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Enhanced Effectiveness of Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate in Combination with Garlic Oil against Experimental Hepatic Injury in Rats and Mice

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Abstract

The present study was designed to evaluate the effects of dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (PMC) in combination with garlic oil against chemical-induced hepatic injury in rats and mice.

Rats insulted with carbon tetrachloride were concomitantly treated with PMC and/or garlic oil (50 and 100 mg kg^{-1}, respectively) for four weeks. Whereas treatment of animals with garlic oil alone was ineffective in suppressing carbon tetrachloride-induced hepatotoxicity, administration of PMC in combination with garlic oil more effectively protected the liver against the carbon tetrachloride-induced insult than PMC alone, as monitored by serum aminotransferase activity. Hepatoprotective effects of the formulation were further supported by the changes in the numbers of Kupffer cells and dead hepatocytes.

Although prior treatment of rats with PMC for three days failed to protect hepatotoxicity elicited by allyl alcohol, the formulation of PMC and garlic oil was capable of blocking allyl alcohol-induced hepatotoxicity by ~ 40%.

To further examine the effect of the agents on lipid metabolism in the liver, hepatic triglycerides and cholesterol contents were assessed in mice after a diet containing PMC and/or garlic oil for one week followed by a single dose of carbon tetrachloride. Garlic oil appeared to be more effective in bringing hepatic lipid levels to those of control than PMC alone. Treatment of animals with PMC in combination with garlic oil synergistically improved chemical-induced impairment of hepatic triglycerides and cholesterol.

These results demonstrated that PMC in combination with garlic oil is effective in protecting (or treating) chemical-induced hepatic injury and that the formulation may be effective against chemical-induced fat-infiltration of the liver.

Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′dicarboxylate (PMC) is a synthetic compound derived from Schizandrin C, a component of *Fructus schizandrae*. PMC protects liver against CCl_4, D-galactosamine-, thioacetamide- and prednisolone-induced liver injuries in rats and mice, and has been found to be effective in improving liver function and symptoms of patients with chronic viral hepatitis. PMC therapy resulted in significant decreases in hepatitis virus B (HVB)-induced elevation of alanine aminotransferase (ALT) levels in patients with hepatitis (Lee et al 1991). Currently, this agent is clinically employed for patients with HVB in Asian countries.

Organosulphur compounds present in *Allium* species (e.g. garlic) including diallyl disulphide, diallyl sulphide and several of their substituted derivatives possess potent inhibitory activity toward colon, liver and oesophageal cancers induced by chemicals (Brady et al 1988a; Reddy et al 1993). The potential chemoprevention of carcinogenesis by the organosulphur compounds is at least partly due to blocking of the initial metabolic activation of chemical carcinogens (Brady et al 1991). Diallyl sulphide, a component of garlic oil, has been shown to possess both suppressive activity of CYP2E1 (Hayes et al 1987; Brady et al 1988b), which modulates the level of toxic or reactive intermediate that can be produced from a wide variety of small organic molecules (Kim et al 1994). It is believed that inhibition of CYP2E1 activity and decrease in the CYP2E1 levels after diallyl sulphide treatment may be associated with the mechanism for its anticarcinogenic properties.

Given these observations that modulation of CYP2E1 levels may elicit decreases in the risk of organ toxicity and in the generation of reactive oxygen species, the present study was initiated to determine whether PMC in combination with garlic oil, is effective in the protection or treatment of chemically induced liver injury.

Because no information is available on the effects of PMC against allyl alcohol-induced hepatitis, both CCl_4 and allyl alcohol (which exert hepatotoxicity through different pathways) were employed in this study to evaluate the hepatoprotective efficacy of the formulation of PMC and garlic oil.

It has been reported that several components of garlic oil have an inhibitory effect on platelet aggregation. Active components for anti-aggregatory activity include dialkyl sulphides (Lawson et al 1992). It has also been shown that serum cholesterol, triglyceride and high-density lipoprotein concentrations decreased to greater extents after a four-week course of garlic treatment in hypercholesteraemic patients. Although PMC is clinically used against HVB patients, little is known on fat accumulation in the liver

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following PMC treatment. Thus, in this study, hepatic triglyceride and total cholesterol levels were also assessed to determine the efficacy of PMC, garlic oil or the combination of both agents against chemical-induced fat infiltration.

Materials and Methods

Materials

Carbon tetrachloride, paracetamol and other reagents in the study were obtained from Sigma Chemical Co. (St Louis, MO). Diagnostic kits for assaying alanine aminotransferase, aspartate aminotransferase, bilirubin, total cholesterol and triglycerides were purchased from Youngdong Pharmaceutical (Seoul, Korea). PMC and garlic oil were provided by Taerim Pharmaceutical Co. (Seoul, Korea).

Animals

Male Sprague-Dawley rats, 200–250 g and ICR mice were obtained from Daehan Laboratory animals (Seoul, Korea) and maintained at a temperature of 20–23°C and a relative humidity of 50%. Animals were caged with a supply of filtered pathogen-free air, with free access to food (Samyang rodent chow, Korea) and water unless otherwise specified.

Collection of blood and tissue samples

Male Sprague-Dawley rats were treated with carbon tetrachloride (0·75 mL kg^{-1}, p.o. twice a week, 50% solution mixed with corn oil) for 4 weeks to induce liver injury. Groups of rats were treated with PMC and/or garlic oil (50 and 100 mg kg^{-1}/day, respectively, p.o., 4 weeks, 6 days per week). PMC was suspended in 0·1% high viscosity carmellose, and garlic oil was diluted with corn oil for administration (1 : 10, v/v). Control rats were treated with vehicle alone. Animals were killed at 24 h following the last treatment, and sera and livers were obtained from individual animals.

When allyl alcohol was used as a hepatotoxicant, rats were treated with PMC and/or garlic oil for 3 days and a single dose of allyl alcohol (40 mg kg^{-1}, i.p.) was injected at 24 h after the last dosing; blood samples were collected at either 6 or 12 h after allyl alcohol treatment. Serum alanine aminotransferase was monitored.

Evaluation of hepatic toxicity

Liver injury was assessed by monitoring serum alanine aminotransferase activities using commercially available kits (Youngdong Pharmaceutical, Korea). Total bilirubin in the sera was also determined using a commercially available kit (Youngdong Pharmaceutical, Korea).

Assessment of hepatocyte death

Cell death was assessed as reported previously (Edwards et al 1993). After perfusion of livers with Krebs–Henseleit bicarbonate buffer, 0·4 mM trypan blue was infused into the liver for 10 min followed by rinsing with Krebs–Henseleit bicarbonate buffer. Subsequently, tissues were fixed by 1% paraformaldehyde perfusion. The number of dead hepatocytes was counted from an area radiating from the pericentral vein, and trypan blue-positive cells were expressed per arbitrary visual field.

Assays of triglycerides and cholesterol

Groups of male ICR mice (20–30 g, 10 mice/group) were fed with the modified diet containing PMC (0·014%) or garlic oil (0·14%) for seven days. When animals were treated with both agents, they received a diet containing 0·007% PMC and 0·07% garlic oil, equivalent to a daily intake of 3 and 30 mg kg^{-1}, respectively. At the end of this diet, animals were treated with a single dose of carbon tetrachloride (50 μg kg^{-1}, i.p.) to induce impairment of liver lipid metabolism, and livers were excised for determination of lipids. Triglycerides and total cholesterol in the liver homogenates (1 : 10, w/v) were determined using commercially available kits (Youngdong Pharmaceutical, Korea).

Results

Carbon tetrachloride hepatotoxicity and protection by PMC and garlic oil

The effects of PMC and garlic oil were assessed in the rats given multiple doses of carbon tetrachloride for four weeks. Daily treatment of rats with PMC at the dose of 50 mg kg^{-1} (6 days/week) was effective in decreasing the increases in serum ALT activity induced by 4-week carbon tetrachloride treatment (Table 1). Improvement in serum ALT activity following PMC treatment was ~ 28%. Administration of garlic oil to animals at the daily dose of 100 mg kg^{-1} failed to exhibit protective effects against the hepatic injury. However, the effectiveness of PMC against the hepatic injury was significantly enhanced following concomitant treatment of animals with both PMC and garlic oil, as monitored by serum alanine aminotransferase activity (Table 1). Treat-

Table 1. The effects of PMC and garlic oil against hepatic injury induced by multiple carbon tetrachloride treatment.

Treatment	Alanine aminotransferase (units L^{-1})	Number of animals	% Control
CCl_4 alone	2270 ± 357	8	100
CCl_4 + PMC (50 mg kg^{-1})	1644 ± 253	8	72
+ garlic oil (100 mg kg^{-1})	2508 ± 223	8	110
+ PMC (50 mg kg^{-1})	814 ± 184[a,b,c]	8	36
+ garlic oil (100 mg kg^{-1})			
Untreated	69 ± 17	4	–

Animals were treated with carbon tetrachloride twice per week for 4 weeks, and PMC and garlic oil was administered by gavage daily (6 days per week). Values represent mean ± s.e.
[a] $P < 0·01$ compared with group treated with CCl_4 alone.
[b] $P < 0·02$ compared with group treated with CCl_4 + PMC alone.
[c] $P < 0·001$ compared with group treated with CCl_4 + garlic oil.

Table 2. The effects of PMC and garlic oil against hepatic injury induced by multiple carbon tetrachloride treatment.

Treatment	Number of Kupffer cells	% control	Number of dead hepatocytes	% control
CCl_4 alone	18·1 ± 3·4 (22)	100	32·4 ± 13·5 (29)	100
CCl_4 + PMC (50 mg kg^{-1})	5·3 ± 1·6 (32)***	29	19·0 ± 6·5 (10)**	59
+ Garlic oil (100 mg kg^{-1})	8·4 ± 2·4 (52)***	46	21·3 ± 6·9 (24)***	66
+ PMC (50 mg kg^{-1}) + garlic oil (100 mg kg^{-1})	4·5 ± 2·5 (29)***	25	11·7 ± 5·9 (27)***[a,b]	36
Untreated	2·0 ± 1·0 (35)	11	0·6 ± 1·4 (8)	2

The values in the parentheses represent number of determinations. Compared with the group treated with CCl_4 alone ** $P < 0·01$ ***, $P < 0·001$). For dead cell counts, the animals treated with PMC + garlic oil exhibited a significant difference from those treated with either CCl_4 + PMC ($P < 0·02$) or CCl_4 + garlic oil ($P < 0·001$). **$P < 0·01$, ***$P < 0·001$ compared with the group treated with CCl_4 alone. [a]$P < 0·02$ compared with the group treated with CCl_4 + PMC. [b]$P < 0·001$ compared with the group treated with CCl_4 + garlic oil.

ment of rats with both PMC and garlic oil led to 64% decrease in CCl_4-induced ALT elevation, which was a twofold improvement in efficacy, as compared with the animals treated with PMC alone (Table 1).

PMC and garlic oil treatment caused 71 and 54% decreases, respectively, in the number of Kupffer cells. Treatment of rats with both agents caused no further enhancement in the inhibition of Kupffer cell infiltration (Table 2).

The enhanced hepatoprotective effect of PMC in combination with garlic oil was further supported by the histochemical evidence. Dead hepatocytes were assessed by counting the number of trypan blue-stained cells in the liver. Four-week treatment of rats with carbon tetrachloride produced marked hepatic necrosis in pericentral regions. Administration of PMC along with carbon tetrachloride reduced the number of dead hepatocytes by 40%. Garlic oil alone also moderately diminished the induced liver injury by 33%. Concomitant treatment of rats with both PMC and garlic oil offered additive therapeutic efficacy (i.e. 64% inhibition) in the number of dead hepatocytes. This enhanced hepatoprotective effect as monitored by histological examinations was consistent with the results obtained from serum alanine aminotransferase (Table 2).

The effects of PMC and garlic oil on serum total bilirubin levels are shown in Table 3. Total bilirubin levels in serum were substantially reduced after both PMC and garlic oil treatment of the animals injured by carbon tetrachloride; nonetheless, the effects were comparable with those treated with PMC alone (Table 3).

Allyl alcohol hepatotoxicity and the effects of PMC and garlic oil

A single dose of allyl alcohol resulted in a 47-fold increase in serum alanine aminotransferase activity, as compared with that in untreated animals. Treatment of rats with PMC for three days failed to block allyl alcohol-induced liver injury (Table 4), but resulted in a 30% increase. Whereas pretreatment of animals with garlic oil before the insult of allyl alcohol inhibited the hepatotoxicity to a small extent (11% at 12 h), concomitant treatment with both agents significantly protected the liver from allyl alcohol-induced injury by 44% ($P < 0·01$). Thus, the two agents, when used in combination, appeared to exert enhanced effectiveness against the hepatotoxicity.

Table 3. The effects of PMC and garlic oil on serum total bilirubin.

Treatment	Total bilirubin (mg dL^{-1})	% control
CCl_4 alone	11·6 ± 5·9 (3)	100
CCl_4 + PMC (50 mg kg^{-1})	3·0 ± 1·3 (4)*	26
Garlic oil (100 mg kg^{-1})	4·9 ± 4·0 (4)	42
PMC (50 mg kg^{-1}) +garlic oil (100 mg kg^{-1})	2·4 ± 0·4 (3)	21
Untreated	nd	

*$P < 0·05$ compared with the group treated with CCl_4 alone. The values in parentheses represent number of determinations. nd = not detectable.

The effects of PMC and garlic oil on carbon tetrachloride-induced fatty liver

Because preliminary studies showed that ICR mice exhibited greater sensitivity to carbon tetrachloride in the genesis of fatty liver as well as in the liver cell injury compared with rats (~ 30-fold, data not shown), mice were employed in this study, and due to the greater sensitivity of mice to carbon tetrachloride, the contents of PMC and garlic oil in the modified diets fed to mice were reduced. The two agents in combination were efficacious in blocking chemical-induced fat infiltration in the liver (Table 5).

Total cholesterol levels were also determined in liver homogenates. Concomitant treatment of both agents exerted a better effect in blocking CCl_4-induced suppression of cholesterol levels (Table 6), with greater increases in liver cholesterol levels being observed in the animals treated with both agents compared with those treated with double doses of the individual agent.

Discussion

PMC is currently employed as an agent against viral or chemically induced hepatic injury. It is effective in lowering the elevated levels of serum alanine aminotransferase activities in animals insulted with carbon tetrachloride, prednisolone or thioacetamide (Liu et al 1979, 1982). The rates of liver regeneration in partial hepatectomized mice are also enhanced by PMC treatment. PMC inhibits lipid peroxidation catalysed by ferrous iron, probably due to an iron-chelating effect or to scavenging of hydroxyl free radicals (Liu & Lesca 1982). Nonetheless, the mechanism of action for the hepatoprotective effects of PMC has not been established.

The hepatoprotective agent, malotilate, suppresses CYP2E1 expression in the absence of transcriptional regulation (Kim et al 1994). The selective suppression of CYP2E1 by hepatoprotective agents may contribute to the protection of organs,

Table 4. The effects of PMC and garlic oil pretreatment of rats against hepatic injury induced 6 or 12 h after a single dose of allyl alcohol (40 mg kg^{-1}).

Treatment	Alanine aminotransferase (units L^{-1})			
	6 h	% control	12 h	% control
Allyl alcohol	499 ± 149	100	2254 ± 409	100
PMC (50 mg kg^{-1}) + Allyl alcohol	486 ± 156	97	2981 ± 546	132
Garlic oil (100 mg kg^{-1}) + allyl alcohol	330 ± 39	66	2916 ± 596	89
PMC (50 mg kg^{-1}) + garlic oil (100 mg kg^{-1}) + allyl alcohol	306 ± 137	61	1263 ± 469**,***	56
Untreated	48 ± 8			

Animals were treated daily with PMC or garlic oil for three days followed by a single dose of allyl alcohol at 24 h after the last dose. Values represent mean ± s.e. from 6 to 8 determinations. **$P < 0.01$ compared with the group treated with allyl alcohol alone, ***$P < 0.001$ compared with the group treated with PMC and allyl alcohol.

particularly liver, from xenobiotic-induced intoxication as well as to the chemoprevention of cancer.

Organosulphur compounds including allyl sulphide and its structurally-related derivatives are present in garlic oil and these sulphur-containing compounds are known to exert chemoprotective effects through the suppression of CYP2E1 or the elevation of phase II enzymes. Garlic oil has been shown to be effective in suppressing chemically-inducible levels of CYP2E1 and in increasing glutathione *S*-transferase expression in rats (data not shown). Allyl disulphide seems to be the most effective in chemically-induced hepatic injury of the compounds in *Allium* species, probably through the mechanism associated with inhibition of CYP2E1-catalytic activity and the suppression of CYP2E1 expression (Brady et al 1988a,b, 1991). Diallyl sulphide blocks the induction of carcinogenic responses in liver and colon which develops to dimethylhydrazine treatment in animals. A plausible mechanism proposed for this suppression in cancer development is the selective inhibition of CYP2E1. Allyl sulphide and other organosulphur compounds present in garlic oil increase glutathione-*S*-transferase expression in rodents, as has been demonstrated previously (Hayes et al 1987; Sparnins et al 1988).

Our preliminary study showed that PMC fails to inhibit CYP2E1 activity or to suppress CYP2E1 expression in rats, nor was PMC capable of increasing the expression of glutathione *S*-transferases. Given these observations the present research was designed to examine the potential additive or synergistic enhancing effects of PMC in combination with garlic oil against experimental hepatic injury. In this study, we found that PMC was more effective in protecting carbon tetrachloride-induced hepatic injury when co-administered with garlic oil.

The synergistic effects of PMC with garlic oil might result from their separate mechanisms of action. PMC induced both CYP2B1 and CYP2B2 levels, as assessed by immunoblot analyses (unpublished data). Induction of CYP2B by PMC was consistent with our previous observation (Liu et al 1981). The increases in CYP2B levels may be one of the potential mechanisms for this synergistic effect. Tuntaterdtum et al (1993) provided evidence that paracetamol overdose produced lower hepatotoxicity in animals treated with phenobarbitone. It has been suggested that the induction of CYP2B1 and CYP2B2 enzymes after phenobarbitone treatment may contribute to the action against paracetamol-induced hepatic necrosis by catalysing the formation of reactive intermediates. In our laboratory, PMC has been shown to induce both CYP2B1 and CYP2B2, although the extent of induction is ~ 50% of that by phenobarbitone. Allyl sulphide, a component of garlic oil, substantially induces CYP2B levels through transcriptional activation with concomitant increases in mRNA levels (Pan et al 1992, 1993). Thus, the induction of CYP2B might be much greater after treatment of animals with both PMC and garlic oil than those in animals treated with the individual agent alone. Nonetheless, the potential additive increases in CYP2B1 and CYP2B2 levels would not be

Table 5. Hepatic triglycerides in mice treated with PMC and garlic oil for seven days followed by a single dose of carbon tetrachloride (50 μg kg^{-1}, i.p.).

Treatment	Triglycerides (mg (g liver)$^{-1}$)	% Control
CCl_4 alone	17·09 ± 0·63	100
CCl_4 + PMC 0·007% + CCl_4	16·12 ± 1·06	87
PMC 0·014% + CCl_4	14·32 ± 1·03	63
Garlic oil 0·07% + CCl_4	14·73 ± 0·88	68
Garlic oil 0·14% + CCl_4	12·57 ± 1·00**	41
PMC 0·007% + garlic oil 0·07% + CCl_4	12.40 ± 0·91**[a]	38
Untreated	9·54 ± 0·86	0

Values represent mean ± s.e. (n = 10). **, $P < 0.01$; ***, $P < 0.001$ compared with the group treated with CCl_4 alone. [a]$P < 0.02$ compared with the group treated with PMC + CCl_4.

Table 6. Hepatic total cholesterol levels in the mice treated with PMC and garlic oil for seven days followed by a single dose of carbon tetrachloride (50 μg kg^{-1}, i.p.).

Treatment	Cholesterol (mg (g liver)$^{-1}$)	% Control
PMC 0·007% + CCl_4	5·55 ± 0·70	53
PMC 0·014% + CCl_4	5·84 ± 0·25	56
Garlic oil 0·07% + CCl_4	5·63 ± 0·24	54
PMC 0·007% + garlic oil 0·07% + CCl_4	6·82 ± 0·40*	66
Untreated	10·41 ± 1·02	100

Values represent mean ± s.e. (n = 10). *$P < 0.05$ compared with the group treated with either garlic oil 0·07% + CCl_4 or PMC 0·014% + CCl_4.

sufficient to explain this synergistic effect of the agents against experimental hepatic injury.

Allyl alcohol injures periportal regions of the liver lobule in-vivo via metabolism by alcohol dehydrogenase (Przybocki et al 1992). We have shown for the first time in this study that PMC was not effective against allyl alcohol-induced chemical injury, although PMC protected liver from the injury caused by a number of hepatotoxicants. Interestingly, however, pretreatment with the formulation of PMC and garlic oil was capable of antagonizing allyl alcohol-induced intoxication. Taken together, the results of these studies support the hypotheses that PMC and garlic oil act through different mechanisms and that these agents in combination exhibit enhanced hepatoprotective effects. It is likely that PMC exerts protective and therapeutic effects against hepatic injury through the mechanism other than that associated with detoxification. Because the formulation of PMC and garlic oil would possess the capability of differentially modulating both CYP2E1 and phase II detoxification enzymes, the formulation may constitute an advance in the development of effective hepatoprotective agents and represents a practical application. These two drugs in combination may sequentially serve as hepatoprotective and therapeutic agents.

The level of triglycerides in the liver reflects a balance between the rates of exogenous supply and endogenous fatty acid synthesis as well as the rates of free fatty acid oxidation and triglyceride secretion from the liver. Oversupply of free fatty acids to the liver is considered to be one of the mechanisms in fatty liver induced by carbon tetrachloride, which triggers lipolysis in the adipose tissue and causes a dramatic increase in circulating free fatty acids. Carbon tetrachloride also interferes with the synthesis of apoprotein, phospholipid and other components including the synthesis of very low density lipoproteins, which is the rate limiting step for the transport of triglycerides from the liver, and causes fatty liver. Prior treatment of animals with either PMC or garlic oil suppressed the accumulation of triglycerides in the liver caused by a single dose of carbon tetrachloride. Thus, the present study provided evidence that PMC as well as garlic oil is active against fat infiltration of the liver. It is likely that carbon tetrachloride insult blocks the synthesis of apoprotein for the transport of triglycerides from the liver, rather than triggers lipolysis in the adipose tissue, and causes a dramatic increase in circulating free fatty acids; this is supported by the finding of normal levels of serum triglycerides in carbon tetrachloride-treated animals (data not shown). When mice were fed with a modified diet containing 0·007% PMC and 0·07% garlic oil, carbon tetrachloride-induced increase in hepatic triglycerides was significantly blocked.

In summary, these results demonstrate that the formulation of PMC and garlic oil is effective in protecting liver against the insult of hepatotoxicant, and that PMC in combination with garlic oil may be employed against chemical-induced fat infiltration.

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