Inchinkoto, a herbal medicine, and its ingredients dually exert Mrp2/MRP2-mediated choleresis and Nrf2-mediated antioxidative action in rat livers

Kosuke Okada,1 Junichi Shoda,1 Masahito Kano,1 Sachiko Suzuki,2 Nobuhiro Ohtake,2 Masahiro Yamamoto,2 Hiroshi Takahashi,3 Hirotoshi Utsunomiya,4 Koji Oda,5 Kimi Sato,6 Ayaka Watanabe,6 Tetsuro Ishii,6 Ken Itoh,7 Masayuki Yamamoto,7 Tsuyoshi Yokoi,8 Katsuosushi Yoshizato,9 Yuichi Sugiyama,10 and Hiroshi Suzuki11

1Department of Gastroenterology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki; 2Pharmacological Department, Central Research Laboratories, Tsumura and Company, Ibaraki; 3Department of Anesthesiology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki; 4Department of Pathology, Wakayama Medical University, Wakayama; 5Division of Surgical Oncology, Department of Surgery, Nagoya University Graduate School of Medicine, Aichi; 6Department of Molecular and Cellular Physiology, Graduate School of Comprehensive Human Sciences, and 7Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Science, University of Tsukuba, Ibaraki; 8Division of Drug Metabolism, Faculty of Pharmaceutical Sciences, Kanazawa University, Ishikawa; 9Department of Biological Science, Developmental Biology Laboratory, Graduate School of Science, Hiroshima University, Hiroshima (Yoshizato Project, Cooperative Link of Unique Science and Technology for Economy Revitalization, Hiroshima Prefectural Institute of Industrial Science and Technology, Hiroshima); and 10Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, and 11Department of Pharmacy, University of Tokyo Hospital, Faculty of Medicine, University of Tokyo, Tokyo, Japan

Okada K, Shoda J, Kano M, Suzuki S, Ohtake N, Yamamoto M, Takahashi H, Utsunomiya H, Oda K, Sato K, Watanabe A, Ishii T, Itoh K, Yamamoto M, Yokoi T, Yoshizato K, Sugiyama Y, Suzuki H. Inchinkoto, a herbal medicine, and its ingredients dually exert Mrp2/MRP2-mediated choleresis and Nrf2-mediated antioxidative action in rat livers. Am J Physiol Gastrointest Liver Physiol 292: G1450–G1463, 2007. First published October 12, 2006; doi:10.1152/ajpgi.00302.2006.—Inchinkoto (ICKT), a herbal medicine, has been recognized in Japan and China as a “magic bullet” for jaundice. To explore potent therapeutic agents for cholestasis, the effects of ICKT or its ingredients on multidrug resistance-associated protein 2 (Mrp2/ MRP2)-mediated choleretic activity, as well as on antioxidative action, were investigated using rats and chimeric mice with livers that were almost completely repopulated with human hepatocytes. Biliary excretion of Mrp2 substrates and the protein mass,acellular localization, and mRNA level of Mrp2 were assessed in rats after 1-wk oral administration of ICKT or genipin, a major ingredient of ICKT. Administration of ICKT or genipin to rats for 7 days increased bile flow and biliary excretion of bilirubin conjugates. Mrp2 protein and mRNA levels and Mrp2 membrane densities in the bile canaliculi and renal proximal tubules were significantly increased in ICKT- or genipin-treated rat livers and kidneys. ICKT and genipin, thereby, accelerated the disposal of intravenously infused bilirubin. The treatment also increased hepatic levels of heme oxygenase-1 and GSH by a nuclear factor-E2-related factor (Nrf2)-dependent mechanism. Similar effects of ICKT on Mrp2 expression levels were observed in humanized livers of chimeric mice. In conclusion, these findings provide the rationale for multidrug resistance-associated proteins; bilirubin; glutathione; heme oxygenase-1; chimeric mice with humanized liver; nuclear factor-E2-related factor MULTIDRUG RESISTANCE-ASSOCIATED PROTEINS (Mrps; also known as ATP-binding cassettes, subfamily C) function as multispecific organic anion transporters of the hepatocellular plasma membrane (10, 21, 27, 31, 37) and mediate the efflux of a wide variety of organic anions, including bilirubin glucuronides, glutathione (GSH), GSH conjugates, and sulfated and glucuronidated bile acids (21, 29, 31). Especially for Mrp2, its mediated excretion of these solutes, i.e., reduced GSH, contributes largely to the bile acid-independent fraction of bile flow (26, 51).

Cholestasis leads to hepatic and systemic accumulation of potentially toxic biliary compounds, resulting in liver damage and jaundice (52). It has been associated with impaired Mrp2-mediated transport (7, 8) as well as downregulation and altered localization of Mrp2 (51). These findings provide the molecular basis for impairment of biliary secretion of a broad range of anionic conjugates and the development of jaundice in human subjects with cholestasis (51). Since intrinsic hepatocellular adaptive induction of transporters in cholestasis is too weak to prevent ongoing liver injury (14, 54), stimulation and restoration of expression and function of Mrp2 may be an important target for specific pharmacotherapeutic interventions (14).

Plants contain abundant bioactive materials. Chinese/Japanese herbal medicines are now manufactured under modern scientific quality controls in Japan and have been ethically administered to a wide range of patients for ~30 yr. Inchinkoto (ICKT), one such medicine, has been recognized as a “magic bullet” for jaundice and has long been used in Japan and China as a choleretic (1) and hepatoprotective (57, 58) agent for various types of liver diseases. Genipin, an intestinal bacterial

Address for reprint requests and other correspondence: J. Shoda, Dept. of Gastroenterology, Graduate School of Comprehensive Human Sciences, Univ. of Tsukuba, 1-1-1 Tennodai, Tsukuba-shi, Ibaraki 305-8575, Japan (e-mail: shodaj@md.tsukuba.ac.jp).

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metabolite of geniposide, a major ingredient of Gardenia fructus in ICKT (2), enhances Mrp2-mediated choleric activity and modulates GSH contents in the liver (1, 43, 47). 6,7-Dimethylesculetin, a major ingredient of Artemisia capillaris spica in ICKT, increases the expression levels of various components involved in bilirubin metabolism in the liver via a constitutive androstane receptor (CAR) (19). Moreover, capillarisin, a major ingredient of A. capillaris spica, exerts potent hepatoprotective action on tert-butylhydroperoxide-induced liver damage by stabilizing the GSH system and quenching free radicals (13).

In this study, we investigated the effects of ICKT and its ingredients on Mrp2/MRP2-mediated choleric activity and renal elimination pathways for bilary constituents as well as on nuclear factor-E2-related factor (Nrf2)-mediated antioxidative actions using rats, chimeric mice with humanized livers, and cultured fibroblasts. The results should contribute to the establishment of new therapeutic strategies aimed at stimulation and restoration of both defective Mrp2/MRP2 expression and function and decreased cytoprotection in various types of cholestatic liver diseases.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 180–220 g were purchased from Nihon SLC (Shizuoka, Japan). Humanized (chimeric) mice developed in the Life Science Research Laboratory of Chugai Technos (Hiroshima, Japan) were used to assess the pharmacological responses of human hepatocytes. The liver of urokinase-type tissue plasminogen activator transgenic severe combine immunodeficient mice treated with an anti-human complement drug were partially repopulated with human hepatocytes as described previously (49). The human hepatocytes in the mouse liver express human liver proteins, including cytochelatin 8/18, various cytochrome P-450 subtypes, and human albumin. Animals were kept under routine laboratory conditions before experiments. They had free access to standard laboratory chow and water. The animal experiments were performed following the guidelines of the Animal Ethics Committee at Tsumura & Co., which were established independently of the research members according to the guidance of the Japanese Society for laboratory animal resources.

Experimental design. ICKT (TJ-135, Tsumura, 2 g·kg⁻¹·day⁻¹) or genipin (100 mg·kg⁻¹·day⁻¹) was suspended in distilled water and orally administered to Sprague-Dawley rats for 7 days. General anesthesia was used in animal experiments. Animals were anesthetized with urethane (1 g/kg body wt ip), and the common bile duct was cannulated with a polyethylene tube (outer diameter 0.6 mm, SP10, Natsume, Tokyo, Japan). From 10 min after the cannulation, bile was collected at 30-min intervals over a period of 90 min. In some experiments, ICKT or geniposide was mixed at a percentage of 1.0 or 0.1 in standard laboratory chow, respectively, and fed to chimeric mice for 7 days.

Biliary analysis. Bile was collected at 30-min intervals into preweighed tubes, and bile flow was calculated by the weight of each specimen. Reduced GSH, total bilirubin, and total bile acid concentrations were determined as previously described (34, 43).

In vivo bilirubin disposal. On the seventh day of treatment, rats were intravenously injected with bilirubin solution (20 mg/kg) via the tail vein or a catheter in the right jugular vein. After the injection, blood and bile were collected over a 120-min period. Urine was collected over a 180-min period using a metabolic cage after an intraperitoneal injection of 5 ml warm saline/rat. Bilirubin solution was used freshly prepared as follows: unconjugated bilirubin (Sigma, St. Louis, MO; 99% purity) was first dissolved in 0.1 N NaOH, adjusted to pH 8.5–9.0 with 1 N HCl, and finally diluted with saline to a concentration of 10 mg/ml. The total bilirubin concentration in plasma, bile, or urine was measured in the same way. Bile and urine were collected into preweighed tubes, and cumulative amounts of bilirubin excretion in bile and urine were calculated by both weight and total bilirubin concentration of each specimen.

Immunoblot analysis. Liver and kidney total homogenates and crude liver membranes were prepared as previously described (11, 41). Transporter protein and antioxidant stress gene product levels in rat livers and kidneys were determined using monoclonal antibodies raised against Mrp2 (M2-III-6, Alexis Biochemicals, Lausen, Switzerland) and P-glycoproteins (C219, Abcam, Cambridge, UK) and polyclonal antibodies raised against Mrp3, Mrp4, bile salt export protein (Bsep), γ-glutamyl cystein synthase (γ-GCS), and heme oxygenase (HO-1) using recently detailed methodology (20, 36, 43). Membranes were reprobed with an antibody raised against actin (Santa Cruz Biotechnology, Santa Cruz, CA) to confirm the specificity of the observed changes in their protein levels. For humanized mice livers, transporter protein levels were determined using monoclonal antibodies raised against MRPI (Alexis Biochemicals) and MDR3 (P-II-J26, Chemicon, Temecula, CA). The specificity of the antibodies was confirmed to distinguish human MRPI and MDR3 from the corresponding murine homologs. Membranes were reprobed with an antibody raised against human cytokeratin (CK)8/18 (Santa Cruz Biotechnology). Blots were treated with an enhanced chemiluminescent (ECL plus) system (Amersham Biosciences, Buckinghamshire, UK) to visualize the antibodies that had bound, followed by scanning using a Typhoon 9410 (Amersham Biosciences). Bands were analyzed using Image Quant TL software (Amersham Biosciences).

Immunohistochemical localization of transporters in the liver and kidney. For experiments on immunohistochemical localizations of Mrp2, Mrp3, and Bsep in rat livers, Mrp2 in rat kidneys, and MRP2, BSEP, and MDR3 in humanized mice livers, liver and kidney specimens (each ~50 mg) were frozen in freon/liquid nitrogen, embedded in OCT compound (Miles, Elkhart, IN), cut into 6-μm-thick sections, and mounted on slides. Tissue sections were immunostained with each polyclonal or monoclonal antibody using recently detailed methodology (36, 43).

Cell cultures. Mouse embryo fibroblasts, prepared from either wild-type mice or Nr2 knockout mice (39), were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS and penicillin-streptomycin (Invitrogen Japan, Tokyo, Japan) at 37°C in 5% CO₂-95% air. Cells were incubated for 5 h with or without 100 μM diethyldiamine (DE), 50 μM genipin, and 50 μM capillarisin.

Real-time quantitative PCR. Total RNA was isolated from tissue specimens and cell pellets of embryo fibroblasts using Isogen (Nippon Gene, Tokyo, Japan). Steady-state mRNA levels in specimens and cell pellets of embryo fibroblasts were determined using Primer Express detailed methodology (36, 43). Primers and probes for hepatocellular plasma membrane transporters and intracellular components involved in bilirubin metabolic pathways were designed using Primer Express (Applied Biosystems) and are shown in Table 1. Data were normalized to the amounts of rRNA present in each specimen and then averaged.

Statistics. Values are given as means ± SE. Means of two groups were compared with an unpaired Student’s t-test or one-way ANOVA, with a post hoc Scheffe’s F-test. Fisher least-significant difference test, or Steel test. P values of <0.05 were defined as statistically significant.

RESULTS

Effects of genipin and ICKT on choleresis and in vivo bilirubin disposal in rats. The bile flow was significantly higher in genipin- and ICKT-treated groups than in the vehicle-treated group (Table 2). In parallel to the increased bile flow, genipin or ICKT treatment resulted in significant increases in both the biliary concentration and rate of secretion of GSH, the
<table>
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<th>Gene</th>
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<th>Reverse Primer</th>
<th>Probe</th>
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<td></td>
<td></td>
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<tr>
<td>Ntcp</td>
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<td>5'-TGCCCTCTGCTGAAGTGAGG-3'</td>
<td>5'-CACTGCGGCAAAAGATGAAAG</td>
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<tr>
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<td>5'-ATCTGGAAACCGTAGGAGACGAA-3'</td>
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**Mouse**

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<td>5'-GCTTGGTGGAAATCGGAATC-3'</td>
<td>5'-GTCGATGGAATCCAC-3'</td>
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*Ntcp, sodium-dependent cholate transporting protein; Oatp, organic anion transporting protein; Mrp, multidrug resistance-associated protein; Bsep, bile salt export protein; Mdr, multidrug resistance; HO, heme oxygenase; γ-GCS, γ-glutamyl cystein synthase; UGT1A1, UDP glucuronosyltransferase 1 family, polypeptide A1; Nrf2, nuclear factor-E2-related factor; CAR, constitutive androstane receptor; AhR, aryl hydrocarbon receptor; PXF, pregnane X receptor. Fluorogenic probes for TaqMan RT-PCR were 5’-FAM and TAMRA-3’ labeled. *Real-time PCR using the SYBR green assay was performed.
Table 2. Comparison of choleretic activities of ICKT and genipin in vivo.

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<th>Total Bilirubin</th>
<th>Bile Acids, Concentration, nmol/min 100 g body wt</th>
<th>Total Bilirubin, Concentration, mmol/l 100 g body wt</th>
<th>Phospholipid, Concentration, mg/dl 100 g body wt</th>
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<td>Vehicle</td>
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<td>2.39 ± 0.16</td>
<td>1.92 ± 0.29</td>
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<td>Genipin</td>
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<td>7.64 ± 0.29</td>
<td>3.67 ± 0.16</td>
<td>2.39 ± 0.29</td>
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<tr>
<td>ICKT</td>
<td></td>
<td>10.22 ± 0.32</td>
<td>4.12 ± 0.16</td>
<td>2.62 ± 0.32</td>
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Values are means ± SE; n = 10 animals/group. Bile was collected over a 30 min period to estimate the choleretic activity and GSH secretion rate. GSH levels of bile were determined as described in M MATERIALS AND METHODS.*

Effects of genipin and ICKT on expression levels of hepatocellular transporters and nuclear receptors in the rat liver.

To clarify the molecular mechanism by which ICKT and its ingredients exhibit potently choleretic activity, expression levels of plasma membrane transporters in liver tissue specimens were determined by analysis of real-time PCR.

For basolateral importing transporters, mRNA levels of organic anion transporting protein (Oatp1), Oatp2, and Oatp4 were significantly increased in ICKT-treated livers compared with levels in vehicle-treated livers, whereas the level of sodium-dependent cholate transporting protein (Ntcp) was unchanged (Fig. 2A). For basolateral membrane exporting transporters, mRNA levels of Mrp1 were significantly increased in ICKT-treated livers and levels of Mrp3 were significantly increased in genipin- and ICKT-treated livers compared with levels in vehicle-treated livers, whereas levels of Mrp4 were unchanged (Fig. 2A). Moreover, for canalicular membrane transporters, mRNA levels of Mrp2 and multidrug resistance 2 (Mdr2; functioning as a phospholipid transporter) were significantly increased in genipin- and ICKT-treated livers compared with levels in vehicle-treated livers, whereas levels of Bsep were unchanged (Fig. 2A).

Based on the results of expression changes in plasma membrane transporters, to further test whether genipin or ICKT regulates classical nuclear receptors and/or transcriptional factors, mRNA levels of pregnane X receptor (PXR), CAR, aryl hydrocarbon receptor (AhR), farnesoid X receptor (FXR), Nrf2, and peroxisome proliferator-activated receptor (PPAR)-α were also determined. There was a significant induction of Nrf2 mRNA in genipin-treated livers (Fig. 2B) and also inductions of Nrf2, CAR, FXR, and AhR mRNAs in ICKT-treated livers (Fig. 2B).

Immunoblot analysis of Mrp2, Mrp3, Mrp4, Bsep, and P-glycoproteins was performed using total homogenates or crude plasma membranes isolated from vehicle-, genipin-, and ICKT-treated rat livers. In parallel to the mRNA levels (Fig. 2A), significant increases in Mrp2 and Mrp3 protein levels were observed in genipin-and ICKT-treated livers compared with levels in vehicle-treated livers (Fig. 3A). Significant increases in protein levels of P-glycoproteins were also observed in ICKT-treated livers. Whereas protein levels of Mrp4 tended to increase in genipin- and ICKT-treated livers, protein levels of Mrp4 and Bsep did not differ significantly (Fig. 3A).

The immunohistochemical localizations of Mrp2, Mrp3, and Bsep in liver tissue sections were studied (Fig. 3B). The immunostaining of Mrp2 was more intensive and more diffuse in the level of which affects “bile acid-independent” choleresis, conjugated bilirubin and phospholipid into the bile (Table 2). Noticeably, the bile flow and excretion rate of these biliary constituents were further potentiated in the ICKT-treated group (Table 2). Following the observations of genipin- or ICKT-induced potent choleresis, rats in each group, which were orally administered for 7 days, were subjected to an acute bilirubin disposal assay by an intravenous infusion of bilirubin. As shown in Fig. 1A, the decay of plasma bilirubin concentrations was suggested to increase in both genipin- and ICKT-treated rats. The plasma concentrations were significantly decreased at 120 min after the injection of bilirubin. On the other hand, both the cumulative amounts of bilirubin excreted into the bile (Fig. 1B) and urine (Fig. 1C) were significantly increased in the drug treatment groups.

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bile canaliculi of genipin- and ICKT-treated liver tissue sections than in those of vehicle-treated liver tissue sections. Moreover, consistent with the increased protein mass of Mrp2 (Fig. 3A), Mrp2 staining was found to be more diffuse in the canaliculi of ICKT-treated liver tissue sections than in genipin-treated liver tissue sections (Fig. 3B). Image analysis of the localization of Mrp2 (Fig. 3C) showed a significant increase in protein levels of Mrp2 in canalicular membranes of genipin-treated liver tissue sections (126 ± 4% of vehicle-treated livers, P < 0.05) and ICKT-treated liver tissue sections (152 ± 4%, P < 0.01).

The immunohistochemical localization of Mrp3 in liver tissue sections showed a remarkable difference between the vehicle-treated group and genipin- or ICKT-treated groups. Weak immunostaining was found mostly in the epithelium of intrahepatic bile ducts, as evidenced by the staining of CK19 as a cholangiocyte marker, whereas intense staining was observed in the bile duct epithelia as well as expanded to hepatocytes surrounding the portal tracts in genipin- and ICKT-treated liver tissue sections (Fig. 3B). The magnitude of these changes was potentiated in ICKT-treated liver tissue sections.

In contrast, the immunostaining of Bsep in genipin- and ICKT-treated liver tissue sections was not different from those of vehicle-treated liver tissue sections (Fig. 3, B and C).

Effects of genipin and ICKT on expression levels of Mrp2, Mrp3, and Mrp4 in the rat kidney. Adaptive induction of renal transporters has been observed in cholestasis, and active renal transport plays a role in the elimination of biliary constituents (50). Therefore, the question of whether Mrp2, Mrp3, and Mrp4 are induced in kidneys by treatment with ICKT and its ingredients should be addressed. As shown in Fig. 4A, expression levels of Mrp2 mRNA and protein were significantly increased in genipin- and ICKT-treated rat kidneys compared with levels in vehicle-treated kidneys. Mrp3 protein levels did not differ significantly. In the case of Mrp4, although its mRNA levels were not different from the levels in vehicle-treated kidneys, protein levels were significantly upregulated in kidneys of the ICKT-treated group.

Immunohistochemical experiments revealed that Mrp2 was expressed on the apical membranes of proximal tubules (Fig. 4C). Consistent with the immunoblot results (Fig. 4B), immunostaining of Mrp2 on the apical membranes was significantly increased in genipin- and ICKT-treated groups compared with the vehicle-treated group.

Effects of genipin and ICKT on expression levels of antioxidative gene products in the rat liver. Supporting previous observations (56), oral administration of genipin or ICKT yielded a significant increase in hepatic GSH levels (Fig. 5A). To elucidate the molecular mechanism responsible for the upregulated GSH synthesis, effects of genipin and ICKT on expression levels of the major rate-limiting factors with respect to GSH synthesis (heavy and light subunits of γ-GCS) as well as antioxidative gene products, HO-1 and UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1), were investigated in genipin- and ICKT-treated livers. mRNA levels of γ-GCS heavy chain, γ-GCS light chain, HO-1, and UGT1A1 and protein levels of γ-GCS and HO-1 were significantly increased in genipin- and ICKT-treated livers (Fig. 5, B and C).

The transcription factor Nrf2 is essential for antioxidant responsive element-mediated gene activation of γ-GCS, HO-1, UGT1A1, and Mrp1, a basolateral membrane efflux transporter for GSH conjugates (16, 17, 22). To investigate the involvement of Nrf2 in the upregulation of GSH synthesis and expression levels of γ-GCS, HO-1, UGT1A1, and Mrp1 in genipin- or ICKT-treated livers, the effects of potent active ingredients of ICKT, genipin and capillarisin, on the expression levels of γ-GCS, HO-1, and Mrp1 were tested using embryo fibroblasts derived from control mice and Nrf2-null mice (Fig. 6). Treatment with 50 μM capillarisin significantly increased mRNA...
and protein levels of γ-GCS, HO-1, and Mrp1 in Nrf2+/+ cells but not in Nrf2−− cells. Treatment with genipin showed effects similar to those of capillarisin but less potent. These results suggest the importance of Nrf2 in the inductive synthesis of antioxidant stress gene products and the upregulation of some ATP binding cassette transporters for cellular detoxication in vivo by treatment with ICKT.

**Effects of geniposide and ICKT on choleresis and expression levels of hepatocellular transporters in chimeric mice with humanized livers.** To investigate the effects of ICKT and its ingredients on human livers, chimeric mice with humanized livers (replacement index of human hepatocytes between 60% and 80%) were fed diets containing geniposide and ICKT for 7 days. Gallbladder sizes of ICKT-treated chimeric mice appeared larger compared with those of vehicle-treated mice, which in turn reflected the potent choleresis induced by ICKT treatment (Fig. 7A). Immunoblot analysis revealed that MRP2 and MDR3 protein levels were significantly increased in human livers of ICKT-treated chimeric mice compared with levels in vehicle-treated mice (Fig. 7B). In the human liver tissue sections that were recognized by immunostaining of CK8/18 (Fig. 7C), MRP2, BSEP, and MDR3 proteins were generally expressed in the bile canaliculi of liver tissue sections. The immunostainings of MRP2 and MDR3 were more intensive and more diffuse in the bile canaliculi of geniposide- and ICKT-treated liver tissue sections than in those of vehicle-treated liver tissue sections (Fig. 7D). Image analysis of the localization of MRP2 and MDR3 (Fig. 7E) showed significant increases in protein levels of MRP2 and MDR3 in the canalicular membranes of geniposide-treated liver tissue sections (138 ± 7% and 146 ± 6% of vehicle-treated livers, respectively) and ICKT-treated liver tissue sections (169 ± 5% and

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Fig. 2. Steady-state mRNA levels of plasma membrane transporters (A) and classical nuclear receptors (B) in livers of vehicle-, genipin-, and ICKT-treated rat groups. Amounts of transporter and nuclear receptor mRNAs were normalized to those of rRNA in each specimen and then averaged. Oatp, organic anion transporting protein; Ntcp, sodium-dependent cholate transporting protein; Mrp, multidrug resistance-associated protein; Bsep, bile salt export protein; Mdr, multidrug resistance; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; FXR, farnesoid X receptor; Nrf, nuclear factor-E2-related factor; PPAR-α, peroxisome proliferator-activated receptor-α; PXR, pregnane X receptor. Data are given as means ± SE; numbers in parentheses are numbers of animals in each group. *P < 0.05 and **P < 0.01, significantly different from the vehicle-treated group.

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G1455 CHOLERESIS AND ANTIOXIDATIVE ACTION BY INCHINKOTO

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The results were quite similar to those obtained for ICKT-treated rats (Fig. 3, B and C).

**DISCUSSION**

Various hormones and drugs upregulate Mrp2 expression levels and stimulate Mrp2-dependent organic anion transport (38). Besides these hormones and drugs, both the results of a recent study (43) on the effects of a single intravenous administration and those obtained in this study on the effects of a long-term oral administration demonstrate that the herbal medicine ICKT or its ingredients enhance Mrp2-mediated choleresis and bilirubin disposal in vivo.
Because even Mrp2-deficient rats such as transport-deficient and Eisai hyperbilirubinemic rats and MRP2-deficient subjects such as those with Dubin-Johnson syndrome do not have severe cholestasis, loss or decreased function of Mrp2/MRP2 is not the major initiating mechanism of cholestasis in acquired hepatobiliary diseases. However, several lines of findings have strongly suggested that Mrp2/MRP2 dysfunction may give serious influences to pathogenesis, progression, and symptoms of cholestatic liver diseases: 1) Mrp2/MRP2 transports, in addition to bilirubin and glutathione, a wide variety of organic anions such as conjugates of leukotrienes, estradiol, and taurolithocholate, whose excretion is very important for the homeostatic regulation of various biological processes; and 2) Mrp2/MRP2 is a major transporter of GSH, which is extraordinarily important for cellular protection of hepatobiliary organs. The maintenance of relevant GSH levels in the liver and bile duct is critical for hepatobiliary function. The depletion of GSH in the liver and bile duct epithelial cells has been known to induce severe liver injury and cholestasis (23, 24); and 3) GSH is the main driving force of bile acid-independent bile formation, which occupies a large part of bile formation, and therefore the dysfunction of Mrp2/MRP2 results in the loss of bile acid-independent bile formation. The lack of bile acid-independent bile formation may not induce severe cholestasis, at least in naive animals. However, in the situation of increased endobiotic and/or xenobiotic loading of Mrp2/MRP2-specific
substrates, Mrp2/MRP2 dysfunction may cause or worsen cholestasis. In accordance with the above discussion, experimental and clinical cholestasis have been associated with impaired Mrp2/MRP2-mediated transport as well as the downregulation and altered localization of Mrp2/MRP2 (8, 42, 55, 57).

Moreover, in cholestasis, while adaptive changes in hepatobiliary transporter and metabolizing enzyme expression are induced, this intrinsic compensation is ineffective in fully preventing cholestatic liver injury (14, 54). Therefore, the findings of this study provide a good rationale for pharmacological interference with bilirubin transport and metabolism via modulations of transport systems in livers and extrahepatic tissues.

Choleretic actions of ICKT and its ingredients. Oral administration of ICKT or genipin results in a potent choleresis in rats (Table 2) through selective upregulation of protein levels of Mrp2 in the liver (Fig. 3A). Analogous to liver tissue sections of rats subjected to acute administration of genipin (43), the density of Mrp2 in the bile canaliculi was significantly increased and the area of bile canaliculi showing immunostaining of Mrp2 was also significantly expanded in liver tissue sections of ICKT- and genipin-treated rats (Fig. 3B). At steady state, the rate of bilirubin excretion is supposed to equal the rate of heme breakdown. Therefore, the increase in the steady-state rate of biliary bilirubin excretion in genipin- and ICKT-treated rats may represent increased heme breakdown, which is compatible with the present data on the upregulation of HO-1 in the liver (Fig. 5B). However, because ICKT and genipin treatment both strongly reduced blood bilirubin to undetectable levels (data not shown), it is certain that the excretory capacity of bilirubin into bile itself is also enhanced in the rats. The results of this study, collectively, appear to indicate that there is an increased bilirubin production but the augmented excretory capacity is more than sufficient to counteract the hyperbilirubinemic effect in the rats, so that the net result is decreased plasma concentrations and increased biliary excretion.
Selective upregulation of Mrp2 levels was also observed in kidneys of genipin- and ICKT-treated rats (Fig. 4), and the immunostaining of Mrp2 was enhanced on the apical membranes of proximal tubules of the kidneys (Fig. 4). Therefore, the increased Mrp2 expression in both liver and kidneys may contribute to an enhanced disposal of bilirubin in vivo (Fig. 1).

In terms of synthesis and trafficking of Mrp2 in hepatocytes, the results of this study do not well clarify the mechanisms by which genipin or ICKT enhance the distribution of Mrp2 protein to the bile canaliculi and increase Mrp2 transcription and protein levels in the liver. Nuclear receptors and/or a transcriptional factor, i.e., Nrf2, play an important role in the transcriptional regulation of various transporters (12, 25). PXR and CAR in rodents or steroid X receptor (SXR) in humans are activators of Mrp2 genes (4). ICKT significantly increased expression levels of AhR, CAR, FXR, and Nrf2 mRNAs, and genipin significantly increased Nrf2 mRNA levels. These results are similar to the results of a recent study (32) showing that AhR, CAR, and Nrf2 activators potently induce Mrp2 transcription in mouse livers. Posttranslational regulations, e.g., the membrane targeting of ATP binding cassette transporters, are mediated via classical second messengers (4). However, we did not find any significant changes in hepatic levels of classical second messengers after genipin or ICKT treatment in rats (cAMP, Ca2+, and PKC).

Moreover, it should be noted that the protein levels of inducible transporters for organic anions and bile acids, Mrp3 and Mrp4, were increased in livers and kidneys of genipin- or ICKT-treated rats. Potent induction of Mrp3 and Mrp4 proteins in rat livers with obstructive cholestasis in the setting of decreased expression and function of Mrp2 had been interpreted as an attempt to minimize cytotoxicities to the hepatobiliary system as cholestatic liver injury progresses (18, 28, 44). As investigated by recent studies on the effects of obstructive cholestasis in mice with targeted disruption of Mrp3 (6, 59) and those with targeted disruption of Mrp4 (35), hepatic Mrp3 is the preferential efflux pump for bilirubin conjugates, whereas Mrp4 is the preferential pump for bile acid conjugates. Both Mrp3 and Mrp4 play a protective role in the adaptive response to obstructive cholestatic liver injury. However, in this study, hepatic Mrp3 and renal Mrp4 were markedly upregulated in ICKT-treated rats with upregulation of Mrp2 expression and function. Although little is known about the mechanism by which changes in the expression levels of Mrp3 and Mrp4 occur, some nuclear receptors and/or transcriptional factors may regulate transcriptional levels of Mrp3 and Mrp4 as recently reported in mouse livers (32). Further studies are required to determine whether the upregulation of Mrp3 and Mrp4 contributes to the accelerated bilirubin disposal in vivo.

In addition, the effects of genipin and ICKT on the hepatic metabolism of bile acid and phospholipid should also be considered. For bile acid, the rate of biliary excretion of bile acids was significantly increased in genipin- and ICKT-treated rats (Table 2), but mRNA, protein, and immunohistochemical
expression levels of Bsep were unchanged between drug- and vehicle-treated groups (Figs. 2 and 3). The details for the mechanism underlying the increased rate of biliary secretion of bile acids remain unknown. On the other hand, for phospholipid, the significant increase in the rate of excretion of phospholipid and in mRNA levels of Mdr2 (Table 2 and Fig. 2) imply that Mdr2 is upregulated in the liver. Furthermore, experiments using chimeric mice were performed to study the effects of geniposide (which is metabolized to genipin by intestinal bacteria) and ICKT on the expression levels and function of human MRP2. Reflecting the induction of potent choleresis, the size of the gallbladder was found to be larger in ICKT-treated mice than in vehicle-treated mice (Fig. 7A). Analogous to rats, expression levels of MRP2 protein were found to be significantly upregulated in humanized livers of ICKT-treated mice. In liver tissue sections, the immunostaining of MRP2 was more intensive and more diffuse in the bile canaliculi of geniposide- and ICKT-treated mice (Fig. 7D). These findings indicate that ICKT exerts potent MRP2-mediated choleric activity in human livers.

Antioxidative actions of ICKT and its ingredients. A study (56) has demonstrated cytoprotective effects of ICKT and its ingredients on liver and bile duct injuries possibly through the stabilizing generation system of GSH, an important endogenous antioxidant. /H9251-Naphtylisocianate (ANIT) selectively injures bile duct epithelia (15) partly because of GSH depletion in the epithelia (5), and an ameliorating effect of ICKT on ANIT-induced cholestasis has already been demonstrated (48). Thus, the intrinsic induction of GSH-mediated antioxidant defense may be potentiated by treatment with either genipin or ICKT.

The molecular mechanisms by which ICKT and its ingredients upregulate GSH synthesis (Fig. 5A) have not yet been well elucidated. The major rate-limiting factors with respect to GSH synthesis are the activity of /H9253-cystathionase, which determines cystine availability, and the activity of /H9253-GCS, which has
regulatory (light) and catalytic (heavy) subunits (30, 53). As revealed by both the analysis of real-time PCR (Fig. 5B) and the immunoblot analysis (Fig. 5C), genipin and ICKT treatment led to increased expression of the mRNAs encoding γ-GCS (both the catalytic and regulatory subunits) and the protein levels.

In addition to GSH, it should be noted that genipin and ICKT upregulate mRNA and protein levels of HO-1 (Figs. 5, B and C), the rate-limiting enzyme that catalyzes the conversion of heme into biliverdin/bilirubin (33). Overexpression of HO-1 protects hepatocytes from inflammation-related apoptotic liver damage in mice (40). Moreover, the products of heme degradation, e.g., biliverdin/bilirubin and carbon monoxide (CO), exert hepatoprotective effects. Biliverdin/bilirubin represents a physiologically important defense against reactive oxygen species (45). In terms of the hepatic microcirculation, CO is important to maintain ample blood supply in sinusoids through a modulatory action on sinusoidal relaxation in the liver (46). In steatotic rat livers subjected to cold ischemia-reperfusion injury, upregulation of HO-1 improved both portal venous flow and bile formation and also decreased hepatocyte injury (3).

Nrf2 regulates expression levels of the antioxidative gene products γ-GCS and HO-1 and the transporter Mrp1 (20, 16). Genipin and capillarisin, other ingredients of ICKT showing potent choleretic and hepatoprotective effects (56), coordinately upregulated mRNA levels of γ-GCS, HO-1, and Mrp1 in embryo fibroblasts but not in corresponding cells with targeted disruption of Nrf2 (Fig. 6). It is therefore likely that ICKT and its ingredients exert hepatoprotective actions by a Nrf2-dependent mechanism. Moreover, since Nrf2 activators induce Mrp2 and other Mrp members in the mouse liver (32), further experiments are required to determine whether ICKT and its ingredients induce Nrf2-mediated coordinated modulation of expressions of plasma membrane transporters in terms of the induction of choleresis and/or detoxification as well as antioxidative actions.

In summary, the results of this study suggest that 1) ICKT and its ingredients enhance the secretory capacity of organic anions of rat livers and kidneys, mainly by both upregulation of Mrp2 gene transcription/protein synthesis (Fig. 8) and stimulation of the redistribution into the bile canaliculi or apical membranes of proximal tubules (this effect on Mrp2 in livers and kidneys may contribute to the acceleration of bilirubin disposal in vivo); 2) the effects of ICKT on MRPS can be traced in humanized livers of chimeric mice; and 3) ICKT and its ingredients upregulate the synthesis of GSH and HO-1 in the liver, probably by a Nrf2-dependent mechanism. The dual properties of ICKT and its ingredients, such as choleresis and antioxidative action, encourage fur-
ther research to establish the rationale for therapeutic options in cholestatic hepatobiliary diseases associated with intractable jaundice (9).

REFERENCES


