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EFFECT OF DITHIOL MALONATE DERIVATIVES (DMDs) ON CARBON TETRACHLORIDE-INDUCED HEPATOTOXICITY IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

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ABSTRACT: *Protective effects of dithiol malonate derivatives (DMDs), YH-100, YH-150 and YH-439 on carbon tetrachloride-induced hepatotoxicity were investigated in primary rat hepatocytes culture. Treatment of DMDs to hepatocytes culture did not affect total cytochrome P-450 content and ECOD and AHH activities. Protein and RNA synthesis was also similar to control. Meanwhile, DMDs significantly decreased LDH release and in vitro lipid peroxidation induced by CCl_4. Accumulation of cellular triglyceride and decreased secretion of VLDL from liver cells by CCl_4 treatment were also significantly protected. Although DMDs themselves did not increase protein synthesis in hepatocytes culture, they effectively recovered CCl_4-induced inhibition of protein synthesis. These results suggest that DMDs may protect the hepatotoxicity induced by CCl_4 without any alteration of the normal function of rat hepatocytes in primary culture.*

Key Words: *Hepatotoxicity protection, dithiol malonate derivative, hepatocytes culture.*

INTRODUCTION

A chemically unique hepatotrophic agent diisopropyl 1,3-dithiol-2-ylidene malonate has been used for the treatment of chronic hepatitis and liver cirrhosis (Imazumi *et al.*, 1982). Recently, series of dithiol malonate derivative were synthesized

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as prodrugs (Park and Lee, 1987). The present study was undertaken to determine hepatoprotective effect of these dithiol malonate derivatives. We used primary rat hepatocyte culture since cultured liver cells have been extensively used as essential components of *in vitro* systems designed to detect potentially toxic chemicals, to elucidate the cellular pathways of metabolism of chemcals, and/or to determine the mechanisms by which active chemicals produce toxic cellular damages (Grisham, 1979).

Carbon tetrachloride (CCl_4) was used as a model hepatotoxic compound. Lipid peroxidation is the most well characterized hepatotoxicity of the CCl_4 and this toxic effect is due to the conversion of the molecule to the highly reactive toxic radical (CCl_3) in the endoplasmic reticulum by the mixed function monooxygenase system (Recknagel and Lombardi, 1961; Alpers *et al.*, 1968; Slater, 1982). The free radicals produced cause autooxidation of the polyenoic fatty acids present within the membrane phospholipids. Then the oxidative decomposition of the lipid is initiated and organic peroxides are formed after reacting with oxygen, which called lipid peroxidation (Cosgrove *et al.*, 1987). In addition to the lipid peroxidation, several more slowly developing consequences have also been well characterized, including hepatic fat accumulations, decreased protein synthesis, glycogenolysis, leakage of cellular enzymes into the blood system, and necrosis (Recknagel, 1967; Hoyumba *et al.*, 1975, Diazani, 1979).

In present study we investigated effects of DMDs on total cytochrome P-450 content, AHH and ECOD activity, protein and RNA synthesis. For CCl4-induced hepatotoxic parameters, we determined *in vitro* lipid peroxidation, accumulation of triglyceride, secretion of VLDL, and protein synthesis in hepatocytes culture.

MATERIALS AND METHODS

Primary Hepatocyte Cultures and DMDs Treatment

Male Sprague-Dawley rats (200~250 g) were used. Hepatocytes were isolated by a collagenase perfusion technique as reported previously (Yang *et al.*, 1983) and cultured in the medium prepared by the method of Dickins and Peterson (1980). DMDs (YH-100, YH-150 and YH-439) were dissolved in dimethyl sulfoxide (DMSO) and added directly to the culture at the designated time. The final concentration to DMSO in the culture was not exceed 0.5% (v/v). DMDs were kindly provided by Dr. J. W. Lee (Yuhan Pharmaceuticals, Korea).

Total Cytochrome P-450 Content and Enzyme Assays

Hepatocytes were cultured for 24 hr with DMDs and whole cell homogenates were prepared by the method of Kim *et al.* (1988). Whole cell homogenates were then centrifuged at 10,000×g for 15 min and the supernatants were diluted with 0.1 M potassium phosphate buffer (pH 7.4). Total cytochrome P-450 content was determined according to the method described by Omura and Sato (1964). Ethoxycoumarin O-deethylase (ECOD) activity was assayed by the method of Greenlee and Poland (1978) and the aryl hydrocarbon hydroxylase (AHH) activity was determined by the method of Nebert and Gelboin (1968). Activity of lactate dehydrogenase (LDH) was determined using Sigma kit (Sigma Chemical Co., St. Louis, MO).

Estimation of Protein and RNA Synthesis

The amount of protein synthesized was determined according to the method of Bonney and Meyer (1974). Culture medium was aspirated off and fresh medium containing ^{3}H-leucine (5 μCi/plate, 0.38 mM) was added to the culture. After 2 hr, the medium was removed and cells were scrapped into a test tube and same volume of 20% trichloroacetic acid (TCA) was added. This was centrifuged at 2, 000$\times$g for 10 min and the pellet was washed twice with 10% TCA. The final pellet was solubilized in 1 ml of 1 N NaOH and radioactivity was determined.

RNA synthesis was determined by measuring the incorporation of ^{3}H-Uridine (15 μCi/plate, 10 mM). The method was similar to that of protein synthesis, but the radioactivity was determined in 1 N KOH hydrolyzable fraction.

In Vitro Lipid Peroxidation

Production of malondialdehyde (MDA) by hepatocytes was measured by the thiobarbituric acid method (Bernheim *et al.*, 1948) using an extinction coefficient of 535 $mM^{-1}cm^{-1}$ (Smith *et al.*, 1982) Incorporation of ^{3}H-Glycerol into Cellular Triacylglyceride and the Release of Lipoprotein (VCDL) into Medium.

Hepatocytes were cultured in the medium containing 5 μCi/ml of 2-^{3}H-glycerol and 1 mM oleic acid for 1 hr, and then CCl_4 was treated for 3 hr. After incubation, triacylglyceride in hepatocytes and VLDL in the medium were extracted and the radioactivity incorporated was counted (Nossen *et al.*, 1984).

Protein Determination

Protein concentration was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin as a standard.

Statistical Analaysis

Results were expressed as the mean $\pm$ S.E. and the significance of the difference between mean values was assessed by Student t-test ($p<0.05$).

RESULTS

To assess the cytotoxic effects of DMDs, hepatocytes were exposed to 1 to 10 μM of DMDs for 24 hr and the release of LDH into culture medium was determined. As shown in Table 1, DMDs did not increase the LDH release up to 10 μM in hepatocytes culture.

Table 2 shows effects of DMDs on total cytochrome P-450 content and its associated monooxygenase activities. Total cytochrome P-450 contents, ECOD and AHH activities were similar to control in DMDs treated group.

Table 3 shows effects of DMDs on protein and RNA synthesis in hepatocytes culture. Treatment of YH-100 and YH-150 tend to increase protein RNA synthesis slightly but was not significant statistically.

Figure 1 shows protective effects of DMDs on CCl_4-induced LDH release in hepatocytes culture. In YH-100 treated group, LDH release was significantly reduced at 1 μM and 10 μM. Meanwhile, YH-150 and YH-439 significantly reduced LDH release from 0.1 μM.

Table 1. Effects of DMDs on LDH release in primary culture of rat hepatocytes

Compound	Concentration (μM)		
	0.1	1	10
YH-100	106.7± 10.2	99.4± 2.7	98.0± 4.6
YH-150	114.6± 1.0	119.9± 2.5	113.8± 4.8
YH-439	99.9± 5.7	106.8± 3.4	97.6± 1.2

LDH activity was measured in the culture medium after 24 hr incubation with DMDs. Each value was expressed as percentage of control cultures.

Table 2. Effects of DMDs on the total cytochrome P-450 content, ECOD and AHH activity in primary cultures of rat hepatocytes

Treatments	Cyt. P-450[a)]	ECOD[b)]	AHH[c)]
Control	40.00	52.16± 1.78[d)]	23.54± 2.72
YH-100			
0.1 μM	ND	50.94± 2.30	20.59± 1.86
1 μM	38.36	56.17± 2.26	23.16± 2.76
10 μM	ND	47.51± 3.44	20.26± 6.36
YH-150			
0.1 μM	ND	50.62± 5.63	24.44± 0.99
1 μM	41.09	77.77± 6.20	24.82± 1.79
10 μM	ND	60.86± 10.29	28.21± 2.74

Hepatocytes were cultured for 24 hr and DMDs were treated for additional 24 hr and the total cytochrome P-450, ECOD and AHH were assayed.
[a)]Total cytochrome P-450, pmole/mg protein, [b)]Ethoxycoumarin O-deethylase, pmole umbelliferone/min/mg protein, [c)]Aryl hydrocarbon hydroxylse, pmole 3-hydroxybenzo(a)pyrene/min/mg protein, [d)]Each value was expressed as mean ± S.E. of three determinations, ND; not determined.

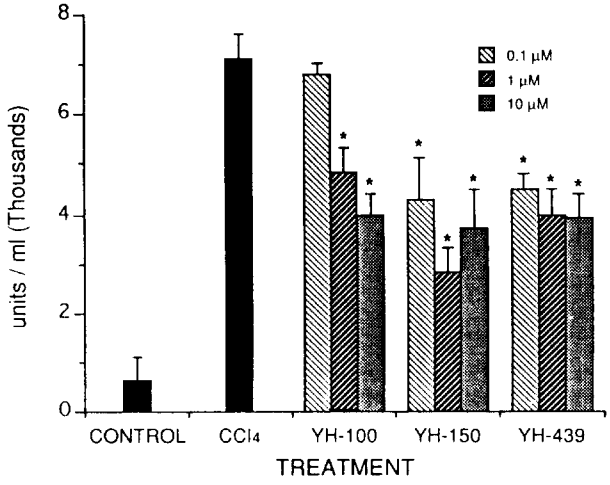

Figure 1. Protective effects of DMDs on LDH release induced by CCl_4. DMDs were added to the culture for 24 hr. After 24 hr, fresh medium containing 4 mM of CCl_4 was added and the cultrues were incubated for 2 hr. An asterisk indicates values significantly different from CCl_4 only ($p<0.05$).

Table 3. Effects of DMDs on protein and RNA synthesis in primary cultures of rat hepatocytes

	Protein synthesis (dpm×10^3/mg protein)	RNA synthesis (dpm×10^3/mg protein)
Control	104.4± 3.4	59.1± 8.5
YH-100	112.7± 5.3	67.1± 9.0
YH-150	105.1± 4.2	62.7± 7.8

Hepatocytes were cultured for 24 hr and DMDs were treated for additional 24 hr. Concentrations of DMDs were 1 μM. Each value was expressed as mean ± S.E. of three determinations.

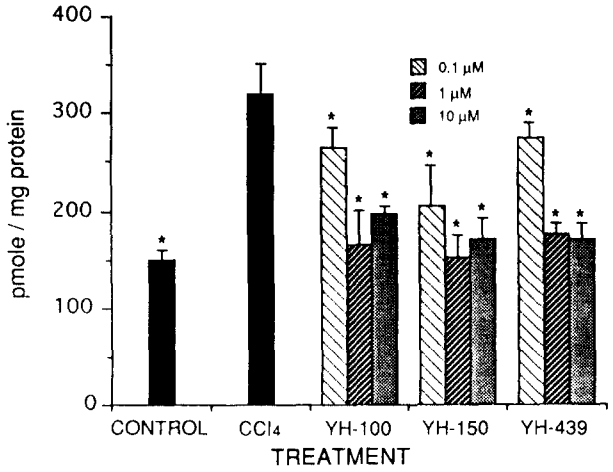

Figure 2. Protective effect of DMDs on CCl_4-induced lipid peroxidation in primary cultures of rat hepatocytes. DMDs were added to the culture for 24 hr. After 24 hr, fresh medium containing 4 mM of CCl_4 was added and the culture were incubated for 2 hr. An asterisk indicates values significantly different from CCl_4 treated group ($p<0.05$).

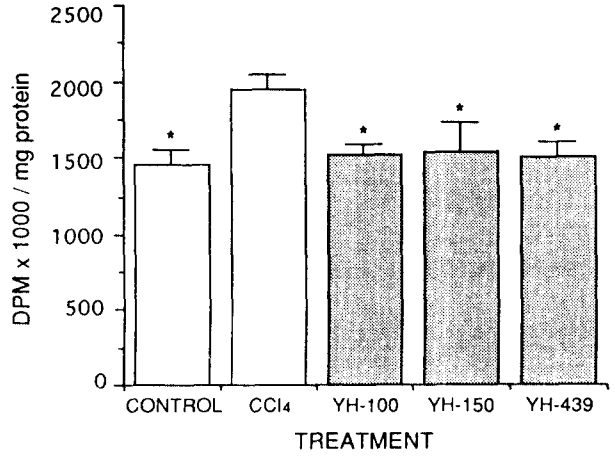

Figure 3. Effects of DMDs on CCl_4-induced increase of ^{3}H-glycerol incorporation into cellular triglyceride. DMDs (10 μM) were added to the cultures for 24 hr. After DMDs treatment, cultures were pulse labelled with ^{3}H-glycerol (5 μCi/ml) for 1 hr. CCl_4 (400 μg/ml) was then treated for 3 hr. An asterisk indicates values significantly different from CCl_4 only ($p<0.05$).

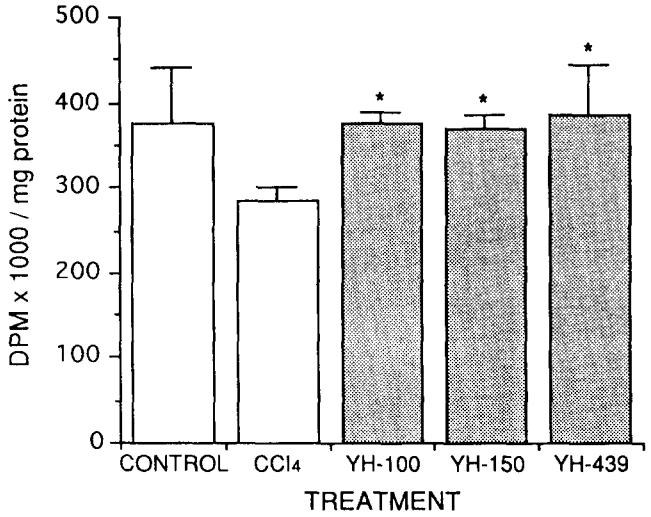

Figure 4. Effects of DMDs on CCl_4-induced suppression of VLDL secretion. Hepatocyte cultures were treated with 10 μM of DMDs for 24 hr. The cultures were then pulse labelled with 3H-glycerol (5 μCi/ml) for 3 hr. CCl_4 (400 μg/ml) was then treated for 3 hr. An asterisk indicates values significantly different from CCl_4 only ($p<0.05$).

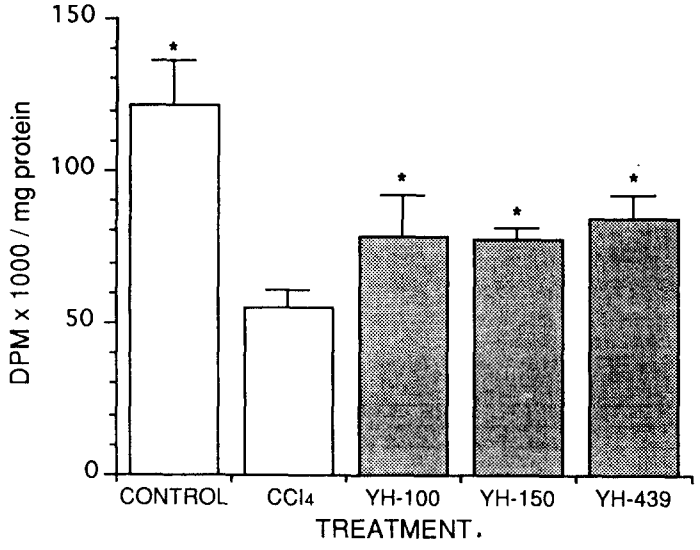

Figure 5. Effects of DMDs on CCl_4-induced suppression of protein synthesis in primary rat hepatocytes. Cultures were treated with 10 μM of DMDs for 24 hr. After DMDs treatment, fresh medium containing ^{3}H-leucine (5 μCi/plate) and CCl_4 (400 μg/ml) was added and the cultures were incubated for 2 hr. An asterisk indicates values significantly different from CCl_4 only ($p<0.05$).

Figure 2 shows protective effect of DMDs on *in vitro* lipid peroxidation induced by CCl_4. The amounts of malondialdehyde produced in control and CCl_4 treated group were 143 and 312 pmole/mg protein, respectively. Treatment of DMDs to hepatocytes culture effectively protected CCl_4-induced lipid peroxidation. The amounts of malondialdehyde produced in 10 μM of YH-100, YH-150 and YH-

439 treated groups were 195, 169 and 170 pmole/mg protein, respectively.

Figure 3 and Figure 4 show effect of DMDs on ^{3}H-glycerol incorporation into cellular triglyceride and VLDL secretion into medium in hepatocyte cultures. Treatment of DMDs effectively reduced CCl_4-induced increase of 3H-glycerol incorporation into cellular triglyceride and increased the secretion of VLDL to control level.

Figure 5 shows effect of DMDs on protein synthesis. Treatment of 10 μM of DMDs significantly recovered protein synthesis inhibited by CCl_4.

DISCUSSION

The results of present study demonstrated that DMDs effectively protected CCl_4-induced hepatotoxicity. The activity of the microsomal enzymatic system in the liver seems to be important in the process of the CCl_4 poisoning, because liver injury by CCl_4 occurs through the metabolic activation of CCl_4 by microsomal cytochrome P-450 system. Inducers such as phenobarbital and PCB are known to potentiate the liver injury by enhancing the activation of CCl_4, whereas inhibitors such as SKF-525A and methoxsalen reversed the hepatotoxic effect of CCl_4 (Lindstom *et al.*, 1978; Garvela *et al.*, 1979; Labbe *et al.*, 1987). In this study, the content of total cytochrome P-450 and the activities of ECOD and AHH were not significantly induced in DMDs treated groups. These results suggest that DMDs may not protect the liver from the CCl_4-induced injury by inhibiting radical formation. Hepatoprotective agents such as tocopherol, glutathione, and cysteine have been reported to suppress the enhanced lipid peroxidation in the liver through acting as antioxidant and radical scavengers, (Slater, 1982). Although the mode of action of DMDs is not clear yet, the fact that DMDs inhibit the lipid peroxidation induced by CCl_4 suggested that DMD itself or its metabolites may act as an antioxidant or radical scavenger in hepatocytes culture. Imazumi *et al.*, (1982) reported that treatment of malotilate increased protein synthesis in rat. However, the results of present study showed that neither protein synthesis nor RNA synthesis was affected by the treatment of DMDs in primary rat hepatocyte culture. The discrepancy may due to the difference of *in vivo* and *in vitro* treatment.

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