percentage of physicians who responded that knowledge of drug interactions and adverse effects were essential for prescribing drugs

Knowledge	Group		
	With CP course (I + II + III) (n=357)	Without CP course (IV) (n=103)	P-value
Drug interactions	65%	45%	<0.01
Adverse effects	50%	39%	<0.05

and III (all were graduates from Jichi Medical School) as discussed in the previous paragraph.

In summary, this study showed that physicians who took a CP course including a drug-interaction laboratory exercise during their undergraduate education were more likely to recognize clinically important drug interactions. Through the personal experience of a drug interaction, undergraduate medical students will be more motivated to recognize drug interactions once in clinical practice.

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