A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway*

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FOOTNOTES

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Abbreviations: 6-HITC, 6-methylsulfinylhexyl isothiocyanate; sulforaphane, 4-methylsulfinylbutyl isothiocyanate; GST, glutathione S-transferase; NQO1, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase 1, EC 1.6.99.2]; γ-GCS, γ-glutamylcysteine synthetase; ARE, antioxidant response element; HPLC, high-performance liquid chromatography; EtOAc, ethyl acetate.
ABSTRACT

Exposure of cells to a wide variety of chemoprotective compounds confers resistance to a broad set of carcinogens. For a subset of the chemoprotective compounds, protection is generated by an increase in the abundance of the protective phase II detoxification enzymes, such as glutathione S-transferase (GST). We have recently developed a cell culture system, using rat liver epithelial RL 34 cells, that potently responds to the phenolic antioxidants resulting in the induction of GST activity [Kawamoto et al. (2000) J. Biol. Chem. 275, 11291-11299]. In the present study, we investigated the phase II inducing potency of an isothiocyanate compound in vitro and in vivo and examined a possible induction mechanism. Based on an extensive screening of vegetable extracts for GST inducer activity in RL34 cells, we found Japanese horseradish, wasabi (Wasabia japonica, syn. Eutrema wasabi), as the richest source and identified 6-methylsulfinylhexyl isothiocyanate (6-HITC), an analogue of sulforaphane (4-methylsulfynlybutyl isothiocyanate) isolated from broccoli, as the major GST inducer in wasabi. 6-HITC potently induced both class α GSTA1 and class π GSTP1 isozymes in RL34 cells. In animal experiments, we found that 6-MSHI was rapidly absorbed into the body and induced hepatic phase II detoxification enzymes more potently than sulforaphane. The observations that (i) 6-HITC activated the antioxidant response element (ARE), (ii) 6-HITC induced nuclear localization of the transcription factor Nrf2 which binds to ARE, and (iii) the induction of phase II enzyme genes by 6-HITC was completely abrogated in the nrf2-deficient mice, suggest that 6-HITC is a potential activator of the Nrf2/ARE-dependent detoxification pathway.
INTRODUCTION

Xenobiotic metabolizing enzymes play a major role in regulating the toxic, oxidative damaging, mutagenic, and neoplastic effects of chemical carcinogens. Mounting evidence has indicated that the induction of phase II detoxification enzymes, such as glutathione S-transferases (GSTs), results in protection against toxicity and chemical carcinogenesis, especially during the initiation phase. The GSTs are a family of enzymes that catalyze the nucleophilic addition of the thiol of reduced glutathione (GSH) to a variety of electrophiles (for a review, see Ref. 1). In addition, the GSTs bind with varying affinities to a variety of aromatic hydrophobic compounds. It is now generally accepted that the GSTs are encoded by at least five different gene families. Four (Classes \( \alpha, \mu, \pi, \) and \( \theta \)) of the gene families encode the cytosolic GSTs, whereas the fifth encodes a microsomal form of the enzyme. It has been shown that the induction of GST is associated with the reduced incidence and multiplicity of tumors (2,3). Recently, two transgenic rodent studies clearly demonstrated that one of the GST isozymes can profoundly alter the susceptibility to chemical carcinogenesis in mouse skin (4) and rat liver (5). Thus, the induction of GSTs is regarded as one of the most important determinants in cancer susceptibility and that its elevated synthesis is required to prevent toxic compounds from accumulating in the cells. The induction of phase II enzymes, such as GSTs, is reported to be evoked by an extraordinary variety of chemical agents, including Michael reaction acceptors, diphenols, quinones, isothiocyanates, peroxides, vicinal dimercaptans, and others (6-8). With few exceptions, these agents are electrophiles, and accordingly, many of these inducers are substrates for phase II detoxification enzymes.

Epidemiologic studies have found that persons who consume a high proportion of green and yellow vegetables in their diet have a decreased risk of developing some types of cancer (9,10). Subsequent laboratory work has led to the isolation of various phase II inducers from fruits and vegetables that reduce the incidence of experimental carcinogenesis in animal models. Among them are included \( \beta \)-carotene from a variety of vegetables and fruits (11), and the monoterpenes D-limonene and D-carvone from various food plants including Citrus species (12). Later, as an approach for the detection of novel
phase II inducing cancer chemoprotective agents, Talalay and his colleagues developed an in vitro assay system using cultured Hepa 1c1c7 murine hepatoma cells (13). They then used this assay to demonstrate that Brassica vegetables are particularly rich sources of phase II inducers and to identify sulforaphane (4-methylsulfinylbutyl isothiocyanate) as the principal phase II inducer in broccoli extracts (14). They also have demonstrated that sulforaphane is a dose-related inhibitor of carcinogen-induced mammary tumorigenesis in rats (15).

We have recently developed a cell culture system, using rat liver epithelial RL34 cells, that potently responds to the already-known phase II inducers, such as phenolic antioxidants and α,β-unsaturated aldehydes, resulting in the induction of the GST activity (16). In the present study, using the RL34 cells, we determined the GST induction potencies of food plants and found that the wasabi extracts induce GST activity with great potency. We provided an analysis of the wasabi extracts which demonstrate an isothiocyanate compound as a principal inducer of phase II enzymes. Moreover, we have investigated the phase II inducing potency of this compound in vitro and in vivo and examined a possible induction mechanism.
EXPERIMENTAL PROCEDURES

Materials — Authentic 6-methylsulfinylhexyl isothiocyanate (6-HITC) and sulforaphane were synthesized by the oxidation of 6-methylthiohexyl isothiocyanate and 4-methylthiobutyl isothiocyanate (kind gifts of Kinjirushi Wasabi, Co., Ltd., Nagoya, Japan), respectively. The 6-HITC-related compounds were synthesized in our laboratory. All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Wasabi (Wasabia japonica, syn. Eutrema wasabi) cultivated in Shizuoka, Japan, was also obtained from Kinjirushi Wasabi Co., Ltd. Low- and high-resolution fast atom bombardment-mass spectrometry (FAB-MS) was measured using a JEOL JMS-700 (MStation) instrument. Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AMX600 (600 MHz) instrument. Ultraviolet absorption spectra were measured with a Hitachi U-Best-50 spectrophotometer, and fluorescence spectra were recorded with a Hitachi F-2000 spectrometer. Liquid chromatography-mass spectrometry (LC-MS) was measured with a JASCO PlatformII-LC instrument.

Cell Culture — RL34 cells were obtained from the Japanese Cancer Research Resources Bank. The cells were grown as monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (588 µg/ml), and 0.16% NaHCO₃ at 37 °C in an atmosphere of 95% air and 5% CO₂.

Extraction and Isolation Procedures — Wasabi roots (water wasabi) harvested in Shizuoka, Japan, were kind gifts from Kinjirushi Wasabi Co, Ltd. The wasabi roots (1.3 kg) were smashed with a grater, and the homogenates were stored at room temperature for 10 min. The homogenates were then sequentially extracted with ethyl acetate (EtOAc, 2 L), n-butanol (BuOH, 2 L) and water (1 L). The EtOAc extract was further separated by silica gel column chromatography (silica gel BM-300, Fuji-Silicia Chem), and the acetone fraction, which showed the most potent activity, was analyzed by reversed-phase HPLC (yield, 70 mg), using a Develosil ODS-HG-5 (8 x 250 mm) column. The flow solvent was
methanol/water = 3/2 (v/v) at a flow rate of 2.0 ml/min. Detection was carried out at 254 nm. Identification of the active compound was done by spectroscopic analyses (17). The NMR and MS data were measured using a Bruker ARX 400 and a JEOL MStation MS-700, respectively. The IR, UV and optical rotation data were measured by a JASCO FT/IR-8300, a Beckman DU7500 and a JASCO DIP-370, respectively. The spectral data of 6-HITC were as follows: $^{13}$C-NMR (CDCl$_3$, TMS), d (ppm): 22.4 (C-4), 26.2 (C-3), 27.9 (C-5), 29.6 (C-2), 38.6 (sulfinyl methyl), 44.9 (C-6), 54.3 (C-1), 130.0 (-NCS); $^1$H-NMR (CDCl$_3$, TMS), d (ppm): 1.4-1.6 (4H, m, H-3 and H-4), 1.73 (2H, tt, J = 6.7, 7.1 Hz, H-5), 1.81 (2H, tt, J = 6.4, 7.4 Hz, H-2), 2.58 (3H, s, sulfinyl methyl), 2.72 (2H, dt, J = 7.1, 13.1 Hz, H-6), 3.54 (2H, t, J = 6.4 Hz); IR (liquid film, CHCl$_3$), $\nu$ max (cm$^{-1}$): 3002 (CH strech), 2108 (-NCS), 1032 (sulfinyl); UV (MeOH), $\lambda$ max (nm): 243 ($\varepsilon$ 1210); [\(\alpha\)]D : -65.0 (c 0.600, CHCl$_3$, 23ºC).

**GLC Analysis of 6-HITC in Wasabi Extracts** — The wasabi extract for the GLC analyses was prepared using dichloromethane (CH$_2$Cl$_2$) as the extracting solvent. The CH$_2$Cl$_2$ was carefully removed under atmospheric pressure in a N$_2$ stream on the ice bath. The residue was redissolved with an adequate amount of CH$_2$Cl$_2$. A 1 µl aliquot of the CH$_2$Cl$_2$ solution of the wasabi extract was injected into the GLC equipment. The quantitative analyses of 6-HITC and 6-methylthiohexyl isothiocyanate were carried out with a Hitachi G-3500 gas chromatograph (column, DB-1, 0.25 mm x 30 m; carrier gas, N$_2$ at 1 ml/min; temp. program, 60ºC to 230 ºC at 5 ºC/min). Each peak was identified by the retention time of each authentic or synthetic sample and the GC-MS analysis using a JEOL MStation MS-700 mass spectrometer linked to a Hewlett-Packard 6890 (column, DB-1, 0.25 mm x 30 m; carrier gas, He at 1 mL/min; temp. program, 60 ºC to 300º C at 8 ºC/min; column inlet split rate, 1/100). These analyses were repeated three times.

**Enzyme Activity Assays** — The total GST activity was measured in cytosolic fractions (105,000 x g) in the presence of 0.1% BSA with 1-chloro-2,4-dinitrobenzene as a substrate (18), whereas quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase 1 (NQO1) activity was determined using menadione as the substrate (19). Cytochrome
P4501A1-mediated ethoxyresorufin O-deethylase activity was measured using the procedures of Kennedy et al. (20). The protein concentration was determined using the bicinchoninic acid protein assay (Pierce).

**Western Blot Analysis** — The homogenates prepared from the cells or animal tissues were treated with SDS-sample buffer (without dye or 2-mercaptoethanol) and immediately boiled for 5 minutes. The protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). One hundred µg of the proteins were separated by SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and electro-transferred onto an Immobilon membrane (Millipore, Bedford, MA). To detect the immunoreactive proteins, we used horseradish peroxidase-conjugated anti-rabbit IgG and ECL blotting reagents (Amersham Japan, Tokyo). The polyclonal antibodies against GSTA1 were the kind gifts from Dr. K. Satoh of Hirosaki University School of Medicine. The polyclonal antibodies against GSTP1 and GSTM1 were obtained from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan) and Oxford Biomedical Research, Inc. (Oxford, MI), respectively. Polyclonal rabbit antisera raised against mouse Nrf2 was used as previously described (21). The anti-nuclear Lamin B antiserum was purchased from Santa Cruz Biotechnology (Palo Alto, CA).

**Nuclear Translocation of Nrf2** — The cells treated with DMSO or 6-HITC were fixed overnight in PBS containing 2% paraformaldehyde and 0.2% picric acid at 4 °C. The membranes were permeabilized by exposing the fixed cells to PBS containing 0.3% Triton X-100. The cells were then sequentially incubated in PBS solutions containing blocking serum (5% normal rabbit serum) and immunostained with the anti-Nrf2 polyclonal antibody (Santa Cruz, Santa Cruz, CA). The cells were then incubated for 1 h in the presence of fluorescein isothiocyanate-labeled rabbit anti-goat (DAKO A/S, Glostrup, Denmark), rinsed with PBS containing 0.3% Triton X-100, and covered with anti-fade solution. Images of the cellular immunofluorescence were acquired using a confocal laser microscope (Bio Rad, Hercules, CA) with a 40x objective (488-nm excitation and 518-nm emission).
**Plasmid Preparation** — The annealed oligonucleotide of GSTA1 ARE (top strand; TCGAGTAGCTTGGAAATGACATTGCTAATGGTGACAAAGCAACTTTG, bottom strand; TCGACAAAGTTGCTTTGTACACCATTAGCAATGTCATTTCCAAGCTAC) was ligated to the XhoI and SalI sites of the Bluescript SK(-) plasmid and the plasmid with three GSTA1 ARE inserts in tandem was selected. The KpnI and HincII fragment of the plasmid was blunted and then subcloned into the SmaI site of the pRBGP3 plasmid (22). The annealed oligonucleotide of the human NQO1 ARE (top strand; CGCGTTGAGGATTTCTAGACTGACATGACTGGCAAAATCG, Bottom strand; CTAGCGGTTTGTGCAAGTCCTGTGACTCTAGACTGAAATCTCTGAA) was ligated to the MluI and NheI site of the pRBGP3.

**RNA Blot Hybridization** — RL34 cells were maintained in Iscoves’s modified Eagle’s medium and 10% FBS. Fifteen µg of the total cellular RNAs extracted by ISOGEN (NipponGene) was electrophoresed and transferred to Zeta-Probe GT membranes (Bio-Rad Japan, Tokyo). The membranes were probed with [32P]-labeled cDNA probes as indicated in the figures. 18S ribosomal RNA cDNA was used as the positive control.

**Transient Transfection Assay** — RL34 cells were maintained in Iscoves’s modified Eagle’s medium supplemented with 10% FBS and seeded in 5 x 10^4 /well in 12-well dishes 24 h before transfection. The cells were transfected with plasmids using Fuegene (BOEHRINGER MANNHEIM) according to the manufacturer’s instructions. Nine hours after the transfection, the medium was changed to the fresh medium and the cells were treated with DMSO or 6-HITC (5 µM). After 36 h, the Luciferase assay was performed by utilizing the Luciferase Assay System (Promega, Madison, WI) following the supplier’s protocol and measured in a Biolumat Luminometer (Berthold, Bad Wildbad, Germany). Transfection efficiencies were routinely normalized by the activity of a co-transfected Renilla luciferase. Normally, three independent experiments, each carried out in duplicate, were performed and the mean values were presented with the Standard Error of Means (S.E.M).
Animal Study

(i) Determination of 6-HITC and Its Dithiocarbamate in the Plasma. Male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) were obtained at 7 weeks of age and individually housed in stainless wire-mesh cages at 23 ± 0.3 °C with a 12 h light cycle. They were fed unrestricted amounts of water and the control diet as follows: 20% casein, 3.5% mineral (93G-MX), 5.0% vitamin (93-VX), 0.2% choline chloride, 5.0% corn oil, 4.0% cellulose powder, 22.1% sucrose, and 44.2% starch. After 7 days of feeding the control diet, food was withheld for 24 h, and then 6-HITC dissolved in sesame oil was orally administered to 4 rats by direct stomach intubation. The rats were killed 1, 2, 4, and 8 h after the administration by withdrawing blood by heart puncture using heparinized needles and syringes under anesthesia with diethyl ether. The plasma was immediately obtained from the collected blood by centrifugation at 1600 x g for 15 min at 4 °C. The plasma separation was finished within 30 min. An aliquot of the plasma was acidified with one-tenth volume of phosphoric acid and stored at – 80 °C until used. The levels of 6-MSHI in plasma were measured by the cyclocondensation assay as previously reported (23).

(ii) 6-HITC-Administered Mice. Female ICR mice (Japan SLC Inc., Hamamatsu, Japan) were obtained at 4 weeks of age and individually housed in plastic cages (five/cage) at 23 ± 0.3 °C with a 12 h light cycle. They were fed for 12 days unrestricted amounts of water and the control diet as follows: 20% casein, 3.5% mineral (93G-MX), 5.0% vitamin (93-VX), 0.2% choline chloride, 5.0% corn oil, 4.0% cellulose powder, 22.1% sucrose, and 44.2% starch. From the thirteenth day, 6-HITC or sulforaphane was administered to these mice by gavage in daily doses of 15 µmole for 5 days (14).

(iii) Nrf2 Knockout Mice. A single dose of 15 µmol 6-HITC was suspended in olive oil and administered to adult female Nrf2 knockout mice (24) or ICR control animals (Nrf2 +/+) by gavage.
RESULTS

Isolation and Identification of a Major GST Inducer from Wasabi — We have previously shown that the ethyl acetate extracts of wasabi exhibited the most significant enhancement of GST activity at the concentration of 2.5 µg/ml (25). Hence, we performed the activity-guiding separation of a principal inducer from wasabi. Wasabi roots (1.3 kg) were extracted with 1.5 L of ethyl acetate at 4 °C, and the extract was fractionated into seven fractions by silica gel chromatography (Fig. 1A). The acetone fraction exhibited the most potent GST-inducing activity (Fig. 1B) was further fractionated into four fractions by preparative HPLC (Fig. 1C). Final purification of the inducer was carried out by preparative HPLC under the same conditions (Fig. 1D); evaporation of pooled active fraction gave 70 mg of a colorless liquid. The spectroscopic data, including 1H- and 13C-NMR, and EI-MS (molecular ion at m/z of 149 [M+], C8H 7NS), of this compound were completely identical to those of 6-methylsulfinylhexyl isothiocyanate (6-HITC) (Fig. 1E). As shown in Fig. 1F, 6-HITC was one of the major components in the wasabi extract. The concentration of 6-HITC in the wasabi was approximately 550 - 556 µg/g wet body weight of wasabi root.

Structure-Activity Relationship — To define the structural features of 6-HITC in the RL34 cells, we synthesized the following analogues of 6-HITC and measured their GST inducer potencies.

I \[\text{CH}_3\text{S-(CH}_2\text{n-NH}_2\text{)}\text{(n = 4, 6, or 8)}\]

II \[\text{CH}_3\text{S-(CH}_2\text{n-N=C=S)}\text{(n = 4, 6, or 8)}\]

III \[\text{CH}_3\text{SO-(CH}_2\text{n-N=C=S} \text{)(n = 4, 6, or 8)}\]

IV \[\text{CH}_3\text{SO}_2\text-(CH}_2\text{n-N=C=S} \text{)(n = 4, 6, or 8)}\]

As shown in Fig. 2, the methylthioalkyl amines (I) had no induction potency whereas the methylthioalkyl isothiocyanate (II) significantly induced GST activity, indicating that the isothiocyanate moiety was essential for the induction of GST activity. It was also found that the inducer potency was influenced by the oxidation state of sulfur and the number of methylene groups in the bridge linking the thiomethyl and isothiocyanate moieties. These
data indicate that, among the isothiocyanates, 6-HITC is one of the most potent phase II inducers.

**In Vitro Induction of Phase II Enzymes by 6-HITC**— As shown in Figs. 3A and 3B, 6-HITC induced GST activities in time- and dose-dependent manners. To examine the GST isozyme responsible for the increase in the GST activity of the 6-HITC-treated RL34 cells, an immunoblot analysis was carried out using the GST class-specific antibodies to confirm the apparent induction of GST proteins. The immunoblot analysis demonstrated a significant increase in the levels of the class α GSTA1 and class π GSTP1 by treatment with 6-HITC (Fig. 3C), while the amount of the class μ isozyme (GSTM1) was nearly unchanged (data not shown). These results indicated that the induction of GST activity by 6-HITC resulted, at least, from the enhanced expression of GSTA1 and GSTP1. The treatment with 6-HITC also resulted in a significant increase in the intracellular GSH levels (data not shown), suggesting the induction of a rate-limiting enzyme of GSH biosynthesis, γ-glutamylcysteine synthetase (γ-GCS), by 6-HITC.

**Absorption of 6-HITC** — Prior to the *in vivo* assessment of induction of the phase II enzymes, we examined the absorption of 6-HITC into the body. For this purpose, the cyclocondensation assay was utilized (Fig. 4A) (23). To validate this assay for measurement of the 6-HITC, the GSH conjugate of 6-HITC (dithiocarbamate) was incubated with 1,2-benzenedithiol and the reaction products were analyzed by reverse-phase HPLC. As shown in Fig. 4B, the reaction product (1,3-benzodithiole-2-thione) linearly increased with the dithiocarbamate concentration. Thus, this assay was demonstrated to provide a valid measurement of 6-HITC and its dithiocarbamate metabolite. To examine the absorption of 6-HITC into the bloodstream, rats were orally administered 6-HITC and the levels of 6-HITC in the plasma were measured by the cyclocondensation assay. As shown in Fig. 4C, the HPLC chromatogram of the plasma obtained from rats administered 6-HITC showed a peak which was assumed to be 1,3-benzodithiole-2-thione that originated from 6-HITC. The peak was identified as 1,3-benzodithiole-2-thione by comparison with the authentic compound based on the retention time in the HPLC analysis, UV-VIS spectrum, and LC-
The plasma 6-HITC concentration reached a maximum within 30 min after the administration, and began to fall within 1 h (Fig. 4D). These results suggest that 6-HITC is absorbed and rapidly enters the circulatory system as is and/or as its GSH conjugate. Interestingly, the subsequent decrease in the level of 6-HITC was relatively slow and it was detected in the plasma even 4 h after a single administration of 6-HITC.

In Vivo Induction of Phase II Enzymes by 6-HITC — 6-HITC was then investigated in dietary studies performed with female ICR mice. To assess the potential of 6-HITC to induce phase II detoxification enzymes, animals were treated with 6-HITC or sulforaphane, a well-known phase II inducer isolated from broccoli (14), by gavage in a daily dose of 15 μmoles for 5 days, and changes in the hepatic GST, QR, and cytochrome P4501A1 activities were examined. There were no significant differences in the body and liver weights (data not shown). As shown in Fig. 5A, hepatic GST and QR activities were more potently induced by 6-HITC than by sulforaphane. Whereas, only slight increases in the GST and QR activities were observed in the lung and kidney (data not shown). It was also observed that both 6-HITC and sulforaphane moderately inhibited cytochrome P4501A1-mediated ethoxyresorufin O-deethylase activity (Fig. 5B); however, this inhibition was not significant in comparison with the control group. Taken together, these data suggest that induction by 6-HITC was monofunctional (induction of phase II and inhibition of phase I). To further determine the GST isozymes responsible for the increase in the hepatic GST activity of the 6-HITC- or sulforaphane-treated mice, an immunoblot analysis was carried out using the GST class-specific antibodies. As shown in Fig. 5C, 6-HITC induced hepatic GSTA1 and GSTP1 isozymes more prominently than sulforaphane. The difference in the GST activity levels between the 6-HITC- and sulforaphane-treated mice may therefore correspond to that in the levels of the GSTA1 isozyme.

Induction of Phase II Enzyme Gene Expression and Activation of ARE — 6-HITC, as well as sulforaphane, induced the gene expression of the phase II enzymes, such as GSTP1, γ-GCS (heavy chain), and γ-GCS (light chain) (Fig. 6). The data also showed that, similar to the data in Fig. 5, the induction of the phase II enzyme gene expression by 6-
HITC was significantly more prominent than by sulforaphane. It has been established that the induction of the phase II enzyme activity occurs at the transcriptional level and is regulated by a *cis*-acting element that is present in the promoters of the phase II enzyme genes defined as the antioxidant response element (ARE) (26) or electrophile response element (27). Hence, we examined the involvement of ARE in the 6-HITC-induced phase II enzyme gene expression and found that 6-HITC potently stimulated the activity of the ARE reporter genes (Fig. 7).

**Activation of Transcription Factor Nrf2** — Several lines of evidence indicate that a member of the basic leucine zipper transcription factor family, Nrf2 (NF-E2-related factor 2), is involved in the activation of ARE (24,28). To determine whether this transcription factor indeed contributes to the 6-HITC-stimulated activation of ARE, we examined the nuclear localization of Nrf2 in the 6-HITC-treated RL34 cells. As shown in Fig. 8A, only cytoplasmic labeling of Nrf2 with no nuclear staining was observed in the non-stimulated cells (panel a), whereas an intense nuclear labeling was observed in the 6-HITC-stimulated cells (panel b). In addition, the 6-HITC treatment of the cells led to a dose-dependent increase in the Nrf2 levels (Fig. 8B). Sulforaphane also showed a similar effect, but its induction potency was significantly lower than that of 6-HITC.

**Effect of nrf2 Genotype and 6-HITC Treatment on Phase II Enzyme Activities** — Finally, to directly show that Nrf2 is involved in the 6-HITC-induced expression of the phase II enzymes, the *nrf2*-deficient mice were exposed to the isothiocyanates, and the induction of the phase II enzyme gene expression was examined. The mRNA levels of the hepatic GSTP1, NQO1, and γ-GCS heavy chain and light chain were measured 28 h after a single administration of 6-HITC (15 µmole) or vehicle to female wild-type and *nrf2*-disrupted mice. As shown in Fig. 9, the mRNA levels of phase II enzymes were significantly increased by treatment with 6-HITC, while this induction was completely abrogated in the *nrf2*-deficient mice. Taken together, these data suggest that 6-HITC induces the ARE-mediated gene expression of the phase II enzymes via a Nrf2-dependent mechanism.
DISCUSSION

Epidemiological studies have demonstrated that the consumption of cruciferous vegetables is associated with a lower incidence of cancers (29-31). An important group of compounds that have this property are organosulfur compounds, such as the isothiocyanates. The isothiocyanates are compounds that occur as glucosinolates in a variety of cruciferous vegetables, such as *Brussica* species. Glucosinolates are found in the cell vacuoles of various plants in the family Cruciferae such as horseradish, mustard, broccoli, and wasabi. When plant cells are damaged, glucosinolates are hydrolyzed by myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) which is also produced in the same family, and produce isothiocyanates. It is known that wasabi contains isothiocyanate components, such as allyl isothiocyanate, 6-HITC, 7-methylthioheptyl isothiocyanate, and 8-methylthiooctyl isothiocyanate (32). These isothiocyanates have been suggested to have important medical benefits. They not only inhibit microbes, but can also help treat or prevent blood clotting and asthma (33). Many isothiocyanates are also effective chemoprotective agents against chemical carcinogenesis in experimental animals. The isothiocyanates have been shown to inhibit rat lung, esophagus, mammary gland, liver, small intestine, colon, and bladder tumorigeneses (34-37). It has been suggested that the anticarcinogenic effects of isothiocyanates are closely associated with their capacity to induce phase II detoxification enzymes and to inhibit phase I enzymes that are required for the bioactivation of carcinogens. Indeed, some of the isothiocyanates have been shown to inhibit cytochrome P450 and increase the carcinogen excretion or detoxification by the phase II detoxification enzymes (38-40). Many natural isothiocyanates derived from cruciferous vegetables and some fruits have been shown to induce phase II enzymes in cultured cells and rodents (41-43). Recently, sulforaphane has been isolated from broccoli as the major inducer of phase II enzymes with potent *in vivo* chemopreventive properties (6,14,15). In addition, sulforaphane was found to inhibit the cytochrome P450 isozyme 2E1, which is responsible for the activation of a variety of genotoxic chemicals (43).

On the basis of its structural similarity to sulforaphane, the GST inducing potencies of 6-HITC and sulforaphane were anticipated to be equally potent. However, the structure-
activity relationship study revealed that the inducing potencies of 6-HITC was significantly greater than that for sulforaphane (Fig. 2). This was also the case in the animal experiments, in which the hepatic GST activity was induced more potently by 6-HITC than by sulforaphane (Fig. 5). The structure-activity relationship study also indicated that the isothiocyanate moiety is essential for the induction of GST activity (Fig. 2). Talalay et al. (7) have suggested that the inductive ability of various alkyl and aromatic isothiocyanates dependent on the presence of one hydrogen on the adjacent carbon to the isothiocyanate group and that tautomerization of the methylene-isothiocyanate moiety to a structure resembling an α,β-unsaturated thioketone may be important for inductive activity. However, the present study demonstrated that the methylthioalkyl isothiocyanates were less potent inducers than their S-oxidized forms (Fig. 2). The methylsulfinyl group, in addition to the isothiocyanate group, of 6-HITC is therefore suggested to be involved in its inductive activity. Although the reduced potency of the methylthioalkyl isothiocyanates may be simply due to their high volatility, it is not unlikely that the electron-withdrawing potentials of both the sulfinyl or sulfonyl groups may affect the signaling mechanism in the phase II induction.

To confirm that 6-HITC is absorbed into the body following its oral administration, the plasma level of 6-HITC and/or its GSH adduct was analyzed by the cyclocondensation assay, which provides a valid measurement of isothiocyanates or their dithiocarbamates, i.e., GSH derivatives (23). As shown in Fig. 4, it was revealed that 6-HITC was utilized very rapidly, reaching a maximum level within 30 min. Thus, 6-HITC is absorbed and rapidly enters the circulatory system. The plasma concentration of the isothiocyanate began to decrease after 30 min; however this decrease was relatively slow. Zhang and Talalay (23) have recently proposed that the induction of phase II enzymes by isothiocyanates depends on their intracellular levels of accumulation in the cells. Therefore, the prolonged accumulation of 6-HITC in the circulatory system may also correlate with its phase II inducer potencies.

It is notable that 6-HITC specifically accelerated the production of GSTA1 and GSTP1 isozymes in vitro and in vivo (Figs. 3 and 5). The increase in GST activity by treatment with 6-HITC may, therefore, be largely attributable to the elevated synthesis of
these isozymes. The class $\alpha$ GSTs represent the most abundant GST isozymes in the liver and kidney. It has been shown that a small increase in the class $\alpha$ GSTs is linked to a 90% decrease in the levels of the DNA adduct formation with aflatoxin B1, a liver carcinogen that is specifically detoxified by these isozymes (44). A recent study using transgenic mice lacking the class $\pi$ GSTP1 has demonstrated that this class of GST is also involved in the metabolism of carcinogens, such as 7,12-dimethylbenz[a]anthracene, in mouse skin and has a profound effect on tumorigenicity (4). These data suggest that both isozymes may represent the important determinants in cancer susceptibility, particularly in diseases where exposure to polycyclic aromatic hydrocarbons is involved.

In early studies of the stress response system, a wide variety of phase II detoxification enzymes inducers were found to be electrophiles. Although the primary target of 6-HITC is still unknown, there is evidence that the intracellular level of GSH, regulating the redox state of the cell, may be an important sensor for the initiation of the cellular response to various compounds. In fact, the intracellular GSH levels of RL34 cells were readily reduced by treatment with 6-HITC (Morimistu, Y., Nakagawa, Y., and Uchida, K., unpublished observation). Interestingly, the amount of GSH began to recover and increased to over the basal level, indicating that the cell responded to the GSH depletion. Because GSH is important in metabolism and enzyme regulation as well as the detoxification of cytotoxic materials, the level of intracellular GSH is a critical parameter for a signaling cascade for the induction of phase II enzymes by 6-HITC. On the other hand, it has also been shown that the gene expression of GSTA1 is related to the intracellular oxidative stress presumably mediated by reactive oxygen species or the pro-oxidative potential of GSTA1 inducers (45,46). In addition to GSTA1, oxidative stress has been reported to enhance the expression of genes encoding other antioxidant enzymes, including the $\gamma$-GCS (47), heme oxygenase (48), and heat shock protein 90 (49). Thus, it is increasingly recognized that an adequate amount of oxidative stress stimulates a variety of signal transduction pathways under circumstances that do not result in cell death.

The transcriptional activation of the phase II enzymes has been traced to a cis-acting transcriptional enhancer called ARE (26), or alternatively, the electrophile response element (27). It has been shown that the transcription factor Nrf2 positively regulates the ARE-
mediated expression of the phase II detoxification enzyme genes. Itoh et al. (24) have recently established by gene-targeted disruption in mice that Nrf2 is a general regulator of the phase II enzyme genes in response to electrophiles and reactive oxygens. More recently, the general regulatory mechanism underlying the electrophile counterattack response has been demonstrated in which electrophilic agents alter the interaction of Nrf2 with its repressor protein (Keap1), thereby liberating Nrf2 activity from repression by Keap1, culminating in the induction of the phase II enzyme genes and antioxidative stress protein genes via AREs (28). It has been suggested that the dissociation of Nrf2 from Keap1 may involve modification of either one of these proteins, and could be achieved by direct or indirect mechanisms. For example, Nrf2 can be phosphorylated by components of the MAP kinase cascade (50), which could result in its dissociation. On the other hand, Dinkova-Kostova et al. (51) have provided an alternative possibility that the dissociation of this complex may be potentiated by the direct interaction of electrophilic agents with reactive thiol residues in either of the two proteins. This hypothesis is supported by the strong relationship between the potency of the agents as inducers of the gene expression through the ARE and their rate of reaction with sulfhydryl groups. This mechanism implies that the inducing agent will become covalently bound either to Keap1 or Nrf2. Thus, our findings that (i) 6-HITC induced a significant increase in specific binding to the ARE (Fig. 7), (ii) 6-HITC activated Nrf2 (Fig. 8), and (iii) negligible inducibility in nrf2-deficient mice was observed (Fig. 9) suggest that 6-HITC may directly or indirectly act on the Keap1/Nrf2 complex and activate ARE.

In conclusion, to identify novel cancer chemopreventive agents from plants, we screened extracts from a variety of commonly consumed vegetables on the basis of the GST-inducing effect and found that wasabi, which is known to have a variety of medical benefits, including the prevention of blood clotting, asthma, and even cancer, was the richest source of inducers. An analysis of the wasabi extracts demonstrated that 6-HITC, the major isothiocyanate compound in wasabi, is the principal GST inducer. Moreover, we established the GST inducing potency of this compound not only in vitro but also in vivo. These and the previous findings that 6-HITC has an inhibitory effect on the growth of human stomach tumor cells and on skin carcinogenesis of mice induced by 12-O-
tetradecanoylphorbol-13-acetate (52) suggested that this isothiocyanate may be a chemoprotector against tumors evoked by a number of chemical carcinogens and can be regarded as a readily available promising new cancer chemopreventive agent.

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FIGURE LEGENDS

Fig. 1. Isolation and identification of a major GST inducer from wasabi. (A) Scheme for fractionation of ethyl acetate extract of wasabi by silica gel chromatography. (B) GST inducer potency of fractions separated by silica gel chromatography. (C) Fractionation of the active fraction (the acetone fraction in panel B) by reverse-phase HPLC and GST inducer potency of each fraction (fractions 1 - 4). (D) Purification of active compound from fraction 3 in panel C. (E) Chemical structure of 6-HITC. (F) GC analysis of wasabi extract. Peaks: a, allylisothiocyanate; b, 6-methylthiohexyl isothiocyanate; c, 6-HITC.

Fig. 2. Induction of GST in RL34 cells by 6-HITC and analogues. The cells were treated with the compounds (25 µM) for 24 h. After incubation, the cytosols were prepared and assayed for GST activity.

Fig. 3. Induction of GST by 6-HITC. (A) Dose-dependent induction of GST activity by 6-HITC. (B) Time-dependent induction of GST activity by 6-HITC. (C) Immunoblot analysis of GST isozymes in the RL34 cells treated with 25 µM 6-HITC. Upper, GSTA1; Lower, GSTP1.

Fig. 4. Absorption of 6-HITC into body. (A) The cyclocondensation reaction of 6-HITC with 1,2-benzenedithiol. (B) Standard curve for the dithiocarbamate derivative (6-HITC-GSH adduct). (C) HPLC chromatogram of rat plasma extracts after oral administration of 6-HITC. Panel a, HPLC chromatogram of plasma extracts from control rats. Panel b, HPLC chromatogram of plasma extracts from 6-HITC-administered rats at 1 h. (D) Concentration of 6-HITC in rat plasma after oral administration of 6-HITC. Values are means ± S.E.M., n=3.

Fig. 5. Effect of 6-HITC and sulforaphane administrations on mouse hepatic detoxification enzyme activities. (A) GST and NQO1 activities. (B) Cytochrome P4501A1 activity. (C) Immunoblot analysis of GSTA1 and GSTP1.
Fig. 6. Induction of phase II enzyme gene expression by 6-HITC and sulforaphane. The cells were treated with 5 µM 6-HITC and sulforaphane for different time intervals as indicated in the figures. 18S ribosomal mRNA represents the control to demonstrate that equal amounts of mRNA were probed in the Northern blot analysis.

Fig. 7. Activation of ARE reporter genes by 6-HITC. (A) Structures of constructs used for transfections. (B) Time-dependent activation of ARE reporter genes by 6-HITC. Either pGSTA1-Luc, pNQO1-Luc or pRBGP3 reporter plasmids were transfected into RL-34 cells. After transfection, the medium was changed to fresh medium and the cells were treated with DMSO or 5 µM 6-HITC. Luc activity of the each reporter transfection in the absence of the chemical reagents was arbitrarily set to 1 and the mean values of three independent experiments, each carried out in duplicate, are shown with the standard error of the means (S.E.M.).

Fig. 8. Activation of Nrf2. (A) Induction of nuclear translocation of Nrf2 by 6-HITC. Panels: a, untreated control; b, 2 h after 6-HITC treatment; c, 4 h after 6-HITC treatment. The RL34 cells were fixed in 2% paraformaldehyde and 0.2% picric acid and immunostained with anti-Nrf2 antibody. Images of the cellular immunofluorescence were acquired using a confocal laser scanning microscope. (B) 6-HITC induces Nrf2 protein accumulation in the RL34 cells. Lamin B represents a control to demonstrate that equal amounts of proteins were probed in the immunoblot analysis.

Fig. 9 6-HITC induces phase II gene expression in mouse liver in an Nrf2-dependent manner. Either the Wild type (lanes 1-6) or nrf2 (-/-) mice were treated with 6-HITC for the indicated periods.
A

Wasabi

|          | Hexane | Hexane/EtOAc (3:1) | Hexane/EtOAc (1:1) | Hexane/EtOAc (1:3) | EtOAc | Acetone | MeOH |
|----------|--------|-------------------|--------------------|--------------------|-------|---------|------|
| 100%     |        |                   |                    |                    |       |         |      |

B

Induction ratio
(sample/control)

C

Fractions

Induction ratio
(sample/control)

AU (254 nm)

D

Retention time (min)

E

6-HITC

OS

FID recorder response

Retention time (min)
Fig. 2

Morimitsu et al.
Fig. 3

Morimitsu et al.
Fig. 4
Morimitsu et al.
Morimitsu et al.

Fig. 5

Control

6-HITC

Sulforaphane

A

GST

B

Induction ratio (sample/control)

Cytchrome P450/1A1

Sulforaphane

6-HITC

Control

Induction ratio (sample/control)

GSTA1

Sulforaphane

6-HITC

Control

Induction ratio (sample/control)

GSTP1

Sulforaphane

6-HITC

Control

Induction ratio (sample/control)

GST NQO1

Cytochrome P450/1A1

Sulforaphane

6-HITC

Control

Induction ratio (sample/control)
Fig. 6
Morimitsu et al.
Fig. 7

Morimitsu et al.
Fig. 8

Morimitsu et al.
Fig. 9

Nrf2 +/+ Nrf2 −/−

0 12 28 0 12 28 (h)

GSTP1  NQO1

18S ribosomal RNA

Morimitsu et al.
A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway
Yasuiro Morimitsu, Yoko Nakagawa, Hiroyuki Hayashi, Hiroyuki Fujii, Takeshi Kumagai, Yoshimasa Nakamura, Toshihiko Osawa, Fumihiko Horio, Ken Itoh, Katsuyuki Iida, Masayuki Yamamoto and Koji Uchida

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