

SHORT COMMUNICATION

Modulation of *N*-nitrosomethylbenzylamine bioactivation by diallyl sulfide *in vivo*

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Diallyl sulfide (DAS), a major component of garlic oil, is an inhibitor of tumorigenesis by various metabolically activated carcinogens. In rats, pretreatment with DAS has been observed to suppress completely the induction of oesophageal neoplasms by *N*-nitrosomethylbenzylamine (NMBzA) (Wargovich *et al.* (1988) *Cancer Res.*, 48, 6872–6875). This communication reports the effects of DAS on overall NMBzA metabolism and on DNA methylation of NMBzA *in vivo* under conditions equivalent to a single treatment of the chemoprevention assay. Male Fischer 344 rats received a single i.g. dose of DAS (200 mg/kg body wt) followed by an s.c. injection of [methyl-¹⁴C]NMBzA (3.5 mg/kg). In controls, exhalation of ¹⁴CO₂ was complete within 5 h (*t*_{1/2max} = 1.2 h), with 50% of the injected radioactivity recovered as ¹⁴CO₂. When DAS was given 3 h prior to [methyl-¹⁴C]NMBzA, 49% of the injected radioactivity was released within 10 h (*t*_{1/2max} = 3 h). When DAS was administered 18 h before the carcinogen, 42% of [methyl-¹⁴C]NMBzA was converted to ¹⁴CO₂, with exhalation complete after 6 h (*t*_{1/2max} = 1.8 h). We further examined the effects of acute doses of 10–200 mg/kg of DAS on DNA methylation by a single dose of NMBzA (3.5 mg/kg; survival time, 6 h) administered 3 h later. At 200 mg/kg, DAS inhibited the formation of *O*⁶-methyldeoxyguanosine (*O*⁶-MEDG) in oesophagus (–26%), nasal mucosa (–51%), trachea (–68%) and lung (–78%). In liver, levels of 7-MEDG were reduced by 43%. Decreases in DNA methylation were proportional to dose for >25 mg/kg of DAS in oesophagus, liver and nasal mucosa, for 25–200 mg/kg in trachea and 10–50 mg/kg in lung. The dose–activity relationship for inhibition by DAS of DNA methylation by NMBzA suggests that short-term modulation of carcinogen bioactivation *in situ* contributes to but may not be sufficient for the chemoprevention of nitrosamine tumorigenesis by DAS.

Diallyl sulfide (DAS*), a major flavour and fragrance component of garlic oil (1), has been shown to be an effective inhibitor of tumorigenesis by diverse metabolically activated chemical carcinogens (2–5). In the most striking example of chemoprevention, Wargovich and co-workers reported (4) that pretreatment with DAS completely prevented the formation in rat oesophagus of both malignant and premalignant lesions by *N*-nitrosomethylbenzylamine (NMBzA), an exceptionally potent and selective oesophageal carcinogen in this species (6). After preadministration of DAS, a significant reduction has been observed in nuclear aberrations induced by NMBzA and dimethylhydrazine (4,7) but not in the nucleotoxicity of the direct-acting methylating

carcinogens *N*-nitrosomethylurea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (7). Studies *in vitro* have shown that DAS also is an inhibitor of oxidative metabolism by cytochrome P450 enzymes of a number of carcinogenic nitrosamines, including NMBzA, *N*-nitrosodimethylamine, *N*-nitrosodiethylamine and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (4,8–10). These findings suggested that DAS may block nitrosamine-induced tumorigenesis by inhibiting nitrosamine bioactivation *in situ*. In the present investigation, we have examined this possibility by assessing the effects of DAS on the overall metabolism of NMBzA and on DNA methylation by NMBzA *in vivo*.

In the protocol developed by Wargovich and co-workers (4), rats received five weekly treatments consisting of an i.g. dose of 200 mg/kg of DAS followed 3 h later by a s.c. injection of 3.5 mg/kg of NMBzA. However, *in vitro* demethylation of NMBzA was significantly lower by hepatic microsomes prepared 18 h after a similar dose of DAS than by microsomes collected 3 h post-treatment (8). In order to determine whether there is a similar difference *in vivo* between immediate and delayed effects of DAS on overall NMBzA metabolism, we monitored the formation of ¹⁴CO₂ and radioactive urinary metabolites from [methyl-¹⁴C]NMBzA administered 3 or 18 h after a single dose of DAS. In addition, we determined the dose–activity relationship of the effects of DAS on NMBzA bioactivation in target and non-target tissues under conditions equivalent to a single treatment of the chemoprevention experiment (4).

[methyl-¹⁴C]NMBzA was synthesized according to Skipper (11). The radiochemical purity was 83%. DAS was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals and reagents were purchased from commercial sources described earlier (12).

Young male Fischer 344 rats (100–140 g body wt) were obtained from Charles River Wiga GmbH, D-8741 Sulzfeld (Germany) and maintained on a standard laboratory diet with tap water *ad libitum*. For determination of overall NMBzA metabolism, three pairs of rats were treated with an i.g. dose (1 ml/140 g) of corn oil (pair 1) or 200 mg/kg of DAS in corn oil (pairs 2 and 3), followed after 3 h (pairs 1 and 2) or 18 h (pair 3) by a single s.c. injection of [methyl-¹⁴C]NMBzA (3.5 mg/kg; 0.49 mCi/mmol). Immediately thereafter, the animals were placed in a metabolic cage (Jencons Metabowl, Hemel Hempstead, UK). Exhaled ¹⁴CO₂ was absorbed in two serially connected Nilox columns, each containing 600 ml of 1 M NaOH (13). Samples (0.5 ml) were collected hourly. Radioactivity was determined by liquid scintillation counting (efficiency, 87.4%) after the addition of 0.5 ml of 5 M NaOH and 10 ml of Hionic Fluor (Packard Instruments, Zurich, Switzerland).

For studies on DNA methylation, groups of six rats were given a single dose of 0, 10, 25, 50, 100 or 200 mg/kg of DAS in corn oil (p.o., 1 ml/140 g), followed after 3 h by a single s.c. injection of NMBzA (3.5 mg/kg). Animals were killed by exsanguination under ether anaesthesia 6 h later. Tissues were pooled (oesophagus, trachea, nasal cavity scrapings) or collected individually (livers, lungs, kidneys), rapidly frozen in liquid

*Abbreviations: DAS, diallyl sulfide; NMBzA, *N*-nitrosomethylbenzylamine; *O*⁶-MEDG, *O*⁶-methyldeoxyguanosine, 7-MEDG, 7-methyldeoxyguanosine.

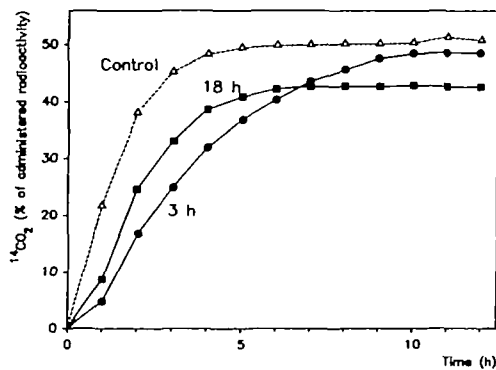


Fig. 1. Effects of DAS on the formation of ¹⁴CO₂ from *N*-nitroso [methyl-¹⁴C]benzylamine. Animals received an i.g. dose (1 ml/140 g body wt) of corn oil (controls) or 200 mg/kg of DAS in corn oil 3 or 18 h prior to a single s.c. injection of *N*-nitroso[methyl-¹⁴C]benzylamine (3.5 mg/kg; 0.49 mCi/mmol). Data are plotted as cumulative percentages of the total radioactivity administered.

nitrogen and stored at -70°C . DNA was isolated by automated phenolic extraction using a Model 340A Nucleic Acid Extractor (Applied Biosystems, Inc., Foster City, CA) as described previously (12), except that tissue homogenates were filtered through nylon gauze (30 μm mesh; Willi Fischer Labortechnik, Frankfurt/Main, Germany). Methylated deoxyguanosines were quantitated by immuno-slot-blot assays using rabbit antiserum NPZ-193 to *O*⁶-methyldeoxyguanosine (*O*⁶-MEDG) (14) and an antiserum to imidazole ring-opened 7-methyldeoxyguanosine (7-MEDG) (15) essentially as described elsewhere (16), with the following modifications. DNA denaturation and conversion of 7-MEDG to the imidazole ring-opened form were carried out at alkaline pH. Briefly, DNA (12 μg in 200 μl of 1 mM EDTA/10 mM Tris, pH 7.8) was treated with 200 μl of 100 mM NaOH at room temperature for 10 min, neutralized by the addition of 200 μl of 15% (v/v) acetic acid, and immediately mixed with 200 μl of 4 M ammonium acetate. Concentrations of methylated deoxyguanosines were calculated using peak areas of the densitometric evaluation. The limits of detection for *O*⁶-MEDG and 7-MEDG were 0.8 and 7.5 $\mu\text{mol/mol}$ dG respectively.

Modulation of the overall metabolism of a single dose (3.5 mg/kg) of [methyl-¹⁴C]NMBzA by a preceding acute dose of DAS (200 mg/kg) is shown in Figure 1. In controls, exhalation of ¹⁴CO₂ was complete within 5 h, with 50% of the injected radioactivity recovered as ¹⁴CO₂; half of the total amount of exhaled ¹⁴CO₂ was released within 1.2 h ($t_{1/2\text{max}}$). Both the rate and the extent of conversion of [methyl-¹⁴C]NMBzA to ¹⁴CO₂ were almost identical to those observed previously (13,18). Recovery of radioactivity from urine after 12 h (20% of the total administered radioactivity) was slightly higher in the present experiment than reported previously (13), which could reflect the excretion of polar radiolysis products of [methyl-¹⁴C]-NMBzA. When [methyl-¹⁴C]NMBzA was given 3 h after DAS, formation of ¹⁴CO₂ was markedly retarded, with a total of 49% of the administered radioactivity released within 10 h ($t_{1/2\text{max}} = 3$ h). In contrast, production of ¹⁴CO₂ from [methyl-¹⁴C]-NMBzA administered 18 h after DAS was complete within 6 h ($t_{1/2\text{max}} = 1.8$ h), whereas the fraction exhaled as ¹⁴CO₂ was decreased to 42%. DAS had no effect on the excretion of radioactive urinary metabolites (data not shown).

As shown in Table I, administration of a single oral dose of DAS (200 mg/kg) 3 h prior to an s.c. injection of NMBzA (3.5 mg/kg; survival time, 6 h) led to decreases in the formation of

Table I. Effects of a single high dose of DAS on DNA methylation by NMBzA in various rat tissues^a

	Corn oil	DAS	Decrease (%)
<i>O</i> ⁶ -MEDG			
Oesophagus	8.4 \pm 1.4 ^b	6.2 \pm 0.1	26
Nasal cavity	9.6 \pm 1.0	3.4 \pm 0.1	51
Trachea	9.1	2.9 \pm 0.4	68
Lung	3.2 \pm 0.4	0.7 \pm 0.4	78
Liver	2.4 \pm 0.6	2.0 \pm 0.6	0
Kidney	1.8 \pm 0.6	2.1 \pm 0.7	0
7-MEDG			
Oesophagus	83.8	58.2	30
Liver	38.5 \pm 0.1	21.8 \pm 6.6	43

^aAdult male Fischer 344 rats received a single dose by gavage (1 ml/140 g body wt) of either corn oil or 200 mg/kg of DAS in corn oil, followed 3 h later by an s.c. injection of NMBzA (3.5 mg/kg; survival time, 6 h).

^b $\mu\text{mol/mol}$ deoxyguanosine. Values are expressed as mean \pm SD of five animals assessed individually (liver, lung, kidney; $n = 3-12$) or of DNA pooled from five animals (oesophagus, nasal mucosa, trachea; $n = 3-6$).

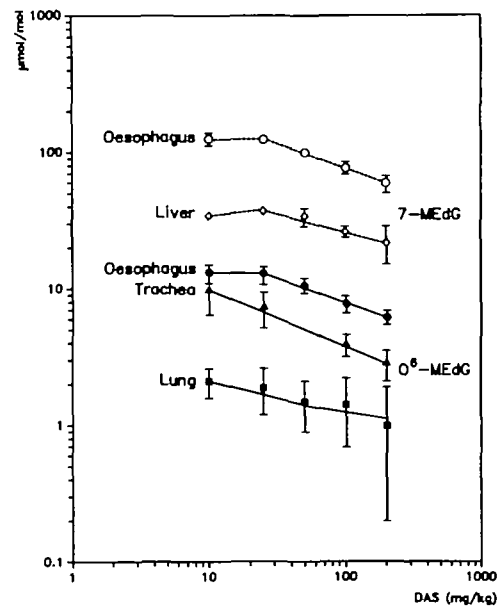


Fig. 2. Dose-dependent inhibition by DAS of DNA methylation by NMBzA in various rat tissues. Animals received a single i.g. dose (1 ml/140 g body wt) of 10–200 mg/kg of DAS in corn oil, followed 3 h later by a s.c. injection of NMBzA (3.5 mg/kg). Concentrations of methylated deoxyguanosines were determined 6 h after administration of the carcinogen. Results are expressed as $\mu\text{mol/mol}$ deoxyguanosine. Each point represents mean \pm SD of determinations in DNA from two animals (liver; $n = 3-12$) or five animals (lung; $n = 3-12$) assessed individually or in DNA pooled from five animals (oesophagus, trachea; $n = 3-6$).

*O*⁶-MEDG in oesophagus (–26%), nasal mucosa (–51%), trachea (–68%) and lung (–78%). In liver, concentrations of *O*⁶-MEDG were close to the limit of quantitation and a significant effect of DAS on its formation was not observed; however, levels of 7-MEDG were decreased by 43%. DAS did not have a detectable effect on DNA methylation in kidney. Dose–activity curves are shown in Figure 2. In liver and oesophagus, inhibition of DNA methylation was directly proportional to dose for >25 mg/kg of DAS. Similar results were obtained in nasal mucosa (data not shown). Trachea was more sensitive to the inhibitory effects of low doses of DAS, with a dose-dependent decrease in the formation of *O*⁶-MEDG observed for 25–200

mg/kg. In lung, there was a significant ($P < 0.001$) trend towards lower levels of methylation with higher doses of DAS for doses ranging from 10 to 50 mg/kg. The linear portions of the double-logarithmic dose–activity curves were parallel in all tissues.

Differential modulation of cytochrome P450 enzyme levels has been proposed to be one of several mechanisms underlying chemoprevention of nitrosamine tumorigenesis by DAS. In rat liver, induction of cytochrome P4502B1, and of ethoxyresorufin and pentoxyresorufin dealkylase activities was detected 5 h after DAS administration, with maximum levels attained within 48 h (8,9). Conversely, preadministration with DAS has been shown to result in inactivation and subsequent removal from the microsomal membrane of hepatic cytochrome P4502E1 within 18 h (8,9). We observed a decrease in both the extent and rate of formation of $^{14}\text{CO}_2$ from [methyl- ^{14}C]NMBzA given 18 h after a single dose of DAS, suggesting that a significant reduction of nitrosamine bioactivation may be achieved after repeated administrations of DAS through modulation of cytochrome P450 enzyme levels in target cells. However, although NMBzA is preferentially bioactivated in the oesophageal mucosa, measurements of overall metabolism in the intact animal primarily reflect hepatic turnover because of the size of the liver (17,18) and cannot necessarily be extrapolated to other organs. Little is known on the effects of DAS on monooxygenase concentrations in extrahepatic tissues. In nasal microsomes, concentrations of P4502E1 and proteins immunologically related to P4502B1 were not affected by pretreatment with DAS (10). The effects of DAS on cytochrome P450 levels in oesophagus have not yet been reported. Stimulation of hepatic nitrosamine metabolism by chronic administration of DAS could indirectly contribute to chemoprevention of nitrosamine tumorigenesis in other tissues even in the absence of an effect on extrahepatic cytochrome P450 levels by increasing first-pass clearance of nitrosamines by the liver and hence reducing extrahepatic concentrations.

Other potentially anticarcinogenic long-term effects of DAS that are not directly related to carcinogen bioactivation have been described. Induction of phase II detoxification enzymes by DAS (2,19,20), could limit the mutagenicity of a wide range of carcinogens by stimulating conjugation and excretion of reactive intermediates. In the present study, formation of urinary metabolites from NMBzA was not significantly altered by an acute dose of DAS administered either 3 or 18 h earlier. However, there is evidence that induction of detoxification pathways may take longer. In mice, hepatic glutathione S-transferase activity was increased from 118% of control levels at 24 h to 178% at 48 h after DAS administration (21). DAS has further been shown to inhibit the induction of ornithine decarboxylase by carcinogens (22) and γ -irradiation (23). Suppression of the proliferative response to genotoxic damage by this or related mechanisms has been proposed to partly account for the decrease in tumour progression (24), in phorbol ester-induced promotion (25,26), and in the tumorigenesis of metabolically activated and direct-acting carcinogens (27) which were observed when DAS or garlic extracts were administered postinitiation.

In vitro studies have revealed that DAS is a competitive inhibitor of cytochrome P4502E1 (8). Our finding that the rate of $^{14}\text{CO}_2$ production from [methyl- ^{14}C]NMBzA was markedly decreased when DAS was given 3 h before the carcinogen but only marginally affected when DAS was administered 18 h prior to NMBzA suggests that nitrosamine metabolism *in vivo* is inhibited by DAS itself or short-lived early metabolite(s). It has recently been demonstrated (28) that DAS is completely metabolized within 18 h *in vivo*. Metabolites include diallyl

sulfoxide, a competitive inhibitor of hepatic cytochrome P4502E1 *in vitro*, and diallyl sulfone, which has been reported to rapidly inactivate cytochrome P4502E1 via competitive suicide inhibition (28). The time course of $^{14}\text{CO}_2$ exhalation has previously been shown to closely parallel the overall metabolism of ^{14}C -labelled nitrosamines (17,29). However, modulation by DAS of enzyme activities involved in the metabolism of the C1 pool may have additionally inhibited the release of $^{14}\text{CO}_2$.

Pretreatment with DAS led to dose-dependent inhibition of DNA methylation by NMBzA in various rat tissues over a wide range of doses. Nevertheless, DAS was not a very potent inhibitor of NMBzA bioactivation. At the highest dose of DAS tested (200 mg/kg), decreases in the extent of DNA methylation ranged from 26% in oesophagus to 78% in lung (Table I). Levels of methylated deoxyguanosines in oesophagus were thus only moderately reduced when DAS and NMBzA were administered at the same time interval and dose ratio at which, after repeated applications, the development of preneoplastic and neoplastic lesions was completely prevented (4). The partial inhibition of NMBzA bioactivation is tantamount to a decrease in the effective dose of NMBzA. It has been shown (6) that even at very small doses of genotoxic carcinogens, there is a clear dose–effect and time relationship for tumor induction, which can be expressed as follows:

$$d \cdot t^n = \text{constant}; n > 1$$

with d being the daily dose, t the median tumour latency period and n representing carcinogenic potency. Therefore, short-term inhibition by DAS of NMBzA bioactivation would be expected to significantly delay but not to prevent tumour induction by NMBzA using the treatment protocol of the chemoprevention assay. However, the possibility remains that multiple doses of DAS could severely reduce the capacity for nitrosamine bioactivation in this tissue if pretreatment with DAS resulted in cumulative and persistent inactivation of oesophageal cytochrome P450 isozymes as a consequence of slow repair or replacement. In the chemoprevention experiments, animals were killed 15 weeks after the first treatment (4). Conceivably, NMBzA-induced oesophageal neoplasms might have been detected had the observation period been extended to account for the increased latency period arising from damage incurred during the initial treatment and the decreased effective dose of carcinogen during subsequent administrations.

In summary, our results strongly suggest that the extent to which NMBzA bioactivation in the oesophagus was inhibited by the immediately preceding dose of DAS during each treatment cycle was not sufficient to elicit the complete suppression of oesophageal tumorigenesis observed in long-term carcinogenicity assays (4). Inhibition of bioactivation is thus very likely only one of multiple mechanisms involved in the suppression of nitrosamine tumorigenesis by DAS. Preliminary investigations have suggested that at low doses of DAS, modulation of cellular responses to genotoxic damage may predominate over effects on carcinogen metabolism (5). This aspect of the chemopreventive properties of DAS may be particularly relevant to human cancer in view of the recent epidemiological study linking consumption of allium vegetables to a reduced incidence of gastric cancer (30).

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