SHORT COMMUNICATION

The clinical antiprotozoal drug nitazoxanide and its metabolite tizoxanide extend *Caenorhabditis elegans* lifespan and healthspan

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Abstract The drugs extending healthspan in clinic have always been searched. Nitazoxanide is an FDA-approved clinical antiprotozoal drug. Nitazoxanide is rapidly metabolized to tizoxanide after absorption *in vivo*. Our previous studies find that nitazoxanide and its metabolite tizoxanide induce mild mitochondrial uncoupling and activate cellular AMPK, oral nitazoxanide protects against experimental hyperlipidemia, hepatic steatosis, and atherosclerosis. Here, we demonstrate that both nitazoxanide and tizoxanide extend the lifespan and healthspan of *Caenorhabditis elegans* through Akt/AMPK/sir 2.1/daf16 pathway. Additionally, both nitazoxanide and tizoxanide improve high glucose-induced shortening of *C. elegans* lifespan. Nitazoxanide has been a clinical drug with a good safety profile, we suggest that it is a novel anti-aging drug.

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1. Introduction

Aging is the progressive decline or loss of organ function with time. Aging is a natural process, but many diseases, such as heart failure, atherosclerosis, neurodegenerative diseases, etc., are related to aging and also called as aging-related diseases[1]. Aging promotes the occurrence of aging-related diseases, for instance, aging of vascular endothelial cells causes leakage of the endothelial barrier, facilitating oxidized low-density lipoprotein to invade the vascular bed and induce atherosclerosis[1]; aging of cardiomyocytes induces cardiac dysfunction and heart failure[2]. The aging process and the occurrence of aging-related diseases often have common mechanisms[3]. Finding ways to combat aging not only extends lifespan, but also provides strategies for treating age-related diseases and extends the healthspan.

Based on the advances in molecular biology technology and the application of model organisms such as *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster*, the mechanisms underlying aging and lifespan regulation have been summarized. The mechanism pathways include[3-5]: insulin-like signaling, AMP-activated protein kinase (AMPK) signaling, Target of rapamycin (TOR) signaling, histone deacetylase sirtuins (SIRT) signaling, circadian clocks mechanism, senescence, chronic inflammation and protein homeostasis. According to the above mechanisms, various drugs and methods with anti-aging effects have been discovered, including metformin, rapamycin, and sirtuin activators, as well as exercise and calorie restriction. These drugs and methods are also therapeutic for aging-related diseases, for instance, metformin improves diabetes and reduces the incidence of cardiovascular diseases by activating AMPK, inhibiting mTORC1, and inhibiting inflammation[6]; rapamycin reverses the decline of cardiac systolic and diastolic function related to aging in dogs by inhibiting mTORC1 signaling[7,8]; calorie restriction improves abnormal lipid metabolism and insulin resistance by activating AMPK, inhibiting mTORC1, and activating SIRT1, and inhibits the occurrence of cardiovascular diseases, diabetes and other age-related diseases[5,9].

Nitazoxanide is an FDA-approved clinical antiprotozoal drug used for diarrhea caused by *Giardia* or *Cryptosporidium*. Nitazoxanide is rapidly metabolized to tizoxanide after absorption *in vivo*. Nitazoxanide exerts antibacterial and antiviral effects[10,11] and clinical trials have been designed to test it for the treatment of chronic hepatitis B, chronic hepatitis C, tuberculosis, severe acute respiratory illness, and COVID-19, etc.[12-16]. Our previous studies found that nitazoxanide and its active metabolite tizoxanide induced mild mitochondrial uncoupling and activated cellular AMPK[17,18], oral nitazoxanide protected against experimental hyperlipidemia, hepatic steatosis, and atherosclerosis[17,18]. Actually, hepatic steatosis and atherosclerosis are aging-related pathological characterizations, indicating that nitazoxanide or its metabolite tizoxanide should show an anti-aging effect. In the present study, by using the *C. elegans* model system, we demonstrated that both nitazoxanide and tizoxanide extended healthspan of *C. elegans* through Akt/AMPK/sir2.1/daf16 pathway. Additionally, both nitazoxanide and tizoxanide improved high glucose-induced shortening of *C. elegans* lifespan dependently on daf-16. Nitazoxanide has been a clinical drug with a good safety profile, we suggest that it is a novel anti-aging drug.

2. Materials and methods

2.1. Reagents

Nitazoxanide (Nit) was purchased from Adamas-beta (Cat# 01088480, Shanghai, China). Tizoxanide (Tiz) was purchased from MedChemExpress (Cat# HY-12687, NJ, USA). The following antibodies were used in this study for Western blot: anti-GFP (Cat# AE012, RRID: AB_2770402, Abclonal, China); p-AMPKα (Cat# 2535, RRID: AB_331250, Cell Signaling Technology, USA); AMPK (Cat# 2532, RRID: AB_330331, Cell Signaling Technology, USA); p-Akt (Cat# 4060, RRID: AB_2315049, Cell Signaling Technology, USA); Akt (Cat# 9272, RRID: AB_329827, Cell Signaling Technology, USA); β-actin (Cat# T0022, RRID: AB_2839417, Affinity Biosciences, USA); β-tubulin (Cat# T0023, RRID: AB_2813772, Affinity Biosciences, USA); GAPDH (Cat# T0004, RRID: AB_2833041, Affinity Biosciences, USA); VDAC1 (Cat# AF5478, RRID: AB_2837960, Affinity Biosciences, USA); LC3B (Cat# L7543, RRID: AB_796155, Sigma−Aldrich, USA); P62/SQSTM1 (Cat# P0067, RRID: AB_1841064, Sigma−Aldrich, USA); IRDye® 800CW Goat anti-Mouse (Cat# 926-32210, LI-COR Biosciences, USA);. HCT116 (human colorectal cell lines) was purchased from Zhongqiaoxinzhou Biotech (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, UT, USA) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Beit Haemek, Israel) and 1% penicillin/streptomycin (P/S, Solarbio, Beijing, China).

2.2. *C. elegans* strains

All strains were grown and maintained at 20 °C on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* strain OP50. The following *C. elegans* strains were used in this study: N2 (*C. elegans* wild isolate, Bristol variety), RB754 (*aak-2 (ok524)* X), GR1307 (*daf-16 (mgDf50)* I), CF1038 (*daf-16 (mu86)* I), RB1206 (*rsks-1 (ok1255)* III), VC199 (*sir-2.1 (ok434)* IV), DA2123 (adIs2122 [*lgg-1p::GFP::lgg-1* + *rol-6 (su1006)*]), TJ356 (zIs356 [*daf-16p::daf-16a/b::GFP* + *rol-6 (su1006)*]), RW1596 (stEx30 [*myo-3p::GFP::myo-3* + *rol-6 (su1006)*]), KN259 (huIs33 [*sod-3::GFP* + *rol-6 (su1006)*]), SJ4103 (zcIs14 [*myo-3::GFP(mit)*]), GR1310 (*akt-1(mg144)* V), RB759 (*akt-1 (ok525)* V), VC204 (*akt-2 (ok393)* X). Some strains were provided by the Caenorhabditis Genetics Center (University of Minnesota, USA), which is funded by NIH office of Research Infrastructure Programs (P40 OD011440).

2.3. Lifespan analysis

All lifespan assays were performed at 20 °C. Experimental plates were prepared from the same batch of NGM agar and *E. coli* strain OP50 except that D-glucose (Cat# 10010518, Sinopharm Chemical Reagent, China) was prepared in agar at a final concentration of 5 mmol/L. Metformin (Cat# M107827, Aladdin, USA) was prepared in agar at a final concentration of 25 mmol/L. Nitazoxanide (Cat# 01088480, Adamas-beta, China) or tizoxanide (Cat# HY-12687, MCE, USA) was prepared in *E. coli* strain OP50 at a final concentration of 10, 100, 500 μmol/L, 1 mmol/L, respectively, from 1, 10, 50, 100 mmol/L solution

stock, and DMSO was prepared in *E. coli* strain OP50 at a final concentration of 1%. After the plates were poured with autoclaved NGM agar and dried, the freshly prepared *E. coli* strain OP50 was evenly smeared on the agar plates and allowed to dry overnight. For each experiment, the synchronized Day 1 adults were placed on five plates with 30 nematodes per plate and allowed to lay eggs for 24 h, then these parental nematodes were removed and the eggs were allowed to develop into L4 larvae. The synchronized L4-stage nematodes were transferred to experimental plates, which was defined as a start time point (Day 0) for lifespan assay. For the lifespan experiments, we transferred nematodes to fresh plates every day in the initial 10-day drug treatment period, and later removed nematodes to fresh plates every 2 days until all the nematodes died. For glucose-related lifespan experiments, D-glucose was prepared in agar at a final concentration of 5 mmol/L. For experimental groups, the synchronized L4-stage nematodes were treated with glucose (5 mmol/L) alone or glucose (5 mmol/L) + Nit (100 μmol/L) or glucose (5 mmol/L) + Tiz (100 μmol/L) for 12 days, and then transferred to agar plates with glucose (5 mmol/L) and fed with fresh OP50 without the drug. For the control group, the synchronized L4-stage nematodes were treated with DMSO for 12 days, and transferred to normal agar plates and fed with fresh OP50 without drug. Nematodes that did not respond to gentle mechanical stimuli were scored as dead. Nematodes that crawled off the plates, died from exploded vulva phenotype and internal hatching were censored. The GraphPad Prism 7.0 software was used to plot and analyze the survival curves. 5′ Fluoro-2′ deoxyuridine was not used in this study.

2.4. Fluorescence microscopy

The synchronized L4-stage nematodes were transferred to the plates. After treatment, the nematodes were placed on slides and anesthetized with 20 mmol/L levamisole (Cat# L812625, Merck, USA). Images of RW1596 (stEx30 [*myo-3p::GFP::myo-3 + rol-6 (su1006)*]) strain, TJ356 (zIs356 [*daf-16::daf-16a/b::GFP + rol-6 (su1006)*]) strain and KN259 (huIs33 [*sod-3::GFP + rol-6 (su1006)*]) strain were photographed by using a fluorescence microscope (Olympus, Japan). Images of SJ4103 (zcIs14 [*myo-3::GFP (mit)*]) strain were photographed by using a fluorescence microscope (Olympus, Japan) equipped with a 100 × 1.3 NA oil lens (Olympus, Japan). The GFP fluorescence intensities of the RW1596 strain and KN259 strain were qualified by using Image J software.

2.5. Fertility assay

The L4-stage nematodes were randomly selected and cultured on agar plates seeded with OP50 with DMSO (1%) or nitazoxanide (100 μmol/L) or tizoxanide (100 μmol/L). The parental nematodes were transferred to fresh plates every day until reproduction ceased, and the number of hatched eggs was counted every day.

2.6. Measurement of age pigment level

The nematodes were placed on slides and anesthetized with 20 mmol/L levamisole. The intestinal spontaneous fluorescence images of nematodes were observed and photographed by using a fluorescence microscope (Olympus, Japan). The age pigment fluorescence intensities were qualified by using Image J software and normalized to the body area of nematodes.

2.7. Measurement of triglyceride level

The triglyceride level was measured by using a Triglyceride Assay Kit (Cat# A110-1-1, Nanjing Jiangcheng, China) as previously described. Briefly, the nematodes were washed three times with M9 buffer and centrifuged at 4000 rpm for 30 s, and then the sediment was boiled at 100 °C for 10 min by adding 30 μL 2% SDS solution. After centrifugation of the sample at 13,500 rpm for 30 min, the supernatant was collected following by adding 2.5 μL sample or standard solution incubated with 250 μL working solution and distilled water at 37 °C for 10 min. The absorbance was immediately measured at 500 nm wavelength by using a microplate reader. The BCA method was used to detect protein concentration. Triglyceride levels were normalized to protein concentration.

2.8. Oil Red O staining assay

0.5% Oil Red O solution (Cat# O1391, Sigma, USA) was diluted in M9 buffer at a concentration of 0.3%. It was then mixed with an equal volume of 2% Triton X-100 and filtered. The nematodes were washed three times with M9 buffer and centrifuged at 4000 rpm for 30 s. Next, they were fixed in 500 μL 1% paraformaldehyde and incubated at 4 °C for 15 min, followed by transfer to −80 °C for 20 min. After that, the nematodes were quickly thawed at 43 °C in a water bath and washed as described above. The nematodes were then stained with 200 μL of the Oil Red O working solution at room temperature for 1 h on a shaker. After staining, the nematodes were washed three times with M9 buffer. Next, they were resuspended in 200 μL M9 buffer, and 20 μL was added to the slide each time for observation under a microscope. The intensity of the Oil Red O staining was quantified using Image J.

2.9. Locomotion assay

Nematodes were randomly selected and picked into 20 μL of M9 buffer on a glass slide. Next, the number of body bends was recorded every 30 s with a camera equipped with a dissecting microscope. At least 30 nematodes were recorded per strain or treatment. The average number of body bends every 30 s for each sample was calculated and graphed by GraphPad Prism 7.00 Software.

2.10. Western blot analysis

At least 100 nematodes were collected from each group, washed three times using M9 buffer, and then centrifuged at 4000 rpm for 30 s. After adding 50 μL RIPA lysis buffer with PMSF and phosphatase inhibitor in each tube, the samples were homogenized by sonication machine and centrifuged at 13,500 rpm at 4 °C for 30 min. The resulting supernatant was collected. The protein content of the samples was quantified by using the BCA assay (Cat# P0010, Beyotime, China). And then the samples were boiled at 100 °C for 7 min, and cooled to room temperature. Samples were electrophoresed and transferred onto the membrane for 90 min at 300 mA. After transferring, the membranes were blocked with 5% skim milk (TBS) for 1 h at room temperature on a shaker. Then, the membranes were washed three times with TBST for 10 min each time on a shaker at room temperature and incubated with a primary antibody at 4 °C overnight and fluorescence-labeled secondary antibodies (LI-COR, USA) for 1 h

at room temperature. The images were captured by using the Odyssey CLx Infrared Imaging System (LI-COR, USA), and the bands were analyzed by using Image Studio. The levels of targeted protein were quantitated relative to GAPDH.

2.11. Measurement of body length and body area of nematodes

The synchronized L4-stage nematodes were randomly selected and cultured on agar plates seeded with OP50 with DMSO, nitazoxanide (100 µmol/L) or tizoxanide (100 µmol/L), which was defined as a start time point (Day 0) for the experiment. To prevent starvation, the nematodes were transferred to fresh plates every day in the initial 10-day treatment period, and later removed to fresh plates every 2 days. The bright field images of nematodes were photographed by using a fluorescence microscope (Olympus, Japan) every day in the initial 4-day treatment period and every 2 days later. Image J software was used to qualify the body length and body area of nematodes.

2.12. Bacterial avoidance and growth assays

For the bacterial avoidance assay, briefly, freshly prepared *E. coli* strain OP50 with DMSO or nitazoxanide or tizoxanide at the indicated concentrations was seeded on both sides of the 90 mm plate in a round shape. Then, the synchronized L4-stage nematodes were transferred to each side with 50 nematodes per side. After 2, 4, 6 h treatment, the number of nematodes in both round areas was counted, respectively. For the bacterial growth assay, LB medium with 1% *E. coli* strain OP50 was incubated with DMSO or nitazoxanide or tizoxanide and shaking for 12 h at 37 °C, and then absorbance (595 nm) was measured by using a 96-well plate reader (SpectraMax iD3, Molecular Device, USA).

2.13. Measurement of mitochondrial oxygen consumption rate

Mitochondrial oxygen consumption assays were performed using the high-resolution respiratory system Oxygraph-2K (Oroboros O2k instruments, Austria). Briefly, 300 nematodes were washed with M9 buffer and spontaneously suspended, and then nematodes were added to the two chambers and basal mitochondrial respiratory was recorded. Datlab 6.0 software was used to qualify basal mitochondrial respiratory.

2.14. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential assay was performed by using the Mitochondrial Membrane Potential Assay Kit with TMRE (Cat# C2001S, Beyotime, China). Briefly, TMRE solution was prepared in OP50 and then evenly smeared on the agar plates and allowed to dry overnight. Nematodes were fed with OP50 containing TMRE solution for 12 h and transferred to NGM agar plates with OP50 containing no TMRE. After 2 h, nematodes were photographed by using a fluorescence microscope (Olympus, Japan). Mitochondrial membrane potential fluorescence was qualified using Image J and normalized to the body area of nematodes.

2.15. Measurement of body-cavity leakage

Body-cavity leakage assay was performed using erioglaucine disodium salt (Cat# 3844-45-9, Sigma, USA). Briefly, erioglaucine disodium salt powder was dissolved in M9 buffer at a concentration of 10% and then prepared in the same volume of OP50 bacteria. Nematodes were cultured in OP50 bacterial with erioglaucine disodium salt for 6 h and then washed three times with M9 buffer. Nematodes were photographed by using a fluorescence microscope (Olympus, Japan). According to body-cavity leakage levels, nematodes were classified into class 1 (Low leakage), class 2 (Medium leakage), and class 3 (High leakage).

2.16. Measurement of ATP content

ATP content was measured by Enhanced ATP Assay Kit (Cat# S0026, Beyotime, China). Briefly, the nematodes were washed three times with M9 buffer and centrifuged at 4000 rpm for 30 s, and then the samples were homogenized by sonication (VCX130, SONICS, USA). After centrifugation of the sample at 13,500 rpm for 30 min, the supernatant was collected following by adding 25 µL sample or standard solution incubated with 100 µL ATP assay reagent. Luminescence was measured immediately using a microplate reader (SpectraMax iD3, Molecular Device, USA). Protein concentration was tested by the BCA method. ATP content was normalized to protein concentration in each group.

2.17. Cytoplasmic and mitochondrial protein extracting assay

The cytoplasmic and mitochondrial protein of cells were extracted and isolated using the Cell Mitochondria Isolation Kit (Cat# C3601, Beyotime, China) according to the manufacturer's protocol. Briefly, HCT116 cells were washed with PBS three times and digested with trypsin-EDTA solution, and then centrifuged at 150 $\times$ g, room temperature for 5 min. After adding 100 µL mitochondrial isolation buffer with PMSF and phosphatase inhibitor in each tube, the samples were gently suspended and bathed in ice for 15 min, and then transferred into a glass homogenizer and rubbed for 15 times. After that, the samples were collected and centrifuged at 800 $\times$ g, 4 °C for 10 min. The supernatant was collected and centrifuged at 3500 $\times$ g, 4 °C for 10 min. The resulting supernatant was cytoplasmic protein and sediment was isolated mitochondria. After adding 30 µL mitochondrial lysis buffer into mitochondrial sediment, the samples were homogenized by sonication machine and centrifuged at 13,500 rpm, 4 °C for 30 min. The supernatant was mitochondrial protein. The protein concentration was qualified using BCA assay (Cat# P0010, Beyotime, China). VDAC1 (Cat# AF5478, RRID: AB_2837960, Affinity, USA) was applied as mitochondrial reference control, GAPDH (Cat# T0004, RRID: AB_2833041, Affinity, USA) was applied as cytoplasmic reference control.

2.18. Statistical analysis

All data were represented as mean $\pm$ standard error of mean (SEM) and analyzed using GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA, USA). Unpaired two-tailed student's *t*-test was used to analyze the statistical significance between the two groups. Log-rank (Mantel-Cox test) test and Gehan−Breslow−Wilcoxon test were applied to analyze the statistical significance of lifespan-related data. The Chi-square test was used to analyze daf16 nuclear translocation measurement assays and body-cavity leakage assay data between the two groups. $P < 0.05$ was considered as significant.

3. Results

3.1. Nitazoxanide (Nit) and tizoxanide (Tiz) extend C. elegans lifespan

Nitazoxanide (Nit) is rapidly deacetylated to tizoxanide (Tiz) after absorption in mammals. In the present study, we investigated the effect of both Nit and Tiz on C. elegans lifespan. Wild-type C. elegans (N2) at the L4 stage were fed with an E. coli OP50 bacteria diet containing indicated concentration of Nit and Tiz. As shown in Supporting Information Fig. S1, Nit (100 μmol/L) and Tiz (100 μmol/L) treatment for the whole worm life significantly extended C. elegans lifespan, but the extension extent at the earlier and late stages was different. We used two analysis methods, the Log-rank test and the Gehan−Breslow−Wilcoxon test. The statistical difference analyzed by using the Gehan−Breslow−Wilcoxon test was more significant than that by using the Log-rank test. The Gehan−Breslow−Wilcoxon test gives more weight to the data at early time points; therefore, it could be inferred that Nit and Tiz-induced lifespan extension was more significant at the earlier stage of the drug treatment. As shown in Supporting Information

Fig. S2A and a certain number of worms exhibited signs of stiffness at about 14 days after Nit (100 μmol/L) and Tiz (100 μmol/L) treatment. The analyzed data of body bends showed that the worm motion increased on Day 10 but tended to decrease in later days (Fig. S2B). Nit itself is an antiprotozoal drug, we speculated that the less effect at the late stage than that at the earlier stage might be due to the drug treatment time of the whole worm life being too long and the drug accumulation induced worm paralysis. Therefore, in the following experiments for lifespan evaluation, Nit (100 μmol/L) and Tiz (100 μmol/L) were administered for 12 days from the L4 stage and stopped. At this experiment condition, Nit (100 μmol/L) and Tiz (100 μmol/L) treatments still significantly extended C. elegans lifespan (Fig. 1A). Both Nit and Tiz have antibacterial activities[19]. We found that Nit and Tiz at 100 μmol/L inhibited E. coli OP50 growth (Fig. 1B). To identify the effect of alive or dead E. coli OP50 on the action of Nit and Tiz, C. elegans were fed with inactivated E. coli OP50 by heat. The inactivated E. coli OP50 diet did not affect Nit and Tiz-induced extension of C. elegans lifespan (Fig. 1C). The worms did not escape from the OP50 diet containing Nit (100 μmol/L) and Tiz (100 μmol/L) (Fig. 1D), indicating that Nit and Tiz-induced C. elegans lifespan extension
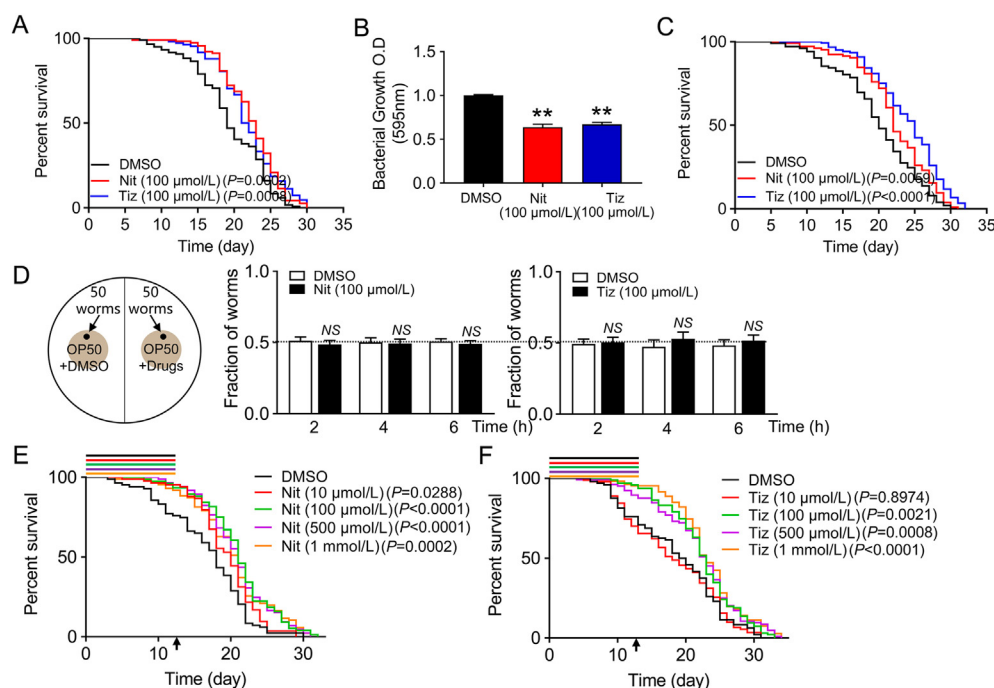

Figure 1 Nitazoxanide (Nit) and tizoxanide (Tiz) prolonged the lifespan of N2 C. elegans (Wild type). (A) Nit or Tiz treatment prolonged the lifespan of N2 C. elegans (Wild type) fed with live OP50 bacteria. The L4-stage nematodes (Day 0) were treated with Nit or Tiz for 12 days and fed with live OP50 bacteria, and then transferred to fresh NGM plates and fed with live OP50 without the drugs. Nit or Tiz was prepared in live OP50 bacteria at a concentration of 100 μmol/L $n = 122, 115, 112$ in DMSO, Nit, and Tiz groups, respectively. Log-rank test was used to calculate the P value vs. DMSO group. (B) Nit or Tiz treatment for 12 h inhibited OP50 bacterial growth. $n = 18$ in each group. **$P < 0.01$ vs. DMSO. (C) Nit or Tiz treatment prolonged the lifespan of N2 C. elegans (Wild type) fed with heat-killed OP50 bacteria. The L4-stage (Day 0) nematodes were treated with Nit or Tiz for 12 days and fed with heat-killed OP50, and then transferred to fresh NGM plates with heat-killed OP50 without the drugs. Nit or Tiz was prepared in heat-killed OP50 bacteria at a concentration of 100 μmol/L $n = 110, 108, 120$ in DMSO, Nit, and Tiz groups, respectively. Log-rank test was used to calculate the P value vs. DMSO group. (D) Nit and Tiz caused no microbial aversion for N2 C. elegans (Wild type). $n = 3$. NS, not significant. (E) The dose−response of the effect of Nit (10 μmol/L, 100 μmol/L, 500 μmol/L, 1 mmol/L) on lifespan of N2 C. elegans (wild-type) fed with live OP50. Nit was applied for 12 days. $n = 83, 84, 76, 85, 86$ in DMSO, Nit (10 μmol/L), Nit (100 μmol/L), Nit (500 μmol/L), and Nit (1 mmol/L) groups, respectively. Log-rank test was used to calculate the P value vs. DMSO group. (F) The dose−response of the effect of Tiz (10 μmol/L, 100 μmol/L, 500 μmol/L, 1 mmol/L) on lifespan of N2 C. elegans (wild-type) fed with live OP50. Tiz was applied for 12 days. $n = 98, 97, 95, 115, 106$ in DMSO, Tiz (10 μmol/L), Tiz (100 μmol/L), Tiz (500 μmol/L), and Tiz (1 mmol/L) groups, respectively. Log-rank test was used to calculate the P value vs. DMSO group. All data are represented as mean ± SEM.

was not due to the diet restriction. Finally, we studied the effect of different doses of Nit (10, 100, 500 μmol/L and 1 mmol/L) and Tiz (10, 100, 500 μmol/L and 1 mmol/L) treatment for 12 days on the lifespan of *C. elegans*. Results showed Nit and Tiz at 100, 500 μmol/L and 1 mmol/L significantly extended *C. elegans* lifespan, but the effect of Nit and Tiz at 100, 500 μmol/L and 1 mmol/L was no significant difference (Fig. 1E and F).

3.2. Nitazoxanide and tizoxanide improve *C. elegans* physical fitness

Nit and Tiz not only extended the lifespan, but also improved the fitness of *C. elegans*. As shown in Fig. 2A−C, Nit (100 μmol/L) and Tiz (100 μmol/L) treatment significantly increased the body length and body area of wild-type *C. elegans*. We further used myo-3 (α-myosin heavy chain homolog)::GFP worms to evaluate the effect of Nit and Tiz on the worm size. Nit (100 μmol/L) and Tiz (100 μmol/L) treatment for 5 days not only increased the body length and body area, but also increased the GFP fluorescence intensity (Fig. 2D and E), which indicated the increased expression of myosin protein. By using GAPDH as the reference protein which was consistent with the total protein quantity in *C. elegans*, we found that both Nit (100 μmol/L) and Tiz (100 μmol/L) treatment significantly increased β-actin and β-tubulin protein expression (Fig. 2F−I). All the myosin, actin, and tubulin are cytoskeletal proteins, the increase of these protein expressions might be involved in Nit and Tiz-induced increase of worm size.

Nit and Tiz treatment increased the body bends, reduced the aging marker lipofuscin content, and improved the body−cavity barrier but showed no significant effect on the progeny number of *C. elegans* (Fig. 3A−D). Mitochondrial dysfunction and mitochondrial fragmentation occur during aging in *C. elegans*[20,21]. Then, the mitochondrial morphology in body wall muscles and the mitochondrial respiration function of *C. elegans* after Nit and Tiz treatment for 10 days were studied. As shown in Fig. 3E, in contrast to the fragmented mitochondria in the control group (DMSO treatment), *C. elegans* treated with Nit and Tiz had more elongated tubular mitochondria. By using the high-resolution respirometry system Oxygraph-2k, the basal mitochondrial respiration is determined by monitoring the oxygen consumption rate (OCR) in the absence of any inhibitors. Results showed that *C. elegans* treated with Nit and Tiz had higher basal OCR (Fig. 3F). Furthermore, Nit and Tiz treatment for 10 days significantly induced an increase of ATP level in *C. elegans* (Fig. 3G).

3.3. Nitazoxanide- and tizoxanide-induced AMPK activation contributes to *C. elegans* lifespan extension

AMPK activation plays a critical role in aging and lifespan regulation[22]. Our previous studies demonstrated that Nit and Tiz activated cellular AMPK through inducing mitochondrial uncoupling[17,18], therefore, we speculated that Nit and Tiz would activate AMPK in *C. elegans*. AMPK activation was not detected when *C. elegans* were treated with Nit (100 μmol/L) and Tiz (100 μmol/L) for one day (Fig. 4A−C). However, when the treatment time was prolonged to five days, AMPK was significantly activated (Fig. 4D and F). We treated the *C. elegans* with Nit (100 μmol/L) and Tiz (100 μmol/L) for five days, then the
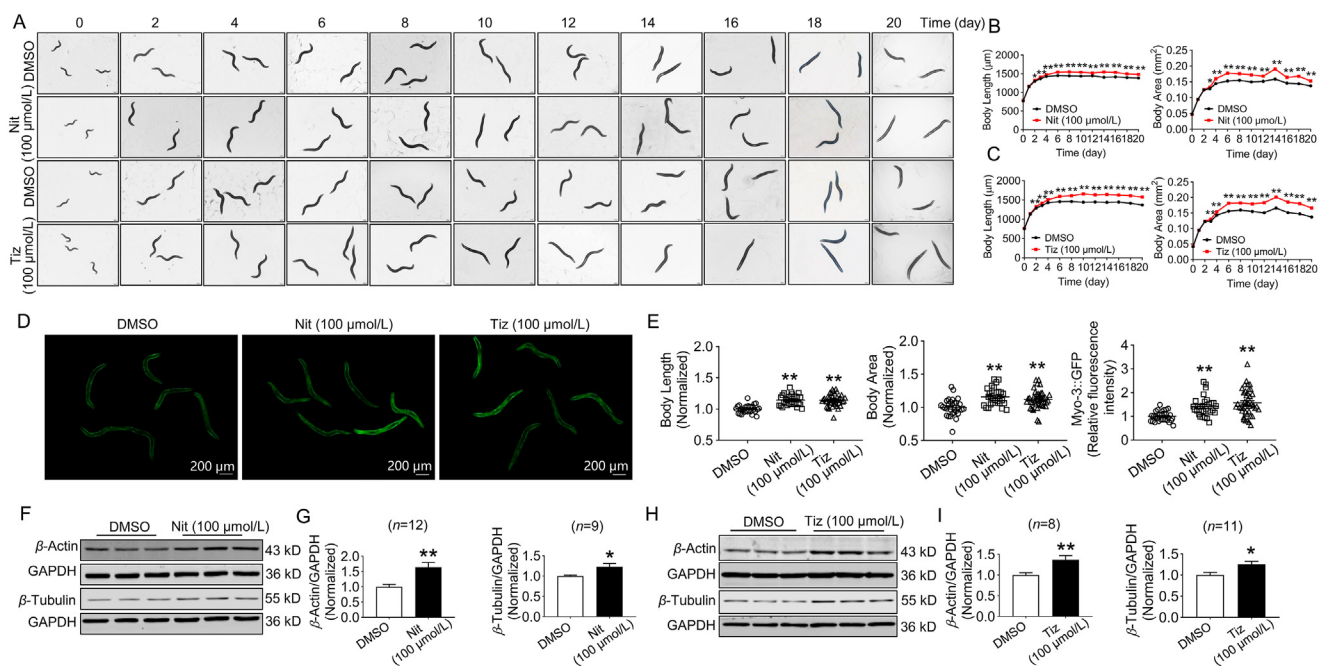

Figure 2 Nitazoxanide (Nit) and tizoxanide (Tiz) increased the worm size and cytoskeletal protein expression in N2 *C. elegans*. (A−C) Nit or Tiz treatment increased the body length and body area of N2 *C. elegans* (wild-type). The L4-stage nematodes (Day 0) were treated with Nit or Tiz for 12 days and fed with live OP50 bacteria, and then transferred to fresh NGM plates and fed with live OP50 without drug. Nit or Tiz was prepared in OP50 bacteria at a concentration of 100 μmol/L *n* = 30−60 in each group. *P < 0.05, **P < 0.01 *vs.* DMSO. (D, E) The representative fluorescent images and summarized data showed that Nit or Tiz treatment for 5 days increased myo-3::GFP fluorescence intensity, body length, and body area of RW1596 strain [myo-3p::GFP::myo-3 + rol (su1006)]. *n* = 30, 30, 42 in DMSO, Nit, and Tiz groups, respectively. **P < 0.01 *vs.* DMSO. (F−I) Nit or Tiz treatment for 5 days increased β-Actin and β-Tubulin protein expression in N2 *C. elegans* (Wild type). *n* = 12, 9, 8, 11 in (G, I) respectively. *P < 0.05, **P < 0.01 *vs.* DMSO. All data are represented as mean ± SEM.
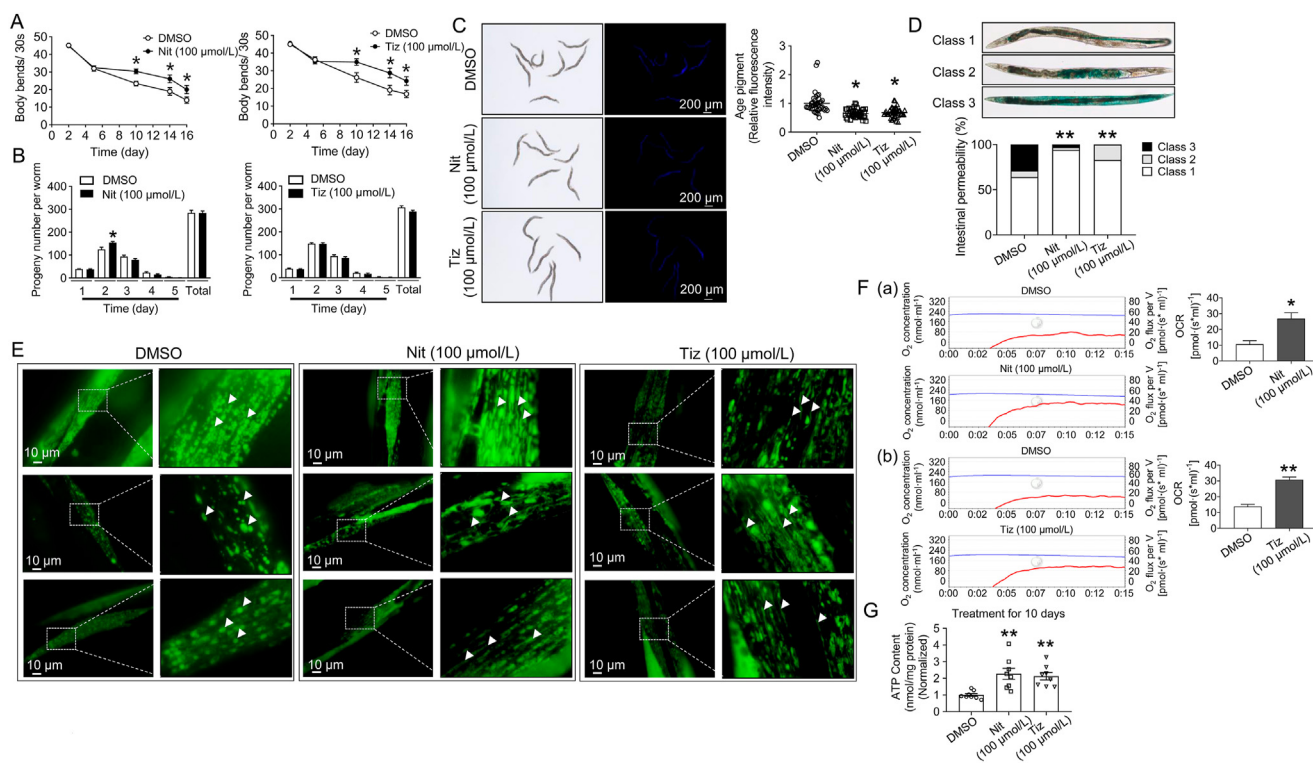
Figure 3 Nitazoxanide (Nit) and tizoxanide (Tiz) prolonged *C. elegans* healthspan. (A) Nit or Tiz treatment improved the body motility of nematodes starting on day 10 of drug treatment. The L4-stage nematodes (Day 0) were treated with Nit or Tiz for 12 days and fed with live OP50 bacteria, and then transferred to fresh NGM plates and fed with live OP50 without drug. Nit or Tiz was prepared in live OP50 bacteria at a concentration of 100 μmol/L $n = 28-33$ in each group. *$P < 0.05$ *vs.* DMSO. (B) Nit or Tiz did not affect the fertility of N2 *C. elegans* (Wild type). $n = 15$ in each group. (C) Nit or Tiz treatment for 7 days inhibited age pigment deposition in N2 *C. elegans* (Wild type). $n = 39, 45, 41$ in DMSO, Nit, and Tiz groups, respectively. *$P < 0.05$ *vs.* DMSO. (D) Nit or Tiz treatment for 5 days improved the body-cavity barrier of nematodes. $n = 28, 34, 29$ in DMSO, Nit, and Tiz groups, respectively. **$P < 0.01$ *vs.* DMSO. (E) The representative fluorescent images of the mitochondrial morphology of SJ4103 [myo-3::GFP (mit)] stain. The L4-stage nematodes (Day 0) were treated with Nit or Tiz for 10 days. $n = 32, 35, 42$ images in DMSO, Nit, and Tiz groups, respectively. (F) Nit or Tiz treatment for 10 days increased mitochondrial oxygen consumption rates (OCR) of nematodes. $n = 300$ worms in each group, three independent experiments. *$P < 0.05$, **$P < 0.01$ *vs.* DMSO. (G) Nit or Tiz treatment for 10 days increased the ATP content of nematodes. $n = 140$ worms in each group, two independent trials. **$P < 0.01$ *vs.* DMSO. All data are represented as mean ± SEM.

treatment was stopped in the next five days (Fig. 4G) and the AMPK activity was further examined. At this condition, AMPK activation was still kept (Fig. 4H and I), indicating that Nit and Tiz-induced AMPK activation in *C. elegans* was long-lasting. In order to prove the contribution of AMPK activation to Nit and Tiz-induced lifespan extension, we examined the effect of Nit and Tiz on aak2 (AMPK homolog) mutant worms. Nit (100 μmol/L) and Tiz (100 μmol/L) treatment did not affect the lifespan of aak2 mutant worms (Fig. 4J and K), indicating that Nit and Tiz-induced extension of *C. elegans* lifespan was dependent on AMPK activation. Metformin, an AMPK activator, has been as a tool to target aging[23]. We also evaluated the effect of metformin in the present experiment. Metformin treatment activated AMPK (Fig. 4L) and prolonged the lifespan in wild type (N2) (Fig. 4M) but not in aak2 mutant worms (Fig. 4N).

3.4. Nitazoxanide and tizoxanide extend C. elegans lifespan through Akt/AMPK/sir2.1/daf16 pathway

We further examined the effect of Nit and Tiz on lifespan of FOXO transcription factor homolog daf-16 (mgDf50) and daf-16

(mu86) mutant, mammalian sirt 1 homolog sir-2.1 (ok434) mutant, ribosomal protein S6-Kinase homolog rsks-1 (1255) mutant strains. Nit (100 μmol/L) and Tiz (100 μmol/L) did not affect the lifespan of daf-16 (mgDf50), daf-16 (mu86), sir-2.1 (ok434) mutant strains except that they still extended the lifespan of rsks-1 mutant strain (Fig. 5A−D), indicating that daf-16 and sir-2.1 were involved in Nit and Tiz-induced lifespan extension in *C. elegans*. AMPK-induced stimulation of FoxO/daf-16 and sirt 1 signaling pathways mediates the lifespan extension in different species[22]. Sir-2.1 binds daf-16 in the nucleus and the resulting complex participates in transcriptional activation of daf-16 target genes involved in lifespan extension[24]. We found that Nit (100 μmol/L) and Tiz (100 μmol/L) treatment activated AMPK in wildtype (N2), daf-16 (mgDf50) and sir-2.1 (ok434) mutants (Fig. 5E−G), promoted nuclear accumulation of daf-16::GFP in daf-16-GFP worms (Fig. 5H and I), and increased daf-16 target gene sod-3 expression in sod-3-GFP worms (Fig. 5J and K). These results indicated that Nit and Tiz-induced AMPK activation was the upstream signal of daf-16 and sir-2.1.

In the *C. elegans* of akt-1 (ok525) and akt-2 (ok393) loss-of-function mutants, both Nit and Tiz treatment did not extend the
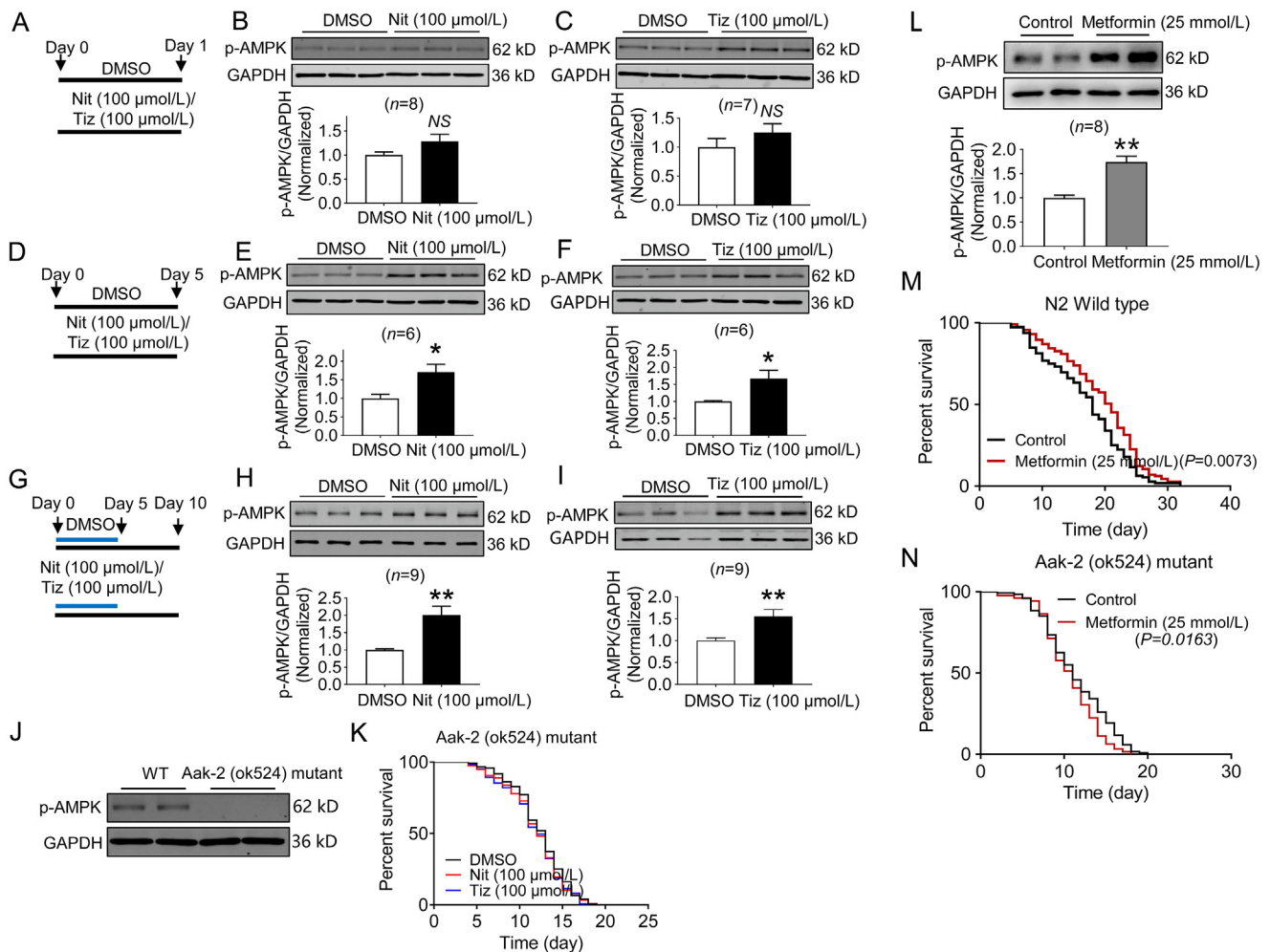
Figure 4 Nitazoxanide (Nit) and tizoxanide (Tiz) prolonged *C. elegans* lifespan through AMPK activation. The L4-stage nematodes were transferred to experimental plates, which was defined as Day 0. Nit or Tiz was prepared in OP50 bacteria at a concentration of 100 μmol/L; metformin was added to the agar at a concentration of 25 mmol/L. (A−C) Nit or Tiz treatment for 1 day from Day 0 to Day 1 had no effect on AMPK activity in nematodes. $n = 8, 7$ in (B, C), respectively. *NS*, not significant. (D−F) Nit or Tiz treatment for 5 days from Day 0 to Day 5 activated AMPK in nematodes. $n = 6$ in each group. $*P < 0.05$ *vs.* DMSO. (G−I) Nit and Tiz were used for 5 days from Day 0 to Day 5 and withdrawal for 5 days from Day 5 to Day 10. AMPK activation was detected in nematodes. $n = 9$ in each group. $**P < 0.01$ *vs.* DMSO. (J, K) AMPK knockout (aak2 mutant) abolished nitazoxanide- and tizoxanide-induced lifespan extension in nematodes. $n = 130, 125, 133$ in DMSO, Nit, and Tiz groups, respectively. (L) Metformin treatment for 5 days activated AMPK in nematodes. $n = 8$ in each group. $**P < 0.01$ *vs.* Control. (M) Metformin prolonged the lifespan of N2 *C. elegans* (Wild type). The L4-stage nematodes were treated with metformin for the worm lifelong time. $n = 117, 122$ in control, and metformin groups, respectively. Log-rank test was used to calculate the *P* value *vs.* Control. (N) AMPK knockout (aak2 mutant) abolished the metformin-induced lifespan extension of nematodes. The L4-stage nematodes were treated with metformin for the worm's lifelong time. $n = 125, 128$ in control and metformin groups, respectively. Log-rank test was used to calculate the *P* value *vs.* Control. All data are represented as mean ± SEM.

lifespan (Fig. 6A and B), but in the *C. elegans* of akt-1 (mg144) gain-of-function mutant, Nit and Tiz treatment significantly extended the lifespan (Fig. 6C). These results indicated that Akt signal was involved in Nit- and Tiz-induced lifespan extension. Previous data had shown that Nit- and Tiz-induced lifespan extension in *C. elegans* was dependent on AMPK activation (Fig. 4), suggesting that there existed an interaction of AMPK and Akt signals in the effect of Nit- and Tiz-induced lifespan extension.

It has been reported that mitochondrial uncoupling induced by UCP overexpression blocks Akt activation[25] and Akt inhibition often occurs concomitantly with AMPK activation when cells are treated with AMPK activators[26]. Nit and Tiz as the mitochondrial uncouplers decreased mitochondrial membrane potential and reduced ATP level after acute treatment (Supporting Information Fig. S3). Nit and Tiz as the AMPK activators inhibited Akt activity in wild-type (N2) worms (Fig. 6D), thus, it is necessary to elucidate the upstream or downstream relationship between Nit- and Tiz-induced AMPK activation and Akt inhibition in *C. elegans*. We performed further experiments and found that: (1) Nit (100 μmol/L) and Tiz (100 μmol/L) treatment inhibited Akt activity in aak2 mutant (Fig. 6E); (2) In *C. elegans* of akt-1 (ok525) and akt-2 (ok393) loss-of-function mutants, the Nit and Tiz-induced AMPK activation was attenuated, but in *C. elegans* of
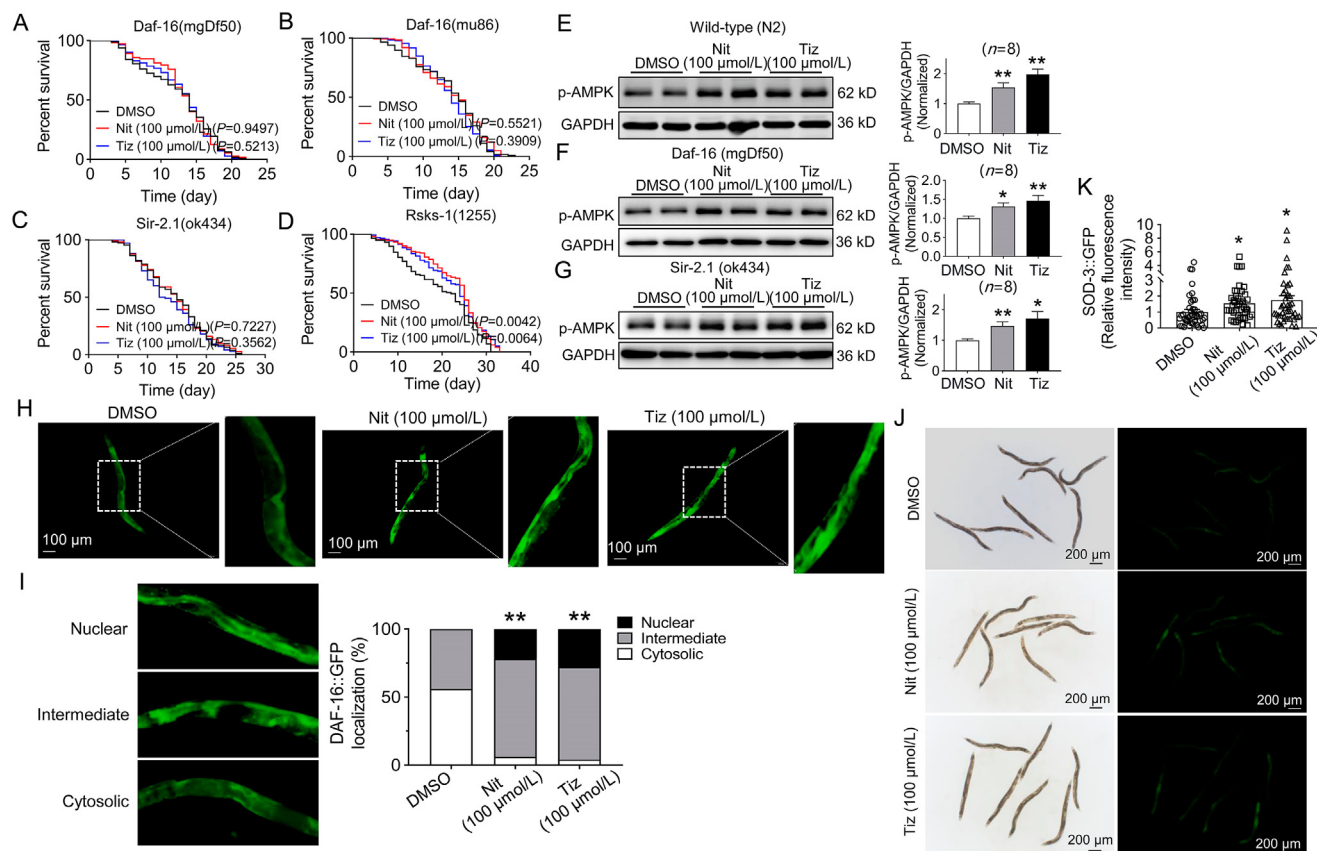
Figure 5 Nitazoxanide (Nit) and tizoxanide (Tiz) prolonged *C. elegans* lifespan through AMPK/Sir2.1/Daf16 pathway. (A) Nit or Tiz treatment did not affect the lifespan of the daf-16 (mgDf50) mutant strain. $n = 120, 119, 121$ in DMSO, Nit, and Tiz groups, respectively. Log-rank test was used to calculate the P value *vs.* DMSO. (B) Nit or Tiz treatment did not affect the lifespan of the daf-16 (mu86) mutant strain. $n = 120, 138, 140$ in DMSO, Nit, and Tiz groups, respectively. Log-rank test was used to calculate the P value *vs.* DMSO. (C) Nit or Tiz treatment did not affect the lifespan of the sir-2.1 (ok434) mutant strain. $n = 116, 114, 106$ in DMSO, Nit, and Tiz groups, respectively. Log-rank test was used to calculate the P value *vs.* DMSO. (D) Nit or Tiz treatment prolonged the lifespan of the rsks-1 (1255) mutant strain. $n = 121, 151, 144$ in DMSO, Nit, and Tiz groups, respectively. Log-rank test was used to calculate the P value *vs.* DMSO. (E−G) Nit or Tiz treatment for 5 days activated AMPK in N2 *C. elegans* (Wild type), daf-16 (mgDf50) and sir-2.1 (ok434) mutant strains. $n = 8$ in each group. *$P < 0.05$, **$P < 0.01$ *vs.* DMSO. (H, I) The representative fluorescent images and summarized data of daf-16 nuclear translocation of TJ356 strain [daf-16p::daf-16a/b::GFP + rol-6 (su1006)]. The L4-stage nematodes were treated with Nit or Tiz for 10 days. $n = 25, 32, 28$ in DMSO, Nit, and Tiz groups, respectively. **$P < 0.01$ *vs.* DMSO. (J, K) Nit or Tiz treatment for 10 days increased sod-3 expression in KN259 strain [sod-3::GFP + rol-6 (su1006)]. $n = 42, 44, 43$ in DMSO, Nit, and Tiz groups, respectively. *$P < 0.05$ *vs.* DMSO. All data are represented as mean ± SEM.

akt-1 (mg144) gain-of-function mutant, Nit and Tiz-induced AMPK activation was enhanced (Fig. 6F−H); (3) Comparison of the basal Akt and AMPK activity in akt-1 (ok525) and akt-2 (ok393) loss-of-function mutants and akt-1 (mg144) gain-of-function mutant showed that the decreased Akt activity in akt-1 (ok525) and akt-2 (ok393) mutants and increased Akt activity in akt-1 (mg144) mutant corresponded to the increased and decreased AMPK activity respectively (Fig. 6I); (4) furthermore, in akt-1 (ok525) and akt-2 (ok393) loss-of-function mutants which had lower level of Akt activity, the Nit and Tiz-induced Akt inhibition could not be significantly detected (Supporting Information Fig. S4A and S4B); however, in akt-1 (mg144) gain-of-function mutants which had higher Akt activity, Nit and Tiz showed significant inhibitory effect on Akt activity (Fig. S4C). Therefore, the above results demonstrated that Nit and Tiz activated AMPK through inhibiting Akt in *C. elegans*. The signaling pathway for Nit- and Tiz-induced lifespan extension in *C. elegans* was summarized in Fig. 6J.

Additionally, we further used cell line (HCT116 cells) to study the effect of Nit and Tiz on Akt and AMPK activity and discern the Akt activity in cellular cytoplasm and mitochondria parts. As shown in Supporting Information Fig. S5, both Nit and Tiz inhibited Akt and activated AMPK activity simultaneously in HCT116 cells. Furthermore, Nit and Tiz inhibited both cytoplasm and mitochondria Akt activity, but only cytoplasm AMPK activation by Nit and Tiz treatments was detected (Supporting Information Fig. S6).

3.5. Nitazoxanide and tizoxanide induce AMPK-dependent autophagy and reduce lipid accumulation in C. elegans

Aging is accompanied by pronounced lipid accumulation[27]. Autophagy induction and autophagy-induced lipid breakdown contribute to lifespan extension of *C. elegans*[28-30]. Since Nit and Tiz extended *C. elegans* lifespan, we examined the effect of Nit and Tiz on lipid deposit and autophagy response in *C. elegans*. Nit (100 μmol/L)
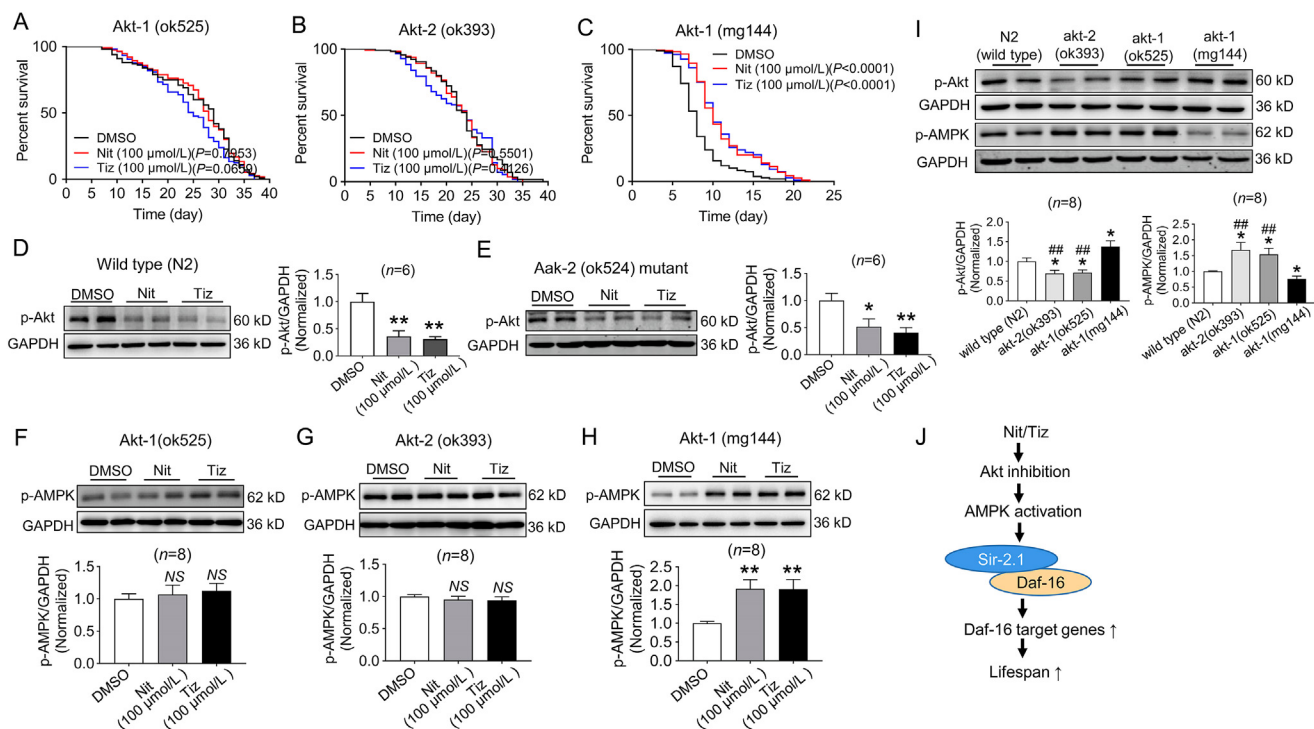
Figure 6 Nitazoxanide (Nit) and tizoxanide (Tiz) activated AMPK through inhibiting Akt in *C. elegans*. (A) Nit or Tiz treatment had no effect on the lifespan of the akt-1 (ok525) strain. $n = 106, 118, 115$ in DMSO, Nit, and Tiz groups, respectively. Log-rank test was used to calculate the P value *vs*. DMSO. (B) Nit or Tiz treatment had no effect on the lifespan of the akt-2 (ok393) strain. $n = 119, 118, 120$ in DMSO, Nit, and Tiz groups, respectively. Log-rank test was used to calculate the P value *vs*. DMSO. (C) Nit or Tiz treatment prolonged the lifespan of the akt-1 (mg144) strain. $n = 116, 119, 116$ in DMSO, Nit, and Tiz groups, respectively. Log-rank test was used to calculate the P value *vs*. DMSO. (D) Nit or Tiz treatment for 5 days inhibited Akt in N2 *C. elegans* (Wild type). $**P < 0.01$ *vs*. DMSO. (E) Nit or Tiz treatment for 5 days inhibited Akt in aak-2 (ok524) mutant strain. $n = 6$ in each group. $*P < 0.05, **P < 0.01$ *vs*. DMSO. (F, G) Akt-1 or Akt-2 knockout abolished Nit- and Tiz-induced AMPK activation. $n = 8$ in each group. *NS*, not significant. (H) Nit or Tiz treatment for 5 days activated AMPK in akt-1 (mg144) strain. $n = 8$ in each group. $**P < 0.01$ *vs*. DMSO. (I) The representative immunoblot images and analyzed data of p-Akt and p-AMPK protein levels in Wild type (N2), akt-1 (ok525), akt-2 (ok393), akt-1 (mg144) strains. $n = 8$ in each group. $*P < 0.05$ *vs*. Wild type (N2); $^{##}P < 0.01$ *vs*. akt-1 (mg144). (J) Schematic diagram of the potential mechanisms of nitazoxanide and tizoxanide-induced lifespan extension in *C. elegans*. All data are represented as mean $\pm$ SEM.

and Tiz (100 μmol/L) treatment reduced lipid content in *C. elegans* as evaluated by Oil red staining and quantification of total tri-glyceride (TG) (Supporting Information Fig. S7A–S7E). Nit (100 μmol/L) and Tiz (100 μmol/L) treatment increased LC3II protein level and LC3II/I ratio, and decreased p62 protein level (Supporting Information Fig. S8A–S8D). GFP::LGG-1 is an autophagy marker in *C. elegans*[31]. We further used transgenic strain (lgg-1p::GFP::lgg-1 + rol-6(su1006) to detect the autophagy level in *C. elegans*. Firstly, the anti-GFP-tag was measured by using Western blot. As shown in Fig. S8E and S8F, Nit (100 μmol/L) and Tiz (100 μmol/L) treatment significantly increased the protein level of GFP-tag. Secondly, fluorescence results showed that Nit (100 μmol/L) and Tiz (100 μmol/L) treatment increased GFP-positive punctate areas in the LGG-GFP worms (Fig. S8G and S8H).

Compared with Nit- and Tiz-induced autophagy induction and reduced lipid deposition in wild type (N2) worms, Nit (100 μmol/L) and Tiz (100 μmol/L) treatment did not increase autophagy response (Supporting Information Fig. S9A and S9B) and did not affect lipid content in aak2 (ok524) mutant worms (Fig. S9C and S9D). These results indicated that Nit and Tiz reduced lipid content and induced autophagy through AMPK activation in *C. elegans*.

3.6. Nitazoxanide and tizoxanide improve high glucose-induced shortening of *C. elegans* lifespan

High glucose shortens *C. elegans* lifespan[32]. We established high glucose (5 mmol/L)-induced lifespan shortening model in *C. elegans* and further studied the effect of Nit and Tiz in this model. As shown in Fig. 7A, glucose (5 mmol/L) shortened *C. elegans* lifespan and the shortening was improved by Nit (100 μmol/L) and Tiz (100 μmol/L) treatment. Nit (100 μmol/L) and Tiz (100 μmol/L) treatment also improved the glucose-induced decrease of body bends and increase of lipofuscin content (Fig. 7B–D). Compared with the control (DMSO) group, glucose (5 mmol/L) treatment induced increase of mitochondrial fragmentation in the body wall muscles of *C. elegans*. Nit and Tiz treatment significantly improved the mitochondrial morphology from the fragmented to elongated tubular state (Fig. 7E). Functionally, Nit (100 μmol/L) and Tiz (100 μmol/L) treatment significantly improved the decreased basal mitochondrial OCR induced by high glucose (5 mmol/L) (Fig. 7F). Besides, high glucose (5 mmol/L) treatment decreased ATP contents of nematodes, which was improved by Nit and Tiz treatment (Fig. 7G).
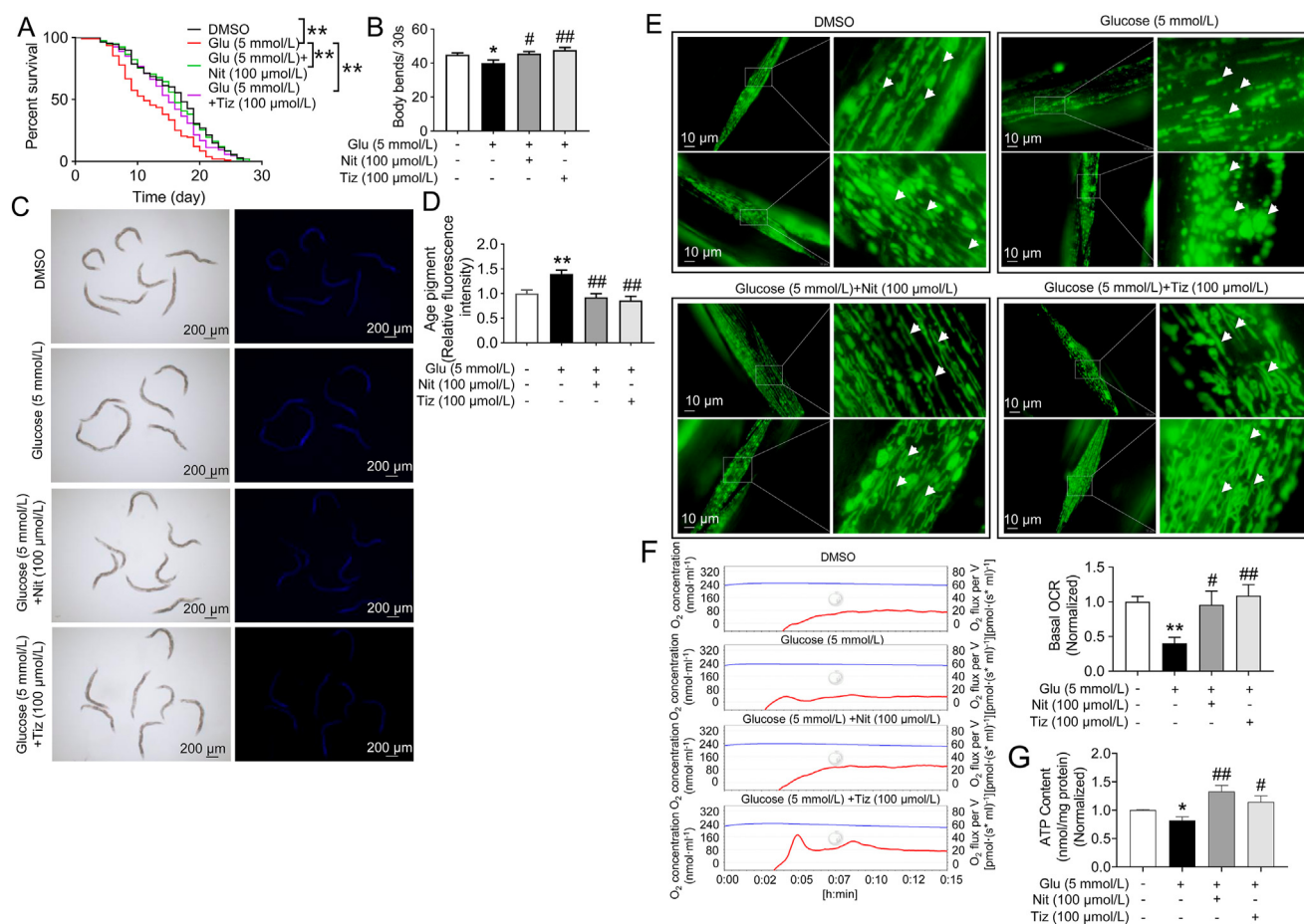
Figure 7 Nitazoxanide (Nit) and tizoxanide (Tiz) improved the lifespan and healthspan of *C. elegans* stimulated by glucose. (A) Nit or Tiz treatment for 12 days prolonged the lifespan of nematodes stimulated by glucose. $n = 115, 115, 130, 123$ in DMSO, Glu, Glu + Nit, and Glu + Tiz groups, respectively. Log-rank test was used to calculate the *P* value. Glu, glucose. **$P < 0.01$. (B) Nit or Tiz treatment for 5 days improved the body motility of nematodes stimulated by glucose. $n = 30, 33, 31, 29$ in DMSO, Glu, Glu + Nit, and Glu + Tiz groups, respectively. *$P < 0.05$ *vs.* DMSO; #$P < 0.05$, ##$P < 0.01$ *vs.* Glu. (C, D) The representative fluorescent images and analyzed data showed that Nit or Tiz treatment for 5 days inhibited glucose-stimulated age pigment deposition of nematodes. $n = 42, 40, 43, 40$ in DMSO, Glu, Glu + Nit, and Glu + Tiz groups, respectively. **$P < 0.01$ *vs.* DMSO; ##$P < 0.01$ *vs.* Glu. (E) The representative fluorescent images of SJ4103 stain [myo-3::GFP(mit)] showed that treatment with Nit or Tiz for 10 days improved mitochondrial morphology of nematodes stimulated by glucose. $n = 49, 46, 49, 33$ images in DMSO, Glu, Glu + Nit, and Glu + Tiz groups, respectively. (F) Nit or Tiz treatment for 10 days increased mitochondrial oxygen consumption rates of nematodes stimulated by glucose. $n = 300$ worms in each group, at least three independent experiments. **$P < 0.01$ *vs.* DMSO; #$P < 0.05$, ##$P < 0.01$ *vs.* Glu. (G) Nit or Tiz treatment for 10 days increased ATP contents of nematodes stimulated by glucose. $n = 140$ worms in each group, at least three independent experiments. **$P < 0.01$ *vs.* DMSO; #$P < 0.05$, ##$P < 0.01$ *vs.* Glu. All data are represented as mean ± SEM.

Because the above results showed that Nit and Tiz-induced lifespan extension was dependent on AMPK activation, we evaluated the effect of Nit and Tiz on AMPK activity in high glucose-treated *C. elegans*

As shown in Fig. 8A, no change of AMPK activity was detected in *C. elegans* treated with high glucose (5 mmol/L) for 2 days, but AMPK was activated in *C. elegans* treated with high glucose (5 mmol/L) plus Nit (100 μmol/L) or Tiz (100 μmol/L) for 2 days. Then, the glucose and drug treatment time was prolonged to 5 days, at this condition, AMPK activity increased not only in high glucose (5 mmol/L) plus Nit (100 μmol/L) or Tiz (100 μmol/L) treatment groups, but also in the only high glucose treatment group (Fig. 8B). These results indicated that, under high glucose condition, Nit and Tiz-induced lifespan extension would not be dependent on AMPK activation in *C. elegans*. In order to prove this hypothesis,

we further used aak2 mutant worms. High glucose (5 mmol/L) reduced the lifespan of aak2 mutant worms and the reduction was improved by Nit (100 μmol/L) and Tiz (100 μmol/L) treatment (Fig. 8C). The above results suggested that there existed AMPK-independent mechanisms for high glucose-induced lifespan reduction and the improvement by Nit and Tiz. We further used daf-16 (mgDf50), daf-16 (mu86), and sir-2.1 (ok434) mutant worms to evaluate the effect of Nit and Tiz on lifespan under high glucose conditions. Results showed that high glucose-induced lifespan reduction and Nit- and Tiz-induced improvement were dependent on daf-16 but not sir-2.1 (Fig. 8D−F). High glucose or hyperglycemia inhibits insulin signaling[33,34], and nuclear daf-16 does not require sir-2.1 for activation under the reduced insulin signaling conditions in *C. elegans*[24]. Therefore, Nit and Tiz-induced lifespan extension was dependent on daf-16 but not
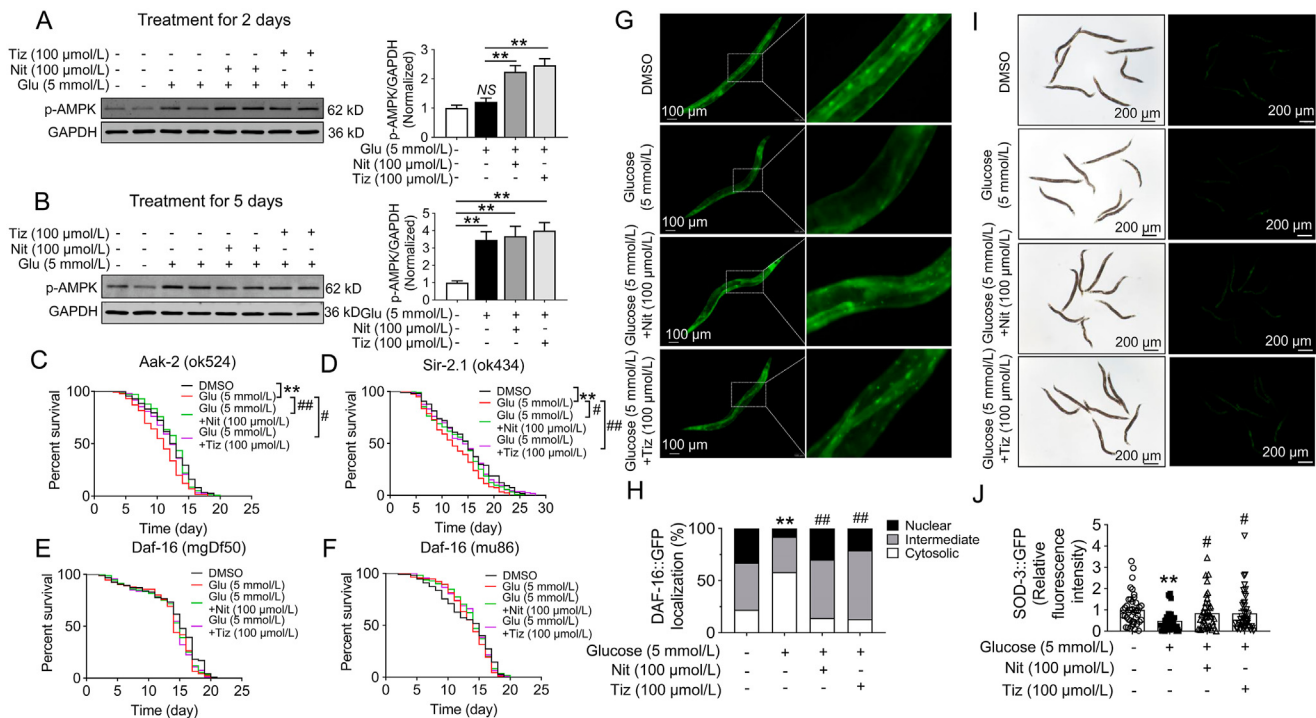
Figure 8 Nitazoxanide (Nit) and tizoxanide (Tiz) improved high glucose-induced shortening of *C. elegans* lifespan by promoting daf-16 nuclear translocation. (A) Nit or Tiz treatment for 2 days activated AMPK in nematodes stimulated with glucose. $n = 6$ in each group. *NS vs* DMSO. *NS*n not significant. **$P < 0.01$ *vs.* Glu. (B) Glu alone or glu + Nit or glu + Tiz treatment for 5 days activated AMPK in nematodes. $n = 6$ in each group. **$P < 0.01$ *vs* DMSO. (C) Nit or Tiz treatment for 12 days prolonged the lifespan of aak-2 (ok524) strain stimulated by glucose. $n = 116, 132, 143, 134$ in DMSO, Glu, Glu + Nit, and Glu + Tiz groups, respectively. **$P < 0.01$ *vs.* DMSO; #$P < 0.05$, ##$P < 0.01$ *vs.* Glu. (D) Nit or Tiz treatment for 12 days prolonged the lifespan of the sir-2.1 (ok434) strain stimulated by glucose. $n = 109, 127, 126, 117$ in DMSO, Glu, Glu + Nit, and Glu + Tiz groups, respectively. **$P < 0.01$ *vs.* DMSO; #$P < 0.05$, ##$P < 0.01$ *vs.* Glu. (E) Nit or tiz treatment for 12 days had no effects on the lifespan of the daf-16 (mgDf50) strain stimulated by glucose. $n = 98, 98, 99, 101$ in DMSO, Glu, Glu + Nit, and Glu + Tiz groups, respectively. (F) Nit or Tiz treatment for 12 days had no effects on the lifespan of the daf-16 (mu86) strain stimulated by glucose. $n = 122, 126, 122, 115$ in DMSO, Glu, Glu + Nit, and Glu + Tiz groups, respectively. (G, H) The representative fluorescent images and analyzed data of TJ356 strain [daf-16p::daf-16a/b::GFP + rol-6 (su1006)]. The L4-stage nematodes were treated with Glu alone or Glu + Nit or Glu + Tiz for 10 days. $n = 54, 50, 50, 56$ in DMSO, Glu, Glu + Nit, and Glu + Tiz groups, respectively. **$P < 0.01$ *vs.* DMSO; ##$P < 0.01$ *vs.* Glu. (I, J) Nit or Tiz treatment for 10 days increased sod-3 expression of nematodes stimulated by Glu (5 mmol/L). $n = 41, 42, 40, 43$ in DMSO, Glu, Glu + Nit, and Glu + Tiz groups, respectively. **$P < 0.01$ *vs.* DMSO; #$P < 0.05$ *vs.* Glu. All data are represented as mean ± SEM.

AMPK and sir-2.1 in *C. elegans* under high glucose stress conditions, the mechanism by which was different from the signals demonstrated in normal conditions (Figs. 4–6). Furthermore, high glucose inhibited daf-16 nuclear translocation and downregulated daf-16-target gene sod-3 expression, and these high glucose-induced effects were restored by Nit and Tiz treatments (Fig. 8G–J).

4. Discussion

Nitazoxanide is bio-transformed into active metabolite tizoxanide after absorption in mammals. Both nitazoxanide and tizoxanide have mitochondrial uncoupling effects[17,18,35,36]. Nitazoxanide has been a clinical antiprotozoal drug with good safety profile, in the present study, we found that nitazoxanide and its metabolite tizoxanide extended the healthspan of wild type *C. elegans*, improved the shortened *C. elegans* lifespan induced by high-glucose. Together with our previous findings that nitazoxanide improved abnormal lipid metabolism and inhibited inflammation *in vivo*[17,18], we thought that nitazoxanide-induced lifespan and healthspan extension and the anti-aging effect were of clinic significance.

4.1. Mitochondrial uncoupling-induced AMPK activation

It is generally acknowledged that mitochondrial uncouplers induce cellular AMPK activation through facilitating proton influx across the mitochondrial inner membrane, reducing mitochondrial membrane potential and inhibiting ATP production. This classical opinion addresses that mitochondrial uncouplers directly reduce the proton motive force for ATP generation. However, comprehensively analyzing our present results and other reports[37,38], the Akt signal should be considered as the crucial factor in the mechanisms of mitochondrial uncoupling-induced AMPK activation. In the present study, we demonstrated that Nit and Tiz-induced Akt inhibition resulted in AMPK activation by using wild type (N2), Akt loss-of-function mutant, and Akt gain-of-function mutant *C. elegans* strains. Akt plays a central role in the insulin signaling pathway. Disrupted insulin/insulin-like growth factor 1 signaling extends *C. elegans* lifespan. Therefore, we thought that both Nit and Tiz-induced Akt inhibition and the subsequent AMPK activation might contribute to Nit and Tiz-induced *C. elegans* lifespan extension. Akt inhibition-induced AMPK activation has also been proven in

mammal cells. It has been reported that Akt resides in the matrix and the inner and outer membranes of mitochondria; when the phosphatidylinositol 3-kinase/Akt signaling is activated, Akt translocates into mitochondria and the mitochondrial Akt is in its phosphorylated, active state[37]. Mitochondrial Akt increases Complex V activity and increases ATP production[38]. On the other hand, the transport of a certain type of proteins into mitochondria was dependent on the mitochondrial membrane potential[39], for instance, mitochondrial uncoupler CCCP reduced mitochondrial membrane potential and inhibited the influx of Akt into mitochondria[37], thus inhibited mitochondrial Akt-dependent complex V activity and ATP production. The above information indicates that Akt activation increases ATP generation and indirectly inhibits AMPK activation; conversely, Akt inhibition reduces ATP generation and activates AMPK. Indeed, it has been demonstrated that ablation of Akt induced AMPK activation in the skeletal muscle of mice[40]. Our present study found that both Nit and Tiz as the mitochondrial uncouplers decreased mitochondrial membrane potential, inhibited Akt activity and activated the downstream AMPK in *C. elegans*, which is consistent with the above Akt-dependent AMPK inhibition pathway. Furthermore, we used HCT116 cells to confirm that not only Nit and Tiz inhibited Akt and activated AMPK activity simultaneously, but also Nit and Tiz inhibited both cytoplasm and mitochondria Akt activity (Figs. S5 and S6). Therefore, a more detailed mechanism for mitochondrial uncoupling-induced AMPK activation was put forward (Supporting Information Fig. S10).

It is accepted that mitochondrial uncouplers activate AMPK by reducing ATP production. Indeed, acute treatment with Nit and Tiz reduced mitochondrial membrane potential and ATP production in *C. elegans* (Fig. S3). However, chronic treatment with Nit and Tiz induced an increase of ATP level (Fig. 3G), which seemed to be controversial to the mitochondrial uncoupler-induced decrease of ATP production. In fact, it was not controversial. The initial action of mitochondrial uncoupler is to reduce ATP level, which triggers AMPK activation. Once activated, AMPK will promote catabolism and augment energy production through multiple signaling pathways, ultimately, restore cellular and whole organismal homeostasis, as manifested by the improved physical fitness, mitochondrial morphology and the increased basal mitochondrial respiration and ATP production in *C. elegans* (Figs. 1−3). Mild mitochondrial uncoupling is protective, namely, it is the mitochondrial uncoupling that induces AMPK activation through reducing ATP level but does not damage the cells. Once the uncoupling induces significant decrease of ATP production and damages cells, the AMPK activation is of no significance.

It was worth mentioning that the protein levels of p-AMPK and p-Akt but not total AMPK and total-Akt were measured in this study, because the total-AMPK and total-Akt protein expression could not be detected in *C. elegans* by the present commercial antibodies. Measurement of p-AMPK protein level to reflect the AMPK activity in *C. elegans* has been reported in other studies[41,42]. In the present study, p-AMPK protein could be detected in N2 wild type but not aak2 mutant worms (Fig. 4J); Compared with that in N2 wild type worms, p-Akt protein expression was significantly higher in akt-1 (mg144) gain-of-function mutants and lower in akt-1 (ok525) and akt-2 (ok393) loss-of-function mutants (Fig. 6I). These results confirmed the measurement of p-AMPK and p-Akt protein levels in *C. elegans* in the present study was reliable.

4.2. Systematic effect of nitazoxanide and tizoxanide

High-glucose treatment significantly induced an increase of the mitochondrial fragmentation in the body wall muscles of *C. elegans*, and the morphological changes were improved by Nit and Tiz treatment. Generally, mitochondrial uncouplers reduce mitochondrial membrane potential and trigger mitochondrial fragmentation *in vitro*[43]. However, Nit and Tiz as the mitochondrial uncouplers improved high glucose-induced increase of mitochondrial fragmentation and decrease of basal mitochondrial OCR, indicating that the effect of Nit and Tiz on mitochondrial morphology and function of *C. elegans* was systematically integrated *in vivo*. Nit and Tiz induced long-lasting AMPK activation in *C. elegans* (Fig. 4), and AMPK acted as a key signaling molecule in regulating mitochondrial dynamics and physical fitness in *C. elegans*[44], therefore, the effect of Nit and Tiz is through a systematic signal network *in vivo*.

Nit and Tiz inhibited Akt and activated AMPK, and as the mitochondrial uncouplers, they also reduced mitochondrial membrane potential and ATP level acutely (Fig. S3). Whatever Akt signal, AMPK signal or mitochondrial membrane potential, changes in these signals will induce a network of signal responses. First, the mitochondrial membrane potential is correlated with *C. elegans* longevity. It has been proven that the long-lived *C. elegans* strains had a lower mitochondrial membrane potential[45] and mitochondrial uncouplers CCCP, FCCP, and DNP which reduced mitochondrial membrane potential and extended the lifespan of *C. elegans*, yeast and mice[45-48]. Here, mitochondrial uncoupling-induced lifespan extension was demonstrated to be due to Akt/AMPK and the downstream signals. Second, AMPK activation extended lifespan of *C. elegans* through an integrated network but not a single signaling, including regulation of SIRT1 signaling, FoxO axis, mTOR/ULK1 signaling and mitochondrial dynamics[22,44].

Another interesting finding of the present study was that Nit and its metabolite Tiz increased *C. elegans* size and β-actin, β-tubulin protein expressions. In the experiments, we had used β-actin and β-tubulin as reference proteins at first, but their protein levels were always not parallel to the total protein quantity in *C. elegans*. Finally, we chose the GAPDH as the reference protein and found that β-actin and β-tubulin protein expressions were increased by Nit and Tiz treatment. Generally, AMPK activation is to decrease anabolic processes and increase catabolism[49], therefore, it was possible that Nit- and Tiz-induced increase of β-actin and β-tubulin protein expressions was attributed to other but not AMPK-related signals.

4.3. The difference of signals in basal and diseased state and the different drug responses

We found that the signaling mechanisms of the effect of Nit and Tiz on *C. elegans* lifespan in normal and high glucose conditions were different. In wild type (N2) *C. elegans* under normal conditions, Nit and Tiz extend *C. elegans* lifespan through Akt/AMPK/sir 2.1/daf16 pathway (Fig. 6J). However, under high glucose state, Nit- and Tiz-induced lifespan extension was dependent on daf-16 but not AMPK and sir-2.1. Previous study demonstrated that sir-2.1 bound daf-16 in the nucleus and participated in transcriptional activation of daf-16 target genes in *C. elegans* following stress; but under low insulin-like signaling conditions, nuclear daf-16 did not require sir-2.1 for activation[24]. Since high glucose or hyperglycemia inhibits insulin

signaling[33,34], we thought that our findings were consistent with the role of sir-2.1 in daf-16 regulation of *C. elegans* lifespan[24].

High glucose shortens *C. elegans* lifespan and the protective effect of AMPK activation against the high glucose-induced toxicity have been extensively studied[50,51]. However, in the present study, we found that high-glucose itself activated AMPK, and Nit and Tiz treatment did not further enhance the AMPK activity in *C. elegans* (Fig. 8A and B). Experiments using aak-2 mutant also evidenced that the role of AMPK under high glucose conditions is negligible for the Nit and Tiz effect (Fig. 8C). In addition to AMPK, high glucose stress altered different key regulators for metabolism in *C. elegans*, including SKN-1/NRF2, HIF-1/HIF1α, SBP-1/SREBP, CRH-1/CREB, CEP-1/p53, and DAF-16/FOXO etc[52]. We thought that these altered signals were compensatory responses to glucose stress. On the other hand, this information suggested that the same signaling might not be at the same level in physiological and pathological conditions, therefore, it was understandable that the drug response under physiological and pathological conditions might be distinct.

4.4. The safety of nitazoxanide and tizoxanide as anti-aging drugs

Another important concern is the safety of nitazoxanide and tizoxanide as anti-aging drugs. Nitazoxanide is completely metabolized to tizoxanide after absorption *in vivo* in mammals. The preclinical and clinical studies had proven that nitazoxanide was safe. The acute oral LD_{50} of nitazoxanide was greater than 10 g/kg in rats, dogs and cats[53], indicating that nitazoxanide was non-toxic. In randomized controlled clinical trials for hepatitis B and hepatitis C treatments, nitazoxanide (500 mg) was administered orally twice daily for up to 48 weeks and well tolerated[12,13]. Nitazoxanide at the doses equivalent to that in human significantly improved the experimental hyperlipidemia, hepatic steatosis and atherosclerosis in hamsters and mice[17,18]. The above information suggested that nitazoxanide as anti-aging drug was feasible.

5. Conclusions

The goal of aging-related studies is to find the interventions that not only extend lifespan, but also improve age-related diseases and extend the healthspan. In the present study, we find that both nitazoxanide and its metabolite tizoxanide extend lifespan, enhance physical fitness, and improve high glucose-induced lifespan shortening in *C. elegans*. Together with our previous findings that nitazoxanide protected against the age-related diseases including hyperlipidemia, hepatic steatosis, and atherosclerosis in mice[17,18], we suggest nitazoxanide as anti-aging drug is of clinic significance.

Acknowledgments

Author contributions

Wenfeng Li: Investigation; Visualization. Shuming Chen: Investigation; Visualization. Jing Lang: Investigation; Visualization. Jing Luo: Investigation; Visualization. Jiahui Chen: Investigation; Visualization. Liping Zhang: Investigation; Visualization. Zhijie Sun: Funding acquisition; Project administration; Supervision; Writing-review & editing. Deli Dong: Funding acquisition; Project administration; Supervision; Writing-review & editing.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2024.03.031.

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