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A Novel 11β-Hydroxysteroid Dehydrogenase Inhibitor Contained in Saiboku-To, a Herbal Remedy for Steroid-dependent Bronchial Asthma

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Abstract—To identify the inhibitor of prednisolone metabolism contained in Saiboku-To, we conducted in-vitro experiments of 11β-hydroxysteroid dehydrogenase (11β-HSD), using rat liver homogenate and cortisol as a typical substrate. We studied the effects of ten herbal constituents on 11β-HSD. Five herbal extracts showed inhibitory activity with *Glycyrrhiza glabra* > *Perillae frutescens* > *Zizyphus vulgaris* > *Magnolia officinalis* > *Scutellaria baicalensis*. This suggests that unknown 11β-HSD inhibitors are contained in four herbs other than *G. glabra* which contains a known inhibitor, glycyrrhizin (and glycyrrhetinic acid). Seven chemical constituents which have been identified as the major urinary products of Saiboku-To in healthy and asthmatic subjects were studied; magnolol derived from *M. officinalis* showed the most potent inhibition of the enzyme (IC50, $1{\cdot}8 \times 10^{-4}$ M). Although this activity was less than that of glycyrrhizin, the inhibition mechanism (non-competitive) was different from a known competitive mechanism. These results suggest that magnolol might contribute to the inhibitory effects of Saiboku-To on prednisolone metabolism through inhibition of 11β-HSD.

Saiboku-To is the most popular anti-asthmatic Chinese herbal medicine (Kampo medicine in Japan) and has been used for corticosteroid-dependent asthma to obtain a steroid-sparing effect in prednisolone therapy (Nagano et al 1988). On the basis of animal experiments, the mechanism of action of Saiboku-To has been attributed to hormonal stimulation of the adrenal cortex (Hiai et al 1981; Shimizu et al 1984) and synergistic adjuvant effects on autacoid secretions (Toda et al 1988) or allergic reactions (type I and IV) (Nishiyori et al 1983, 1985).

Recently, we proposed another mechanism which involves suppression of the systemic elimination of prednisolone (Taniguchi et al 1992). This pharmacokinetic effect seemed to result from 11β-hydroxysteroid dehydrogenase (11β-HSD) metabolic enzyme inhibition, because plasma prednisolone/prednisone ratios following Saiboku-To administration increased significantly (Taniguchi et al 1992). Since other Kampo-preparations containing *Glycyrrhiza glabra* did not show an effect on prednisolone pharmacokinetics (unpublished data), the effect of Saiboku-To could not be explained by known enzyme inhibitors such as glycyrrhizin and its aglycone glycyrrhetinic acid, which are contained in *G. glabra*. These observations suggested that Saiboku-To must contain as yet uncharacterized 11β-HSD inhibitors.

In the present study, we carried out in-vitro experiments of 11β-HSD inhibition using cortisol and rat liver homogenate.

Materials and Methods

Materials

Saiboku-To (TJ-96, Tsumura Co., Tokyo, Japan) consists of fine brownish granules containing ten different herbal extracts (Table 1). Original herbs used for the assay were purchased from Uchida Wakanyaku Co. (Tokyo, Japan). The extracts of Saiboku-To and of original herbs were prepared as follows. One gram Saiboku-To or the crushed herb in 15 mL 35% ethanol was gently refluxed for 1 h on a steam bath. After cooling to room temperature, water was added to make a total volume of 10 mL before centrifugation at 1600 *g* for 10 min. The resulting supernatant was used for the assay.

Glycyrrhizin, glycyrrhetinic acid, wogonin, and baicalein were purchased from Wako Pure Chemicals (Osaka, Japan). Magnolol and honokiol were donated by Professor Y. Sashida of Tokyo College of Pharmacy (Fujita et al 1973). Medicarpin and oroxylin A were kindly contributed by Professor T. Nomura of Toho University School of Pharmacy (Tokyo, Japan) and Tsumura Co., respectively. 8,9-Dihydroxydihydromagnolol was prepared by us from magnolol by osmic acid oxidation (Homma et al 1992). Liquiritigenin was isolated from *G. glabra* according to Shibata & Saitoh (1978). Chemical structures of these compounds are given in Fig. 1. Cortisol and cortisone were purchased from Sigma Chemical Co. (St Louis, MO, USA). Other organic and inorganic reagents were of analytical grade.

Rat liver homogenates were prepared in the usual manner: fresh liver was isolated from a male Wistar rat (freely fed, body weight 250 g) and was cut into small pieces. The pieces were homogenized in 10 vol 0·25 M sucrose in a glass homogenizer with a Teflon piston. The homogenates were frozen at −80°C and stored until incubation.

Instruments

Our HPLC system for determination of glucocorticoids in incubation mixtures consisted of a solvent delivery pump (VIP-I, Jasco, Tokyo), a UV-detector (Uvidec-100-III, Jasco), a single pen recorder (Pantos U-228, Nippon Denshi, Tokyo), a sample injector with a loop volume of 100 μL

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Table 1. Herbal composition of Saiboku-To.

Constituent herb	Family	Composition (%, w/w)
Bupleurum falcatum L.	Umbelliferae	20·6
Pinellia ternata Beitenbach	Araceae	14·7
Poria cocos Wolf.	Polyporaceae	14·7
Scutellaria baicalensis Georgi	Labiatae	8·8
Zizyphus vulgaris Lam.	Rhamnaceae	8·8
Panax ginseng C. A. Meyer	Araliaceae	8·8
Magnolia officinalis	Magnoliaceae	8·8
Glycyrrhiza glabra L.	Leguminosae	5·9
Perillae frutescens Britton var. *acuta* Kudo	Labiatae	5·9
Zingiber officinale Roscoe	Zingiberaceae	3·0

(Model 7125, Rheodyne, CA, USA), and a silica gel column (LiChrosorb Si-60, 5 μm, i.d. 4 mm × 250 mm, Merck, Darmstadt, Germany). The mobile phase was a mixture of water/methanol/dichloromethane/n-hexane (0·1/8·0/30·0/61·9 v/v) with a flow rate of 1·5 mL min^{-1}. Detector sensitivity was set at 0·005–0·01 aufs at 245 nm. We used a disposable syringe minicolumn (Extrashot, Kusano Sci. Co., Tokyo) to perform sample injections (Homma et al 1989; Kouno et al 1990).

Determination of 11β-HSD inhibition activity

We measured 11β-HSD activity in rat liver homogenate incubation mixtures, detecting chemical transformation of cortisol to cortisone in the presence of 11β-HSD inhibitors. Oxidation at the C-11 position of the steroid nucleus was kinetically characterized by measuring the conversion rate of cortisol to cortisone in the presence of $NADP^+$ in rat liver homogenate according to the procedure of Monder et al (1989) with minor modification. The incubation mixtures consisted of 620 μL 0·1 M Tris-HCl buffer (pH 8·5) containing 0·014% Triton-X, 50 μL 5 mM $NADP^+$, 100 μL rat liver homogenate, and 200 μL aqueous solution for Saiboku-To and original herbal extracts or 200 μL buffer solution for each chemical such as the known inhibitors (glycyrrhizin and glycyrrhetinic acid) and our candidates isolated from urine of subjects receiving the preparation. These chemicals were dissolved in a buffer solution directly or after pre-solubilization in a small amount of ethanol with a final concentration in incubation mixtures of less than 2%. After 10 min pre-incubation at 37°C, 200 μL 0·3 mM cortisol was added and the resulting mixtures were further incubated for 10 min. The enzyme reaction was terminated by an addition of 100 μL 5% sulphuric acid. Cortisol and cortisone in the mixtures were determined by HPLC using Extrashot as described in our previous papers (Homma et al 1989; Kouno et al 1990). Briefly, 5 μL incubation mixture and 2 μL sodium hydroxide solution were loaded onto Extrashot which was then attached to the sample-loading injector of the HPLC system.

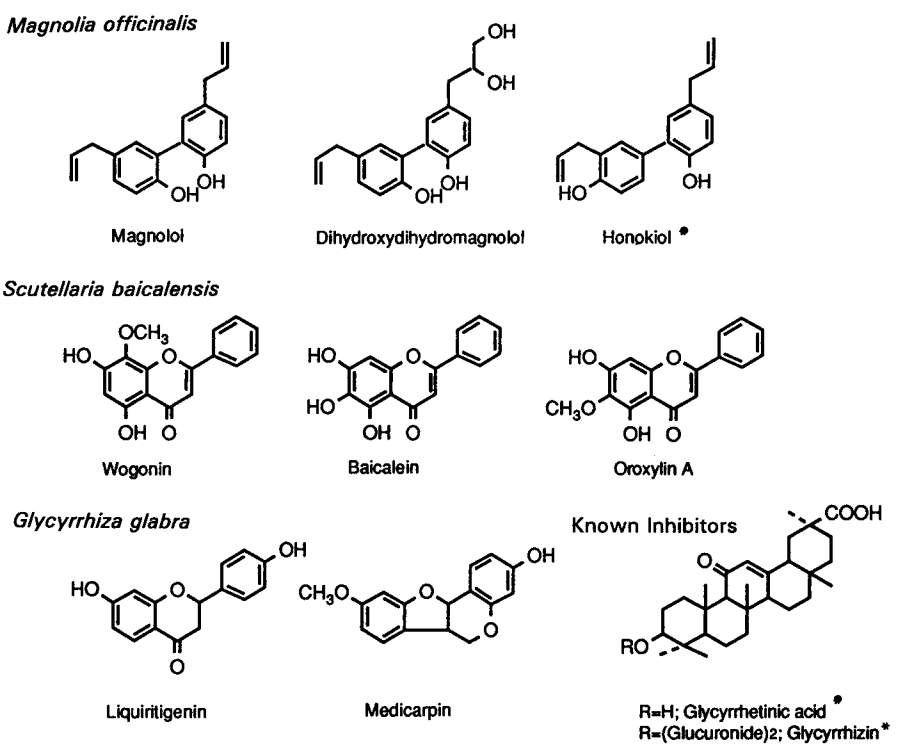

FIG. 1. Chemical structures of test compounds. * These compounds have not been detected in urine following Saiboku-To administration.

Ethanol/dichloromethane (2/98 v/v, 130 μL) was injected into the system through Extrashot using a tuberculin glass syringe. Thus, extraction and injection of the glucocorticoids in the incubation mixtures were achieved simultaneously. The recovery rates of glucocorticoids from the incubation mixture were more than 95% with coefficient of variations less than 5%. Direct peak-height calibration of the test and control mixtures afforded inhibitory activity (% inhibition) of the test materials against 11β-HSD.

Results

Effects of herbal extracts

Effects of original herbal extracts on conversion of cortisol to cortisone by rat liver homogenate were compared with that of Saiboku-To (Table 2). Cortisone production in the reaction mixture was significantly inhibited by Saiboku-To and five original herbal extracts ($P < 0.05$). The magnitude of the inhibition (% inhibition) was in the order Saiboku-To (87·5%) > *G. glabra* (80·8%) > *P. frutescens* (30·9%) > *Z. vulgaris* (27·6%) > *M. officinalis* (19·8%) > *S. baicalensis* (19·1%).

Effects of urinary metabolites of Saiboku-To

Seven candidates (Fig. 1) were tested with respect to the effects on rat liver 11β-HSD at concentrations of 10 and 100 μM. The results were compared with those of the known inhibitors, glycyrrhizin and glycyrrhetinic acid (Table 3). Five of seven candidates showed inhibitory activity at 100 μM, although their activities were weaker than those of the known inhibitors. Dihydroxydihydromagnolol in *M. officinalis* and liquiritigenin in *G. glabra* did not show any activity at the test concentrations. Wogonin, baicalein, and oroxylin A (flavonoids derived from *S. baicalensis*), and medicarpin (a

Table 2. Effects of Saiboku-To and its constituent herbal extracts on 11β-hydroxysteroid dehydrogenase in rat liver homogenate.

	% inhibition[a]	% activity of Saiboku-To
Saiboku-To	87·5±3·4**	100·0
B. falcatum	7·7±5·7	8·8
P. ternata	5·8±4·2	6·6
P. cocos	—	—
S. baicalensis	19·1±11·5*	21·8
Z. vulgaris	27·6±4·0**	31·5
P. ginseng	10·9±6·9	12·5
M. officinalis	19·8±3·7**	22·6
G. glabra	80·8±1·0**	92·3
P. frutescens	30·9±9·6**	35·3
Z. officinale	12·8±8·7	14·6

[a] Data are presented as mean±s.d. of triplicate experiments. *$P < 0.05$, **$P < 0.01$ compared with control.

Table 3. Inhibition of 11β-hydroxysteroid dehydrogenase by urinary metabolites of Saiboku-To and known inhibitors.

	Inhibition (%)	
Inhibitor	10 μM	100 μM
Urinary metabolites of Saiboku-To		
Magnolol	15·1±4·4	43·9±3·0
Dihydroxydihydromagnolol	—	—
Wogonin	—	7·4±0·8
Baicalein	6·8±1·6	14·8±1·6
Oroxylin A	—	5·1±5·5
Liquiritigenin	—	—
Medicarpin	—	12·2±3·3
Known inhibitors		
Glycyrrhizin	81·1±5·4	97·3±1·1
Glycyrrhetinic acid	100·0	

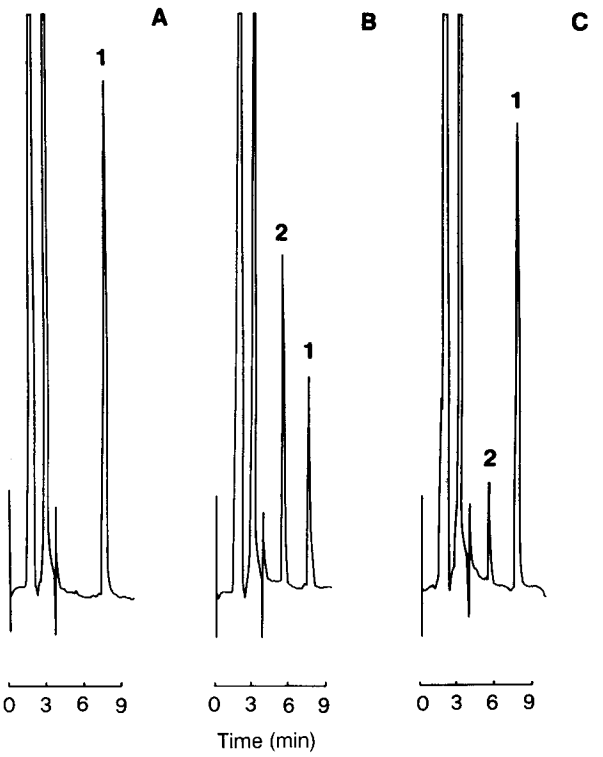

FIG. 2. Chromatographic comparison of the effect of magnolol (100 μM) on transformation of cortisol (peak 1) to cortisone (peak 2) by 11β-hydroxysteroid dehydrogenase. A. Before incubation with magnolol; B. after incubation without magnolol; C. after incubation with magnolol.

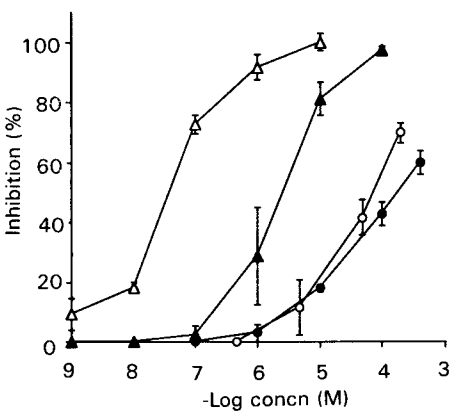

FIG. 3. Dose-dependent inhibitory effects of magnolol (●), honokiol (○), glycyrrhizin (▲), and glycyrrhetinic acid (△) on 11β-hydroxysteroid dehydrogenase. Data are presented as mean±s.d. of triplicate experiments.

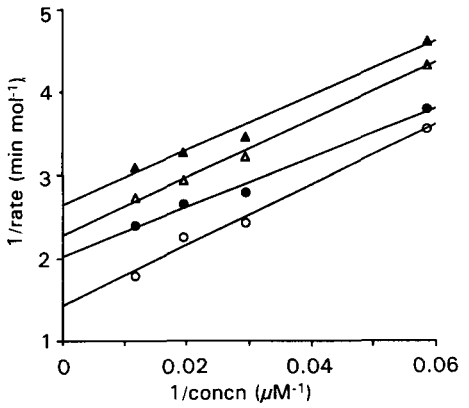

FIG. 4. Lineweaver–Burk double reciprocal plots of initial enzyme velocity and concentration of cortisol in the presence of magnolol at concentrations of 0 (○), 0·1 (●), 0·15 (△), and 0·2 (▲) mM.

homoisoflavonoid in *G. glabra*) showed weak activity. However, considerable inhibition was observed with magnolol, a neolignan derived from *M. officinalis*. A typical chromatogram for determination of the inhibitory activity of magnolol is shown in Fig. 2, where the chemical transformation from cortisol to cortisone was clearly suppressed. The dose-dependent inhibitory effect of magnolol is compared with those of glycyrrhizin and glycyrrhetinic acid in Fig. 3. The IC50 values of magnolol, glycyrrhizin, and glycyrrhetinic acid were $1{\cdot}8\times10^{-4}$, $2{\cdot}6\times10^{-6}$, and $9{\cdot}0\times10^{-8}$ M, respectively. Since *M. officinalis* contains another congener of magnolol, honokiol (not a urinary metabolite), we also examined the effect of honokiol on 11β-HSD and found a dose-dependent inhibitory effect with IC50 of $7{\cdot}0\times10^{-5}$ M (Fig. 3).

Mechanism of magnolol in 11β-HSD inhibition

Fig. 4 shows the inhibitory effects of magnolol on rat liver 11β-HSD. The data were plotted according to the Lineweaver–Burk linear transformation of the Michaelis–Menten equation. The double reciprocal plots on Fig. 4 suggested magnolol has a unique non-competitive inhibitory mechanism. We were unable to estimate an inhibition constant (K_i) of magnolol by the Dixon plot because of this non-competitive inhibition.

Discussion

This paper suggests the presence of several novel inhibitors of 11β-HSD in five constituent herbs. *G. glabra*, *P. frutescens*, *Z. vulgaris*, *M. officinalis* and *S. baicalensis*. Although these inhibitors seem to contribute to in-vitro activity of Saiboku-To, their contributions to prednisolone metabolism during clinical Saiboku-To treatment has been unclear. However, we emphasize the importance of this possibility, since our biologically active compounds in herbal medicine are found in biofluids following administration (Homma et al 1992, 1993a).

In our previous study, we found seven phenolic compounds in urine after oral administration of Saiboku-To (Homma et al 1992, 1993a, b). These compounds seemed to be possible candidates which explain in-vivo effects of Saiboku-To. Five of these compounds showed inhibitory activity against 11β-HSD in-vitro (Table 3). The intensities of those activities were almost equal to those of the corresponding herbal extracts, except that *G. glabra*, containing glycyrrhizin, concealed the effects of liquiritigenin and medicarpin. Magnolol exhibited activity at concentrations higher than 1×10^{-5} M (Fig. 3). Similar activity was also observed in honokiol, a hydroxylated derivative of magnolol isolated from *M. officinalis* but not found as a urinary metabolite of Saiboku-To.

The novel 11β-HSD inhibitors found in this study belong to a class of phenolic compounds, lignans and flavonoids, whose chemical structures are completely different from those of the previously described inhibitors. Unexpectedly, the inhibition mechanism of magnolol seems to be different from those of the known inhibitors, the latter exhibiting competitive inhibition (Monder et al 1989). Although 11β-HSD inhibitors have been considered so far to belong to a limited class of liquorice triterpenoids, the present results suggested that the naturally occurring lignans and flavonoids also possess inhibitory activity through a different mechanism.

Urinary non-conjugated magnolol in responders to Saiboku-To is significantly higher than that in the non-responders (Homma et al 1993a, b). This suggests that magnolol is an important chemical constituent for the clinical effects of Saiboku-To, playing an important role for alteration of prednisolone pharmacokinetics.

The inhibitory effects of liquorice glycosides on 11β-HSD are so marked in animal experiments in-vivo and in-vitro (Monder et al 1989; Mackenzie et al 1990), that Saiboku-To could inhibit 11β-HSD even though the glycyrrhizin content is relatively small. However, the effect of Saiboku-To cannot be explained by glycyrrhizin alone, because another Kampo preparation, Sho-Saiko-To which contains *G. glabra* but not *P. cocos*, *M. officinalis* or *P. frutescens*, did not affect prednisolone pharmacokinetics in healthy subjects (unpublished data). Animal experiments using pure compounds will be needed to clarify the role of lignans and flavonoids on prednisolone metabolism.

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