Hepatoprotective Constituents in Plants 14. Effects of Soyasapogenol B, Sophoradiol, and Their Glucuronides on the Cytotoxicity of *tert*-Butyl Hydroperoxide to HepG2 Cells¹⁾

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The effects of soyasapogenol B, sophoradiol, their glucuronides, and glycyrrhizin on the hepatotoxicity of *tert*-butyl hydroperoxide (*t*-BuOOH) in a human-liver-derived cell line (HepG2 cells) were investigated. Glycyrrhizin showed significant dose-dependent protective effects against the cytotoxicity of *t*-BuOOH. Among soyasapogenol B and its glucuronides, the monoglucuronide showed the most potent hepatoprotective activity, followed by soyasapogenol B itself. Soyasaponin III was weakly protective, while soyasaponin I increased the toxicity of *t*-BuOOH. Among sophoradiol and its glucuronides, sophoradiol itself showed the most potent hepatoprotective activity, which was equal to glycyrrhizin, while the monoglucuronide and kaikasaponin III showed an increase in cytotoxicity. These results were considerably different from those reported previously on the protective effects of these compounds using primary cultures of immunologically injured rat liver cells. Consequently, the hepatoprotective action of the triterpene derivatives investigated would be different in HepG2 cells and in rat primary hepatocyte cultures.

Key words hepatoprotective triterpenoid; HepG2 cell; *tert*-butyl hydroperoxide; soyasapogenol B glucuronide; sophoradiol glucuronide

Saponins are glycosidic compounds present in many edible and inedible plants. Structurally, they are composed of a lipid-soluble aglycone consisting of either a sterol or, more commonly, a triterpenoid and water-soluble sugar residues differing in type and amount of sugars.²⁾ Because of their amphiphilic nature, they are highly surface-active. Their biological activity is closely related to the chemical structures that determine the polarity, hydrophobicity, and acidity of compounds.²⁾ A recent review by Safayhi and Sailer shows that pentacyclic triterpenes, including the oleanane skeleton, might be a rich natural resource of lead compounds for anti-inflammatory drug development.³⁾ Some oleanane-type triterpenoidal saponins are known to exhibit hepatoprotective actions.³⁾ 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-17 and a glucuronic acid moiety at the C-3 hydroxy group of the triterpene.¹⁰⁾ Although glycyrrhizin belongs to OG, soyasaponin I¹¹⁾ is a representative OG.

Puerariae Flos, the flowers of *Pueraria lobata*, is a crude drug used to counteract the overconsumption of alcohol in traditional Japanese and Chinese therapeutic systems.¹²⁾ We have found that the total saponin fraction in this crude drug was effective in an experimental *in vivo* model of hepatic injury.¹³⁾ Abri Herba, the whole plant of *Abrus cantoniensis*, is used as a folk medicine for hepatitis in China. Its efficacy has been substantiated clinically.¹⁴⁾ We reported that the total saponin fraction and soyasaponin I isolated from this plant were effective for experimental liver injuries *in vivo* induced by CCl₄.¹⁵⁾ We have also reported the hepatoprotective activity of soyasaponin I and kaikasaponin III isolated from this drug against CCl₄-induced cytotoxicity in primary cultured

rat hepatocytes.¹⁶⁾

In a series of studies of hepatoprotective drugs, we devised an *in vitro* assay method in which the extent of immunological liver injury was measured in rat primary hepatocytes cultures.¹⁷⁾ We examined the preventive effect of soyasapogenol B^{18,19)} and sophoradiol¹⁾ glucuronides using this method, and elucidated some structure–activity relationships.

However, because of the expense and the time involved, the use of primary cultured rat hepatocytes is not convenient for the screening of a large number of plant extracts and constituents. Therefore, Thabrew *et al.* have devised a reproducible microplate screening assay method, based on the quantitative measurement of cytotoxicity of *tert*-butyl hydroperoxide (*t*-BuOOH) against human-liver-derived HepG2 cells.²⁰⁾ HepG2 cells have been suggested to be a useful *in vitro* model for investigation of the metabolism and toxicity of drugs compared with animal cells, since HepG2 cells retain many of the specialized functions which are characteristic of normal human hepatocytes, including the synthesis and secretion of plasma proteins.²¹⁾ In order to clarify the efficacy of soyasapogenol B and sophoradiol glucuronides and their aglycones on human-liver-derived cell lines, we investigated their hepatoprotective effects against the cytotoxicity of *t*-BuOOH in HepG2 cells.

MATERIALS AND METHODS

Instruments The instruments used in this study were the same as those described in previous papers.^{1,22)}

Materials Soyasapogenol B and sophoradiol and their glucuronides have been isolated from some fabaceous plants according to previous papers.^{1,19)} Glycyrrhizin was purchased from Tokyo Chemical Ind. Co. (Tokyo, Japan). RPMI 1640 medium, fetal bovine serum (FBS), trypsin/EDTA, Dulbec-

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co's phosphate-buffered saline (PBS), and kanamycin sulfate were purchased from Lifetech (Rockville, MD, U.S.A.). 70% *tert*-Butyl hydroperoxide (*t*-BuOOH) solution was obtained from Wako Chemical Co. (Osaka, Japan). AlamarBlue²³ was purchased from Nacalai Tesque (Kyoto, Japan).

Cells HepG2 cells were supplied from the Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University.

Determination of Hepatoprotective Activity The hepatoprotective activity was examined according to the method described by Thabrew.²⁰ HepG2 cells were cultured in 150 cm³ flasks at 37 °C in a humidified atmosphere of 5% CO₂/95% air. RPMI 1640 medium supplemented with 10% (v/v) FBS and kanamycin sulfate (100 µg/ml) was used for cultivation. Cells were routinely grown in monolayer until 80% confluence. Cell suspensions were prepared by rinsing a confluent monolayer with PBS and treating the cell layer with trypsin/EDTA for 4 min. After inactivation of trypsin by FBS, the cells were centrifuged at 150×*g* for 5 min and resuspended in the above medium. The cell suspension (100 µl per well) was placed in 96-well plates (3×10⁴ cells per well), and incubated at 37 °C for 24 h. After cultivation, dimethyl sulfoxide (DMSO) solution at various concentrations of the test samples was added to the wells. The final concentration of DMSO in the solution in each well was 1.6%. The treatment with DMSO only was always used as a control (see below). After incubation for 1 h, the *t*-BuOOH solution (10 µl) was added to the wells. The *t*-BuOOH solution was adjusted to 1.25 mM in ice-cold RPMI 1640 medium immediately prior to addition. After 2 h incubation under the above-mentioned conditions, cell viability was determined using AlamarBlue as described by Lancaster and Fields.²⁴ Cells in the plates were incubated at 37 °C for 3 h after addition of AlamarBlue (12 µl). Cell viability was determined by measuring the absorption of the reduced form of AlamarBlue using a 96-well microtiter plate reader at 570 nm (595 nm reference filter). Results are expressed as percentage protection, *i.e.*, the percentage increase in cell viability compared to the viability of cells treated with the *t*-BuOOH alone. The percent protection is calculated as {(Sample-Control)/(Reference-Control)}×100. Reference is the absorbance value of those not challenged with *t*-BuOOH and samples. Control is the absorbance value of those challenged with *t*-BuOOH and not treated with samples. The viability of HepG2 cells treated with *t*-BuOOH (1.25 mM) for 2 h was 30.1±0.8%.

Statistical Analysis The data are shown as the mean±S.D. of four wells. After analysis of variances, Dunnett's test was employed to determine the significance of differences between control and experimental samples.

RESULTS

We examined the hepatoprotective activity of glycyrrhizin at first, since it has been successfully used to treat chronic hepatitis,³ and it was proved to be hepatoprotective against *in vitro* immunological injury by antiserum in rat primary hepatocyte cultures.¹⁷ Glycyrrhizin showed significant dose-dependent protective effects against the cytotoxicity of *t*-BuOOH in HepG2 cells (Fig. 1). For example, at the highest concentration (500 µM), cell viability was improved significantly (*p*<0.01) up to 52.2±5.7%.

Next, we examined the hepatoprotective activity of several glucuronides which showed potent protective activity to *in vitro* immunological injury of rat primary hepatocyte cultures,^{1,19} together with their aglycones, soyasapogenol B (**1d**) and sophoradiol (**2d**). The glucuronides investigated were soyasaponins I (**1a**), III (**1b**), soyasapogenol B monoglucuronide (SBMG, **1c**), kaikasaponins III (**2a**), I (**2b**), and sophoradiol monoglucuronide (SoMG, **2c**). The results are shown in Table 1.

Soyasaponin I (**1a**), a trisaccharide of **1d**, did not show any hepatoprotective activity against the cytotoxicity of *t*-BuOOH in HepG2 cells, although it showed more potent hepatoprotective activity than glycyrrhizin in *in vitro* experiments using rat primary hepatocyte cultures and antiserum.¹⁷ In a similar manner, kaikasaponin III (**2a**), a trisaccharide of **2d** and the most potent hepatoprotective trisaccharide in the rat model, also did not show any hepatoprotective activity against the cytotoxicity of *t*-BuOOH in HepG2 cells. In contrast, both trisaccharides showed strong hepatotoxicity (Table 1). The disaccharides [soyasaponin III (**1b**) and kaikasaponin I (**2b**)] of **1d** and **2d** exhibited greater effects than the trisaccharides in rat primary hepatocyte cultures.^{1,19} However, **1b** and **2b** showed very weak protection of HepG2 cells against *t*-BuOOH. Similar to the experiment using rat primary hepatocyte cultures, the monoglucuronide (**1c**) of **1d** showed moderate dose-dependent protective effects in HepG2 cells. On the contrary, the monoglucuronide (**2c**) of **2d** demonstrated the strong hepatotoxicity at the higher dose in HepG2 cells, although it was slightly effective at 30 µM. On the other hand, soyasapogenol B (**1d**) itself showed weak protective activity at every dose, while sophoradiol (**2d**) exhibited signifi-

Table 1. Hepatoprotective Activity of Glycyrrhizin, Compounds **1a**—**d** and **2a**—**d**

Sample	Dose (μM)	Protection (%)
Glycyrrhizin	10	1.5 ± 2.2
	30	6.9 ± 2.0
	90	$23.8 \pm 2.6^{**}$
	200	$39.5 \pm 6.6^{**}$
	500	$52.2 \pm 5.7^{**}$
Soyasaponin I (1a)	10	0.3 ± 6.6
	30	0.3 ± 5.1
	90	$-12.4 \pm 4.2^{\dagger}$
	200	$-15.6 \pm 4.0^{\dagger}$
	500	$-32.8 \pm 3.5^{\dagger}$
Soyasaponin III (1b)	10	-2.6 ± 3.2
	30	1.9 ± 7.0
	90	4.6 ± 5.7
	200	$12.6 \pm 7.0^{*}$
	500	8.4 ± 4.4
Soyasapogenol B monoglucuronide (SBMG, 1c)	10	-1.5 ± 4.0
	30	8.5 ± 2.0
	90	$22.9 \pm 8.8^{**}$
	200	$28.5 \pm 4.9^{**}$
	500	$32.4 \pm 10.1^{**}$
Soyasapogenol B (1d)	10	$13.7 \pm 9.4^{**}$
	30	$22.3 \pm 4.2^{**}$
	90	5.9 ± 3.8
	200	$21.3 \pm 4.1^{**}$
	500	$15.9 \pm 2.4^{**}$
Kaikasaponin III (2a)	10	-1.1 ± 4.6
	30	2.6 ± 6.3
	90	$-11.6 \pm 2.5^{\dagger}$
	200	$-22.1 \pm 5.1^{\dagger}$
	500	$-52.3 \pm 2.2^{\dagger}$
Kaikasaponin I (2b)	10	1.1 ± 4.3
	30	1.1 ± 3.2
	90	0.0 ± 3.4
	200	3.8 ± 5.1
	500	8.4 ± 4.9
Sophoradiol monoglucuronide (SoMG, 2c)	10	6.1 ± 7.5
	30	$16.6 \pm 6.7^{**}$
	90	-7.7 ± 5.2
	200	$-41.5 \pm 2.9^{\dagger}$
	500	$-54.2 \pm 1.1^{\dagger}$
Sophoradiol (2d)	10	8.8 ± 4.5
	30	$22.5 \pm 4.1^{**}$
	90	$40.2 \pm 6.1^{**}$
	200	$41.7 \pm 6.1^{**}$
	500	$48.4 \pm 9.5^{**}$

Effect of samples on *t*-BuOOH (1.25 mM)-induced cytotoxicity in HepG2 cells. The percent protection is calculated as $\{(\text{Sample-Control})/(\text{Reference-Control})\} \times 100$. Reference is the absorbance value of those not challenged with *t*-BuOOH and samples. Control is the absorbance value of those challenged with *t*-BuOOH and not treated with samples. Significantly different from control value, effective * $p < 0.05$, ** $p < 0.01$. Significantly different from control value, toxic $^{\dagger}p < 0.01$.

cant dose-dependent protective effects in HepG2 cells. The hepatoprotective activity of **2d** at 500 μM was equal to that of glycyrrhizin.

DISCUSSION

Among soyasapogenol B (**1d**) and its glucuronides (**1a**—**c**), the decreasing order of the hepatoprotective effect on the cytotoxicity of *t*-BuOOH in HepG2 cells was monoglucuronide (**1c**) > aglycone (**1d**) > disaccharide (**1b**). The trisaccharide (**1a**) showed strong hepatotoxicity. In *in vitro* experiments using rat primary hepatocyte cultures and antiserum, the order of potency was **1c** $\geq$ **1b** $\gg$ **1a** $\geq$ **1d**.¹⁹⁾ The trisaccha-

ride (**1a**) did not demonstrate any cytotoxicity in rat hepatocytes.¹⁹⁾ Among sophoradiol (**2d**) and its glucuronides (**2a**—**c**), the aglycone (**2d**) itself showed the most potent hepatoprotective effect on human-liver-derived HepG2 cells that was equal to glycyrrhizin, which is used to treat chronic hepatitis. In the rat model, **2d** showed very weak protective activity that was less than **1d**. Although the trisaccharide (**2a**) did not show any cytotoxicity in rat hepatocytes,¹⁾ it exhibited a strong hepatotoxicity in HepG2 cells similar to **1a**. However, **1a** and **2a** did not exhibit hepatotoxicity in HepG2 cells without *t*-BuOOH (data not shown). Further, **1a** and **2a** showed anticomplementary activity, *i.e.*, inhibition of the hemolysis of human serum against sensitized erythrocytes.²⁵⁾ Consequently, the hepatoprotective action of the triterpenes investigated would be different in HepG2 cells and in rat primary hepatocyte cultures. The difference may be due the difference between human hepatocytes and rat hepatocytes.

Puerariae Flos¹³⁾ and Abri Herba¹⁴⁾ are known to be hepatoprotective crude drugs. Whereas three major saponins obtained from these crude drugs are weakly hepatoprotective kaikasaponin I (**2b**), or strongly hepatotoxic sophoradiol monoglucuronide (**2c**) and kaikasaponin III (**2a**). It seems that there might be some inconsistency in the facts. However, the hepatoprotective properties of these crude drugs could be reasonably attributed to sophoradiol (**2d**) generated from its glucuronides (**2a**—**c**) by hydrolysis by intestinal bacteria.²⁶⁾

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