DDS, 4,4′-diaminodiphenylsulfone, extends organismic lifespan

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DDS, 4,4′-diaminodiphenylsulfone, is the most common drug prescribed to treat Hansen disease patients. In addition to its antibacterial activity, DDS has been reported to be involved in other cellular processes that occur in eukaryotic cells. Because DDS treatment significantly enhances the antioxidant activity in humans, we examined its effect on lifespan extension. Here we show that DDS extends organismic lifespan using *Caenorhabditis elegans* as a model system. DDS treatment caused a delay in aging and decreased the levels of a mitochondrial complex. The oxygen consumption rate was also significantly lowered. Consistent with these data, paraquat treatment evoked less reactive oxygen species in DDS-treated worms, and these worms were less sensitive to paraquat. Interestingly enough, all of the molecular events caused by DDS treatment were consistently reproduced in mice treated with DDS for 3 mo and in the C2C12 muscle cell line. Structural prediction identified pyruvate kinase (PK) as a protein target of DDS. Indeed, DDS bound and inhibited PK in vitro and inhibited it in vivo, and a PK mutation conferred extended lifespan of *C. elegans*. Supplement of pyruvate to the media protected C2C12 cells from apoptosis caused by paraquat. Our findings establish the significance of DDS in lowering reactive oxygen species generation and extending the lifespan, which renders the rationale to examining the possible effect of DDS on human lifespan extension.

First synthesized a century ago, 4,4′-diaminodiphenylsulfone (DDS) is a drug still used to treat many skin diseases. Specifically, DDS is a principal drug in a multidrug regimen recommended by the World Health Organization for the treatment of leprosy (1–4). DDS acts as an antibiotic in a manner similar to sulphonamides by inhibiting bacterial synthesis of dihydrofolic acid through competition with para-aminobenzoic acid (PABA) for the active site of dihydropteroate synthetase (DHPS) (5). In addition to its antibacterial activity, DDS has been reported to be involved in other cellular processes that occur in eukaryotic cells, such as inflammation, migration, and apoptosis (6). However, there has been controversy over the issue of whether DDS acts as a pro-oxidant (especially when a higher dose of DDS is used rather than a standard dose) or antioxidant (7–9). Most studies on the pro-oxidant effects of DDS have been based on its use at high concentrations. For example, in human dermal fibroblasts, a high dose of DDS (1.5 mM) induced oxidative stress and glutathione depletion (10), but in rat livers, DDS administration at a dose of 30 mg/kg body weight resulted in oxidative stress (11). Therefore, the debate regarding the nature of DDS as a pro-oxidant or antioxidant appears to reflect the dosage effect. Another interesting observation is that Hansen disease patients in Korea, who usually have taken DDS for several decades, had a longer lifespan in spite of their socioeconomical disadvantages (12). This finding prompted us to examine whether and how DDS treatment extends an organism's lifespan. We first examined the antioxidant activity in human patients. We then used the nematode *Caenorhabditis elegans* as a model system to examine the DDS effect on lifespan. We then extended our studies to mammalian cells.

Results and Discussion

DDS Increased Antioxidant Activity in Humans. We first examined the effect of DDS on the antioxidant activities in humans by comparing the blood samples from control subjects and Hansen disease patients. To examine the effect of long-term use of DDS, we compared Hansen disease patients who have continuously taken DDS (the DDS-taking group) and those who have ceased taking DDS for at least 1 y at the time of blood sampling (the DDS-nontaking group). We observed a significantly increased antioxidant activity in the blood of the DDS-taking group (Fig. 1*A*). Increased antioxidant activity can result from higher antioxidant levels or lower reactive oxygen species (ROS) generation. To explore the molecular mechanism of the DDS effect, it was necessary to use a model system in which molecular genetic manipulation was feasible. We decided to use the nematode *C. elegans* as a model system to examine the effect of DDS.

DDS Treatment Significantly Increased the Lifespan of *C. elegans*. *C. elegans* is one of the most powerful animal models currently in use for studying the aging process and lifespan (13, 14). Because of the fact that DDS is poorly water-soluble, the worms were placed on the nutrient growth medium (NGM) agar plates seeded with *Escherichia coli* OP50 that had absorbed DDS during culture. In addition, PABA (at a concentration of 10 μM, which allowed for growth at 80% of the control growth rate) (Fig. S1*A*) was added in the culture to reduce the antimicrobial effect of DDS. Treatment with PABA allows bacteria to grow better, preventing calorie restriction effect by starvation of *C. elegans*. The concentration of DDS used for these studies was 2 mM, and the accumulated DDS inside the body of the worms was about 5 mg/kg of the *C. elegans* body weight (Fig. S1*B*). This concentration is comparable to the dose used to treat human patients. When treated with DDS for the entire lifetime or during the adult period after the L4 stage (which is comparable to human adolescence), the worms had a mean lifespan and a maximum lifespan significantly and reproducibly longer than those of the worms that were untreated (Fig. 1 *B* and *C* and

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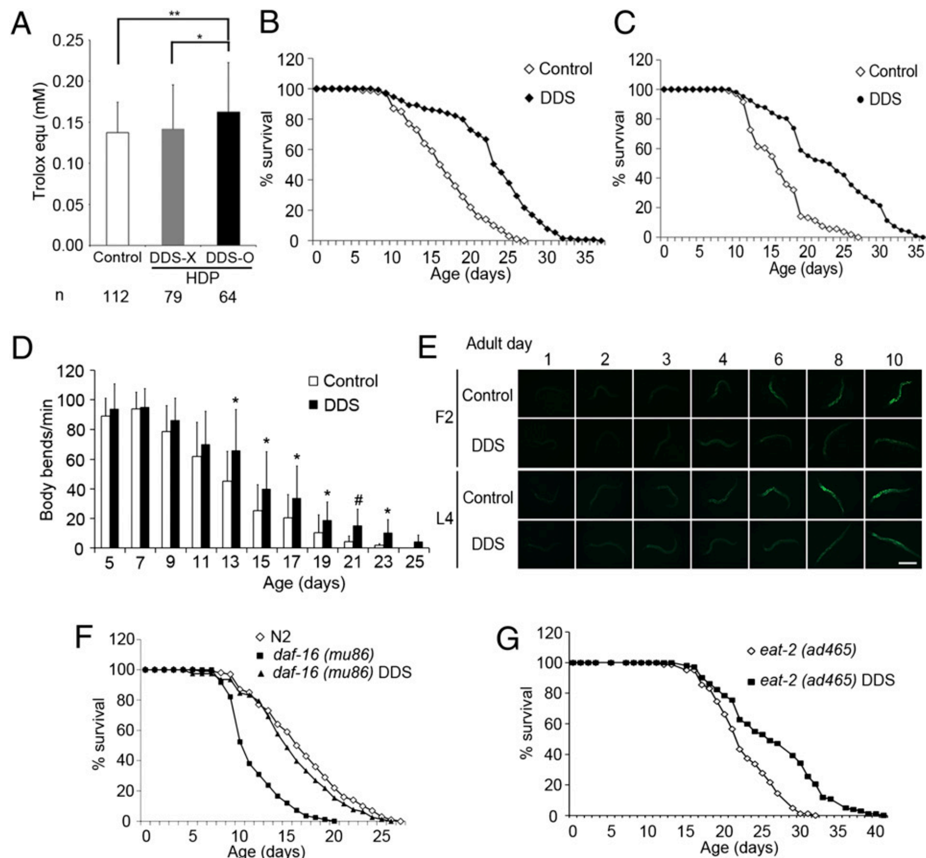

Fig. 1. The DDS effects in humans and *C. elegans.* (*A*) DDS increases antioxidant activity in humans. Blood samples were obtained and analyzed from individuals with Hansen disease (the DDS-taking group, DDS-O, or the DDS-nontaking group, DDS-X) and controls. The total numbers of individuals in each group are marked as "n." The average age of each group was 79.76 ± 10.69, 81.18 ± 9.86, and 79.89 ± 10.08 for the control, DDS-X, and DDS-O group, respectively. Asterisks indicate significant differences: $*P < 0.05$ and $**P < 0.01$. (*B*–*G*) DDS treatment extends the lifespan of *C. elegans.* (*B*) Survival curves of *C. elegans* ($n = 129$) treated with DDS (2 mM) during their entire lifetime compared with worms that were untreated. (*C*) Survival curves of *C. elegans* ($n = 107$) treated with DDS (2 mM) beginning after the L4 stage for the rest of their lifetime. (*D*) Spontaneous movement at different ages of the control and DDS-treated worms. DDS-treated worms had significantly faster body movement than control worms after day 13. At least 20 animals were measured on each day, except for days 23 and 25 in control and day 25 in the DDS-treated group, when enough numbers of live animals were not available. Error bars represent the SD. $*P < 0.05$; $^{\#}P < 0.001$. (*E*) DDS delays the progression of aging. Reduced accumulation of lipofuscin in worms treated with DDS for their entire lifetime (F2) and after the L4 stage (L4) compared with their respective control animals. Images show intestinal lipofuscin autofluorescence. The number of days after reaching the adulthood in the presence or absence of DDS is indicated above each photo. F2 shows worms treated with DDS during the F2 generations and L4 shows worms treated with DDS after the L4 stage. At least 30 animals were measured on each day. (Scale bar, 0.5 mm.) (*F*) DDS-mediated lifespan extension is independent of *daf-16* in *C. elegans*. The graph shows the survival curves of *daf-16* mutant animals either treated with 2 mM DDS ($n = 78$) or left untreated ($n = 84$) throughout their adult life. N2 represents an additional control ($n = 100$). (*G*) DDS-mediated lifespan extension is independent of calorie restriction in *C. elegans*. The graph shows the survival curves of *eat-2* mutant animals either treated with 2 mM DDS ($n = 102$) or untreated ($n = 83$) throughout their adult life.

Table S1). In addition, DDS-treated worms were surprisingly more active in their movements compared with the control animals at old ages (Fig. 1*D* and Fig. S2*A*). To examine whether DDS treatment delayed the initiation of aging or just prolonged the aged stage, the accumulation of lipofuscin was measured (15). The appearance of lipofuscin was delayed for several days in worms treated either for their entire lifetime or only after adolescence compared with untreated control worms (Fig. 1*E* and Fig. S2*B*). These data suggest that worms treated with DDS initiated the aging process more slowly than control worms did. In addition, we observed that treatment of *C. elegans* with DDS at lower concentrations, such as 0.5 mM and 1 mM, were also able to extend the animals' lifespan as effectively as a 2-mM concentration (Fig. S2*C* and Table S2).

DDS Effect Is Independent of Insulin Signaling and Calorie Restriction. There are several major nonoverlapping factors that contribute to lifespan extension in worms, including reduced insulin signaling, dietary restriction, and decreased mitochondrial function (16). To distinguish between these three possible mechanisms of the DDS effect, we first examined whether insulin signaling is involved. As shown in Fig. 1*F* and Table S1, we treated mutant animals defective in *daf-16*, the key regulator in the insulin signaling pathway in *C. elegans* (17), with DDS and observed that these animals lived longer than control *daf-16* worms. This result suggests that the effect of DDS is independent of *daf-16*.

As described above, we added PABA to *E. coli* medium to prevent the inhibition of bacterial growth by DDS (Fig. S1*A*), and worms grown on an *E. coli* strain defective in *folP*, the direct target protein of DDS (18), lived longer when treated with DDS than control worms did (Fig. S3*A* and Table S2). We also found that the lifespan of *eat-2* mutant animals, which exhibit the phenomenon of calorie restriction because of slower pumping (19), was extended by DDS treatment (Fig. 1*G* and Table S1). In addition, DDS did not cause any reduction in the pumping rates of wild-type and *eat-2* mutant animals (Fig. S3*B*). These data strongly suggest that calorie restriction per se is unlikely to be the cause of the lifespan extension observed in DDS-treated worms.

DDS Lowers ROS Generation. Because the DDS-mediated lifespan extension that we observed in *C. elegans* is independent of *daf-16* and dietary restriction, we examined the effect of DDS on the mitochondria. Quantitative densitometry analysis of Western blots detecting mitochondrial complex V from mitochondrial preparations revealed a marked decrease in the protein level in DDS-treated worms (Fig. 2*A*). In addition, a lower ATP level (Fig. 2*B*) and a dramatically reduced oxygen consumption rate (Fig. 2*C*) was observed in DDS-treated worms. All these data indicate that DDS causes a reduction in the generation of ROS. To examine whether DDS could inhibit the generation of ROS, we measured the generation of H_2O_2 in worms treated with paraquat (PQ), an intracellular free-radical generating compound, in the presence or absence of DDS treatment. We found that the treatment of PQ-treated worms with DDS significantly lowered the generation of ROS (Fig. 2*D*). Accordingly, the survival of animals in NGM liquid medium containing PQ (250 mM) was significantly enhanced in the presence of DDS (2 mM) compared with control worms (Fig. 2*E*). These data suggest that

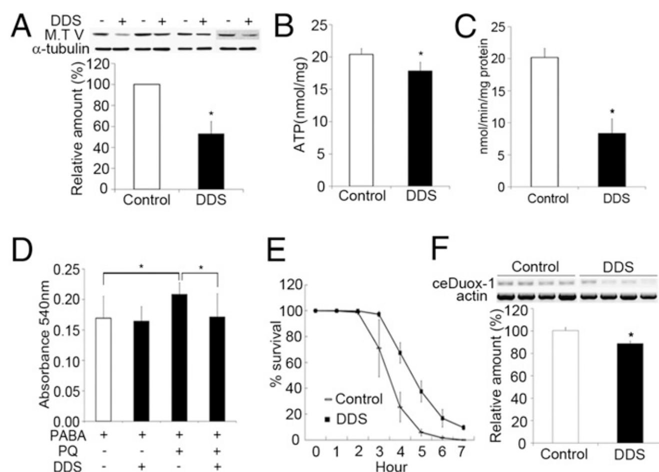

Fig. 2. DDS lowers ROS generation in *C. elegans*. (*A*) Western blot of mitochondrial fractions from untreated control (–) and DDS-treated (+) *C. elegans* for mitochondrial complex V protein levels. The relative level of mitochondrial complex V levels are graphed (*$P < 0.01$). α-Tubulin was used as a loading control. Data are the average of four independent experiments. (*B*) DDS-treated *C. elegans* worms have a lower ATP level (*$P < 0.05$). (*C*) DDS-treated *C. elegans* worms have a lower oxygen consumption rate than control worms. Results are means ± SD (42) and represent the average of four independent experiments (*$P < 0.001$). (*D*) Hydrogen peroxide production by PQ is compromised by DDS treatment. H_2O_2 production was measured using Amplex Red at an absorbance of 540 nm using a plate reader (*$P < 0.05$). (*E*) Survival curve of *C. elegans* on NGM liquid medium containing PQ (250 mM) in the presence or absence of DDS treatment. Data represent the mean ± SD, $P < 0.0001$. (*F*) DDS decreased the amount of the cytosolic oxidative stress-related molecule, *Duox1*. The mRNA level of *ceDoux1* was measured by RT-PCR from *C. elegans* grown on DDS-less or DDS-replete bacterial strains. Actin was probed as a loading control. Data are presented as means ± SD and represent the average of four independent experiments (*$P < 0.005$).

DDS increased the resistance of these animals to oxidative stress. Interestingly, the mRNA level of *ceDuox1*, a *C. elegans* NADPH oxidase, which serves as another source of ROS generation, was also decreased in DDS-treated worms (Fig. 2*F*). These data suggest that the effect of DDS is not limited to the mitochondria but extends to the cytosol. ROS scavenging enzymes, such as SOD1, SOD2, catalase, glutathione peroxidase, and glutathione reductase, were not significantly affected by DDS (Fig. S4), suggesting that DDS does not increase ROS scavenging activities, but lowers ROS generation.

DDS Effect Held True in Mammalian Cells. To examine whether the DDS effect on ROS generation holds true in mammalian cells, we first performed quantitative densitometry analysis of Western blots detecting mitochondrial complexes from mitochondrial preparations of mice treated with DDS for 3 mo. We found that all of the complexes were decreased even at a low-dose treatment of 0.2 mg/kg (Fig. 3*A*). The same effect was observed in C2C12 cells treated with DDS (Fig. 3*B*). Next, we found that ATP levels of muscle cells in both mice (Fig. 3*C*) and C2C12 muscle cells (Fig. 3*D*) were significantly decreased by DDS treatment. We then tested whether DDS could lower ROS generation using C2C12 cells by visually examining H_2O_2 generation and lipid oxidation (Fig. 3*E*). DDS clearly lowered superoxide formation and lipid oxidation. Consistently, oxygen consumption rate was also lowered (Fig. 3*F*). Importantly, DDS treatment was able to protect C2C12 cells from cytotoxicity caused by PQ treatment (Fig. 4*K*). The DDS dosage used in the C2C12 cells (20 μM) was optimized based on the observation that the long-term administration of DDS at standard doses (100 mg/50 kg body weight per day) in patients with Hansen disease usually results in few or no clinically significant side effects, and results in a DDS plasma concentration that ranges from 1 to 20 μM (20). Taken together, our results strongly suggest that the DDS effect is similar in worms and mammals.

Muscular Pyruvate Kinase Is a Major Target of DDS in *C. elegans*. The mechanism by which DDS acts as an antibiotic is well-known. DDS associates with the active site of DHPS, an enzyme required for folic acid synthesis in bacteria. Although yeast has a DHPS homolog, metazoan eukaryotic cells do not possess a DHPS homolog; therefore, DDS does not act by this mechanism to exert an effect on multicellular organisms. Thus, we wanted to identify DDS target proteins in multicellular eukaryotic cells. To do this, we searched the protein structure database for candidate proteins whose structures resemble the putative binding pocket of DHPS with DDS (21). A best candidate we identified was pyruvate kinase (PK) (Fig. 4*A*). The fact that a PK-deficiency in humans results in hemolytic anemia, a most common side effect of DDS in leprosy patients (22, 23), prompted us to examine whether PK is a real target of DDS contributing to lifespan extension in *C. elegans*. Both in vitro and in vivo *C. elegans* and mouse analyses showed that DDS inhibited the biochemical activity of PK (Fig. 4 *B–D*). BIAcore analysis also showed that DDS binds PK in vitro (Fig. S5). We then examined PK genes in *C. elegans*. The *C. elegans* genome contains two PK-encoding genes, *pyk-1* and *pyk-2,* which correspond to F25H5.3 and ZK593.1, respectively. A deletion mutation is available for *pyk-1*: *ok1754*. We found that the lifespan of *pyk-1 (ok1754)* worms without DDS treatment was significantly longer than that of N2 control worms (Fig. 4*E* and Table S1). The *pyk-1 (ok1754)* worms treated with DDS lived longer than untreated *pyk-1* mutant animals, but just as long as the N2 worms treated with DDS (Fig. 4*E* and Table S1). These data suggest that *pyk-1* is a DDS target, but not the only one, and that there must be additional DDS targets for extending lifespan. Because there is no available mutation for *pyk-2*, we examined the RNAi effect of *pyk-2* on lifespan. The decreased mRNA level resulting from *pyk-2* RNAi was confirmed by RT-PCR (Fig. S6). As shown in Fig. 4*F*, *pyk-2* RNAi did not affect lifespan either alone or in conjunction with the *pyk-1(ok1754)* mutation, suggesting that *pyk-2* is not involved in the DDS effect. Previous expression studies (24) showed that *pyk-1* is mainly expressed in the muscles, and *pyk-2* in the intestine, suggesting that the DDS effect on lifespan is through its action of PK in muscles but not in the intestine. Taken together, all of the data presented here strongly suggest that muscular PK is a major target of DDS in *C. elegans*, with the strong possibility that there are other unidentified targets of DDS. It would be of interest to identify DDS targets through searching for proteins that interact with DDS.

An unexpected result was that the level of pyruvate in DDS-treated worms was higher than that in control worms (Fig. 4*G*). The *pyk-1(ok1754)* mutant animals also contained a higher level of pyruvate (Fig. 4*H*). These results are consistent with a report that the level of pyruvate was higher in the leprosy patients who took DDS than in control groups (25). Currently, it is not easy to reconcile how inhibition of PK activity either by DDS or by a mutation leads to an increase in pyruvate levels. One possibility is that autophagy may provide additional amino acids and pyruvate. However, this is unlikely because genes involved in autophagy did not show any change in the transcript levels after DDS treatment and the autophagy marker protein, LGG-1, was not increased by DDS treatment (Fig. S7). A second possibility is that DDS inhibits other enzymes working in the tricarboxylic acid cycle so that pyruvate is less rigorously consumed. Consistent with this, the mitochondrial complex levels and the oxygen consumption rate are decreased by DDS treatment, as described above. Alternatively, the inhibition of PK activity may lead to

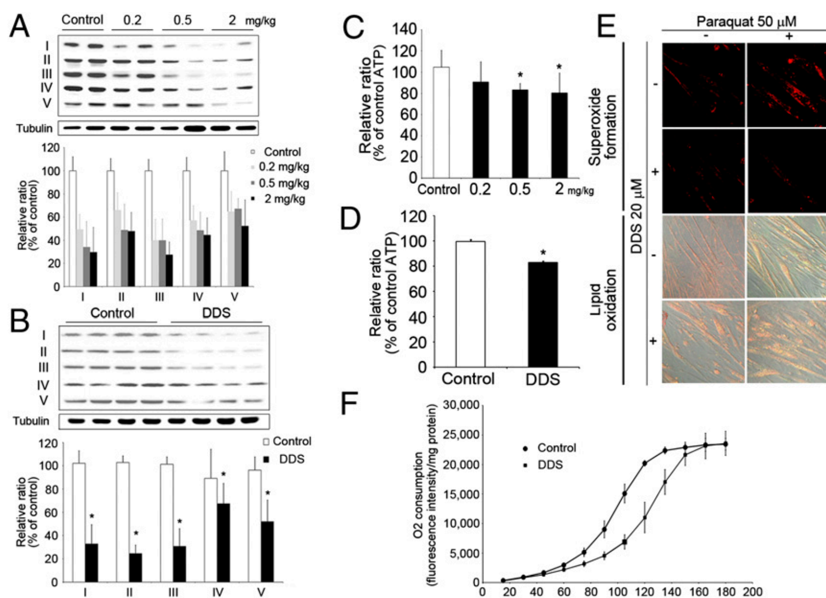

Fig. 3. The DDS effect in mouse whole muscle tissues and C2C12 myotubes. (*A*) Western blot of mitochondrial complexes from untreated control and DDS administered mouse whole muscle tissues. The relative levels of mitochondrial complexes are graphed ($n = 6$; $*P < 0.05$). α-Tubulin was used as a loading control. (*B*) Western blot of mitochondrial complexes from DMSO only (control) or DDS-treated C2C12 myotubes. The relative levels of mitochondrial complexes are graphed ($*P < 0.05$). α-Tubulin was used as a loading control. Data are the average of four independent experiments. (*C*) Whole muscle tissues from DDS administered mice have a lower ATP level ($n = 6$; $*P < 0.05$). (*D*) DDS-treated C2C12 myotubes have a lower ATP level ($*P < 0.05$). Data are the average of four independent experiments. (*E*) DDS decreases PQ-induced ROS production and lipid oxidation in C2C12 myotubes. ROS production in PQ-treated and control cells were measured by dihydroethidium (DHE) fluorescent probe and lipid oxidation measured by ^{11}C-BODIPY$^{581/591}$ fluorescent probe. (*F*) DDS treated C2C12 myotubes have a lower oxygen consumption rate than control C2C12 myotubes. Results are means ± SD (42) and represent the average of three independent experiments.

down-regulation of the entire tricarboxylic acid cycle as a compensatory mechanism. This possibility is consistent with the fact that long-lived *isp-1* mutant animals, in which the mitochondrial complex III is defective (26), also had a higher level of pyruvate (Fig. 4*I*). The pyruvate level was increased in muscle cells of mice treated with DDS for 3 mo (Fig. 4*J*), suggesting that PK is a protein target of DDS in mammals. Furthermore, supplementation of exogenous pyruvate to differentiated C2C12 muscle cells protected them from PQ-induced cytotoxicity (Fig. 4*K*), suggesting that the elevation of pyruvate content in cells may have a causal relationship with the decreased ROS sensitivity. It would be of interest to examine whether the high level of pyruvate per se leads to long lifespan.

Aging is a multifactorial process involving morphological and biochemical changes in single cells and in the whole organism. The exact mechanism underlying aging is not well understood. However, there is a relationship between lifespan and production of free radicals (27, 28). In the present study, we showed that DDS treatment extended the lifespan of *C. elegans*. Although it is unclear whether the reduced ROS generation by DDS treatment has a causal relationship with extended lifespan, our results establish the importance of DDS treatment in lifespan extension in *C. elegans* and possibly in humans. Further studies are necessary to determine whether DDS influences lifespan through changes in other physiological processes, such as immune function, metabolism, or brain function. Long-term administration of DDS at a standard dose (100 mg/d) in normal human patients sometimes results in minor side effects, such as hemolytic anemia (8). Anemia occurs commonly with aging and is a frequent medical problem. In elderly persons, hemoglobin concentrations tend to be slightly low under the lower limit of normal range (29). Because the main side effect of DDS is anemia (30), we monitored hemoglobin levels of each group. We found that the hemoglobin levels of Hansen disease patients were not significantly different from those of the control group (Fig. S8), suggesting that DDS is not life-threatening in humans. Recently, a 5% DDS gel, which achieves a plasma concentration of about 20 μM, was reported to be safe and effective for long-term treatment (31). If DDS is to be used in a safer way, the treatment of DDS at a lower concentration may be more beneficial. Indeed, we observed that treatment of *C. elegans* with DDS at lower concentrations were also able to effectively extend the animal's lifespan (Fig. S2C and Table S2). These data suggest that a lower concentration of DDS should reduce negative side effects, if any, without losing its lifespan extension effect. In conclusion, DDS, a drug commonly used to treat Hansen disease, reduces ROS generation, and enhances longevity in *C. elegans* and possibly in mouse. We suggest that it is worthwhile to examine whether DDS is effective in enhancing longevity in humans as well.

Materials and Methods

Human Subjects and Blood Collection. The peripheral blood samples of Hansen disease patients were obtained from National Sorokdo Hospital, which is the specialized national hospital for Hansen disease in Korea; subjects included those who took DDS (DDS group) and who did not take it at least 1 y before blood sampling (no DDS group). The control subjects were recruited from Jeolla province. This study was approved by the Institutional Review Board of the Seoul National University College of Medicine (IRB approval No. C-0803-023-237). Recommendations of the Declaration of Helsinki for biomedical research involving human subjects were also followed.

Ferric Reducing Ability of Plasma Assay. The ferric reducing ability of plasma (FRAP) assay was carried out based on the method by Benzie and Strain (32). The absorbance was recorded at 593 nm. Absorbance measurement was taken for up 8 min, but the 2-min readings were selected for calculation of FRAP value. The results were expressed as Trolox equivalent.

Nematode Strains. We used the N2 Bristol strain as the wild-type strain. *C. elegans* mutant strains used in this study are of the genotype *daf-16 (mu86)*, *eat-2(ad465)*, *isp-1(qm150)*, *adls2122* [*gfp::lgg-1*, *rol-6(su10060)*], and *pyk-1(ok1754),* all of which were obtained from the Caenorhabditis Genetics Center.

Lifespan Measurement of the Nematode. We measured the lifespan of worms at 20 °C. In all experiments we excluded worms that died because of bursting vulvae, forming worm bags, or climbing up plate walls. We considered a worm dead if the worm did not respond to a platinum pick. Significance was determined by comparing the differences in the mean lifespan by using the log-rank (Mantel-Cox) test (33).

Lipofuscin Autofluorescence Measurement. We photographed lipofuscin autofluorescence in the worms using a 525-nm band-pass filter with an exposure time of 800. At least 30 worms were measured each day to calculate the average and SE of lipofuscin autofluorescence.

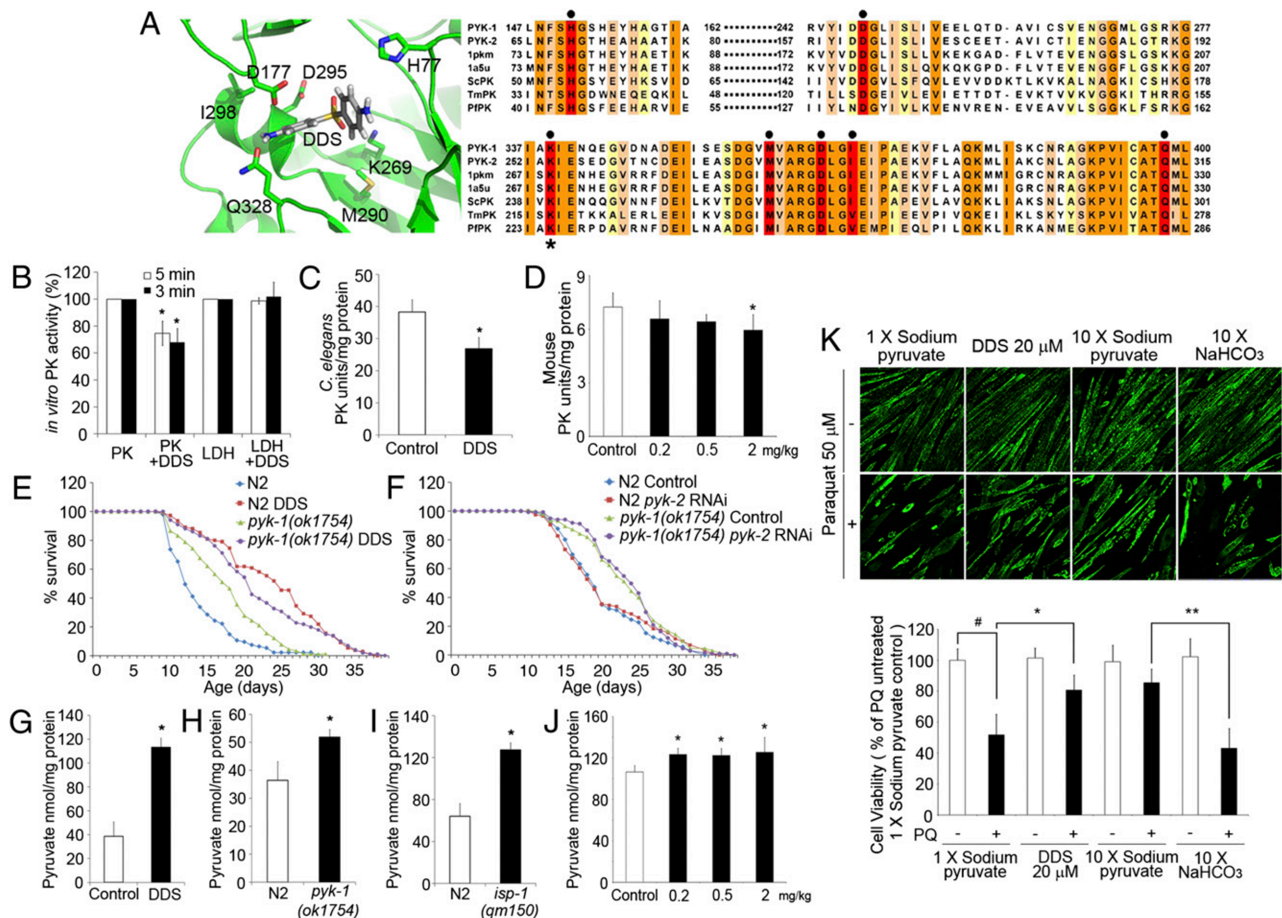

Fig. 4. Pyruvate kinase is a target of DDS. (*A*) Binding model of DDS to pyruvate kinase. (*Left*) A pyruvate kinase (green color, PDB ID: 1PKM) modeled with DDS. The putative interacting residues are indicated. (*Right*) A partial sequence alignment of PKs from different species. The putative interacting residues are colored red and dotted. Residue K269 is marked with an asterisk (*) and is one of the PEP binding residues in typical PK. Abbreviations: 1pkm, cat PK; 1a5u, rabbit PK; Sc, *Saccharomyces cerevisiae*; Tm, *Tamiops maritimus*; Pf, *Pyrococcus furiosus*. (*B–D*) DDS inhibits PK activity in vitro and in vivo. (*B*) Inhibition of PK activity by DDS in vitro. PK activity was measured after reactions were allowed to proceed for 3 min (white bars) or 5 min (black bars). DDS was directly added to the reaction mixture. LDH indicates a reaction in which pyruvate was added instead of PEP and PK was not added. (*C*) Inhibition of PK activity by DDS in vivo. DDS-treated *C. elegans* extracts were added to the reaction mixture (*$P < 0.05$). (*D*) Whole muscle tissue from the DDS-administered mice has a lower PK activity ($n = 6$; *$P < 0.05$). (*E*) Lifespan extension of *pyk-1(ok1754)* mutant animals. N2 and *ok1754* animals were fed with bacteria OP50 containing PABA only or DDS. (*F*) The *pyk-2* RNAi does not affect the lifespan of wild-type N2 and *pyk-1* mutant animals. (*G*) Increase in the pyruvate content in DDS-treated *C. elegans* (*$P < 0.001$). (*H*) Increase in the pyruvate concentration in *pyk-1(ok1754)* mutant animals without DDS treatment. (*I*) Increase in the pyruvate concentration in *isp-1* mutant animals. Data presented in these figures represent an average of at least three independent experiments (*$P < 0.001$). (*J*) Increase in the pyruvate concentration in DDS-administered mice whole muscle ($n = 6$; *$P < 0.05$). (*K*) DDS or pyruvate supplement ameliorated PQ-induced cytotoxicity in C2C12 myotubes. C2C12 myotubes viability was measured with troponin T fluorescent images. The graphs are expressed as percent of each control rate. Marks indicate the significant difference, $P < 0.005$ (#from untreated control cells; *from PQ only-treated cells; **from 10× $NaHCO_3$- and PQ-treated cells).

Animals and Experimental Conditions. Male BALB/c mice (20–25 g body weight, 4 wk old) were acclimatized for 1 wk before use. DDS (0.2, 0.5, 2 mg/kg) was administered to the groups of six mice intragastrically every day for 3 mo and then muscles were separated. All of the animal studies were approved by the Animal Experimentation Committee of Seoul National University.

C2C12 Cell Culture. C2C12 cells were obtained from the American Type Culture Collection. For differentiation, the medium was renewed or changed to DMEM supplemented with 2% FBS (mitogen-poor, differentiation promoting medium) when the cells reached 60 to 70% confluence.

Analysis of *C. elegans* Mitochondrial Fractions. Worms were harvested and homogenized in the S basal buffer. Mitochondrial and cytosolic fractions were frozen, thawed, and treated with 0.8% CHAPS before mitochondria complex V measurement (34).

ATP Measurement. ATP levels were measured as described previously with slight modifications (35).

Amplex Red Assay for H_2O_2 Measurement. Possible ROS scavenging effects of DDS were examined as previously described (36), using an Amplex Red hydrogen peroxide/peroxidase assay kit (Molecular Probes).

DHE Assay for Superoxide Anion Measurement. After 20 μM DDS was treated for 72 h, C2C12 myotubes were treated with 50 μM PQ for 30 min and then incubated with 5 μM DHE (Molecular Probes) for 15 min at 37 °C in the dark. Then ethidium-DNA fluorescence was visualized under a fluorescent microscope with LSM510 META (Carl Zeiss; magnification, 200×).

Measurement of Lipid Oxidation. Lipid oxidation was measured using a fluorescent probe (^{11}C-BODIPY581/591; Molecular Probes) as described previously (37).

Oxidative Stress Resistance Assay. For *C. elegans*, we added 250 mM of PQ in M9 buffer to a 24-well plate containing 5-d-old F2 worms fed with DDS in each well and counted live worms at each time point. For C2C12 myotube cells, myotubes were washed with PBS and placed in either basal media containing 1 mM sodium pyruvate (1×) or test media supplemented with 10 mM sodium pyruvate (10×) for 48 h. C2C12 myotubes were also incubated in media supplemented with 10 mM sodium bicarbonate (10× $NaHCO_3$), to

control for changes in the osmolarity. After 50 μM PQ was treated for 48 h, cell viability was measured using a troponin T (a muscle differentiation marker, clone JLT-12; Sigma), as described previously (38).

Oxygen Consumption Assay. For *C. elegans*, oxygen consumption rates were measured using a Clark-type oxygen electrode (782 Oxygen Meter; Strathkelvin Instruments), as described previously (39, 40). For C2C12 myotubes, the C2C12 myotubes were treated with DDS, and then cells were plated in the fluorescent dye-embedded 96-well microplate of the BD oxygen biosensor system (BD Biosciences). The C2C12 myotubes were incubated for 3 h, and then results were read with a fluorescent microplate reader (Wallac Victor3V 1420 Multilabel Counter; Perkin-Elmer).

Modeling of DDS Binding. DDS was modeled into the PABA binding site of DHPS by superimposing the aminophenyl group of DDS onto the equivalent position of PABA and the structure of cat PK was superimposed to the DHPS.

PK Activity Assay. The PK activity was assayed by a procedure described previously (41). For in vitro experiments, we used 0.003 units/reaction type I PK preparation from rabbit muscle (Sigma).

Pyruvate Measurements. Pyruvate contents were measured using a commercially available fluorescence-based assay kit (BioVision) following the manufacturer's protocol. The pyruvate contents were normalized to total protein and expressed as nmol/mg of protein.

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