Extension of Lifespan in C. elegans by Naphthoquinones That Act through Stress Hormesis Mechanisms

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Abstract

Hormesis occurs when a low level stress elicits adaptive beneficial responses that protect against subsequent exposure to severe stress. Recent findings suggest that mild oxidative and thermal stress can extend lifespan by hormetic mechanisms. Here we show that the botanical pesticide plumbagin, while toxic to C. elegans nematodes at high doses, extends lifespan at low doses. Because plumbagin is a naphthoquinone that can generate free radicals in vivo, we investigated whether it extends lifespan by activating an adaptive cellular stress response pathway. The C. elegans capn’collar (CNC) transcription factor, SKN-1, mediates protective responses to oxidative stress. Genetic analysis showed that skn-1 activity is required for lifespan extension by low-dose plumbagin in C. elegans. Further screening of a series of plumbagin analogs identified three additional naphthoquinones that could induce SKN-1 targets in C. elegans. Naphthazarin showed skn-1-dependent lifespan extension, over an extended dose range compared to plumbagin, while the other naphthoquinones, oxoline and menadione, had differing effects on C. elegans survival and failed to activate ARE reporter expression in cultured mammalian cells. Our findings reveal the potential for low doses of naturally occurring naphthoquinones to extend lifespan by engaging a specific adaptive cellular stress response pathway.

Introduction

As organisms age, cellular proteins, lipids and nucleic acids sustain damage that can lead to functional deficits in tissues and, ultimately, death. The free radical theory of aging proposes that aging results, at least in part, from damage to cellular components by reactive oxygen species (ROS), such as nitrooxides, hydrogen peroxide and superoxide anion. Indeed, oxidative modification is a major form of damage detected in aging tissues [1–3]. ROS occur as byproducts of normal mitochondrial metabolism, but are also produced by environmental sources, including some biological toxins. Levels of oxidative damage correlate with relative age and extent of functional decline, consistent with oxidative damage acting as a contributing force driving tissue decline with age [4,5]. Aging-related diseases, such as Alzheimer’s disease and cancer, have also been linked to oxidative damage [6,7]. Multiple lines of evidence suggest that attenuating stressful insults or increasing stress resistance can delay aging and functional decline in model organisms and in human tissues [8–10]. Such data support the concept that chemicals with prolongevity activity can be identified by their ability to activate stress response pathways.

Stress hormesis occurs when toxic agents elicit beneficial effects at low concentrations and is classically described by an inverted U-shaped dose response curve [11]. Stress hormesis has been observed for both thermal and oxidative stressors. Sublethal thermal stress is protective against subsequent thermal stress in yeast, worms, and flies [12–14]. In Caenorhabditis elegans, sublethal thermal stress can extend adult lifespan, suggesting that thermal stress responses overlap with prolongevity pathways in this organism [13,15,16]. Chemical stress can induce heat shock protein expression and thermotolerance in Saccharomyces cerevisiae [17]. Cultured cells and whole organisms are protected from oxidative stress by pretreatments with hyperbaric oxygen or low levels of free-radical generators such as paraquat or juglone [16,18]. In addition, mild oxidative stress from low concentrations of juglone extended C. elegans lifespan, suggesting that oxidative stress response pathways also overlap with prolongevity pathways in C. elegans [19]. The close link between stress and aging suggests that interventions harnessing hormetic mechanisms may extend lifespan or delay age-associated functional decline. However, challenges for developing hormetic mechanisms into anti-aging therapies include the relatively small dose range providing...
protective benefits and the toxic effects of higher doses. Therefore, studies are needed to determine the feasibility of modifying hormetic agents to extend the beneficial dose range and minimize toxicity.

Here, we report that hormetic chemicals can be modified to optimize beneficial effects and minimize toxicity in C. elegans, a model for studying aging in whole organisms. C. elegans is well-suited to this problem due to the short lifespan, ease of genetic manipulation and transparent anatomy. First, we examined whether lifespan extension is common among biological toxins with various chemical structures and mechanisms of action. In a small screen of natural phytochemicals, we identified two ROS generating compounds, plumbagin and juglone, which extended lifespan at subtoxic doses. Mean lifespan extension by plumbagin was dependent on SKN-1, a cap’n’collar transcription factor that promotes antioxidant gene expression in response to oxidative stress [20]. We further screened a collection of six plumbagin analogs, identifying three additional naphthoquinones that activated expression of a skn-1 target. One of these could extend lifespan over a larger range of doses than plumbagin, demonstrating the utility of stress hormesis mechanisms as promising longevity intervention. The other compounds had differing effects on longevity, possibly reflecting structure-specific alterations in stability and toxicity. This work highlights C. elegans as an experimental approach for identifying lead compounds with the potential to act on conserved targets.

**Results**

A screen for biological toxins with hormetic prolongevity activity

To gain a better understanding about the ability of phytotoxins to extend lifespan through stress hormesis mechanisms, we conducted a small screen of 14 phytochemicals which was derived from a larger collection of 30 phytochemicals used to identify compounds activating stress response pathways in cultured cells [21]. These compounds represented a diverse range of chemicals, including lignins, lipids, alcohols and cyclic compounds, encompassing at least three modes of action, ROS generators, antifeedants and neurotoxins (Table 1). For the C. elegans screen, we examined survival of populations of roughly 40 sterile fem-1(hc17) adults treated with each of the 14 phytochemicals [22]. First, each phytotoxin was tested for toxicity at 200 μM. Four phytotoxins were toxic at this dose (plumbagin, visnagin, eugenol and farnesol) and were retested at 100 μM (Table 1). At 100 μM, toxicity was observed for plumbagin, visnagin and eugenol, while farnesol had no effect. Plumbagin, visnagin and eugenol were further tested at 60, 30 and 10 μM doses. At both 60 and 30 μM, plumbagin demonstrated a prolongevity effect with treated animals surviving longer than controls. A previous study reported that 50 μM plumbagin was toxic to C. elegans [23]. This variation in hormetic and toxic dose ranges for plumbagin may reflect variability in culturing conditions between laboratories. We further tested 300 μM doses for 10 phytotoxins that were not toxic at 200 μM. Of these, juglone, piperine, veratrine and asarone were toxic at 300 μM, although they had shown no benefit or toxicity at 200 μM. We examined these at 100 μM and found that juglone was beneficial and could extend lifespan at this concentration. In an independent study, 40 μM juglone extended C. elegans lifespan in axenic media [19].

The microbial environment can be a source of stress that shortens C. elegans lifespan [24,25]. Therefore, all subsequent experiments were conducted in the presence of 5-fluoro-2’-deoxyuridine (FUDR), a DNA synthesis inhibitor which is both bacteriostatic and bacteriocidal [26,27]. In four trials with wild type C. elegans, treatment with 25 μM plumbagin was associated with an average increase in mean lifespan of 12% ± 2% (p<0.0001, two-way ANOVA) (Fig. 1, Table S1). Treatment with 50 μM plumbagin was also associated with a significant increase of mean lifespan in two of three trials. These doses of plumbagin did not increase maximum lifespan. Plumbagin’s prolongevity effect was lost at 100 μM and doses between 200–500 μM were toxic. Based on this screen, we conclude that plumbagin and juglone possessed hormetic activity that could extend lifespan in C. elegans.

Table 1. Screen for hormetic prolongevity activity in C. elegans.

| Mechanism          | Compound     | Notes                  | Relative survival; (μM dose) |
|--------------------|--------------|------------------------|-----------------------------|
|                    |              |                        | 300 | 200 | 100 | 60 | 30 | 10 |
| ROS generators     | Plumbagin    | antiproliferative yellow pigment | <   | <   | >   | >  | -  |
|                    | Juglone      | herbicidal brown pigment | <   | -   | >   | >  | -  |
|                    | Visnagin     | vasodilator            | <   | <   | <   | <  | >  |
| Neurotoxins        | Eugenol      | TRPV channel agonist    | <   | <   | <   | >  | -  |
|                    | Piperine     | TRPV channel agonist    | <   | -   | -   | -  | -  |
|                    | Veratrine    | Na+ channel antagonist  | <   | -   | -   | -  | -  |
|                    | Anabasine    | Nicotinic agonist       | -   | -   | -   | -  | -  |
|                    | Domoic acid  | Neurotoxin             | -   | -   | -   | -  | -  |
| Antifeedants       | Farnesol     | Membrane disruptor     | -   | -   | -   | -  | -  |
|                    | a-Asarone    | Phenylopropanoid        | <   | -   | -   | -  | -  |
|                    | Marmesin     | Coumarin               | -   | -   | -   | -  | -  |
|                    | Precocene II | Cytotoxin              | -   | -   | -   | -  | -  |
|                    | Sesamin      | Lignin                 | -   | -   | -   | -  | -  |
| Translation inhibitor | L-canavanine | Arginine analog         | -   | -   | -   | -  | -  |

Empty cells, dose not tested; (<) shorter survival than untreated controls; (=) approximately equal survival to untreated controls; (>) longer survival than untreated controls.

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Individual trial data are presented in Table S1.

To identify pathways activated by plumbagin, we performed microarray analysis of C. elegans gene expression after a 2-day treatment with 100 μM plumbagin. This dose is slightly greater than that required for stress hormesis but is only weakly toxic to adult C. elegans (0%, −11%, −16% in 3 trials, Table S1). We chose this dose with the goal of maximizing the expression level of plumbagin-responsive genes. GO analysis revealed that the strongest effect of plumbagin was upregulated expression of oxidoreductase activity, consistent with plumbagin’s action as an oxidative stressor (Fig. 2A) [28,29]. Additionally, plumbagin treatment was associated with repression of growth, development and reproductive processes, indicating a shift from energy expenditure to energy conservation and repair (Fig. 2B). Plumbagin treatment led to the upregulation of several genes for glutathione-S-transferases, cytochrome P450 enzymes and small-molecule dehydrogenases, as was previously observed for juglone and hyperbaric oxygen in C. elegans [30,31].

CNC-family NF-E2 transcription factors direct antioxidant enzyme expression as part of the Phase 2 stress response, an important pathway for cellular oxidative stress resistance [32]. Plumbagin activates NF-E2 target genes in human neuroblastoma cells [21]. Therefore, the C. elegans CNC transcription factor, SKN-1, is one candidate for mediating the beneficial effects of plumbagin. Our microarray analysis surveyed 14 of the top 15 upregulated skn-1 targets [33]. Plumbagin treatment increased expression of 9 of these skn-1 targets by 2-fold or greater (Table 2). A second candidate effect for plumbagin’s benefits is the stress-responsive FoxO transcription factor, DAF-16. In C. elegans, stresses such as growth factor withdrawal, starvation, oxidative and heat stress, trigger DAF-16/FoxO nuclear translocation and expression of target genes [14,34–37]. In C. elegans, lifespan extension by stress hormesis from juglone required daf-16 activity [19]. The activities of skn-1 and daf-16 are interdependent for lifespan regulation [38]. Among 38 upregulated daf-16 target genes [39] surveyed in our experiment, only two were upregulated with 100 μM plumbagin (Table 3). Plumbagin did not alter expression of sod-3, a direct target of DAF-16 [35].

Next, we examined whether lifespan extension by low-dose plumbagin required skn-1 and daf-16 activity. Consistent with a role for skn-1, 25 μM plumbagin did not extend lifespan of skn-1(zu135) animals in four independent trials (Fig. 3B). Additionally, skn-1 RNAi abrogated lifespan extension from treatment with 10, 25 and 50 μM plumbagin (Table S2). Furthermore, skn-1 RNAi enhanced toxicity of plumbagin at doses of 50 μM and above. Thus, skn-1 activity was required both for the longevity benefits of plumbagin, and for normal resistance to plumbagin toxicity.

We also examined the effects of plumbagin on lifespan in both daf-16(mgDf50); daf-2(e1370) and daf-16(mgDf50) backgrounds lacking daf-16 activity (Fig. 3C, D). Lifespan was extended by plumbagin treatment in these daf-16-deficient backgrounds, although the effect was significantly weaker and more variable than in the wildtype background. Together, these results implicate both skn-1 and daf-16 as mediators of lifespan extension by low-doses of plumbagin.

Using GFP reporters, we examined whether activation of skn-1 or daf-16 by low-dose plumbagin could be monitored in vivo. C. elegans strain CL2166 carries a GFP reporter expressed from the gst-4 promoter [40]. The gst-4 promoter contains two predicted SKN-1 binding sites and Pgst-4::GFP expression induced in response to oxidative stress from hyperbaric oxygen and hydrogen peroxide was skn-1-dependent [41]. Treatment with 25 μM plumbagin induced expression of Pgst-4::GFP in the intestine, with weaker expression in the head and body muscles (Fig. 4B). We quantified Pgst-4::GFP fluorescence in the bodies of animals treated for 48 hours with 0-150 μM plumbagin, encompassing a range of beneficial and toxic doses. The increase in Pgst-4::GFP was approximately linear between 0-50 μM plumbagin and appeared to become saturated, but also variable, at higher plumbagin doses (Fig. 4A, Table S2). In three trials, the beneficial extension by stress hormesis from juglone required daf-16 activity [19]. The activities of skn-1 and daf-16 are interdependent for lifespan extension by stress hormesis from juglone required daf-16 activity [19]. The activities of skn-1 and daf-16 are interdependent for lifespan regulation [38]. Among 38 upregulated daf-16 target genes [39] surveyed in our experiment, only two were upregulated with 100 μM plumbagin (Table 3). Plumbagin did not alter expression of sod-3, a direct target of DAF-16 [35].

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A GFP reporter for a SKN-1 transcriptional target is a dose-responsive biosensor of plumbagin exposure

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Figure 2. Transcriptional effects of plumbagin under non-toxic conditions in *C. elegans*. (A,B) Results of GO analysis of molecular function (A) and biological processes (B) for transcripts differently expressed in animals treated with plumbagin versus vehicle (DMSO). Results indicate upregulation of genes involved in oxidative stress resistance functions (A) and biological functions related to electron transport (B), possibly related to electron capture. Furthermore, reductions in growth and developmental processes were identified in plumbagin-treated animals, consistent with a shift of resources to stress response.

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dose of 25 μM plumbagin was associated with a 57+/−13% increase in Pgst-4::GFP fluorescence over background (p<0.001, T-test). 50 μM plumbagin induced a range of Pgst-4::GFP levels, with whole worm fluorescence increased by 96%, 173%, and 241% compared to controls in three trials. We note that 50 μM plumbagin increased lifespan in a subset of trials, suggesting that this concentration may represent the upper end of the beneficial dose range. Pgst-4::GFP induction by plumbagin was skn-1-dependent. In animals treated with skn-1 RNAi, plumbagin failed to induce Pgst-4::GFP expression in most tissues (Fig. 4B; Table 4). The idea that there is an optimal window of skn-1 activity for lifespan extension is consistent with data showing that moderate over-expression of skn-1 extended lifespan while high-copy over-expression of skn-1 was toxic [38].

In cultured mammalian neurons, plumbagin activated expression of target genes containing the antioxidant response element (ARE) in an Nrf2-dependent manner [21]. Nrf2 activity is induced in response to oxidative stress and plumbagin has reported ROS generating activity [42]. We therefore examined whether plumbagin activation of ARE-containing targets could be altered by treatment with glutathione, an antioxidant capable of scavenging plumbagin-generated ROS. Indeed, ARE-reporter induction by plumbagin was significantly diminished by cotreatment with glutathione (Fig. 4C). These data support the hypothesis that plumbagin activates Nrf2 target gene expression through alteration of the cellular redox environment.

In two independent experiments with 20 animals each, 25 μM plumbagin failed to increase nuclear localization of a DAF-16::GFP reporter (Fig. 4D). Therefore, we utilized the Pgst-4::GFP for a biosensor of stress hormesis in all subsequent experiments.

### Analysis of plumbagin analogs for improved benefic/toxicity profiles

Two problems for translating interventions based on stress hormesis mechanisms are the limited dose range for beneficial

| Sequence ID | Gene name | Plumbagin treatment | Fold-change | P  |
|-------------|-----------|---------------------|-------------|----|
| C55B7.4     | acdh-1    | −1.2                | 1.0         | 0.39|
| C46F4.2     | acs-17    | 1.0                 | 1.0         | 0.64|
| F32A5.5     | aop-1     | 1.2                 | 1.2         | 0.02|
| K11D2.2     | asah-1    | 1.0                 | 1.0         | 0.54|
| H22K11.1    | asp-3     | −1.6                | −1.6        | 0.13|
| C50B8.2     | bir-2     | −1.1                | −1.1        | 0.60|
| Y54G11A.6   | ctl-1     | −1.3                | −1.3        | 0.04|
| Y54G11A.5   | ctl-2     | −1.2                | −1.2        | 0.21|
| T10B9.1     | cyp-13A4  | −1.2                | −1.2        | 0.06|
| B0213.15    | cyp-34A9  | −1.3                | −1.3        | 0.23|
| K07C6.4     | cyp-35B1  | −1.4                | −1.4        | 0.41|
| K07E3.3.2   | dao-3     | −1.0                | −1.0        | 0.68|
| C24B9.9.1   | dod-3     | −1.7                | −1.7        | 0.14|
| T20G5.7     | dod-6     | 1.4                 | 1.4         | 0.42|
| F10D2.9     | fat-7     | −1.0                | −1.0        | 0.23|
| C52E4.1     | gcp-1     | −1.0                | −1.0        | 0.36|
| C05E4.9     | gel-7     | 1.2                 | 1.2         | 0.48|
| R12A1.4     | ges-1     | −1.2                | −1.2        | 0.97|
| T28B8.2     | ins-18    | −1.5                | −1.5        | 0.87|

*14 skn-1 targets (Oliveira et al. 2009).

| Sequence ID | Gene name | Plumbagin treatment | Fold-change | P  |
|-------------|-----------|---------------------|-------------|----|
| F38E11.2    | hsp-12.6  | −1.2                | −1.2        | 0.39|
| T27E4.8     | hsp-16.1  | 1.1                 | 1.1         | 0.64|
| Y46H3A.3    | hsp-16.2  | 1.0                 | 1.0         | 0.02|
| C02A12.4    | lys-7     | −3.4                | −3.4        | 0.02|
| C17G10.5    | lys-8     | 1.2                 | 1.2         | 0.68|
| R03E9.1     | mdl-1     | −1.6                | −1.6        | 0.15|
| K11G9.6     | mtl-1     | 2.2                 | 2.2         | 0.01|
| F49E11.9    | scl-1     | −1.6                | −1.6        | 0.76|
| F43D9.4     | sip-1     | 1.0                 | 1.0         | 0.47|
| C08A9.1     | sod-3     | 1.0                 | 1.0         | 0.14|
| K12G11.3    | sodh-1    | 1.4                 | 1.4         | 0.03|
| K12G11.4    | sodh-2    | 1.8                 | 1.8         | <0.001|
| T07C4.4     | spp-1     | −1.0                | −1.0        | 0.26|
| T22G5.7     | spp-12    | 1.7                 | 1.7         | <0.001|
| C06B3.4     | stdh-1    | 1.2                 | 1.2         | 0.33|
| F11A5.12    | stdh-2    | 1.2                 | 1.2         | <0.001|
| F28D1.3     | thn-1     | −1.1                | −1.1        | 0.25|
| AC3.7       | ugt-1     | 1.0                 | 1.0         | 0.17|

* 37 daf-2 pathway targets (Murphy et al. 2003).

| Table 2. Effect of plumbagin on expression of skn-1 targets. |
|-------------|---------------------|---------------------|-------------|----|
| Sequence ID | Gene name | skn-1 target * | Plumbagin treatment | Fold-change | P  |
|-------------|-----------|----------------|---------------------|-------------|----|
| K08F4.7     | gst-4     | √               | 4.9                 | <0.001      |
| C32H11.4    | √         | 2.1             | 0.001               |
| C32H11.12   | √         | 2.0             | 0.006               |
| ZK1085.6    | √         | 2.5             | <0.001              |
| Y45G12C.2   | √         | 2.3             | 0.01                |
| K100D11.1   | √         | 2.7             | <0.001              |
| C32H11.3    | √         | 1.9             | 0.01                |
| Y102A11A.3  | √         | −1.1            | 0.89                |
| T26C5.1     | √         | 1.8             | 0.05                |
| F56D5.3     | √         | 3.8             | <0.001              |
| K10C2.3     | √         | 1.7             | <0.001              |
| F23B2.12    | √         | 1.1             | 0.53                |
| F55G11.2    | √         | 2.0             | <0.001              |
| C35B1.5     | √         | 2.0             | 0.02                |

Average fold-change from 4 biological replicates comparing gene expression in animals treated with 100 μM plumbagin vs DMSO vehicle control using C. elegans 4x4K oligo microarrays (Agilent Technologies, USA).

* 14 skn-1 targets.
effects and toxicity at higher doses. Therefore, we investigated whether plumbagin analogs existed which could activate skn-1 and extend C. elegans lifespan without significant toxicity at higher doses. A group of six plumbagin analogs, consisting of 3 naphthoquinones and 3 tetralones, was screened for activation of Pgst-4::GFP and effects on lifespan in C. elegans. Tetralones, which lack the 4-ketone of plumbagin, did not activate Pgst-4::GFP fluorescence (Table S3) and conferred no survival benefit at any tested dose (Table S1). In addition, the tetralones were generally non-toxic to C. elegans at concentrations up to 500 μM.

In contrast to the tetralones, all three naphthoquinones, menadione, naphthazarin and oxoline, increased Pgst-4::GFP expression, although not as strongly as plumbagin (Fig 5A, B; Table S3). As with plumbagin, increases in Pgst-4::GFP expression in worms treated with naphthazarin and oxoline were abrogated by skn-1 RNAi (data not shown). Plumbagin, but not menadione, was previously shown to induce the Nrf2-mediated Phase 2 response in cultured mammalian cells [21]. We therefore compared activation of the Nrf2-dependent ARE reporter by naphthazarin, oxoline and plumbagin in HepG2 cells. Both plumbagin and naphthazarin induced the ARE reporter, while oxoline failed to activate the ARE reporter at up to 100-fold higher concentrations (Fig. 5C). We also examined survival of HepG2 cells in the presence of each naphthoquinone. Plumbagin and naphthazarin had similar toxicity profiles, while oxoline was approximately 100-fold less toxic than plumbagin (Fig. 5D).

We considered the possibility that naphthoquinones extended lifespan by protective effects against bacterial proliferation. The classical approach for testing this possibility is to measure lifespan in worms fed on a lawn of dead or growth-inhibited bacteria. As mentioned, all lifespan assays were conducted in the presence of FUDR, which is bacteriostatic and bacteriocidal. Consistently, average lifespan was longer when using FUDR-containing medium as compared to medium lacking FUDR (20.2 days vs 15.9 days, respectively; p<0.0001, Log-Rank). This eliminated the possibility that plumbagin, naphthazarin and oxoline extended lifespan by simply inhibiting bacterial growth. We further probed...
this question by testing the effects of these drugs in the presence of heat-killed bacteria. However, these experiments were confounded by the fact that naphthoquinone toxicity was significantly higher in the presence of heat-killed bacteria (Table S4). There are several explanations for this result. One is that bacteria were protective against naphthoquinone toxicity which was necessary to unmask the hormetic effects in C. elegans. Thus, growth on heat-killed bacteria could have shifted the hormetic dose range to doses that were much lower than those we tested. An alternative explanation is that bacteria enzymatically converted the naphthoquinones into products with hormetic activity in C. elegans. We did not further investigate these possibilities.

Naphthazarin and oxoline extended C. elegans lifespan through different mechanisms

We found that naphthazarin and oxoline, but not menadione, could extend C. elegans lifespan. At doses between 100–500 μM, naphthazarin treatment was associated with increased mean and maximum lifespan (e.g., 200 μM, 13+/−6% increase in mean lifespan, P<0.0001 in each of three trials) (Fig. 6A; Table S1). For oxoline, a 500 μM dose extended mean lifespan by 13–15% in 3 of 4 trials and increased maximum lifespan by at least 10% in all 4 trials. 1 mM oxoline was also associated with significant increases in mean and maximum lifespan (Fig. 6B; Table S1). Comparison of relative Pgst-4::GFP fluorescence and lifespan indicated that
Beneficial concentrations of naphthazarin, oxoline and plumbagin were associated with 50–150% increased Pgst-4::GFP expression (Table 5). However, Pgst-4::GFP expression was not strictly correlated with lifespan extension, as menadione, which also induced Pgst-4::GFP within this range, had no effect on lifespan at low concentrations and was toxic at high concentrations (Fig. 6C). This may reflect additional toxic effects of menadione in vivo.

Because beneficial doses of naphthazarin and oxoline could induce skn-1-dependent Pgst-4::GFP expression at levels similar to those induced by beneficial doses of plumbagin, we tested whether lifespan extension by oxoline and naphthazarin were also skn-1 dependent. Animals treated with skn-1 RNAi lived shorter on 200 μM naphthazarin than controls, with an average 10% decrease in mean and maximum lifespan over 4 trials (Fig. 7A; Table S2). The decrease was more severe in skn-1(zu135) mutants, in which lifespan was only 55±/−10% of controls in four trials (Fig. 7C; Table S1). These results indicate that lifespan extension by naphthazarin was dependent on skn-1, and that skn-1 activity was protective against naphthazarin toxicity. In contrast, skn-1 RNAi did not block lifespan extension by oxoline in 3 of 4 trials (Fig. 7B; Table S2). Additionally, 500 μM oxoline increased mean lifespan by 12%+/−8% in skn-1(zu135) (Fig. 7C; Table S1). The finding that oxoline influenced lifespan independently of skn-1 is consistent with the finding that oxoline did not induce the ARE reporter in HepG2 cells. In daf-16(mgDf50);daf-2(e1370) animals, both 200 μM naphthazarin and 500 μM oxoline increased mean lifespan by 11%+/−2%, indicating that lifespan extension by both compounds was independent of daf-16 activity (Fig 7D; Table S1).

### Discussion

Our findings and those of previous studies [43,44] suggest that interventions activating stress response pathways can reduce cellular damage, thereby slowing aging and increasing lifespan. Phytochemicals that exert cytoprotective effects, such as curcumin [43,46] and sulforaphane [47,48], do so by inducing the Phase 2 detoxification response via NF-E2 transcription factors. Here, we characterized the ability of three naphthoquinones, plumbagin, naphthazarin and oxoline, to extend *C. elegans* lifespan through stress hormesis pathways linked to the antioxidant response. In our study, lifespan extension by plumbagin and naphthazarin was dependent on skn-1 activity. In the absence of skn-1 activity, the beneficial effects of plumbagin were lost. In *C. elegans*, lifespan extension from stress hormesis by thermal stress and juglone-induced oxidative stress also require daf-16 [19,49]. Lifespan extension by plumbagin was weaker and more variable in daf-16 mutants than in wildtype animals, suggesting that daf-16 is a component of the hormetic pathway induced by plumbagin. However, we did not detect daf-16 target gene induction or DAF-16::GFP nuclear localization by hormetic doses of plumbagin. Reduced insulin/IGF-1-like signaling (IIS) from the DAF-2/IIS receptor increases nuclear accumulation of SKN-1 and activates a subset of skn-1 dependent genes (including gst-4) independently of DAF-16 [38]. The interactions between daf-16 and skn-1 complicate efforts to tease apart their individual roles in this pathway. For this reason, further study will be necessary to clearly define the daf-16’s role in lifespan extension by low-dose plumbagin.

One aim of this study was to identify derivatives of a hormetic chemical that display broad beneficial dose ranges and lower toxicity. Such properties would be necessary in order to consider clinical translation of hormetic interventions. By comparing the relative effects of plumbagin on longevity and Pgst-4::GFP expression, we predicted that compounds which increased Pgst-4::GFP expression by 50–150% above basal levels would also extend lifespan. Plumbagin doses that activated expression above this level were toxic, possibly due to excessive levels of oxidative stress and cellular damage. We identified three naphthoquinones, naphthazarin, oxoline and menadione, that could induce Pgst-4::GFP. One of these, naphthazarin, behaved similarly to plumbagin with a broader beneficial dose range, as desired. Naphthazarin extended lifespan at doses which induced Pgst-4::GFP expression to the same range as hormetic doses of plumbagin. Overall, naphthazarin was less potent than plumbagin in *C. elegans*.

Although menadione induced Pgst-4::GFP within the predicted beneficial range, it failed to extend lifespan at any concentration tested. One explanation is that menadione affects cells through multiple mechanisms, which overlap partially with mechanisms for plumbagin toxicity. Findings in *Saccharomyces cerevisiae* are consistent with this idea. Low-dose pretreatment with either plumbagin or menadione induced adaptive responses protective against plumbagin toxicity, while low-dose plumbagin failed to protect from menadione toxicity [50]. These data are consistent with the idea that menadione has additional toxic activities that may mask its potential benefits in *C. elegans*.

Oxoline, the third plumbagin derivative we studied, extended *C. elegans* lifespan through *skn-1* - and *daf-16*-independent mechanisms. Oxoline weakly activated Pgst-4::GFP and failed to activate ARE reporter expression in HepG2 cells. We found oxoline to be non-toxic to *C. elegans* and mammalian cells within the tested dose range. Oxoline belongs to the class of bacteriocidal quinoline antibiotics that inhibit DNA topoisomerase activity. Oxoline has been prescribed for treatment of urinary tract infections from gram-negative bacteria and is used for antiviral therapy [51,52]. Based on this evidence, it is possible that oxoline affects *C. elegans* lifespan through antimicrobial effects.

**Table 4.** Effect of skn-1 RNAi on Pgst-4::GFP induction by plumbagin.

| Expt. | Plumbagin (μM) | RNAi | AMPI | Std. Dev. | Ratio | P | n |
|-------|----------------|------|------|-----------|--------|---|---|
| 1     | 0              | skn-1 | 8.2  | 2.0       | −      | .0001 | 30 |
|       | 25             | skn-1 | 12.9 | 2.2       | 1.57   | .0001 | 30 |
| 2     | 0              | skn-1 | 5.3  | 0.9       | −      | .0001 | 34 |
|       | 100            | skn-1 | 32.2 | 8.6       | 6.08   | .0001 | 32 |
| 3     | 0              | skn-1 | 5.8  | 0.9       | −      | .0001 | 33 |
|       | 100            | skn-1 | 23.0 | 5.1       | 3.94   | .0001 | 22 |

Animals (CL2166) were raised from embryo to adulthood on HT115 bacteria containing either the L4440 RNAi vector or a plasmid inducing skn-1 RNAi. Adults were treated for two days with plumbagin or DMSO vehicle control before GFP visualization.

1 AMPI represents the average whole worm background subtracted mean pixel intensity for the treatment group.

2 AMPI ratio for treatment relative to the L4440 vehicle (DMSO) control.

3 T-test compares the AMPI of each worm in the treatment group with those of the control (0 μM plumbagin, L4440) in that experiment.

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Although the oxidative stress response is molecularly conserved between worms and mammals, we uncovered differences between these systems that call for caution when attempting to extrapolate effects of specific longevity interventions across species. For instance, oxoline induced \( \text{Pgst-4}\cdot\text{GFP} \) expression in \( C. \text{elegans} \) but failed to induce an ARE reporter in mammalian cells. Moreover, sulforaphane and plumbagin had different relative effects on expression of ARE reporter and \( \text{Pgst-4}\cdot\text{GFP} \) levels [21]. Numerous differences between \( C. \text{elegans} \) nematodes and cultured mammalian cells could affect the activities of specific compounds in each system. First, compounds are likely to enter cultured cells more easily than \( C. \text{elegans} \) adults, which are surrounded by an impermeable cuticle. Second, intact \( C. \text{elegans} \) may possess a distinct array of metabolizing enzymes from cultured cells which could alter the production of bioactive metabolic intermediates. Third, the systems differ in mechanisms for excreting compounds from the cellular cytoplasm. Finally, the culture conditions for nematodes and cells are different and expose the compounds to significantly different environments.

We propose that \( C. \text{elegans} \) provides a facile approach to identifying families of compounds that can activate targets with conserved anti-aging or stress response functions. However, individual compounds within such families may have distinct activities reflecting differences in uptake, metabolism and environ-
mental conditions. Ideally, a group of related compounds should be compared to identify those with maximal effectiveness in a particular experimental system or therapeutic context. In this light, our findings positively demonstrate that stress hormesis mechanisms can be accessed for longevity benefits in a whole organism, such as *C. elegans*, and that structural analogs of hormetic compounds may have improved toxicity profiles. However, care must be taken when extrapolating findings about specific compounds between experimental systems.

**Materials and Methods**

**Chemicals**

The following chemicals were obtained commercially, as indicated: plumbagin (#P7262), naphthazarin (#388513), menadione (Fluka #67900), visnagin (#254932), eugenol (#55995), farnesol (#F203), alpha-asarone (#231282), piperine (#P49007), L-canavanine (#C1625); domoic acid (#D6152), precocene II (#194913), sesamin (sesamol, #S8518), juglone (#H47003), 5-hydroxy-1-tetralone (#219975) and 5,8-dimethoxy-1-tetralone (#369658) were obtained from Sigma-Aldrich Co., St. Louis, MO; veratrine (#219834) was from MP Biomedicals, Solon, OH; anabasine (#L18484) was from Alfa Aesar, Ward Hill, MA and marmesan (#CDL 0080-0004) was from Chemical Diversity, San Diego, CA. Oxoline was obtained from Wako Chemicals, Richmond, VA (#328-67371). 7-hydroxy-1-tetralone was obtained from Milestone PharmaTech, New Brunswick, NJ (#2A-0017). For each, chemical characterization was performed to confirm the correct chemical structure and, for agents that proved less than 98% pure, recrystallization and purification procedures were performed to obtain high chemical purity. Chemicals were stored at −20°C as 100 mM stock solutions in DMSO. To prepare *C. elegans* media for experimental treatments, chemicals were added from stock solutions to molten NGM agar and then poured into 3.5-cm culture dishes for use, with the exception of the screen for hormetic chemicals.

**C. elegans** strains and growth conditions

*C. elegans* strains Bristol N2 (wild-type), CL2166 (N2; daf-19 (Pgst-4::gfp; rol-6)), TJ356 (N2; zIs356 (daf-16::gfp; rol-6)), EU31 (skn-1(zu135)/DnT1), and CY312 (daf-16(mgDf50);daf-2(e1370)) were utilized in this work. Strains were grown and maintained at 15°C or room temperature (22-24°C) on Nematode Growth Media (NGM) containing 2.1% agar with *E. coli* strain OP50 as a food source [53], except as indicated for RNAi experiments. Age-synchronized populations were obtained by collecting the eggs laid by fertile adults over 5 to 7 hours and allowing them to develop to the young adult stage before transfer to treatment plates.

**Table 5.** Effects of plumbagin, naphthazarin and oxoline on *Pgst-4::GFP* and lifespan in wildtype *C. elegans*.

| Dose (mM) | Plumbagin | Naphthazarin | Oxoline |
|----------|-----------|--------------|---------|
|          | Ratio *Pgst-4::GFP* | Ratio lifespan | Ratio *Pgst-4::GFP* | Ratio lifespan | Ratio *Pgst-4::GFP* | Ratio lifespan |
| 1        | 0.94      | 1.01         |         |         |         |         |
| 2.5      | 0.97      | 1.03         |         |         |         |         |
| 5        | 1.06      | 1.08         |         |         |         |         |
| 7.5      | 1.06      | 1.08         |         |         |         |         |
| 10       | 1.23      |              |         |         |         |         |
| 15       | 1.29      |              |         |         |         |         |
| 20       | 1.57      | 1.12         |         |         |         |         |
| 25       | 1.79      |              |         |         |         |         |
| 35       | 3.97      | 0.77         |         |         |         |         |
| 50       | 2.70      | 1.07         | 1.01    | 1.02    |         |         |
| 100      | 4.00      | 0.91         | 1.43    | 1.10    | 1.17    | 1.04    |
| 150      | 3.97      | 0.77         |         |         |         |         |
| 200      | 0.16      | 1.63         | 1.12    |         |         |         |
| 250      | 0.12      |              |         |         |         |         |
| 300      | 0.14      |              |         |         |         |         |
| 400      | 0.10      |              |         |         |         |         |
| 500      | 0.14      | 1.63         | 1.15    | 1.54    | 1.11    |         |
| 1000     | 2.07      | 1.12         |         |         |         |         |

*Ratio of *Pgst-4::GFP* fluorescence in animals treated with indicated dose versus vehicle. Data for individual trials is presented in Table S3.

†Ratio of adult lifespan in animals treated with indicated dose versus vehicle. Data for individual trials is presented in Table S1.

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Cell culture methods

The stable Nrf2/ARE reporter cell line, ARE-bla HepG2, was purchased from Invitrogen (Carlsbad, CA, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% dialyzed fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM nonessential amino acids, 25 mM HEPES, 1% penicillin/streptomycin, and 5 μg/ml of blasticidin (Invitrogen) at 37°C in a humidified 5% CO2 atmosphere. Blasticidin was removed from the media during experimental treatments. For cell viability and ARE reporter assays, ARE-bla HepG2 cells were plated in 96-well plates and allowed to adhere overnight. Cell viability was assessed 24 hours after incubation with the indicated concentration of plumagin, naphthazarin and oxoline by Celltiter 96 AQUEOUS One Solution reagent (Promega, Madison, WI, USA). Nrf2-transactivation was measured by beta-lactamase activity according to the manufacturer's instructions (Invitrogen). All experiments were performed in triplicate.

Screen for hormetic prolongevity chemicals

For the initial screen, compounds were suspended to 100 mM in either water or EtOH. 20 μL of each stock solution, diluted in water to 150 μL immediately prior to use, was dropped onto the...
surface of 10 mL NGM agar plates supplemented with live OP50 bacterial food. Plates were incubated at room temperature for 2 hours to allow compounds to diffuse into the media to a final concentration of 200 μM. Approximately 40 sterile fem-1(hc17) adults, raised at 25°C, were suspended in water and dropped onto the surface of test plates. Survival was scored daily for the first week, then at 1–3 day intervals until all animals were dead. Survival assays were terminated as soon as an outcome became apparent relative to paired control populations treated with vehicle alone. Chemicals that were toxic at 200 μM were tested at 100 μM. If the compound was toxic at 100 μM, it was further tested at 60 μM, 30 μM and 10 μM. Compounds that were not toxic at 200 μM were tested at 300 μM, and, if toxic, further tested at 100 μM for possible beneficial effects. Vehicle controls (water or ethanol) were included for all concentrations tested.

Lifespan analyses

In all lifespan assays, worms were grown on NGM agar until day 1 of adulthood, the first day following the L4-adult molt. Young adults were transferred to fresh medium supplemented with indicated compounds or 0.2% DMSO as a vehicle control. For most treatments, the final concentration of the DMSO vehicle was <0.2%. Lifespan assays were performed at room temperature (22–25°C) and survival was scored every 2–4 days as the ability to move in response to touch with a platinum wire. 200 μM FUdR was used in lifespan assays to prevent progeny production, except for experiments with fem-1(hc17) hermaphrodites, which are sterile and do not produce progeny [22,54]. FUdR is also bactericidal and bacteriostatic. Maximum lifespan was calculated by the mean lifespan of the oldest 10% cohort in each experiment. Log-Rank P-values within experiments were calculated using the Survival/Reliability function in JMP 5.0.1.2 (SAS, USA). Significance probability P-values over multiple experiments with the same conditions were calculated with the GLM Procedure in SAS 9.2 (SAS, USA), with treatment and experiment number as the two independent factors for two-way ANOVA, and days of life as the dependent variable.

Microarray Analysis

Approximately 1,000 age-synchronized young adults were treated for two days with either 100 μM plumbagin or DMSO as a vehicle control and then washed three times in M9 and flash frozen with dry ice. RNA was isolated with the Absolutely RNA Miniprep Kit (Agilent Technologies, USA). RNA concentration and quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA). Microarray hybridizations were performed with the C. elegans 4x44K Oligo Microarray (Agilent Technologies, USA). Raw microarray hybridization intensity data from four separate experiments were log-transformed and normalized to generate z-scores and subsequent z-ratios [55].

RNA interference

RNA interference (RNAi) was induced by providing a food source of HT115 bacteria expressing double-stranded RNA corresponding to the targeted gene [56]. The RNAi medium was NGM agar supplemented with 100 μg/mL ampicillin and 1 mM isopropyl β-D-thiogalactoside (IPTG) to induce bacterial expression of the dsRNA trigger. Young adult animals (Po) were placed onto RNAi plates for several hours to lay eggs and then removed. Larvae developed to young adulthood on RNAi plates and were then transferred to fresh RNAi plates supplemented with test compounds or vehicle (DMSO). In this way, worms continued feeding on dsRNA-expressing bacteria throughout the experiment.

Analysis of Pgst-4::GFP expression

Young CL2166 adults were transferred onto NGM agar supplemented with test compounds or vehicle (DMSO). After two days at room temperature (22–24°C), animals were mounted on 2% agarose pads and immobilized with 20 mM levamisole. Using a Nikon E900 microscope (Nikon Corporation, Japan), GFP fluorescence was visualized with a mercury illumination source at 100× magnification (10X objective, 10X ocular magnification). Images were captured using Openlab software, v. 5.5.0 (Improvision Inc., USA) controlling a Hamamatsu Orca CCD camera (Hamamatsu Photonics, Japan). Image processing was performed using Adobe Photoshop CS5 (Adobe Systems Inc., USA). Levels of Pgst-4::GFP were measured as average mean pixel intensity (AMPI) over the whole worm using ImageJ software [Rashband, W.S., U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/]. AMPI values were normalized by subtracting the background intensity of the slide as measured by the minimum pixel intensity in each image. Statistical significance was judged using a two-tailed student’s t-test in Excel (v.12.2.4, Microsoft, USA).

Supporting Information

Table S1 Lifespan assays were conducted as described in Methods. Control lifespan varied by experiment, and individual experiments can be identified by control mean lifespan; (+) treatment; (−) DMSO vehicle control. * Mean lifespan of the 90th percentile. ^ Log-Rank Probability.

Table S2 † Animals were grown from embryos on feeding RNAi vector control bacterial strain L4440 or RNAi bacteria expressing skn-1 dsRNA. Day 0 adults were transferred to treatment plates with 200 μM FUdR to prevent progeny production; (+) treatment; (−) DMSO vehicle control. * Mean lifespan of the 90th percentile. ^ Log-Rank Probability.

Table S3 CL2166 animals were grown to adulthood and treated for two days as indicated. (+) Treatment; (−) DMSO vehicle control. * AMPI represents the average whole worm background-subtracted mean pixel intensity for the treatment group. † Ratio given is the AMPI for the treatment group relative to the DMSO control. ^ T-test.

Table S4 † P, plumbagin; O, Oxoline; N, naphthazarin. (+) Bacteria killed at 65°C for 1 hour; (−) Live bacteria spread onto plates; both (+) and (−) plates contained FUdR to prevent progeny production and control bacterial growth.

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Author Contributions

Conceived and designed the experiments: PRH TGS MAW Q-SY WHW YZ KGB NHG MPM SC CAW. Performed the experiments: PRH TGS MAW Q-SY WHW YZ SC. Analyzed the data: PRH TGS MAW YZ KGB NHG MPM SC CAW. Contributed reagents/materials/analysis tools: Q-SY WHW YZ KGB NHG. Wrote the paper: PRH SC CAW.
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