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Protection of Rat Hepatocytes Exposed to CCl_4 In-vitro by Cynandione A, a Biacetophenone from *Cynanchum wilfordii*

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Abstract

To identify hepatoprotective agents from plant sources we use primary cultures of rat hepatocytes injured by CCl_4. The hepatoprotective agents are the compounds that mitigate the injury caused by CCl_4. Using this system we have investigated the biochemical mechanisms involved in the hepatoprotective activity of cynandione A, a biacetopherone, isolated from the roots of *Cynanchum wilfordii* Hemsley (Asclepiadaceae). Cynandione A (50 μM) significantly reduced (approximately 50%) the release into the culture medium of glutamic pyruvic transaminase and sorbitol dehydrogenase from the primary cultures of rat hepatocytes exposed to CCl_4. Glutathione, superoxide dismutase, catalase and glutathione reductase play important roles in the cellular defence against oxidative stress. Cynandione A appeared to protect primary cultured rat hepatocytes exposed to CCl_4 from significant drops in the levels of each of these four specific markers. Cynandione A also ameliorated lipid peroxidation by up to 50% as demonstrated by a reduction in the production of malondialdehyde.

These results suggest that cynandione A protected the hepatocytes from CCl_4-injury by maintaining the level of glutathione and by inhibiting the production of malondialdehyde, due to its radical scavenging properties.

In our search for compounds from natural products that might be valuable in protecting the liver from toxin-induced injury, we found that a methanolic extract of the dried roots of *Cynanchum wilfordii* Hemsley (Asclepiadaceae) showed a significant ability to protect primary cultured rat hepatocytes from the effects of CCl_4 exposure. A primary culture of rat hepatocytes exposed to CCl_4 is the system we use to identifying hepatoprotective compounds (Lee et al 1995; Kim et al 1997). CCl_4 was selected as the toxicant because it is thought that CCl_4 exerts its toxicity by means of its metabolites. Free radicals generated by the cytochrome P450-dependent detoxification step are believed to induce injury either by interacting with the unsaturated fatty acids of cell membranes thereby causing lipid peroxidation, or by binding free radicals covalently to important macromolecules such as proteins, lipids and DNA (Yasuda et al 1980; Slater 1984).

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In Korean traditional medicine *C. wilfordii* has been used as a tonic (Han 1975) but to our knowledge there has been no report on its hepatoprotective activity. In this study, we have documented the isolation and identification of a hepatoprotective compound from *C. wilfordii*. We also attempted to elucidate the mechanism of its hepatoprotective activity by determining its effects on a series of well known biological markers which change within injured hepatocytes, namely, the levels of glutathione and malondialdehyde, and the activities of catalase, superoxide dismutase and glutathione reductase.

Materials and Methods

Animals

Male Wistar rats (200–250 g; Laboratory Animal Center, Seoul National University) were kept on standard rat chow with free access to tap water. They were housed in temperature- and humidity-controlled animal quarters under a 12-h light–dark cycle. All experiments were conducted according

to the guidelines of the Committee on Care and Use of Laboratory Animals of Seoul National University.

Materials

The roots of *C. wilfordii* were purchased from a commercial supplier in Seoul, Korea, and identified by Dr Dae Suk Han, an emeritus professor of the college of Pharmacy, Seoul National University. Waymouth's MB 752/1 medium, supplements for cell culture and other reagents for the evaluation of enzyme activities were obtained from Sigma (St Louis, MO). Chemicals used in the isolation and purification process were obtained from Duksan (Seoul, Korea) and were of the highest purity available.

Extraction and isolation

The air-dried roots of *C. wilfordii* were minced and extracted with 80% CH_3OH. The CH_3OH extract was successively extracted with *n*-hexane, $CHCl_3$ and *n*-BuOH. The $CHCl_3$ layer, which showed the most significant hepatoprotective activity, was fractionated by repeated column chromatography over silica gel using a $CHCl_3$–CH_3OH gradient to elute the active compound. The purified compound was identified as cynandione A by comparison to previously reported spectroscopic data (Lin et al 1997).

Culture of hepatocytes and exposure to CCl_4

Isolated hepatocytes were prepared from male Wistar rats by the collagenase perfusion technique of Berry & Friend (1969) with minor modifications (Kleiman & Hartin 1979; Seglan 1993). Cells were purified by several centrifugations and inoculated at a density of 5×10^5 cells mL^{-1} onto collagen-coated plates. One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to a medium containing 7 mM CCl_4 with or without the samples to be tested for hepatoprotective activity (Kiso et al 1983). After exposure to CCl_4 for 1·5 h, the culture medium was collected and used for the determination of both glutamic pyruvic transaminase and sorbital dehydrogenase activities. After the medium was removed, the cells were harvested in order to determine the intracellular activities of particular antioxidant enzymes as well as the levels of malondialdehyde and glutathione. The morphology of the cells was also examined by phase-contrast microscopy.

Determination of glutamic pyruvic transaminase and sorbital dehydrogenase activities

The activities of glutamic pyruvic transaminase and sorbital dehydrogenase in the culture medium were determined by the methods of Reitman & Frankel (1957) and Gerlach (1965), respectively.

Antioxidant enzyme activity

Mitochondrial fractions were prepared from the cultured rat hepatocytes as described previously (Gibson & Skelf 1988). Superoxide dismutase activity was determined according to the method of McCord & Fridovich (1969) using the xanthine–xanthine oxidase reaction. The absorbance was read at 550 nm. Units of activity were extrapolated from a superoxide dismutase standard curve that was constructed using the human erythrocyte enzyme as a standard. Catalase activity was determined according to the method of Beers & Sizer (1952) based on H_2O_2 decomposition. The rate of decomposition was followed at 240 nm. One unit was equivalent to 1 μmol H_2O_2 consumed min^{-1} (mg protein)$^{-1}$. Glutathione disulphide reductase activity, based on the reduction of glutathione disulphide by glutathione disulphide reductase and NADPH, was measured according to the method of Carlberg & Mannervik (1975). The assay measured the decrease in absorbance at 340 nm as NADPH was converted to NADP. One unit of activity was defined as 1 μmol NADPH oxidized min^{-1} (mg protein)$^{-1}$; NADPH oxidation was followed spectrophotometrically at 340 nm. Glutathione-S-transferase (GST) activity was determined spectrophotometrically by measuring the rate of formation of the glutathione and 1-chloro-2,4-dinitrobenzene conjugate according to the method of Habig et al (1974).

Determination of glutathione and malondialdehyde

The glutathione level was determined spectrophotometrically using enzymatic cycling. Total glutathione (glutathione + glutathione disulphide) was measured in the supernatant by the method of Tietz (1969). To measure glutathione disulphide, glutathione was first removed from the supernatant by reacting it with 4-vinylpyridine. The level of malondialdehyde was determined by the thiobarbituric acid method (Ohkawa et al 1979). Spectrophotometric absorbance was determined at 535 nm; the concentration of malondialdehyde was determined against a 1,1,3,3-tetraethoxypropane standard curve.

Protein assay
The protein content was measured by the method of Lowry et al (1951) with bovine serum albumin as a standard.

Statistical analysis
All data are expressed as the mean ± s.d. The evaluation of statistical significance was determined by one-way analysis of variance.

Results and Discussion

Methanolic extract of the dried roots of *C. wilfordii* Hemsley (Asclepiadaceae) showed a significant ability to protect primary cultured rat hepatocytes from exposure to CCl_4. The methanolic extract was successively extracted with *n*-hexane, $CHCl_3$ and *n*-BuOH and the relative hepatoprotective activities of each of the *C. wilfordii* fractions were determined. As shown in Table 1, the $CHCl_3$ fraction of *C. wilfordii* showed significant protective activity at a concentration of 100 μg mL^{-1}. Repeated column chromatography of the $CHCl_3$ fraction led to the isolation of an active compound, cynandione A, a biacetophenone (Figure 1).

To evaluate the hepatoprotective activity of cynandione A, primary cultured hepatocytes exposed to CCl_4 were exposed simultaneously to cynandione A at concentrations ranging from 1 to 100 μM. When cynandione A was present during exposure to CCl_4, the levels of glutamic pyruvic transaminase and sorbitol dehydrogenase released into the medium from intoxicated primary cultured rat hepatocytes were decreased significantly compared with untreated controls exposed to CCl_4 alone. This effect was most potent at a concentration of 50 μM (Table 2). We tried to further elucidate the biochemical mechanisms involved in the hepatoprotective activity of cynandione A.

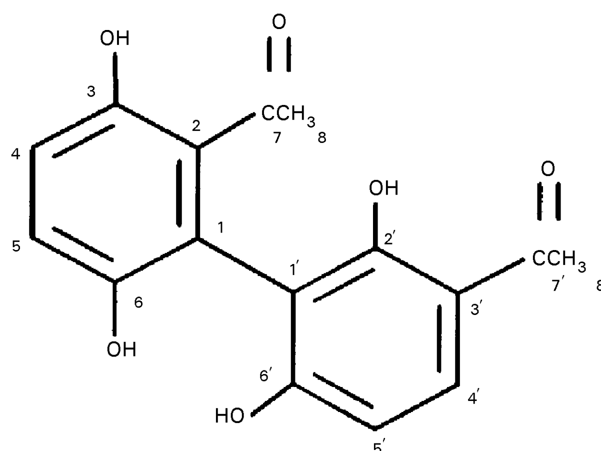

Figure 1. The chemical structure of cynandione A.

Table 1. The effect of each fraction of *C. wilfordii* on glutamic pyruvic transaminase activity released from primary cultured rat hepatocytes exposed to CCl_4.

Fraction of *C. wilfordii*	Glutamic pyruvic transaminase activity (int. units L^{-1})
Control	29·5 ± 2·5
CCl_4	82·4 ± 3·4
Total extract	67·2 ± 2·7
n-Hexane fraction	69·3 ± 6·4
$CHCl_3$ fraction	63·6 ± 4·1*
n-BuOH fraction	75·5 ± 3·9
Aqueous fraction	76·6 ± 6·7

Primary cultured rat hepatocytes were exposed to 7 mM CCl_4 with or without samples. The activity of glutamic pyruvic transaminase was measured as described in Materials and Methods. The concentration of each fraction was 100 μg mL^{-1}. Each value represents the mean ± s.d., n = 3. *$P < 0·05$ compared with cultured hepatocytes exposed to CCl_4 alone.

Glutathione, a ubiquitous tripeptide, is present at a large concentration in the liver and plays a major role in the elimination of a large number of nucleophilic exogenous toxicants. It is important, therefore, in the detoxification of many chemical agents. Glutathione metabolism is also important in quenching the reactive intermediates and radical species generated during oxidative toxicity. The ultimate result of oxidative stress is the formation of glutathione disulphide at the expense of reducing equivalents like NADPH (Huang & Philbert 1995). Glutathione disulphide reductase is necessary for maintaining the cellular homeostasis of reduced glutathione (Meister & Anderson 1983). Enzymes such as glutathioneperoxidase, catalase and superoxide dismutase also play important roles in protecting against free radical damage. A toxicant itself can be a nucleophile or converted to a nucleophile by microsomal enzymes. After the administration of toxicants such as CCl_4, glutathione levels are drastically decreased and, most often, the enzymes responsible for protecting against nucleophilic damage are also affected (Ip et al 1996).

Treatment with 50 μM cynandione A preserved levels of total glutathione significantly and prevented the decrease in glutathione levels caused by CCl_4. This resulted in a decrease in the glutathione disulphide/total glutathione ratio, an index of glutathione redox status (Table 3). Moreover, 50 μM cynandione A significantly preserved the activities of the antioxidative enzymes superoxide dismutase and catalase, but exerted only a slight effect on glutathione disulphide reductase activity (Table 4). These results suggest that the maintenance of

Table 2. The effect of cynandione A on the activities of glutamic pyruvic transaminase and sorbitol dehydrogenase released from primary cultured rat hepatocytes exposed to CCl_4.

Group	Glutamic pyruvic transaminase activity (int. units L^{-1})	Sorbitol dehydrogenase value (units mL^{-1})
Control	29.5 ± 2.5	2.1 ± 0.1
CCl_4	82.4 ± 3.4	48.1 ± 0.5
CCl_4 + cynandione A 1 μM	79.6 ± 3.7	44.9 ± 2.8
CCl_4 + cynandione A 5 μM	75.2 ± 5.4	41.2 ± 2.4
CCl_4 + cynandione A 10 μM	66.3 ± 4.2*	34.3 ± 1.9*
CCl_4 + cynandione A 50 μM	59.2 ± 4.0**	27.5 ± 2.1**
CCl_4 + cynandione A 100 μM	59.9 ± 5.8**	28.3 ± 3.8**
CCl_4 + silybin 100 μM	51.8 ± 2.8***	21.8 ± 1.3***

Primary cultured rat hepatocytes were exposed to 7 mM CCl_4 with or without cynandione A. The activities of glutamic pyruvic transaminase and sorbitol dehydrogenase were measured as described in Materials and Methods. Silybin (100 μM) was used as a positive control. Each value represents the mean ± s.d. (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with cultivated hepatocytes exposed to CCl_4 alone.

Table 3. The effect of cynandione A on glutathione levels in primary cultured rat hepatocytes exposed to CCl_4.

Group	Total glutathione (nmol (mg protein)$^{-1}$)	Reduced glutathione (nmol (mg protein)$^{-1}$)	Glutathione disulphide/ total glutathione
Control	19.3 ± 1.4	14.0 ± 0.8	0.27 ± 0.01
CCl_4	4.7 ± 0.1	2.5 ± 0.1	0.48 ± 0.02
CCl_4 + cynandione A	10.6 ± 2.7*	7.7 ± 0.2*	0.28 ± 0.05**

Primary cultured rat hepatocytes were exposed to 7 mM CCl_4 with or without cynandione A. The glutathione level was measured as described in Materials and Methods. Each value representes the mean ± s.d., n = 3. *$P < 0.05$, **$P < 0.01$ compared with exposure to CCl_4 alone.

Table 4. Effect of cynandione A on hepatic antioxidant enzymes in primary cultured rat hepatocytes exposed to CCl_4.

Group	Glutathione disulphide reductase (μmol NADPH oxidised min^{-1} (mg protein)$^{-1}$)	Catalase (μmol H_2O_2 consumed min^{-1} (mg protein)$^{-1}$)	Superoxide dismutase (munits mL^{-1})	Malondialdehyde (nmol (mg protein)$^{-1}$)
Control	52.7 ± 4.2	682.7 ± 21.9	27.4 ± 1.8	2.40 ± 0.3
CCl_4	22.1 ± 1.7	312.7 ± 9.2	15.3 ± 1.3	3.41 ± 0.2
CCl_4 + cynandione A	31.4 ± 5.7	563.2 ± 37.9**	21.3 ± 1.5**	2.93 ± 0.3*

Primary cultured rat hepatocytes were exposed to 7 mM CCl_4 with or without cynandione A. Each enzyme's activity was measured as described in Materials and Methods. Each value represents the mean ± s.d., n = 3. *$P < 0.05$, **$P < 0.01$ compared with exposure to CCl_4 alone.

reduced glutathione by cynandione A was due mainly to inactivation of reactive oxygen species via retention of antioxidative enzymes such as catalase and superoxide dismutase. It was probably not due to a promotion of the recycling of glutathione from glutathione disulphide—a process catalysed by glutathione disulphide reductase. Cynandione A showed no significant effect on the activity of glutathione-S-transferase (data not shown), an enzyme which plays a pivotal role in the detoxification and excretion of xenobiotics. Depletion of glutathione by CCl_4 ultimately resulted in lipid peroxidation, which is believed to be responsible in part for subsequent hepatocellular damage (Yasuda et al 1980). The effect of cynandione A on lipid peroxidation was quantified by measuring the production of malondialdehyde. The level of malondialdehyde in hepatocytes exposed to CCl_4 plus cynandione was significantly lower when compared with the cultures exposed to CCl_4 alone.

Cynandione A (50 μM) significantly ameliorated lipid peroxidation as demonstrated by a reduction in malondialdehyde production (Table 4).

From these results, we suggest that the hepatoprotective activity of *C. wilfordii* against injury to primary cultured rat hepatocytes induced by exposure to CCl_4 may be due, at least in part, to the hepatoprotective activity of cynandione A. Due to its radical scavenging properties, cynandione A seems to protect hepatocytes from CCl_4-injury by maintaining the level of glutathione and by inhibiting the production of malondialdehyde. Whether this compound has hepatoprotective effects in-vivo requires investigation.

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