3-Monoglucuronyl-glycyrrhetinic acid is a substrate of organic anion transporters expressed in tubular epithelial cells and plays important roles in licorice-induced pseudoaldosteronism by inhibiting 11β-hydroxysteroid dehydrogenase 2

Toshiaki Makino, Kyoko Okajima, Rie Uebayashi, Nobuhiro Ohtake, Katsuhisa Inoue, Hajime Mizukami

Department of Pharmacognosy (T.M., K.O., R.U., H.M.), Department of Pharmacokinetics (K.I.), Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan; Tsumura Research Laboratories, Tsumura Co. Ltd., Japan (N.O.); Department of Pharmacy, Fujita Health University Hospital, Toyoake, Japan (K.O.); Department of Pharmacy, Mie University Hospital, Mie, Japan (R.U.)
Running title: Metabolite of glycyrrhetinic acid and pseudoaldosteronism

Corresponding Author: Toshiaki Makino, Ph. D., Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Mizuho-ku, Nagoya 467-8603, Japan.  E-mail: makino@phar.nagoya-cu.ac.jp. Tel. & Fax: +81 52 836 3416

Text pages: 28
Tables: 0
Figures: 8
References: 26
Abstract: 249 words
Introduction: 407 words
Discussion: 1,195 words

ABBREVIATIONS: BSA, bovine serum albumin; CMC, carboxymethylcellulose; DMSO, dimethyl sulfoxide; EHB, Eisai hyperbilirubinemic rat; ES, estrone sulfate; FBS, fetal bovine serum; GA, glycyrrhetinic acid; GL, glycyrrhizin; HBSS, Hank’s balanced salt solution; 3MGA, 3-monoglucuronyl-glycyrrhetinic acid; Mrp, multi-drug resistance-associated protein; OAT, organic anion transpoter; OATP, organic anion transpoting peptide; PBS; phosphate-buffered saline; PAH, p-aminhippuric acid; PHB, p-hydroxybenzoic acid n-butyl ester; 11β-HSD, 11β-hydroxysteroid dehydrogenase

Recommended section: Metabolism, Transport, and Pharmacogenomics
ABSTRACT

Licorice (glycyrrhiza root) has been used as a herbal medicine worldwide with its main active constituent being glycyrrhizin (GL). Licorice sometimes causes adverse effects such as inducing pseudoaldosteronism by inhibiting type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2) caused by glycyrrhetinic acid (GA), a major metabolite of glycyrrhizin (GL). In this study we compared inhibitory effects of GA, GL, and 3-monoglucuronyl-glycyrrhetinic acid (3MGA), another metabolite of GL, on 11β-HSD2 activity using microsomes and rat kidney tissue slices. GA, 3MGA, and GL inhibited 11β-HSD2 in rat kidney microsomes, with an IC50 of 0.32 µM, 0.26 µM, and 2.2 µM, respectively. However, the inhibitory activity of these compounds was reduced markedly, in the slices, in a medium containing 5% bovine serum albumin (BSA). Assays using HEK293 cells with transient transformation in transporter genes showed that 3MGA is a substrate of human organic anion transporter (OAT) 1, hOAT3, and human organic anion transporting peptide (OATP) 4C1, whereas GA is not. When GA (100 mg/kg/day) was administered orally for 16 days to Eisai hyperbilirubinuria rats (EHBRs), plasma concentrations and urinary excretion of 3MGA were significantly higher, while the activity of 11β-HSD2 in kidney microsomes was significantly lower compared with SD rats. These results suggest that 3MGA is actively transported into tubules through organic anion transporters, resulting in inhibition of 11β-HSD2. As the plasma level of 3MGA is dependent on the function of hepatic transporters, monitoring of 3MGA levels in plasma or urine may be useful for preventing pseudoaldosteronism.
when licorice or GL is prescribed to patients.
Introduction

Glycyrrhizin (GL) is a glycoside of glycyrrhetinic acid (GA) and two molecules of glucuronic acid (Fig. 1), and is one of the active ingredients of licorice. Licorice is obtained from the roots of *Glycyrrhiza glabra* or *G. uralensis* and is prescribed frequently in herbal formulas of Japanese traditional kampo medicine to treat a variety of diseases. GL is also used as a chemical drug to treat chronic hepatitis, gastric ulcers, and allergic diseases in Japan and Europe (Arase et al., 1997; van Rossum et al., 1998; Kumada, 2002), and as a natural sweetener in various foods.

It is well known that licorice and products containing GL sometimes induce adverse effects such as peripheral edema, hypokalemia, and hypertension, named licorice-induced pseudoaldosteronism (Conn et al., 1968). This condition is considered to result from GA, a major metabolite of GL, inhibiting type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2) in renal tubular epithelial cells, resulting in an elevation in cortisol levels, a potent agonist of mineralocorticoid receptors, leading ultimately to increased sodium retention and potassium excretion (Stewart et al., 1987).

In our previous report, we showed that damage in the liver to multidrug resistant-association protein (Mrp) 2, a transporter involved in bile excretion of GL and its metabolites, led to an increase in plasma and urine concentrations of 3-monoglucuronyl-glycyrrhetinic acid (3MGA), another metabolite of GL (Fig. 1). In contrast, urinary excretion of GA was very low regardless of its high plasma concentration when GL was administered orally to rats (Makino et al., 2008). In human beings, GA is also excreted at very
low levels in the urine (Ploeger et al., 2001). Another study reported that the plasma concentration of 3MGA was significantly higher after treatment with GL for longer than 4 weeks in patients with chronic hepatitis and hypokalemia than those with normal potassium levels. However, the plasma GA concentration did not differ between the two groups (Kato et al., 1995). These results attracted attention on the possible role of 3MGA in the licorice-induced pseudoaldosteronism.

In the present investigation, we compared the inhibitory effects of GL, 3MGA, and GA on 11β-HSD2 activity in vitro using rat kidney microsomes and in situ using rat kidney slices. We also examined uptake of GA and 3MGA in rat kidney slices. Finally, we evaluated uptake of GA and 3MGA in cells expressing human anion transporters. We show here that 3MGA plays a crucial role by inhibiting 11β-HSD2 in rat renal epithelial cells, whereas GA does not.
Materials and Methods

Animals. Wistar rats, SD rats, and Eisai hyperbilirubinuria rats (EHBRs) were purchased from Japan SLC (Hamamatsu, Japan). EHBRs are known to express dysfunctional Mrp2 protein as a result of a point mutation in the open reading frame (Ito et al., 1997). The animals received food and water ad libitum under controlled temperature (25°C), humidity, and lighting (12 h light, 12 h dark) conditions. The experimental procedures were approved by the Animal Care Committee at the Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, in accordance with the guidelines of the Japanese Council on Animal Care.

Reagents. GL was purchased from Calbiochem (San Diego, CA, USA), and GA from Tokyo Kasei Kogyo (Tokyo, Japan). 3MGA, cortisone, cortisol, dexamethasone, p-hydroxybenzoic acid n-butyl ester (PHB), carboxymethylcellulose (CMC), penicillin, and streptomycin were purchased from Nacalai tesque (Kyoto, Japan). Subtilisin, Dulbecco’s modified Eagle’s medium, poly-L-lysine, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), human kidney total RNA, pCI-neo mammalian expression vector, Hilly Max reagent with a cationic liposome, and [glycyl-1-14C] p-aminohippuric acid (PAH, 55 mCi/mm mol) were supplied by Invitrogen (Carlsbad, CA, USA), Clontech Laboratories (Mountain View, CA, USA), Promega (Madison, WI, USA), Dojindo Laboratories (Kumamoto, Japan), and American Radiolabeled Chemicals (St. Louis, MO, USA), respectively. [1,2,6,7-3H] Cortisol (79.3 Ci/mmol) and [6,7-3H] estrone sulfate (ES, 45.6 Ci/mmol) were obtained from Perkin Elmer (Waltham, MA, USA). All other chemicals were analytical grade or the highest grade available.
**Protein Assay.** Protein concentrations in various samples were determined using the BCA™ Protein Assay kit (Thermo Scientific, Rockford, IL, USA) with BSA as the calibration standard.

**Determination of *in vitro* 11β-HSD2 Activity Using Rat Kidney Microsomes.** Assays were conducted as described by Diederich et al. (2000) with slight modifications. Male Wistar rats (7-week-old) were sacrificed using CO₂ inhalation and their kidneys were collected. After removal of the capsule, the kidneys were homogenized in 50 mM Tris-HCl buffer (pH 7.8) containing 0.25 mM sucrose and 1 mM MgCl₂. The homogenate was then centrifuged (10,000 × g) at 4°C for 10 min, followed by further centrifugation of the supernatant at 40,000 × g at 4°C for 60 min. The pellet was suspended in a solution containing 50 mM Tris-HCl buffer:glycerol, 2:1 to prepare a kidney microsome fraction. The microsome fraction (1.0 mg protein) was then incubated at 37°C for 30 min in a reaction mixture containing 50 nM [1,2,6,7-³H] cortisol, 1 mM NAD⁺, and 0–12 µM of either GL, 3MGA, or GA in 250 µl of 0.1 M phosphate buffer (pH 6.0). The reaction was terminated by adding 250 µl ethanol containing 1.25 mg cortisol and 1.25 mg cortisone. A 10 µl aliquot of the solution was spotted onto a silica gel 60 F₂₅₄ plate (Merek, Whitehouse Station, NJ, USA) and was developed with chloroform:methanol (9:1). Spots corresponding to cortisone (Rᵢ value, 0.43) were detected under UV light, scraped into a scintillation vial and the radioactivity was measured using a liquid scintillation counter (Hitachi Aloka Medical, Tokyo, Japan).

**Determination of *in situ* 11β-HSD2 Activity Using Rat Kidney Slices.** Male Wistar rats (9-week-old) were sacrificed using CO₂ inhalation, followed by collection of their kidneys.
After removal of the capsule, the kidneys were sliced at approximately 0.5 mm thickness in the frontal section of the organ, using a tissue slicer (Natsume Seisakusyo, Tokyo, Japan) and then cut at the renal hilus into two halves. The slices were pre-incubated at 37°C for 20 min in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) with 0.5 ml of the incubation medium (120 mM NaCl, 16.2 mM KCl, 1 mM CaCl₂, 1.2 mM MgSO₄, 10 mM NaH₂PO₄/Na₂HPO₄, pH 7.4) with or without 5% BSA. The 3MGA and GA solutions were prepared by dissolving the compounds in incubation medium containing 5% BSA or by preparing dimethyl sulfoxide (DMSO) solutions of 3MGA and GA (100 mM) diluted in the incubation medium. After removal of the pre-incubation medium, the slices were incubated at 37°C for 2 hr with 0.5 ml of medium containing 3MGA and GA with or without 5% BSA. The medium was then replaced with 0.5 ml of medium containing 50 µM cortisol in the same composition and the slices incubated further at 37°C for 30 min. A 100 µl aliquot of the medium was transferred into a tube containing 300 µl of dexamethasone solution (30 µg/ml in ethanol containing 0.5% acetic acid), and stored at −20°C until analysis. Cortisone concentrations were measured using the LC/MS/MS system described below. The kidney slices were washed three times with 2 ml of ice-cold 0.15 mM phosphate buffered saline (PBS; pH 7.2) and then homogenized with 600 µl of PBS by sonication. The homogenate was centrifuged at 14,000 × g for 5 min and the protein concentration determined. The activity of 11β-HSD2 in the kidney slices was calibrated by the protein content of the slices. We confirmed that the linear trend in 11β-HSD2 activity under these experimental conditions was dependent on incubation time and the amount of kidney tissue protein.
Uptake of 3MGA and GA by Rat Kidney Slices. Rat kidney slices were pre-incubated at either 4°C or 37°C for 15 min in 24-well plates with 0.5 ml of the incubation medium containing 5% BSA. 3MGA and GA were dissolved in this incubation medium at a concentration of 4 μM. After removal of the pre-incubation medium 0.5 ml of medium containing 3MGA or GA, either pre-cooled on ice, or pre-warmed at 37°C, were added to each well, followed by incubation of the slices at 4°C or 37°C for 30, 60, and 120 min. After removal of the medium, the slices were washed three times with 2 ml ice-cold PBS and homogenized with 600 μl PBS by sonication. The homogenate was centrifuged at 14,000 × g for 5 min and a 100 μl of aliquot of the supernatant then mixed with 20 μl of subtilisin (9.1 U/ml), and incubated at 37°C for 30 min. A 300 μl aliquot of the PHB solution (3 μg/ml in ethanol containing 0.5% acetic acid) was added to the supernatant to stop protein digestion, and stored at −20°C until analysis. The concentrations of 3MGA and GA in the samples were measured using a LC/MS/MS system. The protein concentration of the supernatant was measured, and uptake of 3MGA and GA calibrated against the protein content of the slices.

Uptake of 3MGA and GA by Cells Expressing Organic Anion Transporters. Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO₂. For the transport assay, HEK293 cells were seeded in poly-L-lysine-coated 24-well plates (1.5 × 10⁵ cells/well), and incubated for 24 hr. cDNAs encoding human organic acid transporter (OAT) 1 and OAT3 inserted into pGH19 were generously supplied by Prof. Mitsuru Sugawara, Hokkaido University (Sapporo, Japan), and then subcloned into a pCI-neo mammalian
expression vector. The open reading frame coding for human organic acid transporting peptide (OATP) 4C1 was amplified from human kidney total RNA by an RT-PCR method using PCR primers based on sequences in the DDBJ/EBI/GenBank DNA databases under accession number AB555731, and then subcloned into a pCI-neo mammalian expression vector. The constructs were transfected into HEK293 cells using Hilly Max reagent and a cationic liposome according to the manufacturer’s protocol, and the cells were incubated for 24 hr. The functional expressions of transporter genes were confirmed by estimating the uptake of PAH for OAT1 (Hosoyamada et al., 1999), and ES for OAT3 (Cha et al., 2001), and OATP4C1 (Yamaguchi et al., 2010). The transfected cells were pre-incubated for 15 min at 37°C and 5% CO₂ with 0.25 ml of Hanks’ balanced salt solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 5.5 mM glucose, 4.2 mM NaHCO₃, pH 7.4). DMSO solutions of 3MGA and GA (100 mM) were diluted with HBSS to prepare sample solutions. The cells were then incubated further at 37°C with 0.25 ml of the solutions. The surface of the cells were washed 3 times with 0.5 ml of ice-cold PBS and then extracted with 100 µl of EtOH:AcOH (99.5:0.5) containing PHB (1 µg/ml) at room temperature for 10 min. A 60 µl aliquot of the extract was transferred to a tube containing 60 µl water and the concentration of 3MGA and GA in the extract measured using a LC/MS/MS system. The residual cells were lysed with 0.1 ml of 0.1 M NaOH, and after neutralization with 1 M HCl, the protein contents of the lysate were measured. The uptake of 3MGA and GA by the cells was calibrated against the protein content of the slices.

**Oral Administration of GA in Rats.** Male SD rats or EHBFRs (9-week-old) were
anesthetized by an intraperitoneal injection of urethane (Sigma, 1 g/kg) and their jugular veins were exposed. GA suspended in 0.5% CMC was then administered orally (50 mg/kg) to the unconscious rats and blood samples were collected from the jugular vein at an appropriate time over 12-h period. In another experiment, GA was suspended in 0.5% CMC and administered orally to SD rats and EHBRs (7-week-old), twice a day for 16 days (100 mg/kg/day). Both the control groups of SD rats or EHBRs were administered 0.5% CMC instead of GA. A 300 µl aliquot of blood was collected from the tail vein just before and 3, 7, and 11 days after the start of GA administration. Twenty-four hour urine samples were collected from day 15 to day 16 using metabolic cages. On day 16, 12 hr after the final oral administration of GA, the rats were sacrificed, and blood and kidneys collected. The plasma potassium and urinary sodium concentrations were measured using an ion-selective electrode-based electrolyte analyzer (Nagahama Life Science Laboratory, Nagahama, Japan). Aliquots (10 µl) of plasma or urine were mixed with 20 µl of subtilisin (0.91 U/ml), incubated at 37°C for 30 min, followed by the addition of 70 µl of PHB solution (3 µg/ml in ethanol containing 0.5% acetic acid) and storage at −20°C until analysis. The concentration of 3MGA and GA in plasma or urine was measured using a LC/MS/MS system. The activity of 11β-HSD2 in the kidney microsomes was measured radiometrically.

**LC/MS/MS Analysis.** Concentrations of 3MGA, GA, and cortisone were measured using a LC/MS/MS system (Quattro Premier XE, Waters, Milford, MS, USA). The mass spectrometer used an electrospray ionization source in the positive ion mode with multiple reaction monitoring. The analytical column was an Inertsil ODS-3, 2.1 i.d. × 100 mm, 3 µm
(GL Sciences Inc., Tokyo, Japan). The mobile phase was delivered using a linear gradient elution system, 0.5% AcOH (A): acetonitrile containing 0.5% AcOH (B), at a flow rate of 0.2 ml/min, with the following gradient profile: 48% B (0 to 1 min), increasing from 48% to 80% (1 to 2 min), increasing from 80% to 98% (2 to 5 min), and 98% (5 to 6.5 min). Isocratic elution using 48% B at a flow rate of 0.2 ml/min was used for cortisone and dexamethasone. The transitions (precursor to daughter) monitored and retention times were 647.4 to 453.5 m/z for 3MGA (3.8 min), 471.3 to 91.0 m/z for GA (5.8 min), 195.2 to 139.0 m/z for PHB (3.9 min), 361.3 to 163.2 m/z for cortisone (2.4 min), and 393.2 to 373.5 m/z for dexamethasone (3.4 min).

PHB was used as an internal standard for the measurement of 3MGA and GA, and dexamethasone for cortisone. Standard samples for 3MGA, GA, and cortisone were prepared in rat plasma, urine, kidney homogenate, or water depending on the target samples. Linear regression over the concentration range 6.4 nM–50 µM for 3MGA and GA, and 0.08–10 µM for cortisone was examined using the peak-area ratio of the compounds to their internal standards and the least-squares method ($r^2 > 0.98$).

**Statistics.** Statistical analysis of the data included repeated one-way analysis of variance (ANOVA) and Bonferroni-type multiple $t$-test for comparison of multiple groups for multiple data, and Student’s $t$-test for comparison of two independent groups. The analyses were conducted using the Java program presented on http://chiryo.phar.nagoya-cu.ac.jp/javastat/Bonferro-j.htm. A probability value of less than 0.05 was considered to be statistically significant.
Results

Inhibitory Effects of GL, 3MGA, and GA on 11β-HSD2 Activity in Rat Kidney Microsomes. Comparison of inhibitory effects of GA, 3MGA, and GA on 11β-HSD2 activity in rat kidney microsomes showed all three compounds caused inhibition in a dose-dependent manner (Fig. 2). The IC$_{50}$ values were 2.2 µM for GL, 0.26 µM for 3MGA, and 0.32 µM for GA.

Inhibitory Effects of GL, 3MGA, and GA on 11β-HSD2 Activities in Rat Kidney Slices. Inhibitory effects of GL, 3MGA, and GA on 11β-HSD2 activity in situ were estimated by measuring the formation of cortisone from exogenous cortisol in rat kidney slices. When the slices were pre-incubated with 4 µM of GA for 2 hr in albumin-free condition, the production of cortisone from cortisol decreased to approximately 6% of that measured in control medium. Pre-incubation of the slices with 4 µM of 3MGA and GL caused a small and insignificant decrease in cortisone production, to approximately 75% of the level seen in controls (Fig. 3A). In contrast, neither GL, 3MGA, or GA at concentrations up to 20 µM had an inhibitory effect on 11β-HSD2 activity in slices incubated with 5% BSA (data not shown). At a concentration of 100 µM, both 3MGA and GA, but not GL, caused a small decrease in cortisone production to approximately 80% of that measured in controls (Fig. 3B).

Uptake of 3MGA and GA by Rat Kidney Slices. The uptake of 3MGA and GA by rat kidney slices was monitored in the presence of BSA. When incubated at 4°C, the amount of 3MGA in the slices increased for 60 min and then reached a plateau, while at 37°C, the amount of 3MGA tended to be continued to increase for 120 min (Fig. 4A). In contrast, the amount of
GA in kidney slices reached a maximum regardless of the incubation temperature, with uptake profiles at 37°C and 4°C completely overlapping (Fig. 4B).

**Uptake of 3MGA and GA by Cells Expressing Organic Anion Transporters.** HEK293 cells were transfected with a plasmid containing the open reading frame of OAT1, OAT3, or OATP4C1, and the expressions of these transporters were functionally confirmed by estimating the uptakes of their substrates (Fig. 5A, C, and E). The uptake of 3MGA by HEK293 cells transiently expressing OAT1, OAT3, or OATP4C1 was significantly greater than in mock-transfected cells (Fig. 5B, D, and F, Fig. 6A). However, the uptake profile of GA by cells expressing the transporters was overlapped to that in mock cells (Fig. 6B). Figure 6C shows the concentration-dependency of 3MGA uptake by HEK293 cells expressing organic anion transporters. The initial velocity data were visualized by Eadie-Hofstee plots (Fig. 6D), with the apparent $K_m$ values (mean ± S.E. of triplicate measurements) of 3MGA being 49.0 ± 18.3 µM for OAT1, 30.1 ± 2.9 µM for OAT3, and 41.8 ± 4.8 µM for OATP4C1.

**Effects of GA and Its Metabolites on 11β-HSD2 in Rats.** GA was administered orally at a dosage of 50 mg/kg to SD rats or EHBRs under anesthesia, and changes in plasma concentrations of GA and 3MGA then compared. In SD rats, the plasma GA concentration reached a peak at 90 min and was almost eliminated 12 hr after administration. 3MGA was not detected in the SD rats (Fig. 7A). In EHBRs, plasma clearance of GA was slower than in SD rats, with plasma 3MGA concentration continuing to increase after administration (Fig. 7B). We then orally administered 50 mg/kg of GA to SD rats and EHBRs every 12 hr for 16 days. On day 16, the plasma 3MGA concentration in EHBRs was significantly higher than in the SD rats,
although plasma GA concentrations were similar in the two strains (Fig. 8A). Consistent with 3MGA and GA concentrations in plasma, the urinary excretion of 3MGA in EHBRs was significantly higher than in SD rats, while urinary excretion of GA was not different in the two strains (Fig. 8B). The ratio of urinary excretion of GA over 24 hr to the oral dosage was approximately 0.00033% in SD rats and 0.00071% in the EHBRs. We also showed that 11β-HSD2 activity in kidney microsomes of EHBRs treated with GA was significantly lower than in control EHBRs, whereas activity in SD rats treated with GA was not significantly different from control SD rats (Fig. 7C). Protein levels of 11β-HSD2 in kidney microsomes measured by Western blot analysis showed no differences between the four groups (data not shown). Although the activity of 11β-HSD2 in kidney microsomes was suppressed markedly by GA treatment in EHBRs, there was no significant difference in plasma potassium levels between any of the experimental groups (Fig. 7D). Urinary sodium levels were also unaffected by GA treatment (data not shown).
Discussion

Ingestion of licorice sometimes causes pseudoaldosteronism (Conn et al., 1968), and the mechanism is considered to be based on inhibition of 11β-HSD2 in tubular epithelial cells by GA, whereas GL is thought to have no effect (Stewart et al., 1987). Monder et al. (1989) reported that GA had a 178-fold greater inhibitory activity on 11β-HSD1 (Ki value, 3.8 nM) compared with GL (Ki value, 675 nM) in rat liver microsomes. On the other hand, Kato et al. (1995) evaluated the inhibitory effects of GA and 3MGA on 11β-HSD2 using rat kidney microsomes, and showed an equivalent effect at a concentration of 1 µM, while GA had a marginally greater inhibitory effect than 3MGA at 0.1 µM concentration. The present study evaluated the inhibitory effects of GA, 3MGA, and GL in rat kidney microsomes, and showed that GA and 3MGA had equivalent inhibitory effects on 11β-HSD2, with a IC50 of approximately 0.3 µM. In contrast, the IC50 value of GL on 11β-HSD2 was about 7-fold higher than either GA or 3MGA. To the best of our knowledge, this is the first report comparing the inhibitory effects of GA, 3MGA, and GL on 11β-HSD2.

As 11β-HSD2 is located in the cytoplasm of tubular epithelial cells (Odermatt and Kratschmar, 2011), in order to examine the inhibitory effects of these compounds in vivo, it is necessary to determine the cell membrane transport systems for GA, 3MGA, and GL. When GA, 3MGA, or GL was dissolved in BSA-free medium containing DMSO, GA had significant inhibitory effect on 11β-HSD2 in rat kidney slices at a concentration of 4 µM, but 3MGA and GL did not. It suggests that GA passes easily through cellular membranes because of its hydrophobic structure, thereby resulting in a stronger inhibitory effect than either 3MGA or GL.
that has a hydrophilic sugar moiety in its chemical structure.

It is known that GA and GL bind efficiently to serum albumin (Ishikawa et al., 1985; Ishida et al., 1988; Ishida et al., 1989), and that the pharmacokinetics of GA and GL are affected by the albumin concentration (Koga et al., 2008). As albumin-bound compounds cannot penetrate cellular membranes, the above results may not be reflected by in vivo events. When GA, 3MGA, and GL were dissolved in medium containing BSA, inhibition of 11β-HSD2 was considerably lower than in medium without BSA. Therefore, in order to predict the in vivo pharmacological action of GA, 3MGA, and GL, it is necessary to consider the system used to penetrate the cell membrane and also binding to albumin.

As 3MGA and GA both have a carboxyl group in their chemical structures, it is considered that these compounds may be substrates of organic anion transporters. The major transporters for organic anions found in the basolateral membrane of renal tubular epithelial cells are OAT1 (Hosoyamada et al., 1999), OAT2 (Sun et al. 2001), OAT3 (Motohashi et al., 2002), and OATP4C1 (Mikkaichi et al., 2004). We focused on OAT1, OAT3, and OATP4C1 because the expression level of OAT2 in kidney is much lower than those of OAT1 and OAT3 in rats (Kojima et al. 2002), and also because PAH-transporting capability of human OAT2 is negligible compared with those of OAT1 and OAT3 (Sun et al. 2001). Compared with uptake by mock cells, HEK293 cells expressing these transporters actively transport 3MGA, with $K_m$ values of about 30–50 $\mu$M, while none of these transporters recognize GA as a substrate. When GL was orally administered to the rats with dysfunctional Mrp2, not GA but 3MGA was detected in the urine collected for 24-h, though the plasma concentration of GA was
considerably higher than that of 3MGA (Makino et al., 2008). As both GA and 3MGA in plasma are mainly bound to albumin, these compounds are unlikely to be excreted by glomerular filtration. OAT1, OAT3 and OATP4C1 are expressed in tubules and ascending limb (Ljubojevic et al, 2004; Mikkaichi et al, 2004). Therefore, the present investigation suggests that 3MGA is transported actively via OAT1, OAT3 and OATP4C1 into tubular epithelial cells, thereby inhibiting 11β-HSD2, whereas GA was unable to pass through tubular epithelial cells and to inhibit 11β-HSD2 in vivo.

In order to evaluate the in vivo inhibitory effects of GA and 3MGA on 11β-HSD2, GA was administered orally to SD rats or EHBRs twice a day at a dose of 100 mg/kg/day for 16 days. As expected, plasma concentrations of 3MGA were almost undetectable in SD rats, and were considerably higher in EHBRs. The urinary excretion of 3MGA in EHBRs was also significantly higher than in SD rats, in parallel with higher plasma 3MGA levels. The excretion of GA in urine in both strains of rats corresponded to 0.0003%–0.0007% of the oral dosage over 24 hr. As GA would not be excreted actively through tubular secretion, this value may be derived from the ratio of unbound GA to albumin in plasma with GA appearing in the urine as a result of ultrafiltration through the glomerular membrane or passive transport of free GA in plasma through tubular epithelial cells. The 11β-HSD2 activity in kidney microsomes of EHBRs treated orally with GA was significantly lower than in SD rats. As we did not detect either GA or 3MGA in kidney microsomal fractions, we considered that the significant decrease in 11β-HSD2 activity in GA-treated EHBRs may result from a decrease in 11β-HSD2 levels in the kidney. However, Western blot analysis showed no decrease in 11β-HSD2 protein levels in
kidney microsomes. This suggests that inhibitory effects of GA or 3MGA on \(11\beta\)-HSD2 may be mechanism-based. 3MGA accumulated in the plasma of EHBRs although plasma levels of GA were equivalent to those in SD rats. The difference in \(11\beta\)-HSD2 activities between SD rats and EHBRs therefore reflects the plasma concentration of 3MGA. Although a significant reduction in \textit{in vivo} \(11\beta\)-HSD2 activity was detected in EHBRs, profiles of plasma potassium levels were overlapped in all the groups, and urinary sodium levels were not changed significantly. These results suggest that the activity of renal \(11\beta\)-HSD2 is not influenced by plasma potassium homeostasis in rats. In rats, adrenal hormones have a more central role in plasma potassium homeostasis than in humans (Bia et al., 1982). For example, carbenoxolone, a derivative of GA, causes hypokalemia in adrenolectomized rats (Souness and Morris, 1989). However, we found that GA failed to reduce plasma potassium levels in EHBRs as \(11\beta\)-HSD2 activity in the kidneys was suppressed significantly. It may therefore be difficult for licorice to induce hypokalemia, an important diagnostic criterion of pseudoaldosteronism, in non-adrenalectomized rats even when \(11\beta\)-HSD2 is inhibited.

In conclusion, 3MGA is a substrate of the organic anion transporters, OAT1, OAT3, and OATP4C1, and is actively transported from the plasma into tubular epithelial cells where it inhibits \(11\beta\)-HSD2. GA is not a substrate of these transporters and exists as an unbound portion of GA bound to plasma albumin that diffuses passively into the cells and inhibits \(11\beta\)-HSD2. Therefore, 3MGA, which appears in the circulation when hepatic Mrp2 function is impaired, may have a major role in pseudoaldosteronism induced by ingestion of licorice. In order to prevent pseudoaldosteronism following ingestion of kampo medicines containing licorice it
may be useful to monitor plasma and urine 3MGA concentrations in patients.
Acknowledgements

The authors are grateful to Professor Mitsuru Sugawara, Laboratory of Pharmacokinetics, Faculty of Pharmaceutical Science, Graduate School of Hokkaido University, for providing us with plasmids containing the open reading frame of OAT1 and OAT3.
Authorship Contribution

Participated in research design: Makino, Ohtake, Inoue, and Mizukami.

Conducted experiments: Makino, Okajima, and Uebayashi.

Performed data analysis: Makino

Wrote or contributed to the writing of the manuscript: Makino, and Mizukami.
References


Footnotes

a) This research was supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Young Scientists (B), 0790475 (2008), and 23790748 (2011), and supported partially by a grant from the OTC Self-Medication Promotion Foundation (2008).

b) Part of this work was previously presented in poster form: Okajima, K., Makino, T., Inoue, K., Ohtake, N., Mizukami, H. Mechanisms of pseudoaldosteronism caused by licorice. The 58th Annual Meeting of the Japanese Society of Pharmacognosy held at Tokyo (Sep. 25, 2011, Abstract 2P-73).

c) Toshiaki Makino, Ph. D., Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Mizuho-ku, Nagoya 467-8603, Japan. E-mail: makino@phar.nagoya-cu.ac.jp. Tel. & Fax: +81 52 836 3416
Legends for figures

Fig. 1. Chemical structures of glycyrrhizin (GL), 3-monoglucuronyl-glycyrrhetic acid (3MGA), and glycyrrhetic acid (GA). GlucUA, glucuronic acid.

Fig. 2. Inhibitory effects of GL and its metabolites on 11β-HSD2 in rat kidney microsomes. GL, 3MGA, and GA were added to the reaction mixture containing rat kidney microsomes and cortisol, and the production of cortisone measured. Open square, GL; closed circle, 3MGA; open triangle, GA. Data are expressed as mean of % of control ± S.E. (n = 3). *P < 0.05, **P < 0.01 by Bonferroni-type multiple t-test vs control group.

Fig. 3. Inhibitory effects of GL and its metabolites on 11β-HSD2 in rat kidney slices. The slices were incubated for 2 hr in BSA-free medium containing 4 µM of the samples (A), or in medium containing 5% BSA and 100 µM of the samples (B). Cortisol was then added, followed by incubation for 30 min. Concentrations of cortisone in the medium were measured. Data are expressed as mean of % of control ± S.E. (n = 3). **P < 0.01 by Bonferroni-type multiple t-test.

Fig. 4. Uptake of 3MGA and GA by rat kidney slices. The slices were incubated in medium containing 3MGA (A, closed circle) or GA (B, closed circle) with 5% BSA at 37°C (solid line and closed symbols) or 4°C (dotted line and open symbols). Data are expressed as mean ± S.E. (n = 3).

Fig. 5. Uptake of representative substrates and 3MGA by HEK293 cells transiently transfected with OAT1, OAT3, and OATP4C1. HEK293 cells transfected with mock plasmid or OAT1 were incubated with 0.5 µM [14C] p-aminohippuric acid (PAH) (A) or 20 µM 3MGA (B) for 5 min. HEK293 cells transfected with OAT3 (C, E) or OATP4C1 (E, F) were incubated with 5 nM [3H] estrone sulphate (ES) (C, E) or 20 µM 3MGA (D, F) for 5 min. Then, the uptakes of the compounds were measured. Data are expressed as mean ± S.E. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs mock cells by Student's t-test.
Fig. 6. Uptake of 3MGA and GA by HEK293 cells transfected with OAT1, OAT3, and OATP4C1. HEK293 cells transfected with mock plasmid (open circle), OAT1 (closed square), OAT3 (open triangle), and OATP4C1 (closed circle) were incubated for 3–10 min with 20 µM 3MGA (A) or 1 µM GA (B), and for 5 min with 5–160 µM 3MGA (C). The uptake mediated by the transporters was calculated by subtracting the uptake in mock cells from transporter-transfected cells (C). Data are expressed as mean ± S.E. (n = 3). The uptake kinetics was shown as Eadie-Hofstee plots (D). *P < 0.05 vs mock cells by Bonferroni-type multiple t-test.

Fig. 7. The plasma concentration profiles of GA and 3MGA in SD rats (A) and EHBRs (B) after oral administration of GA. GA (50 mg/kg) suspended in 0.5% CMC was then administered orally to SD rats or EHBRs. Plasma concentrations of GA (closed circle and solid line) and 3MGA (open triangle and dotted line) were measured by LC/MS/MS as described in the Materials and Methods. Data are expressed as mean ± S.E. (n = 4).

Fig. 8. The plasma concentration and urinary excretion of 3MGA and GA and activity of 11β-HSD2 in kidney microsomes and plasma potassium levels in SD rats and EHBRs treated with GA for 16 days. SD rats or EHBRs were administered GA orally twice a day for 16 days. Plasma and 24-hr urine samples were collected. Plasma concentrations (A) and urinary eliminations (B) of GA (open column) and 3MGA (closed column) were measured. 11β-HSD2 activity in kidney microsomes in GA-treated rats (closed column) were expressed as a % of the value in control rats treated with vehicle (open column) (C). The plasma was collected for measurement of potassium levels before (day 0) and 3, 7, 11, and 16 days after the start of GA-treatment. The dotted lines and circles represent data from SD rats, and the solid lines and squares are data from EHBRs (D). Data are expressed as mean ± S.E. (n = 3). *P < 0.05 by Student's t-test.
Figure 1

GL \quad R = \text{glucUA-glcUA}

3MGA \quad R = \text{glucUA}

GA \quad R = H
Figure 2

11β-HSD2 activity (% of control)

Concentration (µM)

GA

3MGA

GL
Figure 3

(A) 11\beta-HSD2 activity (% of control) for different treatments: Control, GL (4 \mu M in BSA-free medium), 3MGA, and GA. The bars indicate the mean ± SEM, and the asterisks denote significance levels with ** indicating a significant difference.

(B) 11\beta-HSD2 activity (% of control) for different treatments: Control, GL (100 \mu M in 5% BSA), 3MGA, and GA. The bars indicate the mean ± SEM, and the asterisks denote significance levels with ** indicating a significant difference.
Figure 5
Figure 6

**A**

Uptake of 3MGA (nmol/mg protein) vs. Incubation time (min)

- Mock
- OAT1
- OAT3
- OATP4C1

**B**

Uptake of GA (nmol/mg protein) vs. Incubation time (min)

- Mock
- OAT1
- OAT3
- OATP4C1

**C**

Uptake of 3MGA (nmol/protein/5 min) vs. 3MGA (μM)

- OAT1
- OAT3
- OATP4C1

**D**

V (pmol/mg protein/min) vs. V/[S] (μl/mg protein/min)

- OAT1
- OAT3
- OATP4C1
Figure 7

A

Plasma GA or 3MGA (µM)

Time after the treatment (hr)

B

Plasma GA or 3MGA (µM)

Time after the treatment (hr)
Figure 8

A) Plasma GA or 3MGA concentration (µM) in SD rat and EHBR.

B) Urinary GA or 3MGA excretion (nmol/kg/day) in SD rat and EHBR.

C) 11β-HSD2 activity (% of control) in SD rat and EHBR.

D) Plasma potassium (mEq/l) over time for SD-control, SD-GA, EHRB-control, and EHRB-GA.