Hepatoprotective Effects of Irisolidone on *tert*-Butyl Hyperoxide-Induced Liver Injury

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To clarify the hepatoprotective effects of kakkalide and its metabolite irisolidone by human fecal microflora, their effects on *tert*-butyl hydroperoxide (*t*-BHP)-injured HepG2 cells and mice were investigated. Irisolidone protected HepG2 cells against cytotoxicity induced by *t*-BHP. However, kakkalide did not protect cytotoxicity. When kakkalide 100 mg/kg was orally administered to mice injured by *t*-BHP, it significantly inhibited the increase in plasma alanine aminotransferase and aspartate aminotransferase activities by 84% and 85% of *t*-BHP-treated control group, respectively. The inhibitory effect of kakkalide is much more potent than that of silybin, a hepatoprotective agent. However, intraperitoneally administered kakkalide did not exhibit hepatoprotective activity. When irisolidone was intraperitoneally administered to mice, it exhibited potent hepatoprotective activity. Based on these findings, irisolidone can be hepatoprotective and kakkalide may be a prodrug transformed to irisolidone.

Key words irisolidone; kakkalide; HepG2 cell; *tert*-butyl hydroperoxide; hepatic injury; intestinal bacteria

tert-Butyl hydroperoxide (*t*-BHP) can be metabolized to free radical intermediates by cytochrome P450 (hepatocytes) or hemoglobin (erythrocytes), which can subsequently initiate lipid peroxidation,[1] affect cell integrity, and form covalent bonds with cellular molecules resulting in cell injury.[2] *t*-BHP is known to cause lactate dehydrogenase and alanine aminotransferase (ALT) leakage in hepatocyte cells.[3,4] Therefore *t*-BHP has been used as a chemical inducer for the preparation of liver-injured animal models.[5,6]

Most herbal medicines are orally administered, and most components of these medicines inevitably come into contact with intestinal microflora in the alimentary tract. Some are transformed by intestinal bacteria before their absorption in the gastrointestinal tract.[1,2] Among these traditional Chinese medicines, Puerariae Flos is used to counteract problems associated with alcohol drinking and liver injury.[7] Niiho *et al.* reported that the isoflavonoid fraction of Puerariae Flos suppressed the increase in the concentration of blood ethanol, acetaldehyde, and ketones induced by ethanol administration and that its isoflavonoid and triterpenoid saponin fractions improved both the abnormal metabolism induced by ethanol and hepatic injuries induced by carbon tetrachloride or high-fat food.[8,9] Yamazaki *et al.* reported that intraperitoneally administered kakkalide, which was isolated from Flos Puerariae, reduced the mortality associated with administration of ethanol and serum ALT and aspartate aminotransferase (AST) activities.[10] Recently, Kinjo *et al.* have also reported that isolated kaikasponins showed *in vitro* hepatoprotective effects in rat primary liver cell cultures and HepG2 cells.[11,12] Han *et al.* reported that, when kakkalide isolated from Puerariae Flos was metabolized to irisolidone by human intestinal microflora, irisolidone reduced the mortality associated with adminstration of ethanol in mice and showed hepatoprotective activity.[13] In addition, Park *et al.* reported that tectoridin isolated from Purerariae Flos could be metabolized to tectorigenin, which showed antiallergic activity more potently than that of tectoridin.[14] However, the hepatoprotective effects of irisolidone on free radical-mediated hepatotoxicity in aminal has not been studied. Therefore we isolated kakkalide from Puerariae Flos and its metabolite irisolidone transformed by human intestinal microflora (Fig. 1) and then investigated their hepatoprotective effects in *t*-BHP-injured HepG2 cells and mice.

MATERIALS AND METHODS

Materials *t*-BHP was purchased from Sigma Co. (St. Louis, MO, U.S.A.). Diagnostic kits for AST and ALT were purchased from Asan Pharmaceutical Co., Ltd. (Korea). Silybin (Fig. 2) was purchased from Carl Roth (Karlsruhe, Germany). Other chemicals used in this study were of analytical reagent grade.

Isolation of Kakkalide and Irisolidone from Flowers of *Pueraria thunbergiana* Kakkalide and irisolidone were isolated according to the previous methods.[13,15] The flowers of *Pueraria thunbergiana* (500 g), which were produced in Korea, were extracted with 2.5 l of boiling water, concentrated in a rotary evaporator, extracted three times with ethyl acetate, and evaporated. The resulting extract (28 g) was loaded on a silica-gel flash column chromatograph and eluted

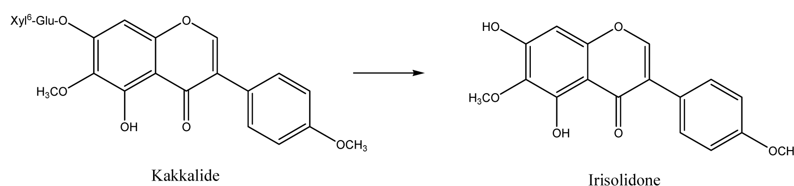

Fig. 1. Proposed Metabolic Pathway of Kakkalide by Human Intestinal Microflora

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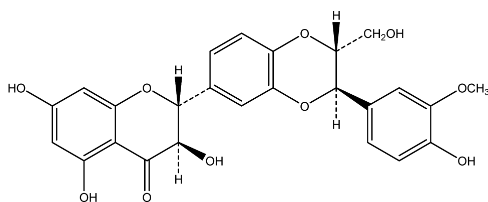

Fig. 2. Structure of Silybin

with $CHCl_3$: MeOH (20 : 1 → 4 : 1). We isolated kakkalide (2.3 g). The kakkalide (1 g) was incubated with human fecal microflora, and then irisolidone (0.2 g) was isolated.

Kakkalide: Pale yellowish needles, mp 251—253 °C. IR (KBr) ν_{max} cm^{-1}: 3500, 3350, 3200 (OH), 1635 (C=O), 1605, 1580, 1510 (phenyl). FAB-MS: 609 $[M+H]^+$.

Irisolidone: Pale yellowish amorphous powder, mp 189—190 °C. IR (KBr) ν_{max} cm^{-1}: 3447 (OH), 1648 (C=O) and 1023 cm^{-1}. FAB-MS: 315$[M+H]^+$.

Animals Mice (ICR, male, 20—25 g) were supplied by the Orient Co., Ltd. (Seoul, Korea) and were maintained on pellet food (Charles River Orient Animal Experimental Animal Breeding Center, Seoul, Korea) and tapwater.

Six mice in each group were used. Animals were orally or intraperitoneally administered kakkalide, irisolidone, and silybin suspended in 1% CMC–Na. The control group was given saline (0.1 ml/20 g) instead of the sample compounds. Samples were orally administered three times (once per day) and intraperitoneally administered ones were given once. Animals were intraperitoneally treated with 1.5 mmol *t*-BHP/kg 24 h after the oral administration of the final sample (or 2 h after intraperitoneal administration). Blood samples were collected 18 h after *t*-BHP administration by cardiac puncture under ether anesthesia and serum was obtained by centrifugation (1000×***g***, 15 min).

All procedures relating to animals and their care conformed with the international guidelines Principles of Laboratory Animals Care (NIH publication no. 85-23, revised 1985).

Enzyme Activity Assay ALT and AST in serum were analyzed according to the manufacturer's instructions.[16)]

Culture of HepG2 Cells and *t*-BHP Cytotoxicity Induction HepG2 cells (hepatocellular carcinoma cell line) donated by the Korean Cell Bank (Seoul, Korea) were cultured in MEM containing 10% fetal bovine serum, 1% antibiotic–antimycologic solution and sodium bicarbonate 1.5 g/l under 5% CO_2 at 37 °C. The protective effects of kakkalide or irisolidone in HepG2 cells injured by *t*-BHP were measured using the 3-(4,5-imethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.[17)] Briefly, HepG2 cells were dispensed into 96-well plates at the concentration of 1×10^4 cells/well. The test compounds were added to the HepG2 cells and preincubated for 2 h. Then the culture media were replaced with media containing *t*-BHP (100 μM), incubated for 3 h and then rinsed with phosphate-buffered saline. The MTT reagent (0.25 mg/ml) was added to the cells and incubated for 1 h. Then the supernatant was discarded, and 100 μl of dimethyl sulfoxide (DMSO) added. Absorbance at 540 nm was measured to estimate surviving cells.

Statistics All the data are expressed as mean±S.D. and statistical significance was determined using one way ANOVA followed by Student–Newman–Keuls test.

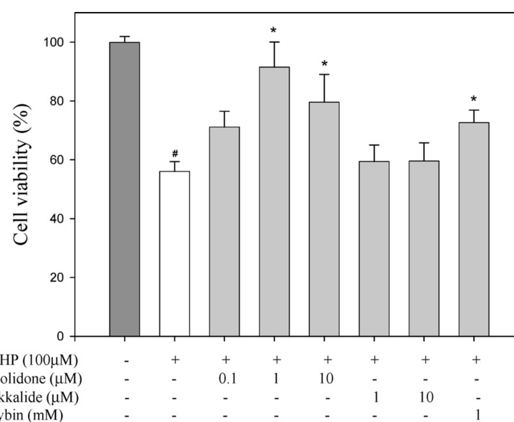

Fig. 3. Cytoprotective Effect of Kakkalide and Irisolidone on *t*-BHP-Toxificated HepG2 Cells

Significantly different compared with the normal control group ($p<0.05$). * Significantly different compared with the *t*-BHP control group ($p<0.05$).

Table 1. Protective Effect of Orally Administered Kakkalide on *t*-BHP-Induced Hepatotoxicity in Mice

Group	*t*-BHP	Dose (mg/kg)	ALT (Karmen unit)	AST (Karmen unit)
Normal control	–	0	39.3±3.1	54.5±6.5
t-BHP control	+	0	94.0±6.4#	120.3±14.1#
Silybin	+	100	52.3±10.1*	60.5±13.7*
Kakkalide	+	100	48.0±8.0*	64.5±8.7*
	+	50	62.8±17.5*	66.0±11.5*
Irisolidone	+	50	52.0±8.1*	66.5±11.6*
	+	25	60.8±20.3*	64.5±15.8*

Significantly different compared with the normal control group ($p<0.05$). * Significantly different compared with the *t*-BHP control group ($p<0.05$).

RESULTS

Protective Effects of Kakkalide and Irisolidone against Cytotoxicity Induced by *t*-BHP To evaluate the hepatoprotective effects of kakkalide, we isolated its metabolite irisolidone transformed by human intestinal microflora and investigated their protective effects against cytotoxicity induced by *t*-BHP in HepG2 cells (Fig. 3). When *t*-BHP alone was added to HepG2 cells, cell viability decreased dose dependently. *t*-BHP at a dose of 100 μM decreased cell viability to 54% of that in the control cells. The pretreatment of HepG2 cells with irisolidone significantly protected against *t*-BHP-induced cytotoxicity. Irisolidone at a dose of 1 μM protected HepG2 cells against *t*-BHP-induced cytotoxicity, with cell viability 92% of that in the control group. The protective effects of irisolidone was more potent than those of silybin, a commercial hepatoprotective agent, which resulted in 73% cell viability compared with the control group at a dose of 1 μM. However, kakkalide at the concentration of 10 μM was not effective against *t*-BHP-induced cytotoxicity. Irisolidone and kakkalide did not show cytotoxicity at a concentration of less than 20 μM.

Hepatoprotective Effects of Kakkalide and Irisolidone on *t*-BHP-Induced Liver Injury in Mice The hepatoprotective effects of kakkalide were also investigated in mice in-

Table 2. Protective Effects of Intraperitoneally Administered Irisolidone and Kakkalide on *t*-BHP-Induced Hepatotoxicity in Mice

Group	*t*-BHP	Dose (mg/kg)	ALT (Karmen unit)	AST (Karmen unit)
Normal control	–	0	42.0±5.9	58.7±10.9
t-BHP control	+	0	89.6±10.0#	123.8±20.0#
Silybin	+	100	54.5±17.1*	70.5±9.0*
Kakkalide	+	50	84.5±18.4	103.3±18.3
Irisolidone	+	50	52.5±7.6*	70.0±12.5*
	+	25	59.0±15.9*	69.8±24.9*

Significantly different compared with the normal control group ($p<0.05$). * Significantly different compared with the *t*-BHP control group ($p<0.05$).

jured by *t*-BHP (Table 1). When *t*-BHP was intraperitoneally administered, serum ALT and AST levels were significantly increased compared with those in the normal control group. The reference agent silybin (100 mg/kg) inhibited the increase in serum ALT and AST levels. Orally administered kakkalide also potently inhibited the increase in serum ALT and AST levels induced by *t*-BHP treatment by 84% and 85% compared with the *t*-BHP control group, respectively.

We evaluated the hepatoprotective effects of intraperitoneally administered irisolidone and kakkalide when *t*-BHP was intraperitoneally administered to mice. Serum ALT and AST levels were significantly increased compared with those in the normal control group (Table 2). The reference agent silybin (100 mg/kg) inhibited the increase in serum ALT and AST levels by 73% and 81% compared with the control group treated with *t*-BHP alone, respectively. However, intraperitoneally administered kakkalide 50 mg/kg did not inhibit the increase in ALT and AST levels induced by *t*-BHP treatment. Intraperitoneally administered irisolidone 25 mg/kg inhibited serum ALT and AST levels by 64% and 83% compared with the *t*-BHP control group, respectively.

DISCUSSION

Most traditional medicinal herbs are orally administered and their components inevitably come into contact with intestinal microflora in the alimentary tract. The chemical components of herbs may be transformed by intestinal bacteria before being absorbed by the gastrointestinal tract. Han *et al.* reported that when kakkalide was administered to rats, irisolidone, but not kakkalide, was detected in blood.[13)]

t-BHP and carbon tetrachloride have been used as chemical inducers for the preparation of liver-injured animal models.[3,4)] *t*-BHP also induces hepatotoxicity. Therefore, to understand the hepatoprotective effects of kakkalide and irisolidone against liver injury, we investigated their hepatoprotective activities in HepG2 cells and mice injured by *t*-BHP.

When their protective effects against cytotoxicity in HepG2 cells injured by *t*-BHP were investigated, *t*-BHP showed potent cytotoxicity against HepG2 cells. However, the pretreatment with irisolidone more potently protected against the cytotoxicity than kakkalide. However, irisolidone at a high dose (10 μM) decreased the protective effect. These results may be originated from the cytotoxicity of *t*-BHP being synergistically increased by irisolidone.

Hepatoprotective effects were evaluated *in vivo.* Orally administered kakkalide to mice injured by *t*-BHP treatment potently protected against hepatototoxicity. However, when kakkalide was administered intraperitoneally, it did not show hepatoprotective effects. However, intraperitoneally administered irisolidone showed hepatoprotective effects. These results support the report that intraperitoneally and orally administered irisolidone potently exhibited hepatoprotective effects in an ethanol-induced liver-injury mouse model. The protective effects of irisolidone were similar to those of silybin, a commercial hepatoprotective agent. To investigate the protective mechanism of irisolidone against *t*-BHP-induced liver injury, the antioxidant activity of irisolidone was examined. However, these compounds did not show potent antioxidant activities, such as radical scavenging and xanthine oxidase-inhibitory activities (data not shown). However, irisolidone inhibited procaspase 3 activation of HepG2 cells injured by *t*-BHP, which induced apoptosis (data not shown).

Based on these findings, we believe that kakkalide may be metabolized to irisolidone in the human intestine by intestinal microflora and when Puerariae Flos extract is administered orally, and the biotransformed irisolidone can protect against liver injury.

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