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Original Article

Nordihydroguaiaretic Acid Extends the Lifespan of *Drosophila* and Mice, Increases Mortality-Related Tumors and Hemorrhagic Diathesis, and Alters Energy Homeostasis in Mice

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Abstract

Mesonordihydroguaiaretic acid (NDGA) extends murine lifespan. The studies reported here describe its dose dependence, effects on body weight, toxicity-related clinical chemistries, and mortality-related pathologies. In flies, we characterized its effects on lifespan, food consumption, body weight, and locomotion. B6C3F1 mice were fed AIN-93M diet supplemented with 1.5, 2.5, 3.5, or 4.5 g NDGA/kg diet (1.59, 2.65, 3.71 and 4.77 mg/kg body weight/day) beginning at 12 months of age. Only the 3.5 mg/kg diet produced a highly significant increase in lifespan, as judged by either the Mantel–Cox log-rank test (p = .008) or the Gehan–Breslow–Wilcoxon test (p = .009). NDGA did not alter food intake, but dose-responsively reduced weight, suggesting it decreased the absorption or increased the utilization of calories. NDGA significantly increased the incidence of liver, lung, and thymus tumors, and peritoneal hemorrhagic diathesis found at necropsy. However, clinical chemistries found little evidence for overt toxicity. While NDGA was not overtly toxic at its therapeutic dosage, its association with severe end of life pathologies does not support the idea that NDGA consumption will increase human lifespan or health-span. The less toxic derivatives of NDGA which are under development should be explored as anti-aging therapeutics.

Key Words: NDGA—Lifespan—Longevity—Dose–response—Therapeutic

Nordihydroguaiaretic Acid (NDGA) is a lignin which constitutes about 12.5% of the dry weight of the leaves and twigs of the creosote bush, Larrea tridentate [Reviewed in (1–4)]. Aqueous extracts of creosote leaves and twigs have been used medicinally by indigenous North American tribes to treat over 50 health disorders, ranging from colds to cancer (2–4). NDGA was once classified as "generally recognized as safe" by the Federal Food and Drug Administration, and used as an antioxidant food additive. This classification was withdrawn after studies in rats showed that NDGA produced serious kidney toxicity and other pathologies, including stunted growth and internal hemorrhages [(5); Reviewed in (6)]. Human consumption of creosote leaf and stem extracts as dietary

supplements led to cases of hepatitis, cirrhosis, and fulminant liver failure (4,7,8). Despite these findings, a recent Google internet search identified multiple vendors selling creosote leaf extracts as medicinal health aids.

In vitro studies have shown that NDGA is an inhibitor of intercellular inflammatory signaling, tumor cell proliferation, insulin-like growth factor-1 (IGFIR) and HER2 receptor activation, and oxidative phosphorylation (4,9). Based on the therapeutic potential suggested by these results, the National Institute of Health Interventions Testing Program (NIH-ITP) undertook studies of the effects of NDGA on murine lifespan (10–12). They found that 2.5 g of NDGA/kg diet produced a significant 12% increase in median lifespan for male mice, but not females (12). A second study found no effect on the lifespan of female mice (11). A third study censored after 70% mortality suggested that multiple NDGA doses may extend the lifespan of male mice (10). No necropsy, pathology, or toxicology results were reported, nor was food consumption reported. In *Drosophila*, a single dose study found a nonsignificant increase in lifespan (13).

Thus, the effects of NDGA dosage on lifespan, and its effects on food consumption, end of life pathologies, energy disposition, and phylogenic conservation of the response are unclear. For these reasons, we conducted dose–response studies of the effects of NDGA on lifespan in mice and *Drosophila melanogaster* (*Drosophila*). We also investigated the effects of NDGA on food intake, body weight, and mortality-related pathologies in mice. In *Drosophila*, we characterized its effects on lifespan, food consumption, body weight, and locomotion Induced caloric restriction (CR) is a possible explanation for lifespan responses when food consumption is not monitored (14).

Methods

Mouse Studies

Mouse lifespan, weight, and food consumption were monitored as described in detail previously (15). Briefly, male B6C3F1 mice (Harlan Breeders; Indianapolis) were randomly assigned to treatment groups at 12 months of age. Two hundred ninety-seven control mice were shifted from ad libitum chow feeding (Diet # 5001, Purina Mills, Richmond, IN) to daily feeding with 13.3 kcal/day/mouse of control diet [American Institute of Nutrition (AIN)-93M diet; Diet No. F05312; Bio-Serv, Frenchtown, NJ]. Four groups of 18 mice each were shifted to daily feeding with an identical quantity of control diet supplemented with NDGA at either 1.5, 2.5, 3.5, or 4.5 g per kg diet (approximately 1.59, 2.65, 3.71, 4.77 mg/kg body weight/day, respectively). All mice were fed daily. Food consumption and health were monitored at the time of feeding, and any uneaten food noted. With few exceptions, all food was eaten each day. NDGA was mixed with the powdered diet and cold-pressed into 1 g pellets by Bio-Serv. The food was stored moisture free at 4 °C until used. The mice had ad libitum access to tap water, which was acidified (pH 4.0) to reduce colonization by *Pseudomonas* (16,17). Mice were weighed bimonthly. The health of the mice was examined twice daily by laboratory staff and weekly by a veterinarian. Dead mice were stored at –20 °C until necropsy. This study was approved by the Institutional Animal Care and Use Committee at the University of California, Riverside.

Statistical Analysis

These lifespan studies utilized an unbalanced statistical design to minimize the number of mice per test group while maintaining statistical power (18). Unbalanced designs have economic and logistic advantages for comparing multiple treatments to a common control (18). The group sizes in this study are similar to those required for a Weibull survival analyses with a 75% probability of detecting an 10% increase in mean lifespan with a 1% probability of a false positive ($\alpha \leq .01$). The Weibull analysis is more stringent than the comparison of Kaplan–Meier survival curves using the Mantel–Cox or Gehan–Breslow–Wilcoxon tests, implemented in GraphPad Prism 5.01, which are used here. The significance of the differences in body weights between the treated and control groups was judged using a linear mixed effects model (19,20) as described previously (15). In brief, we modeled the mean response by a set of fixed effects assumed to be shared by mice and a set of random effects that are unique to a particular mouse. Additionally, our model imposed a common intercept since all mice were on the same diet at the time of the first measurement. To determine which group weights were significantly different than those of the controls, a Bayesian Information Criterion (BIC) model selection criteria, a likelihood ratio test (LRT), and an Akaike's Information Criterion (AIC) model selection criteria were used (Table 1 and *Results*). Food consumption was determined by totaling the amount eaten by each treatment group during each time period, adjusted for the number of mice. This value was divided by the per mouse amount eaten by the control mice. The significance of the necropsy results was determined using Fisher's exact test.

Drosophila Studies

Drosophila (Wild-type Oregon-R-C; Bloomington Drosophila Stock Center, Department of Biology, Indiana University, Bloomington, IN) lifespan determinations were performed as described in detail previously (21,22), using 0, 1, or 3 mg/mL NDGA. Capillary Feeder (CAFE) assays (23) and fecal plaque assays (FPAs (24,25)) were performed as described (26). The flies were replaced every 6 months

Table 1. Summary of the Statistical Analysis of Mouse Group Weights (Figure 2) Using BIC Model Selection Removing Each Diet Individually

Diet	Df*	AIC†	BIC‡	*X*	Chi Df§	Pr ($\chi_{df}^2 > X$)
Control	22	23,281	23,424			
20% CR	20	23,301	23,431	24.081	2	5.9e–06
40% CR	20	23,521	23,651	243.54	2	<2.2e–16
1.5 g/kg diet	20	23,285	23,415	7.7943	2	0.0203
2.5 g/kg diet	20	23,307	23,437	29.71	2	3.537e–07
3.5 g/kg diet	20	23,289	23,419	11.438	2	0.003283
4.5 g/kg diet	20	23,350	23,480	72.912	2	< 2.2e–16

*Degrees of freedom.
†Akaike's Information Criterion.
‡Bayesian Information Criterion.
§Chi-squared degrees of freedom.

with new stocks from the supplier. The effect of NDGA on FPA plaque size was determined as described (21,22). The effects of NDGA on locomotor activity were determined as described previously (26). Briefly, one day old male flies were exposed to 3 mM NDGA in DMSO or vehicle treatments in 95 × 25 mm glass vials for 3 days (at 25 °C with 12 hour light/dark cycle). After 3 days, movement of the flies was monitored for 72 hours using a TriKinetics Locomotor Activity Monitor (LAM 32). Beam breaks were recorded using the accompanying software. Means ± SEM were calculated for 30 minutes time increments over the entire 72 hour trial (144 recordings). An unpaired, two-sample *t* test was used to determine the significance of differences between experimental and control treatments using GraphPad Prism 5.

Results

Lifespan Results

Male mice were fed NDGA in their food at 1.5, 2.5, 3.5, or 4.5 g/kg diet (160, 267, 373, 480 mg/kg bw/d), beginning at 12 months of age. Median lifespan was extended by 12% at 373 mg/kg bw/d (~13.5 mg/mouse/day; Mantel–Cox p = .008; Gehan–Breslow–Wilcoxon p = .009; Figure 1). The Gehan–Breslow–Wilcoxon test gives more weight to deaths at early time points, while the Mantel-Cox test gives equal weight to all time points. Despite its effects on median lifespan, we found no effect of 373 mg of NDGA/kg bw/d (3.5 g/kg diet) on maximum lifespan using the method of Gao et al. (27). Maximum lifespan is variously defined as the lifespan of the longest lived 10% or 20% of a cohort. Here, we eliminated the lifespan data below a pooled quantile of 0.8. The resulting Mann–Whitney U test had a p-value of .8932.

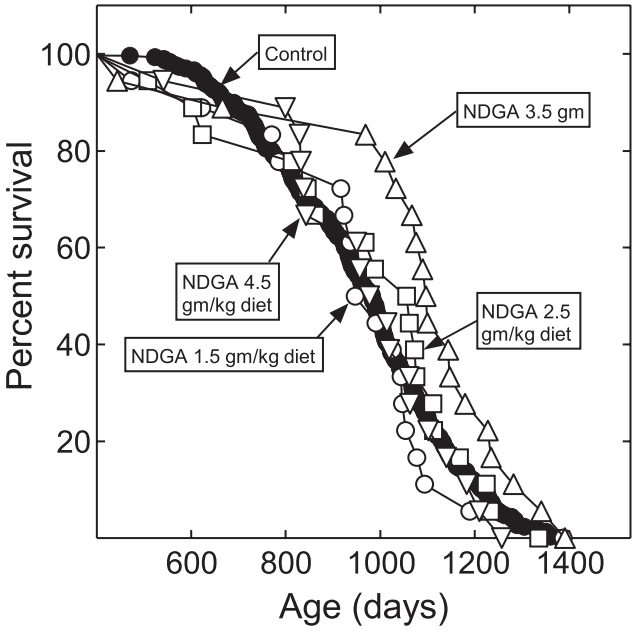

Figure 1. Shown are the lifespans of mice consuming AIN-93M diet without additions (filled circles; median survival 983 days); mice consuming 1.5 g of NDGA/kg AIN-93M diet (open circles; median survival 969 days; Mantel–Cox p = .769; Gehan–Breslow–Wilcoxon p = .991); mice consuming 2.5 g of NDGA/kg diet (open squares; median survival 1059 days; Mantel-Cox p = .528; Gehan–Breslow–Wilcoxon p = .490); mice consuming 3.5 g of NDGA/kg diet (open upward pointing triangles; median survival 1099 days; Mantel-Cox p = .008; Gehan–Breslow–Wilcoxon p = .009); and mice consuming 4.5 g of NDGA/kg diet (open downward pointing triangles; median survival 995 days; Mantel–Cox p = .963; Gehan–Breslow–Wilcoxon p = .647). The controls began with 297 mice and the treatment groups with 18 mice each. The treatments were started at 365 days of age.

The dose of NDGA which extended median lifespan is within 12% of that initially reported to extend the lifespan of the NIH-ITP mice (~417 mg/kg bw/d (12)). In our study, only this dose extended lifespan (Figure 1). Recently, the NIH-ITP reported lifespan extension using censored data at higher and lower dosages. Further study will be needed to resolve these differences.

The statistical design and animal husbandry used in these studies have been described in detail elsewhere (15,18,26,28).

Food Consumption and Body Weight

Food consumption and body mass were monitored throughout the study (Figure 2). The mice ate essentially all their food each day, with the exception of a single excursion by the 1.5 g/kg diet group late in life. This is consistent with the instability of the weights in late life due to the smaller number of mice and the presence of more age-related pathologies.

The weights of the mice were analyzed using a mixed effects model with a BIC model selection criterion, as described previously [Table 1; Reference (15)]. These results suggest that the weights of the 2.5 and 4.5 g/kg diet-treated mice, as well as the 20% and 40% CR mice were reduced significantly relative to those of the control group (Figure 2; Table 1). The differences between the weights of the control and the 1.5 and 3.5 g/kg diet-treated groups were close to significance by this test (Table 1). A LRT suggests that the weights of all the groups except the 1.5 g/kg diet group differed significantly from those of the control group. An AIC model selection criteria suggests that all the diets groups differed significantly from the control group (Table 1).

The group consuming the highest dose of NDGA weighed less than the 20% calorically restricted (20% CR) group at younger ages, when the data were more accurate due to sample sizes. This weight reduction occurred without reduced food intake by the NDGA-treated mice. Thus, NDGA appears to alter either calorie absorption or utilization by the mice.

Pathologies Associated with Mortality

Death-related pathology has not been reported previously (10–12). The results of the necropsies from our study are summarized in Tables 2 and 3. Focusing on the aggregated results for the pathologies for all the groups, there was a highly or very highly significant increase in liver and lung tumors, enlarged thymuses (indicative of thymomas or lymphomas), and hemorrhage into the peritoneum (hemorrhagic diathesis; Table 2). There was not an obvious dose–response relationship for these pathologies, likely due to the relatively small group sizes. However, even the lowest dose of NDGA produced significant pathologies (Table 2). NDGA decreased the size of liver tumors by 24% relative to those found in the control mice, suggesting that it may decrease the rate of tumor growth. However, the total mass of the tumors per mouse was unchanged, suggesting it may increase hepatic tumorigenesis (Tables 2 and 3; see *Discussion* below).

Clinical Chemistries

In view of the clinical evidence of hepatotoxicity and nephrotoxicity in humans and rodents consuming NDGA, we investigated whether NDGA produced signs of toxicity at the concentrations used in these lifespan studies. Male B6C3F1 mice were treated with NDGA or control diet for 9 weeks, and clinical chemistries performed. The results are shown in Table 4. Little evidence for toxicity was found. Alanine

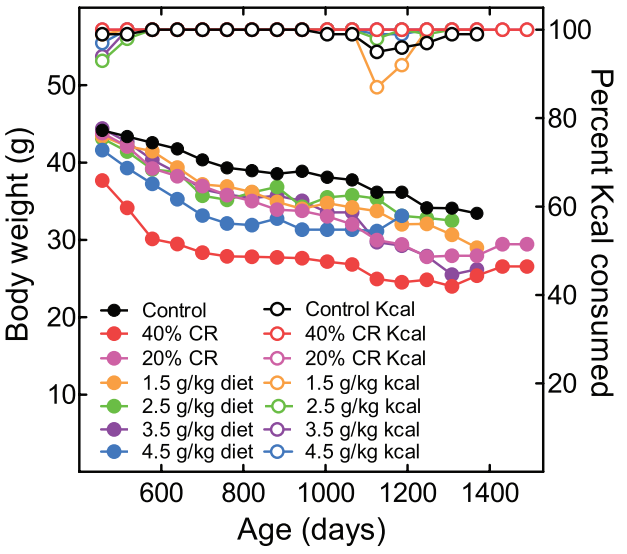

Figure 2. Food consumption and body weight of the mice shown in Figure 1. Shown is the body weight of the control (closed black circles); 40% CR fed (closed orange circles); 20% CR fed (closed fuchsia circles); 1.5 g NDGA/kg diet (closed gold circles); 2.5 g NDGA/kg diet (closed green circles); 3.5 g NDGA/kg diet (closed purple circles); and 4.5 g NDGA/kg diet (closed blue circles). The standard deviations of the weights were omitted for clarity. Food consumption as a percent of the Kcal offered with respect to the Kcals consumed during the preceding month is shown for control, (open black circles); 40% CR fed (open orange circles); 20% CR fed (open fuchsia circles); 1.5 g NDGA/kg diet (open gold circles); 2.5 g NDGA/kg diet (open green circles); 3.5 g NDGA/kg diet (open purple circles); and 4.5 g NDGA/kg diet (open blue circles). The significance of differences in body weights between groups are shown in Table 1. A preliminary report of these data from 365 to 1,063 days of age was published previously (14).

transaminase (ALT) was significantly decreased by the higher doses of NDGA, which is the opposite of what would be expected if the drug was toxic to the liver or kidneys. Aspartate transaminase (AST), alkaline phosphatase, bilirubin, and creatinine levels, which can also be signs of toxicity if elevated, were unchanged.

Triglyceride and glucose levels were elevated. Triglyceride elevation can be a sign of compromised liver or kidney function. However, the elevated triglyceride and glucose levels seem more likely to have resulted from the alterations in energy homeostasis evinced by the decrease in body weight in the absence of a change in caloric consumption (Figure 2; Table 1).

Conservation of the Lifespan Response

To further characterize the effects of the drug, we investigated its effects on the lifespan of *Drosophila*. Optimum oral treatment with NDGA at 3.0 mg/mL medium increased the median lifespan of the flies by approximately 23% (Figure 3). Similar results were replicated in two additional trials. These results suggest that at least some of the target(s) of NDGA action are phylogenically conserved between mice and flies. The relatively short lifespans of the control flies are the result of using large flybottles, which induce more flight time (29), male flies only (30), mildly elevated incubation temperatures (31), and food with a high protein concentration (32–34). Each of these parameters somewhat shortens lifespan, although the flies remain responsive to compounds that lengthen lifespan [e.g. References (21,22,26)].

Because drug induced CR or reduced locomotor activity might increase *Drosophila* lifespan (29,35), we investigated these parameters in NDGA-treated flies. We found no effect of NDGA on food consumption (Tables 5 and 6), body weight (Table 7), or locomotor activity (Table 8). Food consumption was quantified in two ways. We used our modifications of the CAFE assay (Table 5) and the *Fecal Plaque Assay* [FPA; Table 6; References (21,22)]. No effect on food consumption was found by either technique. Long-term NDGA treatment also did not change body weight (Table 7). Locomotor activity was quantified using a LAM 32 Activity Monitor (TriKinetics) as described previously [Table 8; Reference (26)]. There was no significant difference between the activity of flies consuming food containing NDGA or an equivalent volume of vehicle. Together, these results indicate that the effects of NDGA on *Drosophila* lifespan were not due to CR or altered locomotor activity. Further, we did not detect a disequilibrium between food intake and weight in this species as we did in the mice.

Discussion

The results presented here show that NDGA can extend murine and *Drosophila* lifespan. In our study, the therapeutic window for this longevity effect was narrow in mice. NDGA consumption, even at doses below its therapeutic dose, was associated with an increase in multiple tumor types and hemorrhagic diathesis. The drug also increased the lifespan of *Drosophila*, suggesting its target(s) and mechanism(s) of action may be phylogenetically conserved. However, aspects of the response in flies were unlike those in mice. In flies, NDGA did not appear to affect energy uptake or utilization.

Drug Dosages

In our studies, 3.5 g/kg diet (373 mg NDGA/kg bw/d) extended lifespan, but higher and lower dosages were ineffective. The effective dose in *Drosophila* was 3.0 mg NDGA/kg food. The NIH-ITP found that NDGA extended male mouse lifespan at 2.5 g NDGA/kg diet, which they estimated supplied approximately 417 mg/kg bw/d (10,11). However, the actual dose was uncertain because food intake was not continuously measured in this study. More recently, they report that NDGA at 0.8 or 5.0 g/kg food produced lifespan effects similar to that of 2.5 g/kg diet. These data were censored at 30% survival, and thus further study will be required to determine the dose response range of the lifespan effect in mice (12).

Toxicity

The NIH-ITP did not report necropsy or toxicity studies for their NDGA-treated mice (10,12). Based on the mortality-related pathologies we found, there was an association between NDGA consumption and an increased incidence of liver, lung, and thymus tumors, and increased hemorrhagic diathesis. There was little evidence for acute liver or kidney toxicity as measured by serum chemistries. However, others reported that treatment of mice with 5.0 g NDGA/kg diet, a concentration reported to extend lifespan by the NIH-ITP, stunted the growth of male and female mice, and induced inflammatory cecal lesions, hemorrhages, and cysts in rats [reviewed in (6)]. Thus, the doses of NDGA which extend murine lifespan appear to overlap the dosages which produce serious pathologies.

In humans, the consumption of dietary supplements containing creosote leaf and stem extracts resulted in toxic hepatitis, cirrhosis, and fulminant liver failure (4,7,36,37). However, NDGA also was found to suppress signaling through the IGFIR and to suppress the androgen-stimulated growth of human prostate cancer cells in culture (38–40). These studies led to clinical investigation of its safety and efficacy for the treatment of prostate cancer (41,42). In humans, continuous oral dosing at up to 2.5 g/d (~31 mg/kg bw/d), one-tenth of the dose which extended lifespan in this study in mice, was tolerated, except for the development of elevated AST or ALT (*transaminitis*) in some patients

Table 2. Necropsy Results from the Mouse Longevity Studies Shown in Figure 1

		Diet Treatment (*n*)															
		Control (*n* = 72)*		NDGA (1.5 g/kg diet) (*n* = 18)			NDGA (2.5 g/kg diet) (*n* = 18)			NDGA (3.5 g/kg diet) (*n* = 18)			NDGA (4.5 g/kg diet) (*n* = 18)			NDGA (All Groups) (*n* = 54)		
Organ	Pathology	#†	%‡	#	%	*p* value§	#	%	*p* value	#	%	*p* value	#	%	*p* value	#	%	*p* value
Spleen	Enlarged/tumorous	43	59.7	13	72.2	.4199	14	77.8	.1822	13	72.2	.4199	9	50.0	.5947	49	68.1	.3858
Liver	Tumor	22	30.6	10	55.6	.058	9	50.0	.1656	9	50.0	.1656	10	55.6	.0580	38	52.8	**.0109**
	Enlarged/fatty liver	3	4.2	1	5.6	1.0000	0	0.0	1.0000	2	11.1	.2603	1	5.6	1.0000	4	5.6	1.0000
	Hemangioma	7	9.7	0	0	.3371	0	0.0	.3371	1	5.6	1.0000	0	0.0	.3371	1	1.4	.0628
Intestinal	Tumor	9	12.5	1	5.6	.6799	7	38.9	**.0154**	1	5.6	.6799	4	22.2	.2841	13	18.1	.4878
Lung	Tumor	11	15.3	9	50.0	**.0034**	7	38.9	**.0437**	6	33.3	.0975	9	50.0	**.0034**	31	43.1	**.0004**
Penis	Necrosed/inflamed	6	8.3	0	0	.3427	0	0.0	.3427	1	5.6	1.0000	0	0.0	.3427	1	1.4	.1158
Seminal vesicles	Enlarged	3	4.2	4	22.2	**.0275**	1	5.6	1.0000	5	27.8	**.0072**	0	0.0	1.0000	10	13.9	.0777
Bladder	Distended	12	16.7	1	5.6	.4524	2	11.1	.7272	3	16.7	1.0000	0	0.0	.1146	6	8.3	.2069
Kidneys	Enlarged/tumorous	3	4.2	3	16.7	.0917	3	16.7	.0917	2	11.1	.2603	3	0.0	.0917	11	15.3	**.0457**
Thymus	Enlarged	2	2.8	2	11.1	.1772	6	33.3	**.0006**	2	11.1	.2603	3	16.7	.0527	13	18.1	**.0048**
Skin/ abdominal cavity	Fibroma	4	5.6	1	5.6	1.0000	1	5.6	1.0000	1	5.6	1.0000	0	0.0	.5799	3	4.2	1.0000
Peritoneum	Hemorrhage	11	15.3	8	44.4	**.0195**	9	50.0	**.0034**	4	22.2	.4894	5	27.8	.2985	26	36.1	**.0071**

*Number of necropsied mice in each treatment group. Not all mice in the control group were necropsied. The necropsied control mice approximated the distribution of ages in the treatment groups.

†Number of necropsied mice in each treatment group with the indicated pathologies.

‡Percent of the necropsied mice in each treatment group with the indicated pathologies.

§Significance of the differences from the control values were determined using Fisher's exact test. For convenience, values which were significantly different are in bold type.

Table 3. Liver Tumor Mass of the Mice Shown in Table 2 and Figure 1

Liver tumors	Control (n = 72)	NDGA (1.5 g/kg diet) (n = 18)	NDGA (2.5 g/kg diet) (n = 18)	NDGA (3.5 mg/kg diet) (n = 18)	NDGA (4.5 mg/kg diet) (n = 18)	NDGA (All groups) (n = 72)
Mean mass of each tumor ± SEM (g)*	1.06 ± 0.18	0.78 ± 0.23 (p = .0897)†	**0.35 ± 0.17** (p = .0003)	**0.66 ± 0.29**(p = .0062)	0.81 ± 0.27 (p = .1177)	**0.66 ± 0.12** (p = .0010)
Liver tumor mass/number of mice with tumors (g)	1.2	1.2	0.5	1.2	1.4	1.1

*Tumor mass was calculated as $(\pi/6) \times l \times w \times h \times 1.0$g, where l is length, w is width, and h is the height of each tumor. One cm^3 = 1 gram.
†Significance of the difference from the control value, calculated using the Mann–Whitney U test. For convenience, significant changes are in bold.

(42). Drug dosages in rodents are often scaled down approximately 10-fold in mg per kg body weight for investigational studies in humans (14). In a phase II study of men with non-metastatic hormone-sensitive prostate cancer, oral doses of 2.0 g NDGA/d in 28 day cycles produced transaminitis in 8 patients (67%). Transaminitis suggests the treatments produced mild liver or kidney toxicity. The development of less toxic derivatives of NDGA, such as tetra-O-methyl nordihydroguaiaretic acid, are underway for potential use as cancer therapeutics (43,44).

Possible Mechanisms for the Longevity Effects of NDGA

A large number of molecular mechanisms have been proposed to explain the presumed, but as yet largely undocumented, health benefits of NDGA [Reviewed in (4)].

mTOR

Perhaps the most probable mechanism for the longevity effects of NDGA is its inhibitory effects on the activity of mammalian target of rapamycin complex 1 (mTORC1 (45)). mTORC1 inhibition is a well-established mechanism for the extension of mouse and *Drosophila* lifespan (22,46–49). A recent review of the literature argues that rapamycin inhibition of mTORC1 extends mouse lifespan by suppressing cancer formation and growth (50). However, it will be important to discover whether the dosages of NDGA that extend lifespan actually inhibit mTORC1 activity in tissues shown to be important to its longevity effects.

Tumor Growth

In our studies, NDGA decreased the mass of liver tumors by 24% relative to those found in control mice, suggesting that it decreased the rate of liver tumor growth (Table 3). These observations are consistent with in vitro and in vivo studies showing that NDGA inhibits the growth and proliferation, and induces the apoptosis of multiple cancer cell types (51–54). These effects likely result from the inhibitory effects of NDGA on growth factor signaling (38–40). In mammals, NDGA inhibits IGFIR activation, IGFIR and HER2 tyrosine kinase activity, androgen dependent growth of cultured prostate tumor cells, and growth of cultured HER2-overexpressing human breast cancer cells (38–40).

In our studies, NDGA appeared to increased hepatic tumor number (Tables 2 and 3), and to increase the prevalence of lung and thymus tumors (Table 2). Taken together, the data suggest that while NDGA reduces tumor growth rates, it increases tumor formation.

IGF-I

Inhibition of IGFI receptor signaling can enhance rodent lifespan, although these effects are sex and background dependent (55,56). There is less of an effect on lifespan on the C57BL/6J background than on the 129/SvPas background (56). Female mice heterozygous null for the IGF1R exhibit extended longevity, while their male counterparts do not (56,57). In other studies, neither male nor female mice with reduced IGFI levels experience an increase in mean lifespan (55). Together, these results suggest that if a lifespan effect is observed when IGFR activity is inhibited, females, rather than males respond. Thus, this response is the opposite of that found with NDGA, where males rather than females respond. Thus, reduced IGFIR activity alone is unlikely to be the major mechanism by which NDGA extends murine lifespan.

Hormesis

It is possible that NDGA induces a hormetic response which leads to increased lifespan. Hormetic stimuli are thought to produce molecular damage at low doses, which stimulate increased maintenance and

Table 4. Clinical Chemistries on Serum from Control and NDGA Fed Mice*

Test†	CON‡	NDGA 1.5 gm/kg		NDGA 2.5 gm/kg		NDGA 3.5 gm/kg		NDGA 4.5 gm/kg		All		Two Lowest		Two Highest	
		Result	*p*	Result	*p*§	Result	*p*	Result	*p*	Result	*p*	Result	*p*	Result	*p*
Alanine Amino-transferase (U/L)	42.9 ± 13.5	38.4 ± 19.3	.641	26.5 ± 3.6	**.017**	23.4 ± 8.1	**.012**	28.6 ± 8.0	**.042**	29.5 ± 12.1	**.042**	32.4 ± 14.6	.137	26.2 ± 8.1	**.018**
Aspartate Amino-transferase (U/L)	175.7 ± 80.0	134.5 ± 38.7	.261	134.5 ± 38.7	.261	97.6 ± 14.5	**.045**	125.2 ± 43.8	.184	124.0 ± 37.0	.149	134.5 ± 36.9	.239	112.7 ± 35.4	.090
Alkaline Phosphatase (U/L)	92.9 ± 19.7	122.9 ± 25.5	**.043**	110.6 ± 22.0	.159	102.2 ± 16.2	.390	97.1 ± 25.2	.744	108.5 ± 23.5	.107	116.8 ± 23.6	**.033**	99.5 ± 20.7	**.050**
Blood Urea Nitrogen (mg/dL)	19.8 ± 2.2	21.0 ± 4.3	.571	19.2 ± 2.7	.676	23.6 ± 3.4	**.070**	22.6 ± 4.3	.197	21.5 ± 3.9	.161	20.1 ± 3.6	.836	23.1 ± 3.8	**.036**
Cholesterol (mg/dL)	201.3 ± 31.6	228.0 ± 20.8	.098	231.7 ± 32.9	.121	210.5 ± 8.0	.485	191.8 ± 18.5	.519	215.7 ± 26.6	.305	229.9 ± 26.3	.071	200.3 ± 17.1	.941
Creatinine (mg/dL)	0.092 ± 0.024	0.085 ± 0.010	.499	0.087 ± 0.020	.668	0.109 ± 0.020	.226	0.097 ± 0.022	.693	0.094 ± 0.020	.872	0.086 ± 0.015	.554	0.103 ± 0.021	.370
High Density Lipoprotein (mg/dL)	211.8 ± 31.0	229.2 ± 17.2	.233	233.1 ± 27.0	.214	215.6 ± 14.4	.780	202.7 ± 18.2	.528	220.4 ± 22.4	.515	231.2 ± 21.7	.178	208.6 ± 17.1	.808
Low Density Lipoprotein (mg/dL)	23.5 ± 10.3	51.4 ± 17.4	**.011**	46.0 ± 17.2	**.026**	31.1 ± 7.1	.165	23.8 ± 8.8	.954	38.2 ± 17.3	**.013**	48.7 ± 16.7	**.001**	26.8 ± 8.7	.499
Bilirubin (mg/dL)	0.046 ± 0.023	0.061 ± 0.035	.417	0.075 ± 0.019	**.031**	0.058 ± 0.035	.546	0.036 ± 0.018	.368	0.057 ± 0.030	.321	0.068 ± 0.028	.088	0.046 ± 0.028	.964
Total Protein (g/dL)	5.88 ± 0.49	6.18 ± 0.29	.210	6.41 ± 0.29	.042	6.40 ± 0.63	.167	6.06 ± 0.27	.427	6.26 ± 0.39	.103	6.30 ± 0.30	.081	6.22 ± 0.47	.181
Triglyceride (mg/dL)	47.1 ± 7.6	32.9 ± 10.1	**.020**	45.9 ± 15.4	.865	72.1 ± 27.9	.122	70.7 ± 22.8	.060	54.7 ± 25.0	.215	39.4 ± 14.2	.143	71.3 ± 23.9	**.009**
Glucose (mg/dL)	145.7 ± 29.3	184.3 ± 13.2	**.014**	173.0 ± 36.1	.173	210.6 ± 33.6	**.010**	170.0 ± 44.8	.289	183.3 ± 35.2	**.016**	178.7 ± 26.5	**.033**	188.5 ± 43.6	**.025**

Notes. CON = control; *p* = *p*-value.

*Eight male B6C3F1 mice were treated with NDGA at each dosage or with control diet for 8 weeks. At 21 months of age they were bled by cardiac puncture. Clinical chemistries were performed on the 6–8 mice in each group with no obvious signs of pathology at necropsy.

†Blood glucose levels were measured with the FreeStyle Lite Blood Glucose Monitoring System (Abbot Laboratories). The other blood tests were performed by the Comparative Pathology Laboratory, University of California, Davis.

‡All, results and *p*-values for all NDGA treated groups considered together; Two Lowest, the number of pathologies and the *p*-values for the 1.5 and 2.5 g/kg diet fed mice considered together; Two Highest, the number of pathologies and *p*-values for the 3.5 and 4.5 g/kg diet fed mice considered together.

§Calculated using unpaired two-sample *t* tests For convenience, significant changes are indicated in bold.

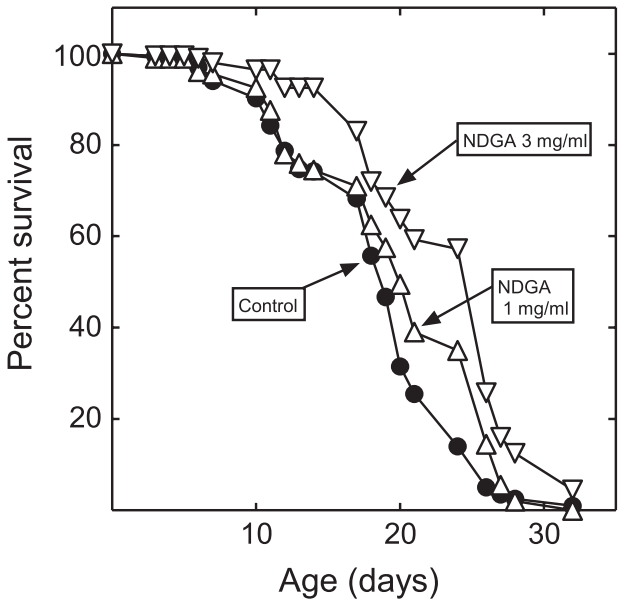

Figure 3. NDGA dose-responsively extends the lifespan of male *Drosophila*. Shown is the survival of *Drosophila* fed diets containing either vehicle alone (closed circles; median survival 20 days), vehicle containing 1.0 mg/mL NDGA (upward pointing triangles), or vehicle containing 3.0 mg/ml NDGA (downward pointing triangles; median survival 26 days; both Mantel–Cox and Gehan–Breslow–Wilcoxon $p < .0001$).

Table 5. NDGA Does Not Alter Food Consumption in *Drosophila* as Measured Using CAFE Assays

Treatment*	Food consumption (µl/fly)†	n‡	p value§
DMSO	0.23 ± 0.045	4	
NDGA	0.16 ± 0.057	4	$p = 0.3734$

*One day old male flies were starved for 2 hours and exposed to capillary tubes containing a solution of 5% sucrose and 5% yeast extract with the addition 1% by volume of either 3 mM NDGA dissolved in DMSO, or DMSO alone. A small volume of red food coloring was added to the food to aid in the visualization of the liquid. For a complete description see *Methods*.

†Consumption per fly is the mean ± SEM of the difference between the initial volume in the capillary pipets at the start and completion of assay conducted with flies present less the average amount of liquid loss to evaporation in control assays conducted in the absence of flies, divided by the number of flies in the assays.

‡Four bottles per condition (50 flies per bottle). Each bottle had four capillary tubes inserted into its sides. Nul control bottles were identical except for the absence of flies.

§The significance of the difference in food consumption between the treated and control flies was determined using an unpaired, two-sample t test.

repair, and thereby increased longevity (58). At high doses, damage becomes too extensive and the agents can reduce lifespan. Masoro and others have proposed that CR is a hormetic agent (35,59,60). In one example, the genic profiles produced by a group of progeroid mutations recapitulate the profiles produced by the prolongevity treatments of CR and GH deficiency in mice (60). The data regarding NDGA are not sufficiently developed to indicate whether it is a hormetic longevity agent. It appears to produce molecular damage at its optimum dosage for increasing lifespan, as evinced by the induction of cancers (Table 2) and modest liver and kidney toxicity in humans (41,42) and mice (Table 4). Thus, NDGA may be a hormetic lifespan agent. An unanswered question is whether the less toxic derivatives of NDGA such as tetra-O-methyl nordihydroguaiaretic acid will also be capable of extending murine and *Drosophila* lifespan (43,44).

Antioxidant Effects

One theory proposes that NDGA exerts positive effects on health because it is a potent scavenger of activated oxygen and nitrogen species in vitro and in vivo (4). However, exogenous antioxidants have not been shown to reproducibly extend mammalian lifespan, and they can shorten it (15,28,61–64). Further, the consumption of agents that increase oxidative stress, rather than reduce it, appear to be associated with enhanced longevity in mammals and other species (65).

Inflammation

NDGA reportedly suppresses proinflammatory gene expression and prostaglandin E 2 production and thereby inhibits arachidonic acid 5-lipoxygenase and cytokine-stimulated activation of microglia and macrophages (66,67). Some data support an inverse correlation between inflammation and lifespan (68). However, correlative evidence has been misleading in the context of oxidative stress (61). In the absence of direct evidence for an association between reduced inflammation and extended lifespan, it is unclear whether inflammation has a direct role in lifespan extension by NDGA.

Oxidative Phosphorylation

NDGA is reportedly an inhibitor of oxidative phosphorylation (9). Inhibition of oxidative phosphorylation can increase rodent and fly lifespan (69,70). However, decreased rates of oxidative phosphorylation are not consistent with the dose responsive loss of body weight observed in our mice (Figure 2; Table 1). Inhibition of oxidative phosphorylation should preserve body weight, since it reduces the utilization of calories. In flies, NDGA had no effect on body weight, physical activity, or food consumption (Tables 5–8). Thus, our data are inconsistent with the extension of lifespan through inhibition of oxidative phosphorylation.

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Table 6. NDGA Does Not Alter Food Consumption in *Drosophila* as Measured Using FPAs

Treatment*	Plaque Number/cm²/fly (mean ± SEM)†	*n*‡	Significance§	Plaque Diameter (mm²) (mean ± SEM) ‖	*n*¶	Significance
DMSO	0.133 ± 0.006	4		0.137 ± 0.021	40	
NDGA	0.134 ± 0.009	4	$p = 0.9346$	0.135 ± 0.020	40	$p = 0.9349$

Notes. NDGA = Meso-nordihydroguaiaretic acid.

*Two day old flies were starved for 2 hours and exposed to capillary tubes containing a solution of 5% sucrose and 5% yeast extract with the addition 1% by volume of either 3 mM NDGA dissolved in DMSO, or DMSO alone. A small volume of red food coloring was added to the food to aid in the visualization of the fecal plaques.

†Fecal plaques were counted on five 4 × 4 cm² sections of each fly bottle.

‡Number of bottles per condition with 50 flies per bottle utilized.

§The significance of the differences between the treated and control groups was determined using an unpaired two-sample *t* test.

‖Plaque sizes were determined as described (22).

¶Number of plaques from each condition used in the determination.

Table 7. Effects of NDGA on the Body Weight of *Drosophila*

Treatment*	*n*†	Mean weight (mg/fly) ± SEM‡	*p* value§
DMSO	7	0.7509 ± 0.007	
NDGA	7	0.7354 ± 0.006	$p = 0.1089$

Notes. NDGA = Meso-nordihydroguaiaretic acid.

*One day old flies were fed for 7 days with food containing either 3 mM NDGA or an equal volume of vehicle (DMSO). Flies were euthanized with CO_2 and frozen at −20 °C. After thawing, the flies were immediately weighed in 7 groups of 25 flies each.

†A total of 7 groups (*n*) of 25 flies were weighed for each treatment condition.

‡Mean weight ± SEM was calculated by dividing the total weight in milligrams of each group of flies by the number of individuals in the group.

§The significance of the differences in weight was determined using unpaired two-sample *t* tests.

Table 8. NDGA Does Not Alter the Locomotor Activity of *Drosophila*

Treatment*	*n*†	Mean ± SEM‡	Significance§
DMSO	10	1,605.9 ± 333.1	
NDGA	10	1,428.5 ± 243.7	$p = .6559$

Notes. NDGA = Meso-nordihydroguaiaretic acid.

*Flies were treated with either 3 mM NDGA, dissolved in DMSO, or medium containing an equal volume of DMSO alone for 3 days, followed by 72 hours of monitoring at 25 °C in a LAM 32 Activity Monitor (Trikinetics).

†Ten flies in each of 10 vials were monitored simultaneously for each condition.

‡The average number of infrared beam disruptions per 72-hour period by the groups of 10 flies.

§The significance of the differences in the mean number of beam breaks for treated and control flies determined using unpaired two-sample *t* tests.

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