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Inhibition of cooked food-induced mutagenesis by dietary constituents: Comparison of two natural isothiocyanates

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ABSTRACT

Sulforaphane(1-isothiocyanato-(4*R*)-(methylsulphinyl)butane), a major constituent of broccoli (Brassica oleracea, var. italica) and a structurally related natural aliphatic isothiocyanate, sulforaphen (4-isothiocyanato-(1*R*)-(methylsulphinyl)-1-(*E*)-butene), found in radish (Raphanus sativus L., Cruciferae) were investigated for their antimutagenic potential against different classes of cooked food mutagens (heterocyclic amines) in the Ames assay using *Salmonella typhimurium* TA98 and TA100 strains in the presence of Aroclor 1254-induced rat liver S9. Results of the in vitro antimutagenicity studies in the TA100 strain strongly suggest that both isothiocyanates were potent inhibitors of the mutagenicity induced by all the tested mutagens. Sulforaphen, possessing unsaturation in the alkyl chain of its structure, was, however, found to be 1.3–1.5 times more active than sulforaphane. These studies strongly warrant further investigations of sulforaphen for its potential as a chemopreventive agent.

1. Introduction

Heterocyclic amines (HCAs) represent a unique class of dietary mutagens and carcinogens to which humans are exposed. These dietary carcinogens are generated in muscle meats, such as beef, pork, fowl and fish, during cooking procedures, such as broiling, frying, barbequing, that employ high temperatures (Felton & Knize, 1991). Potent mutagenic and carcinogenic effects of HCAs are reported in rodents (Wakabayashi, Nagao, Esumi, & Sugimura, 1992), as well as in non-human primates (Adamson, Takayama, Sugimura, & Thorgeirsson, 1994). Studies also indicate the presence of heterocyclic amines in the urine of humans eating a normal non-vegetarian diet, thus illustrating that a certain population eating animal protein is continuously exposed to these carcinogens through diet (Reistad et al., 1997). Epidemiological studies also show that heterocyclic amines intake is associated with the etiology of human cancer (deMeester & Gerber, 1995; Steck et al., 2007).

However, the carcinogenic risk imposed by these probable human carcinogens can be reduced by other dietary factors that influence their uptake and biotransformation. There is sufficient scientific evidence indicating that populations consuming diets rich in fruits and vegetables have a reduced risk of developing several types of cancers (Steinmetz & Potter, 1996; Surh, 2003). Recently, several natural compounds with chemoprotective properties have been identified. Some of these compounds particularly, the isothiocyanates (ITCs) present in widely consumed cruciferous vegetables, such as radish, cabbage, cauliflower, watercress, horseradish, broccoli and mustard, have been shown to inhibit various human cancers (Choi et al., 2006; Hecht, 1999; Kohlmeier & Su, 1997). Among these, sulforaphane (SF), found in broccoli, sprouts and kale (Zhang & Tang, 2007) is one of the most characterised ITCs and is currently under active investigation for its chemopreventive properties against various cancers. It has been reported to induce cell cycle arrest and apoptosis in human colon (Gamet-Payrastre et al., 2000; Nair et al., 2008), prostate (Choi et al., 2006) and mammary cancer cells (Pledgie-Tracy, Sobolewski, & Davidson, 2007; Singletary & MacDonald, 2000). Mechanistic studies have shown that cancer chemopreventive activity of SF is due to a favourable modification of Phase-1 and Phase-2 carcinogen metabolism, resulting in an increased carcinogen excretion or detoxification and decreased carcinogen–DNA interaction (Clapper et al., 1997; Nestle, 1997). In vitro inhibitory studies on various human cytochrome P450 enzymes have shown SF to be an effective inhibitor of a range of cytochrome enzymes that are essential for metabolic activation of many proximate carcinogens (Langouet et al., 2000). Previously we have reported the inhibitory effect of SF against various cooked food mutagens (Shishu & Kaur, 2003). From the large number of reported investigations on this potential chemopreventive phytochemical, it has been observed that it possesses the ability to simultaneously modulate multiple cellular targets involved in cancer development. Taking into account this

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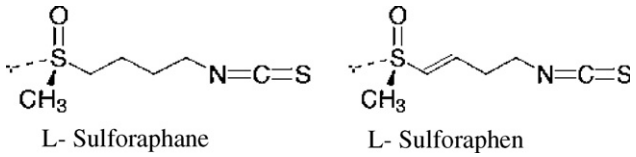

Fig. 1. Structures of sulforaphane and sulforaphen.

evidence and its favourable toxicological profile, SF has been viewed as a conceptually promising agent in cancer prevention and/or therapy.

Although, literature indicates extensive reports on antineoplastic properties of SF (Zhang & Tang, 2007), its natural analogue, sulforaphen (SFN), isolated from radish, has yet not been scientifically explored for its potential in preventing cancer and other diseases. In Asian medicine, the juice of radish is used for treatment of a variety of ailments, including whooping cough, cancer, coughs, gastric discomfort, liver problems, constipation, dyspepsia, gallbladder problems, arthritis, gallstones, kidney stones and intestinal parasites, but its effectiveness in solving these problems has not been scientifically confirmed. Moreover, radish roots are generally relished as a raw vegetable and as a component of salads. Hence they may be more beneficial than other cruciferous vegetables which are consumed after cooking because heating inactivates the enzyme myrosinase essential for liberating active ITCs from their glucosinolate precursors (Conaway et al., 2000). Therefore, it would be of interest to evaluate this SF-related isothiocyanate for its chemoprotective effects against diet-induced cancers.

In the present study, we have investigated and compared the antigenotoxic potential of SF with its natural analogue SFN from radish, using the Ames Salmonella/reversion assay in two different strains of S. typhimurium, namely TA98 and TA100, against various classes of heterocyclic amines (cooked food mutagens) found in human diet. SFN is a structurally related alkyl isothiocyanate and differs from SF in having a double bond in the alkyl chain (Fig. 1).

2. Materials and methods

2.1. Bacterial strains

Histidine-requiring TA98 and TA100 strains of *Salmonella typhimurium* were obtained as free gifts from Dr Bruce N. Ames (University of California, Berkley, USA).

2.2. Chemicals

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]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) acetate were purchased from Toronto Research Chemicals Inc., Canada. 2-Amino-6-methyldipyrido[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. Nohmi, National Institute of Hygenic Sciences, Tokyo, Japan. L-Sulforaphane and L-sulphoraphen were 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.

2.3. 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 Garner, Miller, and Miller (1972).

2.4. Determination of protein concentration of S9

Protein concentration of induced rat liver S9 was determined by the biuret method (Gornall, Bardwill, & David, 1949) and was found to be 54 mg/ml.

2.5. Antimutagenicity testing

The plate incorporation procedure reported by Maron and Ames (1983) was used for antimutagenicity testing with the inclusion of a pre-incubation step (Yahagi et al., 1977). Negative and positive controls were included in each assay (see footnote of Table 1 below). Both SF and SFN in the concentration range 100–500 nmol/plate were 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 in the tested dose range (see footnote of Table 1 for revertant counts).

Table 1
Inhibition of Aroclor-induced S9-mediated mutagenicity of heterocyclic amines by SF and SFN in TA100 strain of *Salmonella typhimurium*

Cooked food mutagen (nmol/plate)	No. of His$^+$ revertants/plate[a] (percent of control)						
	Control	SF (100)	SFN (100)	SF (250)	SFN (250)	SF (500)	SFN (500)
IQ (5)	1457 ± 41 (100)	887 ± 81 (60.88)	1264 ± 58 (86.75)	926 ± 36[c] (63.56)	609 ± 34 (41.80)	519 ± 19 (35.62)	230 ± 09 (15.78)
MeIQ (0.5)	955 ± 28 (100)	846 ± 32 (88.59)	713 ± 30 (74.66)	845 ± 33[c] (88.48)	430 ± 19 (45.03)	357 ± 24 (37.38)	249 ± 05 (26.07)
MeIQx (12)	1277 ± 31 (100)	964 ± 20 (75.49)	908 ± 25 (71.10)	870 ± 19 (68.13)	721 ± 20 (56.46)	572 ± 19 (44.79)	255 ± 08 (19.97)
Trp-P-1 (83)	858 ± 17 (100)	724 ± 24 (84.38)	765 ± 21 (89.16)	481 ± 11 (56.06)	610 ± 17 (71.10)	443 ± 10 (51.63)	209 ± 12 (24.36)
Trp-P-2 (8.3)	799 ± 35 (100)	590 ± 22 (73.84)	640 ± 18 (80.10)	591 ± 24[c] (73.97)	503 ± 33 (62.95)	543 ± 10[d] (67.96)	462 ± 21 (57.82)
PhIP (400)	1087 ± 28 (100)	981 ± 19 (90.25)	747 ± 22 (68.72)	829 ± 27 (76.26)	453 ± 21 (41.67)	572 ± 18 (52.62)	255 ± 15 (23.46)
Glu-P-1 (20)	1360 ± 34 (100)	1368 ± 29[b] (100.59)	1201 ± 28 (88.31)	1331 ± 35[b c] (97.87)	1020 ± 51 (75.00)	1248 ± 48 (91.76)	499 ± 11 (36.69)

With 2-aminofluorene (positive control) revertant count is 2758 ± 67 (n = 15).
The spontaneous revertant counts in the presence of various concentrations of SF and SFN alone are: 129 ± 6 (100 nmol/plate);132 ± 10 (250 nmol/plate); 126 ± 8 (500 nmol/plate) using three plates per point.

[a] All values are expressed as means ± S.D.(n = 6); they include spontaneous revertant count (negative control) of 132 ± 10 (n = 15) and are statistically different from control as well as from each other at $P < 0.05$ (analyzed by Student- Newman–Keuls method).

[b] Mean value is not statistically different from mean value at 0 nmol/plate of SF (control) at $P < 0.05$.

[c] Mean value is not statistically different from mean value at 100 nmol/plate of SF at $P < 0.05$.

[d] Mean value is not statistically different from mean value at 250 nmol/plate of SF at $P < 0.05$.

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 that they resulted in a maximum of about 2000 His^+ revertants/plate (9- or 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 1).

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

2.6. Statistical analysis

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

3. Results and discussion

In the present study, we investigated the antimutagenic potential of two structurally related dietary ITCs found in cruciferous vegetables, namely SF and SFN, against various cooked food mutagens generated during cooking of muscle meats, such as beef, fish and chicken, using a well established short-term genotoxicity assay, i.e. the Ames Salmonella/microsome assay. Results of the in vitro antimutagenicity studies, using base pair substitution sensitive strain TA100, strongly suggest that both SF and SFN are potent inhibitors of the mutagenicity induced by all the tested cooked food mutagens. SF was observed to be a strong inhibitor of the mutagenicity induced by imidazoazaarenes, IQ, MeIQ, MeIQx and PhIP (60% inhibition); however, it was moderately active against pyridoindole derivatives, Trp-P-1 and Trp-P-2 (32–48% inhibition), and ineffective against the dipyridoimidazole derivative, Glu-P-1 (Table 1 and Fig. 2). The ID_{50} values (the dose of SF required to reduce the mutagenicity of a given mutagen by 50%, calculated from corresponding dose response curves), indicate that

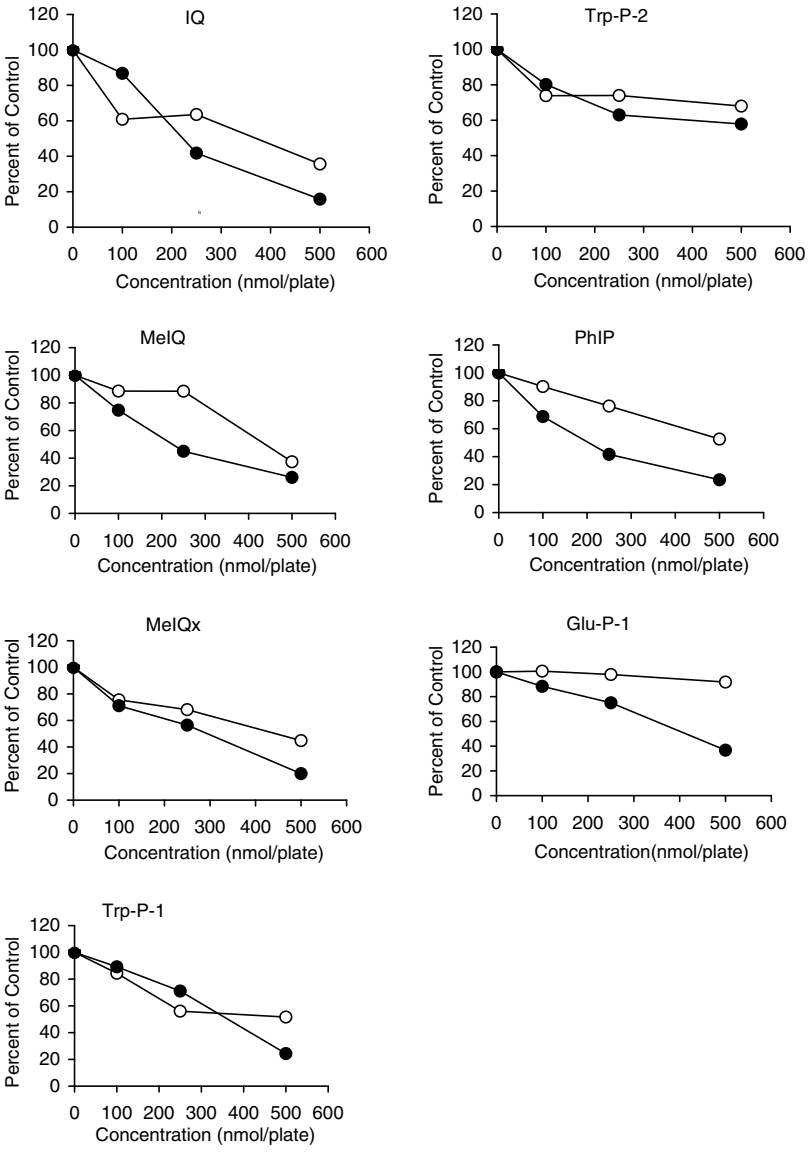

Fig. 2. Comparison of antimutagenic activity of sulforaphane and sulforaphen against various cooked food mutagens in TA100 strain of *Salmonella typhimurium*. For the dose of mutagen see Table 1. (—○—) SF, (—●—) SFN.

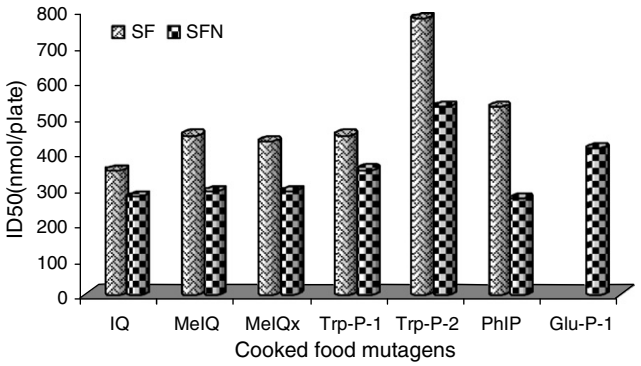

Fig. 3. Comparison of ID50 values of SF and SFN observed against various cooked food mutagens in TA100 strain of *Salmonella typhimurium*.

SF was most active against IQ-induced mutagenicity (Fig. 3). It showed almost similar reduction in mutagenicity induced by PhIP (47% inhibition at 500 nmol/plate) and Trp-P-1 (48% inhibition at 500 nmol/plate). The least inhibitory effect was observed against Trp-P-2 (32% inhibition at 500 nmol/plate).

It was observed that SFN was more active than its analogue SF against mutagenesis induced by all the tested food derived mutagens (Table 1 and Figs. 2 and 3). More potent antimutagenic activity was found against imidazoazaarenes, IQ, MeIQ, MeIQx and PhIP (74–84% inhibition) and pyridoindole derivatives, Trp-P-1 and Trp-P-2 (42–76% inhibition) (Table 1 and Fig. 2). Moreover, it suppressed the mutagenicity induced by the dipyridoimidazole derivative, Glu-P-1 (63% inhibition) as well. The order of antimutagenic potential, quantified as ID_{50} values (in nmol/plate), observed against various cooked food heterocyclic amines was

PhIP (270) > IQ (276) > MeIQ (291) = MeIQx (291) > Trp-P-1 (351) > Trp-P-2 (530) > Glu-P-1 (411).

Comparison of these observations suggests that an unsaturation in the alkyl chain of SFN (Fig. 1) might be responsible for its stronger inhibitory effects against heterocyclic amines. Furthermore, the dose–response effects were linear in the concentration range of 100–500 nmol/plate and no mutagenic or toxic effects were observed against TA100 in this dose range. It was confirmed, by a set of experiments, that decrease in mutagenicity was solely because of the antimutagenic nature of these ITCs (Table 1 and Fig. 2). However, doses higher than 500 nmol/ plate were found to be inhibitory to the bacterial culture.

As the heterocyclic amines are promutagens and need metabolic activation, particularly by cytochrome P4501A2 isozyme (Boobis et al., 1994; Snyderwine et al., 1997), and since, SF is reported to inhibit various cytochrome P450 enzymes, including cytochrome P4501A2 (Langouet et al., 2000), it may be proposed that the potent antimutagenic effects of both ITCs observed against food-derived heterocyclic amines in our studies, were due to the inhibition of metabolic activation of these promutagens. This observation can be further strengthened by an earlier investigation (Barcelo, Gardiner, Gescher, & Chipman, 1996), in which sulforaphane was reported to be ineffective against sodium azide-induced mutagenicity, the latter being a direct acting genotoxicant. On the other hand, a similar pattern of a fairly strong antimutagenic action against heterocyclic amines, strongly supports the involvement of the same mechanism of inhibition, i.e. the suppression of bioactivation of these mutagens.

Against frame shift mutagenesis in the TA98 strain, both ITCs were found to be inactive up to a dose of 100 nmol/plate and higher doses were toxic to the culture (data not shown). Earlier reports also reveal that SF is a potent inhibitor of base substitution mutagenesis induced by NDMA (Barcelo et al., 1996). Although these food-derived heterocyclic amines are reported to be more potent inducers of frameshift mutagenesis in the TA98 strain, these agents do show a significant base substitution mutagenesis in the TA100 strain (Koch, Wu, Cebula, & Felton, 1998; Wakabayashi et al., 1992). In the present studies, both ITCs were found to be effective against base pair mutagenesis induced by the heterocyclic amines.

To summarize, both SF and SFN are potent inhibitors of food-derived heterocyclic amine-induced bacterial mutagenesis in TA100. These inhibitory effects may be due to the suppression of metabolic activation of cytochrome P450 enzymes. SFN, possessing a double bond in the alkyl chain, is a stronger antimutagen (1.3–1.5 times more active) than SF. These preliminary investigations using *Salmonella typhimurium* strains as the test system strongly warrant more intensive evaluation of the chemoprotective effect of SFN.

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