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Compounds that Confer Thermal Stress Resistance and Extended Lifespan

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Abstract

The observation that long-lived and relatively healthy animals can be obtained by simple genetic manipulation prompts the search for chemical compounds that have similar effects. Since aging is the most important risk factor for many socially and economically important diseases, the discovery of a wide range of chemical modulators of aging in model organisms could prompt new strategies for attacking age-related disease such as diabetes, cancer and neurodegenerative disorders (Collins et al., 2006; Floyd, 2006; Gill, 2006; Hefti and Bales, 2006). Resistance to multiple types of stress is a common trait in long-lived genetic variants of a number of species; therefore we have tested compounds that act as stress response mimetics. We have focused on compounds with antioxidant properties and identified those that confer thermal stress resistance in the nematode *Caenorhabditis elegans*. Some of these compounds (lipoic acid, propyl gallate, trolox and taxifolin) also extend the normal lifespan of this simple invertebrate, consistent with the general model that enhanced stress resistance slows aging.

Keywords

antioxidant; stress; aging; thermotolerance; lifespan; compound screen; trolox; taxifolin; propyl gallate; lipoic acid

1. Introduction

Organismal lifespan can be extended by mutation or reduced expression of a large number of single genes. Consequently, it is likely that a wide range of chemical structures will have lifespan-extending properties. The potential *druggable* targets include the many signaling pathways that determine lifespan in metazoans such as insulin/IGF signaling (Kenyon, 2005), cell cycle checkpoint signaling (Olsen et al., 2006a), TOR signaling, JNK signaling (Oh et al., 2005; Wang et al., 2003) and others (Gill, 2006). In addition, metabolic functions such as mitochondrial electron transport (Anson and Hansford, 2004) and protein translation (Hansen et al., 2007; Pan et al., 2007) are also likely drug targets based on the lifespan enhancing effect of lowering the rates of these processes in some circumstances. A range of chemical interventions have been shown to enhance *C. elegans* lifespan, such as superoxide dismutase and catalase mimetics (Melov et al., 2000), resveratrol (Wood et al., 2004), and a range of

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compounds that are commonly used in the treatment of neurological disease including the anticonvulsants drugs ethosuximide (Evason et al., 2005) and valproic acid (Evason et al., 2008), the mood-stabilizing lithium (McColl et al., 2008) and a series of antidepressants (mianserin, mirtazapine, methiothepin and cyproheptadine) (Petrascheck et al., 2007). In addition, some complex plant extracts extend lifespan including Ginkgo biloba extract EGb 761 (Strayer et al., 2003; Wu et al., 2002) and a proanthocyanid-rich fraction of blueberry extract (Wilson et al., 2006). It remains to be seen whether these compounds act on targets known to have a role in lifespan determination or whether they act through novel mechanisms (Collins et al., 2006).

There is a strong correlation between lifespan extension and resistance to multiple environmental stresses. Most genetic manipulations that extend lifespan also confer enhanced stress resistance. Conversely, many genetic modifications that enhance resistance to stress also result in longevity (Lithgow and Walker, 2002). We propose that compounds that enhance stress response (*stress response mimetics*; *SRMs*) would likewise extend lifespan. There are many potential classes of compounds that could act as SRMs. For example, compounds that target regulatory networks which control the expression of stress response factors could improve survival under various stresses.

Such compounds may induce hormetic stress responses that are beneficial for normal lifespan (Lamming et al., 2004) and mimic the effects of mild environmental stresses (Lithgow et al., 1995; Maynard Smith, 1958; Olsen et al., 2006b). A second class could provide more direct protective or detoxification activities. These may include chemical chaperones that stabilize protein conformation or antioxidants that reduce macromolecular damage that results from oxidative stress.

Testing the effects of compounds with antioxidant properties on aging has a long history (Zuckerman and Geist, 1983) following from the related theories that oxygen radicals *cause* or contribute to aging. These theories are challenged by some studies (Van Remmen et al., 2003) but it is clear that oxygen radicals contribute to aging phenotypes and appear to play critical roles in age-related disease (Golden et al., 2002). Genetic evidence from invertebrates suggests that oxidative stress resistance is a common characteristic of long-lived mutants (Larsen, 1993; Lin et al., 1998; Vanfleteren, 1993). Lifespan extension results from the over-expression of some antioxidant enzyme genes and other stress factors (Morrow et al., 2004; Orr et al., 2005; Sohal et al., 1996; Sun et al., 2004; Sun and Tower, 1999; Walker et al., 2006). There is also some evidence from rodent models that genetic modifications resulting in extended lifespan also result in resistance to oxidative stressors at both the whole animal level (Holzenberger et al., 2002; Migliaccio et al., 1999) and the cellular level (Maynard and Miller, 2006; Nemoto and Finkel, 2002; Salmon et al., 2005).

A number of encouraging studies suggest that chemical antioxidants may extend lifespan in some circumstances. For example, treatment of *C. elegans* from hatching with vitamin E (α -tocopherol) extends the mean lifespan at some concentrations (Harrington and Harley, 1988; Ishii et al., 2004; Zuckerman and Geist, 1983) but slowed development appears to contribute to the effect. Vitamin C and vitamin C/E combinations did not extend lifespan (Harrington and Harley, 1988). In contrast, *C. elegans* lifespan was significantly increased by on average 40% with a treatment with synthetic, Mn-containing, catalytic superoxide dismutase (SOD)/catalase mimetics (SCMs) without affecting development or fertility or inducing the cellular stress response (Melov et al., 2000; Sampayo et al., 2003a; Sampayo et al., 2003b). The SCMs reduce oxidative stress in a range of disease models (Melov, 2002) and render the worms resistant to paraquat (a superoxide radical generator) (Keaney et al., 2004; Sampayo et al., 2003b) and intriguingly, thermal stress (Sampayo et al., 2003b). Another group was unable to observe increased survival in the absence of oxidative stress and even observed a dose dependant

reduction in lifespan (Keaney and Gems, 2003; Keaney et al., 2004). and the reasons for the dramatic differences in lifespan between these studies are not understood although some differences in treatment regimens are known to exist. The SCM-associated lifespan increase has been replicated under the conditions previously published (Lithgow et al., 2005). Interestingly, Mn alone acts as an antioxidant in budding yeast (Sanchez et al., 2005) and is sufficient to confer thermotolerance and increase the lifespan of a short lived *mev-1* mutant in *C. elegans* (Lin et al., 2006); *mev-1* encodes succinate-coenzyme Q oxidoreductase of mitochondrial complex II and the mutants are sensitive to oxygen, show an increase in oxidative damage to protein and accumulate damaged mitochondria (Ishii et al., 1990; Matsuo, 1993; Senoo-Matsuda et al., 2003; Senoo-Matsuda et al., 2001). The lifespan of the *mev-1* mutant is also increased by treatment with electron carrier Q₁₀ which also reduced superoxide anion production and lowered apoptosis rates (Ishii et al., 2004). Q₁₀ treatment of wild type worms resulted in an 18% increase in lifespan. Thus it appears that chemicals with antioxidant properties can slow aging rates but the effects of Q and other antioxidants on *C. elegans* lifespan are complex and the exact mechanisms at play are elusive (Anson and Hansford, 2004).

These studies stand in contrast to reports of failures of antioxidant compounds to extend invertebrate lifespan particularly when the trials are conducted with insects. For example, Magwere et al. demonstrated that whilst *Drosophila* mutants with compromised antioxidant defenses benefited from treatment with either SOD/catalase mimetics or mitochondria-targeted ubiquinone (MitoQ), these compounds appeared toxic to normal flies and no lifespan increase was observed (Magwere et al., 2006). Furthermore, SOD/catalase mimetics had no effect on housefly lifespan (Bayne and Sohal, 2002).

There are a number of possible explanations for the limited success of antioxidant compounds in lifespan extension. First, very few of the invertebrate studies make any pharmacokinetic measures and consequently the levels of compound in specific tissues is seldom known; a failure to extend lifespan may arise from a failure in bioavailability or be the result of a toxic build up of the compound or a modified by-product. Second, it may be that the antioxidant functions of a compound may not be sufficient to reduce macromolecular damage during normal aging. Third, perhaps resistance to oxidative stress alone is not sufficient for slowed aging; only compounds that render organisms resistant to “multiplex” stress are able to extend lifespan. Note that the catalytic superoxide dismutase(SOD)/catalase mimetics protect against both oxidative and thermal stress(Sampayo et al., 2003b). The need for multiplex stress resistance for lifespan extension is also suggested by genetic studies. In *C. elegans*, a conserved neuroendocrine axis is, in part, responsible for determining survival during stress and also for normal aging (Kenyon, 2005; Lithgow and Walker, 2002). Partial loss-of-function mutations in the genes encoding an insulin/IGF-1-like signaling (ILS) pathway extend normal lifespan 70–100% (Kenyon, 2005) but also confer resistance to multiple stress; for example, mutation of the phosphoinositide-3-OH kinase (PI3K) gene (*age-1*) makes worms resistant to thermal stress (Lithgow et al., 1995), reactive oxygen species (Larsen, 1993; Vanfleteren, 1993), ultraviolet radiation (Murakami and Johnson, 1996), heavy metal toxicity (Barsyte et al., 2001), bacterial pathogens (Garsin et al., 2003) and hypertonic stress (Lamitina and Strange, 2005). This is likely due to the fact that ILS coordinately regulates the expression of antioxidant enzyme, molecular chaperone, metallothionein and other stress response genes with each perhaps contributing to lifespan extension (Barsyte et al., 2001; Honda and Honda, 1999; Hsu et al., 2003; Larsen, 1993; Lithgow, 1996; McElwee et al., 2003; Murphy et al., 2003; Vanfleteren, 1993; Walker and Lithgow, 2003; Walker et al., 2001; Yanase et al., 2002).

We hypothesized that agents that could confer resistance to multiple stresses might extend lifespan. We surveyed compounds with known antioxidant functions and determined whether they could confer protection against a thermal stress in *C. elegans*. We find that a number of classes of antioxidant compounds provide protection; those that do, also extend lifespan.

2. Materials and Methods

2.1 Nematode Culture and Handling

Bristol N2 (wild-type), TJ1060 [*spe-9(hc88)I*; *fer-15(b26)II*], CL2070 [dvIs70(*hsp-16.2::gfp*; *rol-6(su1006)*)], CL2166 [dvIs19(pAF15(*gst-4::GFP::NLS*))], CF1038 [muIs84[pAD76 (*sod-3::GFP*)], CYP35A3 [*cyp-35::GFP*], and *E. coli* OP50 were obtained from the *Caenorhabditis* Genetic Center at the University of Minnesota. Worms were maintained on 10cm or 6cm nematode growth medium (NGM) agar plates carrying a lawn of *E. coli* OP50 or in liquid culture (S-medium) supplemented with *E. coli* OP50 at 5×10^7 cells/ml. Culture plates were maintained at 20°C, unless otherwise stated. Eggs were isolated by hypochlorite treatment of gravid adults.

2.2. Methods

2.2.1 Treatment of *C. elegans* with Compounds—Compounds were obtained from Sigma-Aldrich (St. Louis, MO) and EMD Biosciences (San Diego, CA) except Euk-189 which was a gift from Simon Melov at the Buck Institute (Novato, CA). Compounds were dissolved in the appropriate vehicle and diluted to working concentrations. For thermotolerance treatments, 50 µl of diluted compound was added to a 10ml week old agar plate spotted with 200 µl of concentrated *E. coli* OP50. The plates were set aside for 2–5 hr to allow the compound to disperse. Age-synchronous worm cultures were prepared by hypochlorite treatment of fertile adults as previously described (Brooks et al., 1994) and grown to three days of age at 20°C in the absence of compound. The worms were washed off, resuspended in a small volume of S-basal and approximately 1000 worms were spotted onto each compound plate and incubated at 20°C for 24 hr prior to thermotolerance assays or GFP reporter quantitation as described in the text. For lifespan treatments, 15 µl of compound dilution was added to a 3ml week old agar plate spotted with 50 µl of an overnight culture of OP50. The plates were set aside for 2–5 hr to allow the compound to disperse.

2.2.2. Longitudinal Automated Thermotolerance Assay—Microplate assays were undertaken as previously described (78). Briefly, worms were washed off treatment plates, rinsed, and introduced to the sample cup of a COPAS BIOSORT at a concentration of approximately 1 worm per µl. Worms were dispensed, 1 animal per well, into 384-well black low-volume microtitre plates (Greiner Bio-One Inc, FL) containing 20 µl S-medium, OP50 at 5×10^7 cells per ml, and 1 µM SYTOX. The plate was sealed using VIEWseal plate sealing film (Greiner Bio-One Inc, FL) to prevent evaporation. Fluorescence was measured using a Fluoroskan Ascent fluorometer (Thermo Labsystems, MA) with the temperature set to 35°C and fluorescence was measured in each well every 30 min over a 20–24 hr period, with a 20 millisecond integration time for each well. For SYTOX green fluorescence the excitation wavelength was set to 485nm and the emission wavelength 538nm. Differences in thermotolerance were assessed using the Mantel–Haenszel Logrank test as implemented in Prism (GraphPad Software Inc.). Kaplan–Meier survival curves were generated using Prism survival analysis.

2.2.3. Survival during normal aging—Compound plates were prepared as described above (2.2.1.). All lifespan assays were performed using sterile TJ1060 worms grown and maintained at 25°C. Synchronous populations of hermaphrodite worms were prepared by picking eggs from a plate of fertile adults and moving to a fresh NGM plate. Worms were moved to plates containing compound, 30–40 per plate, on the first day of adulthood. The worms were transferred to fresh compound-containing plates every 2–3 days. Worms were scored for touch-provoked movement as previously described (Olsen et al., 2006a). Differences in survival were assessed using the Mantel–Haenszel Logrank test as implemented in Prism (GraphPad Software Inc.). Kaplan–Meier survival curves were generated by using Prism

survival analysis. Maximum survival was calculated as the average survival of the oldest 10% of the population. Differences in maximum lifespan were assessed using the Kruskal-Wallis test with Dunn's post-test comparison.

2.2.4. Induction of Stress Response Reporters—Young adult worms containing inducible GFP reporters for *gst-4*, *hsp-16.2*, *cyp-35*, and *sod-3* were placed onto compound treatment plates for 24 or 48 hrs. The strains used were CL2070 Worms were mounted on slides and immobilized with levamisole. GFP expression was scored at 24 and 48 hours on 20 randomly selected worms per condition at 20x magnification using GFP filters and a one second exposure time.

Images are opened with ImageJ and the color image converted to 8-bit greyscale. The pixel threshold is set at 25–255. Measure total fluorescence as integrated density of pixels over threshold (*cyp-35*, n>9 for each treatment group; *sod-3*, n>8 for each treatment; *gst-4*, n>4)

2.2.5. Measurement of Hermaphrodite Self Fertility—Fertility measurements were undertaken as previously described (Lithgow et al., 1994). Briefly, synchronous N2 eggs were collected from a one hour egg lay and incubated at 20°C. Young adults were transferred to individual treatment plates on day 3 and each day throughout the fertile period. Developing progeny were counted for 11 individuals per condition. Comparisons to vehicle-treated worms were made using a one-way ANOVA test followed by multiple comparison pair wise testing with Bonferroni correction.

3. Results

3.1. Antioxidant compounds confer thermotolerance in *C. elegans*

We selected a group of compounds for testing based on previously described antioxidant properties either *in vitro* or in biological systems. We tested the compounds at two concentrations which were based on the compound's solubility. We pursued compounds that induced thermotolerance in two of three replicate experiments (six out of the eleven compounds). The assay itself was somewhat variable; the average median time of death of the control populations was 10.2±1.4 giving a CV of 13%. There was also variability of effect size with the same compounds. Despite this, some compounds exhibited consistent effects between experiments; these are discussed as functional classes.

3.1.1. Catalytic antioxidants—It has been proposed that catalytic antioxidants are likely to be more beneficial than other antioxidants such as spin traps. Some hit compounds fell into this category and some proved highly variable between trials. EUK-189, an SOD/catalase mimetic, gave variable results between trials (data not shown). A second antioxidant with catalytic activity mimicking SOD (Reddan et al., 2003), propyl gallate (PG) conferred thermotolerance reproducibly at high concentrations (1.3 mM) but gave variable results at lower concentrations including in one instance a significant sensitivity to heat shock at 53 µM.

3.1.2. Non-catalytic antioxidants—We tested a series of non-catalytic free radical scavengers. α -lipoic acid (LA, 1,2-dithiolane-3-pentanoic acid) has broad antioxidant activity, inhibits NF-kappa B activation (Suzuki et al., 1992) and nitric oxide formation (Demarco et al., 2004) and has beneficial effects on the glutathione pool (Bharat et al., 2002; Han et al., 1997). Treatment of adult *C. elegans* with LA lead to a high level of thermotolerance (Figure 1, Table 1) in a dose dependant manner in six of seven trials.

Notably α -tocopherol, the most bioactive of the naturally occurring vitamin Es, failed to protect against thermal stress but trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid),

a cell-permeable, water-soluble derivative of vitamin E, provided significant protection against thermal stress in five of six trials (Table 1; Figure 1). We did not observe a clear dose response to trolox across the trials.

3.1.3. Polyphenols—There is considerable interest in the potential beneficial effects of polyphenols during aging. Many polyphenols are associated with antioxidant activities; hence we surveyed some of the many thousand polyphenolic chemical species. We found that the flavanoid, rutin hydrate (RH) protects strongly at high concentration (211 μ M) in two of three trials but either has no effect or causes sensitivity at low concentrations (8 μ M) reminiscent of that observed for PG (Figure 1; Table 1). A second flavanoid, taxifolin gave variable effects but protected in two of three trials at 657 μ M. Purpurogallin, catechin hydrate and resveratrol did not confer thermotolerance (Table 1).

3.2. Antioxidants that enhance lifespan

We tested the effects of compounds that most reproducibly conferred thermotolerance for effects on lifespan. Hermaphrodites were transferred to compound containing plates on the first day of adulthood and were exposed to compounds throughout normal aging (Figure 2; Table 3). α -lipoic acid increased survival in five of six trials at a concentration of 1mM (log rank test; $p \leq 0.0043$). Median lifespan was increased by on average 21%. This result is consistent with the lifespan increases observed in *Drosophila* (Bauer et al., 2004) and *C. elegans* (Brown et al., 2006) with this compound. Interestingly, there appears to be little effect on maximum survival leading to a “squaring” of the survival curve. A similar trend was apparent for worms treated with PG which increased lifespan in two of three trials at 1mM ($p < 0.003$) and at 50 μ M in one trial ($p = 0.018$). Where significant, PG-treatment increased median lifespan by 12%.

Other compounds increased both median and maximum lifespan. Trolox increased median lifespan on average by 31% in all four trials at concentrations from 0.6mM to 3mM ($p < 0.0001$). Maximum lifespan was also significantly increased (Figure 2; Table 3). Taxifolin at 820 μ M increased median lifespan on average by 51% in all three trials ($p < 0.0001$) and also increased median lifespan in two other trials at lower concentrations ($p \leq 0.032$) (Figure 2; Table 3).

3.3. Effects of antioxidant on stress response gene expression

We tested for a general cellular stress response directly by examining the expression from the transcriptional promoter of a gene encoding heat shock protein (HSP)-16.2. The HSP-16.2 protein is encoded by a classical stress gene that is induced by heat and a range of environmental toxins including oxidants (Jones and Candido, 1999; Jones et al., 1989). We examined worms transgenic for the *hsp-16.2* transcriptional promoter fused to a green fluorescent protein marker (*hsp-16.2::GFP*). 24 hr exposure to the antioxidants at concentrations that extend lifespan failed to induce expression of the *hsp-16* marker construct (data not shown) consistent with an absence of general toxic effects or a constitutive up-regulation of the cellular stress response following antioxidant treatment. This is consistent with our previous observation that the catalytic antioxidants, EUK-8 and EUK-134 confer protection to stress but do not induce the cellular stress response (Sampayo et al., 2003b).

We then tested the effects of compound treatment to two genes up-regulated upon oxidative stress; *gst-4* and *sod-3* (Figure 3). 24hr treatment with 1mM doses of LA and PG seemed to lower *gst-4::GFP* levels (Figure 3A) although following correction for multiple comparison, none of the difference reached significance. Likewise, no significant differences were observed with *sod-3::GFP* reporter (Figure 3B). Finally, we tested a marker of xenobiotic responses *cyp-35*, (Menzel et al., 2001; Menzel et al., 2005) as some mutants with extended lifespan exhibit some gene expression profile similarities to cells exposed to exogenous toxic chemicals. We

treated worms carrying a *cyp-35::GFP* reporter construct and observed a significant 2-fold increase in the expression when the worms were exposed to 3mM Trolox. There appeared to be a decrease in expression induced by LA and PG treatment but these did not reach significance following multiple comparison correction.

3.4. Fertility and antioxidant treatment

To further investigate whether lifespan-enhancing antioxidant treatment is the result of a response to a toxic effect, we measured hermaphrodite self-fertility, a trait that is very sensitive to stress (Lithgow et al., 1994). We found no significant differences in *total* fertility for any of the treatments. Interestingly the 1mM lipoic acid treatment showed a delay in fertility with a significant 17% decrease in fertility during the first 48 hour period (Figure). Also the 1mM doses of lipoic acid and propyl gallate showed growth effects on the progeny. EUK-189 had no statistical effect on fertility consistent with our previous assessment of other catalytic antioxidants, EUK-134 and EUK-8 (Melov et al., 2000; Sampayo et al., 2003b).

4. Discussion

The identification of a range of chemical compounds that slow aging in multiple species is highly desirable both as a tool for further research but also as potential therapeutic avenues for age-related disease. One way of obtaining such compounds is to target proteins known to influence aging in *in vitro* activity assays or in cell based screens. The promising compounds can then be tested *in vivo* for anti-aging properties (Howitz et al., 2003; Yang et al., 2007). An alternative approach is to screen compounds *in vivo* for anti-aging properties, thus simultaneously testing the *druggability* of hundreds of potential aging targets. In using thermal stress as a surrogate, in much the same way as it has been used to identify novel aging genes (Munoz and Riddle, 2003; Olsen et al., 2006a), we have been able to find a number of compounds that extend lifespan.

We have demonstrated that some compounds that act as antioxidants in biological systems can confer longevity, in some cases by increasing both mean and maximum lifespan (trolox) but in others, by increasing median lifespan only (LA). However the exact mechanisms at play have still to be elucidated. We postulated that compounds that had antioxidant properties in biological systems may confer protection to a range of stresses (multiplex stress resistance). Here we show that a range of antioxidants confer thermotolerance. Perhaps the compounds are affecting stress signaling pathways that regulate thermal stress resistance factors such as heat shock proteins. However we find no compelling evidence of constitutive up-regulation of a small *hsp-16* gene, an *sod* gene or a glutathione-S transferase gene, each of which are up-regulated by various stresses. One of the four compounds that extend lifespan (trolox) did however up-regulate the expression of a gene (*cyp-35*) associated with a xenobiotic response. Although the two fold induction is less than that observed with some organic compounds (Menzel et al., 2005), it does indicate that a xenobiotic response may contribute to the observed lifespan extension and stress resistance.

An alternative mechanism of action of these compounds is the direct detoxification of ROS. Indeed worms subjected to a heat shock exhibit dramatically increased production of ROS (Sampayo and Lithgow, unpublished data) therefore survival death from thermal stress may be mediated by oxidative stress. There are multiple protective mechanisms described in the literature for some of the compounds. For example, LA may function in *C. elegans* reducing mitochondrial superoxide production. Naturally occurring LA (R-LA) is found in the mitochondria and acts as a co-enzyme for pyruvate dehydrogenase and α -ketoglutarate and treatment of aged rodents improves mitochondrial function (Hagen et al., 1999; Hagen et al., 2002; Packer et al., 1997). R-LA acts to prevent glutathione depletion in neuronal culture and preserves mitochondrial complex I activity which is normally reduced as a consequence of

GSH pool depletion (Bharat et al., 2002) (probably through reduction of GSSG to GSH). There is evidence that LA can also act as a mitochondrial uncoupler thus reducing ROS production (Dicter et al., 2002). Moreover, it activates insulin receptor activity from hepatocytes by binding directly to the receptor (Diesel et al., 2007). However, this would be predicted to shorten lifespan. PG is also associated with increased glutathione levels (Reddan et al., 1999) and has also been shown to be an SOD mimetic (Reddan et al., 2003). Trolox is a scavenger of peroxy and alkoxy radicals and is commonly used as a standard in assays of antioxidant capacity (Huang et al., 2005). Taxifolin provides protection in a reperfusion injury model and has multiple effects on cell signaling pathways (Wang et al., 2006; Wang et al., 2004).

In summary, the antioxidants that extend lifespan may function by direct scavenging of radical species or may function through modulating endogenous antioxidant functions and intracellular signaling pathways. In addition to single gene mutations, antioxidants can confer resistance to multiple stresses and extend lifespan consistent with the mechanistic commonality between stress and aging.

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α -lipoic acid **propyl gallate** **α -tocopherol** **resveratrol** **trolox** **taxifolin**

Figure 1. Survival during Acute Thermal Stress of *C. elegans* Treated with Antioxidant Compounds
 3-day-old adult hermaphrodite worms were treated with compound or control vehicle (water, DMSO, or ethanol) for 24 hr on agar plates then dispensed using a COPAS Biosort wormsorter

1 worm per well into a 384 well plate. Survival of individuals at 35°C was measured via SYTOX-Green fluorescent staining every 30 min for 20 hr. All curves have $n > 50$. * Drug treatment varied significantly from vehicle control (p-value < 0.001).

Figure 2. Survivorship during Normal Aging of *C. elegans* Treated with Antioxidants

3-day-old adult hermaphrodite worms were added to plates containing either compound or vehicle control. Worms were transferred to fresh compound-containing agar plates every 2–3 days. Survival was scored by means of touch provoked movement and pumping of the pharynx. Kaplan-Meier survival curves were generated using the GraphPad Prism version 2, GraphPad Software Inc., San Diego Ca.

Figure 3. Effect of Antioxidant Treatment on Stress Gene Expression

3-day-old adult hermaphrodite worms were added to plates containing compound or DMSO vehicle control. After 24 hr, worms were mounted on slides and immobilized with levamisole. GFP expression was scored at 24 on up to 20 randomly selected worms and fluorescence quantified (mean±SEM). The data were analyzed by 1 way ANOVA in GraphPad Prism™ and were subject to Bonferroni's multiple comparison test. (*= p<.05).

Figure 4. Average Daily Fertility of *C. elegans* Treated with 1mM lipoic acid

Young adult worms were transferred to compound or vehicle control plates each day during the fertile period. Progeny was counted for 11 worms per condition. Comparisons were made using one-way ANOVA test in GraphPad PrismTM. 48 hr fertility totals are 260 ± 16 and 194 ± 25 for DMSO and 1mM lipoic acid respectively (p-value <0.05).

Table 1

Compound	Experiment ⁺	Dose	Median Survival (hrs)	p-value [*]
<i>α</i>-lipoic acid	Trial 1	-	10	#
		47μM	8.3	<0.0001
	Trial 2	233μM	10	ns
		1mM	15.5	<0.0001
		-	10.5	
		47μM	9.5	ns
	Trial 3	233μM	14	<0.0001
		1mM	15	<0.0001
		-	11.8	
		228μM	10	#
	Trial 4	1.13mM	11	#
		-	10	
	Trial 5	242.3μM	10	ns
		1.2mM	13.5	<0.0001
propyl gallate	Trial 1	-	11	
		53μM	9.5	#
	Trial 2	1.3mM	15.3	▲
		-	11	
		53μM	11.5	ns
		1.3mM	15.5	▲
<i>α</i>-tocopherol	Trial 1	-	15	
		160μM	12	#
	Trial 2	4mM	12.5	#
		-	10	
		160μM	10	ns
		4mM	10.5	ns
	Trial 3	-	11.8	
		160μM	11	#
		4mM	14	ns
resveratrol	Trial 1	-	10	
		18μM	8.5	ns
	Trial 2	438μM	8.5	ns
		-	10	
		18μM	9	#
		438μM	9.5	#
trolox	Trial 1	-	11	
		116μM	10.3	#
		579μM	10.8	ns
		2.9mM	11.5	ns

Compound	Experiment ⁺	Dose	Median Survival (hrs)	p-value [*]
	Trial 2	-	9.8	ns
		116µM	9.5	
		579µM	10	
	Trial 3	2.9mM	11	<0.0001
		-	10	
		116µM	9.5	
	Trial 4	579µM	10	ns
		2.9mM	11.5	
		-	9.5	
	Trial 5	200µM	11.3	0.0009
		1mM	10	
		-	8.5	
	Trial 6	200µM	9.5	0.0422
		1mM	8.8	
		-	8	
taxifolin	Trial 1	200µM	9	0.0428
		1mM	8.5	
		-	9.5	
	Trial 2	33µM	10	ns
		164µM	10	
		657µM	12	
	Trial 3	-	10	<0.0001
		33µM	9.5	
		164µM	9.8	
		657µM	10	0.0002
		-	10.5	
		33µM	10	
		164µM	10	0.0022
		657µM	10	
		-	11.5	

^{*} log-rank test as implemented in Prism.
⁺ >50 worms in each treatment group in each trial.
▲ Significant increase in thermotolerance. P-value <0.05.
Significant decrease in thermotolerance. P-value <0.05.

Table 2

Effect of antioxidant treatment on hermaphrodite self-fertility

Compound	Dose	Total Fertility
DMSO control	-	311 ± 32
Lipoic acid	500μM	298 ± 45
	1mM	280 ± 21
Trolox	500μM	293 ± 35
	3mM	287 ± 27
Propyl gallate	500μM	284 ± 20
	1mM	295 ± 25
Taxifolin	150μM	287 ± 25
	820μM	290 ± 26

Values are average of 11 individuals ± sd.

Table 3

Effect of antioxidant treatment on *C. elegans* lifespan.

Compound	Experiment	Dose	Deaths	Censored	Median survival (days)	p-value	90 th Percentile Survival
α -lipoic acid	Trial 1	-	150	10	11	-	19.7
		1mM	149	11	16 *	<0.0001	19.5
	Trial 2	-	129	3	15	-	23.2
		50 μ M	123	9	16	ns	22.3
	Trial 3	-	128	14	17	ns	21.7
		1mM	196	5	15	-	21.8
propyl gallate		100 μ M	124	4	16	ns	23.9
		1mM	123	5	17 *	0.0004	22.1
	Trial 4	-	97	4	15	-	20.3
		1mM	89	15	17 *	0.0043	21.2
	Trial 5	-	107	1	14	-	21.1
		1mM	115	11	16 *	<0.0001	22.8
trolox	Trial 6	-	203	7	14	-	22.2
		200 μ M	61	0	14 *	0.01	23.7
		1mM	64	3	18 *	<0.0001	22.3
		5mM	61	5	11	0.0013	15.7
	Trial 1	-	129	3	15	-	23.2
		50 μ M	119	9	17 *	0.018	23.5
taxifolin		1mM	125	12	16	ns	22.2
	Trial 2	-	196	5	15	-	21.8
		100 μ M	138	10	16	ns	22.9
		1mM	139	5	17 *	0.0003	22.6
	Trial 3	-	97	4	15	-	20.3
		1mM	93	13	17 *	<0.0001	21.9
trolox	Trial 1	-	97	4	15	-	20.3
		600 μ M	118	6	17 *	<0.0001	22.1
		3mM	137	7	20 *	<0.0001	22.6
	Trial 2	-	95	5	12	-	20.4 **
		3mM	110	5	17 *	<0.0001	24.6
	Trial 3	-	107	1	14	-	21.1 **
taxifolin		1mM	154	5	19 *	<0.0001	24.9
	Trial 1	-	74	1	12	-	19.6
		820 μ M	108	0	17 *	<0.0001	22.3
	Trial 2	-	64	1	10	-	22.2
		205 μ M	62	5	13 *	0.012	24.7
		820 μ M	54	3	20 *	<0.0001	23
taxifolin	Trial 3	-	84	1	14	-	25.8
		150 μ M	88	0	15	0.032	28.9
		820 μ M	84	0	21 *	<0.0001	28.3

* p-value <0.01 with Mantel-Haenszel Logrank test

** p-value <0.05 with Dunn's multiple comparison test