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Review

Antimutagenic and Anticarcinogenic Properties of *Kyo-yasai*, Heirloom Vegetables in Kyoto

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Heirloom vegetables in Kyoto, termed *Kyo-yasai*, have had their seeds preserved by traditional cultivation methods. These heirloom vegetables offer a more distinctive flavor than conventional vegetables, and extracts from some *Kyo-yasai* are known to decrease ultraviolet light induced mutations in *Escherichia coli* B/r WP2 (*trpE65*) significantly more than extracts from their counterpart of conventional vegetables. 4-Methylthio-3-butenyl isothiocyanate which causes the pungency in daikon (*Raphanus sativus*), and 3-methylthiopropionic acid ethyl ester, which causes melon-like odor, were identified from heirloom vegetables in Kyoto to be antimutagens in *Escherichia coli* mutagenicity assays. These two chemicals also demonstrated *in vivo* animal cancer prevention, and induced differentiation, a chemotherapeutic strategy, in an *in vitro* human colon-cancer cell system. The heirloom daikon varieties in Kyoto produced 2.0–11.5 times higher levels of 4-methylthio-3-butenyl isothiocyanate as compared to the conventional Aokubi variety, because the conventional variety is grown for consumer preferences of milder flavor, which is corresponding to both quantity of 4-methylthio-3-butenyl isothiocyanate and quality associated with its antimutagenicity. The heirloom pickling melon in Kyoto, Katsura-uri (*Cucumis melo* var. *conomon*) began to produce 3-methylthiopropionic acid ethyl ester between the midripening to fully ripening stage of fruit development. Shiro-uri, a conventional variety for Katsura-uri, did not contain 3-methylthiopropionic acid ethyl ester. Results also indicate that antimutagenic and anticarcinogenic properties change over the ripening stage quantitatively. In this review, we discuss the value of retaining the original phenotypes of vegetables, including the flavors, to maximize the anticarcinogenic properties of these food products.

Key words: antimutagen, vegetable, isothiocyanate, daikon, melon

Introduction

In 1997, the World Cancer Research Fund and American Institute for Cancer Research released ''Food, Nutrition and the Prevention of Cancer: a global perspective, First Expert Report'' that did a comprehensive review of published research studies from around the world, leading to fifteen specific recommendations to reduce the risk of cancer (1). The Second Expert Report published in 2007 also leaded to eight intensive recommendations to the general population and two recommendations to special populations (2). One major recommendation was to establish a predominantly plant-based diet that includes eating 400–800 *g*/d of a variety of vegetables and fruits, and 600–800 *g*/d of a variety of grains, legumes and root crops (1). ''Kenko-Nippon 21'' a guideline to promote health for the Japanese population was published, and 350 *g* of vegetables a day is recommended to reduce the risk of cancer (3). Vegetables are known to contain naturally beneficial compounds called phytochemicals in addition to the well known nutrients and vitamins that can contribute to human heath. Many of these phytochemicals have chemopreventive properties, thus, the consumption of a variety of vegetables could greatly lower the risk of cancer.

In the last several decades, the consumer demands by the Japanese population resulted in the breeding of new varieties of traditional vegetables with milder flavors and odors. Most phytochemicals contribute strong flavors such as the astringency of flavonoids, pungency of isothiocyanates, and unpleasant odors of sulfur compounds, thus contemporary varieties of traditional Japanese vegetables tend to have greatly reduced levels

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of phytochemicals that can be beneficial to human health. Although the consumption of the recommended levels of fruits, vegetables and whole grains can contribute to the prevention of cancer, eating vegetables and fruits with stronger, but often less desirable flavors, could further improve the benefits to human health. In addition to consumer driven preferences to decrease the strong flavor associated with conventional vegetables, the following reasons also contributed to the current varieties of vegetables: 1. Producer driven preferences for higher yield, fewer days to harvest and disease resistance; 2. Distributor driven preferences for transport tolerance; 3. Vender driven preferences to decrease the rates of spoilage at the supermarket. Breeding by cross-fertilizing has been successful in this respect and resulted in the present conventional vegetables with a milder flavor and easier handling.

Kyoto was the seat of the imperial court of Japan from 794 to 1868, and during this period many novel varieties of vegetables were gathered as an offering to the emperors, and as a result of this historical period, current day Kyoto Prefecture has an extensive collection of various heirloom vegetables that have been termed ''*Kyo-yasai*''. The seeds of *Kyo-yasai* have been preserved by traditional cultivation methods, and typically offer a more distinctive flavor than conventional vegetables. In 1998, we reported that some heirloom vegetables in Kyoto have greater bioantimutagenic activity than their conventional counterpart as determined by decreased mutagenic rates in ultraviolet irradiated-*Escherichia coli* B/r WP2 (*trpE65*) (4). The original phenotypes of *Kyo-yasai*, including their flavors, have been maintained by plant breeders for more than 300 years.

Bioantimutagen

Compounds that decrease gene mutations are generically called antimutagens. Antimutagens can be divided into desmutagens and bioantimutagens depending on their action of mechanisms (5). Desmutagens can suppress mutations by decreasing levels of DNA lesions through various mechanisms such as preventing or decreasing the conversion of a pre-mutagen to a mutagen (Fig. 1A), or by chemically degrading a mutagen (Fig. 1B), or by inducing enzymes that will detoxify the mutagen before it reaches the cell's DNA (Fig. 1C). In contrast, bioantimutagens, which is a term sometimes misunderstood as an antimutagen derived from a biological source, can decrease the effects of preexisting DNA lesions by increasing the level of error-free DNA repair (Fig. 1D), or increasing the opportunity for DNA repair by delaying DNA replication and mutation fixation (Fig. 1E). Bioantimutagens identified so far are less than 10% of all known antimutagens. However, when we consider antimutagen in vegetables, the bioantimutagen, which can act even after the cell has acquired a DNA lesion, is as important as desmutagen.

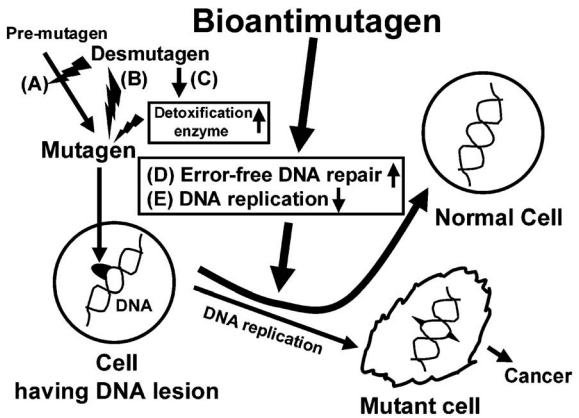

Fig. 1. Concept of antimutagens includes desmutagens and bioantimutagens. The action of mechanisms of desmutagen (A–C): suppression of mutation before mutagens reach the cell's DNA either by preventing or decreasing the conversion of a pre-mutagen to a mutagen (A), by chemically degrading a mutagen (B), by inducing detoxification enzymes for a mutagen (C). The action of mechanisms of bioantimutagen (D, E): suppression of mutation either by increasing the level of error-free DNA repair for preexisting DNA lesions (D), by increasing the opportunity for DNA repair by delaying DNA replication and mutation fixation (E).

Comparative Bioantimutagenicity of Conventional Vegetables and *Kyo-yasai*, Heirloom Vegetables in Kyoto

We reported that some *Kyo-yasai* have greater bioantimutagenicity than their counterpart of conventional vegetables as determined by an UV-induced mutation assay that typically causes thymine dimers, which is a bulky DNA byproduct (4). The assay was carried out as following: cell suspensions of *E. coli* B/r WP2 (*trpE65*, repair-proficient) were irradiated with UVC (254 nm; 20 J/m^2). The cell suspensions (1.5×10^9 cells/mL) were diluted with PBS 10 times and 1×10^6 times the original concentration and plated to detect revertants and survivors, respectively. Fifty microliters of the sample solution to be tested for bioantimutagenicity was dissolved in dimethyl sulfoxide, and mixed with 0.2 mL of UV-irradiated bacterial cells at both dilution levels. This mixture was added to plates that contained 2 mL of 0.7% molten agar that was mixed with 0.5 mL of PBS, which was poured onto a semi-enriched minimal agar medium (SEM). The numbers of revertants and survivors were counted as colony-forming units on the same organized SEM plates after incubation at 37°C for 2 days. The antimutagenicity was evaluated by determining the relative mutagenic activity (RMA), which was expressed as a percent of control, *i.e.* the mutagenic activity adjusted by sample toxicity, as calculated using the formula $[(Mt/Mc)/(St/Sc)] \times 100$, where *Mt* is the number of

Table 1. Bioantimutagenic potency of conventional and heirloom vegetables in Kyoto

Fraction	Conventinal type			Heirloom Kyoto type			Relative value of yield/IC_{50}
	IC_{50} (mg/plate)	Yield (mg/kg)	Yield /IC_{50}	IC_{50} (mg/plate)	Yield (mg/kg)	Yield /IC_{50}	
Egg plant	(Senryo)			(Kamo)			
n-Hexane	1.43 ± 0.42	221	155	0.79 ± 0.32	423	535	3.5
Chloroform	2.76 ± 1.51	252	91.3	2.01 ± 0.34	338	168	1.8
Ethyl acetate	2.73 ± 1.21	259	94.9	0.70 ± 0.41	408	610	6.4
Pickling melon	(Shiro)			(Katsura)			
n-Hexane	0.90 ± 0.47	30.2	33.4	0.78 ± 0.36	303	391	12
Chloroform	2.91 ± 1.59	251	86.3	1.41 ± 0.35	347	246	2.9
Ethyl acetate	2.87 ± 0.39	174	60.6	1.24 ± 0.42	646	521	8.6
Pumpkin	(Seiyo)			(Shishigatani)			
Chloroform	Inactive	1557	—*	4.53 ± 0.41	241	53.2	—*
Ethyl acetate	Inactive	1042	—*	1.78 ± 0.30	638	258	—*

IC_{50} values are presented with the 95% confidence interval.
The relative value of yield/IC_{50} is the ratio of the yield/IC_{50} value for the heirloom type against that for conventional type.
—* Unable to calculate.

revertant colonies in the presence of the test sample; *Mc* is the number of revertant colonies in the absence of the test sample; *St* is the number of surviving colonies in the presence of the test sample, and *Sc* is the number of surviving colonies in the absence of the test sample. To identify an active sample, we used a criterion ''IC_{50}'' determining the lowest dose needed to acquire a 50% RMA that was calculated from a linear regression derived from at least 15 points taken over five doses. A fraction was determined active if it met this criterion.

Extracts of the four different solvent fractions (*n*-hexane, chloroform, ethyl acetate, and aqueous) collected from the following heirloom vegetables, Kamo-nasu eggplant (*Solanum melongena*), Shishigatani-kabocha pumpkin (*Cucurbita moschata*), and Katsura-uri pickling melon (*Cucumis melo* var. *conomon*), had higher antimutagenic potencies (lower IC_{50} and higher yield) than their counterparts in conventional vegetables using the *E. coli* assay (4, 6; Table 1). To rank the bioantimutagenic potency, we used the second criterion that is ''yield/IC_{50}''. The criterion allowed us to compare the comprehensive bioantimutagenicity on the quantitative aspect of the fractions. On the basis of the second criterion, great differences can be seen between heirloom and conventional vegetables.

Bioantimutagenicity of Pungent Compound in Daikon

Daikon (*Raphanus sativus*, Japanese white radish) root is usually referred to by its Japanese name ''daikon'' throughout the world, although the cultivar originated from the Mediterranean area. Daikon is one of the Japanese vegetables that have been affected by the consumer-driven preferences to decrease the strong pungent flavor, and have gradually disappeared from the market during the recent decade in Japan. The pungent compound is 4-methylthio-3-butenyl isothiocyanate (MTBITC; chemical structure shown in Fig. 2A), which has been known for more than 50 years. The biological effect of MTBITC was first identified in 1982 as an antimicrobial compound against *E. coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae* and *Aspergillus orizae* (7). Because no other biological or health effects have been identified for this compound between 1982 and 2001, there was no motivation by plant breeders to prevent the reduction in MTBITC levels in daikon. Breeding programs to reduce the pungency of this vegetable has been quite successful resulting in Aokubi, which is the most commonly used conventional daikon used in Japanese cuisine. Ironically, the bioantimutagenic property of MTBITC was found in 2001, which was after Aokubi variety was developed and produced and distributed throughout Japan (8). Anticarcinogenic activity for MTBITC was demonstrated in 2005 by this compound's ability to reduce *N*-nitrosobis(2-oxopropyl) amine (BOP)-induced pancreatic carcinogenesis in hamsters (our unpublished data).

In contrast, heirloom daikon varieties in Kyoto were preserved without selective breeding or hybridization, retaining their original phenotypes including morphology (8). In fact, the level of MTBITC in Aokubi was 71.0 ± 1.3 μmol/100 *g* in 1999–2000 (8), whereas in 2004–2006 the level had decreased to 36.7 ± 3.5 μmol/100 *g* (9). This verifies that the aim of Aokubi selective breeding toward less MTBITC pungency has been successful in meeting the demands of human taste, but probably resulted in a lower benefit to human health. To reverse the current breeding practice of reducing pungency, we hope that these results could be used to encourage growers and consumers that the cultivation and consumption

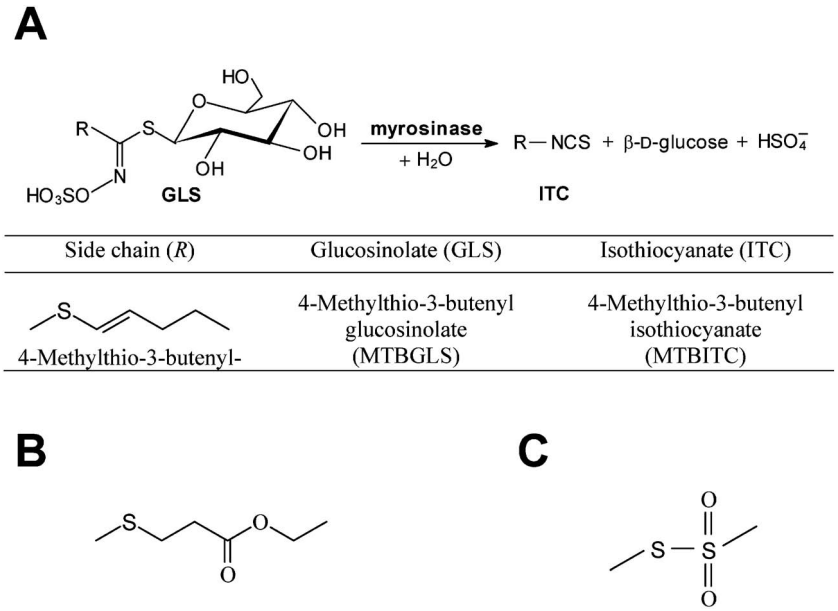

Fig. 2. Chemical structures of 4-methylthio-3-butenyl isothiocyanate (MTBITC), 3-methylthiopropionic acid ethyl ester (MTPE), and *S*-methyl methanethiosulfonate (MMTS). (A) Proposed pathway to produce isothiocyanate from glucosinolate. (B) Chemical structure of MTPE. (C) Chemical structure of MMTS.

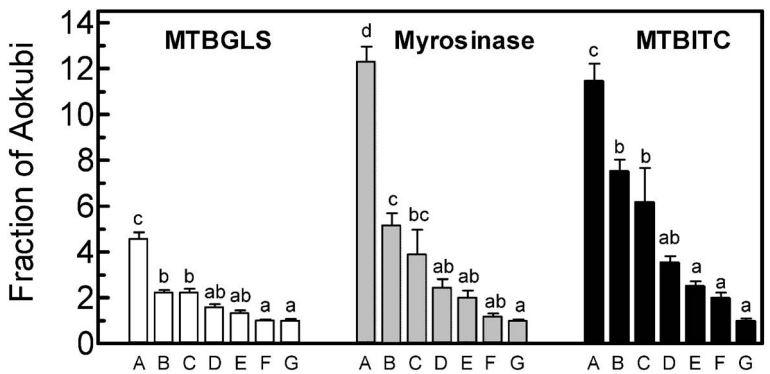

Fig. 3. Concentration of MTBGLS, activity of myrosinase, and production of MTBITC in seven daikon varieties. The data was plotted as a fraction of the value of Aokubi for each parameter. The value of concentration was measured on a fresh weight basis (100 *g*) of 4–22 different daikons of the same variety (A: Karami, B: Momoyama, C: Sabaga, D: Kuki, E: Tokinashi, F: Shogoin, G: Aokubi). Those values (mean ± SEM) in Aokubi were 278 ± 20.7 μmol (MTBGLS), 44.0 ± 2.7 units (myrosinase), and 36.7 ± 3.5 μmol (MTBITC). Values followed by different letters (among a–d) are significantly different ($p < 0.05$) by multiple-comparison test of Scheffe's PLSD.

of the heirloom varieties would be a healthier alternative.

Optimization of the Level of MTBITC in Daikon Dish

MTBITC is produced from glucosinolate (4-methylthio-3-butenyl glucosinolate; MTBGLS) through the enzymatic hydrolysis by myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147) (Fig. 2A). Glucosinolates are compartmentalized in the vacuoles of non-specific cells and myrosinases in the vacuoles of myrosin cells (10,11), and the rupture of the vacuole is critical to assure production of MTBITC in daikon tissues. Because Japanese prefer grating or cutting the daikon prior to consumption, production of MTBITC is likely to occur. The pungent taste in daikon caused by high levels of MTBITC is due to high levels of its precursor MTBGLS or on myrosinase, the enzyme that produces MTBITC from MTBGLS, or on both of these factors. Although the conventional Aokubi daikon has a lower amount of MTBITC than the heirloom varieties, however these heirloom varieties preserved in Kyoto are unfortunately limited in number.

The six heirloom varieties in Kyoto (Karami, Momoyama, Sabaga, Kuki, Tokinashi, Shogoin) produced 2.0–11.5 times higher levels of MTBITC as com-

pared to the conventional variety, Aokubi (9; Fig. 3). The levels of MTBGLS and myrosinase were correlated with the levels of MTBITC. We conclude that choosing heirloom daikon varieties in food preparation will augment the level of MTBITC in daikon dish.

The MTBGLS is widely distributed throughout the root tissue in Aokubi, but myrosinase is located exclusively in the outer epidermal layer (9). Although the skin is a potentially rich source of myrosinase in Aokubi, the skin is usually peeled off when preparing daikon for cooking. We therefore propose new practices for the preparation of daikon roots that avoid peeling of the skin to avoid removing the enzyme needed to convert MTBGLS to MTBITC.

To increase the level of the demonstrated anticarcinogenic MTBITC, future breeding programs will need to rely on the various heirloom strains of daikon. Thus, understanding which varieties contain the highest levels of myrosinase. The level of myrosinase mRNA, (*RMB1* and *RMB2*), is correlated with the activity of the myrosinase enzyme in which the two varieties, Karami and Momoyama, had the highest and Aokubi and Shogoin had the lowest levels of myrosinase activity (9). The levels of *RMB1* and *RMB2* were 85.5 and 100.5 times higher in Karami, and 6.6 and 8.4 times higher in Momoyama as compared to the conventional variety, Aokubi (9). To optimize the level and spatial distribution of MTBITC, which has potential health benefits, plant breeders can use PCR determination of myrosinase for selecting cultivars that would maximize the production of MTBITC that may match or surpass the levels found in the two heirloom varieties (Karami and Momoyama).

Bioantimutagenicity of Fragrant Compound in Katsura-uri, Japanese Pickling Melon

The oleophilic fraction of fully ripened Katsura-uri Japanese pickling melon (*C. melo* var. *conomon*) exhibited bioantimutagenic activity assessed by UV-induced mutation assays using *E. coli* B/r WP2, at levels twelve times higher than that from the conventional type, Shiro-uri (4, 6; Table 1). This fraction also induced differentiation in a RCM-1 human colon cancer cell line (12). Some of the bioantimutagenic compounds identified from vegetables in this assay also exhibited anticarcinogenic activities in animal experiments (13,14). Thus these results warranted further investigation into the anticarcinogenic properties of Katsura-uri. We further purified this fraction using silica-gel column and silica-gel thin layer chromatography procedures *via* a bioassay-guided fractionation scheme, which was based on the induction of differentiation in a RCM–1 human colon cancer cell line. The most potent fraction contained a compound identified as 3-methylthiopropionic acid ethyl ester (MTPE; chemical structure shown in Fig. 2B). Katsura-uri began to produce MTPE between the midripening to fully ripening stage of fruit development. Shiro-uri, a conventional variety for Katsura-uri, did not contain MTPE. These results indicate that compounds associated with health benefits may quantitatively change over the ripening stages. Previously, the role of MTPE was considered to be an odor producing compound in many fruits, and not considered for any potential medical benefits.

Interestingly, MTPE is a food additive authorized by the Ministry of Health, Labour and Welfare in Japan to add a melon-like odor to food products. Recent published results indicating the chemopreventative and chemotherapeutic benefits of MTPE suggests that supplementing foods with this compound or its use as a drug can benefit human health. We have recently started a study to characterize the differentiation potential of this compound on 1,2-dimethylhydrazine (DMH)-dextran sodium sulfate (DSS)-induced colorectal carcinogenesis rat model at the Division of Pathology, National Institute of Health Sciences. If MTPE shows chemotherapeutic potential by inducing differentiation of colorectal cancer cells into Paneth cells in DMH-DSS-induced regenerative mucosa, then MTPE may expand for potentially used in colon cancer therapy (15).

Is the Assay on UV-induced Mutation in *E. coli* B/r WP2 Only Useful in Detecting Bioantimutagens?

We identified several bioantimutagenic compounds isolated from vegetables: MTBITC from daikon (8), *S*-methyl methanethiosulfonate (MMTS; chemical structure shown in Fig. 2C) from cauliflower (16,17), and MTPE from Katsura-uri (our unpublished data) in UV-induced mutation assays using *E. coli* B/r WP2. These compounds also exhibited anticarcinogenic activities in *in vivo* experiments (13,14,18,19) or *in vitro* rat liver cells (our unpublished data) and human colon cancer cells (12).

Oral administration of MTBITC reduced BOP-induced pancreatic carcinogenesis in hamsters at the initiation stage (our unpublished data). The bioantimutagenic effect in *in vitro* UV-induced *E. coli* mutation assay system and the anticarcinogenic effect on the initiation stage in animals are biological events that are likely related to each other, particularly since the initiating phase of cancer require mutational events, although we have not confirmed whether bioantimutagenicity of MTBITC could be observed in *in vitro* BOP-induced *E. coli* mutation assay system. However, MTBITC induced increased levels of mRNA of UGT1A6 (phase II enzyme for nitrosamine), but did not induce DNA-repair associated with DDB (damaged-DNA binding protein) and GADD45 (growth arrest and DNA damage inducible 45 protein) in an *in vitro* rat liver cell system (our unpub-

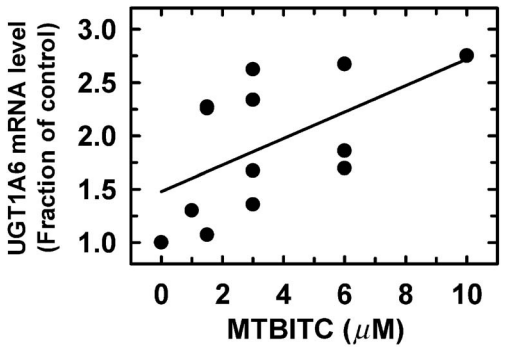

Fig. 4. UGT1A6 mRNA levels in rat liver cells. The F344 rat liver epithelial cell line (WB cells, 5×10^4) were plated in 35-mm diameter culture plates with 2 mL of 5% FBS-DMEM and cultured overnight, and then treated with MTBITC (as 2.5 μL of acetonitrile solution) in 2 mL of the same medium for 24 h. Quantitative RT-PCR was performed with the DyNAmo SYBR Green quantitative PCR kit (Finnzymes, Espoo, Finland). Each point represents the relative ratio compared to control. mRNA levels of DDB and GADD45 were not changed in same treatment of MTBITC (data not plotted).

lished data; Fig. 4). The anticarcinogenic effect of MTBITC in hamster was probably coincident with the bioantimutagenic effect in *in vitro* UV-induced *E. coli* mutation assay system, and most likely corresponding with phase II enzyme induction for BOP.

Oral administration of MMTS reduced mitomycin C-induced somatic mutation and recombination in *Drosophila melanogaster* and micronuclei in mice (18); and aflatoxin B1- or methyl methanesulfonate-induced chromosome aberrations in rat bone marrow cells (19). These effects appear to well correspond with the bioantimutagenicity found in the *in vitro* UV-induced *E. coli* mutation assay system and the antimutagenic effect found in the *in vivo* system. In contrast, a decrease in cancer incidence was observed by oral administration of MMTS at the post initiation step in azoxymethane-induced colon carcinogenesis in rats (13), or at the promotion step in diethylnitrosamine-initiated and phenobarbital-promoted hepatocarcinogenesis in rats (14). These effects do not appear to correspond with the bioantimutagenicity found in the *in vitro* UV-induced *E. coli* mutation assay system and the anticarcinogenic effect found in the *in vivo* system. The anticarcinogenic properties of MMTS may be involved in the multiple steps of carcinogenesis, including the initiation and promotion steps of cancer.

MTPE is now being orally administered similar to the MMTS experiments described above to further determine the anticarcinogenic potential of this compound. The justification for these *in vivo* experiments is based on the results from the bioantimutagenic assays using *in vitro* UV-induced *E. coli* mutation assay system, and on the effect of this compound to induce differentiation in human colon cancer cells.

We consequently anticipate that the *in vitro* UV-induced *E. coli* B/r WP2 mutation assay system can extensively detect compounds showing plural types of anticarcinogenic properties other than bioantimutagenicity, thus indicating a need for further experimentation using *in vitro* mammalian cell or *in vivo* animal model systems. Interestingly, the three bioantimutagens identified above all have in common a methylthio-moiety structure (Fig. 2).

Conclusions

Beginning in the 1970s, Japanese breeding programs made a drastic change from tradition and began to select genetic characteristics that allowed for large scale production and distribution of vegetables rather than characteristics that met the demands of local growers and consumers. By meeting the demands of a larger population, acquired tastes of local communities gave way to more average, milder tastes as well as slowed fruit ripening characteristics needed for longer transport and storage. This unfortunately led to varieties with reduced phytochemical content and ripening factors that could contribute to human health. Reconsidering the circumstance, heirloom vegetables without undergoing any these forces of selective breeding, and traditional organic farming methods is becoming more important. The trend of this agricultural direction should make Japanese vegetables better in flavor and health promotion. Although some phytochemicals in vegetables, which promote our health, carry some off-flavors, the beneficial health effects of these phytochemicals may motivate consumers to acquire a taste for them. Identifying all beneficial chemicals in vegetables may be impractical but vegetables that are richer in flavor may be a good guide in selecting the best breeds for human health.

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