

Protective Effects of Diallyl Sulfide against Thioacetamide-Induced Toxicity: A Possible Role of Cytochrome P450 2E1

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Abstract

Effects of diallyl sulfide (DAS) on thioacetamide-induced hepatotoxicity and immunotoxicity were investigated. When male Sprague-Dawley rats were treated orally with 100, 200 and 400 mg/kg of DAS in corn oil for three consecutive days, the activity of cytochrome P450 (CYP) 2E1-selective p-nitrophenol hydroxylase was dose-dependently suppressed. In addition, the activities of CYP 2B-selective benzyloxyresorufin O-debenzylase and pentoxyresorufin O-depentylyase were significantly induced by the treatment with DAS. Western immunoblotting analyses also indicated the suppression of CYP 2E1 protein and/or the induction of CYP 2B protein by DAS. To investigate a possible role of metabolic activation by CYP enzymes in thioacetamide-induced hepatotoxicity, rats were pre-treated with 400 mg/kg of DAS for 3 days, followed by a single intraperitoneal treatment with 100 and 200 mg/kg of thioacetamide in saline for 24 hr. The activities of serum alanine aminotransferase and aspartate aminotransferase significantly elevated by thioacetamide were protected in DAS-pretreated animals. Likewise, the suppressed antibody response to sheep erythrocytes by thioacetamide was protected by DAS pretreatment in female BALB/c mice. Taken together, our present results indicated that thioacetamide might be activated to its toxic metabolite(s) by CYP 2E1, not by CYP 2B, in rats and mice.

Key Words: Diallyl sulfide, Thioacetamide, CYP, Toxicity, Metabolic activation

INTRODUCTION

Thioacetamide is a potent hepatotoxicant which requires metabolic activation by the mixed-function oxidases. For its toxicity, thioacetamide requires oxidation to its S-oxide and then further to reactive S,S-dioxide form which ultimately attacks lipids and proteins (Hajovsky *et al.*, 2012). To date, cytochrome P450 (CYP) and flavin-containing monooxygenases have been implicated to metabolize thioacetamide to its toxic metabolites (Hunter *et al.*, 1977; Chieli and Malvaldi, 1984). In addition, it was suggested that the intermediate metabolites of thioacetamide might bind to cellular proteins with the formation of acetylimidolysine derivatives (Dyroff and Neal, 1981). Of particular interest is that thioacetamide can also stimulate the DNA synthesis and mitosis in livers for hepatic regeneration (Mangipudy *et al.*, 1995). More recently, Wang *et al.* (2000) reported that thioacetamide could be metabolized to its

hepatotoxic form(s) by CYP 2E1 in diabetic rats.

Allin (S-allylcysteine sulfoxide), a major sulfur-containing constituent in garlic (*Allium sativum*), can be converted to various metabolites including diallyl sulfide (DAS) by food processing and drug-metabolizing enzymes in body. DAS is a flavor compound present in the garlic. The effects of DAS on CYP expression have been studied in a number of groups, with particular interests in its anticancer activities. DAS showed protective effects against the growth of *in vitro* tumor cell cultures and chemical-induced tumorigenesis in various animal models (Hong *et al.*, 1992; Wang *et al.*, 1996; Chen *et al.*, 1998). DAS can be metabolized by CYP enzymes to diallyl sulfoxide (DASO) and, subsequently, to diallyl sulfone (DASO₂) which act as competitive inhibitors to CYP 2E1 (Brady *et al.*, 1991a; Brady *et al.*, 1991b).

Many other investigators have also reported several toxicants - DAS interactions in animals by inhibiting CYP 2E1 en-

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zyme. For example, acetaminophen-induced hepatotoxicity and nephrotoxicity could be protected by treatment with DAS, because DAS inhibited the metabolic activation of acetaminophen to its toxic metabolites (Wang *et al.*, 1996). In addition, the activity of dimethylnitrosamine demethylase, a selective marker enzyme for CYP 2E1, could be inhibited by DAS, and the inhibitory effect on CYP 2E1 was more potent by a DAS metabolite, DASO₂ (Brady *et al.*, 1991b). Likewise, DAS and its metabolites could inhibit, another selective marker for CYP 2E1, p-nitrophenol hydroxylase activity (Brady *et al.*, 1991a). Moreover, hepatotoxicity induced by carbon tetrachloride, a potent hepatotoxicant which requires metabolic activation by CYP 2E1, could be protected by pretreatment with DAS (Yang *et al.*, 1990). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced tumorigenesis was reduced when DAS was pretreated to animals (Hong *et al.*, 1992). When rats were fed with DAS-containing diets, it was demonstrated that the level of CYP 2E1 was significantly reduced (Haber *et al.*, 1994; Dav-enport and Wargovich, 2005). Therefore, all the reports exemplified above collectively suggest that the inhibitory effects of DAS on CYP 2E1 might be very obvious. However, effects of DAS on other CYP-associated monooxygenase activities have not been investigated extensively.

The objectives of our present studies were two folds: first, to characterize the effects of DAS on CYP-associated monooxygenase activities in rats; secondly, to investigate the possible role of CYP 2E1 in thioacetamide-induced hepatotoxicity and immunotoxicity. In this regard, CYP 2B has been believed to activate thioacetamide to its hepatotoxic form(s) (Hunter *et al.*, 1977). However, more recently, it has also been reported that thioacetamide might be metabolized by CYP 2E1 (Wang *et al.*, 2000). Fortunately, during our present studies with DAS, we found that DAS could also induce the activities of CYP 2B-selective pentoxyresorufin O-depentylyase (PROD) and benzyloxyresorufin O-debenzylase (BROD) in liver microsomes isolated from DAS-treated rats. From these results, it was postulated that the possible role of CYP 2E1 can be studied in DAS-pretreated animals, because DAS can not only suppress CYP 2E1, but also induce CYP 2B.

MATERIALS AND METHODS

Materials

DAS, thioacetamide, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, benzyloxyresorufin, resorufin, p-nitrophenol, erythromycin, glucose 6-phosphate, NADPH, glucose 6-phosphate dehydrogenase and the kits for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assays were purchased from Sigma Chemical Company (St. Louis, MO, USA). The primary antibodies against individual CYP proteins were purchased from Easy-bio system (Seoul, Korea). Earle's balanced salt solution (EBSS) and guinea pig complements were purchased from GIBCO (Grand Island, NY, USA). Sheep red blood cells (SRBCs) were obtained at the College of Natural Resources at Yeungnam University. All other chemicals were of reagent grade commercially available and used as received.

Animals

Specific pathogen-free male Sprague-Dawley (SD) rats and female BALB/c mice were obtained from the Orient (Seoul,

Korea). The animals received at 4 weeks of age were acclimated for 2 weeks. Upon arrival, animals were randomized and housed five per cage. Animals were freely provided with pelleted LabDiet® (Purina Mills, MO, USA) and tap water ad libitum. Six weeks old mice were used in this study. The animal quarters were strictly maintained at 23 ± 3°C and 40 - 60% relative humidity. A 12-hr light/dark cycle was used with an intensity of 150-300 Lux. This study was performed with the permission of Institutional Animal Care and Use Committee of Yeungnam University College of Pharmacy, based on the recommended Guiding Principles in the Use of Animals in Toxicology by the Society of Toxicology (Reston, VA, USA).

Animal treatment

For the induction study, DAS in corn oil at 100, 200 or 400 mg/kg was treated orally for 3 consecutive days. Twenty four hr after the last dosing, all animals were subjected to necropsy. For the hepatotoxicity study, DAS at 400 mg/kg was pretreated orally for 3 consecutive days. Twenty four hr after the last pretreatment, animals were treated intraperitoneally with thioacetamide at 100 or 200 mg/kg in 10 ml saline. Twenty four hr later, all animals were subjected to necropsy. For the immunotoxicity study, female BALB/c mice were pretreated orally with DAS in corn oil at 400 mg/kg once a day for 3 consecutive days, followed by an intraperitoneal treatment with 200 mg/kg of thioacetamide in saline 24 hr after the last treatment with DAS. Thirty min later, mice were sensitized intraperitoneally with 5×10⁸ SRBCs per mouse in 0.5 ml of EBSS. The antibody-forming cells (AFCs) in spleen were enumerated four days later.

Hepatotoxicity parameters

For assaying the activities of serum ALT and AST, the serum was prepared by a centrifugation of the blood at 2,500×g for 10 min at 4°C on the day of necropsy. The sera were stored at -80°C until use. The activities were determined under the instruction manual supplied by the manufacturer.

Preparation of liver microsomal fractions

Livers were removed and homogenized with four volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4. The liver homogenates were centrifuged at 9,000×g for 20 min at 4°C. The resulting post mitochondrial S-9 fractions were centrifuged at 105,000×g for 60 min at 4°C. The microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol. The aliquots were stored at -80°C until use. The protein content in microsomal fraction was determined using bovine serum albumin as a standard (Lowry *et al.*, 1951).

Assay of monooxygenase activities

Ethoxyresorufin O-deethylase (EROD) activity was determined as described by Blank *et al.* (1987) with a slight modification. The reaction mixture (2 ml) consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mg/ml of bovine serum albumin, 10 μM dicumarol, 5 mM glucose 6-phosphate, 1 U of glucose 6-phosphate dehydrogenase, 5 μM NADPH and 2.5 μM 7-ethoxyresorufin. The formation of resorufin was monitored fluorometrically at an excitation maximum of 550 nm and an emission maximum of 585 nm. Methoxyresorufin O-demethylase (MROD), PROD and BROD activities were determined by the method of Lubet *et al.* (1985) with a slight

modification. All reaction components and assay procedures were exactly the same as the EROD assay, except that the substrates were 2.0 μ M. p-Nitrophenol hydroxylase (PNPH) activity was determined as described by Koop (1986). The reaction mixture (1.0 ml) was composed of 0.1 M potassium phosphate buffer, pH 7.4, containing 100 μ M p-nitrophenol, 1 mM NADPH and an enzyme source. The amount of 4-nitro-catechol formed was measured spectrophotometrically at 512 nm. Erythromycin N-demethylase (ERDM) activity was determined by measuring the amount of formaldehyde formed, as described previously (Nash, 1953; Lee *et al.*, 2004). The reaction mixture (1.5 ml) consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.8 mM NADPH, 5.0 mM magnesium chloride, 3.0 mM glucose 6-phosphate, 1 U of glucose 6-phosphate dehydrogenase and 7.5 mM semicarbazide. The final concentration of substrate in the reaction mixture was 400 μ M.

Western immunoblotting

Ten μ g/well microsomal proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were transferred to nitrocellulose filters. The filters were incubated with 3% gelatin for 1 hr to block the non-specific binding, and then were incubated with rabbit polyclonal antibodies against either rat P450 2B1/2, 2E1 or 3A1/2, followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody. Immunostaining was done as previously described (Kim *et al.*, 2004).

Antibody response to SRBCs

Single cell suspensions of splenocytes were prepared in 3 ml of EBSS, washed and resuspended in 3 ml of EBSS. Spleen cells were then diluted 30-fold by resuspending a 100 μ l aliquot of each suspension in 2.9 ml of EBSS. The number of AFCs was determined using a modified Jerne plaque assay, as described previously (Kaminski *et al.*, 1990).

Statistics

The results were expressed as the mean \pm S.E. and Dunnett's *t*-test was used to compare statistical significance of data. The significant values at either $p < 0.05$ (*) or $p < 0.01$ (**) were represented as asterisks.

RESULTS

Fig. 1 shows the effects of DAS on CYP-associated monooxygenase activities in rat liver microsomes. Male SD rats were treated orally with DAS in corn oil for 3 consecutive days. DAS could suppress CYP 2E1-selective PNPH activity dose-dependently. In addition, DAS could induce other CYP enzymes, such as EROD, PROD, BROD and ERDM. Among them, CYP 2B-selective activities were induced most significantly. As aforementioned in the Introduction, thioacetamide has long been believed to be activated by CYP 2B to its hepatotoxic metabolites (Hunter *et al.*, 1977). Therefore, DAS could be a model CYP modulator in the study of thioacetamide-induced hepatotoxicity, because DAS has dual effects in modulating the expression of CYP isozymes; suppression of CYP 2E1 and/or induction of CYP 2B. From the same studies in female BALB/c mice, it was also found that DAS could suppress CYP 2E1 and/or induce CYP 2B (data not shown). In the

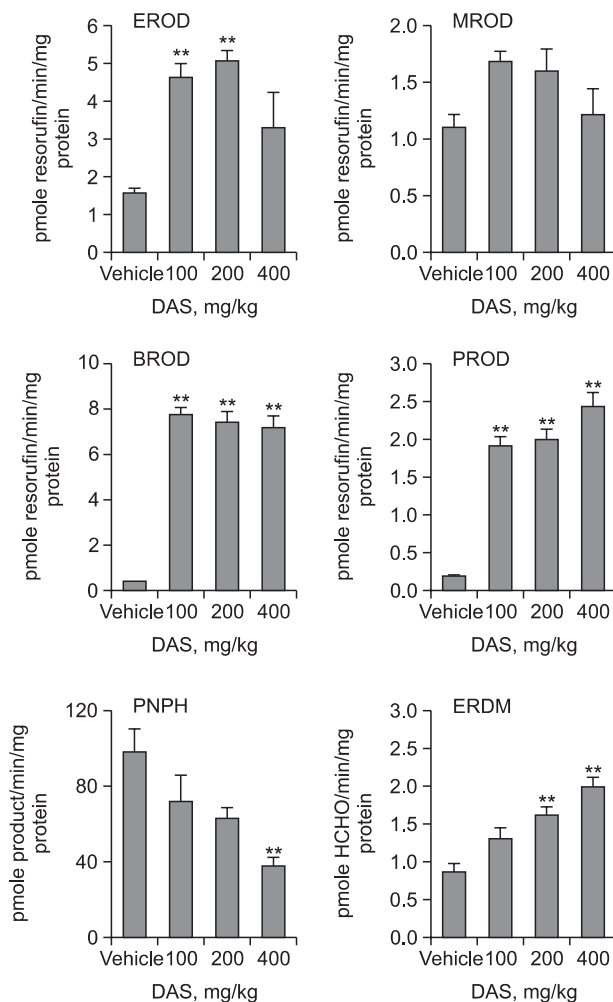


Fig. 1. Effects of DAS on CYP-associated monooxygenase activities in male SD rats. Rats were treated orally with 0, 100, 200 and 400 mg/kg DAS in corn oil for 3 consecutive days. Twenty four hr after the last treatment, livers were removed to prepare the microsomes. Each bar represents the mean activity \pm S.E. of five animals. The asterisks indicate the values significantly different from the vehicle-treated control at $p < 0.01$ (**).

subsequent studies, DAS was used as a model CYP modulator to investigate the possible role of CYP 2E1 and CYP 2B in thioacetamide-induced toxicity in rats and mice.

The liver microsomes prepared from DAS-treated rats in Fig. 1 were electrophoresed and immunoblotted with polyclonal antibodies against either one of the CYPs 2B1/2, 3A1/2 or 2E1 (Fig. 2). DAS clearly induced CYP 2B1/2 and CYP 3A1/2 proteins in liver microsomes. Meanwhile, CYP 2E1 proteins were decreased by the treatment with DAS. From these results, it was concluded that DAS might be useful to modulate CYP 2E1, 2B and 3A enzymes in rats.

As shown in Fig. 3, thioacetamide-induced elevation of serum ALT and AST was significantly protected when rats were pretreated with DAS for 3 consecutive days. In addition, as in Fig. 4, CYP-associated monooxygenase activities were assayed in the livers isolated from the same animals used in Fig. 3. CYP 2B-selective BROD activities were clearly induced by pretreatment with DAS and CYP 2E1-selective PNPH activi-

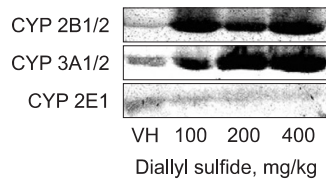


Fig. 2. Western immunoblotting analyses for CYPs 2B1/2, 3A1/2 and 2E1 with DAS-treated rat liver microsomes. The liver microsomal proteins (10 µg/well) prepared in Fig. 1 were resolved on 10% SDS-PAGE. Lane 1, vehicle (VH) control; lane 2, DAS at 100 mg/kg; lane 3, DAS at 200 mg/kg; lane 4, DAS at 400 mg/kg.

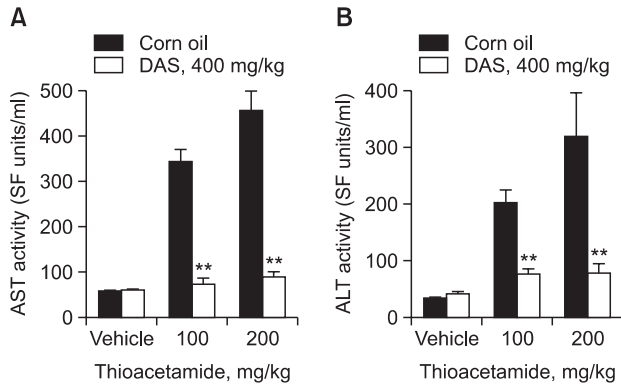


Fig. 3. Effects of DAS on thioacetamide-induced elevation of serum ALT (A) and AST (B) activities in male SD rats. Animals were pretreated orally with 400 mg/kg of DAS for 3 consecutive days, followed by an intraperitoneal treatment with 100 and 200 mg/kg of thioacetamide in 10 ml/kg of saline for 24 hr. The blood was collected for assaying the serum ALT and AST activities. Each bar represents mean activity ± S.E. of five animals. The asterisks indicate the values significantly different from the vehicle control at $p < 0.01$ (**).

ity was significantly suppressed in animals treated with DAS. Thioacetamide only marginally affected the enzyme activities studied.

In the subsequent studies, effects of pretreatment with DAS on thioacetamide-induced immunosuppression were also studied in mice. Following pretreatments of female BALB/c mice with 400 mg/kg of DAS for 3 days, mice were treated intraperitoneally with 200 mg/kg of thioacetamide. 30 min after the thioacetamide treatment, mice were immunized with SRBCs. The antibody response to SRBCs was enumerated 4 days later. Thioacetamide significantly suppressed the antibody response to SRBCs, which was protected by the pretreatment with DAS. In addition, DAS treatment could not only induce PROD and BROD activities, but also suppress PNP activity in livers, as observed in rats (data not shown).

Taken together, our present results indicated that thioacetamide-induced hepatotoxicity and immunotoxicity could be protected by the pretreatment of animals with DAS, and that thioacetamide might be metabolically activated by CYP 2E1, not by CYP 2B, to its toxic metabolites although supportive data are needed to elucidate mechanisms of action by CYP 2E1 and CYP 2B in thioacetamide-induced toxicity.

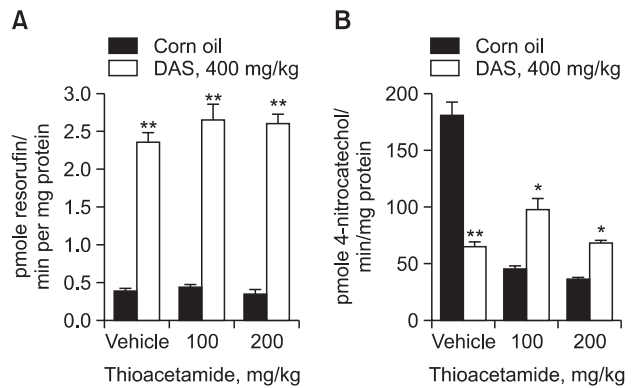


Fig. 4. Effects of DAS and thioacetamide on CYP-associated enzyme activities in liver microsomal fractions. CYP enzymes were assayed using the liver microsomes prepared in Figure 3. Each value represents mean activity ± S.E. of five animals. The asterisks indicate the values significantly different from the vehicle control at either $p < 0.05$ (*) or $p < 0.01$ (**). (A) BROD, (B) PNP.

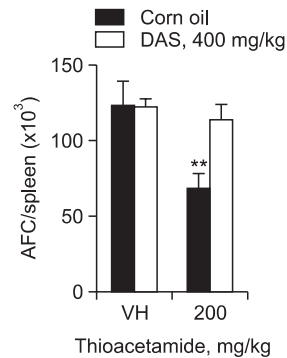


Fig. 5. Effects of DAS on thioacetamide-induced suppression of antibody response to SRBCs. Female BALB/c mice were pretreated orally with 400 mg/kg of DAS for 3 consecutive days prior to the treatment with 200 mg/kg thioacetamide. Thirty min later, mice were immunized with SRBCs. Four days later, the number of antibody-forming cells (AFCs) was enumerated. Each bar represents the mean ± S.E. of five animals. The asterisks indicate the values significantly different from vehicle (VH) control at $p < 0.01$ (**).

DISCUSSION

It has generally been accepted that chemical toxicants including carcinogens require metabolic activation to become ultimately toxic or carcinogenic metabolites (Guengerich and Shimada, 1991). Due to this reason, toxicity induced by chemicals can be modulated by many factors that are capable of modulating the expression of drug-metabolizing enzymes. The factors that can modulate the expression of drug-metabolizing enzymes include drugs, industrial chemicals, environmental pollutants, natural products, and so on. Of particular interests, compounds originated from foods are very important, because we consume them on a regular basis. In addition, the modulation of drug-metabolizing enzymes by such compounds may modulate not only the toxicity induced by chemicals requiring metabolic activation, but also pharmacokinetic properties of certain drugs that are metabolized by the affected enzymes.

In this regard, DAS is one of those examples.

Garlic (*Allium sativum*) has a strong taste and flavor, and has been used in traditional remedy and food processing in oriental countries (Yun *et al.*, 2014). An epidemiological study showed that, in China and Italy where the garlic consumption is relatively high, the incidence of stomach cancer is significantly lower than that in others (Buiatti *et al.*, 1989). In addition, studies using laboratory animals also showed results that garlic extracts could protect the incidence of various tumor formation induced by chemical carcinogens in skin, cervix, stomach, lung, colon and esophagus (Belman, 1983; Wargovich, 1987; Sathna *et al.*, 1988; Spornins *et al.*, 1988; Wargovich *et al.*, 1988; Hussain *et al.*, 1990; Singh and Shukla, 1998a; Singh and Shukla, 1998b).

Garlic contains numerous ingredients, particularly sulfur-containing compounds. Alliin, an oxidized form of γ -glutamyl cysteine that is contained in intact garlic bulbs, is transformed to other sulfur-containing compounds, such as DAS, diallyl disulfide, diallyl trisulfide, and so on (Amagase *et al.*, 2001). Taken all these reports together with the results that DAS, a major component in garlic, selectively inhibit the CYP 2E1 enzyme, the chemoprotection of DAS from cancer formation by chemical carcinogens would be resulted from the selective inhibitory effect of DAS to carcinogen activation by CYP 2E1 (Yang *et al.*, 1990; Brady *et al.*, 1991a; Brady *et al.*, 1991b). In other words, DAS is capable of inhibiting CYP 2E1, by which the procarcinogens requiring metabolic activation could not be activated to their toxic and/or carcinogenic reactive metabolites. However, the studies on DAS have primarily been focused on the CYP 2E1 which has been believed to be important in metabolizing small molecular weight compounds. Therefore, the effects of DAS on other CYP isozymes remained to be characterized.

In the present studies, treatment of rats with DAS could not only suppress liver microsomal CYP 2E1-selective PNP activity, but also induce CYP 2B-selective BROD and PROD activities (Fig. 1). The changes in CYP-associated monooxygenase activities were proportional to the changes in the level of individual CYP proteins (Fig. 2). The suppression of CYP 2E1 protein was consistent with a previous report (Davenport and Wargovich, 2005). In addition, the induction of CYP 2B by DAS might be mediated by the transcriptional activation through an accumulation of a DNA-protein complex binding NR1 that is associated with the constitutively activated receptor, so called CAR (Zhang *et al.*, 2006). Using this model, thioacetamide was employed to determine the possible role of CYP enzymes in the activation of thioacetamide in the present study. The results indicated that thioacetamide-induced hepatotoxicity and immunotoxicity were significantly protected by the pretreatment of animals with DAS at the same dose that modulated CYP enzymes (Fig. 3, 5). Although the possible role of flavin-containing monooxygenase in the activation of thioacetamide should be further investigated, the present results suggested that CYP 2E1 might have a critical role, at least in part, in metabolic activation of thioacetamide. In fact, the possible role of CYP 2E1 in thioacetamide bioactivation becomes more obvious, because a recent report indicated that thioacetamide-induced toxicity was blocked by CYP 2E1 inhibitors in rat hepatocytes (Hajovsky *et al.*, 2012).

Finally, it should be emphasized that DAS might be a useful modulator of CYP enzymes with many advantages: it neither elevated the serum activities of ALT and AST nor

suppressed the antibody responses at the doses that modulate CYP enzymes (data not shown). To investigate the role of metabolic activation in chemical-induced toxicity, it is very convenient to employ nontoxic model inducers and/or inhibitors for drug-metabolizing enzymes *in vivo*. In this regard, the number of nontoxic CYP modulators is very limited, although some inducers and inhibitors have been known to date. For examples, 3-methylcholanthrene and β -naphthoflavone, well-known inducers of CYP 1A enzymes, could not be used as model inducers in studying the role of metabolism in toxicant-induced toxicity *in vivo* due to their intrinsic toxicity (White *et al.*, 1985). Ethanol and dexamethasone, inducers of CYP 2E1 and CYP 3A enzymes, respectively, are also immunosuppressive themselves (Sabbele *et al.*, 1987; Holsapple *et al.*, 1993). Therefore, developing new CYP inducers capable of inducing specific CYP enzyme(s) without or with low toxicity *in vivo* at the dose for CYP induction is of importance. In this context, our research has been focused on natural products because many natural products are reportedly capable of modulating CYP enzymes, and because natural products have long been used, at least in part, without severe toxicity (Jeong *et al.*, 1995; Kim *et al.*, 2004; Lee *et al.*, 2004).

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