Hepatoprotective and Hepatotoxic Activities of Sophoradiol Analogs on Rat Primary Liver Cell Cultures[1)]

Junei KINJO,*[,2)] Tsuyoshi IKEDA, Masafumi OKAWA, Manabu UDAYAMA, Tomoki HIRAKAWA, Yuichi SHII, and Toshihiro NOHARA

Faculty of Pharmaceutical Sciences, Kumamoto University, 5–1 Oe-Honmachi, Kumamoto 862–0973, Japan.
Received February 14, 2000; accepted May 11, 2000

As a part of our studies of hepatoprotective drugs, we prepared kaikasaponin I (2), sophoradiol monoglucuronide (SoMG, 3) and sophoradiol (4) from kaikasaponin III (1). We examined the hepatoprotective effects of these analogs, using immunologically-induced liver injury in primary cultured rat hepatocytes and found that compound 1 was more effective than soyasaponin I (1a) while 2 was more effective than 1. On the other hand, 3 was less effective than 2 at 30—200 μM. Further, compound 3 was strongly cytotoxic at 500 μM while 4 exhibited hepatoprotective activity at the same dose, although less potent. When the cytotoxicity toward hepatocytes of these analogs was tested, only 3 was cytotoxic at doses of 200 and 500 μM. This is the first example of an oleanene glucuronide (OG) which is cytotoxic toward hepatocytes. Compound 3 exhibited hepatoprotective activity at 200 μM, while it was also cytotoxic at the same dose without antiserum. Therefore, the hepatoprotective activity of OG represents a balance between a hepatoprotective action and its cytotoxicity toward hepatocytes.

Key words hepatoprotective activity; cytotoxicity; primary cultured rat hepatocyte; immunological liver injury; triterpenoidal saponin; oleanene glucuronide

Some oleanane-type triterpenoidal saponins are known to exhibit hepatoprotective action[3)] and saikosaponins[4—6)] and glycyrrhizin[7—9)] are the most well-known of these. In Japan, glycyrrhizin has been widely used as a drug to treat hepatitis.[3)] Oleanene glucuronide (OG) is a triterpenoidal saponin of the olean-12-ene type, with a methyl group at C-28 and a glucuronic acid moiety at the C-3 of the triterpene.[10)] OG is widely distributed in leguminous plants and the seeds of legumes[10)] and soyasaponin I (**1a**)[11)] is a very common OG. In a series of studies of hepatoprotective drugs, we devised an *in vitro* assay method[12)] in which the extent of immunological liver injury[13,14)] was measured in rat primary hepatocytes cultures. The mechanism of immunological liver injury is complement-mediated cell damage.[13,14)] We reported the preventive effect of soyasaponins I, II, III (**2a**) and IV which have the same aglycone, soyasapogenol B (**4a**).[15)] The order of the preventive effect, soyasaponin III>IV≫I>II, suggested that the disaccharide group has a greater effect than the trisaccharide group. In order to obtain further information on the structure–activity relationship, the monoglucuronide of soyasapogenol B (SBMG, **3a**) was prepared by partial hydolysis of **1a**.[16)] Although **3a** showed similar hepatoprotective activity to that of **2a**, the hepatoprotective effect of **4a** and glucuronic acid was weak or completely absent. Although a mixture of the latter two compounds was tested, the hepatoprotective action of the mixture did not change, compared with **4a**. Therefore, we concluded that the linkage between glucuronic acid and soyasapogenol B enhances the hepatoprotective activity. By similar examination of OGs isolated from Puerariae Flos, we confirmed that the hydroxyl group at C-24 reduces the hepatoprotective activity.[17)] A similar result has been also observed in another liver injury model induced by CCl_4, whose sophoradiol glycoside was found to be much more effective than soyasapogenol B glycoside.[18)]

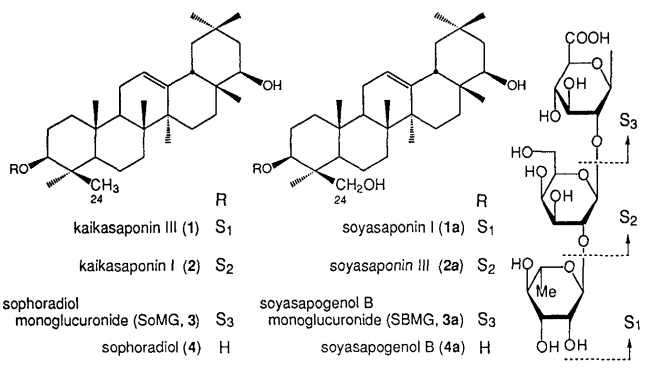
In order to clarify in more detail the structure–hepatoprotective relationship of OGs, we investigated the hepatoprotective effects of the hydrolytic products of kaikasaponin III (**1**), *i.e.*, kaikasaponin I (**2**), sophoradiol monoglucuronide (SoMG, **3**) and sophoradiol (**4**). Here, we present not only the hepatoprotective action of sophoradiol derivatives but also their cytotoxicity toward liver cells, and discuss the structure–activity relationships involved.

MATERIALS AND METHODS

Instruments and Reagents The instruments and reagents used in this study were the same as those described in previous papers.[12,15)] Compounds **1a**—**4a** were prepared according to the previous paper.[16)]

Preparation of Kaikasaponin III (1) The leaves of *Pueraria lobata* (5.3 kg) were extracted with MeOH under reflux. The extract was concentrated and defatted with *n*-hexane. The MeOH layer was diluted to 40% MeOH with H_2O and extracted with EtOAc. The 40% MeOH layer was subjected to Diaion HP-20 column chromatography using H_2O→MeOH to give a saponin fraction (5.7 g). This fraction was further purified with Sephadex LH-20 (MeOH) and SiO_2 [$CHCl_3$–MeOH–H_2O (7 : 3 : 0.5)] to provide **1** (366 mg), which was identical to an authentic sample.[19)]

Preparation of Kaikasaponin I (2) and Sophoradiol Monoglucuronide (3) A solution of kaikasaponin III (150 mg) in 1.0 N HCl–dioxane (1 : 1, 10 ml) was heated at



* To whom correspondence should be addressed. e-mail: kinjojun@fukuoka-u.ac.jp

75—80 °C for 3 h. The reaction mixture was diluted with H_2O and desalted using a MCI gel CHP-20P column, successively eluting with H_2O and MeOH. The MeOH eluate was evaporated *in vacuo*, and the residue (122 mg) was chromatographed over SiO_2 ($CHCl_3$–MeOH–H_2O=8 : 2 : 0.2→7 : 3 : 0.5) to give **3** (30 mg) and **2** (25 mg) as amorphous powders.

Kaikasaponin I (2) A white amorphous powder, $[\alpha]_D^{19}$ +25.6° (c=0.10, MeOH). Positive FAB-MS *m/z*: 819 $[M+K]^+$, 803 $[M+Na]^+$. ^{1}H-NMR (in pyridine-d_5) δ: 0.90, 1.00, 1.02, 1.15, 1.24, 1.29, 1.30, 1.35 (each 3H, s, *tert*-Me×8), 5.05 (1H, d, J=7.0 Hz, glc A H-1), 5.26 (1H, d, J=7.6 Hz, gal H-1), 5.33 (1H, br s, H-12). ^{13}C-NMR (in pyridine-d_5) δ: 38.8, 26.7, 89.1, 39.6, 55.7, 18.4, 33.1, 40.0, 47.9, 36.8, 23.8, 122.5, 144.8, 42.3, 26.4, 28.6, 38.0, 45.3, 46.8, 30.9, 42.3, 75.6, 28.6, 15.7, 16.8, 17.1, 25.8, 21.2, 33.3, 28.1 (C-1—30), 105.3, 83.9, 77.3, 74.8, 77.7, 172.6 (glc A C-1—6), 107.2, 73.1, 74.9, 69.5, 76.9, 61.3 (gal C-1—6).

Sophoradiol Monoglucuronide (3) A white amorphous powder, $[\alpha]_D^{19}$ +21.2° (c=0.12, MeOH). Positive FAB-MS *m/z*: 641 $[M+Na]^+$. ^{1}H-NMR (in pyridine-d_5) δ: 0.90, 1.01, 1.03, 1.04, 1.24, 1.31×2, 1.35 (each 3H, s, *tert*-Me×8), 5.05 (1H, d, J=7.4 Hz, glc A H-1), 5.34 (1H, br s, H-12). ^{13}C-NMR (in pyridine-d_5) δ: 38.8, 26.6, 89.0, 39.5, 55.7, 18.5, 33.1, 40.0, 47.9, 36.8, 23.8, 122.5, 144.8, 42.4, 26.4, 28.6, 38.0, 45.3, 46.8, 30.9, 42.3, 75.5, 28.6, 15.7, 17.0, 17.1, 25.8, 21.2, 33.3, 28.2 (C-1—30), 107.3, 75.5, 78.1, 73.4, 77.8, 172.9 (glc A C-1—6).

Sophoradiol (4) A solution of **1** (50 mg) in 2 N HCl (5 ml) was refluxed for 2 h. The reaction mixture was filtered and the precipitate was washed with H_2O and dried *in vacuo*. The residue (20 mg) was chromatographed over SiO_2 (*n*-hexane–AcOEt=1 : 1) to give **4** (12 mg) as an amorphous powder, which was identified by comparison with an authentic sample.[20]

Animals Male Wistar rats (6 weeks old, body weight 150—160 g) and a male New Zealand white rabbit (body weight 3 kg) were used.

Preparation of Primary Cultured Rat Hepatocytes Liver cells were isolated according to a procedure developed by Berry and Friend.[21] The detailed procedure is described in a previous paper.[12,18]

Preparation of Antiserum against Rat Hepatocytes The antiserum was prepared according to the method of Shiki *et al.*[13] An antibody to rat hepatocytes was raised in rabbits, first by injection of 1×10^8 cells, followed by four injections of 5×10^7 cells over a period of 4 weeks. The antiserum to the rat hepatocytes was prepared by the method of Harboe and Ingild.[22]

Determination of Hepatoprotective Activity of Saponins toward *in Vitro* Immunological Liver Injury One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to the above medium (300 μl) containing the antiserum against rat plasma membranes (80 μl/ml) and a dimethyl sulfoxide (DMSO) solution (4 μl) of the test samples or glycyrrhizin [final concentration 0 [reference (Ref.)]; 10; 30; 90; 200; 500 μM]. Forty min after the antiserum was administered, the medium was withdrawn for determination of ALT (Alanine aminotransferase). The control is hepatocytes not treated with antiserum. The percentage protection is calculated as {1−(sample-control)/(reference-control)}×100. The reference is the value for hepatocytes treated with antiserum but not with the test samples. The percentage protection of glycyrrhizin (positive control) was 37% at 500 μM.

Determination of Cytotoxicity of Saponins toward Hepatocytes (without Antiserum) In the same way as above, the cultured cells were exposed to the above medium (300 μl) containing the DMSO solution (4 μl) of the test samples [final concentration 0 (Ref.); 10; 30; 90; 200; 500 μM]. Forty min after the test samples were administered, the medium was withdrawn for determination of ALT. The percentage cytotoxicity is calculated as (sample/reference)×100. The reference is the value of hepatocytes not treated with the test samples.

ALT Assay The ALT activity was assayed by autoanalyzer, COBAS MIRA (Roche), using commercial kits based on the ALT assay method.[23]

Statistical Analysis The data are shown as means±S.D. (n=4). After analysis of variance, Sheffe's test was employed to determine the significance of differences between reference and experimental samples.

RESULTS AND DISCUSSION

Kaikasaponin III (**1**) was prepared from the leaves of *Pueraria lobata* according to a previous paper.[19] It was partially hydrolyzed with 0.5 N HCl to afford kaikasaponin I (**2**) and sophoradiol monoglucuronide (SoMG, **3**); sophoradiol (**4**) was obtained by acid hydolysis of **1** with 2 N HCl.

Next, we examined the hepatoprotective action of these analogs and **1** against immunologically-induced liver injury in primary cultured rat hepatocytes. In a previous paper,[12] we reported that the activity of ALT in the medium was in good agreement with the extent of hepatocyte damage induced by immunological liver injury. Therefore, the cell damage was evaluated by means of ALT activity. The results of the hepatoprotective effect of **1**—**4** are shown in Table 1.

Compound **1** was more effective than **1a**. This information supported previously obtained structure–hepatoprotective relationship data, namely, that the methyl group at C-24 enhances the hepatoprotective activity.[17] Furthermore, **2** was more effective than **1**. Compound **2** showed hepatoprotective activity even at 30 μM. On the other hand, **3** was less effective than **2** at 30—200 μM. Further, compound **3** exhibited strong cytotoxic activity at 500 μM while the aglycone (**4**) exhibited hepatoprotective activity at the same dose, although less potent.

Since **3** was strongly cyototoxic at the highest dose, the cytotoxicity of **1**—**4** together with that of soyasapogenol B analogs (**1a**—**4a**) toward liver cells was also examined without antiserum (Table 2).

Only **3** was cytotoxic toward hepatocytes at doses of 200 and 500 μM. This is the first example of OG being cytotoxicity toward hepatocytes although some glucuronides of oleanolic acid have exhibited such toxicity.[24]

Compound **3** was hepatoprotective at 200 μM, and was also cytotoxic at the same dose without antiserum. Therefore, the hepatoprotective activity of OG could represent a balance between a hepatoprotective action and its cytotoxicity toward hepatocytes. A similar observation has been obtained from experiments with oleanolic acid-type glucuronides.[24]

Table 1. Hepatoprotective Activity of **1**—**4** and **1a**

Substances	Dose (μM)	ALT	
		IU/l	Protection (%)
Control	—	5.15±1.2	—
Kaikasaponin III (**1**)	0 (Ref.)	71.50±1.7	—
	10	68.50±3.7	5
	30	69.50±2.6	3
	90	65.00±4.5	10
	200	49.00±2.4**	34
	500	18.00±1.6**	81
Kaikasaponin I (**2**)	0 (Ref.)	72.25±3.8	—
	10	70.25±3.1	3
	30	60.75±4.1*	17
	90	21.25±2.9**	76
	200	9.75±1.7**	93
	500	9.50±2.1**	94
Sophoradiol monoglucuronide (SoMG, **3**)	0 (Ref.)	72.50±1.3	—
	10	69.50±5.1	4
	30	68.50±4.4	6
	90	72.25±3.0	0
	200	60.50±2.5*	18
	500	105.30±2.2 †	−49
Sophoradiol (**4**)	0 (Ref.)	69.50±2.4	—
	10	69.50±3.7	0
	30	67.25±1.3	3
	90	68.25±2.8	2
	200	69.25±2.2	0
	500	61.00±2.0*	13
Soyasaponin I (**1a**) (Positive control)	0 (Ref.)	70.75±1.7	—
	10	71.25±2.6	−1
	30	71.00±3.8	0
	90	70.75±5.2	0
	200	61.75±5.3	14
	500	32.00±2.2**	59

Hepatoprotective activity of compounds **1**—**4** and **1a** toward *in vitro* immunological liver injury in primary cultured rat hepatocytes. Significantly different from Reference, effective * $p<0.01$, ** $p<0.001$, toxic † $p<0.001$.

Table 2. Cytotoxicity toward Hepatocytes of **1**—**4**, **1a**, **2a**, **3a** and **4a**

Substances	Dose (μM)	ALT	
		IU/l	Cytotoxicity (%)
Kaikasaponin III (**1**)	0	5.00±0.8	—
	10	4.75±1.0	95
	30	4.50±1.0	90
	90	4.00±0.0	80
	200	4.75±1.0	95
	500	3.75±0.5	75
Kaikasaponin I (**2**)	0	4.00±0.0	—
	10	4.50±1.0	113
	30	5.75±1.0	144
	90	4.50±0.6	113
	200	5.50±1.3	138
	500	6.25±1.9	156
Sophoradiol monoglucuronide (SoMG, **3**)	0	5.00±0.8	—
	10	5.75±1.7	115
	30	5.25±0.5	105
	90	5.75±1.0	115
	200	17.50±1.0 †	350
	500	33.00±3.8 †	660
Sophoradiol (**4**)	0	5.50±1.3	—
	10	4.50±0.6	82
	30	4.50±1.0	82
	90	5.00±0.8	91
	200	5.25±1.5	95
	500	5.50±0.6	100
Soyasaponin I (**1a**)	0	4.75±1.0	—
	10	5.75±0.5	121
	30	4.50±0.6	95
	90	4.50±0.6	95
	200	5.00±0.0	105
	500	4.25±0.5	89
Soyasaponin III (**2a**)	0	4.25±1.0	—
	10	3.50±0.6	82
	30	4.25±1.0	100
	90	4.50±0.6	106
	200	4.25±0.5	100
	500	6.00±0.8	141
Soyasapogenol B monoglucuronide (SBMG, **3a**)	0	5.00±0.8	—
	10	4.50±1.0	90
	30	4.25±1.0	85
	90	4.00±0.8	80
	200	4.25±0.5	85
	500	5.50±1.3	110
Soyasapogenol B (**4a**)	0	4.25±1.3	—
	10	5.00±0.0	118
	30	3.50±1.0	82
	90	4.50±0.6	106
	200	4.75±1.3	112
	500	4.25±1.0	100

Cytotoxicity of compounds **1**—**4**, **1a**, **2a**, **3a** and **4a** in primary cultured rat hepatocytes. Significantly different from Reference, toxic † $p<0.001$.

On the other hand, Shiraki reported that glycyrrhizin inhibited the replication of hepatitis B virus after penetrating hepatocyte membranes.[25] Since soyasaponin I (**1a**) also exhibited antiviral effects against several viruses,[26] OG might exhibit not only a protective action on hepatocyte membranes but also an anti-hepatitis virus action after penetrating the membranes.

Acknowledgement This research was supported by Grants-Aid for Scientific Research from Suntory Institute for Bioorganic Research.

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