Zoledronate extends healthspan and survival via the mevalonate pathway in a FOXO-dependent manner

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ABSTRACT

Over recent decades, increased longevity has not been paralleled by extended healthspan, resulting in more years spent with multiple diseases in older age. As such, interventions to improve healthspan are urgently required. Zoledronate is a nitrogen containing bisphosphonate, which inhibits the farnesyl pyrophosphate synthase (FPPS) enzyme, central to the mevalonate pathway. It is already used clinically to prevent fractures in osteoporotic patients, who have been reported to derive unexpected and unexplained survival benefits. Using *Drosophila* as a model we determined the effects of Zoledronate on lifespan, parameters of healthspan (climbing ability and intestinal dysplasia) and the ability to confer resistance to oxidative stress using a combination of genetically manipulated *Drosophila* strains and Western blotting. Our study shows that Zoledronate extended lifespan, improved climbing activity and reduced intestinal epithelial dysplasia and permeability with age. Mechanistic studies showed that Zoledronate conferred resistance to oxidative stress and reduced accumulation of X-ray-induced DNA damage via inhibition of FPPS. Moreover, Zoledronate was associated with inhibition of pAKT in the mTOR pathway downstream of the mevalonate pathway and required dFOXO for its action, both molecules associated with increased longevity. Taken together, our work indicates that Zoledronate, a drug already widely used to prevent osteoporosis and dosed only once a year, modulates important mechanisms of ageing. Its repurposing holds great promise as a treatment to improve healthspan.

Keywords aging, lifespan, bisphosphonates, DNA damage, *Drosophila*

INTRODUCTION

It has been estimated that by 2050, Europe, North America, and eight countries across the other continents will have more than 30% of their population over the age of 60(1). However, this increase in life expectancy has not translated into extended healthspan(2). More than 60% of those over the age of 65 suffer from multimorbidities – defined as the co-existence of multiple chronic health conditions(3). These multimorbidities impose quality-of-life and care management challenges associated with treating multiple conditions individually and are frequently associated with increased costs, reduced efficacy and increased likelihood of adverse events due to polypharmacy(4). Targeting pathways underpinning central mechanisms of ageing, which are common to multiple disorders offers new opportunities to treat multimorbidity and overcome these problems.

Zoledronate (Zol) is a nitrogen containing-bisphosphonate used for the treatment of skeletal disorders, including osteoporosis. It inhibits the mevalonate pathway through inhibition of the enzyme farnesyl pyrophosphate synthase (FPPS)(5) and thereby inhibits bone resorption by inhibition of osteoclast activity. Due to its high affinity binding to hydroxyapatite crystal mineralisation, Zol is strongly enriched in bone and released following bone breakdown conferring long lasting activity(6). In clinical practice, Zol is administered by intravenous infusion once a year in post-menopausal women to prevent fractures(7, 8). Recently, retrospective analysis of several clinical trials showed that patients treated with Zol had a significant decrease in mortality rates(9). In addition, among patients admitted to intensive care units, those previously treated with Zol had increase survival despite overall higher level of multimorbidities and older age(10). It is unknown whether other mechanisms independent of bone protection may be involved in the Zol-dependent extension of survival.

The mevalonate pathway is an important metabolic pathway responsible for the production of cholesterol and protein prenylation. One key group of isoprenylated proteins are small GTPases(11), important signalling molecules which play central roles in multiple cellular processes, including

cellular morphology, integrin function and longevity-associated pathways such as mTOR(12). In this study we examined whether Zol is able to extend lifespan and healthspan independent of its effect on bone using *Drosophila*, a model widely used for ageing studies. While retaining evolutionarily conserved components of both the mevalonate and mTOR pathways, *Drosophila* does not feature the bone-like mineralisation present in mammalian models(13). This approach allows us to exclude mechanisms, which have been hypothesized to explain Zol-mediated increases in human survival. These include the bone acting as a regulator of other tissues' homeostasis(10) through the release of hormones such as osteocalcin a regulator of glucose homeostasis(14) . In doing so, our approach allows osteo-protective and gero-protective activities to be dissected *in vivo*. Our results uncovered a previously unrecognised role of Zol extending both lifespan and healthspan and improving survival in the presence of oxidative stress. The extension in survival by Zol is associated with a reduction in pAKT expression and requires dFOXO signalling, a conserved pathway well known for its positive effects on longevity.

EXPERIMENTAL METHODS

Fly Stocks and Husbandry

The *white*$^{\text{Dahomey}}$ (*w*^{Dah}) stock has been maintained in large population cages with overlapping generations since 1970(15) and was a kind gift of the Partridge lab. *y*^{1},*w*^{67c23}*; P(lacW)Fpps*^{k06103}*/CyO* and *y*^{1},*w*^{67c23}*; raw*^{k03514}*, P(lacW)Fpps*^{k03514}*/CyO* (ref (16)) and *w*^{1118}*; foxo*$^{\Delta 94}$*/TM6B, Tb*^{1} (ref(17)) were obtained from the Bloomington *Drosophila* Stock Centre. *w; Su(H)-lacZ, esg-Gal4,UAS-GFP/CyO* expresses GFP in intestinal stem cells and progenitor cells and was a kind gift of Leanne Jones(18). *w*$^{+}$*; GMR-Gal4,UAS-white*^{RNAi} is a recombinant between *GMR-Gal4* (ref(19)) and GD30033, an *in vivo* hairpin loop RNAi construct targeting *white* mRNA from the Vienna *Drosophila* Resource Center(20).

All flies were maintained in a 12:12h light-dark cycle on standard yeast, molasses, cornmeal, agar food at 18°C or 25°C. Drug-containing food was prepared using freshly cooked molten fly food cooled to 60°C. Zol (kindly provided by Hal Hebetino, University of Rochester), GGOH (Sigma Aldrich, Dorset, U.K.) and FOH (Sigma Aldrich, Dorset, U.K.) or carrier(s) were added, mixed thoroughly and immediately poured into standard vials and bottles so as to minimize exposure of drug to high temperatures. The continued activity of Zol following brief exposure to this temperature was confirmed molecularly (data not shown). Drug food was stored at 4°C until use for a maximum of 3 weeks.

For lifespan experiments overnight embryo collections from approximately 200 adult flies were collected on apple juice agar plates, washed with water and 32µl of embryos transferred into food bottles. Flies that subsequently ecclosed within the first 24hrs of the first to emerge were discarded with those subsequently ecclosing over an 16-20 hour overnight collection window being used for experiments. These later flies were transferred into new food bottles without the use of CO_2 and incubated for 3-4 days. Flies were then sorted by gender and counted while minimizing CO_2 exposure.

Longevity Assay and H_2O_2 Survival Assays

For longevity assays, 20 gender matched flies were maintained in vials of food and transferred into new vials every 2-3 days. At every transfer numbers of dead or censored flies were recorded. Unless specifically specified 100 flies were analysed for each experimental with three independent experimental replicates. To test survival in presence of hydrogen peroxide, adult flies of the appropriate genotype and pre-treatment were first starved in vials containing 1% agar for 3 hours and then transferred to standard food containing 5% H_2O_2 and the appropriate drugs / vehicle. Death was then scored at 12, 24 and 36 hours and then every

2 hours for the next 48 hours. If 100% mortality was not reached by that point, scoring was continued every 12 hours.

Rapid iterative negative geotaxis (RING) assays

For rapid iterative negative geotaxis (RING) assay flies we kept in groups of ~150. The day before an experiment, flies were sorted into groups of 20 and transferred into separate vials following brief CO_2 anaesthesia. Flies were then transferred into 25ml strippette (Fisher Scientific) and after 1 minute, the strippette was tapped to knock flies to the bottom and initiate the negative geotaxic response. After 15 seconds a photograph was taken and the position of each fly, and hence the distance climbed, was recorded. Twenty flies were assessed from each condition with each experiment repeated 3 times. Flies that climbed above 10cm were classified as 'high climbers'.

Gut barrier integrity and food uptake assays

For gut barrier integrity assays *Drosophila* food containing 2.5% (w/v) erioglaucine disodium salt (Sigma Aldrich) and drugs (as appropriate) was fed to flies for 9 hours. For gut integrity assays flies were scored for uniform blue coloration beyond the GI tract ('Smurf-ness').

Food uptake assays were undertaken as described in (21) using food containing 1% (w/v) erioglaucine disodium salt and 11.7µM Zol as appropriate. Ten adult flies between 8-11d old were placed in vials for 22h and allowed to feed *ad libitum* at 25°C. Flies were frozen and then dissociated in dH_2O before centrifugation to pellet debris and extract colour from internalised food. In addition, colour present in faecal matter deposited on the walls of the vial was recovered by washing with dH_2O. Colour, and hence quantity of food consumed was calculated on the basis of OD 630nm measurements and reference to a standard curve of dye dilutions (eFig 4A). Extracts from flies fed on undyed food were used as a blank. Ten groups

of 10 flies were tested for each condition and gender and tested by one-way ANOVA. Final values are expressed as µg food per fly per day (correcting for the 22h sampling period).

DNA damage assay

An overnight egg collection of w^+, *GMR-Gal4 UAS-white*^{RNAi} adults outcrossed to OreR were allowed to develop for 96 hours at 25°C. These heterozygous third instar larvae were then irradiated with 200Gy using a Torrex Cabinet X-ray system (Faxitron X-ray, Arizona, USA) and adult flies that subsequently ecclosed were scored for the frequency of red w^+ clones.

***Drosophila* midgut analysis**

Midguts from *Su(H)-lacZ; esg-Gal4,UAS-GFP/CyO* female adults were dissected in cold PBS and fixed in PBS+4% formaldehyde and blocked in PBST (PBS, 0.1% Triton X-100 and 1% BSA). Antibodies included Chicken anti-GFP 1:4000 (Abcam, Cambridge, UK) and anti-Phospho-histone H3 1:100 (New England Biolabs, Ipswich, USA) and secondary antibodies AlexaFluor™ 488 goat anti-chicken and AlexaFluor™ 594 goat anti-rabbit IgG (Life Technologies, Oregon, USA) were used. Stained guts were mounted with Fluoroshield™ with DAPI (Sigma Aldrich) and Grace Bio-Labs SecureSeal™ imaging spacers. Samples were imaged using Perkin Elmer Spinning Disk confocal microscope with a 40X objective. Images were then processed using Image J.

Protein extraction and western blot

Thirty flies of the appropriate genotype were snap frozen in liquid N_2 and crushed using an Eppendorf pestle in 200µl ice-old lysis buffer (50mM Tris HCl pH 7.4, 250mM NaCl, 5mM EDTA and 0.003% Triton X-100) with protease inhibitor (cOmplete Mini, EDTA-free, ROCHE, Sussex, UK). After 30mins incubation at 4°C extracts were centrifuged at 13,000rpm, 4°C for 30mins and supernatants stored at -20°C.

Proteins (40-60µg) were electrophoresed through 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, Hertfordshire, UK) and transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P Membrane, Millipore, Hertfordshire, UK) using standard techniques. Primary antibodies used were anti-pAKT 1:500, anti-AKT 1:500, anti-tubulin 1:1000 (Cell Signaling Technology, Denver, Massachusetts). HRP conjugated secondary antibodies were used at 1:10,000 and visualised using ECL (GE Healthcare, Buckinghamshire, UK), high performance chemiluminescence film (Amersham Hyperfilm ECL, GE Healthcare) and an Optimax 2010 X-ray film processor (PROTEC, Oberstenfeld, Germany).

Statistical Analysis

Statistical analysis was performed on GraphPad Prism 7. For single comparisons data were analysed using non-parametric unpaired Student's t-test. For multiple comparisons, ordinary one-way analysis of variance (ANOVA) was used followed by Sidak's multiple comparison post-hoc tests. For the analysis of RING assay Two Way ANOVA was used followed by Dunnet's multiple comparison test to determine the effect of dose of the treatment at the

different ages. Survival data was analysed using Log-rank (Mantel-Cox) test to identify significant differences in organism survival between treatment groups. For the analysis of the Smurf assay Chi-squared test was used. All data is expressed as mean ± standard deviation (SD). A difference was stated to be statistically significant if the p value was <0.05 (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

RESULTS

Zol increases lifespan in *Drosophila*

To determine whether Zol might have beneficial effects on the lifespan of *Drosophila*, both male and female w^{Dah} flies were maintained on standard fly food supplemented with 1µM or 10µM Zol from day 4 of adult life onwards (Fig. 1 A&B and eFig.1). In multiple experimental replicates, the survival of males fed with 1µM Zol was significantly increased compared to the vehicle-treated group (Fig. 1A and eFig.1). By contrast, females, showed no significant beneficial effect when treated with Zol at 1µM throughout their lives and 10µM of Zol had adverse effects on female survival (Fig. 1B).

As Zol modulates the mevalonate pathway through inhibition of FPPS, we next tested the lifespan of two heterozygous FPPS mutants ($Fpps^{k06103}$/+ and $Fpps^{k03514}$/+) – two independently generated homozygous-lethal loss-of-function alleles containing transposon insertions within the FPPS gene[15]. Following at least 7 generations of outcrossing into the w^{Dah} genetic background, both mutant strains showed a statistically significant increase in overall survival in males and females compared to w^{Dah} controls (Fig. 1 C&D and eFig2) suggesting that reduced FPPS activity mediates an extension in lifespan.

To limit potential side effects due to long term drug treatment, we next assessed the effects of Zol on lifespan when starting administration at day 40 of adult life (middle age) (red arrow in Fig. 1 E&F and

eFig.3). This late treatment led to a significant increase in survivorship in both males and females for both 1 and 10μM Zol compared to controls (Fig. 1 E&F and eFig.3). This lifespan extension was significant for both males and females with 1μM Zol producing average median lifespans increases of 1.79% (±0.10%) and 11.53% (±2.10%)(N=3) for male and females respectively, while with 10μM Zol, survival was increased in males by 4.67% (±1.03%) and females by 16.51% (±4.94%) (N=3). Finally, to verify that these results were not influenced by calorific restriction caused by unpalatable food we exposed the flies to drug-laced food containing a non-toxic food colorant via which the volume of food both present within flies and excreted can be measured. No significant difference in food uptake was observed in presence of Zol in either males or females (eFig. 4 n=10/trial, 10 trials per condition), excluding the possibility that the life extending effects were the result of caloric restriction.

Zol increases healthspan in *Drosophila*

To determine whether Zol improved signs of healthspan we next tested its effects in *Drosophila* by assessing their ability to climb and by the presence of signs of intestinal epithelial dysplasia and intestinal permeability. Displaying strong negative geotactic responses, climbing activity is a widely used assay of *Drosophila* health and activity(22) and was assessed using the rapid iterative negative geotaxis (RING) assay (Fig. 2). We classed as “high climbers” flies able to climb over 10cm in 15 seconds. As expected, the percentage of high climbers decreased significantly with age in both males (Fig. 2 A&C) and females (Fig. 2 B&D). However, a significant improvement in climbing ability was observed in both males and females receiving Zol from day 4 of adult life (Fig. 2 A&B) or starting at 40 days of life (Fig. 2 C&D) when as assessed at 42 and 56 days of age respectively. Two-way ANOVA analysis showed that, despite a significant interaction between age and treatment (Fig. 2 for details of statistical analysis), no significant effect of Zol was observed at a later time point in any of the conditions analysed by post hoc test. These data show there is an overall improvement in climbing ability at middle age, which is independent of the effects on lifespan.

As flies age, intestinal barrier dysfunction and epithelial dysplasia develop in female flies, a development associated with increased mortality and a development considered to be a good marker of healthspan(23). To determine the effects of Zol on epithelial dysplasia we examined intestinal morphology at day 7, 42 and 63 of adult life following treatment with vehicle or Zol starting at either day 4 or 40 of adult life. Using *Su(H)-lacZ; esg-Gal4, UAS-GFP* reporters we labeled intestinal stem cells and enteroblasts (progenitor cells) on the basis of GFP expression driven by the *escargot* promoter - a marker associated with stemness. Epithelial dysplasia in the *Drosophila* gut is characterised by hyperproliferation, as identified by the G2/M marker phospho-Histone3 (pH3)(24) and mis-differentiation of intestinal stem cells(25). As expected, we observed an increase in intestinal stem-cell proliferation with age in vehicle treated flies as shown by the significant increase in the number of GFP+ / pH3 positive cells with age (Fig. 3 A&B) and an increase in the overall proportion of GFP+ cells (Fig. 3 C&D). Both parameters are significantly decreased in female flies treated with Zol (Fig. 3 A-D).

One of the key roles played by the intestinal epithelia is to provide an impermeable barrier to the exterior environment in which damaged or dying epithelial cells are replaced with new cells generated by the ISCs. A loss of barrier function in the intestinal epithelium has been reported with age in both flies and humans. To assess intestinal integrity we performed the "Smurf assay"(26) in which blue colouring added to regular food is able to cross a compromised epithelium and stain the entire fly blue. Consistent with previous findings, the percentage of flies presenting the 'Smurf' phenotype was increased with age and is attenuated by treatment with Zol under the all-life feeding regime (Fig. 3 E&F).

Taken together, these data suggest that treatment with Zol throughout adult life reduces intestinal dysplasia and permeability in *Drosophila*.

Zol increases lifespan of flies exposed to oxidative stress through FOXO

To elucidate the mechanism of action mediating the beneficial effects of Zol and considering the role of GTPases in longevity we hypothesized that resistance to oxidative stress was partly responsible for the increased survivorship. Therefore, flies were challenged with food containing 5% H_2O_2, a treatment that reduces absolute lifespan and acts as a source of oxidative stress. Groups of adult, 11-day old female w^{Dah} flies were subjected to 5% H_2O_2 at the same time as either Zol or Rapamycin used as a positive control(27). We also tested another group of flies pre-treated with Zol for 7 days before exposure to 5% H_2O_2 (Fig. 4A). While the survival of flies treated with Rapamycin increased significantly compared to vehicle control, Zol-treatment did not improve survival when administered at the same time as the 5% H_2O_2 (Fig. 4B). By contrast, survival in response to H_2O_2 was significantly increased following pre-treatment with Zol for 7 days (Fig. 4B). To determine whether the increase was due to inhibition of FPPS, flies pre-treated with Zol were also treated with the downstream metabolites of the mevalonate pathway, Farnesyl farnesol (FOH) and Geranyl geranyol (GGOH), which bypass the block in FPPS inhibition. As expected, the survival advantage provided by Zol pre-treatment was abrogated in the presence of FOH and GGOH demonstrating that the extension in survival is due inhibition of the mevalonate pathway (Fig. 4C). To further support the hypothesis that inhibition of FPPS is responsible for the resistance to oxidative damage, $Fpps^{k06103}/+$ *and* $Fpps^{k03514}/+$ heterozygotes outcrossed into a w^{Dah} genetic background were also subjected to 5% H_2O_2 and their survival assessed. In these flies, survival was also significantly increased compared to w^{Dah} controls following exposure to H_2O_2-induced oxidative stress (Fig. 4D). Taken together these data suggest that the Zol confers resistance to oxidative stress and demonstrates that these effects are mediated by inhibition of the FPPS enzyme in the mevalonate pathway.

To determine the mechanism of action downstream of the mevalonate pathway, we hypothesized that Zol may be affecting the activity of dFOXO, a factor regulating oxidative stress signals. To determine

whether Zol protects *Drosophila* from oxidative damage through dFOXO, heterozygous $dFOXO^{\Delta 94}$/+ loss of function mutations(17) and w^{Dah} controls were pre-treated with either Zol at the highest dose of 10μM or vehicle control-containing food for 7 days before exposure to food containing 5% H_2O_2. While w^{Dah} pre-treated with Zol showed a significant increase in lifespan, $dFOXO^{\Delta 94}$/+ flies treated in the same way did not show any beneficial effect (Fig. 4E), suggesting dFOXO is required for Zol action on survival.

Given that pAKT is a known regulator of FOXO(28) we next determined levels of pAKT, following treatment with Zol and observed a significant decrease (Fig. 4F and eFig. 5). Furthermore, these effects were reversed by the addition of FOH and GGOH (Fig. 4 F&G), suggesting that inhibition of pAKT is dependent on the mevalonate pathway.

Taken together these data suggest that Zol confers resistance to oxidative damage via inhibition of the mevalonate pathway through a mechanism that requires dFOXO for its activity.

Zol enhances DNA damage repair in *Drosophila* upon irradiation

DNA damage and the resulting mutations induced by ionizing radiation are at least partly caused by increased oxidative stress(29). We therefore wanted to determine whether the ability of Zol to protect against H_2O_2-induced oxidative stress would also reduce the frequency of DNA damage induced by X-ray irradiation. In order to visualise DNA damage in an *in vivo* environment, we developed an assay in which endogenously expressed mRNA from the wild type *white* locus is knocked down by an *in vivo* RNAi hairpin-loop expressed within the cells of the future eye. Where knock down is successful, *white* mRNA is destroyed and very little pigment is produced, resulting in pale yellow eye pigmentation. Where the DNA encoding components of the *GMR-Gal4,UAS-white*^{RNAi} expression

system are mutated, *white* mRNA is not destroyed and wild type levels of red pigment are produced to give a readily recognisable red eye clone (Fig. 5A) in adult *Drosophila*. In order to assess DNA damage levels using this reporter, larvae heterozygous for the *GMR-Gal4,UAS-white*^{RNAi} reporter were raised on food containing either carrier controls, 1μM Zol, 10μM Zol or a combination of 10μM Zol and 33μM FOH and 33μM GGOH. Larvae were irradiated 96 hours after hatching using one dose of 200Gy of X-ray irradiation (Fig. 5C). Strikingly, *GMR-Gal4,UAS-white*^{RNAi}/+ flies contained significantly lower frequency of red-marked mutated cells when grown on 1 or 10μM Zol-containing food compared to controls. However, when *Drosophila* were treated with 10μM Zol in combination with FOH and GGOH, a partial reversal of this effect was observed (Fig. 5C). These data suggest that Zol is also able to protect individuals from the accumulation of mutations, via mechanism(s) that, at least in part, depend on the mevalonate pathway.

DISCUSSION

In this study we show that Zol has properties of a geroprotector, an activity mediated by its inhibition of FPPS. We show that Zol extends the lifespan and healthspan of *Drosophila* in absence of mineralised bone-like structures and demonstrate that it confers resistance to oxidative damage via the inhibition of FPPS in the mevalonate pathway. The median extension of lifespan by Zol is in the same range of other geroprotectors including rapamycin, with Zol extending the median lifespan of females by 14-18% when given from middle age. Rapamycin, one of the most studied geroprotectors, which positively impacts on the immune system in older patients by boosting their ability to improve flu vaccine responses(30) and also improves several healthspan parameters in mice(31), increases lifespan by 9-14% in female mice when fed late in life(32). A similar level of extension was shown in other studies testing rapamycin in *Drosophila*(33).

Our results suggest that Zol requires dFOXO for its action and is associated with reduction in the levels of pAKT. These *in vivo* findings are in line with our previous work in human mesenchymal stem cells (hMSC) where we have shown that Zol was able to reduce the accumulation of DNA damage caused by cellular ageing or irradiation via a mechanism requiring FOXO3a, as well as reducing the accumulation of senescent markers p16 and p21 (ref(34)). In addition, Zol was able to increase the translocation of pFOXO3a to the nucleus in human mesenchymal stem cells and this was reversed by the addition of FOH and GGOH, suggesting an action on the activation of FOXO3a(34). Molecules such as Rheb and Ras are small GTPases prenylated via the mevalonate pathway and upstream of TORC1 and TORC2 and therefore are likely candidates to mediate the regulation of pAKT and FOXO by Zol(35, 36). FOXO is a key player in ageing and has been shown to regulate several of its hallmarks including DNA damage, senescence, changes in mitochondrial function and mutation rates(37). Whilst *Drosophila* have a single FOXO gene (dFOXO), the human genome is more complex and encodes four different FOXO proteins, with polymorphisms in FOXO3a having been associated with exceptional longevity(38, 39). In addition, FOXO3a activity has been shown to reduce the effects of reactive oxygen species (ROS) production in multiple ways. For example, its expression improves the fidelity of DNA damage repair by arresting the cell cycle to allow the repair of damaged DNA(40, 41). In addition, FOXO3a activation results in the repression of a large number of nuclear-encoded genes with mitochondrial function(42). As most intrinsic ROS are produced by the respiratory complexes located in the inner mitochondrial membrane, these changes in mitochondrial activity may directly influence the levels of ROS production *in vivo*. More work is required to understand in detail which of these mechanisms and molecular pathways Zol modulates via FOXO. In addition, it will be important to determine which of the many small GTPases modulated by the mevalonate pathway are responsible for FOXO activity. A detailed analysis of each individual tissue in a mammalian system is also required as there are important differences in response to oxidative stress not only among tissues but even within regions of the same tissues(43).

A similar molecular mechanism mediating extension of lifespan has been described following administration of statins in *C. elegans.* Statins inhibits HMG-Co-A reductase in the mevalonate pathway and extends lifespan via DAF16/FOXO3a(44). However, statins are administered daily as opposed to Zol which is given once a year in patients affected by osteoporosis. This can be an advantage in terms of cost-effectiveness, especially when considering preventive interventions.

One observation we made is that the effects of Zol varied depending on time of administration, dose and sex differences that may be partly due to differences in drug uptake by males and females. In females, egg production requires higher levels of nutritional input than required by males, resulting potentially in increased food consumption and therefore increased drug uptake with consequent signs of toxicity(45). By contrast, in the FPPS mutants, where the action of the enzyme is disrupted independently of drug uptake, similar lifespan extension is observed in both sexes suggesting that the effect of mevalonate pathway inhibition on lifespan is unlikely to be sexually dimorphic. However, further work is required to understand this aspect of the work.

In addition to differences in lifespan extension, it is intriguing to note that indicators of improved health such as climbing ability and intestinal dysplasia occur even at high doses of Zol that reduce absolute lifespan (compare Fig. 1B 10µM with Fig. 3A, 63day 10µM), demonstrating that drug treatment is able to increase healthspan independently of absolute lifespan. This observation is in line with the finding of others which shows that healthspan and lifespan are not necessarily related(46). For example, Nicotinamide has recently been shown to improve aspects of healthspan but not lifespan(47). This disconnect between healthspan and lifespan may indicate that organisms can cope with the accumulation of a certain number of defects which are sufficient to negatively affect healthspan but which do not lead directly to death. Being able to reduce these healthspan-associated deficits is of particular interest from a translational perspective as they contribute to morbidity and poor health and are responsible for significant healthcare costs.

Another notable aspect of Zol treatment in *Drosophila* is the improvement in lifespan and healthspan measures following treatment that begins only in middle age (Fig. 1 E&F). It is unclear why the effects are more prominent when the treatment starts at middle age, particularly in females. The treatment profile observed in these flies mirrors that of women affected by osteoporosis who also generally begin Zol treatment post-menopausally. In both *Drosophila* and humans, the effect of Zol on survival is only detectable sometime after initial treatment(48). On average, it takes 16 months before an improvement in survival is observed in postmenopausal women(48) and approximately 14 days in *Drosophila* (Fig. 1F). The reasons for this delayed phenotypic response are unclear but may reflect an ability by Zol to improve biological processes only when they are mildly dysregulated. However, when those mechanisms are either working at healthy levels or when their dysregulation exceeds a compensatory threshold then Zol treatment is either not needed or no longer sufficient to maintain function. Consistent with this model, Zol did not have any effect on climbing activity in geriatric flies at 70 days of age – when no activity was detected in any of the populations tested. It is possible that a similar process may also explain why a Zol-mediated extension of lifespan was not observed in *Zmpste24*$^{-/-}$ mice. In this model of Hutchinson-Guilford progeria syndrome Zol alone does not modify premature ageing while combined treatment with both Zol and statins (which inhibit the same pathway) is able to extend lifespan(49).

While evidence collected to date suggests that the effects of Zol on lifespan and healthspan in humans may be limited, it should be noted that the effects observed follow treatment with just a single yearly dose. None-the-less, retrospective analysis of patients taking Zol has not only shown a marked increase in survival but also a reduction in the frequency of death by pneumonia and cardiovascular events, suggesting broader effects unrelated to the musculoskeletal system(9). In addition, patients treated with Zol and admitted to hospital for intensive / critical care for a condition not related to osteoporosis showed reduced mortality rates relative to controls (5.2% vs 9.1% respectively).

Furthermore, the patients previously treated with Zol required 30% shorter in-patient care despite being older than the control group and having higher comorbidity index(10).

Older people with frailty and multimorbidity have a reduced ability to respond to adverse events and often lose independence following major health-related incidents. Such events have significant consequences for follow up health and social care costs(50, 51). Indeed, there has been an increase of 18% in the number of emergency admissions of older patients between 2010/11 and 2014/15 in the UK with those patients over the age of 65 now accounting for 62% of total bed days spent in hospital. Considering average cost for a patient to stay in an NHS ward is up to £400 per day, the financial and societal benefits of improving resilience in older people and reducing length of hospital stay are huge(51).

In conclusion we have shown that inhibition of FPPS by Zol modulates mechanisms of ageing to extend lifespan and healthspan *in vivo* – an effect that is independent of its effects on bone. These findings are in line with the unexplained improved survival rates that have been reported recently for patients being treated with Zol – findings that highlight the substantial benefit Zol can potentially provide with only a single yearly treatment. Studies in mammalian models are now required to understand the effects of Zol on specific tissues, to define which of the FOXO-regulated mechanisms are at play and whether an infrequent administration of Zol is the best approach to elicit the strongest beneficial effects. Given that Zol is off-patent, available at low cost and displays a well understood safety profile featuring minimal side effects, we suggest that repurposing studies seeking to widen the use of Zol are likely to identify great potential for the improvement of healthspan and resilience in older people.

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Conflicts of interest

None

Author Contributions

IB, MZ conceived the idea, analysed the data, wrote the manuscript ZC, MZ and AA performed the experiments, analysed the data and wrote the manuscript. All authors designed the experiments, reviewed and approved the manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legend

Fig. 1 Administration of Zol affects lifespan of flies

Percentage survival of (A) male and (B) female w^{Dah} flies fed with food in presence or absence of Zol (1 or 10μM) throughout their lives; percentage survival of (C) male and (D) female heterozygote FPPS mutant flies and w^{Dah} fed with standard *Drosophila* food; percentage survival of (G) male and (F) female w^{Dah} flies fed with food in presence or absence of Zol (1 or 10μM) from 40 days of age. Average of 3 experiments with 100 flies/group/experiment. Log-rank (Mantel-Cox) test in Graphpad Prism used to statistically analyse the survival curves, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. All experiments were repeated 3 times.

Fig. 2 Flies fed with Zol shows a significant increase in climbing ability at middle age.

Lifelong treatment with Zol: (A) Percentage of male flies climbed above 10cm (high climbers) within 15 seconds. (B) Percentage of female flies climbed above 10cm (high climbers) within 15 seconds. Flies treated with Zol from midlife: (C) Percentage of male flies climbed above 10cm (high climbers) within 15 seconds. (D) Percentage of female flies climbed above 10cm (high climbers) within 15 seconds. Data were analysed by Two-Way ANOVA and Dunnet's multiple comparison test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ **** $p \leq 0.0001$

Fig. 3 Treatment with Zol reduces epithelial dysplasia and intestinal permeability in *Drosophila*

(A-B) Quantification of pH3+ cell in the intestine of *Drosophila*. Flies were treated with or 1 or without 10μM of Zol from 4 days of age (A) or 40 days of age (B). At least 7 midguts were assessed per condition and per time point. (C-D) Quantification of $\%GFP^{+}$ cells (normalised to DAPI) in the intestine of flies treated with or without 1 or 10μM of Zol from 4 days of age (C) or 40 days of age

(D). (E-F) Percentage of flies Smurf positive following treatment with or without 1 or 10µM of Zol starting from 4 days of age (E) or 40 days of age (F) (n>65 per condition per timepoint). Data were analysed with one-way ANOVA and Sidak's post-hoc test (number of PH3+ cells and %GFP^+ cells) and Chi square test for the analysis of the smurf assay. *p<0.05 , **p<0.01, ***p<0.001, ****p<0.0001

Fig. 4 Zol increases lifespan of flies under oxidative stress through FOXO

(A) A schematic representation of the experimental design. (B) Survivorship of female w^{Dah} flies fed on food containing 5% H_2O_2. The flies were treated with 5% H_2O_2 in combination with PBS (vehicle) or 10µM Zol or 200µM rapamycin (Rap) both given at the same time than H_2O_2. Two groups of flies were pretreated for 7-days with 10µM Zol (d4) or PBS (d4) as vehicle before addition of H_2O_2 (C) Survivorship of female w^{Dah} flies fed with 5% H_2O_2. The flies were pre-treated PBS+EtOH (Vehicles), 10µM Zol, or 10µM Zol in combination with 330µM FOH & 330µM GGOH. (D) Survivorship of heterozygote female FPPS mutants: $Fpps^{k06103}$/+ and $Fpps^{k03514}$/+ fed with 5% H_2O_2. w^{Dah} flies either pre-treated PBS or 10µM Zol, were used as control (E) Survivorship of female dFOXO $^{\Delta 94/+}$ flies treated with 5% H_2O_2. w^{Dah} flies either pre-treated with 10µM Zol or PBS (vehicle) were used as control. For all survival tests, 100 flies were used in each treatment group per test, experiments were repeated 3 times with different cohorts of flies. (F) A representative example of AKT and pAKT expression in whole flies fed with Zol (100µM) for 10 days, in presence or absence of FOH (330µM), GGOH (330µM). PBS and ethanol (EtOH) were used as vehicle control. (G) Quantification of expression level of pAKT normalised to tubulin in presence or absence of Zol (100µM), FOH (33µM) and GGOH (33µM) for 10 days analysed by ImageJ (n=3).

Fig. 5 Zol enhances DNA damage repair in *Drosophila* upon irradiation

(A) A representative image of an eye from a *w*$^{+}$*; GMR-Gal4, UAS-white RNAi* fly (left) and one containing a clone of cells in the eye in which DNA damage has led to the production of red pigment (right). (B) Time line of experiment. (C) Percentage of eyes with red colonies upon 200Gy irradiation in homozygous *w+; GMR-Gal4,UAS-white RNAi* flies when treated with PBS (control), 1μM Zol, 10μM Zol, or 10μM Zol with 330μM FOH and GGOH. Data analysed by one way-ANOVA and Sidak post-hoc test. ***P<0.001, ****P<0.0001

Fig.1

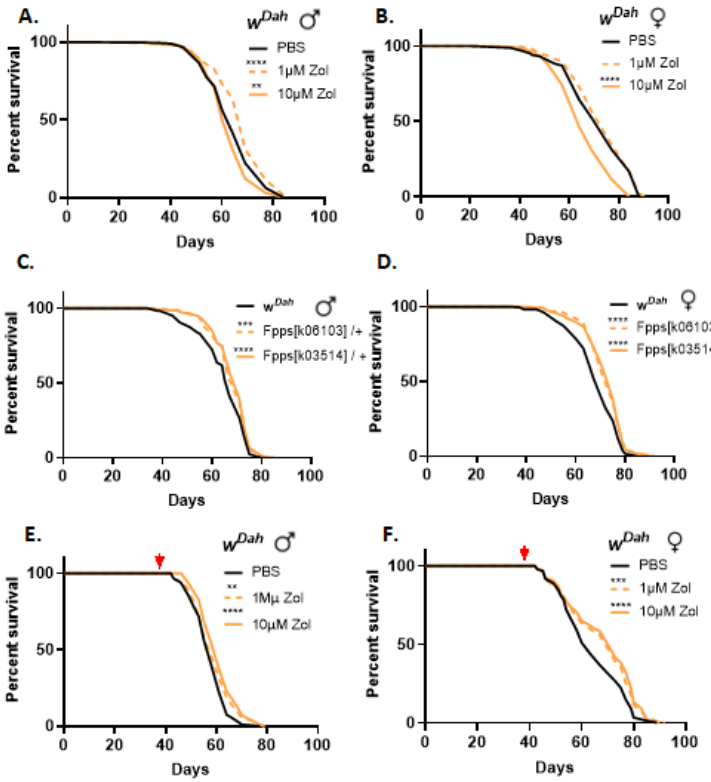
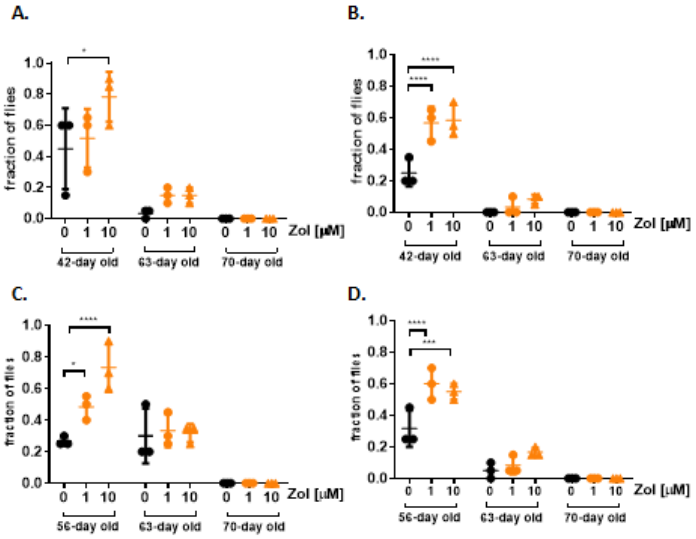


Fig. 2



Two-way ANOVA		Df	F value	P value
Male flies treated with Zol throughout their life (Fig. 2A)	Age	2	57.56	<0.0001*
	Treatment	2	3.414	0.161
	Interaction	4	1.858	0.0553
Female flies treated with Zol throughout their life (Fig. 2B)	Age	2	162.9	<0.0001*
	Treatment	2	13.52	0.0003*
	Interaction	4	8.238	0.0006*
Male flies treated with Zol starting at middle age (Fig. 2C)	Age	2	68.51	<0.0001*
	Treatment	2	7.090	0.0054*
	Interaction	4	6.433	0.0021*
Female flies treated with Zol starting at middle age (Fig. 2D)	Age	2	166.3	<0.0001*
	Treatment	2	10.33	0.0010*
	Interaction	4	5.833	0.0034*

Fig.3

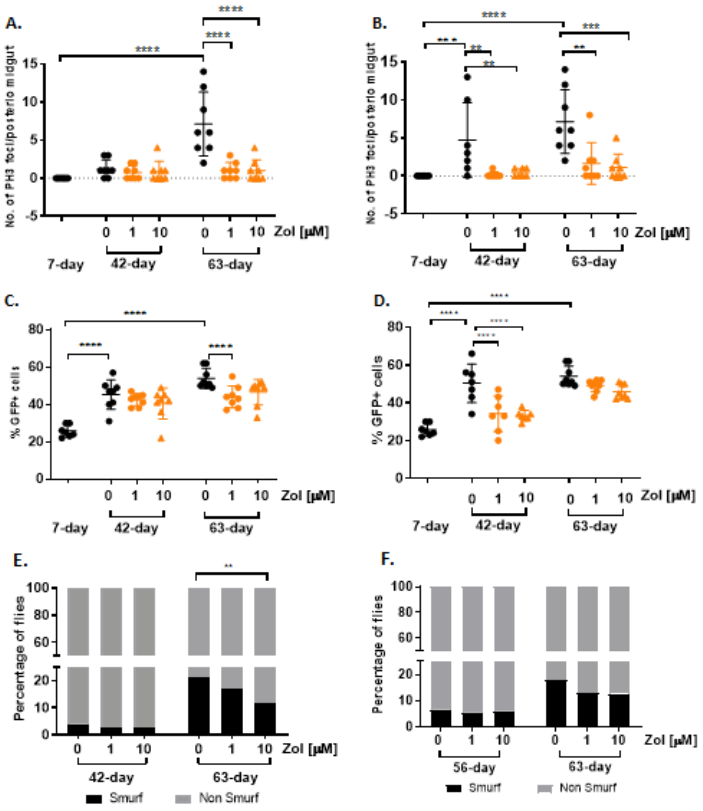


Fig.4

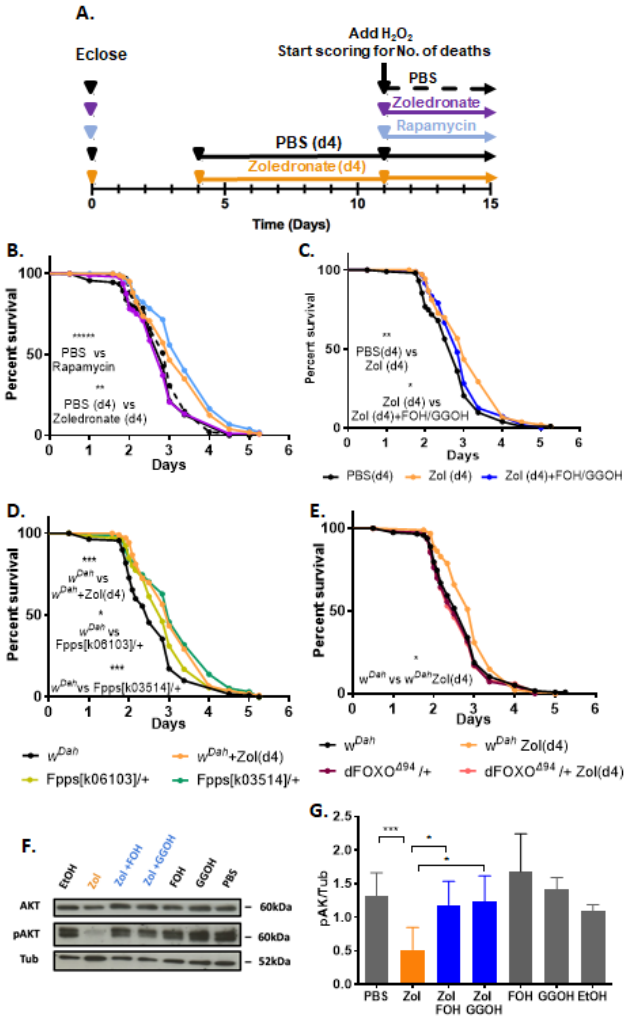


Fig. 5