Inhibition of mutagenicity of food-derived heterocyclic amines by sulforaphane — A constituent of broccoli

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Sulforaphane, a constituent of broccoli was investigated for its antimutagenic potential against different classes of cooked food mutagens (heterocyclic amines). These include imidazoazarennes such as 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP); pyridomide derivatives such as 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2); and, dipyrroimidizazole derivative such as 2-amino-6-methylpyrido[1,2-a:3',2'-d]imidazole (Glu-P-1). Tests were carried out by Ames Salmonella typhimurium TA98 (frame shift mutation sensitive) and TA100 (base pair mutation sensitive) bacterial strains in the presence of AtTCor 1254-induced rat liver S9. Results of these in vitro antimutagenicity studies strongly suggest that sulforaphane is a potent inhibitor of the mutagenicity induced by imidazoazarennes such as IQ, MeIQ and MeIQx (~60% inhibition) and moderately active against pyridomide derivatives such as Trp-P-1 and Trp-P-2 (32-48% inhibition), but ineffective against dipyrroimidizazole derivative (Glu-P-1) in TA100.

Recently, much attention has been focused on the role of diet in the etiology of cancer. Diet is a complex mixture of chemical entities and may contain substances that cause cancer as well as agents that can inhibit or modulate the development of neoplasia. Common cooking procedures such as broiling, frying, barbecuing, heat processing and pyrolysis of protein rich foods such as beef, fish and chicken induce the formation of potent mutagenic and carcinogenic compounds that are of heterocyclic amines1-3. There is considerable evidence to indicate that man is exposed to heterocyclic amines through diet and is susceptible to the carcinogenic effects of these highly potent mutagens and reported rodent and non-human primate carcinogens4,5. The carcinogenic risk imposed by these probable human carcinogens is also modulated by other dietary factors that influence their uptake and biotransformation. Researchers are now investigating specific phytochemicals found in fruits and vegetables that may serve as weapons against cancer and other diseases. There is ample scientific evidence indicating that populations consuming a diet rich in fruits and vegetables have a reduced risk of developing several types of cancers6-10.

Isothiocyanates represent an important class of dietary constituents that are of potential value in the prevention of cancer11-14. These are widely distributed in cruciferous vegetables such as broccoli, watercress, horseradish, cabbage, cauliflower, and Brussels sprouts etc.15. Over the past 40 years more than 20 naturally occurring as well as synthetic isothiocyanates have been assessed for their chemopreventive activities16. A large number of studies on dietary isothiocyanates have shown that these isothiocyanates play an important role in the prevention of chemically induced cancers in laboratory animals and might also be chemoprotective in humans1,17,18.

Sulforaphane, an alkyl isothiocyanate, recently isolated from broccoli19 and a potent inducer of Phase-2 detoxification enzymes20-22 and inhibitor of Phase-1 enzymes24,25 is currently under active investigation for its potential cancer chemopreventive properties. Although, several isothiocyanates including phenyl isothiocyanate (PITC), BITC, PEITC and phenolpropyl isothiocyanate (PPITC) have been reported to reduce the genotoxicity of cooked food mutagens such as IQ, MeIQx, PhIP and 2-amino-3,8-dipyrroimidizazole (Glu-P-2) in S. typhimurium/ reversion assay in the presence of liver microsomes23 there are no reports regarding antigenotoxic effects of sulforaphane against cooked food mutagens. Thus, sulforaphane appears to be a potential candidate for
investigation of in vitro inhibitory activities towards cooked food mutagens.

In view of these findings it was considered important to investigate the antimutagenic potential of sulforaphane (1-isothiocyanato-(4R)-(methylsulfinyl)butane) using Ames Salmonella/reversion assay in two strains of S. typhimurium namely TA98 and TA100 against various classes of heterocyclic amines found in human diet.

Materials and Methods

Bacterial strains— A set of histidine requiring TA98 and TA100 strains of Salmonella typhimurium were obtained as a kind gift from Dr Bruce N. Ames (University of California, Berkley, USA).

Chemicals— 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (Mel), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MelQx), 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) acetate and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) were purchased from Toronto Research Chemicals Inc., Canada. 2-Amino-6-methylidipiridyl[1,2-a:3',2'-d]imidazole (Glu-P-1) hydrochloride (monohydrate) was purchased from Wako Pure Chemicals, Japan. 3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) acetate was kindly gifted by Dr T. Nomhi, National Institute of Hygienic Sciences, Tokyo, Japan. L-Sulforaphane was kindly gifted by LKT laboratories, USA. Albumin, bovine and nicotinamide adenine dinucleotide phosphate (NADP) sodium salt, were purchased from Sisco Research Laboratories, Bombay, India. D-Glucose-6-phosphate monosodium salt and d-biotin were purchased from Sigma Chemical Company, USA, Oxoid, nutrient broth no.2 was purchased from Oxoid Ltd., Basingstoke, Hampshire, England. Nutrient agar was purchased from Hi media Lab. Pvt. Ltd., India. All other reagents used were of AR grade.

Preparation of liver homogenate S9 fraction— The S9 fraction was prepared from the pooled liver homogenate of 2 male Sprague-Dawley rats previously induced with Aroclor 1254, by the method of Garnett et al.32

Determination of protein concentration of S9 — Protein concentration of induced rat liver S9 was determined by biuret method 28 and was found to be 54 mg/ml.

Antimutagenicity testing — The plate incorporation procedure given by Maron and Ames28 was used for antimutagenicity testing with the inclusion of preincubation step26. Negative and positive controls were included in each assay (Table I). L-sulforaphane in the concentration range of 100-500 nmoles/plate was also checked for possible toxic or mutagenic effects in both TA98 and TA100 strains and no change in spontaneous revertant count indicated absence of any mutagenic/toxic effects of L-sulforaphane in the tested dose range (see footnote of Table I for revertant counts). A suitable dose of the test mutagens was selected from the linear portion of the dose-response curve of the respective mutagen for both TA98 and TA100. Further, mutagens were applied to the test in such doses which resulted in a maximum of about 2000 His+ revertants/plate (9-12 fold increase over spontaneous count), so as to ensure accurate counting, since at this count overlapping of bacterial colonies is avoided and inhibition or enhancement by modulators can be detected with a minimum statistical variation (for dose of mutagens see Table I).

All assays were carried out in duplicate/triplicate on separate occasions. Results are expressed as mean±SD of His+ revertants per plate (uncorrected for spontaneous count) for each dose.

Statistical analysis — All the data were statistically analysed by one way analysis of variance (ANOVA) followed by Student-Newman-Keuls method. Linear regression was used to test for linearity of dose-response relationship.

Results and Discussion

The ability of sulforaphane to inhibit genotoxicity of heterocyclic amine mutagens derived from cooked meat was assessed in S. typhimurium TA100. Results as shown in Table I, clearly indicate that sulforaphane effectively inhibited S9-mediated mutagenicity of all the tested cooked food mutagens, except Glu-P-1, in a dose-dependent manner, over the entire concentration range of 100-500 nmoles/plate (r=0.8-0.9). Only 8% reduction in the mutagenicity of Glu-P-1 was achieved at a dose of 500 nmoles/plate. The IC50 values (the dose of sulforaphane required to reduce the mutagenicity of a given mutagen by 50%, calculated from corresponding dose response curves) for IQ, MelQ, and MelQx, were found to be 371, 440 and 443 nmoles/plate, respectively, indicating that sulforaphane was most active against IQ induced mutagenicity. It showed almost similar reduction in mutagenicity induced by PhIP (47% inhibition at 500 nmoles/plate) and Trp-P-2 (48% inhibition at 500 nmoles/plate). The least inhibitory effect was observed against Trp-P-1 (32% inhibition at 500 nmoles/plate). Doses greater than 500 nmoles/plate were found to inhibitory to the strain TA100.
of biological strongly suggest that sulforaphane is a potent inhibitor of food-derived heterocyclic amines such as IQ, MelIQ and MelQx (~60% inhibition) and moderately active against pyridonoindole derivatives like Trp-P-1 and Trp-P-2 (32–48% inhibition), but ineffective against dipyridoindazole derivative, Glu-P-1. In the tested dose range sulforaphane did not show any mutagenic or toxic effects against TA100 strain of S. typhimurium. However, doses higher than 500 nmoles/plate were found to be toxic to the bacterial culture.

As the heterocyclic amines are promutagens and need metabolic activation particularly by cytochrome P450A2 isozyme \( \text{P450A2} \) and since, sulforaphane is reported to inhibit various cytochrome P450 enzymes including cytochrome P450A2 \( \text{P450A2} \), it may be proposed that the potent antimutagenic effects of sulforaphane observed against cooked food mutagens in our studies, were due to the inhibition of metabolic activation of these promutagens. This proposition can be further strengthened by an earlier investigation \( \text{Sulforaphane has been reported to be ineffective against sodium azide-induced mutagenicity, latter be-} \)

<table>
<thead>
<tr>
<th>Cooked food mutagen (nmoles/plate)</th>
<th>L-Sulfuraphane (nmoles/plate)</th>
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<tbody>
<tr>
<td><strong>No. of His(^+) revertants/plate</strong></td>
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<tr>
<td>IQ (5)</td>
<td>1457 ± 41</td>
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<tr>
<td>MeIQ (0.5)</td>
<td>887 ± 81</td>
</tr>
<tr>
<td>MelQx (12)</td>
<td>926 ± 36</td>
</tr>
<tr>
<td>Trp-P-1 (83)</td>
<td>519 ± 19</td>
</tr>
<tr>
<td>Trp-P-2 (8.3)</td>
<td>100</td>
</tr>
<tr>
<td>PhiP (400)</td>
<td>100</td>
</tr>
<tr>
<td>Glu-P-1 (20)</td>
<td>100</td>
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</table>

*All values are expressed as mean ± SD (n = 6), include spontaneous revertant count (negative control) of 132 ± 10 (n = 15) and are statistically different from control as well as from each other at \( P < 0.05 \) (analysed by Student-Newman-Keuls method).

*Mean value is not statistically different from mean value at 0 nmoles/plate of L-sulfuraphane (control) at \( P < 0.05 \).

*Mean value is not statistically different from mean value at 250 nmoles/plate of L-sulfuraphane at \( P < 0.05 \).

With 2-aminoflourene (positive control) revertant count is 2758 ± 67 (n = 15).

The spontaneous revertant count in presence of various concentrations of L-sulfuraphane alone is: 129 ± 6 (100 nmoles/plate); 132 ± 10 (250 nmoles/plate); 126 ± 8 (500 nmoles/plate) using three plates per point.

Only a very narrow dose range upto 100 nmoles/plate of sulforaphane could be tested in TA98 strain as doses of 250 nmoles/plate and higher showed toxicity to the culture. It did not show any significant inhibitory effects against induced mutagenicity of any of the tested cooked food mutagens upto the dose of 100 nmoles/plate (data not shown).

Results of these in vitro antimutagenic studies using base pair mutation sensitive strain TA100 strongly suggest that sulforaphane is a potent inhibitor of bacterial mutagenicity induced by imidazooazacenes such as IQ, MeIQ and MelQx (~60% inhibition) and moderately active against pyridoindole derivatives like Trp-P-1 and Trp-P-2 (32–48% inhibition), but ineffective against dipyridoindazole derivative, Glu-P-1. In the tested dose range sulforaphane did not show any mutagenic or toxic effects against TA100 strain of S. typhimurium. However, doses higher than 500 nmoles/plate were found to be toxic to the bacterial culture.

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ing a direct acting genotoxicant. On the other hand, a similar pattern of a fairly strong antimutagenic action (32–60% inhibition) against heterocyclic amines, strongly support the involvement of the same mechanism of inhibition i.e. the suppression of bioactivation of these mutagens.

Against frame shift mutagenesis in TA98 strain sulforaphane was found to be inactive upto dose of 100 nmoles/plate and higher doses were toxic to the culture. Earlier reports also indicate that sulforaphane is a potent inhibitor of base substitution mutagenesis induced by NDMA \( \text{NDMA} \). Although these food derived heterocyclic amines are reported to be more potent inducers of frameshift mutagenesis in TA98 strain at the same time these agents do show a significant base substitution mutagenesis in TA100 strain of the order of 3200 revertants/µg with Glu-P-1 \( \text{Glu-P-1} \). In the present study sulforaphane has been found to be effective against base pair mutagenesis induced by various heterocyclic amines.

To summarise, these findings suggest that, sulforaphane is a potent inhibitor of food-derived heterocyclic amines induced base substitution mutagenesis. These inhibitory effects may be due to the suppression of metabolic activation of cytochrome P450 enzymes. Even though these studies only supplement the previous investigations with this putative candidate, they do open a new channel of thought where they strongly warrant more intensive evaluation of the chemoprotective effect of sulforaphane against diet induced carcinogenesis as till now it has been evaluated as an agent against chemical induced carcinogenesis.
References