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Dihydroisotanshinone I Protects Against Menadione-Induced Toxicity in a Primary Culture of Rat Hepatocytes

Abstract

Dihydroisotanshinone I is a phenanthrenequinone derivative isolated from the roots of *Salvia trijuga* Diels. The present study demonstrated the hepatoprotective effect of dihydroisotanshinone I against menadione-induced cytotoxicity in a primary culture of rat hepatocytes. Pretreating the cells with dihydroisotanshinone I at concentrations ranging from 2.5 μM to 20 μM for 24 hours caused dose-dependent protection against hepatotoxicity induced by menadione. Intracellular glutathione level and activity of DT-diaphorase have been suggested to play important roles in menadione-induced cytotoxicity. However, treating the hepatocytes with 20 μM dihydroisotanshinone I for 24 hours did not cause a significant change in glutathione level and DT-diaphorase activity. On the contrary, adding dihydroisotanshinone I to freshly isolated hepatocytes at concentrations between 50 nM to 200 nM inhibited NADH-induced superoxide production dose-dependently as indicated by the decrease of lucigenin-amplified chemiluminescence. In addition, dihydroisotanshinone I at concentrations ranging from 5 μM to 20 μM inhibited *tert*-butyl hydroperoxide-induced lipid peroxidation dose-dependently in isolated hepatocytes as indicated by the level of malondialdehyde. These results suggest that the protective action of dihydroisotanshinone I against menadione-induced hepatotoxicity is attributed to its antioxidant properties including the free radical scavenging activity and inhibition of lipid peroxidation.

Key words
Salvia trijuga · Lamiaceae · dihydroisotanshinone I · menadione liver damage · free radical scavenger · lipid peroxidation

Abbreviations

DTD: DT-diaphorase
GSH: glutathione
LDH: lactate dehydrogenase
MDA: malondialdehyde
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TBHP: *tert*-butyl hydroperoxide

Introduction

Menadione (2-methyl-1,4-naphthoquinone, vitamin K3) has been used as an anticancer drug [1]. Menadione-induced toxicities include lipid peroxidation, oxidative damage to cellular macromolecules, disruption of calcium homeostasis, depletion of cellular thiols, and apoptosis. The cytotoxicity of menadione is associated with the generation of reactive oxygen species such as superoxide, singlet oxygen, and hydrogen peroxide [2]. Therefore, menadione has been utilized in assay models of oxidative injury. Menadione can undergo one-electron reduction, resulting in the formation of a semiquinone radical. Semiquinone is a reactive intermediate capable of transferring one electron to molecular oxygen to form the superoxide anion radical. This is well known as a redox cycling reaction [2]. The semiquinone radical itself may also cause cellular damage by reacting directly with

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cellular constituents. On the other hand, menadione can be converted to hydroquinone through a two-electron reduction catalyzed by DT-diaphorase (NAD(P)H:quinone oxidoreductase 1; EC 1.6.99.2). This reaction has been suggested to be a detoxification pathway of menadione since it avoids the formation of semiquinone radical [3].

Dihydroisotanshinone I is a phenanthrenequinone derivative (Fig. **1**) isolated from the roots of *Salvia trijuga* Diels, a medicinal plant used as an antiphlogistic, antibacterial and antituberculosis drug in China. Most studies on this plant have focused on the biological activities of a major derivative, tanshinone IIA [4], [5]. Little information can be found for dihydroisotanshinone I. The present study is the first report to demonstrate the hepatoprotective effect of dihydroisotanshinone I against menadione-induced cytotoxicity in a primary culture of rat hepatocytes. The hepatoprotective action of dihydroisotanshinone I may be attributed to its antioxidant properties including the free radical scavenging activity and inhibition of lipid peroxidation.

Materials and Methods

Preparation of dihydroisotanshinone I

Salvia trijuga was collected in Lijiang, Yunnan province, China. The plant was identified by Professor Xi-wen Li of Kunming Institute of Botany, Chinese Academy of Science, at which a voucher specimen (KIB 86-8-16 Zheng) was deposited. The air-dried plant material was ground into a powder and extracted with 75% acetone. The extract was dissolved in water and partitioned with hexane. The hexane extract was chromatographed on a silica gel column (230–400 mesh, 4 cm × 60 cm) and eluted stepwise with hexane/ethyl acetate mixtures. The fractions of hexane/ethyl acetate (6:4) containing dihydroisotanshinone I were combined and purified by crystallization. The chemical structure of dihydroisotanshinone I was determined by comparison with the reported values of NMR and MS data [6]. The optical rotation value of the compound at 20 °C with sodium D line was determined to be –110.5°.

Primary culture of hepatocytes

SD rats (220–250 g) were maintained on a 12-hour light/dark cycle at 22 °C. Food and water were allowed *ad libitum*. Hepatocytes were prepared from the rats by a two-step collagenase perfusion as described previously [7]. The cells were purified by Percoll gradient centrifugation and cell viability was over 95% as determined by the trypan blue exclusion method. The purified hepatocytes were seeded onto 24-well tissue culture plates at 2 × 10^5 cells per well in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), insulin (0.3 μM) and 10% (v/v) fetal calf serum. The cells were maintained at in humidified atmosphere in 5% CO_2 at 37 °C.

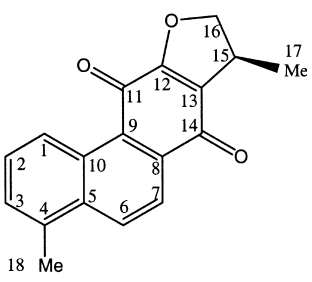

Fig. **1** Chemical structure of dihydroisotanshinone I.

Pretreatment with dihydroisotanshinone I and antioxidants

Dihydroisotanshinone I was dissolved in ethyl acetate and mixed with the culture medium. The final concentration of ethyl acetate was 0.1% (v/v). Twenty-four hours after seeding, the cells were treated with dihydroisotanshinone I, ascorbic acid, and deferoxamine mesylate, respectively. The controls were treated with equal amount of vehicle.

Menadione treatment

Twenty-four hours after drug pretreatment, the cells were washed with Hanks' balanced salts solution. Menadione was dissolved in 0.1% (v/v) DMSO at a final concentration of 30 μM. The cells were treated with menadione in the culture medium for 24 hours at 37 °C. The same amount of DMSO was added to the control cells.

Measurements of cellular damage

Intracellular enzymes such as lactate dehydrogenase (LDH) are released from damaged cells into the culture medium. After menadione treatment, an aliquot of the culture medium was taken for measurement of LDH activity. LDH was assayed using an assay kit from Sigma Chemical Co. (St. Louis, MO, USA). Cell viability is quantified by MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using a Cell Proliferation Assay Kit (Roche, Germany). MTT yielded a blue formazan product in living cells, which was measured spectrophotometrically at 560 nm.

Biochemical analysis

After dihydroisotanshinone I treatment, the hepatocytes were washed by Hanks' balanced salts solution and harvested by scraping. Hepatic glutathione (GSH) level was measured by an HPLC assay method [8]. Hepatic DT-diaphorase activity was assayed by measuring the reduction of acetylated cytochrome c as previously described [9]. The dicumarol inhibitable activity in the assay system was regarded as a measure of the DT-diaphorase activity.

NADH-induced superoxide production in isolated hepatocytes

Superoxide formation was measured by lucigenin-amplified chemiluminescence [10]. Isolated hepatocytes (1 × 10^5 cells/ml) were suspended in phosphate buffer saline (pH 7.4) either in the absence or presence of various concentrations of dihydroisotanshinone I. NADH-stimulated superoxide production was initiated by mixing with 0.25 mM lucigenin and 0.1 mM NADH. Chemiluminescence was recorded and integrated by a luminometer (TD-20/20, Promega). Superoxide level was expressed as relative light units (RLU)/min/1 × 10^5 cells.

TBHP-induced lipid peroxidation in isolated hepatocytes

tert-Butyl hydroperoxide (TBHP)-induced lipid peroxidation was assayed in isolated hepatocytes [11]. Isolated hepatocytes (2 × 10^6 cells/ml) were suspended in phosphate buffer saline (pH 7.4) either in the absence or presence of various concentrations of dihydroisotanshinone I. Lipid peroxidation was induced by adding 1 mM TBHP. The reaction mixture was incubated at

37 °C for 30 min. Following the incubation, the reactions were terminated by 10% (w/v) trichloroacetic acid. After centrifugation, the supernatant was assayed for malondialdehyde (MDA) by mixing 0.8 ml supernatant with 0.4 ml thiobarbituric acid reagent (0.5% (w/v) in 25 mM NaOH). The mixture was incubated at 90 °C for 15 min and the level of MDA was quantitated by HPLC method as described previously [12].

Statistical analysis

Data were analyzed by one-way ANOVA followed by Duncan's multiple range test in order to detect inter-group differences. Significant difference from the control was determined when $p < 0.05$.

Results and Discussion

Chemotherapeutic agents such as menadione possessing a quinone structure play a prominent role in cancer treatment [1]. However, cytotoxicity was observed in different cell types. As shown in Table **1**, exposing a primary culture of hepatocytes to menadione led to decreased cell viability. The toxicity of menadione is believed to be a consequence of oxidative stress induced by its reactive intermediates such as semiquinone radicals and reactive oxygen species. The decrease of cell viability in menadione-treated hepatocytes was associated with an increase of LDH leakage from the cells (Table **1**). However, pretreating the hepatocytes with dihydroisotanshinone I at concentrations ranging from 2.5 μM to 20 μM for 24 hours increased cell viability and reduced LDH leakage dose-dependently.

The cytotoxicity of menadione can be suppressed by free radical scavengers [13] and by stimulation of the detoxification systems [14]. Indeed, treating the hepatocytes with ascorbic acid led to a dose-dependent protection against hepatotoxicity induced by menadione (Table **1**). Previous studies have shown that pretreating cultured hepatocytes with a high dose of deferoxamine (20 mM) inhibited the acute toxicity of menadione (250 μM) after a short period of time (3 hours) [15], [16]. However, the deferoxamine treatment might have only delayed the menadione-induced hepatotoxicity. The present study showed that deferoxamine mesylate, at concentrations ranging from 40 μM to 320 μM, did not produce any protection against hepatotoxicity induced by 30 μM menadione (Table **1**). It suggests that dihydroisotanshinone I may act as a free radical scavenger instead of an ion chelator in the process of hepatoprotection against menadione-induced oxidative injury.

The role of glutathione (GSH) level in menadione-induced cytotoxicity has been demonstrated in many studies. Intracellular GSH level was found to be two-fold greater in menadione-resistant cells (MRc40) than in the parental cells (CHO-K1) [17]. In addition, depletion of GSH by an inhibitor of glutathione synthesis (buthionine sulfoximine) has been found to enhance menadione cytotoxicity in menadione-resistant K300 cells [18]. However, treating the hepatocytes with dihydroisotanshinone I for 24 hours did not cause a significant change in glutathione level (Table **2**). It therefore excludes the role of GSH in the hepatoprotective effect of dihydroisotanshinone I against menadione toxicity.

DT-diaphorase (DTD) catalyzes the two-electron reduction of menadione to hydroquinone without the formation of reactive oxygen species. Hydroquinone can form glucuronide or sulphate conjugates before being excreted. Therefore, the two-electron reduction of menadione catalyzed by DTD is generally regarded as a major detoxification pathway for menadione. It has been demonstrated that over-expression of DTD could protect against the toxic effects of menadione [19]. However, dihydroisotanshinone I treatment did not cause any significant increase of DTD activity in the primary culture of rat hepatocytes (Table **2**). It suggests that the hepatoprotective effect of dihydroisotanshinone I could not be attributed to the activation of DTD in the cells.

In order to understand the antioxidant mechanism involved in the hepatoprotective action of dihydroisotanshinone I against

Table **1** Effect of Dihydroisotanshinone I, Ascorbic acid, and Deferoxamine Mesylate on Menadione-induced Hepatotoxicity

	Cell viability % of control	*LDH leakage % of control*
Menadione-treated Control		
	62.8 ± 4.1	184 ± 10
Dihydroisotanshinone I		
2.5 μM	77.4 ± 5.2*	155 ± 9.0*
5.0 μM	82.9 ± 5.8*	149 ± 8.1*
10 μM	91.4 ± 6.7*	102 ± 6.3*
20 μM	95.4 ± 7.2*	95.9 ± 6.0*
Ascorbic acid		
200 μM	66.0 ± 5.2	175 ± 10
400 μM	80.0 ± 6.3*	159 ± 8.2*
600 μM	87.5 ± 7.0*	144 ± 7.8*
800 μM	89.5 ± 7.1*	133 ± 7.3*
Deferoxamine		
40 μM	56.9 ± 5.6	166 ± 12
80 μM	58.0 ± 5.8	158 ± 10
160 μM	59.7 ± 5.9	160 ± 10
320 μM	58.0 ± 5.8	200 ± 14*

Freshly isolated rat hepatocytes were seeded onto 24-well tissue culture plates with 2×10^5 cells per well. Twenty-four hours after seeding, the cells were treated with dihydroisotanshinone I, ascorbic acid, and deferoxamine mesylate at the indicated concentrations for 24 hours. Cell viability was assessed by MTT assay and hepatocellular damage was measured as leakage of lactate dehydrogenase (LDH) from the cells. Values given are the mean ± SEM, with n = 5. *Significantly different from the menadione-treated control.

Table **2** Effect of Dihydroisotanshinone I on Glutathione Level and DT-Diaphorase Activity in a Primary Culture of Hepatocytes

	Glutathione level (nmol/mg)	*DT-diaphorase activity (mU/mg)*
Control	48.2 ± 2.5	14.5 ± 1.2
Dihydroisotanshinone I	46.8 ± 2.6	15.6 ± 1.3
N-Acetylcysteine	57.5 ± 3.0*	–
1,2-Dithiole-3-thione	–	18.3 ± 1.3*

Freshly isolated rat hepatocytes were seeded onto 24-well tissue culture plates with 2×10^5 cells per well. The cells were treated with 20 μM dihydroisotanshinone I for 24 hours. *N*-Acetylcysteine and 1,2-dithiole-3-thione (100 μM each) were used as positive controls for intracellular glutathione level and DT-diaphorase activity, respectively. Values given are the mean ± SEM, with n = 5. *Significantly different from the control.

menadione toxicity, the effects of dihydroisotanshinone I on NADH-induced superoxide production and TBHP-induced lipid peroxidation were investigated. Addition of dihydroisotanshinone I to a primary culture of hepatocyte at concentrations between 50 nM to 200 nM inhibited NADH-induced superoxide production dose-dependently as indicated by the decrease of lucigenin-amplified chemiluminescence (Fig. **2**). In addition, dihydroisotanshinone I at concentrations of 5 μM to 20 μM inhibited TBHP-induced lipid peroxidation dose-dependently in isolated hepatocytes as indicated by the levels of malondialdehyde, an end product of lipid peroxidation (Fig. **3**).

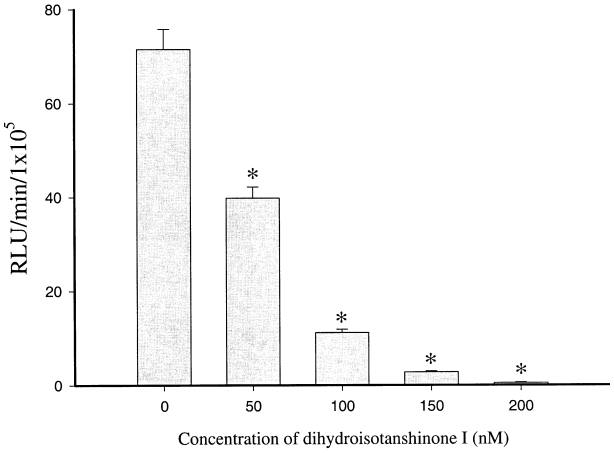

Fig. **2** Effect of dihydroisotanshinone I on NADH-induced superoxide production in isolated hepatocytes. Isolated hepatocytes (1×10^5 cells/ml) were suspended in phosphate buffer saline (pH 7.4) either in the absence or presence of various concentrations of dihydroisotanshinone I. Superoxide production was initiated by mixing with 0.25 mM lucigenin and 0.1 mM NADH. Superoxide level was determined as lucigenin-enhanced chemiluminescence and expressed as relative light units (RLU)/min/1×10^5 cells. Values given are the mean ± SEM, with n = 5. *Significantly different from the control.

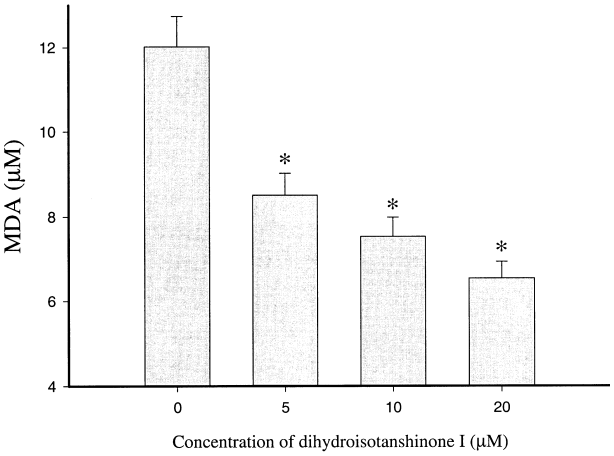

Fig. **3** Effect of dihydroisotanshinone I on TBHP-induced lipid peroxidation in isolated hepatocytes. Isolated hepatocytes (2×10^6 cells/ml) were suspended in phosphate buffer saline (pH 7.4) either in the absence or presence of various concentrations of test samples. Lipid peroxidation was induced by adding 1 mM TBHP. Lipid peroxidation was quantitated in terms of malondialdehyde (MDA) concentration as described under Materia and Methods. Values given are the mean ± SEM, with n = 5. *Significantly different from the control.

In conclusion, the results suggest that the protective action of dihydroisotanshinone I against menadione-induced hepatotoxicity is not related to its actions on intracellular glutathione level and the activity of hepatic DTD. On the contrary, the hepatoprotection is attributed to its free radical scavenging activity and inhibition of lipid peroxidation.

Previous studies have demonstrated the cytotoxicity of dihydroisotanshinone I in tumor cells lines such as HeLa, Colon 205, and Hep-2 [20]. Interestingly, the present study showed that dihydroisotanshinone I suppressed the cytotoxicity of menadione (an antitumor drug) in rat hepatocytes. The combination therapy of dihydroisotanshinone I with menadione in clinical treatment of cancer is worthwhile to be evaluated since dihydroisotanshinone I not only inhibits the growth of cancer cells, but also reduces the cytotoxicity caused by quinone-type antitumor drugs.

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