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A deuterohemin peptide extends lifespan and increases stress resistance in *Caenorhabditis elegans*

SHUWEN GUAN[1], PENGFEI LI[1], JING LUO[2], YUANYUAN LI[3], LEI HUANG[1], GUAN WANG[1], LIMIN ZHU[1], HONGKUAN FAN[1], WEI LI[1] & LIPING WANG[1,4]

[1]*College of Life Science, Jilin University, Changchun, PR China,* [2]*Chongqing Medical and Pharmaceutical College, Chongqing, PR China,* [3]*Jilin Province Product Quality Supervision Test Institute, Changchun, PR China, and* [4]*China-Japan Union Hospital, Jilin University, Changchun 130033, China.*

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Abstract

This group has invented a novel deuterohemin containing peptide deuterohemin- AlaHisThrValGluLys (DhHP-6), which has various biological activities including protection of murine ischemia reperfusion injury, improving cell survival and preventing apoptosis. It was hypothesized that DhHP-6 is beneficial on the lifespan of *Caenorhabditis elegans* (*C. elegans*) and increases their resistance to heat and oxidative stress. *C. elegans* were treated with different concentrations of DhHP-6. Survival time and sensitivity to heat and paraquat were investigated. The data demonstrated that the mean survival time of *C. elegans* was significantly increased ($p < 0.05$) in the DhHP-6 treated group compared with the control group. The maximum lifespan was not affected by DhHP-6 treatment. DhHP-6 improved the survival rate of *C. elegans* in the acute heat stress (35°C) and rescued the *C. elegans'* sensitivity to paraquat in acute oxidative stress. Superoxide dismutase 3 (SOD-3) protein was up-regulated by DhHP-6 treatment. It was further demonstrated that stress resistance genes such as *hsp-16.1*, *hsp-16.49* and *sir-2.1* were regulated by DhHP-6. DAF-16 and SIR-2.1 genes are essential for the beneficial effect of DhHP-6. Therefore, the investigation into the beneficial effect of DhHP-6 on *C. elegans*' lifespan has the potential to develop novel drugs to prevent ageing.

Keywords: *Ageing, oxidative damage, lifespan, Caenorhabditis elegans*

Introduction

Ageing is characterized by progressive and widespread degenerative changes in tissues. Four categories of compounds have been reported to extend lifespan of *C. elegans* [1]: resveratrol or other sirtuins (sir2-like proteins) [2,3]; compounds or complex mixtures derived from natural products [4–6]; heterocyclic anticonvulsants which affect neural activity [1,7,8]; and antioxidant compounds [9–11] or low concentration of ROS generator [12]. A recent report demonstrated that over-expression of catalase genes targeted to mitochondria that confer resistance to oxidation lead to extended lifespan of mice. These studies suggest that reducing mitochondrial ROS level may be important in determining mammalian longevity [13].

DhHP-6 contains a deuterohemin prosthetic group together with the six amino acid residues (AlaHisThrValGluLys). Because the peptide chain reduced the problem of aggregation in aqueous solution, it is highly soluble in aqueous buffers. In the previous study, we found that DhHP-6 could efficiently catalyse substrate oxidations using hydrogen peroxide (H_2O_2) and protect cultured rat lens crystallina from cataracts induced by galactose [14,15]. These studies indicate that DhHP-6 had potential effects on age-related diseases caused by reactive oxygen species (ROS).

To further investigate the effects of DhHP-6 on ageing, we hypothesized that DhHP-6 can delay ageing and prolong lifespan in *C. elegans*. The free living soil

Correspondence: Liping Wang, College of Life Science, Jilin University Changchun, PR China. Tel: +86-431-88499505. Email: wanglp@jlu.edu.cn

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nematode *Caenorhabditis elegans* (*C. elegans*) is a suitable experimental organism due to its short lifespan (2–3 weeks) and rapid ageing of invertebrates [16]. Here, we report that DhHP-6 extends *C. elegans* lifespan and delays age-related degenerative changes in body movement. Importantly, we found that DhHP-6 could significantly improve the survival time of *C. elegans* under stress conditions. These results suggest that DhHP-6 might provide strong protection against stress and extend the survival time of *C. elegans* and up-regulate the expression of *sir-2.1*. We believe that these findings will provide insight into the anti-ageing study and age-related disease.

Material and methods

Materials

Deuterohemin and deuterohemin-AlaHisThrValGluLys (DhHP-6) were prepared following an optimized procedure [14]. Stock solutions were sterilized using 0.2 m filters, and aliquots were stored at 4°C. A 10 mM aqueous stock solution of DhHP-6 was used to prepare a series of concentrations in a suspension of *E. coli* strain OP50 in S medium [17]. All reagents used in lifespan experiments (DhHP-6, S medium, etc.) were taken from the same stock solutions. Catalase assay kit was purchased from Beyotime Company (Jiangsu, China). All other regents were of highest purity available and used as received.

Strains used in this study were: N2, Bristol (wild-type); TK22, *mev-1 (kn1)*; GR1307, *daf-16 (mgDf50) I*; VC199, and *sir-2.1 (ok434) IV*. All these strains were provided by the Caenorhabditis Genetics Center (CGC) and grown at 20°C on a lawn of *Escherichia coli* strain OP50 or in S-medium [18]. Synchronization of worm cultures was performed as described [17].

Determination of lifespan and age-related changes of physiological functions

Synchronous L4 stage hermaphrodites were picked and placed singly into U- bottom wells of 96-well plates, each well containing 50 µl of treatment medium: S-medium with DhHP-6 and *E.coli* OP50 to OD = 0.3. *C. elegans* were transferred daily to new wells with fresh treatment medium during the reproductive period to avoid progeny overgrowth. Hatching was the starting point for the determination of lifespan, which is defined as the time from day 0 to the last day of survival. *C. elegans* were scored as dead by two experimenters if they failed to respond to the prodding by worm picks. All nematodes were followed from hatching to death and only lost, bagged and exploded *C. elegans* were excluded from the data.

The measurements of age-related changes of physiological functions (self-fertile reproduction, fast body movement and pharyngeal pumping) were used to define the stages of ageing [19]. All experiments were carried out by the blind method: one group of experimenters marked the strains and another group performed the counting.

Assays of thermotolerance and resistance to paraquat

Thermotolerance assays were measured at 35°C using young adult *C. elegans*. Synchronously cultured L4 stage *C. elegans* were transferred to 3 cm plates along with 2 ml S-medium (200 µM DhHP-6, *E.coli* OP50, 50 µM FUdR) and allowed to culture at 20°C for 1 day, then incubated at 35°C for 10 h. *C. elegans* surviving at the end of the assay were counted. Paraquat-induced oxidative stress assays were performed as described above, except that paraquat was added to the S-medium to a 2 mM final concentration [20]. *C. elegans* were transferred every day and survival number was noted.

Fluorescence quantification and visualization

Overall GFP fluorescence of GFP-expressing populations was assayed using a BioTek Synergy HT Multi-Mode Microplate Reader. Adult worms were treated with or without 100 µM DhHP-6 for 2 days. One hundred control or treated adult animals of the indicated age were transferred in 100 µl of PBS to a well of a Costar 96-well microtiter plate (black, clear, flat-bottom wells) and total GFP fluorescence was measured using 485 nm excitation and 530 nm emission filters [21]. Quadruple populations were used for each determination.

For fluorescence microscopy, the worms were mounted with a drop of heavy mineral oil placed on a coverslip covered with 2% agarose. The GFP pictures of transgenic worms were taken on an Olympus BX51 microscope [22].

Gene expression analysis by real time RT-PCR

Adult *C. elegans* were treated with or without 100 µM DhHP-6 for 8 days. Approximately 200 worms were collected from culture. Total RNA was isolated and purified by an EZ spin column mRNA Purification kit (BS583) from Sangon Bio. Inc. (China) as recommended by the manufacturer. Purified RNA was dissolved in 30 µl RNAse-free water. First strand cDNA was prepared with the RT-PCR kit (TaKaRa, Japan). Real-time qPCR was performed in a Prism 7500 Real Time PCR System (ABI, USA), using SYBR® PCR kit (TaKaRa, Japan) with 0.3 µM primers and 1 µL cDNA in a 25-µL reaction volume. The $2^{-\Delta\Delta CT}$ value was calculated to reflect the relative expression gene affected by DhHP-6 treatment. Each experiment was repeated two or three times [23].

The gene-specific primers used for amplification were as follows: *gpd-1 (T09F3.3)*, 5′ TCGGAATCAACGGTATCGGAAGT 3′ and 5′GACGGAAA-

Table I. Effect of DhHP-6 on the wild type *C. elegans* at 20°C.

Trial	Strain	Treatment (μM DhHP-6)	Mean lifespan ± SE (*n*)	% Change	Max lifespan	*p*-value*
1	N2	*E.coli* (0)	16.1 ± 0.54 (48)	—	25	—
		E.coli (200)	19.6 ± 0.43 (57)	21.7	28	< 0.001
2	N2	*E.coli* (0)	16.2 ± 0.55 (48)	—	25	—
		E.coli (50)	19.4 ± 0.31 (89)	19.8	27	< 0.001
		E.coli (200)	19.6 ± 0.27 (95)	21.0	28	< 0.001
		E.coli (500)	19.2 ± 0.31 (76)	18.5	28	< 0.001
3	N2	*E.coli* (0)	16.0 ± 0.56 (44)	—	25	—
		E.coli (5)	16.7 ± 0.25 (79)	0.04	26	0.221
		E.coli (50)	19.6 ± 0.32 (79)	22.5	27	< 0.001
		E.coli (500)	19.3 ± 0.27 (83)	20.6	27	< 0.001

*p-value (log rank test) compared with untreated control.

CATCTGGTGTAGGGA3′; *hsp-12.6* (*F38E11.2*), 5′ CAGTGATGGCTGACGAAGGAA 3′ and 5′ TTTGGGAGGAAGTTATGGGCT 3′; *hsp16.49* (*T27E4.8*), 5′ ATGCTCCGTTCTCCATTTTCTG 3′ and 5′ CCATGCTCTGATTTTTTTTCTTGA 3′ 5; *ins-17* (*F56F3.6*), 5′ TTTTGGGACGGA-CAATGAATATG 3′ and 5′ GCACGGTAGAA-CAAGATGATGGA 3′; *ins-18* (*T28B8.2*), 5′TTCAACAAGCTGACGGACGC 3′ and 5′ GACG-GATTGATGGGGCGA 3′; *2H371* (*T02G5.5*), 5′ CTTCGCCCTGCTCCGTTAC 3′ and 5′ ATCAC CGTTCCTCCTCCAAAAT 3′; *sir-2.1* (*R11A8.4*), 5′ AAATCTTCCCAGGACAGTTCGTA 3′ and 5′ ATGGGCAACACGCATAGCA 3′.

Statistical analysis

The Kaplan–Meier method was utilized to generate survival curves for all survival experiments using the GraphPad Prism Software (Version 5.01, GraphPad Software, Inc. La Jolla, CA). Differences in survival between treatment groups were compared using the non-parametric Log-Rank test, where $p < 0.05$, $p < 0.01$ or $p < 0.001$ indicates a statistically significant difference between experimental and control groups (see Table I). The logistic regression (http://statpages.org/logistic.html) on ordinal response variable lifespan was used to analyse the prediction of age at death by variables like self-fertile reproductive span, fast body movement span, fast pharyngeal pumping span and pharyngeal pumping span [19]. The two-way analysis of variance (ANOVA) was used to determine whether age-related genes expression by treatment were significantly different from these genes expression untreated control. A value of $p < 0.05$ was considered statistically significant.

Results

DhHP-6 significantly extends the lifespan of wild type C. elegans N2

The mean lifespan of wild type *C. elegans* is 16.1 days and the maximum lifespan is 25 days under our laboratory conditions at 20°C. Treatment with DhHP-6 at a final concentration of 50 μM, 200 μM and 500 μM increased the average lifespan of wild type *C. elegans* by 19.8%, 21.0% and 18.5%, respectively ($p < 0.001$, Figure 1 and Table I).

Three physiological functions—self-fertile reproduction, body movement and pharyngeal pumping—are positively correlated with each other and with the

Table II. The effect of genetic variables upon lifespan in the presence of DhHP-6.

Trial	Strain	Treatment (μM DhHP-6)	Mean lifespan ± SE (*n*)	% Change	Max lifespan	*p*-value*
1	*mev-1 (kn1)*	*E.coli* (0)	11.7 ± 0.49 (50)	—	20	—
		E.coli (200)	15.0 ± 0.42 (45)	28.2	20	< 0.001
2	*sir-2.1 (ok434)*	*E.coli* (0)	22.04 ± 0.36 (48)	—	28	—
		E.coli (100)	22.44 ± 0.42 (50)	0.02	28	0.1653
3	*daf-16(mgDf50)*	*E.coli* (0)	12.9 ± 0.52 (66)	—	20	—
		E.coli (50)	11.0 ± 0.49 (55)	−0.14	19	< 0.01
		E.coli (100)	11.2 ± 0.48 (52)	−0.13	18	< 0.01
4	*daf-16(mgDf50)*	*E.coli* (0)	12.62 ± 0.56 (55)	—	21	—
		E.coli (100)	11.16 ± 0.61 (38)	0.11	18	0.0531

*compared with untreated *daf-16* gene mutation control.

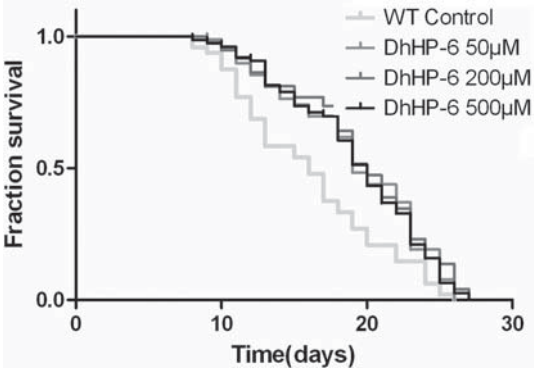

Figure 1. Treatment with DhHP-6 extended lifespan of wild type *C. elegans* grown at 20°C. $p < 0.001$, Log rank for wild type (0) vs DhHP-6 (50 μM, 200 μM, 500 μM).

lifespan of *C. elegans* [19,24]. The measurement of the age-related decline of physiological processes was used to define the stages of ageing [24]. DhHP-6 treatment significantly delayed the age-related decline of fast body movement, but it had no effect on the decline of pharyngeal pumping and self-fertile reproduction (Figure 2).

As shown in Figure 2C, DhHP-6 extended stage II, the period characterized by vigorous motor activity.

DhHP-6 improves the heat and oxidative stress resistance of C. elegans

According to the free radical theory of ageing, enhanced cellular stress resistance should help retard ageing [25]. Several studies have shown that increased longevity is closely associated with improved survival under conditions of heat and oxidative stress [26]. To study the possible mechanism for DhHP-6's lifespan extension effect on *C. elegans*, we examined its impact on stress resistance. DhHP-6 significantly increased thermotolerance. In wild type *C. elegans*, treatment with 200 μM DhHP-6 increased the survival rate from 8% (untreated) to 42% (treated) at 35°C (Figure 3A). DhHP-6 also significantly increased resistance to oxidation stress. Paraquat exerts toxicity through a redox-cycling mechanism that produces superoxide and other ROS, *in vivo* [27], and has been used successfully to induce oxidative stress in various organisms. Treatment with

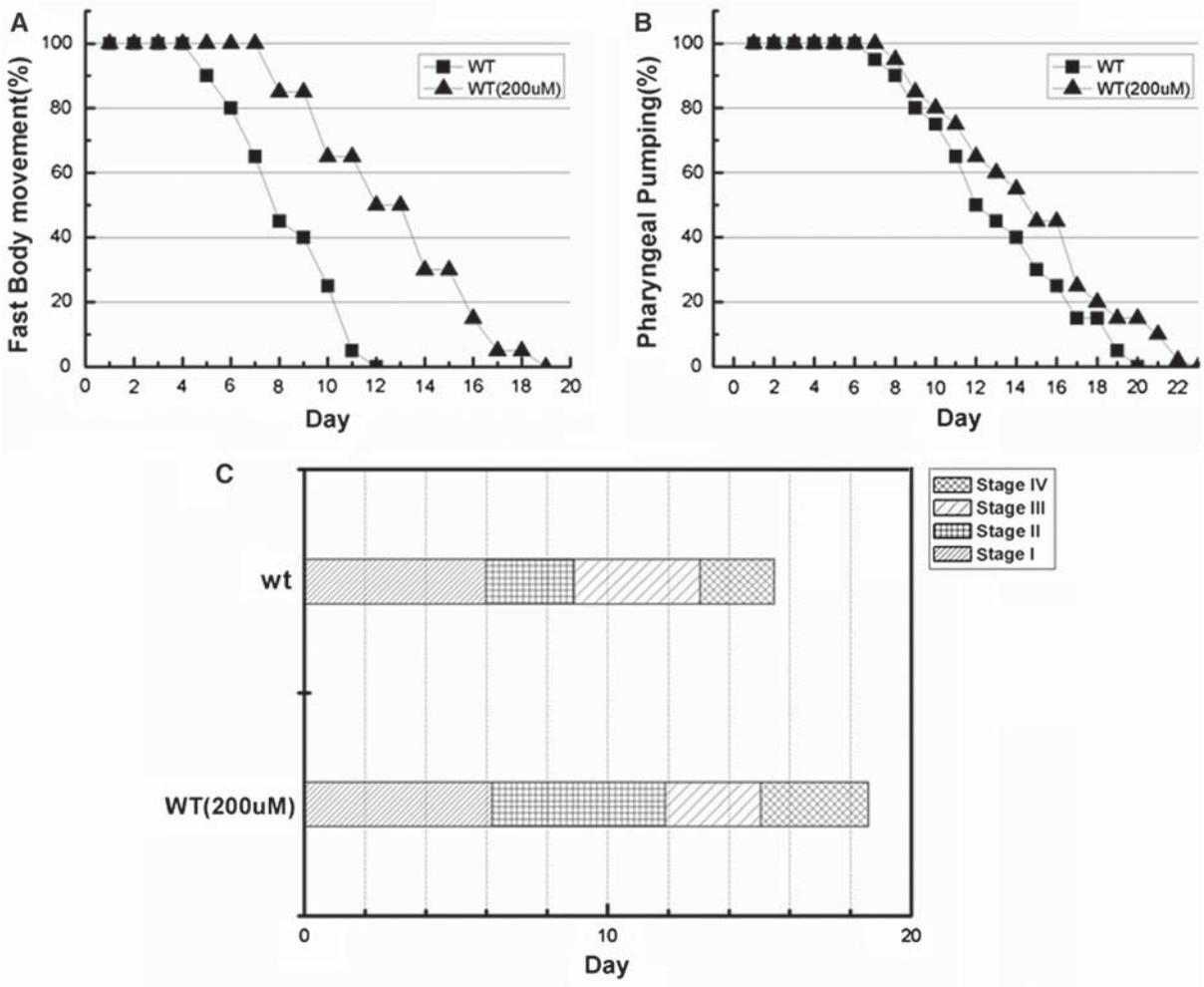

Figure 2. Effect of DhHP-6 on age-related changes in physiolog ical functions. (A) The time from L4 to cessation of fast body movement. (B) The time from L4 to the cessation of all pharyngeal pumping (< 1/10 s). (C) Stages I–IV, from L4 to dead. WT (0): wild type worms, no treatment. WT (200): wild type worms, treated with 200 μM of DhHP-6.

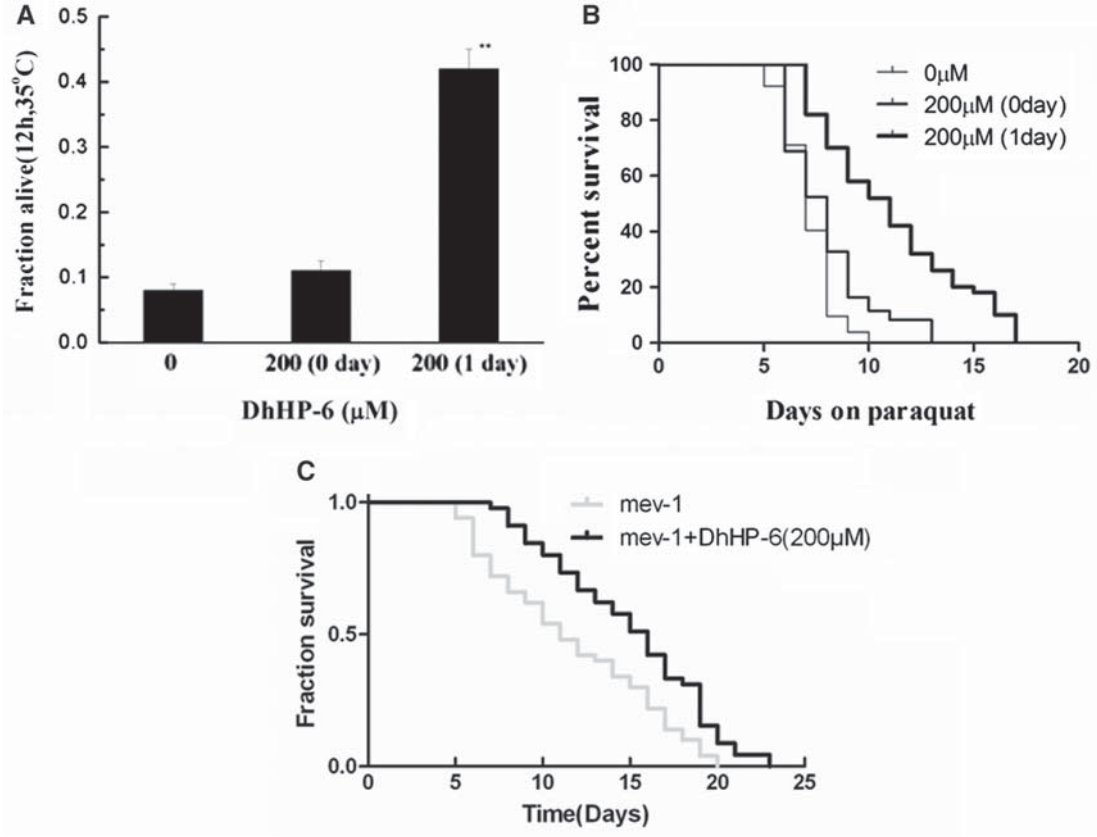

Figure 3. Treatment with DhHP-6 improved oxidative stress resistance. (A) Fraction which survived at 35°C at day 4. Shown is average survival from three experiments with 30–50 animals per experiment; $^{**}p < 0.01$, *t*-test. WT (0) vs DhHP-6 (200 μM). (B) Survival rate of worms administered 2 mM paraquat along with DhHP-6. 200 μM (0 day) represents simultaneous treatment with DhHP-6 and the stress injury; 200 μM (1 day) indicates treatment with DhHP-6 for 1 day, followed by the stress injury. $P < 0.001$, log rank, for paraquat vs paraquat+DhHP (200 μM) for 1 day. (C) Effects of DhHP-6 on lifespan in *mev-1 (kn1)* animals. $p < 0.01$, log rank, treated vs untreated controls.

200 μM DhHP-6 significantly reduced the *C. elegans*' sensitivity to paraquat and extended the lifespan of the treated *C. elegans* (Figure 3B).

Importantly, the increased thermo and oxidative tolerance was observed only in *C. elegans* treated at least 1 day before acute injury (Figures 3A and B), suggesting that DhHP-6 might obtain this effect by enhancing the ability of scavenging free radicals *in vivo*. To further examine whether DhHP-6 could protect against acute oxidative stress, we examined *mev-1(kn1) C. elegans*. In *C. elegans*, *mev-1* encodes cytochrome b, a large subunit of the Complex II enzyme succinate-CoQ oxidoreductase. This mutation is hypersensitive to oxidative stress and ages precociously, probably because of elevated superoxide anion production in mitochondria [28]. Treatment with DhHP-6 increased the lifespan of *mev-1* null mutated *C. elegans*, consistent with the finding that DhHP-6 protect against extrinsic oxidative stress (Figure 3C).

DhHP-6 up-regulates SOD-3 expression

To further study the protective effects of DhHP-6 in *C. elegans*, SOD-3::GFP reporter gene expression in transgenic CF1553 was investigated. Compared with the control group, treatment with DhHP-6 significantly increased the intensity of SOD-3::GFP in pictures taken with Olympus BX51 microscope (Figure 4). In a following study, we quantified the SOD-3::GFP expression by a BioTek Synergy HT Multi-Mode Microplate Reader. The data showed that DhHP-6 at 100 μM could significantly up-regulated SOD-3::GFP expression by 24.9% in transgenic CF1553 (Figure 4D), indicating that treatment with DhHP-6 could increase expression of SOD-3 in *C. elegans*.

DhHP-6 regulates the mRNA expression of age-related genes in wild type C. elegans

A number of genes, such as *sir-2.1*, *hsp12.6*, *hsp16.1*, *ins-17*, *ins-18* and *T02G5.5* (members of the TC3 transposase family) undergo statistically significant changes in transcription levels during ageing in *C. elegans* [29,30]. Using real-time RT-PCR, we confirmed that the expression of *hsp12.6*, *hsp16.1*, *ins-17*, *ins-18* and *T02G5.5* (members of the TC3 transposase family) increased, while *sir-2.1* (R11A8.4, encodes SIR2 homologue) decreased on day 8 (day 0 comes from the day when *C. elegans* reaches adulthood) (Figure 5).

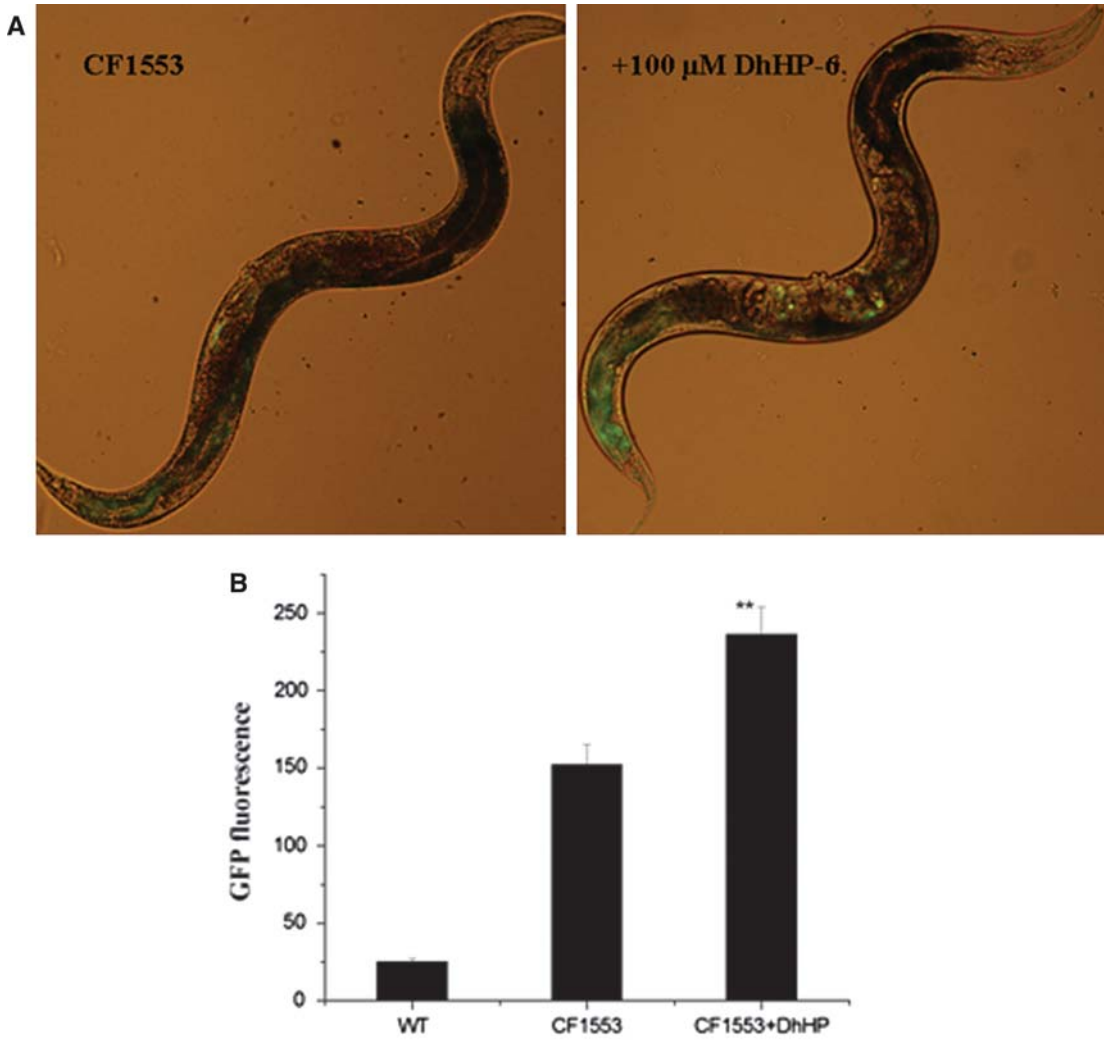

Figure 4. DhHP-6 up-regulates SOD-3::GFP expression in transgenic *C. elegans* CF1553. (A) Image of SOD-3::GFP expression in transgenic *C. elegans* CF1553 (fluorescence and light microscopic pictures) after 1 day of incubation without (control) or with DhHP-6. The GFP pictures of transgenic worms were taken on an Olympus BX51 microscope. (B) Quantified GFP intensity (± SE) in CF1553 from three experiments in each group, with 30 worms in each experiment. $^{**}p < 0.01$.

We examined the effect of DhHP-6 on these age-related genes. We found that DhHP-6 treatment blocked the increase in the expression of *hsp12.6* ($p < 0.01$), *hsp16.1* ($p < 0.01$) and the decrease of *sir-2.1* ($p < 0.01$), but it had no effect on the increase of *ins-17*, *ins-18* and *T02G5.5* (Figure 5). *Sir-2.1* gene encodes a histone deacetylase-like protein and over-expression of *sir-2.1* gene was shown to play a crucial role in transmitting longevity and resistance to heat and oxidative stress in *C. elegans* [31]. The mRNA levels of *sir-2.1* increased six times higher than the untreated worms at day 0. This indicated that DhHP-6 treatment increased stress resistance in *C. elegans* during the worm's ageing process and consequently extended *C. elegans*' lifespan.

Genetic requirements for the lifespan extension by DhHP-6 treatment

Pathways for induction of stress-response genes that affect lifespan have been identified in *C. elegans*. Because DhHP-6 treatment might improve survival by acting through these genes, we examined whether mutations in the two major stress response genes: *sir-2.1* and *daf-16* (Table II). As DhHP-6 could increase significantly the expression of *sir-2.1* gene, we first examined the possibility that DhHP-6 treatment might improve survival by acting through *sir-2.1*. DhHP-6 treatment could not further extend lifespan of *sir-2.1 (OK434) C. elegans* which lacked *sir-2.1* gene activity (Figure 6A), showing that DhHP-6 act through *sir-2.1* to extend the lifespan of *C. elegans*.

In *C. elegans*, *daf-16* gene encodes DAF/FOXO transcription factors in the insulin/IGF-1 signalling pathway. The DAF/FOXO transcription factor promotes expression of genes that confer extended longevity and enhanced stress resistance [31,32]. DhHP-6 treatment did not extend the lifespan of *C. elegans* with non-functional mutant of *daf-16 (mgDf50)* (Figure 6B), indicating that DAF-16 is indispensable for its beneficial effects of DhHP-6 on lifespan extension in *C. elegans*.

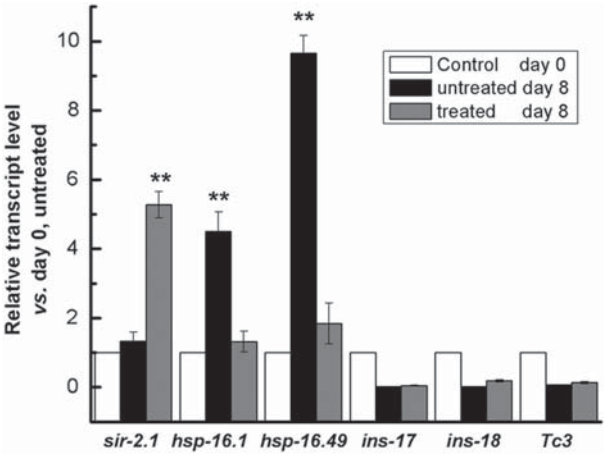

Figure 5. Effect of DhHP-6 on expression of age-related genes in wild type *C. elegans* N2. Expression levels, relative to *gpd-1* (a type of housekeeping gene), were determined by quantitative real time RT-PCR. Error bars indicate SE. $^{**}p < 0.01$ vs day 0 control.

Discussion

If ageing is associated with the accumulation of molecular damage caused by free radicals, antioxidants are expected to delay ageing. Recent studies have also demonstrated important roles of antioxidative proteins in signalling and suggested a complex regulatory network controlling ROS-resistance and longevity [33]. Here, we observed that DhHP-6 could extend lifespan of wild type *C. elegans* by 19% *in vivo* and increase its stress resistance in our laboratory culture condition. As mentioned, DhHP-6 is a novel peptide containing deuterohemin, with an *in vitro* ability to scavenge hydroxyl free radicals at ~ 94% that of microperoxidase-11 [15]. One reasonable mechanism is that DhHP-6 directly scavenges free radicals and reduces the level of hydrogen peroxide. Hydrogen peroxide has been reported to be one agonist of insulin and IGF-1 signalling pathway and it could suppress DAF-16 and reduce the lifespan of *C. elegans* [34]. Furthermore, because SOD-3 is the downstream effector of DAF-16 and can serve as a stress sensitive reporter to predict longevity in *C. elegans* [35], the effect of DhHP-6 on the expression of SOD-3 was investigated. We found that SOD-3 expression was up-regulated in transgenic *C. elegans* with SOD-3::GFP, which might help to explain why DhHP-6 could significantly increase the survival of *C. elegans* under heat and oxidative stress. Considering the studies by Gems et al. [33] that SOD has, at most, a minor effect on the lifespan of *C. elegans*, DhHP-6 might increase stress resistance and extend lifespan in different ways.

By using *C. elegans* for these experiments, a genetic analysis of the beneficial effects of DhHP-6 was possible. We showed that DhHP-6 could protect *C. elegans* against the elevated oxidative stress imposed by the *mev-1 (kn1)* mutation, consistent with our finding that DhHP-6 protected *C. elegans* against oxidative stress by heat or paraquat. In addition, we tested the effect of DhHP-6 upon longevity and ageing using the *daf-16 (mgDf50) I* and *sir-2.1 (ok434) IV* null mutant strains of *C. elegans*. The treatment with 200 μM DhHP-6 failed to prolong the lifespan of animals with defects in the DAF-16 and SIR-2.1, suggesting that the effects of DhHP-6 treatment are mediated, or at least in part, through these genes. On the other hand, we also noted that DhHP-6 shortened the lifespan of the DAF-16 mutant. This may be due to DhHP-6's hormesis effect that depends on the *daf-16* gene. The toxicity of DhHP-6, however, is expected to be insignificant for higher level animals. Safety assessment results for DhHP-6 show that it has no acute toxicity in mice when injected peritoneally (LD50 = 4.797 g/kg), and no long-term toxicity in rabbits and rats (dose range from 42–10 g/kg) [15].

In conclusion, the longevity improving effects of DhHP-6 in the nematode *C. elegans* might be attributed to its direct or indirect ROS scavenging activity through up-regulating expression of antioxidative enzymes SOD and stress resistance associated genes such as *sir-2.1*. As a stable peptide mimetic of low toxicity, DhHP-6 has the potential to be a useful clinical tool in delaying ageing and ameliorating oxidation and age-related disease.

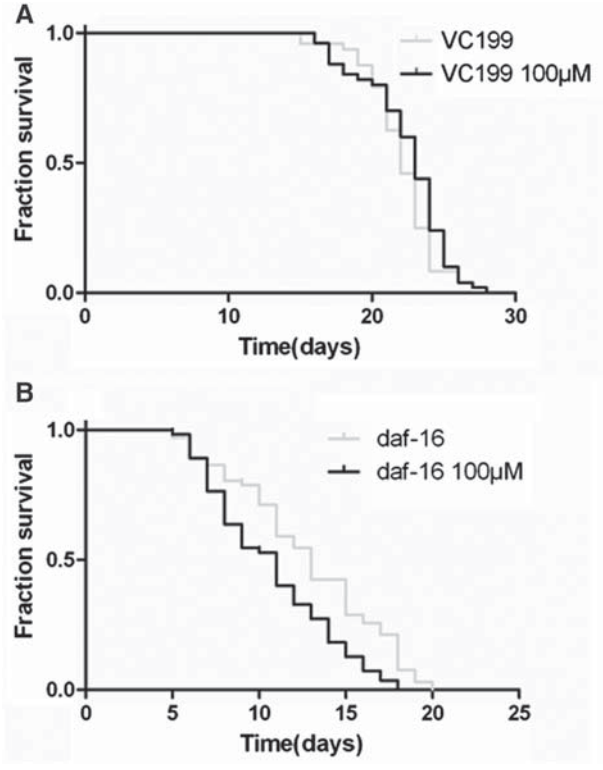

Figure 6. Increased lifespan from DhHP-6 treatment is blocked by *daf-16* and *sir-2.1* mutations. (A) *sir-2.1 (OK434)*. (B) *daf-16 (mgDf50)*. Lifespan survival statistics are contained in Table II.

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