Possible Interaction of Thiol Groups of Proteins with Antimutagens Containing a Conjugated Carbonyl Structure[†]

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Further support to the hypothesis that antimutagenic activities of α,β -unsaturated carbonyl compounds against UV-induced mutagenesis of *E. coli* may be due to an interaction with thiol groups were obtained by an experiment with the supplement of glutathione to the assay medium. Antimutagenic activity against MNNG induced mutation was also observed.

Since finding cinnamaldehyde as the first naturally occurring organic antimutagen,¹⁾ we have been involved in screening and chemical studies of such biologically active substances.^{2,3)} Currently, antimutagenic actions observed in the *Escherichia coli* system are thought to be affected either by a selective killing effect of cells having premutational lesion of DNA, by inhibition of the error-prone SOS repair system, or by enhancement of the error-free DNA repair system.^{4~6)} However, most of the mechanism studies on antimutagenesis have been based on the genetic aspects. We have given attention to this problem from slightly different chemical aspects by studying structure-activity relationship and developing of novel synthetic antimutagens.³⁾ It has been suggested that some of these antimutagens seem to be chemically reactive Michael acceptors, and that the antimutagenic action of these compounds may be due to interaction with thiol groups of the target proteins.³⁾ This hypothesis was further examined and several supporting results are described in this paper.

MATERIALS AND METHODS

Synthetic antimutagens were prepared as described previously.³⁾ *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was purchased from Tokyo Kasei Kogyo Co., Ltd. A reduced form of glutathione was from Wako Pure Chemical Industries Ltd. Other chemicals and the ingredients of culture media were obtained as described previously.³⁾ Nuclear magnetic resonance (NMR) experiments were conducted with a Hitachi R-24 spectrometer. A sample (0.1 mmol) and a thiol were dissolved together in 0.4 ml of solvent (CDCl_3 or CD_3OD) in a 5 mm diameter tube. Spectra were recorded from time to time at room temperature either with or without triethylamine (0.1 mmol), the signal intensity of the olefinic protons of samples being measured by integration. The half-life of a compound was determined by the period after which time a signal intensity has decreased to one half of the original.

Antimutagenic activities were assayed by the previously described soft agar method with minor modifications.^{1,3)} An antimutagenic chemical was dissolved and diluted to an appropriate concentration in dimethyl sulfoxide, which was then mixed with either the UV-treated or the untreated cell suspension of *Escherichia coli* WP2 B/r *trp E* in a soft agar medium. Glutathione of the reduced form (0.1 ml) in a 1/15 M phosphate buffer (pH 7.0) was added to the soft agar mixture to a final concentration of 0.3 mg/ml or 0.03 mg/ml and was well mixed, the whole then being spread on to an MB plate. After 2 days of incubation

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tion at 37°C, the tryptophan prototrophic revertant colonies were counted.

The antimutagenic activity against MNNG mutation was determined in a similar manner, using a method adapted from that of Kada *et al.*⁷⁾ Cells of *E. coli* WP2 B/*trp E* obtained from an overnight culture were suspended in a phosphate buffer (1/15 M, pH 7.0), to which was added MNNG dissolved in the same phosphate buffer to a final concentration of 50 µg/ml. The cells were harvested by centrifugation. After being washed twice with the same phosphate buffer, the mutagenized cells were subjected to the soft agar assay in a similar way to that already described for the UV-induced mutagenesis.

RESULTS AND DISCUSSION

It was speculated by our studies that most of the naturally occurring antimutagens commonly contain a conjugated α,β -unsaturated carbonyl system and probably exert their antimutagenic activity by trapping the thiol groups of appropriate proteins.³⁾ The recently described antimutagenic activity of a sesquiterpenoid α -methylene lactone, cnicin, supported this hypothesis.⁸⁾

To substantiate the high reactivity of synthetic antimutagens to a thiol functionality, an antimutagen was mixed with a thiol in an NMR tube, and the disappearance of olefinic proton signals was monitored. A typical time-

dependent reaction profile is illustrated in Fig. 1. These results clearly demonstrate the chemical reactivity of synthetic antimutagens with thiol groups. The approximate half-lives of the tested compounds using α -toluenethiol are summarized in Table I, along with the antimutagenic activity (AD_{50}), the definition of which was described in a previous paper.³⁾ Although the half-lives, which are supposed to correspond to the reactivity of the individual compounds to thiol groups, were not fully precise under these conditions, rather good correlation was observed between the half-life and the antimutagenic activity, at least for biologically active compounds. Thus, the modestly active compounds **3**, **4** and **5** were estimated to have half-lives of 1~2 hr with the aid of a triethylamine catalyst. In the absence of the catalyst, these compounds did not react with the thiol. Highly active protoanemonin (**6**) reacted with a thiol very rapidly in the presence of triethylamine, whereas the most active vinylfuranone (**7**) was extremely reactive to show its half-life of 20 min in CD_3OD , even in the absence of the catalyst. Biologically less active butenolide (**2**) also showed similar reactivity to those of such modestly active compounds as **3**, **4** and **5**, but biologically inactive furanone (**1**) was found to be the least reactive,

FIG. 1. Time-dependent Alteration of the 1H -NMR Spectrum of **7** by the Reaction with 1-Propanethiol.

TABLE I. CORRELATION OF ANTIMUTAGENIC ACTIVITY (AD_{50}) AND CHEMICAL HALF-LIFE IN THE REACTION WITH α -TOLUENETHIOL

Compound
	1	2	3	4
AD_{50} (µg/ml)	inactive	550	180	95
$t_{1/2}$	19 day	1.5 hr	2 hr	1 hr
Compound
	5	6	7	
AD_{50} (µg/ml)	70	2.8	0.9	
$t_{1/2}$	1 hr	< 1 min	20 min ^{b)}	

^a The reaction was carried out in $CDCl_3$ in the presence of triethylamine at room temperature unless otherwise noted.

^b In the absence of triethylamine in CD_3OD .

i.e., its half-life was more than two weeks. It was also observed that some of the biologically inactive compounds such as 2-penten-4-olide and 2,2-dimethyl-5-styryl-2(3*H*)-furanone were fairly reactive toward the thiol, and their half-lives were estimated to be less than a few hours. This fact would suggest that high reactivity with a thiol compound is not satisfactory but is a prerequisite.

The *in vivo* effect of a thiol compound on the antimutagenic action of the synthetic antimutagen was next examined by a soft agar method supplemented with a reduced form of glutathione. This kind of supplementation experiment was preceded.^{9,10} Kupchan *et al.*^{11,12} and others¹³ were able to suggest the positive involvement of thiol trapping by the action of quite a number of natural cytotoxic products with an α,β -unsaturated carbonyl functionality.

As can be seen from the Table II, the reduced form glutathione showed a preventive effect against highly antimutagenic vinylfuranone (7). The ratio of the emergence of trp⁺ revertant colonies was greatly increased by adding the reduced-form glutathione. Antimutagenic activity began to appear from a dosage of slight excess and at a higher molar ratio of vinylfuranone (7) to glutathione. Thus, under the supplement condition of 0.3 mg/ml, antimutagenic activity of vinylfuranone (7) was observed at a dosage of 25 μ g/

ml, and this critical concentration was changed to around 1.5 μ g/ml of vinylfuranone (7) against 0.03 mg/ml of glutathione. Molecules of vinylfuranone (7) seemed to react rapidly with glutathione in the medium, if glutathione existed in excess. We also examined the supplementation effect of moderately antimutagenic vinylbutenolide (4), but in this case, a definitive effect beyond experimental error was not observed, probably due to the low chemical reactivity of 4 toward glutathione in the medium.

These results may further reinforce the early hypothesis that the above-mentioned antimutagens exert their activity through the reaction of thiol groups under physiological conditions, and that a likely candidate of such thiol groups is the natural cysteinyl residue of certain proteins.^{3,14}

A direct interaction of these antimutagens as Michael acceptors with nitrogen nucleophiles, *e.g.*, base portions of nucleic acid, cannot be ruled out at present. However, the known chemical reactions of nucleic acid, mostly DNA, are mostly those with rather hard electrophiles such as epoxides, aziridines, alkyl halides, alkyl sulfonates and diazoalkanes.¹⁶⁻¹⁹ Because of the hardness of the nucleophilic centers of nucleic acids, it seems less likely that nucleic acids react directly *in vivo* with such rather soft electrophiles as these conjugated carbonyl compounds, which would

TABLE II. ANTIMUTAGENIC EFFECT OF VINYLFURANONE (7) IN THE PRESENCE OR ABSENCE OF GLUTATHIONE

Glutathione (mg/ml)	UV-induced revertants/plates ^a						
	Concentration of 7 (μ g/ml)						
	0	0.75	1.5	3.1	6.25	12.5	25
None ^b	102 (100)	55 (54)	25 (25)	18 (18)	2 (2)	0 (0)	0 (0)
0.03	209 (100)	173 (83)	166 (79)	140 (67)	94 (45)	17 (8)	0 (0)
0.3	204 (100)	197 (97)	216 (105)	213 (104)	210 (103)	207 (101)	167 (82)

^a Data are the average of triplicates. The numbers in parentheses are the percentage ratio to the control.

^b Data are from the previous studies.

prefer to react with soft nucleophiles such as thiols.²⁰⁾ In fact, it has been reported that no reaction occurred between guanine, the most likely nucleophilic site of nucleic acid, and helenalin²¹⁾ or butenolides.²²⁾

Some rather strange observations have been recently reported. Although its biochemical mechanism is not well understood, cinnamaldehyde, another α,β -unsaturated carbonyl antimutagen,¹⁾ is known to suppress the *umuC*-dependent mutagenesis induced by UV-mimic chemical mutagens.⁶⁾ In contrast, ester derivatives of cinnamic acid, which are also α,β -unsaturated carbonyl compounds, have been reported to enhance UV-induced mutagenesis rather than antimutagenesis due to the inhibition of DNA excision repair.²³⁾ Further investigations may be required to shed light on these compounds.

It was further examined whether vinylfuranone (7) and vinylbutenolide (4) show antimutagenic action against the mutagenesis of MNNG in the same *E. coli* strain. It turned out that these α,β -unsaturated compounds

were active against the MNNG induced mutagenesis in rather narrow dose ranges (Table III), and the AD₅₀ values of 4 and 7 were estimated to be 230 $\mu\text{g}/\text{ml}$ and 4.4 $\mu\text{g}/\text{ml}$, respectively.

UV-induced mutagenesis is supposed to be fixed by an active cellular system of error-prone SOS repair that processes premutagenic lesion of DNA triggered mostly by dimerization of pyrimidine bases, whereas the damaged DNA by alkylating agents including MNNG may be processed by adaptive response.^{24,25)} It is also known, however, that both mutageneses induce SOS response, which causes simultaneous expression of various genes under the SOS regulatory system.²⁶⁾

From the results already described, those synthetic antimutagens are quite active against two different types of mutagenesis and repair system. This may reflect the high reactivity of the antimutagens and/or the rather less specific nature of the reaction between the antimutagens and proteins through thiol trapping. Conversely, although it seems less likely, this might suggest the involvement of similar kinds of proteins in both SOS and adaptive responses.

In any event, most studies on the antimutagenic mechanism have been so far based on the genetic aspect, and much less effort have been directed to the biochemical and molecular bases. However, since studies on the biochemistry of proteins involved in the mutagenesis and repair systems of *E. coli* have been expanding rapidly,²⁷⁾ further investigations from the chemical aspect would facilitate such studies.

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TABLE III. ANTIMUTAGENIC ACTIVITY OF α,β -UNSATURATED CARBONYL COMPOUNDS AGAINST MNNG INDUCED MUTATION OF *E. coli* WP2 B/r *trp E*

Compound	Dose ($\mu\text{g}/\text{ml}$)	Revertant colonies	Viable cells	Mutation frequency ($\times 10^{-6}$)
4	0	704	113	20.11
	7.5	650	94	17.92
	15	646	112	18.40
	31	676	118	19.70
	62.5	672	97	16.37
	125	546	105	17.03
	250	290	114	9.35
	500	0	97	—
7	0	824	172	11.58
	0.75	822	150	9.20
	1.5	720	170	8.66
	3.1	642	172	7.03
	6.25	398	177	4.46
	12.5	12	159	0.15
	25	0	0	—
	50	0	0	—

Data are the average of triplicates.

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