博士論文

Studies on the antioxidant effects of aged garlic extract (AGE) using a model organism *Caenorhabditis elegans*

（モデル生物・線虫 *Caenorhabditis elegans* を用いた熟成ニンニク抽出液（aged garlic extract：AGE）の抗酸化作用解明研究）

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主論文

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Introduction

The human body is constantly exposed to reactive oxygen species (ROS), which are generated by aerobic respiration in the mitochondria and as byproducts of diverse metabolic reactions in cells. Overproduction of ROS causes damage to cellular proteins, lipids and DNA, eventually contributing to various chronic diseases including cancer, diabetes, Parkinson's and Alzheimer's diseases, cardiovascular disease and chronic inflammation (Fridovich, 1999). Therefore, cumulative oxidative damage to the cells may also influence aging. It is known that antioxidant vitamins C and E existing in a wide variety of foods act cooperatively to protect cells from lipid peroxidation by directly neutralizing harmful hydroxyl radicals (Leung *et al*., 1981). Additionaly, sulforaphane, a natural dietary isothiocyanate produced in cruciferous vegetables such as broccoli and broccoli sprouts, has been shown to induce phase II detoxification genes, *e.g.* Heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase, γ-glutamylcysteine synthetase and glutathione *S*-transferases (GSTs), through activating Nrf2 (NF-E2-related factor) signaling (Guerrero-Beltrán *et al*., 2012). The induction of these enzymes by sulforaphane protects cells from damage associated to oxidative stress in diverse *in vivo* and *in vitro* experimental conditions (Guerrero-Beltrán *et al*., 2012). Therefore, intake of these natural compounds through diet could help to prevent pathogenesis of chronic diseases and contribute to slow aging, or in other words, extend health span of organisms.

Since ancient times, garlic has been widely used as food and folk medicine. A number of studies have indicated that garlic possesses diverse pharmacological properties, such as antimicrobial (Hughes *et al*., 1991), anticancer (Sumiyoshi *et al*., 1989: Milner, 1996), antithrombotic (Makheja *et al*., 1979), antihyperlipidemic (Kamanna *et al*., 1982: Lau *et al*., 1987), hepatoprotective (Hikino *et al*., 1986) and antioxidant activity (Wei *et al*., 1998). Many of these beneficial effects have been shown to be attributed to garlic-characteristic organosulfur compounds (OSC), including *S*-allylcysteine (SAC) and *S*-allylmercaptocysteine (SAMC).

SAC is one of the major water-soluble thioallyl compounds naturally occurring during aging process of garlic. Welch *et al*. (1992) reported that SAC treatment inhibited growth of human neuroblastoma cell (LA-N-5) in a time- and dose-dependent manner. Another study also reported that the incidence and frequency of colon tumors

induced by the carcinogen dimethylhydrazine were significantly inhibited by pretreatment with SAC (Sumiyoshi *et al*., 1990). Cardioprotective effect of SAC was reported by Chuah *et al*., (2007) where SAC significantly lowered mortality and reduced infarct size of acute myocardial infarction in mice by enhancing cystathionine-γ-lyase activity in left ventricle followed by increased plasma H_2S concentration. SAC treatment also promoted the survival of rat hippocampal neurons *in vitro* and the axonal branching of cultured neurons (Moriguchi *et al*., 1997). Hsu *et al*. (2006) reported that SAC and *S*-propylcysteine protected Balb/cA mice from acetaminophen-induced hepatotoxicity, such as depleted glutathione content, increased oxidative stress and elevated alanine aminotransferase and aspartate aminotransferase activities. Some *in vitro* studies have demonstrated that SAC can scavenge hydrogen peroxide and protect pulmonary endothelial cells from oxidized low-density lipoproteins (Ox-LDL)-induced injury by removing peroxides and preventing GSH depletion (Ide *et al*., 1996: Ide *et al*., 1999).

SAMC is also water-soluble unique constituent produced in aged garlic extract and is not present in fresh raw garlic or in various garlic preparations. Some studies have demonstrated that SAMC has also been shown to have antiproliferative effects on colon cancer cells (Shirin *et al*., 2001) and induce apoptosis in human prostate cancer cells, breast cancer cells, colon cancer cells and gastric cancer cell line (Pinto *et al*., 2001: Lee *et al*., 2011). Sumioka *et al*. (1998) reported that SAMC treatment protected male ddY mice from acetaminophen-induced liver damage by a reduction in alanine aminotransferase activity that is enhanced by acetaminophen treatment. SAMC has been demonstrated to be able to scavenge hydrogen peroxide, and also shown to inhibit the chain oxidation induced by a hydrophilic radical initiator (Ide *et al*., 1996). These ameliorating effects of SAC and SAMC on these pathological conditions are, at least in part, thought to be due to their strong antioxidant abilities. However, despite the abundant evidence of the effects of SAC and SAMC, it is not understood how they confer antioxidant and other effects *in vivo*.

Since the finding in *Caenorhabditis elegans* that reduction in signaling through the conserved insulin/IGF-I signaling (IIS) pathway results in more than double the mean lifespan compared with wild-type (Kenyon *et al*., 1993), aging has become a particularly active area of research. Further studies have identified genes and molecular

mechanisms involved in stress responses and longevity. For example, the lifespan extension caused by reduced IIS requires the activity of DAF-16, the FOXO (Forkhead box O) orthologue, which induces entry into larval diapause but also promotes longevity in adults (Kenyon *et al*., 1993). When IIS is reduced under conditions where dauer-associated processes are inactive in adults, lifespan extension also requires SKN-1, the Nrf1/2/3 orthologue (Ewald *et al*., 2015), which increases resistance to various stresses (Sykiotis *et al*., 2010). In addition, reduced IIS causes each of these proteins to accumulate in nuclei, leading to upregulation of target genes involved in longevity, stress responses, metabolism, and the extracellular matrix (Murphy *et al*., 2003: Lee *et al*., 2003: Tullet *et al*., 2008: Ewald *et al*., 2015). In *C. elegans*, SKN-1 is required for lifespan to be extended by a variety of different interventions (Bowerman *et al*., 1992: An *et al*., 2003: Tullet *et al*., 2008: Oliveira *et al*., 2009: Robida-Stubbs *et al*., 2012: Mizunuma *et al*., 2014: Ewald *et al*., 2015). Under oxidative stress conditions, PMK-1, a p38 mitogen-activated protein kinase (MAPK), phosphorylates SKN-1, leading to its nuclear accumulation and target gene expression (Inoue *et al*., 2005). In addition to the longevity modulating effect of SKN-1, recent studies have also demonstrated its critical roles in protein homeostasis under conditions of reduced translation or proteasome activity (Wang *et al*., 2010: Li *et al*., 2011) or increased endoplasmic reticulum (ER) stress (Glover-Cutter *et al*., 2013). SKN-1 then selectively induces distinct but partly overlapping set of its downstream target genes under these diverse conditions.

A previous study have shown that there are numerous organosulfur compounds (OSCs), such as *S*-alk(en)ylcysteines, *S*-alk(en)ylcysteine sulfoxides, *γ*-glutamyl-*S*-alk(en)ylcysteines, and allylsulfides, in garlic (Liu *et al*., 2000). Some of those compounds have also been shown to have versatile pharmacological properties, such as radical scavenging activity, chemopreventive activity, hepatoprotective activity, neurotropic activity, and lipid reducing activity (Sumiyoshi *et al*., 1990: Moriguchi *et al*., 1997: Liu *et al*., 2001: Hsu *et al*., 2006: Argüello-García *et al*., 2010). Indeed, a recent study demonstrated that diallyl trisulfide (DATS), one of the major lipophilic allylsulfides in garlic, could induce *gst-4* gene expression dependent of *skn-1* and extend longevity of *C. elegans* (Powolny *et al*., 2011).

The structure activity relationship analysis is a method for investigation of the

relationship between the chemical structure of a molecule and its biological activity. This approach has been widely used in drug discovery area to explore a compound with aimed criteria against its target molecule and to change or modify its chemical structure for optimization of its biological activity. Some previous studies, in which relationships between structures of OSCs from garlic and chemopreventive- (Hatono *et al*., 1997), neurotropic- (Moriguchi *et al*., 1997), enzyme inhibitory- (Gupta *et al*., 2001: Camargo *et al*., 2007), and radical scavenging-activity (Argüello-García *et al*., 2010) were investigated, indicated that the thioallyl group and the number of sulfur atom confer their activities.

In this doctoral thesis, I tried to clarify the molecular mechanism of the effect of small OSCs especially focused on SAC and SAMC on lifespan and resistance against oxidative stress of whole organism and their underlying molecular mechanisms by using *C. elegans*. In chapter I, I investigated whether SAC and SAMC affect lifespan and oxidative stress resistance of *C. elegans*. Indeed, I attempted to clarify whether SAC and SAMC could activate the DAF-16/FOXO and SKN-1/Nrf pathways and could mimic dietary restriction (DR)-like conditions. The results indicated that SAC and SAMC activate the SKN-1/Nrf pathway by presumably sustaining or stabilizing the amount of SKN1 protein, thus leading to its target gene induction especially responsible for oxidative stress resistance, such as *gst-4*. In chapter II, I evaluated the effect of 23 garlic-derived OSCs and their analogs on induction of *gst-4::GFP* reporter transgene to see whether there are any relationships between their structures and inducible activity of the reporter gene. The results showed that thioallyl structure is essential and the number of disulfide bond are positively correlated with *gst-4p::GFP* induction.

Chapter I

Studies on the molecular mechanisms of antioxidant action of *S*-allylcysteine (SAC) and *S*-allylmercaptocysteine (SAMC) using a model organism *Caenorhabditis elegans* (*C. elegans*)

1.1. Abstract

Identification of biologically active natural compounds that promote health and longevity, and understanding how they act, will provide insights into aging and metabolism, and strategies for developing agents that prevent chronic disease. The garlic-derived thioallyl compounds *S*-allylcysteine (SAC) and *S*-allylmercaptocysteine (SAMC) have been shown to have multiple biological activities, including anti oxidant activity.

In this chapter, I show that SAC and SAMC increase lifespan and stress resistance in *Caenorhabditis elegans* and reduce accumulation of reactive oxygen species (ROS). These compounds do not appear to activate DAF-16 (FOXO orthologue) or mimic dietary restriction (DR) effects, but selectively induce SKN-1 (Nrf1/2/3 orthologue) targets involved in oxidative stress defense. Furthermore, their treatments do not facilitate SKN-1 nuclear accumulation, but slightly increased intracellular SKN-1 levels. Taken together, these results indicate that SAC and SAMC possibly modulate SKN-1 by enhancing its stability followed by inducing target gene expression associated with oxidative stress resistance that contributes to the increased lifespan of *C. elegans*.

1.2. Introduction

Reactive oxygen species (ROS) are generated by aerobic respiration in the mitochondria, and as byproducts of diverse metabolic reactions in cells. Oxidative stress reflects a balance between production and reduction of ROS toward the pro-oxidative state (Finkel *et al*., 2000). Because ROS causes damage to cellular components, accumulation of those oxidative damages to the cells and tissues is thought to affect the aging process of organisms. On the other hand, ROS has been shown to play key roles in host defense (Segal, 2005) and in cell signaling to maintain cellular homeostasis (Hancock *et al*., 2001).

Garlic (*Allium sativum* L.) has been widely used as a food and folk medicine at least for a thousand years. A number of studies have indicated that garlic possesses diverse pharmacological potentials related to chronic diseases, such as anticancer (Sumiyoshi *et al*., 1989: Milner, 1996), antithrombotic (Makheja *et al*., 1979), hypolipidemic (Kamanna *et al*., 1982: Lau *et al*., 1987) and hepatoprotective activity

(Hikino *et al*., 1986). Many of these beneficial effects have been shown to be attributed to garlic characteristic organosulfur compounds (OSC), such as SAC and SAMC (Sumiyoshi *et al*., 1990: Moriguchi *et al*., 1997: Sumioka *et al*., 1998: Shirin *et al*., 2001: Hsu *et al*., 2006: Chuah *et al*., 2007). SAC and SAMC are the major water-soluble OSCs naturally occurring during aging process of garlic, and known to act as free radical scavengers (Ide *et al*., 1999: Thomson *et al*., 2003). Therefore, some of these protective effects of SAC and SAMC could potentially be explained by their radical scavenging activity. While some studies have demonstrated that SAC and SAMC inhibited growth of human cancer cells *in vitro* (Li *et al*., 1995: Shirin *et al*., 2001), and development of chemically induced cancers or growth of implanted tumors *in vivo* along with increasing levels of GSTs (Sumiyoshi *et al*., 1990: Hatono *et al*., 1996). GSTs play a key role in the phase II detoxification response, which provides a conserved defense against oxidative stress (McMahon *et al*., 2001). More recent study demonstrated that SAC treatment protected primary cultured neurons and mice against oxidative insults and middle cerebral artery occlusion-induced ischemic damages, respectively, through increase in Nrf2 protein and target expressions, such as γ-glutamylcysteine synthetase catalytic subunit (GCLC), γ-glutamylcysteine synthetase modulatory subunit (GCLM) and HO-1 (Shi *et al*., 2014). In addition, treatment of human umbilical vein endothelial cells with aged garlic extract that contains SAC and SAMC induced the accumulation of Nrf2 into the nucleus in a time- and dose-dependent manner and increased the gene expression and polypeptide level of HO-1 and GCLM (Hiramatsu *et al*., 2015). Because development of cancer, oxidative stress response and apoptosis are strongly associated with aging, I considered the question of whether SAC and SAMC can retard aging. However, the ability of SAC and SAMC to modulate organismal aging and the potential mechanisms involved has not been reported.

In this chapter, to examine the effects of SAC and SAMC on oxidative stress defenses and aging in a whole organism, I used the nematode *Caenorhabditis elegans* as an *in vivo* model. *C. elegans* has been used in studies on aging and longevity, because of it relatively short lifespan and well-defined genetic pathways including those affecting lifespan and oxidative stress response. At first, SAC and SAMC were tested for their ability to increase lifespan and oxidative stress resistance of *C. elegans*. Next, I

investigated whether they affect the DAF-16/FOXO and SKN-1/Nrf pathways because those pathways play an important role in regulating longevity and stress resistance (Bowerman *et al*., 1992: Kenyon *et al*., 1993: Lin *et al*., 2001: An *et al*., 2003: Lee *et al*., 2003: libina *et al*., 2003: Inoue *et al*., 2005). In addition, I tested whether SAC and SAMC could mimic dietary restriction (DR) like conditions, which have been demonstrated to associate with longevity of wide range of species including *C. elegans* (Weindruch *et al*., 1986: Partridge *et al*., 1987: Jiang *et al*., 2000: Walker *et al*., 2005: Bioshop *et al*., 2007). I found that treatment with SAC and SAMC increased *C. elegans* lifespan and resistance to oxidative- and heat-stress with reduced intracellular ROS. In addition, these compounds augmented intracellular abundance of SKN-1 proteins followed by induction of its target genes, such as *gst-4*, but neither affected to DAF-16 pathway nor caused DR-mimic conditions. These results suggest that garlic-derived thioallyl compounds, SAC and SAMC, increase stress resistance and longevity by modulating SKN-1 activity.

1.3. Materials and Methods

1.3.1. Reagents

SAC and SAMC (Figure 1A) were synthesized as in Nagae *et al*., 1994 and Hikino *et al*., 1986, respectively, stored in water solution and added to culture medium at various concentrations.

1.3.2. Strains and culture of *C. elegans*

Nematode strains used in this study are listed in Table 1. Each strain was maintained at 20ºC on nematode growth medium (NGM) agar plates carrying a lawn of *E. coli* OP50 (*Caenorhabditis* Genetics Center) (Brenner, 1974). Unless otherwise stated, animals for each assay were raised according to the following procedure. Briefly, to synchronize growth of *C. elegans*, gravid hermaphrodites were treated with sodium hypochlorite and resulting eggs were kept overnight at 20ºC for hatching in S-complete liquid medium. Synchronized L1 animals were then transferred to a 96-well plate in S-complete liquid medium containing amphotericin B (0.1 μg/mL) and the UV-killed *E. coli* OP50 (1.2 x 10^9 bacteria/mL), sealed to prevent evaporation, and kept at 20ºC (Solis *et al*., 2011). UV killing of *E. coli* OP50 was done using a stratalinker (9999 J/m^2, Stratagene, La Jolla, CA) to exclude any effects of the test compounds on bacterial growth, and

unexpected metabolism of these compounds by live bacteria (Smith *et al*., 2008). 5-fluoro-2'-deoxyuridine (FUdR, 0.12 mM) was added 42-45 hours after seeding to prevent self-fertilization. Thirty micro liters of SAC or SAMC solution, or H_2O as solvent control were added on the first day of adulthood at final concentrations ranging from 1 to 100 μM, respectively.

1.3.3. Lifespan assays

All lifespan assays were started on the first day of adulthood and performed at 20ºC. To avoid starvation, an adequate amount of the UV-killed OP50 was added to each well during assays. Counting of surviving or dead animals was performed daily using a microscope on the basis of movement until all animals had died. Before counting each plate was shaken for one minute on a plate shaker to facilitate observation of movement.

1.3.4. Stress resistance assays

Synchronized day-1 wild-type adults were pretreated with H_2O, SAC or SAMC (10 μM each) for 48 hours at 20ºC. For the oxidative stress assays, the animals were washed with phosphate-buffered saline with 1% Tween 20 (PBST) three times before treating with a ROS generator, juglone (250 μM, Sigma-Aldrich, St. Louis, MO), for 2 hours at 20ºC. For the thermo-tolerance assays, the animals were incubated at 35ºC for 7 hours, and then washed with PBST three times. After a 16 hours recovery period on NGM agar, the survival was determined by touch-provoked movement. Animals were scored as dead when they failed to respond to touching with a platinum wire pick.

1.3.5. Measurement of intracellular ROS in *C. elegans*

To measure intracellular ROS accumulation level in animals after both the oxidative- and the heat-stress treatment, the surviving animals were incubated in the presence of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA, 50 μM, Invitrogen, Carlsbad, CA) in PBST for 1 hour at 20ºC. CM-H_2DCFDA is a cell permeable substance which is intracellularly converted to H_2DCFs. This nonfluorescent probe can be oxidized by interaction with intracellular ROS to yield the fluorescent dye DCF. After washing with PBST, the animals were mounted onto microscope slides coated with 2% agarose, anesthetized with tetramisole (5 mM), and capped with cover slides. Fluorescence images were collected with a BIOREVO BZ-9000 fluorescent microscope (KEYENCE, Osaka, Japan) using the GFP-BP filter set with excitation at 470 nm and emission at 535 nm. The fluorescence

intensity of whole body was quantified as mean pixel density by using ImageJ software (NIH, Bethesda, MD).

1.3.6. Transgenic reporter assays

Synchronized day-1 adults of the transgenic strains carrying an inducible green fluorescence protein (GFP) reporter transgene for *sod-3* (CF1553), *hsp-16.2* (CL2070) or *gst-4* (CL2166 or CL691(*skn-1(zu67)*)) were treated with H_2O, SAC or SAMC (10 or 100 μM each) for 24 hours at 20ºC. Juglone (10 or 100 μM) was used as positive control. GFP fluorescence images were collected with randomly selected animals as described in the measurement of intracellular ROS. For the *sod-3p::GFP* and *hsp-16.2p::GFP* reporters, GFP fluorescence from pharynx was quantified by ImageJ. For the *gst-4p::GFP* reporter, GFP fluorescence from whole body was quantified.

1.3.7. Quantitative real-time reverse transcription PCR (qRT-PCR)

Synchronized day-1 adults of wild-type or KU4 (*sek-1(km4)*) strains were treated with H_2O, SAC or SAMC (10 or 100 μM each) for 6 or 24 hours at 20ºC. Total RNA was extracted from about 50 animals with TRIzol (Invitrogen). Complementary DNA was produced using random 6-mer and oligo (dT) primer. qPT-PCR was performed using SYBR green as the detection method. Expression levels of each mRNA relative to *act-1* gene were calculated with the comparative $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study are follows;

act-1 forward 5'-accatgtacccaggaattgc-3' and reverse 5'-tggaaggtggagagggaag-3'

sod-3 forward 5'-agcatcatgccacctacgtga-3' and reverse 5'-caccaccattgaatttcagcg-3'

hsp-16.2 forward 5'-ctcaacgttccgtttttggt-3' and reverse 5'-cgttgagattgatggcaaac-3'

ctl-2 forward 5'-tccgtgaccctatccacttc-3' and reverse 5'-tgggatccgtatccattcat-3'

gst-4 forward 5'-cgttttctatggaagtgacgc-3' and reverse 5'-tcagcccaagtcaatgagtc-3'

gcs-1 forward 5'-tgttgatgtggatactcggtg-3' and reverse 5'-tgtatgcaggatgagattgtacg-3'

gst-10 forward 5'-gtctaccacgttttggatgc-3' and reverse 5'-actttgtcggcctttctctt-3'

atf-5 forward 5'-ccatcaatcttatcaacagcatcat-3' and reverse 5'-ctggtggaaccgaagtg-3'

haf-7 forward 5'-gacgtggaaaagctgagagg-3' and reverse 5'-gcagggaaaatgtgaggaaa-3'

rpt-3 forward 5'-cccaagaggagttctcatgta-3' and reverse 5'-atgaaggaagcagcagtatt-3'

rpn-12 forward 5'-ctgccaacagattgtccg-3' and reverse 5'-ggcgtagagatgtaagcg-3'

pas-4 forward 5'-cgagccatctggagcttacta-3' and reverse 5'-tcctcaaggtattcacgcac-3'

pbs-6 forward 5'-tggacagagccatctcatt-3' and reverse 5'-cttcagcgatgaccaagtg-3'

skn-1 forward 5'-agtgtcggcgttccagatttc-3' and reverse 5'-gtcgacgaatcttgcgaatca-3'.

1.3.8. Feeding RNAi

RNAi was performed in a 96-well plate format by feeding *E. coli* HT115 expressing RNAi for either *wdr-23* (clone ID: CUUkp3300D063Q, Source BioScience, Nottingham, UK) or control (pL4440) to nematodes. Synchronized L1 animals were raised in S-complete liquid medium containing amphotericin B (0.1 μg/mL), ampicillin (100 μg/ml), isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and 1.2 x 10^9 bacteria/mL of an overnight culture of RNAi bacteria induced by IPTG for 1 hour. The animals were grown at 20°C throughout the assay. FUdR (0.12 mM) was added 42-45 hours after seeding. On the first day of adulthood, the animals were treated with H_2O, SAC or SAMC (10 μM each) for 24 hours at 20ºC, and expression levels of *gst-4* mRNA were determined by qRT-PCR.

1.3.9. Nuclear localization DAF-16 or SKN-1

Synchronized day-1 adults of the strains LD1482 or LD001 carrying a transgene that expresses DAF-16A::GFP or SKN-1B/C::GFP fusion protein, respectively, were treated with H_2O, SAC or SAMC (10 or 100 μM each) at 20ºC. For the DAF-16A::GFP reporter, each treatment was performed for 24 hours. For the SKN-1B/C::GFP reporter, synchronized L4 animals were treated with H_2O, SAC or SAMC (10 μM each) for 16 hours at 20ºC. The following day, eggs were harvested by hypochlorite treatment, and progeny were further treated with same compound as each parent and allowed to develop to the L4 stage. After washing with PBST the animals were additionally challenged without or with 2% NaN_3 for 10 min. As a control experiment, synchronized L1 animals of LD001 strain were treated with either control or *wdr-23* RNAi as described above, and then analyzed on the first day of adulthood.

Subcellular distributions of DAF-16A::GFP or SKN-1B/C::GFP were microscopically-classified into "Low", no visible nuclear localization, "Medium", nuclear localization visible only in anterior and/or posterior of body, or "High", strong nuclear localization visible throughout the body or intestine, respectively.

1.3.10. 26S proteasome activity assays

The 26S proteasome activity in whole animal lysate was measured as previously described (Kisselev *et al*., 2005). Briefly, after treating L1 larvae with H_2O (control), SAC or SAMC (10 μM each) for 4 days at 20ºC, adult animals were sonicated in 4

volumes of lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM MgCl2, 2 mM ATP, 1 mM dithiothreitol and 0.5 mM EDTA) with a Bioruptor UCW310 (BM Equipment, Tokyo, Japan). Lysate was centrifuged at 14,000 X g for 10 min at 4ºC. To measure chymotrypsin-like proteasome activity, 25 μg of whole animal lysate was transferred to a 96-well microtitre plate, then incubated with a fluorogenic peptide substrate (100 μM Suc-Leu-Leu-Val-Tyr-AMC, Boston Biochemicals, MA) in proteasome activity assay buffer (50 mM Tris-HCl, pH 7.5, 40 mM KCl, 5 mM MgCl2, 0.5 mM ATP, 1 mM dithiothreitol and 0.05 mg/mL BSA) at 25ºC. The fluorescence intensity was measured at 380 nm for excitation and 460 nm for emission using an EnVision 2104 multilabel reader (PerkinElmer, Waltham, MA) every 5 min for 1 hour at 25ºC. The assay was performed in the absence or presence of proteasome inhibitor (40 μM Epoxomicin, Peptide Institute, Osaka, Japan) to calculate the 26S proteasome-specific activity.

1.3.11. Western blot analysis

Synchronized wild-type L4 animals were treated with H_2O, SAC or SAMC (10 μM each) for 16 hours at 20ºC. The following day, eggs were harvested by hypochlorite treatment, and progeny were further treated with same compound as parent and allowed to develop to the L4 stage. The animals (~1,000 animals per condition) were sonicated in 10 volumes of buffer (50 mM Tris-HCl, pH7.6, 50 mM NaCl, 1% sodium dodecyl sulfate and 1x Halt protease and phosphatase inhibitor cocktail (Thermo scientific)) with a Bioruptor UCW310 (BM Equipment, Tokyo, Japan). Homogenates of total protein were harvested after centrifugation at 16,100 X g for 5 min. Protein concentrations were determined with a XL-Bradford kit (APRO science, Tokushima, Japan) after diluted in SDS-PAGE sample buffer. Fifteen μg of protein samples were applied and separated by SDS-PAGE, and detected by immunoblotting with a polyclonal antibody against SKN-1 (1:2000; JDC7, (Glover-Cutter *et al*., 2013)) and *β*-tubulin (1:1000; 014-25041; Wako). As control experiments, whole lysates from the *rrf-3(pk1426)* mutant treated with either control or *wdr-23* RNAi from L1 state or the *skn-1(zu135)* mutant were prepared on day-1 adulthood and analyzed. Blots were visualized with a ChemiDoc MP (BioRad, Hercules, CA) and densitometrical analysis was performed using Image Lab software (BioRad).

1.3.12. Reproduction assays

Synchronized wild-type L4 animals were individually transferred to wells containing H_2O, SAC or SAMC (10 μM each), and allow laying eggs for 24 hour at 20°C. The adult animals were transferred to new wells daily until reproduction period was ceased. The number of progeny from individual animal was counted when they raised to the L2 or L3 stage.

1.3.13. Body length and food consumption assays

Synchronized wild-type day-1 adults were treated with H_2O, SAC or SAMC (10 μM each) for 8 days at 20°C. For the body length assays, the animals were collected, and photographs were taken. The body length of individual animal was analyzed using ImageJ. For the food consumption assays, the liquid medium containing total 50 animals was collected and values of optical density at 620 nm were measured with a multiskan spectrophotometer (Labsystems, Helsinki, Finland).

1.3.14. Statistical analysis

Statistical analysis was performed using KyPlot 5.0 software (KyPlot, Tokyo, Japan). For the lifespan assays, *P*-values were determined by log-rank test. For the nuclear localization of DAF-16A::GFP or SKN-1B/C::GFP, a chi^2 test was used. One-way analysis of variance (ANOVA) with Tukey's post hoc analysis was used for other assays. Differences were considered significant at $P<0.05$.

1.4. Results

1.4.1. SAC and SAMC extend *C. elegans* lifespan under normal conditions

I first evaluated whether SAC and SAMC (Fig. 1A) influence the lifespan of wild-type *C. elegans* under normal conditions. To eliminate the possibilities that these compounds could affect growth of *E. coli* OP50, and *vice versa* live bacteria could metabolize these compounds, I used UV-killed *E. coli* OP50 in the lifespan and the following assays. In our lifespan assays, SAC- and SAMC-treatment were begun on the first day of adulthood with concentrations at 1, 10, and 100 μM at 20ºC. As a result, SAC produced significant increase in the mean lifespan of adult animals (7.5% for 1 μM ($P<0.001$), 17.0% for 10 μM ($P<0.001$) and 15.6% for 100 μM ($P<0.001$), Fig. 1B, Table 2). Similarly, SAMC-treatment also significantly increased the mean lifespan (5.8% for 1 μM ($P<0.05$), 19.7% for 10 μM ($P<0.001$) and 20.9% for 100 μM ($P<0.001$), Fig. 1C, Table 2). Given that the significant extension of the mean lifespan

of wild-type *C. elegans* was achieved at 10 and 100 μM of each compound, I performed the following experiments at these concentrations.

1.4.2. SAC and SAMC enhance stress resistance and reduce ROS levels under oxidative- and heat-stress conditions

In *C.elegans*, increased lifespan is sometimes associated with improved survival under conditions of oxidative or heat stress (Lithgow *et al*., 1995: Muñoz *et al*., 2003). To investigate whether SAC and SAMC could enhance resistance to stress, I pretreated wild-type adults with 10 μM of SAC or SAMC for 2 days at 20 ºC, followed by exposure to oxidative (250 μM juglone for 2 hours, an intracellular ROS generator) or heat stress (35ºC, 7 hours). Both SAC- and SAMC-pretreatment increased survival after juglone exposure (Fig. 2A) and heat stress (Fig. 2B) at significantly higher ratio than untreated control. These results indicate that both compounds exert protective roles against oxidative and heat stress in *C. elegans*. Because both juglone treatment and heat shock cause cellular damage by accumulation of ROS, I next investigated whether SAC and SAMC could lower the intracellular ROS level under stress conditions by using CM-H_2DCFDA, a fluorescent probe that reacts with ROS. The results showed that pretreatment with SAC or SAMC significantly suppresses oxidative or heat stress-induced accumulation of ROS compared to untreated control (Figs. 2C and 2D), suggesting that the increase in lifespan and stress resistance by SAC- or SAMC-treatment is associated with reduced ROS levels.

Since SAC and SAMC have been shown to act as radical scavengers (Thomson *et al*., 2003), the increased lifespan and stress resistance by SAC and SAMC could be at least in part due to the direct antioxidant properties. On the other hand, there is increasing evidence that SAC and SAMC modulate pathways involved in oxidative stress response (Hatono *et al*., 1996: Shi *et al*., 2014). Therefore, I investigated whether the SAC- and SAMC-mediated increase in stress resistance and longevity in *C. elegans* could be produced by activating pathways particularly associated with oxidative stress responses and longevity.

1.4.3. SAC and SAMC do not affect DAF-16/FOXO activity

In *C. elegans*, the evolutionarily conserved DAF-16/FOXO transcription factor regulates many biological processes including stress resistance and longevity (Murphy *et al*., 2003: Lee *et al*., 2003). Therefore, I examined whether SAC and SAMC could

have any effect on DAF-16 signaling. I first monitored expression of transgenes in which promoter for DAF-16 target genes *sod-3* (superoxide dismutase) or *hsp-16.2* (small heat shock protein) is fused to green fluorescent protein (GFP), respectively. As shown in Fig. 3A, juglone (positive control) upregulated the expression of both *sod-3p::GFP* and *hsp-16.2p::GFP* transcriptional reporters, whereas no induction of these reporters was observed by SAC- and SAMC-treatment (100 μM each for 24 hours). I also examined expression of endogenous *sod-3*, *hsp-16.2*, and *ctl-2* (catalase) mRNAs by quantitative RT-PCR (qRT-PCR), and found that neither of these genes was activated by these compounds (100 μM each for 24 hours) (Fig. 3B).

To further investigate the effect of SAC and SAMC on DAF-16 signaling, I examined whether SAC and SAMC could promote accumulation of a DAF-16A::GFP translational fusion protein in the nucleus. Like other transcription factors, nuclear localization of DAF-16 is associated with its transcription-activating activity. Exposure to juglone and heat stress resulted in remarkable nuclear localization of DAF-16A::GFP, whereas no nuclear localization of DAF-16A::GFP was observed in animals treated with SAC or SAMC (100 μM each for 24 hours) (Fig. 3C).

To further elucidate the involvement of DAF-16 signaling in the effects of SAC and SAMC on nematodes, I performed the lifespan assays using the *daf-16(mgDf47)* mutant. I found that treatments with SAC and SAMC at 10 and 100 μM appeared to prolong survival of the *daf-16(mgDf47)* mutant in early stage of adult life (Figs. 3D and 3E, Table 3), and when I combined three independent assays, significant extension of the mean lifespan was observed in treatments with 10 and 100 μM of these compounds, although this lifespan extension was reduced compared to wild-type (Table 3). Taken together, our results show that SAC- and SAMC-mediated increase in lifespan and stress resistance appears to be in part independent of DAF-16 signaling.

1.4.4. SAC- or SAMC-mediated lifespan extension is dependent on *skn-1*

In *C. elegans*, the transcription factor SKN-1/Nrf plays a critical role in promoting oxidative stress resistance and longevity by upregulating numerous genes, including phase II detoxification enzymes (An *et al*., 2003: Inoue *et al*., 2005: Kahn *et al*., 2008: Tullet *et al*., 2008: Choe *et al*., 2009). To investigate whether SAC- and SAMC-treatment could modulate SKN-1 activity, I first examined the effect of SAC and SAMC on expression of *gst-4* (glutathione S-transferase) gene, one of the key phase

II enzyme genes that is strongly activated in response to oxidative stress (Kahn *et al*., 2008: Choe *et al*., 2009). I treated the transgenic animals, which contains a *gst-4p::GFP* transcriptional reporter transgene, with juglone (positive control), SAC and SAMC. All these treatments resulted in a dramatic increase in GFP expression compared to untreated control (Fig. 4A). To confirm whether the *gst-4p::GFP* induction by SAC- and SAMC-treatment could require SKN-1, I treated the *skn-1(zu67)* mutant, which carries the *gst-4p::GFP* transgene, with SAC and SAMC. I found that no induction of *gst-4p::GFP* was observed by these compounds in this mutant (Fig. 4B), indicating that the induction of *gst-4p::GFP* by SAC and SAMC is completely dependent upon SKN-1.

It has been demonstrated that *skn-1* loss-of-function mutants have shortened lifespans, and in contrast, that increased expression or activity of SKN-1 increases *C. elegans* lifespan (Tullet *et al*., 2008). I next examined whether SAC- and SAMC-mediated extension of lifespan requires SKN-1. I treated the *skn-1(zu135)* mutant with SAC and SAMC, and found that both compounds failed to increase the mean lifespan of this mutant compared to untreated control (Figs. 4C and 4D, Table 4). Instead, this mutation shortened the mean lifespan in the presence of 10 or 100 μM SAC or SAMC (Figs. 4C and 4D, Table 4). Together, these results suggest that *skn-1* is required for the SAC- and SAMC-mediated lifespan extension.

1.4.5. SAC and SAMC promote longevity by modulating SKN-1

In *C. elegans*, SKN-1 is activated in response to diverse interventions, such as oxidative- and ER-stress, and reduced translation and proteasome activity, leading to partially overlapping but distinct set of target gene expression (An *et al*., 2003: Oliveira *et al*., 2009: Wang *et al*., 2010: Li *et al*., 2011: Glover-Cutter *et al*., 2013). To investigate how SAC- and SAMC-treatment could affect expression of SKN-1 target genes, I examined mRNA levels of some SKN-1 targets related to response against oxidative- or ER-stress, and reduced translation elongation or proteasome activity. SAC- and SAMC-treatment on wild-type animals significantly induced some oxidative stress defense genes, *gst-4* and *gcs-1* (*γ*-glutamylcysteine synthase heavy chain (An *et al*., 2003: Inoue *et al*., 2005), except *gst-10* (Oliveira *et al*., 2009) (Figs. 5A and 5B). Additionally, the *skn-1*-dependent ER and oxidative stress-related transcription factor *atf-5* (a mammalian bZIP transcription factors ATF4 (Oliveira *et al*., 2009:

Glover-Cutter *et al*., 2013) was also induced by these compounds (Figs. 5A and 5B). On the other hand, SAC and SAMC did not increase transcription of *hsp-4* (heat shock protein) and *haf-7* (an ortholog of human ATP-binding cassette B9, ABCB9) (Figs. 5A and 5B), which are induced by SKN-1 in response to ER stress and reduced translation, respectively (Oliveira *et al*., 2009: Wang *et al*., 2010: Li *et al*., 2011: Glover-Cutter *et al*., 2013).

Knockdown of some proteasome subunit genes by RNAi induces *skn-1*-dependent expression of endogenous *gst-4* and *gst-10* (Li *et al*., 2011). Additionally, the amyloid-binding dye Thioflavin T (ThT) has been shown to extend *C. elegans* lifespan dependent upon *skn-1* and also *hsf-1* (heat shock factor 1), which promotes protein homeostasis (Alavez *et al*., 2011). ThT also suppresses aggregation of Amyloid-β(3-42) peptide and polyglutamine, which are associated with Alzheimer's disease and several neurological conditions, respectively, in *C. elegans* models (Alavez *et al*., 2011). One possibility is that undesirable accumulation of aggregated or misfolded proteins in cells might activate SKN-1 to induce its targets associated with protein homeostasis. In contrast, our data indicated that SAC and SAMC did not substantially affect mRNA levels of various components of the proteasomal complex; *rpt-3* (an ATPase subunit of the 19S proteasome), *rpn-12* (a non-ATPase subunit of the 19S proteasome), *pas-4* (an alpha-rings subunit of the 20S proteasome), and *pbs-6* (a beta-rings subunit of the 20S proteasome) (Li *et al*., 2011) (Figs. 5A and 5B). I further examined the effect of SAC and SAMC on the 26S proteasome activity and found that these compounds had no effect on its activity (Fig. 5C), suggesting that these compounds appear to activate SKN-1 through a mechanism uncoupled from protein homeostasis. Taken together, these results suggest that SAC and SAMC may act primarily on oxidative stress response genes regulated by SKN-1, and that this may confer the increased lifespan and stress resistance associated with SAC and SAMC treatment.

I also tested the possibility of whether SAC and SAMC could induce expression of *skn-1* mRNA itself, thus leading to induction of its target genes. Results showed that these compounds had no effect on *skn-1* mRNA expression (Fig. 5A and 5B). Therefore, I next examined whether SAC and SAMC could modulate SKN-1 activity at the protein level. Under oxidative stress conditions, SKN-1 is activated by

p38 MAPK pathway signaling (Inoue *et al*., 2005). p38 MAPK directly phosphorylates specific sites within SKN-1, which then accumulates in the nucleus and activates oxidative stress defense genes such as *gcs-1* (An *et al*., 2003, Inoue *et al*., 2005). Downstream of or in parallel to this regulation, WDR-23 (WD40 repeat protein) physically interacts with SKN-1 and CUL-4/DDB-1 ubiquitin ligase complex in the nucleus, which presumably ubiquitinylates SKN-1 protein and targets it for proteasomal degradation (Choe *et al*., 2009). To elucidate how SAC and SAMC modulate SKN-1 activity, I first examined the effect of these compounds on the p38 MAPK pathway. I treated the *sek-1(km4)* mutant, a gene encoding p38 MAPKK that function is essential for the p38 MAPK pathway, with SAC and SAMC and examined the effect of these compounds on *gst-4* mRNA expression. As a result, these compounds also activated transcription of *gst-4* in this mutant as well as that of wild-type (Fig. 5D), suggesting that the SAC- and SAMC-mediated activation of *gst-4* transcription, which requires *skn-1* (Fig. 4B), might be independent of the p38 MAPK pathway.

I next assessed the possibility whether SAC and SAMC could activate SKN-1 and its target expressions through regulation by WDR-23. To test this idea, I examined the effect of *wdr-23* knockdown by RNAi on the SAC- and SAMC-induced *gst-4* mRNA expression. As shown in Fig. 5E, *wdr-23* RNAi drastically caused *gst-4* mRNA expression in untreated control animals compared with that of control RNAi, and no additional increase of *gst-4* expression was observed in the SAC- or SAMC-treated animals. This suggests that SAC and SAMC might modulate SKN-1 activity by regulating WDR-23 or its interaction with SKN-1, or possibly by stabilizing SKN-1.

Loss of WDR-23 function causes nuclear accumulation of SKN-1 in intestine, and increases SKN-1 protein levels, leading to activation of target genes (Choe *et al*., 2009). Therefore, I next assessed the possibility whether SAC- and SAMC-treatment could promote nuclear accumulation of SKN-1. I examined the effect of these compounds on subcellular distribution of a SKN-1B/C::GFP translational fusion protein that encodes two of three SKN-1 isoforms. I treated L4 animals with SAC or SAMC, and then measured nuclear accumulation of SKN-1B/C::GFP at L3 or L4 stages of the next generation. Results showed that SAC- and SAMC-treatment did not detectably increase nuclear accumulation of SKN-1B/C::GFP under normal conditions (Fig. 5F), suggesting that these compounds do not substantially affect nuclear localization of

SKN-1. On the other hand, it is also possible that hypochlorite treatment for the preparation of L1 animals of the next generation may affect the inducibility of nuclear SKN-1 or levels of SKN-1 protein in later larval stages, leading to a failure of detection of SKN-1B/C::GFP nuclear localization. To address this possibility, I treated L4 animals of the next generation with acute oxidative stress, 2% NaN_3 (as a positive control of SKN-1B/C::GFP nuclear localization) for 15 min after pretreatments with SAC or SAMC. Results showed that this acute oxidative stress caused drastic nuclear accumulation of SKN-1B/C::GFP as indicated in Kahn *et al*., 2008, and population of animals with nuclear SKN-1B/C::GFP slightly but reproducibly increased after exposure to 2% NaN_3 when they were pretreated with SAC or SAMC (Fig. 5F). Taken together, these results implicate that SAC and SAMC do not cause nuclear accumulation of SKN-1 directly under normal conditions, but may facilitate nuclear accumulation of SKN-1 in response to acute oxidative stress by possibly defending it against degradation through WDR-23 regulation.

Consistent with our observation, some studies demonstrated that reduced mTORC1 (mammalian target of rapamacin complex) and tunicamicin-induced ER stress also upregulated SKN-1 targets without robust accumulation of this transcription factor in the nucleus (Robida-Stubbs *et al*., 2012: Glover-Cutter *et al*., 2013). In addition, tunicamicin treatment also causes increase of intracellular abundance of SKN-1 protein (Glover-Cutter *et al*., 2013). Therefore, I investigated whether SAC- and SAMC-treatment could increase intracellular SKN-1 protein levels. To test this idea, total amount of SKN-1 protein in SAC- or SAMC-treated animals was assessed by western blotting using a polyclonal SKN-1 antibody, which was raised against SKN-1c isoform and should detect all of main SKN-1 isoforms (SKN-1a, 1b and 1c). This antibody recognized multiple bands, and four of these increased by *wdr-23* RNAi and decreased in the *skn-1(zu135)* mutant, suggesting that these four bands might correspond to each SKN-1 isoform (1a~ 1d) (Fig. 5G left). As shown in Fig. 5G (middle and right), SAC and SAMC slightly but reproducibly increased protein levels of SKN-1b (2.0~2.1-fold) and SKN-1d (2.0~2.1-fold) isoforms, respectively. SKN-1b is principally expressed in ASI neurons, a set of cells in head that sense food availability and influence metabolism, and is involved in dietary restriction induced longevity (An *et al*., 2003: Bishop *et al*., 2007). Even though the transcript of *skn-1d* has been

informed in WormBase, neither expression nor function of the smallest isoform has been published in detail. At this moment, the underlying mechanisms of the selective increase in these two SKN-1 isoforms by SAC and SAMC are still unclear. However, through a series of experiments addressing a mode of action of SAC and SAMC on SKN-1 activation, our findings implicated that SAC and SAMC could sustain SKN-1 protein levels, which is already present in the nucleus under normal conditions or entering into the nucleus in response to oxidative stress, by presumably suppressing the interaction of SKN-1 with WDR-23, or could also act on an unidentified cofactor that also regulates SKN-1 activity.

1.4.6. SAC and SAMC do not affect body size and reproduction, but enhance food intake of wild-type *C. elegans*

It has been revealed that reducing food intake (dietary restriction, DR) extends lifespan of a wide range of species, including *C. elegans* (Weindruch *et al*., 1986: Partridge *et al*., 1987: Jiang *et al*., 2000: Walker *et al*., 2005: Bioshop *et al*., 2007). In *C. elegans*, DR-induced extension of lifespan appears to require *skn-1b* particularly in the ASI neurons (An *et al*., 2003: Bioshop *et al*., 2007). Our findings also indicated that SAC and SAMC substantially increased levels of SKN-1b isoform (Fig. 5G). Therefore, I considered the possibility that SAC and SAMC might activate SKN-1 in the ASI neurons to produce DR-like state, leading to lifespan extension. Since diet-restricted animals also exhibit reduced brood size, extended reproductive period, and smaller body size (Bioshop *et al*., 2007), I examined the influence of SAC- and SAMC-treatments on reproductive capacity. The results showed that the animals treated with SAC or SAMC for 8 days exhibited a significant increase in progeny production on the 1st and 2nd day of reproductive period, although the total number of progeny was not statistically significant compared to untreated animals (Fig. 6A). Furthermore, neither SAC nor SAMC affected the reproductive period of *C. elegans* (Fig. 6A), suggesting that SAC and SAMC do not affect the reproductive capacity of *C.elegans*. I next examined whether SAC and SAMC could affect *C. elegans* body size. The result showed that wild-type animals raised in the presence of either SAC or SAMC for 8 days did not exhibited any differences in body length compared to untreated animals (Fig. 6B), suggesting that SAC and SAMC also seem to be unrelated with DR with respect to body length.

To assess whether SAC- or SAMC-treatment could cause reduced food intake, I examined the level of food (UV-killed *E. coli* OP50) consumption by measuring the optical density (OD) of wells containing equal numbers of animals (n=50) after 8 days of treatments with SAC or SAMC. The mean values of OD 620 nm of wells without *C. elegans* were comparable among treatments, suggesting that these compounds do not affect food concentration directly (Fig. 6C). On the other hand, SAC- and SAMC-treated animals showed a significant increase in food consumption compared to untreated control (Fig. 6C). This phenomenon became visually apparent after about 5 days of treatment. Taken all together, these results indicate that, at least for parameters investigated here, SAC and SAMC do not extend *C. elegans* lifespan by producing a DR-like state.

1.5. Discussion

SAC and SAMC are garlic-derived water-soluble thioallyl compounds and have been shown to have a variety of biological and physiological activities, including antioxidant activity. However, little is known about their molecular mechanisms. In this study, I used *C. elegans* as an *in vivo* model to study the effects of SAC and SAMC treatments on lifespan and resistance to oxidative- and heat-stress. Additionally, involvement of these compounds with key antioxidant pathways (DAF-16 and SKN-1) was also investigated.

I found that SAC and SAMC (1 ~ 100 μM) could significantly extend mean lifespan (7.5 ~ 15.6% for SAC, 5.8 ~ 20.9% for SAMC) of the nematode *C. elegans* (Figs. 1B and 1C, Table 2). Furthermore, a remarkable lifespan extension was also observed in yeast *Saccharomyces cerevisiae*, which has also been widely used as the model organism by treatment with 100 μM SAC (data not shown). Although effects of dose of these compounds cannot be simply comparable between *C. elegans* and higher organism, these concentrations of SAC and SAMC seem to be clinically practical because treatments with higher doses of SAC and SAMC than this study exhibit no toxicity in some animal studies (Sumiyoshi *et al*., 1990: Hatono *et al*., 1996: Sumioka *et al*., 1998). Because many biological processes have been conserved from yeast to mammals, these results suggest that SAC and SAMC would presumably have potentials to increase lifespan in higher species.

Even though SAC and SAMC extended mean lifespan of *C. elegans*, these compounds did not affect the maximum lifespan of wild-type and the *daf-16(mgDf47)* mutant (Table 2 and Table 3). These results would suggest that these compounds influence the death of younger but not older animals. One possible reason for the lack of effects of these compounds on the maximum lifespan is attributed to decreased stability and/or persistence of effects of these compounds because SAC and SAMC were only added to *C. elegans* on the first day of the lifespan experiments. Therefore, it is possible that additional treatments with fresh SAC and SAMC during middle or late period of the lifespan experiments may conceivably affect the maximum lifespan.

In addition to their lifespan extending effects, SAC and SAMC also protected nematodes from oxidative- and heat-stress-induced death (Figs. 2A and 2B) by suppressing production of intracellular ROS (Figs. 2C and 2D). These suggest that the increase in lifespan and stress resistance by SAC- or SAMC-treatment is associated with reduced ROS levels. Since SAC and SAMC have been shown to act as radical scavengers (Thomson *et al*., 2003), the increased lifespan and stress resistance by SAC and SAMC could be at least in part due to the direct antioxidant properties. On the other hand, because treatments with lower dose (10 μM) of SAC or SAMC could markedly enhance survival after challenging against high dose (250 μM) of oxidant, juglone, that causes death to most populations of the animals in few hours, I assumed that not only direct radical scavenging activities but also indirect activation of antioxidant defense systems may be involved in the increased resistance against oxidative stress. In addition to my findings, there is increasing evidence that SAC and SAMC modulate pathways involved in oxidative stress response (Hatono *et al*., 1996: Shi *et al*., 2014). Therefore, I investigated whether the SAC- and SAMC-mediated increase in stress resistance and longevity in *C. elegans* could be produced by activating pathways particularly associated with oxidative stress responses and longevity.

In *C. elegans*, the evolutionarily conserved DAF-16/FOXO transcription factor regulates many biological processes including stress resistance and longevity (Murphy *et al*., 2003: Lee *et al*., 2003). Therefore, I examined whether SAC and SAMC could have any effect on DAF-16 signaling. SAC and SAMC treatments did not induce DAF-16 target gene expressions (Figs. 3A and 3B) and DAF-16A::GFP nuclear localization (Fig. 3C), indicating that these compounds do not basically affect to

DAF-16 signaling. However, the percentage of lifespan extension of the *daf-16(mgDf47)* mutant by SAC and SAMC (12.8% for 10 μM SAC, 11.1% for 10 μM SAMC) (Table 3) were smaller than those obtained with wild-type strain (17.0% for 10 μM SAC, 19.7% for 10 μM SAMC) (Table 2). These results suggest that SAC and SAMC may conceivably have a mild impact on this signaling under long-term treatment.

I next investigated the effects of SAC and SAMC on SKN-1/Nrf signaling, which is another important regulatory mechanism involving with stress resistance and longevity of *C. elegans* (An *et al*., 2003: Inoue *et al*., 2005: Kahn *et al*., 2008: Tullet *et al*., 2008: Choe *et al*., 2009: Mizunuma *et al*., 2014). SAC and SAMC showed a remarkable induction of *gst-4* (Fig. 4A, 5B and 5C), one of the key SKN-1 target genes that is strongly activated in response to oxidative stresses (Kahn *et al*., 2008: Choe *et al*., 2009). Furthermore, the SAC- and SAMC-mediated increases of the *gst-4* induction and lifespan extension required *skn-1* (Fig. 4B, 4C and 4D, Table 4). In addition, this mutation shortened the mean lifespan in the presence of 10 or 100 μM SAC or SAMC (Figs. 4C and 4D, Table 4). This may indicate that SAC and SAMC have caused toxicity to the *skn-1(zu135)* mutant. Even in wild-type *C. elegans*, SAC and SAMC might partly act as mild stressors. On the other hand, the toxic effects of these compounds might be offset by activation of SKN-1, leading to induction of stress defense genes such as *gst-4* and eventually extension of mean lifespan. It can be also considered that SAC and SAMC may not function as radical scavenger at least in *C. elegans*, because if these compounds elicit their ability as the direct antioxidants, it should partly give longer lifespan even in the *skn-1(zu135)* mutant.

I next examined the effect of SAC and SAMC on the p38 MAPK pathway by investigating *gst-4* mRNA expression in the *sek-1(km4)* mutant, a gene encoding p38 MAPKK that function is essential for the p38 MAPK pathway. I found that these compounds also induced *gst-4* expression in this mutant as well as that of wild-type (Fig. 5D). This was surprising because induction of *gcs-1* is drastically inhibited in the *sek-1* (p38 MAPKK) and *pmk-1* (p38 MAPK) mutants (Inoue *et al*., 2005). On the other hand, it is also indicated that transcription of *gcs-1* is activated in the *sek-1(km4)* mutant when several genes (e.g. C48B6.2, *phi-43* or *wdr-23*) are knocked down by RNAi (Wang *et al*., 2010). In addition, another study also demonstrated that *wdr-23* RNAi robustly

induced *gst-4* transcription in the *sek-1(km4)* mutant (Choe *et al*., 2009).

The result obtained from the knock down of WDR-23 with RNAi (Fig. 5E) implicates the possibility that SAC and SAMC could inhibit WDR-23 function, thus leading to activation of SKN-1 and its downstream target induction. Furthermore, the result of western blot analysis to see whether SAC- and SAMC-treatment could increase intracellular SKN-1 protein levels indicated that SAC and SAMC slightly increased intracellular levels of some SKN-1 isoforms (Fig. 5G). The underlying mechanisms activating SKN-1 by SAC and SAMC are still unclear. However, our data possibly suggested that SAC and SAMC stabilize SKN-1, which is already present in the nucleus under normal conditions or entering into the nucleus in response to acute oxidative stress, by presumably suppressing the SKN-1/WDR-23 interaction, or could also act on an unidentified cofactor that also regulates SKN-1 activity, thus leading to selective activation of a set of target genes involved in oxidative stress defense.

In mammals, Kelch-like ECH-associated protein 1 (Keap1) directly binds to Nrf2, and targets it for polyubiquitination by a Cullin3-based E3 ligase complex, leading to its proteasomal degradation (Sykiotis *et al*., 2010). This inhibition of Nrf2 by Keap1 is abolished under oxidative stress conditions (Sykiotis *et al*., 2010). In addition, some electrophilic compounds have been identified to bind covalently to Keap1 through some cysteine residues (C151, C273, C288 and C434), and thus leading to release of active Nrf2 (Wakabayashi *et al*., 2004: Uruno *et al*., 2011). Therefore, Keap1 functions not only as inhibitor of Nrf2 but also as a sensor of oxidants and electrophiles. *C. elegans* lacks a Keap1 ortholog (Choe *et al*., 2012), and instead WDR-23 directly binds to SKN-1 and targets it for degradation (Choe *et al*., 2009). Similar to Keap1, WDR-23 also contains cysteines adjacent to basic amino acids, which is a characteristic of cysteine highly reactive to electrophiles (Choe *et al*., 2009). Therefore, direct binding of electrophiles to these cysteines of WDR-23 could inhibit the interaction of WDR-23 and SKN-1. Recent study demonstrated that treatment with aged garlic extract or SAC treatment protected primary cultured neurons and mice against oxidative damages through increase in Nrf2 protein levels and subsequent target expressions, such as GCLC, GCLM and HO-1 (Shi *et al*., 2014). In addition, treatment of human umbilical vein endothelial cells with aged garlic extract that contains SAC and SAMC induced the accumulation of Nrf2 into the nucleus followed by induction of HO-1 and GCLM

(Hiramatsu *et al*., 2015). These reports may suggest that SAC and SAMC modulate Nrf2 activity at protein level through the same mechanism as observed in *C. elegans*. Although I have obtained no direct evidence of SAC or SAMC to bind to WDR-23 protein, further approaches will help to unravel the mechanisms underlying SAC- and SAMC-mediated SKN-1 activation.

Including *C. elegans*, dietary restriction (DR) has been reported to extend lifespan of a wide range of species (Weindruch *et al*., 1986: Partridge *et al*., 1987: Jiang *et al*., 2000: Walker *et al*., 2005: Bioshop *et al*., 2007). In addition, DR-induced extension of lifespan of *C. elegans* appears to require *skn-1b* particularly in the ASI neurons (An *et al*., 2003: Bioshop *et al*., 2007). Because SAC and SAMC treatment produced slight increase in intracellular level of SKN-1b isoform (Fig. 5G), I examined the involvement of DR with SAC- and SAMC-mediated extension of mean lifespan. The results showed that SAC and SAMC have no effect on reproductive property (Fig. 6A) or body size (Fig. 6B) those are common parameters caused by DR in *C. elegans* (Bioshop *et al*., 2007). These results suggest that SAC and SAMC do not extend lifespan by producing a DR-mimic state. Interestingly, I found that SAC and SAMC treatments accelerated food consumption of *C. elegans* (Fig. 6C). While further analyses are needed to understand how SAC and SAMC accelerate food consumption, one likely explanation for this phenomenon is that SAC- and SAMC-mediated activation of SKN-1 could slow aging of *C. elegans*, and this health promoting effects of these compounds may lead to the elevated food consumption of this organism, despite enhanced food consumption itself produces more ROS in cells in general.

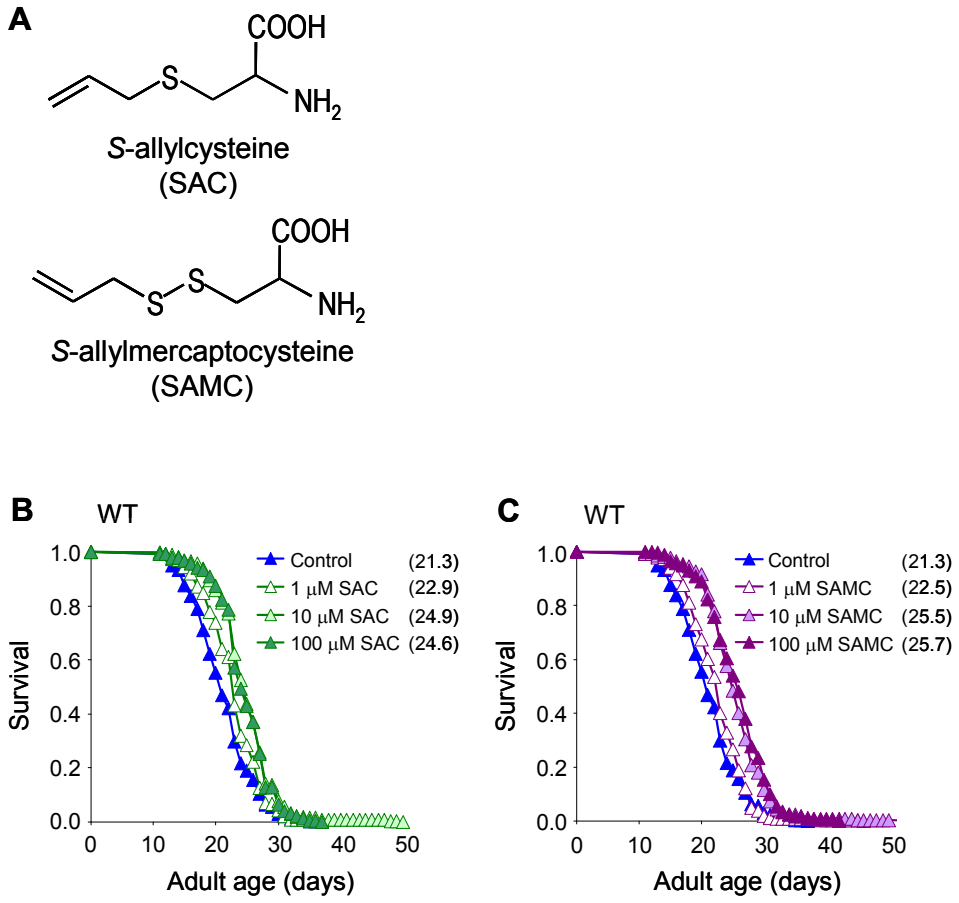

Figure 1. SAC and SAMC increase lifespan of wild-type *C. elegans*. (A) Chemical structures of SAC and SAMC. (B, C) Survival curves of wild-type adults treated with SAC (B) or SAMC (C) at 20ºC. Composites of four replicates are shown respectively, with mean and maximum lifespans indicated in parentheses. Statistics are provided in Table 2.

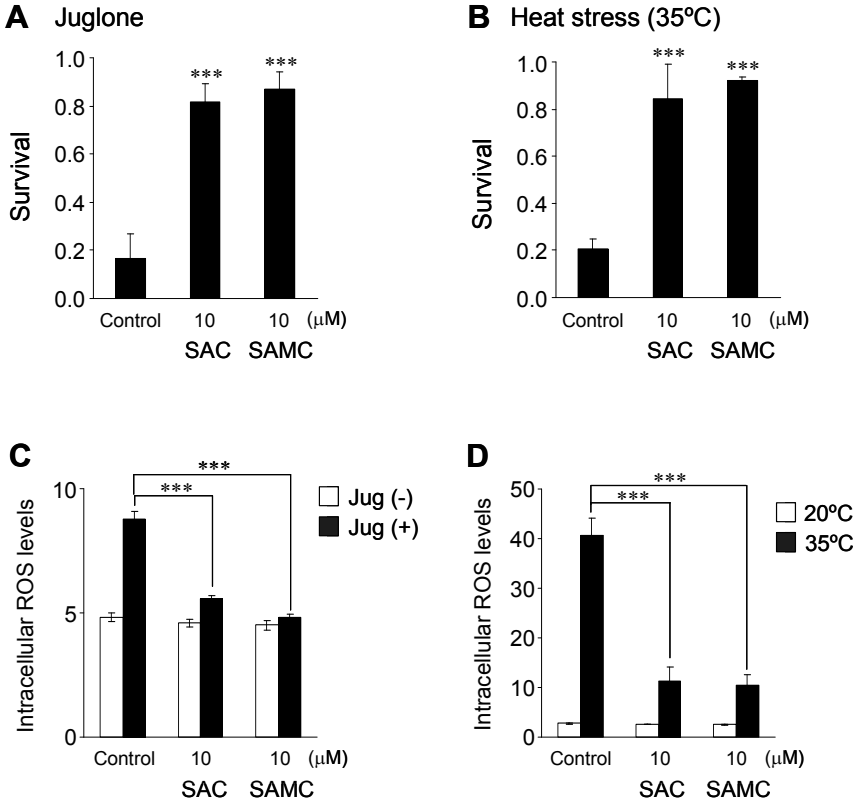

Figure 2. SAC and SAMC increase resistance to oxidative- or heat-stress and reduce intracellular ROS in wild-type *C. elegans*. (A-D) Synchronized day-1 wild-type adults were treated with H_2O (control), SAC or SAMC for 48 hours at 20ºC and then subjected to oxidative stress (250 μM juglone (Jug) for 2 hours at 20ºC) or heat stress (35ºC for 7 hours). (A, B) Survivals after each stress treatment were scored after a 16 hours recovery on NGM agar seeded with *E. coli* OP50. Data are represented as mean ± SD from three independent experiments. Total number of animals tested: for the oxidative stress assays (Control, n=218; SAC, n=211; SAMC, n=226) and for the heat stress assays (Control, n=208; SAC, n=226; SAMC, n=220). (C, D) Intracellular ROS accumulation in individual animal was measured by using CM-H_2DCFDA. The mean fluorescence intensity of at least 20 animals for each group with or without stress treatment is shown. Error bars represent SEM. ***P<0.001 (one-way ANOVA with Tukey's post hoc test).

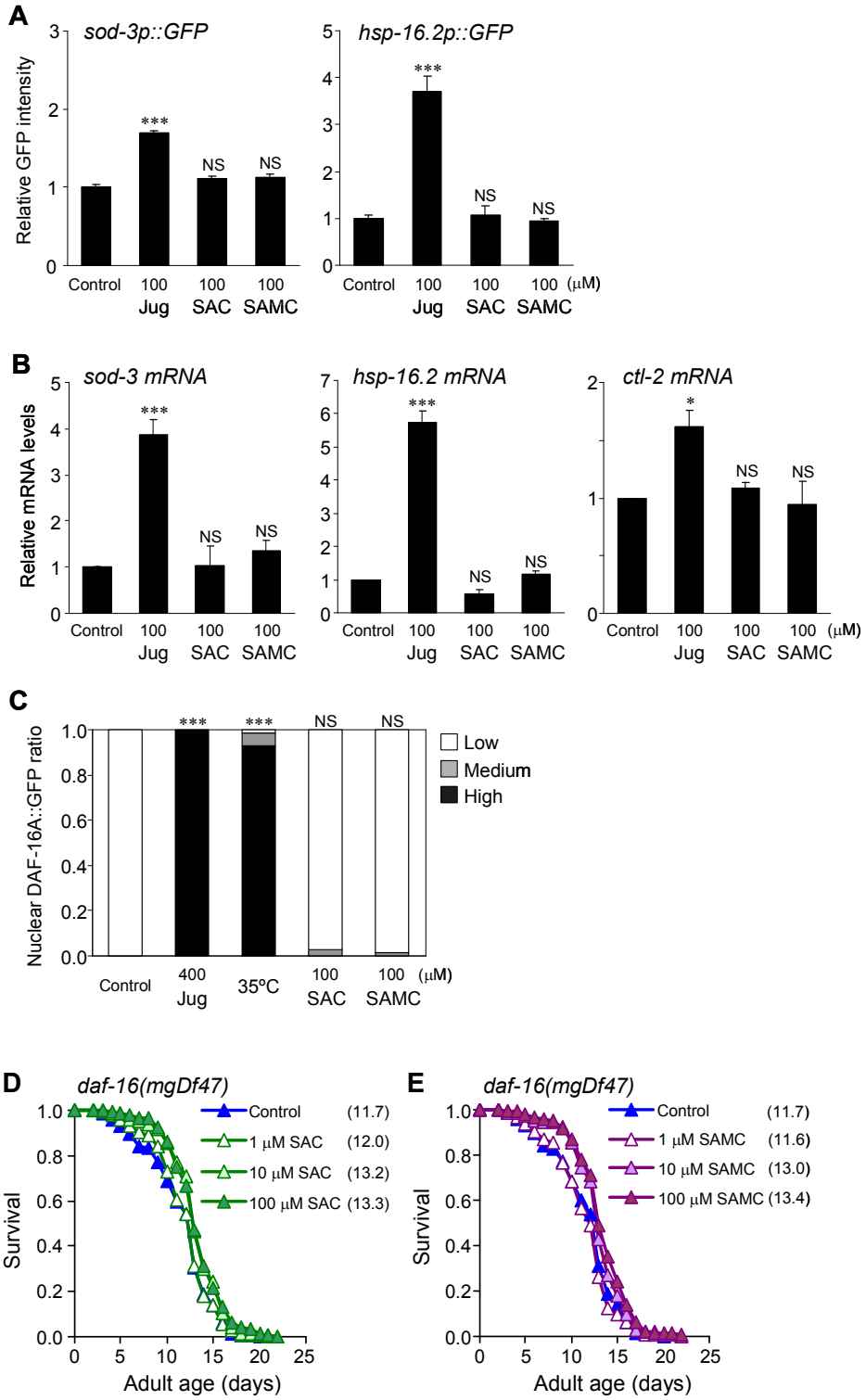

Figure 3. SAC and SAMC do not affect DAF-16 pathway. (A) Induction of the *sod-3p::GFP* or *hsp-16.2p::GFP* transgene in animals treated with juglone, SAC or SAMC for 24 hours. GFP intensity in pharynx was quantified by ImageJ. Data are represented as relative fluorescence intensity with SEM (n≥16 for each group). (B)

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Relative mRNA levels of *sod-3* (left), *hsp-16.2* (middle) and *ctl-2* (right) in day-1 wild-type adults treated with juglone, SAC or SAMC for 6 hours (n=3 of 50 animals) were determined by qRT-PCR. Data are represented as mean ± SEM from three independent experiments normalized to the levels in control. (C) Nuclear localization of DAF-16A::GFP in animals treated with H_2O (control; n=73), SAC (100 μM; n=66) or SAMC (100 μM; n=63) for 24 hour. Juglone (400 μM for 1 hour; n=63) or heat stress (35ºC for 1 hour; n=73) were used as positive controls. Nuclear localization of DAF-16A::GFP throughout whole body was classified into High, Medium or Low. ***P<0.001; NS: not significant (chi^2 test). (D, E) Survival curves of the *daf-16(mgDf47)* mutant treated with SAC (D) or SAMC (E) at 20ºC. Composites of three replicates are shown respectively, with mean lifespans indicated in parentheses. Statistics are provided in Table 3. *P<0.05; ***P<0.001; NS: not significant (one-way ANOVA with Tukey's post hoc test).

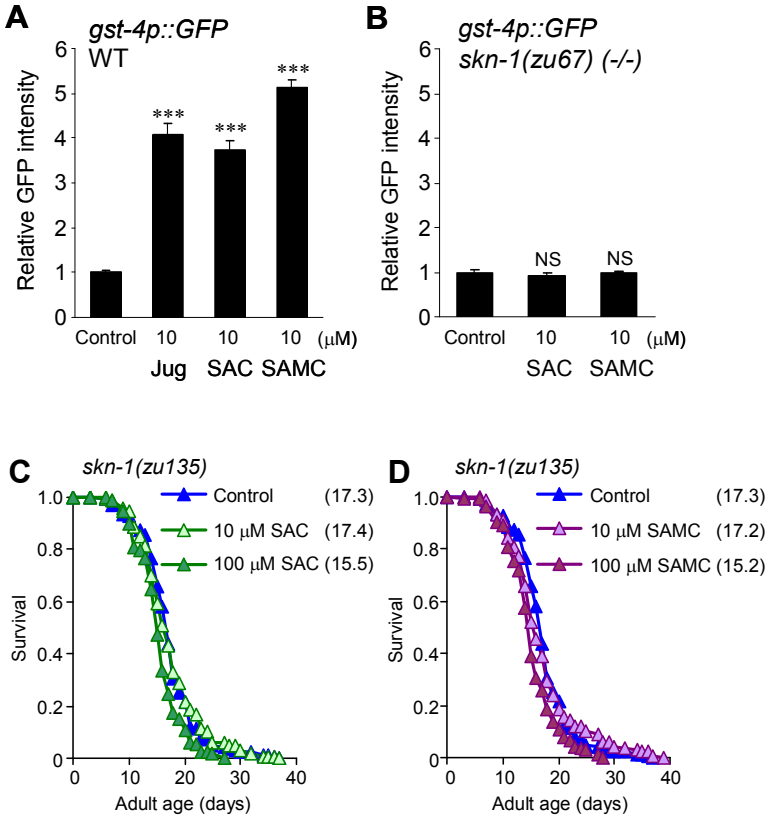

Figure 4. SAC and SAMC induce *gst-4p::GFP* transgene and prolong mean lifespans in a *skn-1* dependent manner. (A, B) Induction of *gst-4p::GFP* transgene in day-1 adults of the wild-type background (A) or the *skn-1(zu67)* mutant (B) treated with juglone, SAC or SAMC for 24 hours. GFP intensity throughout whole body was quantified by ImageJ. Data represent relative fluorescence intensity with SD (n≥20). (C, D) Survival curves of the *skn-1(zu135)* mutant treated with SAC (C) or SAMC (D) at 20ºC. Composites of three replicates are shown, with mean lifespans indicated in parentheses. Statistics are provided in Table 4. ***P<0.001; NS: not significant (one-way ANOVA with Tukey's post hoc test).

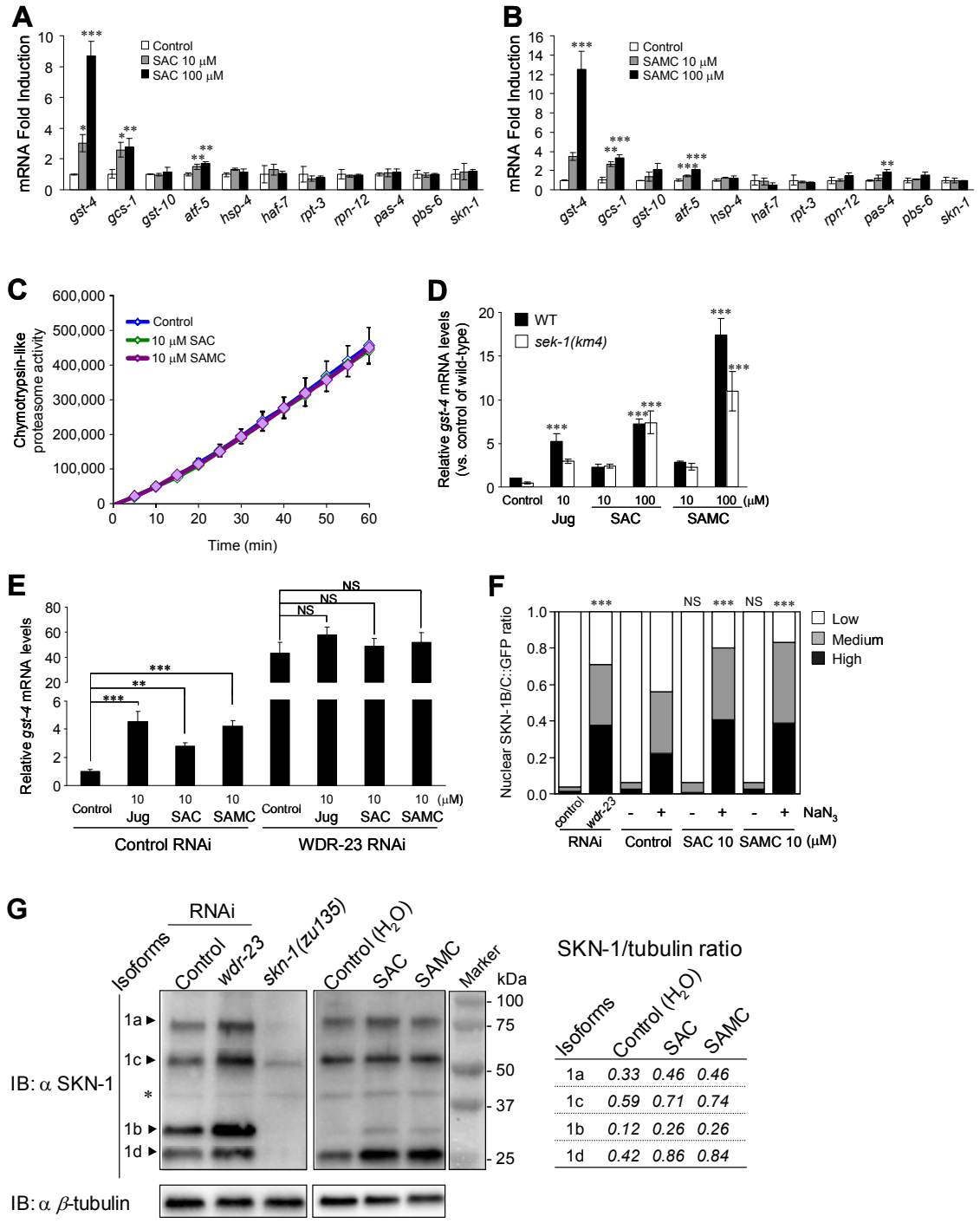

Figure 5. SAC and SAMC modulate SKN-1 pathway. (A, B) Relative mRNA levels of the indicated SKN-1 targets in day-1 wild-type adults treated with SAC (A) or SAMC (B) for 24 hours. Data represent mean ± SD (n=3 of 50 animals). (C) The 26S proteasome activity in whole lysate (25 μg/sample) prepared using about 1,000 wild-type animals treated on the L1 stage with H_2O, SAC or SAMC for 4 days at 20ºC.

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Data represent mean ± SD (n=3 of 1,000 animals). (D) Relative *gst-4* mRNA levels in day-1 adults of the *sek-1(km4)* mutant treated with juglone, SAC or SAMC for 24 hours. Data represent mean ± SD (n=3 of 50 animals). (E) Effect of *wdr-23* RNAi on endogenous *gst-4* mRNA levels in day-1 wild-type adults treated with juglone, SAC or SAMC for 24 hours. Data represent mean ± SD (n=3 of 50 animals). (F) Nuclear localization of SKN-1B/C::GFP in L4 animals pretreated with SAC or SAMC from the L4 stage of parental generation, followed by treatment with or without 2% NaN_3. *wdr-23* RNAi was used as a positive control. Nuclear localization of SKN-1B/C::GFP in intestine was classified into High, Medium or Low. ***P<0.001 (for *wdr-23* RNAi, n=106 vs. Control RNAi, n=102, for the NaN_3 treatment, SAC, n=142; SAMC, n=166 vs. Control, n=151), NS: not significant (without NaN_3, Control, n=130; SAC, n=131; SAMC, n=135) (chi^2 test). (G) Immunoblotting of endogenous SKN-1. (Left) Whole lysates (4.6 μg/lane) from 300 day-1 adults of the *rrf-3(pk1426)* mutant treated with either control or *wdr-23* RNAi from the L1 stage, or of the *skn-1(zu135)* homozygous mutant were analysed. (Middle) Whole lysates (15.0 μg/lane) from 1,000 L4 wild-type treated with SAC or SAMC from the L4 stage of parental generation were used. The blots detected with antibodies against SKN-1 (top) or β-tubulin (bottom). Predicted SKN-1 isoforms (1a ~ 1d) are indicated according to their estimated molecular weights informed in WormBase. (Right) Relative band intensity against β-tubulin (mean of two experiments). *: Non-specific band. α: antibody against. *P<0.05; **P<0.01; ***P<0.001; NS: not significant (one-way ANOVA with Tukey's post hoc test).

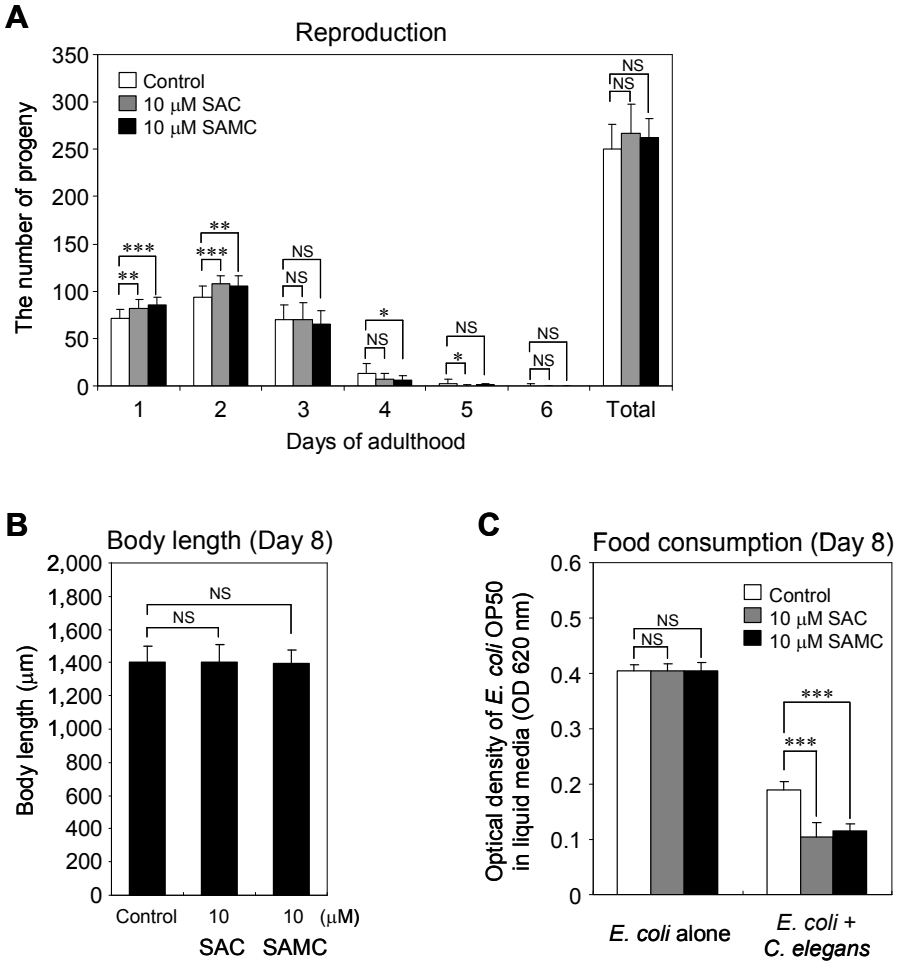

Figure 6. SAC and SAMC do not affect body size and reproduction, but enhance food intake of wild-type *C. elegans*. (A) For the reproduction assays, wild-type L4 animals were treated with H_2O (control; n=17), SAC (n=19) or SAMC (n=22) until reproduction period was ceased. Data represent the mean value of daily or total number of progeny from individual animals with SD. (B) The body length of animals treated with H_2O (control; n=85), SAC (n=87) or SAMC (n=91) for 8 days was measured by ImageJ. Data represent mean ± SD. (C) For the food consumption assays, after 8 days of treatment with H_2O (control), SAC, or SAMC, OD 620 nm of liquid medium containing total 50 animals was measured with a spectrophotometer. Data represent mean ± SD (n=4 of 50 animals). **P*<0.05; ***P*<0.01: ****P*<0.001; NS: not significant (one-way ANOVA with Tukey's post hoc test).

Table 1. Nematode strains used in this study.

Number	Genetic background	Transgene	Array number	Source	Referenced
N2	Wild-type			CGC	
CF1553	N2	Is[*sod-3p::GFP*]		CGC	Libina *et al*., 2003
CL2070	N2	Is[*hsp-16.2p::GFP*]		CGC	Link *et al*., 1999
CL2166	N2	Is[*gst-4p::GFP*]		CGC	Link *et al*., 2002
	daf-16(mgDf47)			Dr. Blackwell	Ogg *et al*., 1997
LD1482	*daf-16(mu86)*	Is[*DAF-16A::GFP*]		Dr. Blackwell	Lin *et al*., 2001
CL691	*skn-1(zu67)*	Is[*gst-4p::GFP*]		CGC	Rea *et al*., 2007
EU31	*skn-1(zu135)*			CGC	Bowerman *et al*., 1992
LD001	N2	Is[*SKN-1B/C::GFP*]	007	Dr. Blackwell	An *et al*., 2003
KU4	*sek-1(km4)*			Dr. Matsumoto	Tanaka-Hino *et al*., 2002
NL2099	*rrf-3(pk1426)*			Dr. Blackwell	Simmer *et al*., 2002

Some strains were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH office of Research Infrastructure Programs (P40 OD010440). KU4 was generous gift from Dr. Kunihiro Matsumoto (Nagoya university, Japan). *daf-16(mgDf47)*, LD1482, LD001 and NL2099 were generously provided from Dr. T. Keith Blackwell (Joslin Diabetes Center, Harvard Medical School).

Table 2. Lifespans of wild-type shown in Figs. 1B and 1C.

Trial	Treatment (μM)	No. animals	Censored	Mean lifespan [1]			Maximum lifespan [2]	
				days ± SEM	% extension	*P* value vs. control [3]	days ± SEM	*P* value vs. control [4]
1st	Control	81	3	20.9 ± 0.6	N/A	N/A	35	
	SAC (1)	85	4	22.3 ± 0.5	6.5%	0.171	32	
	SAC (10)	80	3	23.8 ± 0.6	13.8%	0.003	48	
	SAC (100)	70	9	23.8 ± 0.6	13.5%	0.001	37	
2nd	Control	106	4	21.6 ± 0.4	N/A	N/A	32	
	SAC (1)	100	2	23.1 ± 0.4	7.3%	0.080	37	
	SAC (10)	83	5	24.1 ± 0.4	11.6%	0.001	37	
	SAC (100)	92	11	23.7 ± 0.4	10.1%	0.007	32	
	SAMC (1)	94	3	22.8 ± 0.4	5.7%	0.136	32	
	SAMC (10)	94	11	25.1 ± 0.5	16.4%	<0.001	43	
	SAMC (100)	90	10	24.9 ± 0.5	15.3%	<0.001	42	
3rd	Control	54	0	21.9 ± 0.7	N/A	N/A	35	
	SAC (1)	61	2	23.6 ± 0.6	7.7%	0.196	31	
	SAC (10)	62	7	26.2 ± 0.7	20.0%	<0.001	50	
	SAC (100)	56	7	26.1 ± 0.5	19.3%	0.002	34	
	SAMC (1)	69	3	23.4 ± 0.6	7.1%	0.262	46	
	SAMC (10)	68	3	25.5 ± 0.8	16.6%	0.002	58	
	SAMC (100)	60	5	24.9 ± 0.6	13.9%	0.012	33	
4th	Control	53	0	20.7 ± 0.7	N/A	N/A	37	
	SAC (1)	49	2	22.5 ± 0.8	8.9%	0.159	35	
	SAC (10)	59	0	26.2 ± 0.6	26.7%	<0.001	33	
	SAC (100)	61	3	25.4 ± 0.7	23.2%	<0.001	35	
	SAMC (1)	50	2	21.5 ± 0.7	4.1%	0.521	30	
	SAMC (10)	58	4	26.1 ± 0.6	26.1%	<0.001	37	
	SAMC (100)	63	3	27.2 ± 0.6	31.6%	<0.001	37	
5th	Control	49	2	21.3 ± 0.6	N/A	N/A	32	
	SAMC (1)	59	1	21.9 ± 0.6	2.7%	0.683	31	
	SAMC (10)	54	5	25.6 ± 0.7	20.0%	<0.001	38	
	SAMC (100)	55	6	26.3 ± 0.7	23.5%	<0.001	38	
Combined	Control	343	9	21.3 ± 0.3	N/A	N/A	34.8 ± 1.0	N/A
(Trial 1~5)	SAC (1)	295	10	22.9 ± 0.3	7.5%	<0.001	33.8 ± 1.4	0.989
Fig. 1b and c	SAC (10)	284	15	24.9 ± 0.3	17.0%	<0.001	42.0 ± 4.1	0.171
	SAC (100)	279	30	24.6 ± 0.3	15.6%	<0.001	34.5 ± 1.0	0.999
	SAMC (1)	272	9	22.5 ± 0.3	5.8%	0.024	34.8 ± 3.8	1.000
	SAMC (10)	274	23	25.5 ± 0.3	19.7%	<0.001	44.0 ± 4.8	0.236
	SAMC (100)	268	24	25.7 ± 0.3	20.9%	<0.001	37.5 ± 1.8	0.971

1) Mean lifespan is the day when 50% of worms survived.

2) Maximum lifespan is the day when the last surviving worm died.

3) *P*-values for mean lifespan were obtained by log-rank test by comparing the control

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and other treated groups.

4) *P*-values for maximum lifespan were calculated by one-way ANOVA with Tukey's post hoc test from means of the maximum lifespan of each condition.

Table 3. Lifespans of the *daf-16(mgDf47)* mutant shown in Figs. 3D and 3E.

Trial	Treatment (μM)	No. animals	Censored	Mean lifespan [1]			Maximum lifespan [2]	
				days ± SEM	% extension	*P* value vs. control [3]	days ± SEM	*P* value vs. control [4]
1st	Control	41	2	12.3 ± 0.5	N/A	N/A	18	
	SAC (1)	41	2	12.8 ± 0.4	4.2%	0.873	18	
	SAC (10)	42	1	13.8 ± 0.3	12.2%	0.089	20	
	SAC (100)	41	0	13.9 ± 0.3	13.4%	0.048	20	
	SAMC (1)	42	3	12.4 ± 0.5	1.3%	0.719	20	
	SAMC (10)	41	3	12.8 ± 0.5	4.8%	0.537	22	
	SAMC (100)	42	3	13.2 ± 0.3	7.7%	0.368	18	
2nd	Control	80	0	11.7 ± 0.4	N/A	N/A	18	
	SAC (1)	79	0	12.2 ± 0.4	4.3%	0.551	21	
	SAC (10)	75	0	13.4 ± 0.4	14.2%	0.008	18	
	SAC (100)	80	4	12.7 ± 0.4	8.2%	0.148	19	
	SAMC (1)	85	0	11.4 ± 0.4	-3.1%	0.501	18	
	SAMC (10)	81	1	13.0 ± 0.3	10.6%	0.081	20	
	SAMC (100)	80	1	13.1 ± 0.3	11.6%	0.039	19	
3rd	Control	69	0	11.4 ± 0.4	N/A	N/A	20	
	SAC (1)	66	0	11.3 ± 0.4	-0.6%	0.607	18	
	SAC (10)	68	2	12.7 ± 0.4	11.2%	0.061	22	
	SAC (100)	70	3	13.7 ± 0.4	20.4%	<0.001	22	
	SAMC (1)	75	0	11.4 ± 0.4	-0.3%	0.578	22	
	SAMC (10)	86	0	13.1 ± 0.3	15.3%	0.004	21	
	SAMC (100)	80	3	13.8 ± 0.4	21.5%	<0.001	23	
Combined	Control	190	2	11.7 ± 0.3	N/A	N/A	18.7 ± 0.7	N/A
(Trial 1~3)	SAC (1)	186	2	12.0 ± 0.2	2.6%	0.756	19.0 ± 1.0	0.9941
Fig. 2d and e	SAC (10)	185	3	13.2 ± 0.2	12.8%	<0.001	20.0 ± 1.2	0.7538
	SAC (100)	191	7	13.3 ± 0.2	13.8%	<0.001	20.3 ± 0.9	0.6156
	SAMC (1)	202	3	11.6 ± 0.2	-1.2%	0.481	20.0 ± 1.2	0.8081
	SAMC (10)	208	4	13.0 ± 0.2	11.1%	0.002	21.0 ± 0.6	0.4470
	SAMC (100)	202	7	13.4 ± 0.2	14.5%	<0.001	20.0 ± 1.5	0.8081

1) Mean lifespan is the day when 50% of worms survived.

2) Maximum lifespan is the day when the last surviving worm died.

3) *P*-values for mean lifespan were obtained by log-rank test by comparing the control and other treated groups.

4) *P*-values for maximum lifespan were calculated by one-way ANOVA with Tukey's post hoc test from means of the maximum lifespan of each condition.

Table 4. Lifespans of the *skn-1(zu135)* mutant shown in Figs. 4C and 4D.

Trial	Treatment (μM)	No. animals	Censored	Mean lifespan [1]			Maximum lifespan [2]	
				days ± SEM	% extension	*P* value vs. control [3]	days ± SEM	*P* value vs. control [4]
1st	Control	32	0	17.3 ± 1.1	N/A	N/A	37	
	SAC (10)	32	0	18.0 ± 1.1	3.6%	0.779	34	
	SAC (100)	32	1	16.4 ± 0.8	-5.7%	0.405	27	
	SAMC (10)	32	0	18.5 ± 1.4	6.7%	0.469	39	
	SAMC (100)	32	2	15.8 ± 1.0	-9.1%	0.337	28	
2nd	Control	49	0	15.9 ± 0.7	N/A	N/A	28	
	SAC (10)	50	1	16.7 ± 0.8	5.0%	0.409	37	
	SAC (100)	53	1	14.5 ± 0.5	-9.1%	0.041	23	
	SAMC (10)	51	0	16.4 ± 0.9	2.6%	0.704	37	
	SAMC (100)	52	0	13.9 ± 0.6	-12.8%	0.020	28	
3rd	Control	59	0	18.5 ± 0.6	N/A	N/A	36	
	SAC (10)	53	4	17.8 ± 0.6	-3.9%	0.525	32	
	SAC (100)	57	6	15.9 ± 0.5	-13.8%	0.002	27	
	SAMC (10)	54	3	17.2 ± 0.6	-7.1%	0.137	34	
	SAMC (100)	56	1	16.1 ± 0.5	-12.7%	0.004	27	
Combined	Control	140	0	17.3 ± 0.4	N/A	N/A	33.7 ± 2.8	N/A
(Trial 1~3)	SAC (10)	135	5	17.4 ± 0.5	0.5%	0.853	34.3 ± 1.5	0.970
Fig. 3c and d	SAC (100)	142	8	15.5 ± 0.3	-10.8%	<0.001	25.7 ± 1.3	0.067
	SAMC (10)	137	3	17.2 ± 0.5	0.9%	0.855	36.7 ± 1.5	0.525
	SAMC (100)	140	3	15.2 ± 0.4	-12.3%	<0.001	27.7 ± 0.3	0.134

1) Mean lifespan is the day when 50% of worms survived.

2) Maximum lifespan is the day when the last surviving worm died.

3) *P*-values for mean lifespan were obtained by log-rank test by comparing the control and other treated groups.

4) *P*-values for maximum lifespan were calculated by one-way ANOVA with Tukey's post hoc test from means of the maximum lifespan of each condition.

Chapter II

Structure activity relationship study
with garlic-derived organosulfur compounds and their analogs

2.1. Abstract

In garlic and its aged extract, there are numerous organosulfur compounds (OSCs), such as *S*-alk(en)ylcysteines, *S*-alk(en)ylcysteine sulfoxides, *γ*-glutamyl-*S*-alk(en)ylcysteines, and allylsulfides, including SAC and SAMC. However, little is known whether these OSCs affect SKN-1 activity in *C. elegans* and whether their structures might be correlated with the activity. In this chapter, I examined the effect of 23 garlic-derived OSCs and their analogs on induction of *gst-4p::GFP* transgene. The results indicate that thioallyl structure and the number of disulfide bond are important factors for *gst-4p::GFP* induction. On the other hand, the oxidative or heat stress assay by using the *gst-4p::GFP* inducible compounds suggested that the number of disulfide bond does not correlate with stress resistance capacity of *C. elegans*.

2.2. Introduction

Other than SAC and SAMC, versatile OSCs, such as *S*-alk(en)ylcysteines, *S*-alk(en)ylcysteine sulfoxides, *γ*-glutamyl-*S*-alk(en)ylcysteines, and allylsulfides, have been identified from garlic (Liu *et al*., 2000). Some of those including SAC and SAMC have been shown to have diverse pharmacological properties, such as radical scavenging activity, chemopreventive activity, hepatoprotective activity, neurotropic activity, and lipid reducing activity (Sumiyoshi *et al*., 1990: Moriguchi *et al*., 1997: Lie *et al*., 2001: Hsu *et al*., 2006: Argüello-García *et al*., 2010). Moreover, a recent study reported that diallyl trisulfide (DATS), one of the oil-soluble allylsulfides from garlic, is able to induce *gst-4* gene expression under control of *skn-1* with extended longevity of *C. elegans* (Powolny *et al*., 2011).

The structure activity relationship analysis can be useful to determine the chemical groups responsible for a target biological activity among compounds tested. Some previous studies investigating relationships between structures of OSCs from garlic and chemopreventive- (Hatono *et al*., 1997), neurotropic- (Moriguchi *et al*., 1997), enzyme inhibitory- (Gupta *et al*., 2001: Camargo *et al*., 2007), and radical scavenging-activity (Argüello-García *et al*., 2010), indicated that the thioallyl group and the number of sulfur atom are important factors affecting their activities.

In this chapter, I examined the *gst-4p::GFP* inducible activity of 23

garlic-derived OSCs and their analogs to see of whether these OSCs affect SKN-1 activity in *C. elegans* and whether their structures might be correlated with the activity. As results, I found that i) the thioallyl structure is essential for the *gst-4p::GFP* induction; ii) an increasing number of sulfur atoms in sulfide bonds leads to enhanced activity; iii) the sulfur atom adjacent to the allyl group and iv) cysteine structure are also important factors influencing the activity. Interestingly, there was no positive correlation between the number of disulfide bonds and stress resistance capacity presumably due to their own toxicity on *C. elegans*.

2.3. Materials and Methods

2.3.1. *gst-4p::GFP* transgenic reporter assays

Synchronized day-1 adults of the transgenic strains carrying an inducible GFP reporter transgene for *gst-4* (CL2166) were raised in S-complete liquid medium as described in the strains and culture of *C. elegans* in chapter I (1.3.2.) and treated with 23 garlic-derived OSCs and their analogs shown in Fig. 7 (10 μM each) for 24 hours at 20ºC. GFP fluorescence images were collected with randomly selected animals as described in the measurement of intracellular ROS in chapter I (1.3.5.). GFP fluorescence from whole body was quantified by ImageJ.

2.3.2. Stress resistance assays

Synchronized day-1 wild-type adults were pretreated with H_2O, DMSO (as control for DADS and DATS), DADS, DATS, SAC or SAMC (10 μM each) for 48 hours at 20ºC. For the oxidative stress assays, the animals were washed with PBST three times before treating with a ROS generator, juglone (250 μM, Sigma-Aldrich, St. Louis, MO), for 2 hours at 20ºC. For the heat stress assays, the animals were incubated at 35ºC for 7 hours, and then washed with PBST three times. After a 16 hours recovery period on NGM agar, the survival was determined by touch-provoked movement. Animals were scored as dead when they failed to respond to touching with a platinum wire pick.

2.4. Results

The thioallyl structure and disulfide bond in garlic-derived OSCs are important for SKN-1 activation

Here, I considered the question of whether other OSCs in garlic might activate

SKN-1/Nrf as well as SAC and SAMC, and whether their structures might be correlated with this activity. To address this possibility, I tested the effect of 23 garlic-derived OSCs and their analogs (Fig. 7) on induction of *gst-4p::GFP* transgene. As shown in Table 5, of 23 compounds tested, 5 compounds (SAC; 4.9-fold (P<0.001), SAMC; 8.1-fold (P<0.001), DADS (diallyldisulfide); 3.4-fold (P<0.001), DATS; 9.1-fold (P<0.001), GSAMC (γ-glutamyl-*S*-allylmercaptocysteine); 3.0-fold (P<0.001)) produced a significant increase in *gst-4p::GFP* expression compared to untreated control. Importantly, all these compounds commonly have the thioallyl structure. Moreover, there was a positive correlation between the number of disulfide bonds and *gst-4p::GFP* induction levels as in the case of SAC < SAMC, and DAS < DADS < DATS.

Among compounds containing the allyl structure, alliin (*S*-allylcysteine sulfoxide), in which the sulfur atom of SAC forms sulfoxide group, and OAS (*O*-allylserine), in which the sulfur atom of SAC is substituted by oxygen, exhibited no *gst-4p::GFP* induction. In addition, GSAC (γ-glutamyl-*S*-allylcysteine) and GSAMC, in which glutamic acid is attached to α-amino group of cysteine, exhibited weaker *gst-4p::GFP* inducible activity than SAC and SAMC, respectively. Similarly, SAHC (*S*-allylhomocysteine) and SAMHC (*S*-allylmercaptohomocysteine) had no significant effect on the activity.

The finding that the increasing number of disulfide bonds correlates with the *gst-4p::GFP* inducible activity raises the question of whether the number of disulfide bonds in these compounds might also correlate with their protective effect. To address this, we performed the oxidative-stress resistance assay using SAC, SAMC, DADS and DATS (10 μM each), and found significantly higher survivals after treatment with DADS (41.9 ± 3.1%; P<0.001 by oneway-ANOVA with Tukey's post hoc test) and DATS (41.4 ± 2.6%; P<0.001) compared to DMSO control (16.0 ± 3.4%) (Figure 8A). However, the DADS- and DATS-produced higher survivals were significantly lower than that of SAC (83.2 ± 6.2%; P<0.001 vs. DADS and DATS) or SAMC (90.2 ± 4.0%; P<0.001 vs. DADS and DATS) (Figure 8A). Similar results were obtained in the heat stress assays (Figure 8B), indicating that there is no positive correlation between the number of disulfide bonds and stress resistance capacity.

Taken together, I found out the following structurally important factors that

affect *gst-4p::GFP* inducible activity; i) the thioallyl structure is essential; ii) an increasing number of sulfur atoms in sulfide bonds leads to enhanced activity; iii) the sulfur atom adjacent to the allyl group is important; and iv) cysteine structure is also important factors influencing the activity. Given that SAC and SAMC possibly stabilize SKN-1 by suppressing the interaction between SKN-1 and WDR-23 through binding to reactive cysteines in either of these proteins, it would be interesting to see whether these activity-related factors are closely linked to this event.

2.5. Discussion

In this chapter, to address whether OSCs derived from garlic or aged garlic extract affect SKN-1 activity in *C. elegans* and whether their structures are correlated with the activity, I tested the effect of 23 garlic-derived OSCs and their analogs on induction of *gst-4p::GFP* transgene. The result indicated that, among compounds tested, only thioallyl compounds such as SAC, SAMC, DADS and DATS produced significant increases in expression of *gst-4p::GFP* transgene (Table 5). I also found that number of sulfur atoms (or disulfide bond) positively correlate with the *gst-4p::GFP* inducible activity (Table 5).

When we consider the underlying mechanisms of the *gst-4* induction by SAC and SAMC in *C. elegans*, we should take into account of following points; (i) how each compound can be ingested into cells of *C. elegans*, (ii) whether these compounds are metabolized in cells, (iii) what is the stability of the ingested compound and/or its metabolite. To address these considerations, more simple *in vitro* experiments using cell cultures and oral administration study using laboratory animal are thought to be required. Interestingly, consistent with our findings, some previous studies using garlic-derived OSCs also reported the importance of the thioallyl structure and/or the number of sulfur atoms in sulfide bonds on diverse biological activities. For example, the study investigating chemopreventive activity of *S*-alk(en)ylcysteines and these disulfide derivatives indicated that thioallyl compounds, including SAC, were the most effective for colon cancer prevention (Hatono *et al*., 1997). Other study investigating neurotropic activity of *S*-alk(en)ylcysteines, *S*-alk(en)ylcysteine sulfoxides, γ-glutamyl-*S*-alk(en)ylcysteine, and their analogs also indicated that only thioallyl compounds, such as SAC, SAMC, DAS, DADS, alliin, and GSAC, were effective on

the survival of cultured rat hippocampal neurons (Moriguchi *et al*., 1997). The study of radical scavenging capacity of some OSCs also revealed that thioallyl structure and the number of the sulfur atoms contribute to the activity (Argüello-García *et al*., 2010). Although, direct target(s) of these thioallyl compounds and their underlying mechanisms are still unclear, the notable consistency of observations derived from these and our studies suggests that the thioallyl compounds in garlic play important roles in diverse biological processes including the SKN-1/Nrf pathway.

As shown in Fig. 8, the *gst-4p::GFP* inducible compounds (SAC, SAMC, DADS and DATS) produced significant increase in survival fraction after both oxidative and heat stress treatment. However, the increase in survivals by DADS and DATS were significantly lower than those obtained by SAC and SAMC (Fig. 8). Because DATS treatment at higher concentration (100 μM) caused death of adult animals within 24 hours, this toxicity by DATS might lead to the lower survivals in the oxidative or heat stress resistance assays in spite of its highest *gst-4p::GFP* inducible activity. On the other hand, treatment with SAC and SAMC at 100 μM still caused the increased mean lifespan (Figs. 1B and C) and higher survivals (88.2 ± 10.4% for SAC, 91.1 ± 9.3% for SAMC (N=3 experiments using more than 50 animals each)) in the oxidative stress assays. These results implicate that SAC and SAMC can be treated at higher concentrations with less toxicity, thus leading to superior protective effect compared to DATS. These features of SAC and SAMC suggest that these compounds may have potentials for application to SKN-1/Nrf activators.

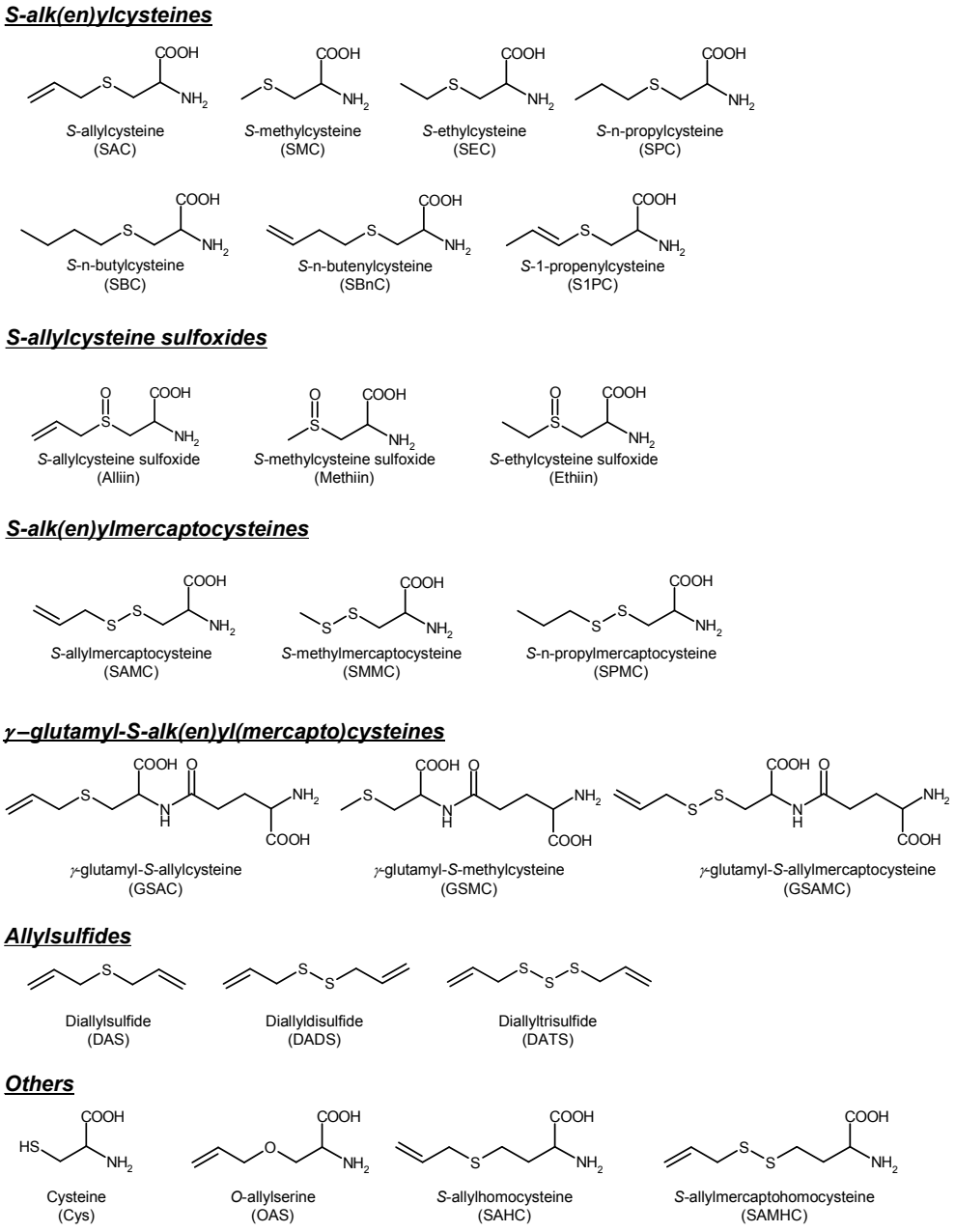

Figure 7. Garlic-derived organosulfur compounds and their analogs used in the structure-activity relationship study. Chemical structure of total 23 compounds indicated in Table 5 were categorized into "*S*-alk(en)ylcysteines", "*S*-alk(en)ylcysteine sulfoxides", "*S*-alk(en)ylmercaptocysteines", "γ-glutamyl-*S*-alk(en)yl(mercapto)cysteines", "Allylsulfides", and "Others" on the basis of their structures.

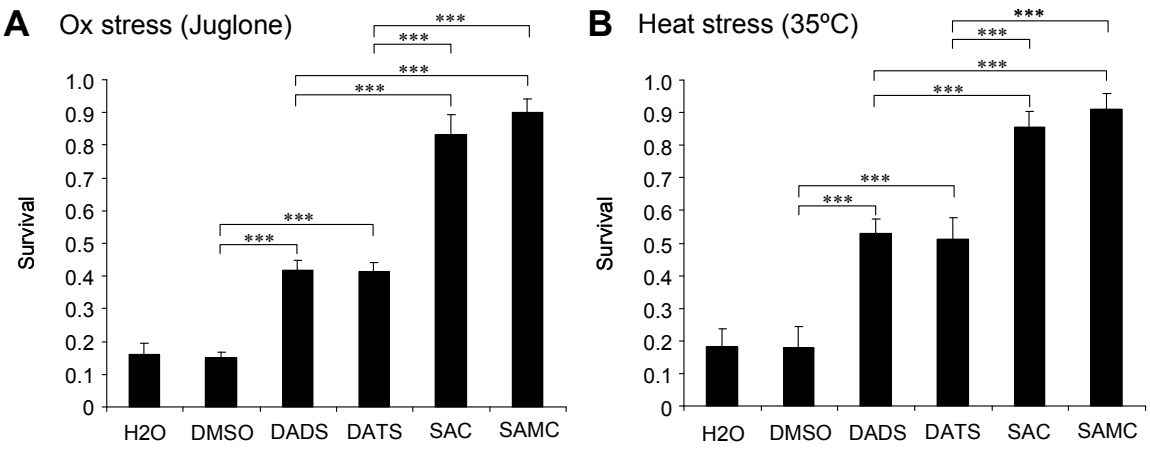

Figure 8. Effects of OSCs on oxidative or heat stress resistance. Synchronized day-1 wild-type adults were treated with H_2O (control for SAC and SAMC), 0.02 % DMSO (control for DADS and DATS), DADS, DATS, SAC or SAMC for 48 hours at 20ºC and then subjected to oxidative stress (250 μM juglone for 2 hours at 20ºC) or heat stress (35ºC for 7 hours). Each compound was treated at 10 μM. Survivals after the oxidative stress treatment (A) or the heat stress treatment (B) were scored after a 16 hours recovery on NGM agar seeded with *E. coli* OP50. Data are represented as mean ± SD from three independent experiments. Total number of animals tested: for the oxidative stress assays (H_2O, n=185; DMSO, n=199; DADS, n=198; DATS, n=200; SAC, n=217; SAMC, n=242) and for the heat stress assays (H_2O, n=173; DMSO, n=166; DADS, n=194; DATS, n=211; SAC, n=231; SAMC, n=224). ***P<0.001 (one-way ANOVA with Tukey's post hoc test).

Table 5. Relative *gst-4p::GFP* inducible activity of garlic-derived organosulfur compounds and their analogs.

Compounds*	*gst-4p::GFP* levels ± SEM (n) †	Compounds*	*gst-4p::GFP* levels ± SEM (n) †
***S*-alk(en)ylcysteines**		***γ*-glutamyl-*S*-alk(en)yl(mercapto)cysteines**	
S-allylcysteine (SAC)	4.9 ± 0.2 (16)‡	*γ*-glutamyl-*S*-allylcysteine (GSAC)	1.7 ± 0.1 (22)
S-methylcysteine (SMC)	1.0 ± 0.1 (16)	*γ*-glutamyl-*S*-methylcysteine (GSMC)	1.3 ± 0.1 (13)
S-ethylcysteine (SEC)	1.1 ± 0.1 (10)	*γ*-glutamyl-*S*-allylmercaptocysteine (GSAMC)	3.0 ± 0.1 (21)‡
S-n-propylcysteine (SPC)	1.3 ± 0.0 (16)	***Allylsulfides***	
S-n-butylcysteine (SBC)	1.0 ± 0.1 (10)	Diallylsulfide (DAS)	1.2 ± 0.1 (17)
S-n-butenylcysteine (SBnC)	1.1 ± 0.1 (13)	Diallyldisulfide (DADS)	3.4 ± 0.2 (16)‡
S-1-propenylcysteine (S1PC)	1.1 ± 0.1 (15)	Diallyltrisulfide (DATS)	9.1 ± 0.4 (16)‡
***S*-alk(en)ylcysteine sulfoxides**		***Others***	
S-allylcysteine sulfoxide (Alliin)	1.1 ± 0.1 (13)	Cysteine (Cys)	1.1 ± 0.1 (11)
S-methylcysteine sulfoxide (Methiin)	1.0 ± 0.1 (10)	*O*-allylserine (OAS)	1.1 ± 0.1 (15)
S-ethylcysteine sulfoxide (Ethiin)	1.1 ± 0.1 (14)	*S*-allylhomocysteine (SAHC)	1.1 ± 0.1 (19)
***S*-alk(en)ylmercaptocysteines**		*S*-allylmercaptohomocysteine (SAMHC)	1.5 ± 0.1 (18)
S-allylmercaptocysteine (SAMC)	8.1 ± 0.4 (15)‡		
S-methylmercaptocysteine (SMMC)	1.3 ± 0.1 (12)		
S-n-propylmercaptocysteine (SPMC)	1.4 ± 0.1 (18)		

* Treated at 10 μM each for 24 hours at 20°C.

† Relative fluorescence intensity with SEM. The number of animals tested in parentheses.

‡ $P<0.001$ by one-way ANOVA with Tukey's post hoc test.

Concluding remarks

Garlic (*Allium sativum* L) has been widely used as food and folk medicine for more than a thousand years. A number of studies have indicated that garlic possesses diverse health benefits, such as antimicrobial, anticancer, antithrombotic, antihyperlipidemic, hepatoprotective and antioxidant activity. In addition to the studies focused on garlic, many researchers have also investigated pharmacological potentials of its constituents as represented by sulfur-containing compounds. *S*-allylcysteine (SAC) and *S*-allylmercaptocysteine (SAMC) are the major water-soluble thioallyl compounds naturally occurring during aging process of garlic and have been indicated to have similar biological properties to garlic. Although the health beneficial effects of SAC and SAMC have been demonstrated, at least in part, to be due to their strong antioxidant abilities, little is well understood how they confer antioxidant and other pharmacological effects *in vivo*. In this study, to clarify the mode of action of SAC and SAMC related to organismal aging and stress resistance capacities *in vivo*, I used the model organism *C. elegans* that has been widely used to study effects of diverse compounds on lifespan and their underlying molecular mechanisms.

In Chapter I, I first examined effects of SAC and SAMC on lifespan and oxidative stress resistance of wild-type *C. elegans* under normal conditions. The results indicated that SAC and SAMC could extend mean lifespan of *C. elegans* and also protect the nematode from acute and severe oxidative- and heat-stress with reduced intracellular ROS. I next assessed whether SAC and SAMC affect the DAF-16/FOXO and SKN-1/Nrf pathways to fulfill lifespan extension and oxidative stress resistance of *C. elegans*. The results suggested that SAC- and SAMC-induced extension of lifespan would be independent of the DAF-16/FOXO pathway. On the other hand, these compounds may stabilize SKN-1 protein that is already present in the nucleus or entering into the nucleus in response to acute oxidative stress by presumably suppressing the interaction between SKN-1 and WDR-23. Alternatively, SAC and SAMC could also act on an unidentified cofactor that also regulates SKN-1 activity. This activation of SKN-1 might lead to selective activation of a set of target genes involved in oxidative stress defense. Finally, I also tested whether SAC and SAMC could mimic a dietary restriction (DR) status, which has been confirmed to link to longevity of various species including *C. elegans*. The results suggested that SAC and

SAMC do not extend *C. elegans* lifespan by mimicking a DR-like state.

In Chapter II, I used the structure activity relationship analysis to examine the *gst-4p::GFP* inducible activity using 23 garlic-derived OSCs and their analogs to see of whether those compounds affect SKN-1 activity in *C. elegans* and whether their structures might be correlated with the activity. I found out the following rules that affect *gst-4p::GFP* induction; i) the thioallyl structure is a critical factor for the *gst-4p::GFP* induction; ii) an increasing number of disulfide bonds positively correlate with enhanced activity; iii) the sulfur atom adjacent to the allyl group negates the inducible activity and iv) cysteine structure are also important factors influencing the activity. It is noteworthy that there was no positive correlation between the number of disulfide bonds and oxidative- or heat-stress resistance capacity, presumably due to their own toxicity on *C. elegans*.

Taken together, I demonstrated that two thioallyl compounds, SAC and SAMC, could enhance antioxidant activity of *C. elegans* through enhancing stability of SKN-1 transcriptional factor, thus leading to lifespan extension of this organism. In addition, I also clarified that among thiol-containing compounds in garlic, SAC and SAMC could specifically induce *gst-4* gene expression, a target of SKN-1 responsible for oxidative stress defense. This study will provide the possibility of applications of thioallyl compounds to the development of nutraceutical products and drugs targeting Nrf signaling.

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公表論文

(1) Natural thioallyl compounds increase oxidative stress resistance and lifespan in *Caenorhabditis elegans* by modulating SKN-1/Nrf

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Natural thioallyl compounds increase oxidative stress resistance and lifespan in *Caenorhabditis elegans* by modulating SKN-1/Nrf

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Identification of biologically active natural compounds that promote health and longevity, and understanding how they act, will provide insights into aging and metabolism, and strategies for developing agents that prevent chronic disease. The garlic-derived thioallyl compounds *S*-allylcysteine (SAC) and *S*-allylmercaptocysteine (SAMC) have been shown to have multiple biological activities. Here we show that SAC and SAMC increase lifespan and stress resistance in *Caenorhabditis elegans* and reduce accumulation of reactive oxygen species (ROS). These compounds do not appear to activate DAF-16 (FOXO orthologue) or mimic dietary restriction (DR) effects, but selectively induce SKN-1 (Nrf1/2/3 orthologue) targets involved in oxidative stress defense. Interestingly, their treatments do not facilitate SKN-1 nuclear accumulation, but slightly increased intracellular SKN-1 levels. Our data also indicate that thioallyl structure and the number of sulfur atoms are important for SKN-1 target induction. Our results indicate that SAC and SAMC may serve as potential agents that slow aging.

The human body is constantly exposed to reactive oxygen species (ROS), which are generated by aerobic respiration in the mitochondria and as byproducts of diverse metabolic reactions in cells. Overproduction of ROS causes damage to cellular proteins, lipids and DNA, eventually contributing to various chronic diseases including cancer, diabetes, Parkinson's and Alzheimer's disease, cardiovascular disease and chronic inflammation[1]. Therefore, cumulative oxidative damage to the cells may also influence aging. It is known that antioxidant vitamins C and E existing in a wide variety of foods act cooperatively to protect cells from lipid peroxidation by directly neutralizing harmful hydroxyl radicals[2]. Additionaly, sulforaphane, a natural dietary isothiocyanate produced in cruciferous vegetables such as broccoli and broccoli sprouts, has been shown to induce phase II detoxification genes, e.g. Heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase, γ-glutamylcysteine synthetase and glutathione *S*-transferases (GSTs), through activating Nrf2 (NF-E2-related factor) signaling[3]. The induction of these enzymes by sulforaphane protects cells from damage associated to oxidative stress in diverse *in vivo* and *in vitro* experimental conditions[3]. Therefore, intake of these natural compounds through diet could help to prevent pathogenesis of chronic diseases and contribute to slow aging, or in other words extend health span of organisms.

Garlic (*Allium sativum* L.) has been widely used as a food and folk medicine since ancient times. A number of studies have indicated that garlic possesses diverse pharmacological potentials related to chronic diseases, such as anticancer[4], antithrombotic[5], hypolipidemic[6] and hepatoprotective activity[7]. Many of these beneficial effects have been shown to be attributed to garlic characteristic organosulfur compounds (OSC), including *S*-allylcysteine (SAC) and *S*-allylmercaptocysteine (SAMC)[8–13]. SAC and SAMC are the major water-soluble OSCs naturally occurring during aging process of garlic, and known to act as free radical scavengers[14]. Therefore, some of these protective effects of SAC and SAMC could potentially be explained by their radical scavenging activity. While some studies have demonstrated that SAC and SAMC inhibited growth of human cancer cells *in vitro*[12,15], and development of chemically induced cancers or growth of implanted tumors *in vivo* along with increasing levels

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of GSTs[8,16]. GSTs play a key role in the phase II detoxification response, which provides a conserved defense against oxidative stress[17]. More recent study demonstrated that SAC treatment protected primary cultured neurons and mice against oxidative insults and middle cerebral artery occlusion-induced ischemic damages, respectively, through increases in the levels of Nrf2 protein and target genes expressions, such as γ-glutamylcysteine synthetase catalytic subunit (GCLC), γ-glutamylcysteine synthetase modulatory subunit (GCLM) and HO-1[18]. Because development of cancer, oxidative stress response and apoptosis are strongly associated with aging, we considered the question of whether SAC and SAMC can retard aging. However, the ability of SAC and SAMC to modulate organismal aging and the potential mechanisms involved have not been reported.

Since the finding in *Caenorhabditis elegans* that reduction in signaling through the conserved insulin/IGF-I signaling (IIS) pathway results in more than double the mean lifespan compared with wild-type[19], aging has become a particularly active area of research. Further studies have identified genes and molecular mechanisms involved in stress responses and longevity. For example, the lifespan extension caused by reduced IIS requires the activity of DAF-16, the FOXO (Forkhead box O) orthologue, which induces entry into larval diapause but also promotes longevity in adults[19]. When IIS is reduced under conditions where dauer-associated processes are inactive in adults, lifespan extension also requires SKN-1, the Nrf1/2/3 orthologue[20], which increases resistance to various stresses[21]. In addition, reduced IIS causes each of these proteins to accumulate in nuclei, leading to upregulation of target genes involved in longevity, stress responses, metabolism, and the extracellular matrix[20,22–24]. In *C. elegans*, SKN-1 is required for lifespan to be extended by a variety of different interventions[20,24–29]. Under oxidative stress conditions, PMK-1, a p38 mitogen-activated protein kinase (MAPK), phosphorylates SKN-1, leading to its nuclear accumulation and target gene expression[30]. In addition to the longevity modulating effect of SKN-1, recent studies have also demonstrated its critical roles in protein homeostasis under conditions of reduced translation or proteasome activity[31,32] or increased endoplasmic reticulum (ER) stress[33]. SKN-1 then selectively induces distinct but partly overlapping set of its downstream target genes under these diverse conditions.

In this study, we have investigated how SAC and SAMC affect lifespan and oxidative stress resistance of *C. elegans*. In addition, we examined their effects on pathways regulated by the DAF-16/FOXO and SKN-1/Nrf transcription factors. We also tested whether SAC and SAMC could mimic a dietary restriction (DR)-like environment, which is strongly linked to longevity of various species including *C. elegans*. Finally, we investigated effect of various OSCs from garlic and their analogs on induction of a *gst-4p::GFP* transgene, an indicator of SKN-1 activity.

Results and Discussion

SAC and SAMC extend *C. elegans* lifespan under normal conditions. We first evaluated whether SAC and SAMC (Fig. 1a) influence the lifespan of wild-type *C. elegans* under normal conditions. To eliminate the possibilities that these compounds could affect growth of *E. coli* OP50, and vice versa live bacteria could metabolize these compounds, we used UV-killed *E. coli* OP50 in the lifespan and the following assays. In our lifespan assays, SAC- and SAMC-treatment were begun on the first day of adulthood with concentrations at 1, 10, and 100 μM at 20 °C. As a result, SAC produced significant increase in the mean lifespan of adult animals (7.5% for 1 μM ($P < 0.001$), 17.0% for 10 μM ($P < 0.001$) and 15.6% for 100 μM ($P < 0.001$), Fig. 1b, Supplementary Table S1). Similarly, SAMC-treatment also significantly increased the mean lifespan (5.8% for 1 μM ($P < 0.05$), 19.7% for 10 μM ($P < 0.001$) and 20.9% for 100 μM ($P < 0.001$), Fig. 1c, Supplementary Table S1). On the other hand, SAC and SAMC did not affect the maximum lifespan of wild-type *C. elegans* (Supplementary Table S1). These results would suggest that these compounds influence the death of younger but not older animals. One possible reason for the lack of effects of these compounds on the maximum lifespan is attributed to decreased stability and/or persistence of effects of these compounds because SAC and SAMC were only added to *C. elegans* on the first day of the lifespan experiments. Therefore, it is possible that additional treatments with fresh SAC and SAMC during middle or late period of the lifespan experiments might affect the maximum lifespan. Given that the significant extension of the mean lifespan of wild-type *C. elegans* was achieved at 10 and 100 μM of each compound, we performed the following experiments at these concentrations.

SAC and SAMC enhance stress resistance and reduce ROS levels under oxidative- and heat-stress conditions. In *C.elegans*, increased lifespan is sometimes associated with improved survival under conditions of oxidative or heat stress[34,35]. To investigate whether SAC and SAMC could enhance resistance to stress, we pretreated wild-type adults with 10 μM of SAC or SAMC for 2 days at 20 °C, followed by exposure to oxidative (juglone, an intracellular ROS generator) or heat stress (35 °C). Both SAC- and SAMC-pretreatment increased survival after juglone exposure (Fig. 1d) and heat stress (Fig. 1e) at significantly higher ratio than untreated control. These results indicate that both compounds exert protective roles against oxidative and heat stress in *C. elegans*. Because both juglone treatment and heat shock cause cellular damage by accumulation of ROS, we next investigated whether SAC and SAMC could lower the intracellular ROS level under stress conditions by using CM-H_2DCFDA, a fluorescent probe that reacts with ROS. The results showed that pretreatment with SAC or SAMC significantly suppresses oxidative or heat stress-induced accumulation of ROS compared to untreated control (Fig. 1f,g), suggesting that the increase in lifespan and stress resistance by SAC- or SAMC-treatment is associated with reduced ROS levels. Since SAC and SAMC have been shown to act as radical scavengers[14], the increased lifespan and stress resistance by SAC and SAMC could be at least in part due to the direct antioxidant properties. On the other hand, there is increasing evidence that SAC and SAMC modulate pathways involved in oxidative stress response[8,16,18]. Therefore, we investigated whether the SAC- and SAMC-mediated increase in stress resistance and longevity in *C. elegans* could be produced by activating pathways particularly associated with oxidative stress responses and longevity.

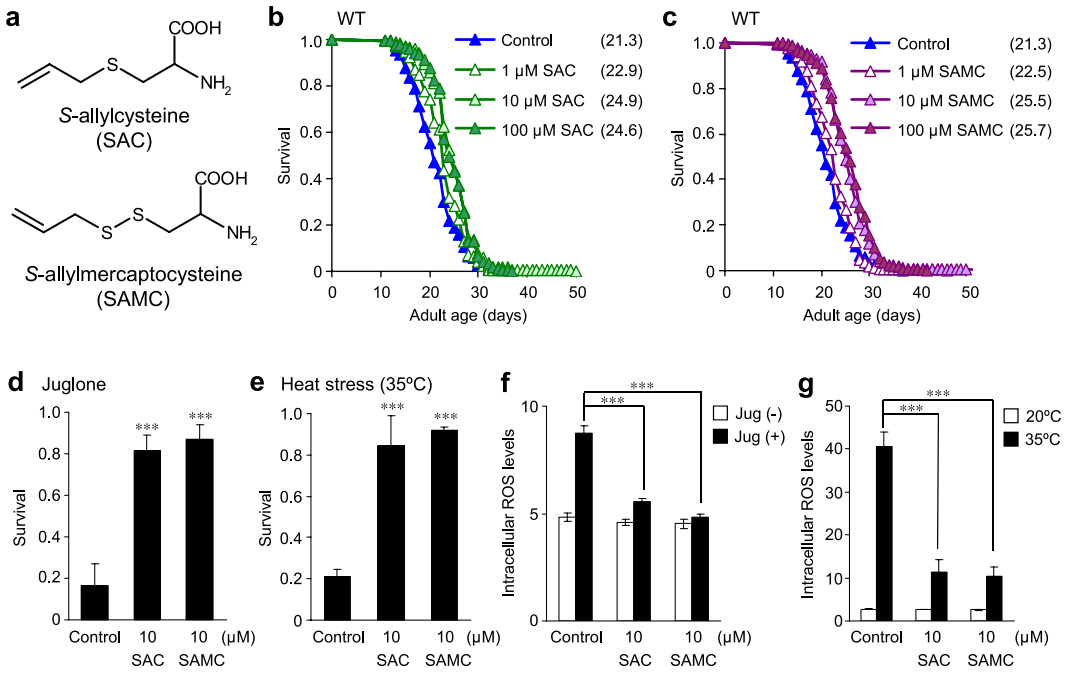


Figure 1. SAC and SAMC increase lifespan and resistance to oxidative- or heat-stress of wild-type *C. elegans*. (**a**) Chemical structures of SAC and SAMC. (**b,c**) Survival curves of wild-type adults treated with SAC (**b**) or SAMC (**c**) at 20 °C. Composites of four replicates are shown respectively, with mean lifespans indicated in parentheses. Statistics are provided in Supplementary Table S1. (**d–g**) Synchronized day-1 wild-type adults were treated with H_2O (control), SAC or SAMC for 48 hours at 20 °C and then subjected to oxidative stress (250 μM juglone (Jug) for 2 hours at 20 °C) or heat stress (35 °C for 7 hours). (**d,e**) Survivals after each stress treatment were scored after a 16 hours recovery on NGM agar seeded with *E. coli* OP50. Data are represented as mean ± SD from three independent experiments. Total number of animals tested: for the oxidative stress assays (Control, n = 218; SAC, n = 211; SAMC, n = 226) and for the heat stress assays (Control, n = 208; SAC, n = 226; SAMC, n = 220). (**f,g**) Intracellular ROS accumulation in individual animal was measured by using CM-H_2DCFDA. The mean fluorescence intensity of at least 20 animals for each group with or without stress treatment is shown. Error bars represent SEM. ***$P < 0.001$ (one-way ANOVA with Tukey's post hoc test).

SAC and SAMC do not affect DAF-16/FOXO activity. In *C. elegans*, the evolutionarily conserved DAF-16/FOXO transcription factor regulates many biological processes including stress resistance and longevity[22,23]. Therefore, we examined whether SAC and SAMC could have any effect on DAF-16 signaling. We first monitored expression of transgenes in which promoter for DAF-16 target genes *sod-3* (superoxide dismutase) or *hsp-16.2* (small heat shock protein) is fused to green fluorescent protein (GFP), respectively. As shown in Fig. 2a, juglone (positive control) upregulated the expression of both *sod-3p::GFP* and *hsp-16.2p::GFP* transcriptional reporters, whereas no induction of these reporters was observed by SAC- and SAMC-treatment (100 μM each for 24 hours). We also examined expression of endogenous *sod-3*, *hsp-16.2*, and *ctl-2* (catalase) mRNAs by quantitative RT-PCR (qRT-PCR), and found that neither of these genes was activated by these compounds (100 μM each for 24 hours) (Fig. 2b).

To further investigate the effect of SAC and SAMC on DAF-16 signaling, we examined whether SAC and SAMC could promote accumulation of a DAF-16A::GFP translational fusion protein in the nucleus. Like other transcription factors, nuclear localization of DAF-16 is associated with its transcription-activating activity. Exposure to juglone and heat stress resulted in remarkable nuclear localization of DAF-16A::GFP, whereas no nuclear localization of DAF-16A::GFP was observed in animals treated with SAC or SAMC (100 μM each for 24 hours) (Fig. 2c). To further elucidate the involvement of DAF-16 signaling in the effects of SAC and SAMC on nematodes, we performed the lifespan assays using the *daf-16(mgDf47)* mutant. We found that treatments with SAC and SAMC at 10 and 100 μM appeared to prolong survival of the *daf-16(mgDf47)* mutant in early stage of adult life (Fig. 2d,e, Supplementary Table S2), and when we combined three independent assays, significant extension of the mean lifespan was observed in treatments with 10 and 100 μM of these compounds, although this lifespan extension was reduced compared to wild-type (Supplementary Table S2). In addition, SAC and SAMC did not extend the maximum lifespan of this mutant presumably due to the same reason as observed in wild-type (Supplementary Table S2). Taken together, our results show that SAC- and SAMC-mediated increase in lifespan and stress resistance appears to be in part independent of DAF-16 signaling.

SAC and SAMC promote longevity by modulating SKN-1. In *C. elegans*, the transcription factor SKN-1/Nrf plays a critical role in promoting oxidative stress resistance and longevity by upregulating numerous

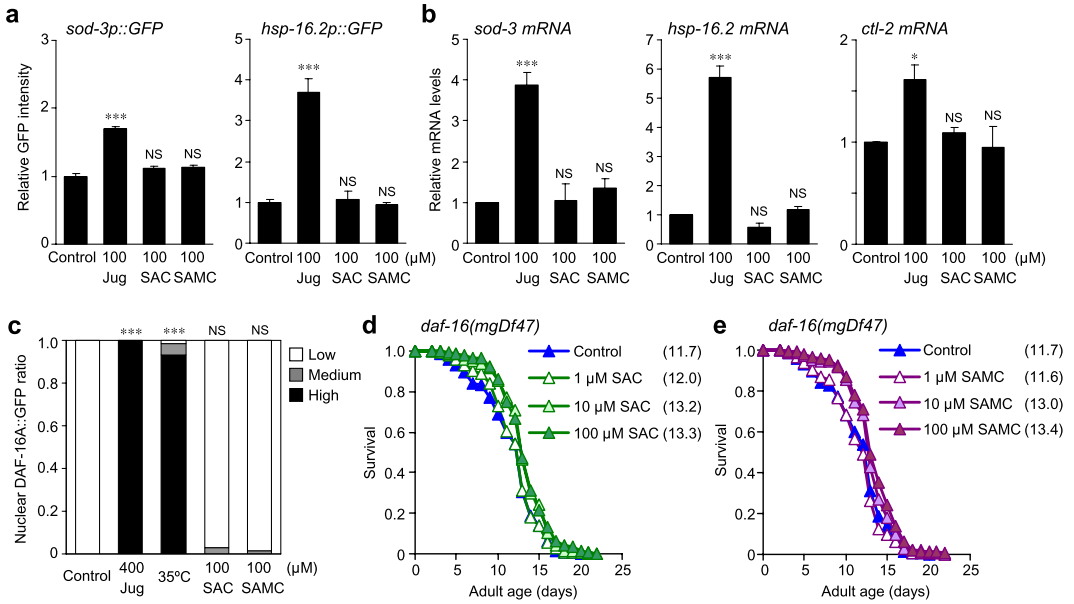


Figure 2. SAC and SAMC do not affect DAF-16 pathway. (**a**) Induction of the *sod-3p::GFP* or *hsp-16.2p::GFP* transgene in animals treated with juglone, SAC or SAMC for 24 hours. GFP intensity in pharynx was quantified by ImageJ. Data are represented as relative fluorescence intensity with SEM ($n \geq 16$ for each group). (**b**) Relative mRNA levels of *sod-3* (left), *hsp-16.2* (middle) and *ctl-2* (right) in day-1 wild-type adults treated with juglone, SAC or SAMC for 6 hours ($n = 3$ of 50 animals) were determined by qRT-PCR. Data are represented as mean ± SEM from three independent experiments normalized to the levels in control. (**c**) Nuclear localization of DAF-16A::GFP in animals treated with H_2O (control; $n = 73$), SAC (100 μM; $n = 66$) or SAMC (100 μM; $n = 63$) for 24 hour. Juglone (400 μM for 1 hour; $n = 63$) or heat stress (35 °C for 1 hour; $n = 73$) were used as positive controls. Nuclear localization of DAF-16A::GFP throughout whole body was classified into High, Medium or Low. $^{***}P < 0.001$; NS: not significant (chi^2 test). (**d,e**) Survival curves of the *daf-16(mgDf47)* mutant treated with SAC (**d**) or SAMC (**e**) at 20 °C. Composites of three replicates are shown respectively, with mean lifespans indicated in parentheses. Statistics are provided in Supplementary Table S2. $^{*}P < 0.05$; $^{***}P < 0.001$; NS: not significant (one-way ANOVA with Tukey's post hoc test).

genes, including phase II detoxification enzymes[24,26,30,36,37]. To investigate whether SAC- and SAMC-treatment could modulate SKN-1 activity, we first examined the effect of SAC and SAMC on expression of *gst-4* (glutathione *S*-transferase) gene, one of the key phase II enzyme genes that is strongly activated in response to oxidative stress[36,37]. We treated the transgenic animals, which contains a *gst-4p::GFP* transcriptional reporter transgene, with juglone (positive control), SAC and SAMC. All these treatments resulted in a dramatic increase in GFP expression compared to untreated control (Fig. 3a). To confirm whether the *gst-4p::GFP* induction by SAC- and SAMC-treatment could require SKN-1, we treated the *skn-1*(*zu67*) mutant, which carries the *gst-4p::GFP* transgene, with SAC and SAMC. We found that no induction of *gst-4p::GFP* was observed by these compounds in this mutant (Fig. 3b), indicating that the induction of *gst-4p::GFP* by SAC and SAMC is completely dependent upon SKN-1.

It has been demonstrated that *skn-1* loss-of-function mutants have shortened lifespans, and in contrast, that increased expression or activity of SKN-1 increases *C. elegans* lifespan[24]. We next examined whether SAC- and SAMC-mediated extension of lifespan requires SKN-1. We treated the *skn-1*(*zu135*) mutant with SAC and SAMC, and found that both compounds failed to increase the mean lifespan of this mutant compared to untreated control (Fig. 3c,d, Supplementary Table S3). Instead, this mutation shortened the mean lifespan in the presence of 100 μM SAC or SAMC (Fig. 3c,d, Supplementary Table S3). This may indicate that SAC and SAMC have caused toxicity to the *skn-1(zu135)* mutant. Even in wild-type *C. elegans*, SAC and SAMC might partly act as mild stressors. On the other hand, the toxic effects of these compounds might be offset by activation of SKN-1, leading to induction of stress defense genes such as *gst-4* and eventually extension of mean lifespan. Together, these results suggest that *skn-1* is required for the SAC- and SAMC-mediated lifespan extension.

In *C. elegans*, SKN-1 is activated in response to diverse interventions, such as oxidative- and ER-stress, and reduced translation and proteasome activity, leading to partially overlapping but distinct set of target gene expression[26,27,31–33]. To investigate how SAC- and SAMC-treatment could affect expression of SKN-1 target genes, we examined mRNA levels of some SKN-1 targets related to response against oxidative- or ER-stress, and reduced translation elongation or proteasome activity. SAC- and SAMC-treatment on wild-type animals significantly induced some oxidative stress defense genes, *gst-4* and *gcs-1* (γ-glutamylcysteine synthase heavy chain[26,30]), except *gst-10*[27] (Fig. 3e,f). Additionally, the *skn-1*-dependent ER and oxidative stress-related transcription factor *atf-5* (a mammalian bZIP transcription factors ATF4[27,33]) was also induced by these compounds (Fig. 3e,f). On the other hand, SAC and SAMC did not increase transcription of *hsp-4* (heat shock protein) and *haf-7* (an

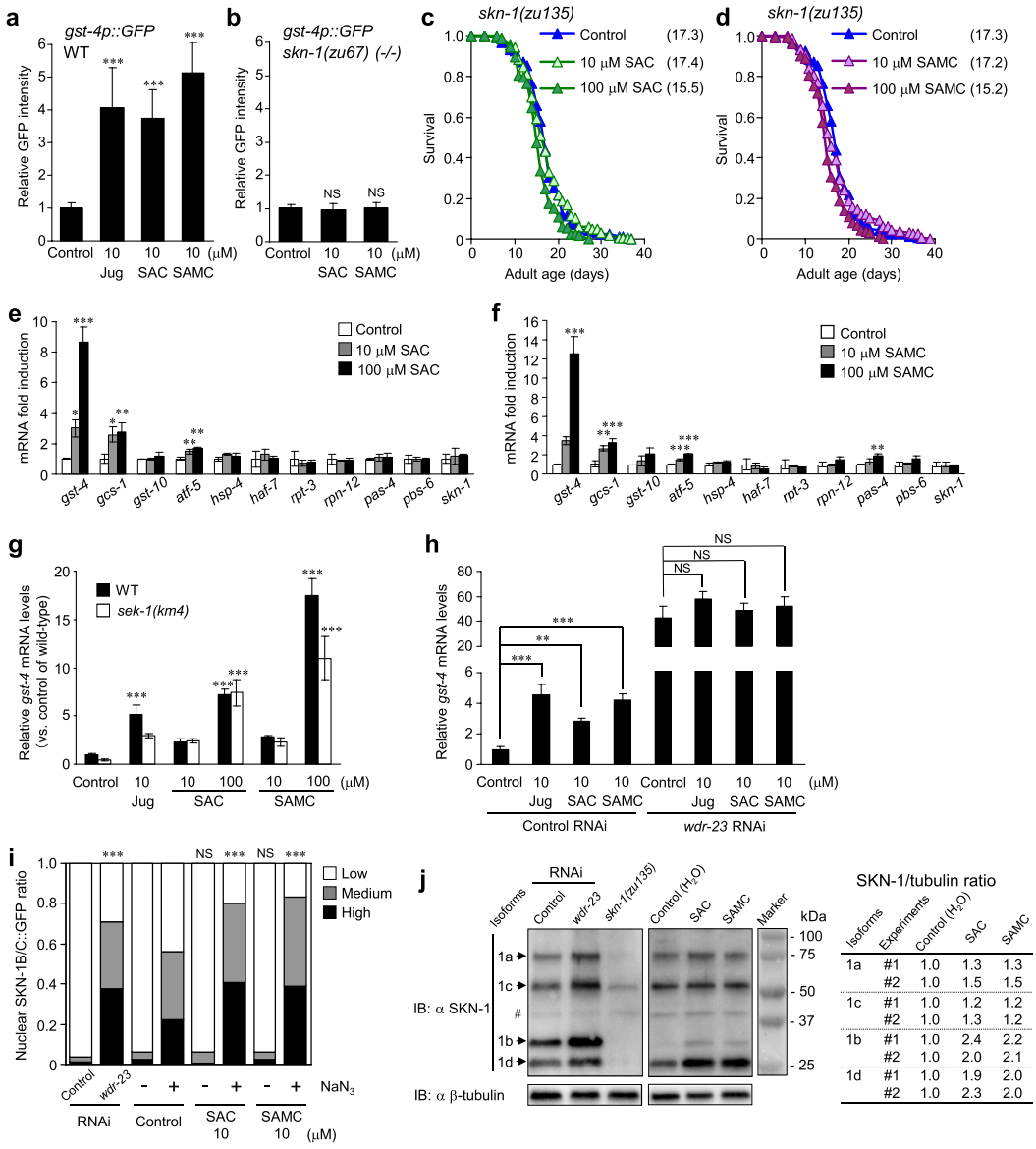


Figure 3. SAC and SAMC modulate SKN-1 pathway. (**a**,**b**) Induction of *gst-4p::GFP* transgene in day-1 adults of the wild-type background (**a**) or the *skn-1(zu67)* mutant (**b**) treated with juglone, SAC or SAMC (24 h). Data represent relative fluorescence intensity throughout whole body with SD ($n \geq 20$). (**c**,**d**) Lifespan of the *skn-1(zu135)* mutant treated with SAC (**c**) or SAMC (**d**) at 20 °C. Composites of three replicates with mean lifespans in parentheses. Statistics are provided in Supplementary Table S3. (**e**,**f**) Relative mRNA levels of the indicated SKN-1 targets in day-1 wild-type adults treated with SAC (**e**) or SAMC (**f**) (24 h). (**g**) Relative *gst-4* mRNA levels in day-1 adults of the *sek-1(km4)* mutant treated with juglone, SAC or SAMC (24 h). (**h**) Effect of *wdr-23* RNAi on endogenous *gst-4* mRNA levels in day-1 wild-type adults treated with juglone, SAC or SAMC (24 h). (**e**–**h**) Data represent mean ± SD ($n = 3$ of 50 animals). (**i**) Nuclear localization of SKN-1B/C::GFP in L4 animals pretreated with SAC or SAMC from the L4 stage of parental generation, followed by treatment with or without NaN_3. *wdr-23* RNAi was used as a positive control. SKN-1B/C::GFP in intestinal nuclei was classified into High, Medium or Low. $^{***}P < 0.001$ (for *wdr-23* RNAi, $n = 106$ vs. Control RNAi, $n = 102$, for the NaN_3 treatment, SAC, $n = 142$; SAMC, $n = 166$ vs. Control, $n = 151$), NS: not significant (without NaN_3, Control, $n = 130$; SAC, $n = 131$; SAMC, $n = 135$) (chi^2 test). (**j**) Immunoblotting of endogenous SKN-1. (Left) Whole lysates (4.6 μg/lane) from 300 day-1 adults of the *rrf-3(pk1426)* mutant treated with control or *wdr-23* RNAi, or of the *skn-1(zu135)* homozygous mutant. (Middle) Whole lysates (15.0 μg/lane) from 1,000 L4 wild-type treated with SAC or SAMC from the L4 stage of parental generation. The blots detected with antibodies against SKN-1 (top) or β-tubulin (bottom). Predicted SKN-1 isoforms (1a–1d) are indicated according to their estimated molecular weights reported in WormBase. (Right) Relative band intensity against β-tubulin of two experiments normalized to the levels in control of each isoform. The blot are data of experiment-1. #: Non-specific band. α: antibody against. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS: not significant (one-way ANOVA with Tukey's post hoc test).

ortholog of human ATP-binding cassette B9, ABCB9) (Fig. 3e,f), which are induced by SKN-1 in response to ER stress and reduced translation, respectively[27,31–33].

Knockdown of some proteasome subunit genes by RNAi induces *skn-1*-dependent expression of endogenous *gst-4* and *gst-10*[32]. Additionally, the amyloid-binding dye Thioflavin T (ThT) has been shown to extend *C. elegans* lifespan dependent upon *skn-1* and also *hsf-1* (heat shock factor 1), which promotes protein homeostasis[38]. ThT also suppresses aggregation of Amyloid-β (3-42) peptide and polyglutamine, which are associated with Alzheimer's disease and several neurological conditions, respectively, in *C. elegans* models[38]. One possibility is that undesirable accumulation of aggregated or misfolded proteins in cells might activate SKN-1 to induce its targets associated with protein homeostasis. In contrast, our data indicated that SAC and SAMC did not substantially affect mRNA levels of various components of the proteasomal complex; *rpt-3* (an ATPase subunit of the 19S proteasome), *rpn-12* (a non-ATPase subunit of the 19S proteasome), *pas-4* (an alpha-rings subunit of the 20S proteasome), and *pbs-6* (a beta-rings subunit of the 20S proteasome)[32] (Fig. 3e,f). We further examined the effect of SAC and SAMC on the 26S proteasome activity and found that these compounds had no effect on its activity (Supplementary Fig. S1), suggesting that these compounds appear to activate SKN-1 through a mechanism uncoupled from protein homeostasis. Taken together, these results suggest that SAC and SAMC may act primarily on oxidative stress response genes regulated by SKN-1, and that this may confer the increased lifespan and stress resistance associated with SAC and SAMC treatment.

We also tested the possibility of whether SAC and SAMC could induce expression of *skn-1* itself, thus leading to induction of its target genes. Results showed that these compounds had no effect on *skn-1* mRNA expression (Fig. 3e,f). Therefore, we next examined whether SAC and SAMC could modulate SKN-1 activity at the protein level. Under oxidative stress conditions, SKN-1 is activated by p38 MAPK pathway signaling[30]. p38 MAPK directly phosphorylates specific sites within SKN-1, which then accumulates in the nucleus and activates oxidative stress defense genes such as *gcs-1*[26,30]. Downstream of or in parallel to this regulation, WDR-23 (WD40 repeat protein) physically interacts with SKN-1 and CUL-4/DDB-1 ubiquitin ligase complex in the nucleus, which presumably ubiquitinylates SKN-1 protein and targets it for proteasomal degradation[37]. To elucidate how SAC and SAMC modulate SKN-1 activity, we first examined the effect of these compounds on the p38 MAPK pathway. We treated the *sek-1(km4)* mutant, a gene encoding p38 MAPKK that function is essential for the p38 MAPK pathway, with SAC and SAMC and examined the effect of these compounds on *gst-4* mRNA expression. As a result, these compounds also activated transcription of *gst-4* in this mutant as well as that of wild-type (Fig. 3g), suggesting that the SAC- and SAMC-mediated activation of *gst-4* transcription, which requires *skn-1* (Fig. 3b), might be independent of the p38 MAPK pathway. This was surprising because induction of *gcs-1* is drastically inhibited in the *sek-1* (p38 MAPKK) and *pmk-1* (p38 MAPK) mutants[30]. On the other hand, it is also indicated that transcription of *gcs-1* is activated in the *sek-1(km4)* mutant when several genes (e.g. *C48B6.2*, *phi-43* or *wdr-23*) are knocked down by RNAi[31]. In addition, another study also demonstrated that *wdr-23* RNAi robustly induced *gst-4* transcription in the *sek-1(km4)* mutant[37].

Therefore, we next assessed the possibility that SAC and SAMC could activate SKN-1 and its target expressions through regulation by WDR-23. To test this idea, we examined the effect of *wdr-23* knockdown by RNAi on the SAC- and SAMC-induced *gst-4* mRNA expression. As shown in Fig. 3h, *wdr-23* RNAi drastically caused *gst-4* mRNA expression in untreated control animals compared with that of control RNAi, and no additional increase in *gst-4* expression was observed in the SAC- or SAMC-treated animals. This suggests that SAC and SAMC might modulate SKN-1 activity by regulating WDR-23 or its interaction with SKN-1, or possibly by stabilizing SKN-1.

Loss of WDR-23 function causes nuclear accumulation of SKN-1 in intestine, and increases SKN-1 protein levels, leading to activation of target genes[37]. Therefore, we next assessed the possibility whether SAC- and SAMC-treatment could promote nuclear accumulation of SKN-1. We examined the effect of these compounds on subcellular distribution of a SKN-1B/C::GFP translational fusion protein that encodes two of three SKN-1 isoforms. We treated L4 animals with SAC or SAMC, and then measured nuclear accumulation of SKN-1B/C::GFP at L3 or L4 stages of the next generation. Results showed that SAC- and SAMC-treatment did not detectably increase nuclear accumulation of SKN-1B/C::GFP under normal conditions (Fig. 3i), suggesting that these compounds do not substantially affect nuclear localization of SKN-1. On the other hand, it is also possible that hypochlorite treatment for the preparation of L1 animals of the next generation may affect the inducibility of nuclear SKN-1 or levels of SKN-1 protein in later larval stages, leading to a failure of detection of SKN-1B/C::GFP nuclear localization. To address this possibility, we treated L4 animals of the next generation with acute oxidative stress, 2% NaN_3 (as a positive control of SKN-1B/C::GFP nuclear localization) for 15 min after pretreatments with SAC or SAMC. Results showed that this acute oxidative stress caused drastic nuclear accumulation of SKN-1B/C::GFP as indicated in ref. 36, and population of animals with nuclear SKN-1B/C::GFP slightly but reproducibly increased after exposure to 2% NaN_3 when they were pretreated with SAC or SAMC (Fig. 3i). Taken together, these results implicate that SAC and SAMC do not cause nuclear accumulation of SKN-1 directly under normal conditions, but may facilitate nuclear accumulation of SKN-1 in response to acute oxidative stress by possibly defending it against degradation through WDR-23 regulation.

Consistent with our observation, some studies demonstrated that reduced mTORC1 (mammalian target of rapamacin complex) and tunicamicin-induced ER stress also upregulated SKN-1 targets without robust accumulation of this transcription factor in the nucleus[28,33]. In addition, tunicamicin treatment also causes increase of intracellular abundance of SKN-1 protein[33]. Therefore, we investigated whether SAC- and SAMC-treatment could increase intracellular SKN-1 protein levels. To test this idea, total amount of SKN-1 protein in SAC- or SAMC-treated animals was assessed by western blotting using a polyclonal SKN-1 antibody, which was raised against SKN-1c isoform and should detect all of main SKN-1 isoforms (SKN-1a, 1b and 1c). This antibody recognized multiple bands, and four of these increased by *wdr-23* RNAi and decreased in the *skn-1(zu135)* mutant, suggesting that these four bands might correspond to each SKN-1 isoform (1a~1d) (Fig. 3j left). As shown in Fig. 3j (middle and right), SAC and SAMC slightly but reproducibly increased protein levels of species that may correspond to the SKN-1b (2.0~2.4-fold) and SKN-1d (1.9~2.3-fold) isoforms, respectively. SKN-1b is principally

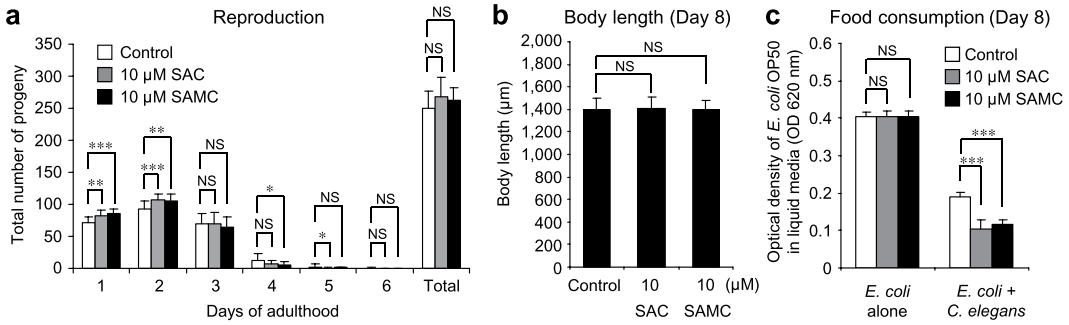


Figure 4. SAC and SAMC do not affect body size and reproduction, but enhance food intake of wild-type *C. elegans*. (**a**) For the reproduction assays, wild-type L4 animals were treated with H_2O (control; n = 17), SAC (n = 19) or SAMC (n = 22) until reproduction period was ceased. Data represent the mean value of daily or total number of progeny from individual animals with SD. (**b**) The body length of animals treated with H_2O (control; n = 85), SAC (n = 87) or SAMC (n = 91) for 8 days was measured by ImageJ. Data represent mean ± SD. (**c**) For the food consumption assays, after 8 days of treatment with H_2O (control), SAC, or SAMC, OD 620 nm of liquid medium containing total 50 animals was measured with a spectrophotometer. Data represent mean ± SD (n = 4 of 50 animals). $^*P < 0.05$; $^{**}P < 0.01$: $^{***}P < 0.001$; NS: not significant (one-way ANOVA with Tukey's post hoc test).

expressed in the ASI neurons, which sense food availability and influence metabolism, and are involved in dietary restriction-induced longevity[26,39]. Even though the transcript of *skn-1d* has been reported in WormBase, neither expression nor function of the smallest isoform has been described. At the moment, the underlying mechanisms of the selective increase in these two SKN-1 isoforms by SAC and SAMC are still unclear.

In mammals, Kelch-like ECH-associated protein 1 (Keap1) directly binds to Nrf2, and targets it for poly-ubiquitination and then proteasomal degradation[21]. Some electrophilic compounds including sulforaphane have been demonstrated to bind to some cysteines in Keap1, and thus leading to release of active Nrf2[40]. *C. elegans* lacks a Keap1 ortholog[41], and instead WDR-23 directly interacts with SKN-1 and targets it for proteasomal degradation[37]. Currently, we have been investigating the possibility whether these compounds could bind to redox-reactive cysteines in WDR-23 or SKN-1 itself.

SAC and SAMC do not affect body size and reproduction, but enhance food intake of wild-type *C. elegans*. It has been revealed that reducing food intake (dietary restriction, DR) extends lifespan of a wide range of species, including *C. elegans*[39,42–45]. In *C. elegans*, DR-induced extension of lifespan appears to require *skn-1b* particularly in the ASI neurons[26,39]. Our findings also suggest that SAC and SAMC increased levels of the SKN-1b isoform (Fig. 3j). Therefore, we considered the possibility that SAC and SAMC might activate SKN-1 in the ASI neurons to produce DR-like state, leading to lifespan extension. Since diet-restricted animals also exhibit reduced brood size, extended reproductive period, and smaller body size[39], we examined the influence of SAC- and SAMC-treatments on reproductive capacity. The results showed that the animals treated with SAC or SAMC for 8 days exhibited a significant increase in progeny production on the 1st and 2nd day of reproductive period, although the total number of progeny was not statistically significant compared to untreated animals (Fig. 4a). Furthermore, neither SAC nor SAMC affected the reproductive period of *C. elegans* (Fig. 4a), suggesting that SAC and SAMC do not affect the reproductive capacity of *C.elegans*. We next examined whether SAC and SAMC could affect *C. elegans* body size. The result showed that wild-type animals raised in the presence of either SAC or SAMC for 8 days did not exhibited any differences in body length compared to untreated animals (Fig. 4b), suggesting that SAC and SAMC also seem to be unrelated with DR with respect to body length.

To assess whether SAC- or SAMC-treatment could cause reduced food intake, we examined the level of food (UV-killed *E. coli* OP50) consumption by measuring the optical density (OD) of wells containing equal numbers of animals (n = 50) after 8 days of treatments with SAC or SAMC. The mean values of OD 620 nm of wells without *C. elegans* were comparable among treatments, suggesting that these compounds do not affect food concentration directly (Fig. 4c). On the other hand, SAC- and SAMC-treated animals showed a significant increase in food consumption compared to untreated control (Fig. 4c). This phenomenon became visually apparent after about 5 days of treatment (unpublished data). One likely explanation for this phenomenon is that SAC- and SAMC-mediated activation of SKN-1 could slow aging of *C. elegans*, and this health promoting effects of these compounds may lead to the elevated food consumption of this organism, despite enhanced food consumption itself produces more ROS in cells in general. Taken all together, these results indicate that, at least for parameters investigated here, SAC and SAMC do not extend *C. elegans* lifespan by producing a DR-like state.

The thioallyl structure and disulfide bond in garlic-derived OSCs are important for SKN-1 activation. Including SAC and SAMC, numerous OSCs, such as *S*-alk(en)ylcysteines, *S*-alk(en)ylcysteine sulfoxides, γ-glutamyl-*S*-alk(en)ylcysteines, and allylsulfides, have been identified from garlic[46]. Some of those have been shown to have diverse pharmacological properties as SAC and SAMC, such as radical scavenging activity, chemopreventive activity, hepatoprotective activity, neurotropic activity, and lipid reducing activity[8,10,11,47,48]. Moreover, a recent study reported that diallyl trisulfide (DATS), one of the allylsulfides derived from garlic, is also able to induce *gst-4* gene expression under control of *skn-1* with extended longevity in

C. elegans[49]. Therefore, we considered the question of whether other OSCs in garlic might activate SKN-1/Nrf as well as SAC and SAMC, and whether their structures might be correlated with this activity. To address this possibility, we tested the effect of garlic-derived OSCs and their analogs on induction of *gst-4p::GFP* transgene. As shown in Table 1, of 23 compounds tested, 5 compounds (SAC; 4.9-fold ($p < 0.001$), SAMC; 8.1-fold ($p < 0.001$), DADS (diallyldisulfide); 3.4-fold ($p < 0.001$), DATS; 9.1-fold ($p < 0.001$), GSAMC (γ-glutamyl-*S*-allylmercaptocysteine); 3.0-fold ($p < 0.001$)) produced a significant increase in *gst-4p::GFP* expression compared to untreated control. Importantly, all these compounds commonly have the thioallyl structure. Moreover, there was a positive correlation between the number of disulfide bonds and *gst-4p::GFP* induction levels as in the case of SAC < SAMC, and DAS < DADS < DATS.

Among compounds containing the allyl structure, alliin (*S*-allylcysteine sulfoxide), in which the sulfur atom of SAC forms sulfoxide group, and OAS (*O*-allylserine), in which the sulfur atom of SAC is substituted by oxygen, exhibited no *gst-4p::GFP* induction. In addition, GSAC (γ-glutamyl-*S*-allylcysteine) and GSAMC, in which glutamic acid is attached to α-amino group of cysteine, exhibited weaker *gst-4p::GFP* inducible activity than SAC and SAMC, respectively. Similarly, SAHC (*S*-allylhomocysteine) and SAMHC (*S*-allylmercaptohomocysteine) had no significant effect on the activity.

Taken together, we found out the following structurally important factors that affect *gst-4p::GFP* inducible activity; i) the thioallyl structure is essential; ii) an increasing number of sulfur atoms in sulfide bonds leads to enhanced activity; iii) the sulfur atom adjacent to the allyl group and iv) cysteine structure are also important factors influencing the activity. Given that SAC and SAMC possibly stabilize SKN-1 by suppressing the interaction between SKN-1 and WDR-23 through binding to reactive cysteines in either of these proteins, it would be interesting to see whether these activity-related factors are closely linked to this event.

The finding that the increasing number of disulfide bonds correlates with the *gst-4p::GFP* inducible activity raises the question of whether the number of disulfide bonds in these compounds might also coorelate with their protective effect. To address this, we performed oxidative stress assays using SAC, SAMC, DADS and DATS (10 μM each), and found significantly higher survivals after treatment with DADS ($41.9 \pm 3.1\%$; $P < 0.001$) and DATS ($41.4 \pm 2.6\%$; $P < 0.001$) compared to DMSO control ($16.0 \pm 3.4\%$) (Table 1, Supplementary Fig. S2). However, neither DADS nor DATS treatment increased survival as robustly as SAC ($83.2 \pm 6.2\%$; $P < 0.001$ vs. DADS and DATS) or SAMC ($90.2 \pm 4.0\%$; $P < 0.001$ vs. DADS and DATS) (Table 1, Supplementary Fig. S2), indicating that there is no positive correlation between the number of disulfide bonds and stress resistance capacity. Similar results were obtained in the heat stress assays (Table 1, Supplementary Fig. S3). Because DATS treatment at higher concentration (100 μM) caused death of adult animals within 24 hours, this toxicity by DATS might lead to the lower survivals in the oxidative stress assay in spite of its highest *gst-4p::GFP* inducible activity. Alternatively, it is also possible that there is an optimal level of SKN-1 activation which, if exceeded may be deleterious. On the other hand, treatment with SAC and SAMC at 100 μM still caused the increased mean lifespan (Fig. 1b,c) and higher survivals ($88.2 \pm 10.4\%$ for SAC, $91.1 \pm 9.3\%$ for SAMC ($N = 3$ experiments using more than 50 animals each)) in the oxidative stress assays. These results implicate that SAC and SAMC can be treated at higher concentrations with less toxicity, thus leading to a superior protective effect compared to DATS.

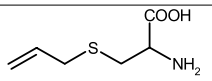
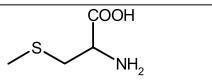
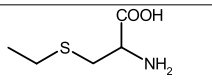
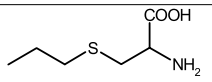
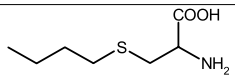
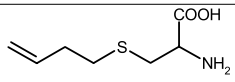
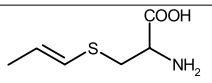
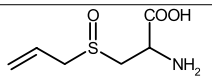
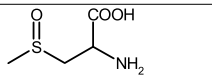
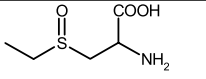
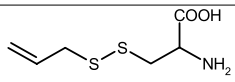
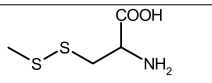
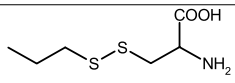
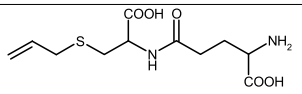
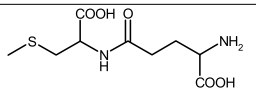
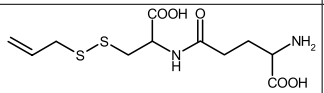
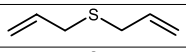
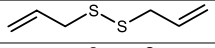
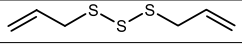
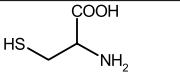
Interestingly, consistent with our findings, some previous studies using garlic-derived OSCs also reported the importance of the thioallyl structure and/or the number of sulfur atoms in sulfide bonds on diverse biological activities. For example, the study investigating chemopreventive activity of *S*-alk(en)ylcysteines and these disulfide derivatives indicated that thioallyl compounds, including SAC, were the most effective for colon cancer prevention[50]. Another study investigating neurotropic activity of *S*-alk(en)ylcysteines, *S*-alk(en)ylcysteine sulfoxides, γ-glutamyl-*S*-alk(en)ylcysteine, and their analogs also indicated that only thioallyl compounds, such as SAC, SAMC, DAS, DADS, alliin, and GSAC, were effective on the survival of cultured rat hippocampal neurons[10]. The study of radical scavenging capacity of some OSCs also revealed that thioallyl structure and the number of the sulfur atoms contribute to the activity[47]. Although direct target(s) of these thioallyl compounds and their underlying mechanisms are still unclear, the notable consistency of observations derived from these and our studies suggests that the thioallyl compounds from garlic play important roles in diverse biological processes including the SKN-1/Nrf pathway.

In conclusion, we have reported that SAC and SAMC increase resistance to oxidative stress and longevity of the nematode *C. elegans*. These beneficial effects of SAC and SAMC are most likely conferred by modulation of SKN-1/Nrf activity and selective activation of its downstream targets involved in oxidative stress defense. Taken together our findings suggest that at least a portion of the multiple health promoting activities of garlic and its constituents, especially those from thioallyl compounds, could be explained by SKN-1/Nrf activation. Furthermore, our study may provide the possibility of applications of natural thioallyl compounds to the development of nutraceutical products and drugs targeting Nrf pathway.

Methods

Reagents. SAC and SAMC were synthesized as in refs. 51 and 7, respectively, stored in water solution and added to culture medium at various concentrations.

Strains and culture of *C. elegans*. Nematode strains used in this study are listed in Supplementary Table S4. Each strain was maintained at 20 °C on nematode growth medium (NGM) agar plates carrying a lawn of *E. coli* OP50 (*Caenorhabditis* Genetics Center) according to ref. 52. Unless otherwise stated, animals for each assay were raised according to the following procedure. Briefly, to synchronize growth of *C. elegans*, gravid hermaphrodites were treated with sodium hypochlorite and resulting eggs were kept overnight at 20 °C for hatching in S-complete liquid medium. Synchronized L1 animals were then transferred to a 96-well plate in S-complete liquid medium containing amphotericin B (0.1 μg/mL) and the UV-killed *E. coli* OP50 (1.2×10^9 bacteria/mL), sealed to prevent evaporation, and kept at 20 °C[53]. UV killing of *E. coli* OP50 was done using a stratalinker (9999 J/m^2, Stratagene,

Compounds*	Structures	*gst-4p::GFP* levels ± SEM (n)†	Survival rates (%) after oxidative/heat stress treatment (Figs S2 and S3)
S-alk(en)ylcysteines			
1 *S*-allylcysteine (SAC)		4.9 ± 0.2 (16)‡	83.2 ± 6.2‡/85.6 ± 4.9‡
2 *S*-methylcysteine (SMC)		1.0 ± 0.1 (16)	
3 *S*-ethylcysteine (SEC)		1.1 ± 0.1 (10)	
4 *S*-n-propylcysteine (SPC)		1.3 ± 0.0 (16)	
5 *S*-n-butylcysteine (SBC)		1.0 ± 0.1 (10)	
6 *S*-n-butenylcysteine (SBnC)		1.1 ± 0.1 (13)	
7 *S*-1-propenylcysteine (S1PC)		1.1 ± 0.1 (15)	
S-alk(en)ylcysteine sulfoxides			
8 *S*-allylcysteine sulfoxide (Alliin)		1.1 ± 0.1 (13)	
9 *S*-methylcysteine sulfoxide (Methiin)		1.0 ± 0.1 (10)	
10 *S*-ethylcysteine sulfoxide (Ethiin)		1.1 ± 0.1 (14)	
S-alk(en)ylmercaptocysteines			
11 *S*-allylmercaptocysteine (SAMC)		8.1 ± 0.4 (15)‡	90.2 ± 4.0‡/91.1 ± 4.9‡
12 *S*-methylmercaptocysteine (SMMC)		1.3 ± 0.1 (12)	
13 *S*-n-propylmercaptocysteine (SPMC)		1.4 ± 0.1 (18)	
γ-*glutamyl-S-alk(en)yl(mercapto)cysteines*			
14 γ-glutamyl-*S*-allylcysteine (GSAC)		1.7 ± 0.1 (22)	
15 γ-glutamyl-*S*-methylcysteine (GSMC)		1.3 ± 0.1 (13)	
16 γ-glutamyl-*S*-allylmercaptocysteine (GSAMC)		3.0 ± 0.1 (21)‡	
Allylsulfides			
17 Diallylsulfide (DAS)		1.2 ± 0.1 (17)	
18 Diallyldisulfide (DADS)		3.4 ± 0.2 (16)‡	41.9 ± 3.1‡/52.8 ± 4.5‡
19 Diallyltrisulfide (DATS)		9.1 ± 0.4 (16)‡	41.4 ± 2.6‡/51.1 ± 6.5‡
Others			
20 Cysteine (Cys)		1.1 ± 0.1 (11)	

Continued

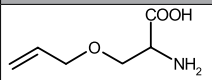
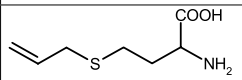
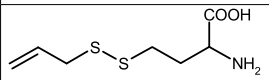
Compounds*	Structures	*gst-4p::GFP* levels ± SEM (n)†	Survival rates (%) after oxidative/ heat stress treatment (Figs S2 and S3)
21 *O*-allylserine (OAS)		1.1 ± 0.1 (15)	
22 *S*-allylhomocysteine (SAHC)		1.1 ± 0.1 (19)	
23 *S*-allylmercaptohomocysteine (SAMHC)		1.5 ± 0.1 (18)	

Table 1. Relative *gst-4p::GFP* inducible activity and stress resistance capacity of garlic-derived organosulfur compounds and their analogs. *Treated at 10 μM each for 24 hours at 20 °C. †Relative fluorescence intensity with SEM. The number of animals tested in parentheses. ‡$P < 0.001$ vs control by one-way ANOVA with Tukey's post hoc test.

La Jolla, CA) to exclude any effects of the test compounds on bacterial growth, and unexpected metabolism of these compounds by live bacteria[54]. 5-fluoro-2′-deoxyuridine (FUdR, 0.12 mM) was added 42–45 hours after seeding to prevent self-fertilization. Thirty micro liters of SAC or SAMC solution, or H_2O as solvent control were added on the first day of adulthood at final concentrations ranging from 1 to 100 μM, respectively.

Lifespan assays. All lifespan assays were started on the first day of adulthood and performed at 20 °C. To avoid starvation, an adequate amount of the UV-killed OP50 was added to each well during assays. Counting of surviving or dead animals was performed daily using a microscope on the basis of movement until all animals had died. Before counting each plate was shaken for one minute on a plate shaker to facilitate observation of movement.

Stress resistance assays. Synchronized day-1 wild-type adults were pretreated with H_2O, SAC or SAMC (10 μM each) for 48 hours at 20 °C. For the oxidative stress assays, the animals were washed with phosphate-buffered saline with 1% Tween 20 (PBST) three times before treating with a ROS generator, juglone (250 μM, Sigma-Aldrich, St. Louis, MO), for 2 hours at 20 °C. For the thermo-tolerance assays, the animals were incubated at 35 °C for 7 hours, and then washed with PBST three times. After a 16 hours recovery period on NGM agar, the survival was determined by touch-provoked movement. Animals were scored as dead when they failed to respond to touching with a platinum wire pick.

Measurement of intracellular ROS in *C. elegans*. To measure intracellular ROS accumulation level in animals after both the oxidative- and the heat-stress treatment, the surviving animals were incubated in the presence of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA, 50 μM, Invitrogen, Carlsbad, CA) in PBST for 1 hour at 20 °C. CM-H_2DCFDA is a cell permeable substance which is intracellularly converted to H_2DCFs. This nonfluorescent probe can be oxidized by interaction with intracellular ROS to yield the fluorescent dye DCF. After washing with PBST, the animals were mounted onto microscope slides coated with 2% agarose, anesthetized with tetramisole (5 mM), and capped with cover slides. Fluorescence images were collected with a BIOREVO BZ-9000 fluorescent microscope (KEYENCE, Osaka, Japan) using the GFP-BP filter set with excitation at 470 nm and emission at 535 nm. The fluorescence intensity of whole body was quantified as mean pixel density by using ImageJ software (NIH, Bethesda, MD).

Transgenic reporter assays. Synchronized day-1 adults of the transgenic strains carrying an inducible green fluorescence protein (GFP) reporter transgene for *sod-3* (CF1553), *hsp-16.2* (CL2070) or *gst-4* (CL2166 or CL691(*skn-1(zu67)*)) were treated with H_2O, SAC or SAMC (10 or 100 μM each) for 24 hours at 20 °C. Juglone (10 or 100 μM) was used as positive control. GFP fluorescence images were collected with randomly selected animals as described in the measurement of intracellular ROS. For the *sod-3p::GFP* and *hsp-16.2p::GFP* reporters, GFP fluorescence from pharynx was quantified by ImageJ. For the *gst-4p::GFP* reporter, GFP fluorescence from whole body was quantified.

Quantitative real-time reverse transcription PCR (qRT-PCR). Synchronized day-1 adults of wild-type or KU4 *(sek-1(km4))* strains were treated with H_2O, SAC or SAMC (10 or 100 μM each) for 6 or 24 hours at 20 °C. Total RNA was extracted from about 50 animals with TRIzol (Invitrogen). Complementary DNA was produced using random 6-mer and oligo (dT) primer. qPT-PCR was performed using SYBR green as the detection method. Expression levels of each mRNA relative to *act-1* gene were calculated with the comparative $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study are listed in Supplementary Table S5.

Feeding RNAi. RNAi was performed in a 96-well plate format by feeding *E. coli* HT115 expressing RNAi for either *wdr-23* (clone ID: CUUkp3300D063Q, Source BioScience, Nottingham, UK) or control (pL4440) to nematodes. Synchronized L1 animals were raised in S-complete liquid medium containing amphotericin B (0.1 μg/mL), ampicillin (100 μg/ml), isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and 1.2×10^9 bacteria/mL of

an overnight culture of RNAi bacteria induced by IPTG for 1 hour. The animals were grown at 20 °C throughout the assay. FUdR (0.12 mM) was added 42–45 hours after seeding. On the first day of adulthood, the animals were treated with H_2O, SAC or SAMC (10 μM each) for 24 hours at 20 °C, and expression levels of *gst-4* mRNA were determined by qRT-PCR.

Nuclear localization DAF-16 or SKN-1. Synchronized day-1 adults of the strains LD1482 or LD001 carrying a transgene that expresses DAF-16A::GFP or SKN-1B/C::GFP fusion protein, respectively, were treated with H_2O, SAC or SAMC (10 or 100 μM each) at 20 °C. For the DAF-16A::GFP reporter, each treatment was performed for 24 hours. For the SKN-1B/C::GFP reporter, synchronized L4 animals were treated with H_2O, SAC or SAMC (10 μM each) for 16 hours at 20 °C. The following day, eggs were harvested by hypochlorite treatment, and progeny were further treated with same compound as each parent and allowed to develop to the L4 stage. After washing with PBST the animals were additionally challenged without or with 2% NaN_3 for 10 min. As a control experiment, synchronized L1 animals of LD001 strain were treated with either control or *wdr-23* RNAi as described above, and then analyzed on the first day of adulthood.

Subcellular distributions of DAF-16A::GFP or SKN-1B/C::GFP were microscopically-classified into "Low", no visible nuclear localization, "Medium", nuclear localization visible only in anterior and/or posterior of body, or "High", strong nuclear localization visible throughout the body or intestine, respectively.

Western blot analysis. Synchronized wild-type L4 animals were treated with H_2O, SAC or SAMC (10 μM each) for 16 hours at 20 °C. The following day, eggs were harvested by hypochlorite treatment, and progeny were further treated with same compound as parent and allowed to develop to the L4 stage. The animals (~1,000 animals per condition) were sonicated in 10 volumes of buffer (50 mM Tris-HCl, pH7.6, 50 mM NaCl, 1% sodium dodecyl sulfate and 1× Halt protease and phosphatase inhibitor cocktail (Thermo scientific)) with a Bioruptor UCW310 (BM Equipment, Tokyo, Japan). Homogenates of total protein were harvested after centrifugation at 16,100 × g for 5 min. Protein concentrations were determined with a XL-Bradford kit (APRO science, Tokushima, Japan) after diluted in SDS-PAGE sample buffer. Fifteen μg of protein samples were applied and separated by SDS-PAGE, and detected by immunoblotting with a polyclonal antibody against SKN-1 (1:2000; JDC7[33]) and β-tubulin (1:1000; 014-25041; Wako). As control experiments, whole lysates from the *rrf-3(pk1426)* mutant treated with either control or *wdr-23* RNAi from L1 state or the *skn-1(zu135)* mutant were prepared on day-1 adulthood and analyzed. Blots were visualized with a ChemiDoc MP (BioRad, Hercules, CA) and densitometrical analysis was performed using Image Lab software (BioRad).

Reproduction assays. Synchronized wild-type L4 animals were individually transferred to wells containing H_2O, SAC or SAMC (10 μM each), and allow laying eggs for 24 hour at 20 °C. The adult animals were transferred to new wells daily until reproduction period was ceased. The number of progeny from individual animal was counted when they raised to the L2 or L3 stage.

Body length and food consumption assays. Synchronized wild-type day-1 adults were treated with H_2O, SAC or SAMC (10 μM each) for 8 days at 20 °C. For the body length assays, the animals were collected, and photographs were taken. The body length of individual animal was analyzed using ImageJ. For the food consumption assays, the liquid medium containing total 50 animals was collected and values of optical density at 620 nm were measured with a multiskan spectrophotometer (Labsystems, Helsinki, Finland).

Statistical analysis. Statistical analysis was performed using KyPlot 5.0 software (KyPlot, Tokyo, Japan). For the lifespan assays, *P*-values were determined by log-rank test. For the nuclear localization of DAF-16A::GFP or SKN-1B/C::GFP, a chi^2 test was used. One-way analysis of variance (ANOVA) with Tukey's post hoc analysis was used for other assays. Differences were considered significant at $P < 0.05$.

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Author Contributions

T.O., Y.K., T.K.B. and M.M. conceived and designed the experiments. Y.K. prepared the compounds used in this study and figures. T.O. carried out the experiments. T.O., T.K.B. and M.M. analyzed the data. T.O. and M.M. wrote the manuscript. All authors discussed the data and edited the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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Supplementary Information

Natural thioallyl compounds increase oxidative stress resistance and lifespan in *Caenorhabditis elegans* by modulating SKN-1/Nrf

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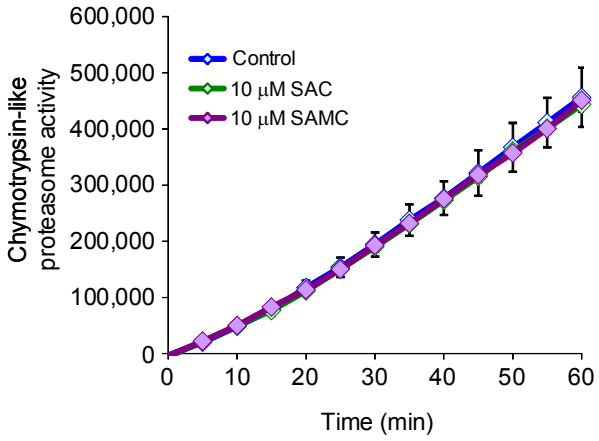

Figure S1. Effect of SAC and SAMC on the proteasome activity. The 26S proteasome activity in whole lysate (25 μg per sample) prepared using about 1,000 wild-type animals treated on the L1 stage with H_2O (control), SAC or SAMC for 4 days at 20ºC. Data represent mean ± SD (n = 3 of 1,000 animals).

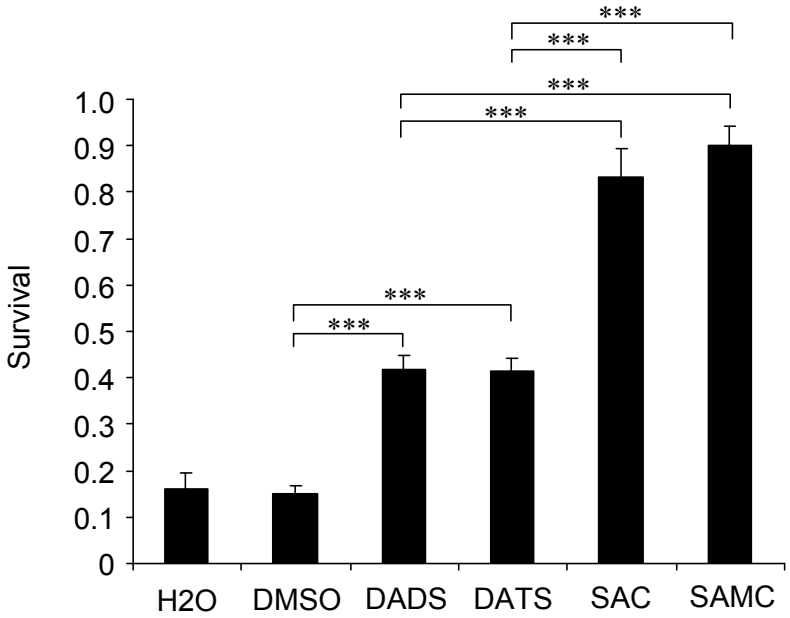

Figure S2. Effect of OSCs on oxidative stress resistance. Synchronized day-1 wild-type adults were treated with H_2O (control for SAC and SAMC), 0.02 % DMSO (control for DADS and DATS), DADS, DATS, SAC or SAMC for 48 hours at 20ºC and then subjected to oxidative stress (250 μM juglone for 2 hours at 20ºC). Each compound was treated at 10 μM. Survivals after the oxidative stress treatment were scored after a 16 hours recovery on NGM agar seeded with *E. coli* OP50. Data are represented as mean ± SD from three independent experiments. Total number of animals tested (H_2O, n=185; DMSO, n=199; DADS, n=198; DATS, n=200; SAC, n=217; SAMC, n=242). ***P<0.001 (one-way ANOVA with Tukey's post hoc test).

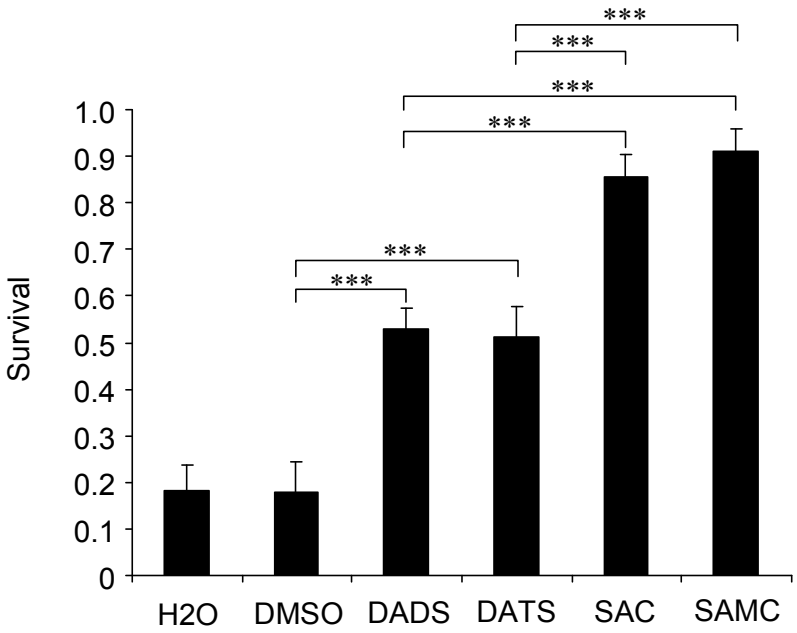

Figure S3. Effect of OSCs on heat stress resistance. Synchronized day-1 wild-type adults were treated with H_2O (control for SAC and SAMC), 0.02 % DMSO (control for DADS and DATS), DADS, DATS, SAC or SAMC for 48 hours at 20ºC and then subjected to heat stress (35ºC for 7 hours). Each compound was treated at 10 μM. Survivals after the oxidative stress treatment were scored after a 16 hours recovery on NGM agar seeded with *E. coli* OP50. Data are represented as mean ± SD from three independent experiments. Total number of animals tested (H_2O, n=173; DMSO, n=166; DADS, n=194; DATS, n=211; SAC, n=231; SAMC, n=224). ***P<0.001 (one-way ANOVA with Tukey's post hoc test).

Table S1. Lifespans of wild-type shown in Figs. 1b and 1c.

Trial	Treatment (μM)	No. animals	Censored	Mean lifespan [1)]			Maximum lifespan [2)]	
				days ± SEM	% extension	*P* value vs. control [3)]	days ± SEM	*P* value vs. control [4)]
1st	Control	81	3	20.9 ± 0.6	N/A	N/A	35	
	SAC (1)	85	4	22.3 ± 0.5	6.5%	0.171	32	
	SAC (10)	80	3	23.8 ± 0.6	13.8%	0.003	48	
	SAC (100)	70	9	23.8 ± 0.6	13.5%	0.001	37	
2nd	Control	106	4	21.6 ± 0.4	N/A	N/A	32	
	SAC (1)	100	2	23.1 ± 0.4	7.3%	0.080	37	
	SAC (10)	83	5	24.1 ± 0.4	11.6%	0.001	37	
	SAC (100)	92	11	23.7 ± 0.4	10.1%	0.007	32	
	SAMC (1)	94	3	22.8 ± 0.4	5.7%	0.136	32	
	SAMC (10)	94	11	25.1 ± 0.5	16.4%	<0.001	43	
	SAMC (100)	90	10	24.9 ± 0.5	15.3%	<0.001	42	
3rd	Control	54	0	21.9 ± 0.7	N/A	N/A	35	
	SAC (1)	61	2	23.6 ± 0.6	7.7%	0.196	31	
	SAC (10)	62	7	26.2 ± 0.7	20.0%	<0.001	50	
	SAC (100)	56	7	26.1 ± 0.5	19.3%	0.002	34	
	SAMC (1)	69	3	23.4 ± 0.6	7.1%	0.262	46	
	SAMC (10)	68	3	25.5 ± 0.8	16.6%	0.002	58	
	SAMC (100)	60	5	24.9 ± 0.6	13.9%	0.012	33	
4th	Control	53	0	20.7 ± 0.7	N/A	N/A	37	
	SAC (1)	49	2	22.5 ± 0.8	8.9%	0.159	35	
	SAC (10)	59	0	26.2 ± 0.6	26.7%	<0.001	33	
	SAC (100)	61	3	25.4 ± 0.7	23.2%	<0.001	35	
	SAMC (1)	50	2	21.5 ± 0.7	4.1%	0.521	30	
	SAMC (10)	58	4	26.1 ± 0.6	26.1%	<0.001	37	
	SAMC (100)	63	3	27.2 ± 0.6	31.6%	<0.001	37	
5th	Control	49	2	21.3 ± 0.6	N/A	N/A	32	
	SAMC (1)	59	1	21.9 ± 0.6	2.7%	0.683	31	
	SAMC (10)	54	5	25.6 ± 0.7	20.0%	<0.001	38	
	SAMC (100)	55	6	26.3 ± 0.7	23.5%	<0.001	38	
Combined	Control	343	9	21.3 ± 0.3	N/A	N/A	34.8 ± 1.0	N/A
(Trial 1~5)	SAC (1)	295	10	22.9 ± 0.3	7.5%	<0.001	33.8 ± 1.4	0.989
Fig. 1b and c	SAC (10)	284	15	24.9 ± 0.3	17.0%	<0.001	42.0 ± 4.1	0.171
	SAC (100)	279	30	24.6 ± 0.3	15.6%	<0.001	34.5 ± 1.0	0.999
	SAMC (1)	272	9	22.5 ± 0.3	5.8%	0.024	34.8 ± 3.8	1.000
	SAMC (10)	274	23	25.5 ± 0.3	19.7%	<0.001	44.0 ± 4.8	0.236
	SAMC (100)	268	24	25.7 ± 0.3	20.9%	<0.001	37.5 ± 1.8	0.971

1) Mean lifespan is the day when 50% of worms survived.

2) Maximum lifespan is the day when the last surviving worm died.

3) *P*-values for mean lifespan were obtained by log-rank test by comparing the control and other treated groups.

4) *P*-values for maximum lifespan were calculated by one-way ANOVA with Tukey's post hoc test from means of the maximum lifespan of each condition.

Table S2. Lifespans of *daf-16(mgDf47)* shown in Figs. 2d and 2e.

Trial	Treatment (μM)	No. animals	Censored	Mean lifespan [1]			Maximum lifespan [2]	
				days ± SEM	% extension	*P* value vs. control [3]	days ± SEM	*P* value vs. control [4]
1st	Control	41	2	12.3 ± 0.5	N/A	N/A	18	
	SAC (1)	41	2	12.8 ± 0.4	4.2%	0.873	18	
	SAC (10)	42	1	13.8 ± 0.3	12.2%	0.089	20	
	SAC (100)	41	0	13.9 ± 0.3	13.4%	0.048	20	
	SAMC (1)	42	3	12.4 ± 0.5	1.3%	0.719	20	
	SAMC (10)	41	3	12.8 ± 0.5	4.8%	0.537	22	
	SAMC (100)	42	3	13.2 ± 0.3	7.7%	0.368	18	
2nd	Control	80	0	11.7 ± 0.4	N/A	N/A	18	
	SAC (1)	79	0	12.2 ± 0.4	4.3%	0.551	21	
	SAC (10)	75	0	13.4 ± 0.4	14.2%	0.008	18	
	SAC (100)	80	4	12.7 ± 0.4	8.2%	0.148	19	
	SAMC (1)	85	0	11.4 ± 0.4	-3.1%	0.501	18	
	SAMC (10)	81	1	13.0 ± 0.3	10.6%	0.081	20	
	SAMC (100)	80	1	13.1 ± 0.3	11.6%	0.039	19	
3rd	Control	69	0	11.4 ± 0.4	N/A	N/A	20	
	SAC (1)	66	0	11.3 ± 0.4	-0.6%	0.607	18	
	SAC (10)	68	2	12.7 ± 0.4	11.2%	0.061	22	
	SAC (100)	70	3	13.7 ± 0.4	20.4%	<0.001	22	
	SAMC (1)	75	0	11.4 ± 0.4	-0.3%	0.578	22	
	SAMC (10)	86	0	13.1 ± 0.3	15.3%	0.004	21	
	SAMC (100)	80	3	13.8 ± 0.4	21.5%	<0.001	23	
Combined	Control	190	2	11.7 ± 0.3	N/A	N/A	18.7 ± 0.7	N/A
(Trial 1~3)	SAC (1)	186	2	12.0 ± 0.2	2.6%	0.756	19.0 ± 1.0	0.994
Fig. 2d and e	SAC (10)	185	3	13.2 ± 0.2	12.8%	<0.001	20.0 ± 1.2	0.754
	SAC (100)	191	7	13.3 ± 0.2	13.8%	<0.001	20.3 ± 0.9	0.616
	SAMC (1)	202	3	11.6 ± 0.2	-1.2%	0.481	20.0 ± 1.2	0.808
	SAMC (10)	208	4	13.0 ± 0.2	11.1%	0.002	21.0 ± 0.6	0.447
	SAMC (100)	202	7	13.4 ± 0.2	14.5%	<0.001	20.0 ± 1.5	0.808

1) Mean lifespan is the day when 50% of worms survived.

2) Maximum lifespan is the day when the last surviving worm died.

3) *P*-values for mean lifespan were obtained by log-rank test by comparing the control and other treated groups.

4) *P*-values for maximum lifespan were calculated by one-way ANOVA with Tukey's post hoc test from means of the maximum lifespan of each condition.

Table S3. Lifespans of *skn-1(zu135)* shown in Figs. 3c and 3d.

Trial	Treatment (μM)	No. animals	Censored	Mean lifespan [1]			Maximum lifespan [2]	
				days ± SEM	% extension	*P* value vs. control [3]	days ± SEM	*P* value vs. control [4]
1st	Control	32	0	17.3 ± 1.1	N/A	N/A	37	
	SAC (10)	32	0	18.0 ± 1.1	3.6%	0.779	34	
	SAC (100)	32	1	16.4 ± 0.8	-5.7%	0.405	27	
	SAMC (10)	32	0	18.5 ± 1.4	6.7%	0.469	39	
	SAMC (100)	32	2	15.8 ± 1.0	-9.1%	0.337	28	
2nd	Control	49	0	15.9 ± 0.7	N/A	N/A	28	
	SAC (10)	50	1	16.7 ± 0.8	5.0%	0.409	37	
	SAC (100)	53	1	14.5 ± 0.5	-9.1%	0.041	23	
	SAMC (10)	51	0	16.4 ± 0.9	2.6%	0.704	37	
	SAMC (100)	52	0	13.9 ± 0.6	-12.8%	0.020	28	
3rd	Control	59	0	18.5 ± 0.6	N/A	N/A	36	
	SAC (10)	53	4	17.8 ± 0.6	-3.9%	0.525	32	
	SAC (100)	57	6	15.9 ± 0.5	-13.8%	0.002	27	
	SAMC (10)	54	3	17.2 ± 0.6	-7.1%	0.137	34	
	SAMC (100)	56	1	16.1 ± 0.5	-12.7%	0.004	27	
Combined	Control	140	0	17.3 ± 0.4	N/A	N/A	33.7 ± 2.8	N/A
(Trial 1~3)	SAC (10)	135	5	17.4 ± 0.5	0.5%	0.853	34.3 ± 1.5	0.970
Fig. 3c and d	SAC (100)	142	8	15.5 ± 0.3	-10.8%	<0.001	25.7 ± 1.3	0.067
	SAMC (10)	137	3	17.2 ± 0.5	0.9%	0.855	36.7 ± 1.5	0.525
	SAMC (100)	140	3	15.2 ± 0.4	-12.3%	<0.001	27.7 ± 0.3	0.134

1) Mean lifespan is the day when 50% of worms survived.

2) Maximum lifespan is the day when the last surviving worm died.

3) *P*-values for mean lifespan were obtained by log-rank test by comparing the control and other treated groups.

4) *P*-values for maximum lifespan were calculated by one-way ANOVA with Tukey's post hoc test from means of the maximum lifespan of each condition.

Table S4. Nematode strains used in this study.

Number	Genetic background	Transgene	Array number	Referenced
N2	Wild-type			
CF1553	N2	Is[*sod-3p::GFP*]		(1) Libina *et al*., 2003
CL2070	N2	Is[*hsp-16.2p::GFP*]		(2) Link *et al*., 1999
CL2166	N2	Is[*gst-4p::GFP*]		(3) Link and Johnson, 2002
	daf-16(mgDf47)			(4) Ogg *et al*., 1997
LD1482	*daf-16(mu86)*	Is[*DAF-16A::GFP*]		(5) Lin *et al*., 2001
CL691	*skn-1(zu67)*	Is[*gst-4p::GFP*]		(6) Rea *et al*., 2007
EU31	*skn-1(zu135)*			(7) Bowerman *et al*., 1992
LD001	N2	Is[*SKN-1B/C::GFP*]	007	(8) An & Blackwell, 2003
KU4	*sek-1(km4)*			(9) Tanaka-Hino *et al*., 2002
NL2099	*rrf-3(pk1426)*			(10) Simmer *et al*., 2002

Table S5. Primer sequences used in qRT-PCR analysis.

Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
act-1	ACCATGTACCCAGGAATTGC	TGGAAGGTGGAGAGGGAAG
sod-3	AGCATCATGCCACCTACGTGA	CACCACCATTGAATTTCAGCG
hsp-16.2	CTCAACGTTCCGTTTTTGGT	CGTTGAGATTGATGGCAAAC
ctl-2	TCCGTGACCCTATCCACTTC	TGGGATCCGTATCCATTCAT
gst-4	CGTTTTCTATGGAAGTGACGC	TCAGCCCAAGTCAATGAGTC
gcs-1	TGTTGATGTGGATACTCGGTG	TGTATGCAGGATGAGATTGTACG
gst-10	GTCTACCACGTTTTGGATGC	ACTTTGTCGGCCTTTCTCTT
atf-5	CCATCAATCTTATCAACAGCATCAT	CTGGTGGAACCGAAGTG
haf-7	GACGTGGAAAAGCTGAGAGG	GCAGGGAAAATGTGAGGAAA
rpt-3	CCCAAGAGGAGTTCTCATGTA	ATGAAGGAAGCAGCAGTATT
rpn-12	CTGCCAACAGATTGTCCG	GGCGTAGAGATGTAAGCG
pas-4	CGAGCCATCTGGAGCTTACTA	TCCTCAAGGTATTCACGCAC
pbs-6	TGGACAGAGCCATCTCATT	CTTCAGCGATGACCAAGTG
skn-1	AGTGTCGGCGTTCCAGATTTC	GTCGACGAATCTTGCGAATCA

Supplementary Methods

26S proteasome activity assays

The 26S proteasome activity in whole animal lysate was measured as previously described (11). Briefly, after treating L1 larvae with H_2O (control), SAC or SAMC (10 μM each) for 4 days at 20ºC, adult animals were sonicated in 4 volumes of lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 2 mM ATP, 1 mM dithiothreitol and 0.5 mM EDTA) with a Bioruptor UCW310 (BM Equipment, Tokyo, Japan). Lysate was centrifuged at 14,000 X g for 10 min at 4ºC. To measure chymotrypsin-like proteasome activity, 25 μg of whole animal lysate was transferred to a 96-well microtitre plate, then incubated with a fluorogenic peptide substrate (100 μM Suc-Leu-Leu-Val-Tyr-AMC, Boston Biochemicals, MA) in proteasome activity assay buffer (50 mM Tris-HCl, pH 7.5, 40 mM KCl, 5 mM $MgCl_2$, 0.5 mM ATP, 1 mM dithiothreitol and 0.05 mg mL^{-1} BSA) at 25ºC. The fluorescence intensity was measured at 380 nm for excitation and 460 nm for emission using an EnVision 2104 multilabel reader (PerkinElmer, Waltham, MA) every 5 min for 1 hour at 25ºC. The assay was performed in the absence or presence of proteasome inhibitor (40 μM Epoxomicin, Peptide Institute, Osaka, Japan) to calculate the 26S proteasome-specific activity.

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