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RESEARCH NOTE

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Mixed-type Inhibition of Human Hepatic Cytochrome P450 1-Catalyzed Ethoxyresorufin *O*-deethylation by Volatile Allyl Sulfides

Hyun Jung Kim and Hyang Sook Chun*

Korea Food Research Institute, San 46-1, Baekhyun-dong, Bundang-gu, Sungnam-si, Gyeonggi-do 463-746, Korea

Abstract Effects of allyl sulfides on kinetic behavior of cytochrome P450 1 (CYP1)-catalyzed ethoxyresorufin *O*-deethylase (EROD) activity were studied using microsomes from benzo[a]pyrene-treated human hepatoma cells. Apparent K_m and V_{max} values were calculated as 2.8 μM and 3.0 μmol resorufin/min/mg protein based on Lineweaver-Burk plot of microsomal EROD activity, respectively. Diallyl disulfide (DADS) and diallyl trisulfide (DATS) affected K_m and V_{max} values of EROD activity and acted as mixed-type inhibitors for CYP1 isozymes. Apparent Ki values of DADS and DATS were calculated as 1.07 and 0.88 mM, respectively, by re-plotting slopes of Lineweaver-Burk plot and inhibitor concentrations.

Key words: allyl sulfides, benzo[a]pyrene, cytochrome P450 1, human hepatoma cell, mixed-type inhibition

Introduction

Allyl sulfides, the volatile flavor components of *Allium* vegetables, are one of the most promising bioactive phytochemicals (1-3). Particularly, they have been shown to have inhibitory potency on chemically induced carcinogenesis as revealed in various experimental models (2, 4-5). Understanding the inhibitory mechanism of anticarcinogenic action, structure-activity relationship, and regulation of target enzyme is essential for the development of nutraceutical or chemopreventive agent from naturally occurring phytochemicals. In this regard, the inhibition of cytochrome P450 1 (CYP1) was proposed as one of the mechanisms involved in the anticarcinogenic action of allyl sulfides (6-7). In our previous report, the suppression of benzo[a]pyrene (B[a]P)-inducible CYP1A2 protein from the immunoblot results, and the reduced levels of 7,8-diol, 9,10-diol and 4,5-diol formed as toxic metabolites of B[a]P by allyl sulfides supported the proposed mechanism for chemoprevention (6). From the structure-activity relationships of allyl sulfides based on their inhibitory potency against B[a]P-induced CYP1 activity, we also demonstrated the necessity of the allyl group and two or more S atoms as an essential molecular moiety (8).

Species-specific differences are observed in the regulation and substrate specificity of mammalian CYP enzymes (9-10). Therefore, analysis of the kinetic mechanism of enzyme inhibition in human cells, based on these differences, may provide useful information on the regulation of enzyme activity in humans. However, types of inhibition by allyl sulfides on the CYP1 activity have not yet been reported.

In this respect, inhibitions of B[a]P-induced CYP1 activity by diallyl sulfide [DAS, $(CH_2=CHCH_2)_2S$], diallyl disulfide [DADS, $(CH_2=CHCH_2)_2S_2$], and diallyl trisulfide [DATS, $(CH_2=CHCH_2)_2S_3$] were studied using microsomes from human liver-derived HepG2 cells. The types of inhibitions were characterized, and the inhibition constants were determined.

*Corresponding author: Tel: 82-31-780-9273; Fax: 82-31-709-9876
E-mail: hschun@kfri.re.kr
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Materials and Methods

Chemicals DAS and DADS were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). DATS was provided by LKT Laboratory (St. Paul, MN, USA). B[a]P, bovine serum albumin (BSA), 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide, ethoxyresorufin, resorufin, dimethylsulfoxide, and hydroxypropyl-β-cyclodextrin (HP-β-CD) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The protein assay kit was obtained from Pierce Chemical Co. (Rockford, IL, USA). Fetal bovine serum, minimum essential media, and antibiotics were purchased from Life Technologies, Inc. (Grand Island, NY, USA). All other chemicals were of analytical grade.

Cell culture Human hepatoma HepG2 subline provided by the American Type Culture Collection (Rockville, MD, USA) was used as the *in vitro* model. HepG2 cells were routinely grown in the minimum essential media supplement with 10% fetal bovine serum and antibiotics, and were subcultured every 7 days at a split ratio of 1:6~1:10 under 5% CO_2 and saturated humidity at 37°C.

Preparation of microsomes HepG2 cells were harvested, after 24 hr incubation with 4 mM B[a]P in ice-cold 10 mM phosphate buffered saline (PBS, pH 7.4), and homogenized for 30 sec with 5 sec pauses in between using an ultrasonic apparatus (Vibracell, Sonics & Materials Inc., Danbury, CN, USA). Cell homogenates were fractionated through serial centrifugation at 12,000×g for 30 min and 100,000×g for 60 min. The resulting pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.8) containing 20% glycerol and used for microsomal preparation. Protein concentration of the microsome was measured using the bicinchoninic acid protein assay kit with BSA as the standard.

Enzyme assays Inhibition of allyl sulfides on the B[a]P-

induced microsomal CYP1 activity was assessed by determining the ethoxyresorufin *O*-deethylase (EROD) activities, reactions catalyzed by the CYP1 isozymes, in the presence of DAS, DADS, and DATS using hepatic microsomes. EROD activity was assayed by fluorometrically measuring the rate of resorufin formation using the method of Sinclair *et al.* (11) with minor modification. EROD activities were expressed as μmol resorufin/min/mg protein. Dose of allyl sulfide resulting in 50% inhibition of B[a]P-induced EROD activity (IC_{50}) was determined. Inhibition of microsomal EROD activities by allyl sulfides was calculated as follows: Relative EROD activity (%)=[(B-A)/B]×100, where A and B are EROD activities (μmol resorufin/min/mg protein) of B[a]P-treated microsomes in the presence and absence of allyl sulfides, respectively.

The inhibitory potency of allyl sulfides on EROD activity was also measured in cultured HepG2 cells directly as previously described with the aid of HP-β-CD as an inclusion vehicle to prevent the loss of volatile allyl sulfides (6). Protein contents of the intact cells were measured using the bicinchoninic acid protein assay kit. EROD activities in each treatment were normalized to protein content and were expressed as μmol resorufin/min/mg protein. The IC_{50} of allyl sulfides on the B[a]P-induced EROD activity was calculated using the same method applied to the microsomal activity. All experiments were performed at least three times.

Inhibition study To characterize the kinetic behavior of the EROD activity in the presence of allyl sulfides, allyl sulfides were applied during the pre-incubation step of EROD assays for 5 min. The residual enzyme activities in the presence of allyl sulfides were analyzed at indicated concentrations of allyl sulfides (0, 1, and 2 mM) as inhibitors, and ethoxyresorufin (1.11, 2.22, 4.44, 6.66, and 8.88 mM) as a substrate. Type of inhibition and apparent Ki were determined based on the double reciprocal plot of 1/v vs. 1/[S] and the secondary plot of the slopes of the Lineweaver-Burk plot vs. inhibitor concentrations.

Results and Discussion

In vitro effects of allyl sulfides on human hepatic microsomal EROD activity, a marker enzyme for CYP1 isozymes, were determined to investigate the possible mechanism underlying the inhibitory effects in human. The microsomal EROD activity induced by B[a]P was inhibited by allyl sulfides. IC_{50} of allyl sulfides clearly displayed a structure-activity relationship; DAS showed no inhibitory potency on EROD activity, and the degree of inhibition was proportional to the number of sulfur atoms (Table 1). When EROD activity was assayed in intact cells using HP-β-CD as an inclusion vehicle for volatile allyl sulfides, all allyl sulfides showed lower IC_{50} values than those determined in microsomes, indicating higher inhibition by allyl sulfides in intact cells. Allyl sulfides are well-known volatile phytochemicals, and at least 80% loss of DAS and DADS was reported after 3 hr incubation under routine culture conditions. Therefore, to prevent the loss of allyl sulfides, use of cyclodextrin as an inclusion vehicle was suggested (12). However, cyclodextrin was not applied to our microsomal EROD activity assay system, because the possibility of the non-specific inclusion of hydrophobic reagents in the assay mixture in the cyclodextrin cavity could not be excluded. The volatile nature of allyl sulfides and differences in experimental conditions such as the use of inclusion vehicle could be the major reasons for discrepancies in the inhibitory potency of allyl sulfides assayed in the microsomal or intact cell systems.

Table 1. Inhibition kinetics of microsomal cytochrome P450 1 isozymes of human hepatoma cells by allyl sulfides

Inhibitor	Ethoxyresorufin *O*-deethylase activity			
	IC_{50} (mM)[1]		App Ki (mM)	Type of inhibition
	Intact cells	Microsomes		
Diallyl sulfide	-[2]	-	-	-
Diallyl disulfide	0.58	1.05	1.07	Mixed
Diallyl trisulfide	0.40	0.90	0.88	Mixed

[1]The calculated IC_{50} (mM) means the dose of allyl sulfide resulting in 50% inhibition of benzo[a]pyrene (B[a]P)-induced ethoxyresorufin *O*-deethylase (EROD) activity assayed in intact cells or microsomes.
[2]No effects on B[a]P-induced EROD activity even at the highest dose treated. Inhibition parameters were determined using microsomes obtained from B[a]P-treated human hapatic cells. EROD activity, the marker enzyme for cytochrome P450 1 isozymes, was measured using 7-ethoxyresorufin as a substrate. The apparent Ki values were determined by re-plotting the slope of the double reciprocal plot of the Michaelis-Menten equation obtained in Fig. 1 *versus* the concentration of allyl sulfides (inhibitors). A Ki (intercept with x-axis) was extrapolated applying linear regression analysis.

To investigate the inhibition type of allyl sulfides, inhibition kinetic studies were performed using microsomal CYP1 from human hepatoma cells. Under the experimental conditions, kinetic parameters of microsomal CYP1 were calculated as apparent K_m and V_{max} values of 2.8 μM and 3.0 μmol resorufin/min/mg protein based on the Lineweaver-Burk plot of microsomal EROD activity, respectively (Fig. 1). A significant decrease in the apparent V_{max} values (reciprocal of intercept with y-axis) and a significant increase in the apparent K_m values (negative reciprocal of intercept with x-axis) were observed by applying DADS and DATS. This result indicated that DADS and DATS caused a mixed-type inhibition (competitive-noncompetitive) of CYP1-mediated EROD activity. A mixed-type inhibition by DADS and DATS indicates they compete for the substrate-binding site with ethoxyresorufin, although their binding sites are different from that of the substrate (13). DAS is known as a suicide inhibitor for CYP4502E1-mediated *p*-nitrophenol hydroxylase (14), however, the inhibition types of allyl sulfides on CYP1 have not yet been reported. Our study, showing a mixed-type inhibition by allyl sulfides, could be the first to elucidate the inhibition type of allyl sulfides toward CYP1.

DADS and DATS showed Ki values of 1.07 and 0.88 mM, respectively, as determined by re-plotting the slopes of the Lineweaver-Burk plots versus the concentration of allyl sulfides as inhibitors (Table 1). However, inhibition type and Ki value of DAS could not be determined owing to the very low inhibitory potency of DAS on EROD activity. Ki values of DADS and DATS toward CYP1 were quite high compared with other CYP1 inhibitors (15). The relatively high values of Ki could be explained by the highly volatile nature of allyl sulfides and assay condition that is vulnerable to the loss of volatile

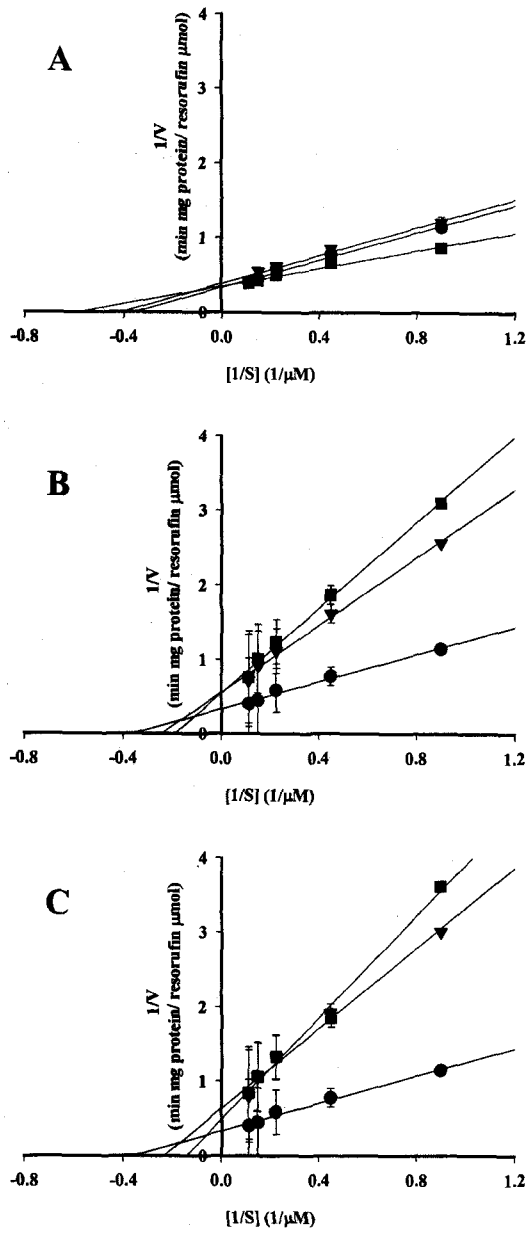

Fig. 1. Lineweaver-Burk plots for inhibition of microsomal cytochrome P450 1 isozymes from human hepatoma cells by allyl sulfides. Cytochrome P450 1-catalyzed ethoxyresorufin *O*-deethylase activity of microsomes from benzo[a]pyrene-treated HepG2 cells were measured in the presence of diallyl sulfide (A), diallyl disulfide (B) and diallyl trisulfide (C) using 7-ethoxyresorufin as a substrate. Allyl sulfides were treated at 0 mM (●), 1 mM (▼) and 2 mM (■). Each value represents the mean of triplicate determinations.

chemicals.

Inhibitory potency of the mixed-type inhibitors of CYPs was primarily controlled by the strength of the bond between their lone pair of electrons and the heme atoms, the hydrophobic and structural characteristics of the nonligating portion of the inhibitor, and the congruence between the geometry of the inhibitor and the active site (16). The increased inhibitory potency of allyl sulfides proportional to the number of S atoms, as observed in our study, could be explained through the hypothesis that it is related to the strength of the bond between the electron pair and the heme atoms of CYP1 isozymes. Sulfur-containing compounds are powerful nucleophiles, because the S atom is larger and more polarizable than the O atom. In the allyl group, the unpaired electron of the allyl radical and the two electrons of the π bond are delocalized over all three carbons (8). Delocalization of the unpaired electron and the resonance structure allow particularly stable allyl group. Due to their chemical features, a stronger nucleophilic nature resulted from the increasing number of S atoms in the allyl sulfides.

Mixed-type inhibitors have also been reported as more effective inhibitors than the agents that only interact either competitively or noncompetitively (17). Although this result was obtained using microsomes from human hepatoma cells, pattern of B[a]P-inducible CYP1 isozymes was similar to that of human liver microsomal samples. In both systems, CYP1A2 isozyme is predominant, and CYP1A1 and 1B1 isozymes were rarely detectable (6, 18-19). Assessment of the inhibition type and kinetic parameters of CYP1 by allyl sulfides could provide meaningful information in developing nutraceuticals having chemopreventive potency, such as appropriate dosage of allyl sulfides that could be calculated based on Ki values.

Conclusion

DADS and DATS, both well-known volatile components of *Allium* vegetables having chemopreventive potentials, inhibited microsomal CYP1 in human hepatoma cells. Under our experimental conditions, apparent K_m and V_{max} of microsomal CYP1 with ethoxyresorufin as a substrate were determined to be 2.8 μM and 3.0 μmol resorufin/min/mg protein based on the Lineweaver-Burk plot, respectively. Application of DADS and DATS affected both V_{max} and K_m values and caused a mixed-type inhibition of CYP1-dependent EROD activity. The Ki values of DADS and DATS were calculated as 1.07 and 0.88 mM, respectively, by re-plotting the slopes of the Lineweaver-Burk plot and inhibitor concentrations. Inhibition type and Ki of allyl sulfides on EROD activity could provide useful information on the inhibition of CYP1 isozyme by allyl sulfides *in vivo*.

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