— Research Letter —

Safety Evaluation of Black Rice Vinegar (Kurosu) from a Jar on Food-drug Interaction: 30-day Ingestion Study on Expressions of Drug Metabolism Enzymes and Transporters in Rats

Yoshihiko Shibayama,*, [a] Masanobu Nagano,[b] Akira Fujii,[b] Miyuki Taguchi,[c] Yasuo Takeda,[c] and Katsushi Yamada[c]

[a]Education Research Center for Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Hokkaido University, Nishi 6, Kita 12, Kita-ku, Sapporo 060–0812, Japan, [b]Sakamoto Brewing Co., Ltd., 21–15 Uenosnono-cho, Kagoshima 890–0052, Japan and [c]Clinical Pharmacy and Pharmacology, Graduate School of Medical and Dental Sciences, Kagoshima University, 8–35–1 Sakuragaoka, Kagoshima 890–8520, Japan

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Black rice vinegar (kurosu) made from a jar is a traditional vinegar in Japan. Kurosu has been commonly used as a healthcare supplement; however, it is not clear whether kurosu has a treatment effect on the expression of drug metabolism enzymes and transporters, whose expression alterations may induce food-drug interaction. Alteration of the principal drug metabolism enzymes and expression following kurosu and moromi, the residue from black vinegar brewing, for 30-day treatment was evaluated. Water, 0.23% acetic acid, concentrated kurosu (containing 0.23% acetic acid) and moromi was administered to Wister rats. The treatment did not significantly affect the biochemical parameters of serum, alanine aminotransferase, alkaline phosphatase, total protein, creatinine in the serum, and body weight. The treatment also did not affect the expression levels of cytochrome P450 (Cyp) 1a2, Cyp2b1/2, Cyp3a1, glutathione *S*-transferase, p-glycoprotein, multidrug resistance protein 2, breast cancer resistance protein, organic anion transport polypeptide 2, and organic anion transport 2 and 3 in the liver. In conclusion, the present study suggests that kurosu from a jar does not have toxic effect and does not alter expression levels of principal drug metabolism enzymes and transporters.

Key words —— black rice vinegar, cytochrome P450, transporter

INTRODUCTION

Food-drug interaction evidence has been accumulated; natural products can interact with drugs by the same mechanisms as drugs. Clinically important interactions appear to involve effects on drug metabolism via cytochrome P450 (Cyp) isoenzymes, impairment of hepatic or renal function, and other possible mechanisms.[1)] For example, a healthcare supplement St. John's Wort, interacts with prescription drugs.[1, 2)] The family of ATP binding cassette (ABC) transporters, for example, p-glycoprotein (P-gp), multidrug resistance protein 2 (Mrp2) and breast cancer resistance protein (Bcrp), also has important roles in the detoxification and excretion of xenobiotics.[3)] In hepatocytes, organic anion transport polypeptide 2 (Oatp2) plays important roles in the vector transport of bile acids from blood to bile. Bile acid is a regulation factor of hepatic and intestinal transporters.[4)] The organic anion transporter (Oat) family also plays important roles in the elimination of a variety of endogenous substances, xenobiotics and their metabolites from the body.[5)]

Black rice vinegar (kurosu) from a jar is a traditional vinegar in Kagoshima, Japan.[6)] It has been reported that kurosu improved hypertension, allergy, hypercholesterol, carbohydrate metabolism and tumor growth.[6, 7)] Kurosu has been commonly used as a healthcare supplement; however, it is not clear whether kurosu treatment effects on the expression of drug metabolism enzymes and transporters.

To evaluate the food-drug interaction with kurosu, the authors investigated the effect of kurosu treatment on the expression of principal drug metabolism enzymes and transporters.

MATERIALS AND METHODS

Chemicals —— Primary antibodies for Western

*To whom correspondence should be addressed: Education Research Center for Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Hokkaido University, Nishi 6, Kita 12, Kita-ku, Sapporo 060–0812, Japan. Tel. & Fax: +81-11-706-3705; E-mail: sibayama@pharm.hokudai.ac.jp

Table 1. Primary Antibody Properties and Immunodetection Condition

	Molecular size (kDa)	Clone or catalog No.	Host	Protein (μg)	Dilution ratio (primary antibody)
β-actin	43	SIGMA-ALDRICH, AC-74	Mouse	10	10,000
Cyp1a2	58	MILLIPORE, AB10089	Rabbit	20	2,000
Cyp2b1/2	56	BIOMOL, CR 3290	Rabbit	20	1,000
Cyp3a1	58	BIOMOL, CR 3310	Rabbit	10	3,000
GST[a)]	26	ROCKLAND, 3D4	Mouse	10	2,000
Mrp2	170	MONOSAN, M_2III-6	Mouse	20	400
P-gp	160	Calbiochem, C219	Mouse	20	1,000
Bcrp	60	Kamiya Biomedical, bxp-21	Mouse	20	500
Oatp2	90	Santa Cruz, sc-33610	Rabbit	20	500
Oat2	60	TransGenic, KE031	Rabbit	50	250
Oat3	130	TransGenic, KE035	Rabbit	50	250

Whole cell homogenate of the liver was separated with SDS-PAGE. The amount of protein and the dilution ratio of the primary antibodies are indicated. Clone name of mouse monoclonal antibodies or catalog number of polyclonal rabbit antibody is shown in the "Clone or catalog No." lane. *a*) GST: glutathione *s*-transterase.

blotting detection were purchased from Sigma-Aldrich Japan (Tokyo, Japan), Nihon Millipore K.K. (Tokyo, Japan), BIOMOL (Plymouth Meeting, PA, U.S.A.), ROCKLAND (Gilbertsville, PA, U.S.A.), MONOSAN (AM Uden, The Netherlands), Calbiochem (San Diego, CA, U.S.A.), Kamiya Biomedical (Seattle, WA, U.S.A.), Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and TransGenic (Kumamoto, Japan). The clone names and catalog numbers of the primary antibodies are summarized Table 1. The second antibodies, anti-rabbit and anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP), were purchased from Nacalai Tesque (Kyoto, Japan). Nitrocellulose membranes, ECL Western blotting detection system and Hyperfilm ECL were purchased from GE Healthcare U.K. Ltd. (Buckinghamshire, U.K.). Protease inhibitor cocktail tablets (Complete®, EDTA free) were purchased from Roche Diagnostics GmbH (Basel, Switzerland). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Concentrated black vinegar from a jar (10-fold concentrated, pH. 4.41, acetic acid distilled) and moromi (residue from black vinegar brewing) were obtained from Sakamoto Brewing Co., Ltd. (Kagoshima, Japan). Concentrated black vinegar contains 0.23% acetic acid, 3.23% lactic acid and 1.99% ash.

Animals —— Male Wistar rats (body weight, 90 ± 5 g, mean ± S.D., Kyudo, Kumamoto, Japan) were housed under standard conditions (21 ± 2°C, ventilated rooms, 12 hr light/dark cycle). The animals were fed rat chow and allowed free access to water during the experimental period. Rats were treated by oral gavage once a day as follows: vehicle group, distilled water was given at a dose of 10 ml/kg; acetic acid group, 1.5 ml of 0.23% acetic acid (pH 3.5) was diluted with 8.5 ml distilled water, and the diluted solution was given at a dose of 10 ml/kg; black vinegar group, 1.5 ml concentrated black vinegar solution was diluted with 8.5 ml distilled water, and the diluted solution was given at a dose of 10 ml/kg; moromi group, 50 mg moromi was suspended in 10 ml distilled water, and the suspension was given at a dose of 10 ml/kg. The rats were killed by exsanguination after blood collection. As a positive control treatment, dexamethasone was intraperitoneally injected at a dose of 50 mg/kg for consecutive 4 days[8,9] and methotrexate was intraperitoneally injected once at a dose of 150 mg/kg.[10,11] Dexamethasone-treated rats were killed the day after 4-day treatment. Methotraxate-treated rats were killed on day 4 after treatment. Target tissues were promptly removed and stored at −30°C until Western blot analysis. Serum prepared from each rat was stored at 4°C until analysis. Hemolysate samples were excluded from samples for analysis. Animal experiments were performed in accordance with the criteria of Kagoshima University for the care and use of experimental animals.

Assays —— Creatinine, alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined with the clinical reagents for diagnosis (Shino-test, Tokyo, Japan). Protein concentration was determined by DC protein assay (Bio-Rad Japan, Tokyo, Japan). Optical densities (ODs) were measured by the public domain program Image J (developed by the U.S. National Institutes

of Health and available on the Internet at http://rsb.info.nih.gov/ij/download.html). Individual exposures were scanned into TIFF images with a GT9000 color scanner (Seiko Epson, Tokyo, Japan). Overall differences among treatments were evaluated by one-way analysis of variance (ANOVA). Differences among groups in vehicle-, acetic acid-, black vinegar- and moromi-treated rats were evaluated using ANOVA.

Western Blotting —— Whole cell homogenate was prepared from the rat liver. Tissues obtained from individual animals of each group were cryopreserved and homogenized in buffer containing 250 mM sucrose and 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-tris(hydroxymethyl)-aminomethane (Tris, pH 7.4) with an ultrasonic homogenizer. Protease inhibitor tablets were dissolved in the above-mentioned homogenizing buffer at appropriate concentrations. Protein samples were mixed with an equal volume of sodium dodecyl sulfate (SDS) sample buffer containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol and 0.005% bromophenol blue. Protein samples for P-gp, Mrp2, Bcrp, Oat2 and Oat3 were not heat treated.[12] Western blotting for P-gp, Mrp2 and Oat3 detection was separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). The other protein samples were separated by 12% SDS-PAGE. Western blotting and primary antibody dilution are summarized in Table 1. After SDS-PAGE separation, proteins were transferred to a nitrocellulose membrane. After transfer, the membranes were incubated with 3% skim milk in buffer A (0.35 M NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% Tween 20) for 1 hr at room temperature for blocking. The membranes were then incubated with primary antibody under the same conditions as for blocking. After washing three times with buffer A, the membranes were further incubated with 1000-fold diluted secondary antibody conjugated with HRP. Finally, membranes were rinsed once for 15 minutes and four more times for 5 minutes with buffer A. For detection, the ECL Western blotting detection system was used.

RESULTS AND DISCUSSION

Rats were administered water, acetic acid, black vinegar and moromi once a day for 30 consecutive days. It was reported that the dose of black vinegar and moromi corresponded to the human dose and that black vinegar treatment improved blood sugar levels in a diabetic model mice.[6] The biochemical parameters of serum, ALT, ALP, total protein, creatinine were measured to estimate general liver and kidney functions. Acetic acid, black vinegar and moromi treatments showed no significant differences in the biochemical parameters of serum and body weight between water treatments (Table 2). This result suggests that acetic acid, black vinegar and moromi treatment have no toxic effect.

An example of Western blot analysis is shown in Fig. 1. β-actin, Cyp2b1/2, Cyp3a1, P-gp, Mrp2 and Bcrp were detected as 43, 56, 58, 160, 170 and 60 kDa bands, respectively. As a positive control treatment for up-regulation of Cyp2b1/2 and Cyp3a1, dexamethasone treatment increased the expression levels of Cyp2b1/2 and Cyp3a1, but the treatment reduced expression levels of P-gp and Bcrp.[8,9] As a positive control treatment for down-regulation of P-gp, Mrp2 and Bcrp, methotrexate treatment also reduced expression levels of P-gp, Mrp2 and Bcrp (Fig. 1).[10,11] Expression levels of the protein are described as a percentage of the corresponding water-treated group (Table 3). Acetic acid, black vinegar and moromi treatments showed

Table 2. Biochemical Parameters of Serum and Body Weight after 30 Consecutive Days Treatment in Rats

	Water	Acetic acid	Black vinegar	Moromi
Body weight (g)	359.8 ± 6.4	343.2 ± 6.8	341.3 ± 10.6	342.5 ± 4.6
Total protein (g/dl)	3.35 ± 0.30	3.22 ± 1.08	3.21 ± 0.41	3.49 ± 0.78
Creatinine (mg/dl)	0.69 ± 0.13	0.85 ± 0.23	0.56 ± 0.18	1.05 ± 0.24
ALP (IU/l)	264 ± 38	307 ± 34	355 ± 46	355 ± 39
ALT (IU/l)	30.5 ± 4.5	25.4 ± 3.3	24.7 ± 2.5	18.3 ± 1.3

Each group was treated as follows: water, distilled water was given at a dose of 10 ml/kg; acetic acid, 1.5 ml of 0.23% acetic acid (pH 3.5) was diluted with 8.5 ml distilled water, and the diluted solution was given at a dose of 10 ml/kg; black vinegar, 1.5 ml concentrated black vinegar solution (pH 3.5) was diluted with 8.5 ml distilled water, and the diluted solution was given at a dose of 10 ml/kg; moromi, 50 mg moromi suspended in 10 ml distilled water, and the suspension was given at a dose of 10 ml/kg. Data are the means ± S.E. ($N = 6$).

Table 3. Relative Expression Levels of the Drug Metabolism Enzymes and Transporters after Kurosu Treatment with Consecutive 30-days

	Water	Acetic acid	Black vinegar	Moromi
Actin	100.0 ± 5.1	98.5 ± 7.3	101.5 ± 9.1	116.4 ± 7.3
Cyp1a2	100.0 ± 10.1	116.1 ± 12.5	89.3 ± 9.5	102.6 ± 8.1
Cyp2b1	100.0 ± 4.5	112.9 ± 4.2	112.7 ± 6.2	95.5 ± 2.9
Cyp3a1	100.0 ± 11.8	81.1 ± 7.9	85.8 ± 5.2	89.4 ± 7.0
GST	100.0 ± 16.1	97.3 ± 7.2	108.2 ± 22.5	118.7 ± 12.2
P-gp	100.0 ± 7.7	81.9 ± 6.1	82.7 ± 7.2	79.7 ± 9.6
Mrp2	100.0 ± 7.5	99.1 ± 2.5	85.7 ± 2.5	78.9 ± 3.2
Bcrp	100.0 ± 9.9	90.1 ± 12.6	90.8 ± 6.0	77.2 ± 11.4
Oatp2	100.0 ± 6.8	85.2 ± 6.4	93.7 ± 9.2	83.4 ± 7.2
Oat2	100.0 ± 9.6	80.8 ± 3.1	79.3 ± 10.5	87.7 ± 11.4
Oat3	100.0 ± 7.8	85.2 ± 15.4	90.0 ± 8.0	113.2 ± 22.7

Rats were killed after 30-day treatment, and the preparation of samples for Western blot analysis was similar to that described in Fig. 1. Each group was treated as follows: water, distilled water was given at a dose of 10 ml/kg; acetic acid, 1.5 ml of 0.23% acetic acid (pH 3.5) was diluted with 8.5 ml distilled water, and the diluted solution was given at a dose of 10 ml/kg; black vinegar, 1.5 ml concentrated black vinegar solution (pH 3.5) was diluted with 8.5 ml distilled water, and the diluted solution was given at a dose of 10 ml/kg; moromi, 50 mg moromi suspended in 10 ml distilled water, and the suspension was given at a dose of 10 ml/kg. Values are the mean ± S.E. (percentage) of normalized ODs, compared with vehicles, $N = 6$. There were no significant differences in expression levels. Statistics analysis was performed by ANOVA.

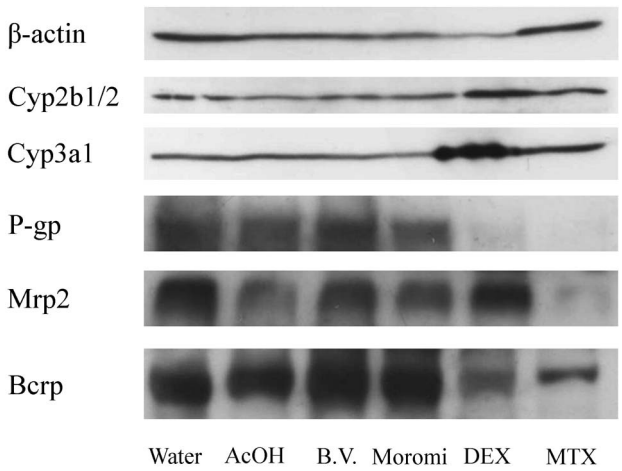

Fig. 1. Effect of Kurosu and Moromi Treatment on Expression Levels of β-actin, Cyp2b1/2, Cyp3a1, P-gp, Mrp2 and Bcrp

Rats were gavaged orally for consecutive 30 days. β-actin, Cyp2b1/2, Cyp3a1, P-gp, Mrp2 and Bcrp were detected as 43, 56, 58, 160, 170 and 60 kDa bands, respectively. As a positive control treatment for up-regulation of Cyp2b1/2 and Cyp3a1, dexamethasone was intraperitoneally injected at a dose of 50 mg/kg for consecutive 4 days. As a positive control treatment for down-regulation of P-gp, Mrp2 and Bcrp, methotrexate was intraperitoneally injected once at a dose of 150 mg/kg. Whole-cell homogenate from the liver was prepared and loaded onto SDS-PAGE. The detailed procedure for Western blot analysis is described in 'Materials and Methods.' AcOH: 0.23% acetic acid, B.V.: black vinegar, DEX: dexametasone, MTX: methotrexate.

no significant difference in the expression levels of the metabolism enzymes and transporters between water treatments (Table 3).

Oguma *et al*. reported that combined administration of kurosu increased maximum concentration of itraconazole in healthy adults with decrease gastric acidity and showed high blood concentration of itraconazole in a pulmonary aspergillosis patient.[13] Iwao *et al*. reported that physicochemical membrane permeability of glibenclamide was reduced in the presence of kurosu.[14] These reports demonstrated that acidic physicochemical property of kurosu may affect absorption of drugs. However, it is not clear whether kurosu induce drug interaction through alteration of drug metabolism enzymes and transporters.

In conclusion, the present study suggest that kurosu from a jar has no toxic effect and does not alter the expression levels of the principal drug metabolism enzymes and transporters.

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