Protection of Nuclear DNA by Lifespan-Extending Compounds in the Yeast *Saccharomyces cerevisiae*

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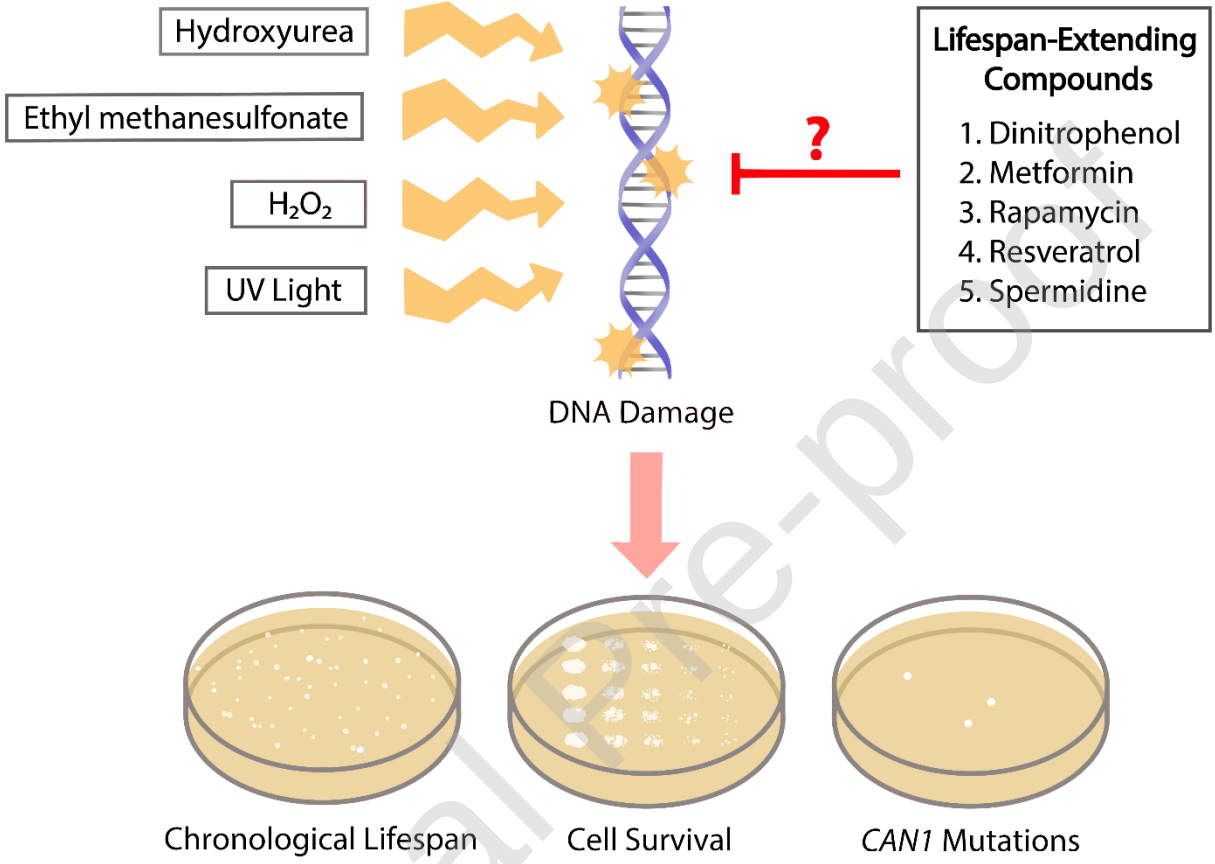
Graphical Abstract



HIGHLIGHTS

- Rapamycin and spermidine decreased *CAN1* mutation rates in yeast
- Dinitrophenol, metformin, and resveratrol decreased EMS-induced mutations
- Spermidine sensitized cells to H_2O_2 insult

- Only spermidine increased lifespan in cells growth-arrested with hydroxyurea

ABSTRACT

DNA damage has been hypothesized to be a driving force of the aging process. At the same time, there exists multiple compounds that can extend lifespan in model organisms, such as yeast, worms, flies, and mice. One possible mechanism of action for these compounds is a protective effect against DNA damage. We investigated whether five of these lifespan-extending compounds, dinitrophenol, metformin, rapamycin, resveratrol, and spermidine, could protect nuclear DNA in the yeast *Saccharomyces cerevisiae* at the same doses under which they confer lifespan extension. We found that rapamycin and spermidine were able to decrease the spontaneous mutation rate at the *CAN1* locus, whereas dinitrophenol, metformin, and resveratrol were able to protect yeast against *CAN1* mutations induced by ethyl methanesulfonate (EMS). We also tested whether these compounds could enhance survival against EMS, ultraviolet (UV) light, or hydrogen peroxide (H_2O_2) insult. All five compounds conferred a protective effect

against EMS, while metformin and spermidine protected yeast against UV light. Somewhat surprisingly, none of the compounds were able to afford a significant protection against H_2O_2, with spermidine dramatically sensitizing cells. We also examined the ability of these compounds to increase lifespan when growth-arrested by hydroxyurea; only spermidine was found to have a positive effect. Overall, our results suggest that lifespan-extending compounds may act in part by protecting nuclear DNA.

1. Introduction

Many ideas have been proposed to explain the aging process. One of them is the DNA damage theory of aging, which was originally proposed as early as 1958 [1-3]. There is significant support for the role of DNA damage in the aging process. In many studies, both DNA damage and mutations have been shown to increase with age, whereas DNA repair capabilities have been found to decrease with age

[4, 5]. Mouse models compare well with human diseases where impairment of DNA repair has been shown to result in age-accelerated phenotypes [6]. In humans, the genetic disease Werner' s syndrome exhibits multiple features of accelerated aging-like clinical signs, such as muscle atrophy, impaired wound healing, atherosclerosis, and osteoporosis, with a median lifespan of about 46 years [7, 8]. The gene responsible was found to encode a DNA helicase [9], and its loss of function results in an increased occurrence of mutations, stalled replication forks, and DNA double-strand breaks [7, 8]. More strikingly, Hutchinson–Gilford progeria syndrome (HGPS) results in a rapid age-accelerated phenotype with a median lifespan of about 13 years [10]. This is due to mutations in the LMNA gene, encoding prelamin A, which causes the nuclear envelope to be poorly formed [11]. While not directly related to DNA damage, mutation in the LMNA protein results in elevated levels of genetic instability [10].

Despite the demonstrated ability of impaired DNA metabolism to cause a shortening of lifespan, there appears to be a lack of data showing the opposite, that is the ability of improved DNA repair to increase lifespan. However, there are multiple treatments that can extend lifespan in laboratory models. The most robust is dietary restriction (DR), which consists of a decreased caloric intake without malnutrition [12]. This treatment has been demonstrated to increase lifespan and improve health in a very broad range of species, and has been shown to result in decreased levels of DNA damage, increased DNA repair capabilities, and decreased mutations and rates of cancer [13, 14]. In addition to DR, there are several compounds that are also able to increase lifespan in model organisms. These mainly cluster in a group that could be commonly termed nutrient sensing or dietary restriction mimetics [15]. One of the most notable is rapamycin. Initially identified from the bacterium *Streptomyces hygroscopicus* on the island of Rapa Nui as an antimycotic, rapamycin has since found use as an immunosuppressant and anti-cancer agent [16, 17]. Rapamycin inhibits the target of rapamycin (TOR) complex, which is a master cell growth regulator, and has been shown to extend

the lifespan of yeast, flies, and mice [18-20]. Another well-known molecule, resveratrol, was identified in a screen searching for yeast silent information regulator (SIR) 2 activators; it had been earlier discovered that increased expression of SIR proteins in yeast could extend lifespan [21, 22]. Resveratrol has since been found to extend lifespan in worms and flies, and in mice fed a high-fat diet [23, 24]. The free radical theory of aging has been a longstanding theory of aging, and more recently modified to the mitochondrial theory of aging [25, 26]. Dinitrophenol is a mitochondrial uncoupler, and allows protons to leak across the inner mitochondrial membrane, bypassing complex V, which has the effect of decreasing free radical production [27]. This compound also extends lifespan in yeast, and in mice [27, 28]. Metformin is a drug commonly prescribed for type 2 diabetes, and has too been shown to extend lifespan in yeast and mice [29, 30]. Metformin has also been proposed to treat aging in humans [31]. Its molecular mechanism is not clear; however, it has been shown to act in a manner dependent on AMP kinase [32]. Thus, one may presume metformin is modulating cellular energy levels. The final compound tested here, spermidine, acts to induce

autophagy, which is the turnover of cellular proteins, and has been shown to extend lifespan in yeast, worms, and flies [33].

In this work, our goal was to ask whether compounds that are able to increase lifespan could also protect DNA in the yeast *Saccharomyces cerevisiae.* This was accomplished by examining spontaneous mutation rates and induced mutation frequencies at the *CAN1* locus. We also examined whether these compounds could enhance cellular survival against known DNA damaging agents.

2. Materials and methods

2.1. Yeast and media

The *Saccharomyces cerevisiae* strains, BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and S288C (*MATα SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*) were acquired from Dharmacon (Layfayette, CO). The yeast-peptone-dextrose

(YPD) media was made with 10 g/L yeast extract from Fisher BioReagents, 20 g/L peptone and 20 g/L dextrose from Difco Laboratories. The YNB + Nitrogen, SC-Arg-Leu-Lys-Ura, and associated amino acids used to make the synthetic complete media that lacked arginine (SC-Arg) were purchased from Sunrise Science (San Diego, CA) and Sigma Aldrich (St. Louis, MO), respectively.

2.2. Chemicals

Canavanine was purchased from Sigma-Aldrich. The YPD plates were made with the addition of 2% Bacto Agar from Difco Laboratories. The SC-Arg canavanine plates were made from SC-Arg media with 340 µM canavanine and 800 µL of potassium hydroxide (KOH) per 1L SC-Arg canavanine media and 2% Bacto Agar. Tetracycline (Tet), provided by Sigma-Aldrich, was the antibiotic used in all the experiments and was made to a concentration of 27 mM in 70% ethanol. Dinitrophenol (DNP), resveratrol (RES), and spermidine (SPD) were purchased from Sigma-Aldrich and rapamycin (RAP) from Fisher Scientific (Waltham, MA).

Metformin (MET) was purchased from the Tokyo Chemical Industry (Tokyo, JPN). Resveratrol and RAP were prepared in DMSO to a 10 mM and 10 µM concentration, respectively. Dinitrophenol was dissolved in H_2O at a 10 µM concentration. The mutagens hydrogen peroxide (H_2O_2) and ethyl methanesulfonate (EMS) were purchased from Sigma-Aldrich. Ultraviolet irradiation (253 nm) was provided by a UV Transilluminator (Model: TE-254S) from Spectroline (West Layfayette, IN). Hydrogen peroxide was diluted to a 150 mM concentration in H_2O. Potassium phosphate buffer pH 7.0 (KPB) was made from K_2HPO_4 and KH_2PO_4, and were purchased from Fisher Chemical. Sodium thiosulfate was obtained from Fisher Chemicals.

2.3. Growth rate assays

Cultures were set up with and without 0.3 M hydroxyurea, and were treated individually with 10 nM DNP, 10 µM MET, 10 nM RAP, 10 µM RES, or 4 mM SPD. Eight hundred µL of the saturated S288C yeast culture was pipetted into each flask

and incubated at 150 rpm and 30 °C for 14 hours (18 hours for the MET and SPD groups). The absorbance was recorded every hour and used to calculate the doubling time for each time period. The fastest doubling time for each individual culture was used.

2.4. Mutation rate assays

Single yeast clones were picked as colonies and grown to saturation for two days in YPD +Tet media and then diluted 10,000-fold with SC-Arg+Tet media. Each experiment contained a treatment of 10 nM DNP, 10 µM MET, 10 nM RAP, 10 µM RES, or 4 mM SPD. For the final yeast dilution, 100 µL was divided into 48 wells that were then incubated at 30° C for two days. After incubation, 42 of the 48 wells were spotted onto canavanine SC-Arg +Tet plates. The remaining six wells were combined and then diluted 200,000 times in YPD +Tet media. For the final yeast concentration, 100 µL was spread on YPD +Tet plates. The plates were incubated at 30°C for two days and examined afterward to identify cell numbers. The spots

plated onto canavanine SC-Arg +Tet plates were analyzed after two days to determine the mutation rate. Mutation rates were calculated via fluctuation analysis [34] as $-\ln(P_0)/N_f$, where P_0 is the proportion of spots on canavanine which do not exhibit any growth, and N_f is the final number of cells which approximates the number of divisions in each spot.

2.5. Induced Mutagenesis assay

A mutagenesis assay was conducted for EMS using five 1 mL samples of saturated yeast, individually containing the drug treatments of 10 nM DNP, 10 µM MET, 10 nM RAP, 10 µM RES, or 4 mM SPD. Each sample was centrifuged at 5000 × g for 1 minute to form a pellet, which was washed twice with 4 mL of KPB and resuspended in an additional 4 mL of KPB. Of the EMS, 120 µL was added to each sample and mixed every 5 minutes for 60 minutes at room temperature. After 60 minutes, 4 mL of sodium thiosulfate was added, and the samples were centrifuged at 5000 × g. Cells were then plated on control plates as a 10-fold dilution series in

order to determine cell number, and on canavanine plates to count the number of *can1* mutants.

2.6. Survival assays

2.6.1. H_2O_2 survival assay

Yeast were grown to saturation for 2 days in YPD +Tet media containing 10 nM DNP, 10 µM MET, 10 nM RAP, 10 µM RES, or 4 mM SPD, and then serial diluted to 10^6, 10^5, 10^4, 10^3, 10^2 cells using YPD +Tet media. The diluted yeast cultures were incubated with 15 mM of H_2O_2 for 1 hour, and then 10 µL of each sample was spotted onto YPD +Tet plates. The plates were incubated at 30° C for 2 days and then analyzed.

2.6.2. EMS survival assay

Individual 1 mL aliquots of the saturated yeast cultures containing the 10 nM DNP, 10 µM MET, 10 nM RAP, 10 µM RES, or 4 mM SPD were centrifuged at 5000 × g

for 1 minute to form a pellet. The pellets were then washed twice with 4 mL of KPB and resuspended in 500 µL of KPB. Five-hundred µL of EMS was added to each sample and mixed for 5 minutes. After 5 minutes of incubation, 4 mL of 10% sodium thiosulfate was added to each sample, which were then centrifuged at 5000 × g for 1 minute. The sample was resuspended in 1 mL YPD and then diluted to 10^6, 10^5, 10^4, 10^3, 10^2 cells in YPD +Tet media. For each sample, 10 µL was spotted onto individual YPD +Tet plates. The plates were incubated at 30° C for 2 days and then analyzed.

2.6.3. UV survival assay

Saturated yeast cultures that contained the drug treatments of 10 nM DNP, 10 µM MET, 10 nM RAP, 10 µM RES, or 4 mM SPD were diluted to 10^6, 10^5, 10^4, 10^3, 10^2 cells in YPD +Tet. Then, 10 uL of each sample was spotted onto individual YPD

+Tet plates that were then exposed to UV radiation (253 nm) for 2 seconds. The plates were incubated at 30° C for 2 days and then analyzed.

2.7. Lifespan in hydroxyurea

Each hydroxyurea survival assay was set up with six 50 mL SC+Tet cultures and S288C yeast was added to give an initial absorbance reading of 0.2. All six flasks were incubated at 150 rpm and 30° C. Absorbance was recorded hourly with the Epoch microplate reader. When the absorbance reached about 0.8, each culture was treated with 10 nM DNP, 10 µM MET, 10 nM RAP, 10 µM RES, or 4 mM SPD. When the absorbance reached about 0.9, 2.66 g of HU and 0.8 g of dextrose were added into each flask. After adding the HU, the absorbance was recorded and serial dilution was used to determine the proportion of yeast alive in the culture every day until the serial dilutions showed no colonies on the agar plates. We replenished the dextrose every other day to prevent yeast from becoming energy depleted.

2.8 Statistical Analyses

Statistical analyses were conducted using Prism software (GraphPad, La Jolla, CA). The tests used and sample sizes for each experiment are indicated in the figure captions. A *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Spontaneous and induced mutations in CAN1

We first questioned whether these lifespan-extending chemicals could decrease spontaneous mutation rates at the *CAN1* locus. The *CAN1* gene encodes arginine permease, an amino acid transporter, which is capable of transporting the toxic

amino acid canavanine [35, 36]. The transfer RNA for arginine cannot distinguish between arginine and canavanine very well, and will often incorrectly insert canavanine into newly synthesized proteins [37]. This causes the synthesis of defective proteins, resulting in cell death. Mutations in *CAN1* prevent the uptake of canavanine in the media, permitting the yeast to survive on its own synthesized arginine. Only rapamycin and spermidine were able to decrease spontaneous mutation rates at the *CAN1* locus (Fig. 1A). The alkylating agent and mutagen ethyl methanesulfonate (EMS) was used to induce mutations. This resulted in an approximate 23-fold increased mutation frequency at the *CAN1* locus over untreated control (Fig. 1B). Interestingly, for the EMS-induced mutation frequencies, we found a result opposite that of spontaneous mutation rates. Dinitrophenol, metformin, and resveratrol were able to protect against EMS-induced mutations, but rapamycin and spermidine were not (Fig. 1B). Rapamycin is known to slow cell growth, which may contribute to its ability to extend yeast lifespan. Due to this effect, we then investigated whether the other compounds would also exhibit a similar response. As expected, rapamycin increased cell

doubling time by more than 2.5-fold (Fig. 1C). However, the other four compounds had no statistically significant effect on doubling time (Fig. 1C).

3.2. Protection against EMS, UV light, and H_2O_2

We then determined whether these lifespan-extending compounds could increase survival when exposed to known DNA mutagens (Fig. 2). All five compounds afforded some protection against EMS when compared to the control (Fig. 2B). Metformin, resveratrol, and spermidine had the greatest effects, with those of dinitrophenol and rapamycin more marginal (Fig. 2B). With respect to the UV light challenge, cells supplemented with metformin and spermidine had better survival than the control, whereas dinitrophenol, rapamycin, and resveratrol seemed to have no marked effect (Fig. 2C). For the H_2O_2 challenge, none of the compounds provided any large protective effect. Cells supplemented with rapamycin and resveratrol fared as well as the control (Fig. 2D). However, dinitrophenol,

metformin, and particularly spermidine, appeared to sensitize yeast to H_2O_2 (Fig. 2D).

3.3. Lifespan in the presence of hydroxyurea

Finally, we examined whether the ability of these lifespan-extending compounds could protect against replicative stress caused by hydroxyurea. Hydroxyurea is a compound that can be used to treat cancer and slows cell growth by inhibiting ribonucleotide reductase [38]. When this enzyme is inhibited, the cell can no longer make deoxyribonucleotides and thus cannot synthesize DNA. Hydroxyurea significantly shortened yeast survival relative to the control cells (Fig. 3A). When supplemented with the lifespan-extending compounds, only spermidine had a positive effect on survival (Fig. 3B and 3C). For spermidine, median survival was extended to 12 days, compared to the control value of 9 days (Fig. 3C). As for *CAN1* mutation rates, we wished to determine whether slowed growth rates contributed to survival in hydroxyurea supplemented cells. Hydroxyurea markedly

slowed cell growth with a doubling time of 230 ± 16 min vs 94 ± 3 min for the control (mean ± SEM, $P < 0.0001$, unpaired t test, n = 6 and 7) (Fig. S1). The growth rate of each compound in the presence of 0.3 M hydroxyurea was measured; only rapamycin had any effect on cell growth, where it completely arrested the cells at the dose tested. (Fig. 3D).

4. Discussion and conclusions

Damage to nuclear DNA is thought to be one of the driving forces of aging [1-3]. At the same time, there exists several compounds which have been shown to increase lifespan in laboratory models, including the yeast, *Saccharomyces cerevisiae* [18, 21, 27, 29, 33]. In this study, we were interested in determining whether these compounds could decrease the occurrence of mutations in nuclear DNA, and protect yeast against known mutagens. Our rationale was that if DNA damage is an important contributor in aging, compounds that extend lifespan may act in part, by protecting DNA against damage. This could then be visualized as a

decrease in the occurrence of mutations in nuclear DNA and/or enhanced survival in the presence of mutagens. This idea was applied to five lifespan-extending compounds, dinitrophenol, metformin, rapamycin, resveratrol, and spermidine, using the same doses that conferred an extended lifespan in yeast.

Of the five compounds tested, we found that only rapamycin and spermidine were able to decrease spontaneous mutation rates at the *CAN1* locus (Fig. 1A). The action of rapamycin could be related to the slowed growth rate at the dose we tested (Fig. 1C). One could imagine that by slowing the cell cycle, cells would simply have more time to repair any damage before DNA polymerase converted it into mutations during replication. An alternative possibility is through the regulation of mitophagy, or turnover of damaged mitochondria. This is a subclass of autophagy, which is a turnover of cellular components caused by autophagy-related (Atg) proteins that are suppressed by TOR [39]. Inhibition of TOR could then induce mitophagy, which would result in the destruction of defective respiratory chains and a decrease in the production of reactive oxygen species

[40]. If autophagy explained the ability of rapamycin to decrease mutation rates in our experiment, it is not surprising that spermidine would also have a positive effect on mutation rate too. Spermidine has been argued to increase lifespan through the induction of autophagy, and more specifically through mitophagy [33], and could thus protect DNA by the same mechanism. Like metformin, spermidine has been proposed as an anti-aging treatment in humans [41].

While rapamycin and spermidine both had positive effects on *CAN1* mutation rates, they surprisingly did not protect against mutations induced by EMS (Fig. 1B), a compound that is an alkylating agent and powerful mutagen [42]. The mechanism of action of EMS itself may explain why rapamycin and spermidine did not work. That is, autophagy and mitochondrial turnover may simply not play a role in repairing EMS-induced adducts. Paradoxically, dinitrophenol, metformin, and resveratrol were found to protect against EMS-induced mutations, while not lowering the spontaneous mutation rate at *CAN1* (Fig. 1A and 1B). This suggests that these compounds may act more by helping the cell to adapt to stress-induced

DNA damage as opposed to enhancing protective or repair activities under normal physiological conditions. This may be most apparent for resveratrol, where it has been shown to extend lifespan, but only in the case of excessive caloric intake [23]. Alternatively, the differing activity of these three compounds could be related to their more direct interaction with mitochondrial function. That is, an increase in energy production and/or decrease in free radical production could allow the cell to have enhanced DNA repair capabilities.

With respect to survival against EMS insult, all five compounds provided some positive benefit (Fig. 2B), which does support a role of nuclear DNA damage in their abilities to extend lifespan. While not quite as robust, challenge to UV light is also consistent, since metformin and spermidine were very protective, while the other three compounds showed a marginal protective effect (Fig. 2C). Our most surprising findings occurred with the H_2O_2 challenge (Fig. 2D). Cells supplemented with rapamycin survived about as well as the control. However, the other four compounds appeared to actually sensitize cells to H_2O_2 exposure. The effect of

dinitrophenol is the most surprising. Dinitrophenol is a mitochondrial uncoupler, which should result in decreased free radical production [27]. We are perplexed at this result and can only speculate on its cause. One possibility is that dinitrophenol lowers endogenous free radical production to such a degree that antioxidant defenses are also lowered, which then permits the cells to be overwhelmed by any excess oxidative insult. Spermidine had the greatest apparent negative effect on cellular survival when exposed to H_2O_2. This exactly the opposite of what Eisenberg et. al., observed [33]. However, they did test yeast aged for 24 days, whereas we examined the effect of spermidine fed to exponentially growing cultures. It may be that spermidine is very effective in growth arrested or nutrient-depleted cells and potentially harmful to rapidly replicating, nutrient sufficient cells. Alternatively, spermidine oxidase produces H_2O_2 [43, 44]. In the case of our experiments, the addition of exogenous H_2O_2 may have simply overwhelmed the endogenous defenses within each cell.

Aging in yeast is commonly measured by the chronological lifespan assay, which has been argued to model the aging of postmitotic cells [45]. In this model, yeast are allowed to grow until glucose depletion, at which point they arrest and then age [45]. However, this seems to be more reminiscent of starvation rather than aging. By comparison, many postmitotic cells in humans, such as neurons, are extremely energy demanding [46]. To address this, we wished to arrest cell growth in a glucose-independent manner. This was done using the cancer drug hydroxyurea, which blocks the synthesis of deoxynucleotides, and hence DNA replication [38]. Hydroxyurea has also been found to cause the formation of micronuclei [47, 48] and possibly 8-hydroxy-2’ -deoxyguanosine [49], and is thus a driver of replicative stress and DNA damage. Under these conditions, four of the five compounds no longer extended lifespan, and spermidine, which exhibited a dramatic lifespan extension in the chronological yeast aging model [33], had only a modest effect here. These results suggest that the ability of these compounds to protect cells against DNA damage may be limited under nonreplicating conditions.

In summary, there were four main results from this study. First, all of the compounds tested afforded at least some protective effect for nuclear DNA, either a decreased spontaneous mutation rate, or a protective effect against EMS insult. Second, at the doses used, none of the compounds protected cells against H_2O_2 insult, thereby suggesting a potentially limited role of oxidative stress in their lifespan-extending action. Third, among the compounds tested, spermidine seemed to be the most robust. It exhibited a positive effect in four of the six experiments, and was the only one to increase survival in the presence of hydroxyurea. Lastly, arresting cells via hydroxyurea resulted in a markedly different effect than the glucose depletion used in traditional chronological lifespan assays. Only spermidine had a positive effect against hydroxyurea, and it was modest relative to its action under glucose depletion.

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AUTHOR DISCLOSURE STATEMENT

No conflicts of interest exist for any of the authors.

References

1. Failla, G., *The aging process and cancerogenesis.* Ann N Y Acad Sci, 1958. **71**(6): p. 1124-40.

2. Szilard, L., *On the Nature of the Aging Process.* Proc Natl Acad Sci U S A, 1959. **45**(1): p. 30-45.

3. Freitas, A.A. and J.P. de Magalhaes, *A review and appraisal of the DNA damage theory of ageing.* Mutat Res, 2011. **728**(1-2): p. 12-22.

4. Niedernhofer, L.J., et al., *Nuclear Genomic Instability and Aging.* Annu Rev Biochem, 2018. **87**: p. 295-322.

5. Vijg, J. and Y. Suh, *Genome instability and aging.* Annu Rev Physiol, 2013. **75**: p. 645-68.

6. Gurkar, A.U. and L.J. Niedernhofer, *Comparison of mice with accelerated aging caused by distinct mechanisms.* Exp Gerontol, 2015. **68**: p. 43-50.

7. Norwood, T.H., et al., *Cellular aging in Werner's syndrome: a unique phenotype?* J Invest Dermatol, 1979. **73**(1): p. 92-6.

8. Oshima, J., G.M. Martin, and F.M. Hisama, *Werner Syndrome*, in *GeneReviews((R))*, M.P. Adam, et al., Editors. 1993: Seattle (WA).

9. Gray, M.D., et al., *The Werner syndrome protein is a DNA helicase.* Nat Genet, 1997. **17**(1): p. 100-3.

10. Gonzalo, S., R. Kreienkamp, and P. Askjaer, *Hutchinson-Gilford Progeria Syndrome: A premature aging disease caused by LMNA gene mutations.* Ageing Res Rev, 2017. **33**: p. 18-29.

11. Eriksson, M., et al., *Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome.* Nature, 2003. **423**(6937): p. 293-8.

12. Speakman, J.R. and S.E. Mitchell, *Caloric restriction.* Mol Aspects Med, 2011. **32**(3): p. 159-221.

13. Heydari, A.R., et al., *Caloric restriction and genomic stability.* Nucleic Acids Res, 2007. **35**(22): p. 7485-96.

14. Meynet, O. and J.E. Ricci, *Caloric restriction and cancer: molecular mechanisms and clinical implications.* Trends Mol Med, 2014. **20**(8): p. 419-27.

15. de Cabo, R., et al., *The search for antiaging interventions: from elixirs to fasting regimens.* Cell, 2014. **157**(7): p. 1515-26.

16. Arriola Apelo, S.I. and D.W. Lamming, *Rapamycin: An InhibiTOR of Aging Emerges From the Soil of Easter Island.* J Gerontol A Biol Sci Med Sci, 2016. **71**(7): p. 841-9.

17. Li, J., S.G. Kim, and J. Blenis, *Rapamycin: one drug, many effects.* Cell Metab, 2014. **19**(3): p. 373-9.

18. Choi, K.M., et al., *Enhancement of mitochondrial function correlates with the extension of lifespan by caloric restriction and caloric restriction mimetics in yeast.* Biochem Biophys Res Commun, 2013. **441**(1): p. 236-42.

19. Bjedov, I., et al., *Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster.* Cell Metab, 2010. **11**(1): p. 35-46.

20. Harrison, D.E., et al., *Rapamycin fed late in life extends lifespan in genetically heterogeneous mice.* Nature, 2009. **460**(7253): p. 392-5.

21. Howitz, K.T., et al., *Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan.* Nature, 2003. **425**(6954): p. 191-6.

22. Kennedy, B.K., et al., *Mutation in the silencing gene SIR4 can delay aging in S. cerevisiae.* Cell, 1995. **80**(3): p. 485-96.

23. Wood, J.G., et al., *Sirtuin activators mimic caloric restriction and delay ageing in metazoans.* Nature, 2004. **430**(7000): p. 686-9.

24. Baur, J.A., et al., *Resveratrol improves health and survival of mice on a high-calorie diet.* Nature, 2006. **444**(7117): p. 337-42.

25. Kauppila, T.E.S., J.H.K. Kauppila, and N.G. Larsson, *Mammalian Mitochondria and Aging: An Update.* Cell Metab, 2017. **25**(1): p. 57-71.

26. Sun, N., R.J. Youle, and T. Finkel, *The Mitochondrial Basis of Aging.* Mol Cell, 2016. **61**(5): p. 654-666.

27. Barros, M.H., et al., *Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in Saccharomyces cerevisiae.* J Biol Chem, 2004. **279**(48): p. 49883-8.

28. Caldeira da Silva, C.C., et al., *Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity.* Aging Cell, 2008. **7**(4): p. 552-60.

29. Kazi, R.S., et al., *Glycation inhibitors extend yeast chronological lifespan by reducing advanced glycation end products and by back regulation of proteins involved in mitochondrial respiration.* J Proteomics, 2017. **156**: p. 104-112.

30. Martin-Montalvo, A., et al., *Metformin improves healthspan and lifespan in mice.* Nat Commun, 2013. **4**: p. 2192.

31. Barzilai, N., et al., *Metformin as a Tool to Target Aging.* Cell Metab, 2016. **23**(6): p. 1060-1065.

32. Rena, G., D.G. Hardie, and E.R. Pearson, *The mechanisms of action of metformin.* Diabetologia, 2017. **60**(9): p. 1577-1585.

33. Eisenberg, T., et al., *Induction of autophagy by spermidine promotes longevity.* Nat Cell Biol, 2009. **11**(11): p. 1305-14.

34. Luria, S.E. and M. Delbruck, *Mutations of Bacteria from Virus Sensitivity to Virus Resistance.* Genetics, 1943. **28**(6): p. 491-511.

35. Hoffmann, W., *CAN1-SUC2 gene fusion studies in Saccharomyces cerevisiae.* Mol Gen Genet, 1987. **210**(2): p. 277-81.

36. Unk, I. and A. Daraba, *Measuring UV-induced Mutagenesis at the CAN1 Locus in Saccharomyces cerevisiae.* Bio Protoc, 2014. **4**(20).

37. Rosenthal, G.A., *The biological effects and mode of action of L-canavanine, a structural analogue of L-arginine.* Q Rev Biol, 1977. **52**(2): p. 155-78.

38. Singh, A. and Y.J. Xu, *The Cell Killing Mechanisms of Hydroxyurea.* Genes (Basel), 2016. **7**(11).

39. Sampaio-Marques, B., W.C. Burhans, and P. Ludovico, *Longevity pathways and maintenance of the proteome: the role of autophagy and mitophagy during yeast ageing.* Microb Cell, 2014. **1**(4): p. 118-127.

40. Eisenberg, T., et al., *Cardioprotection and lifespan extension by the natural polyamine spermidine.* Nat Med, 2016. **22**(12): p. 1428-1438.

41. Madeo, F., et al., *Spermidine: a physiological autophagy inducer acting as an anti-aging vitamin in humans?* Autophagy, 2019. **15**(1): p. 165-168.

42. Sega, G.A., *A review of the genetic effects of ethyl methanesulfonate.* Mutat Res, 1984. **134**(2-3): p. 113-42.

43. Wang, Y., et al., *Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure.* Cancer Res, 2001. **61**(14): p. 5370-3.

44. Vujcic, S., et al., *Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin.* Biochem J, 2002. **367**(Pt 3): p. 665-75.

45. Longo, V.D., et al., *Replicative and chronological aging in Saccharomyces cerevisiae.* Cell Metab, 2012. **16**(1): p. 18-31.

46. Watts, M.E., R. Pocock, and C. Claudianos, *Brain Energy and Oxygen Metabolism: Emerging Role in Normal Function and Disease.* Front Mol Neurosci, 2018. **11**: p. 216.

47. Santos, J.L., et al., *Mutagenic and genotoxic effect of hydroxyurea.* Int J Biomed Sci, 2011. **7**(4): p. 263-7.

48. Flanagan, J.M., et al., *Assessment of genotoxicity associated with hydroxyurea therapy in children with sickle cell anemia.* Mutat Res, 2010. **698**(1-2): p. 38-42.

49. Sakano, K., et al., *Hydroxyurea induces site-specific DNA damage via formation of hydrogen peroxide and nitric oxide.* Jpn J Cancer Res, 2001. **92**(11): p. 1166-74.

FIGURE CAPTIONS

Fig 1. The effect of lifespan-extending chemicals on **(A)** *CAN1* mutation rates, **(B)** *CAN1* EMS-induced-mutation frequencies, and **(C)** doubling times. CON, untreated control; DNP, dinitrophenol; MET, metformin; RAP, rapamycin; RES, resveratrol; SPD, spermidine. Values are shown as means + SD (n = 10 for (A), except for MET where n = 8; n = 10 for (B); N = 7 for (C). *P*-values were calculated by the Dunn's multiple comparison test compared to the control. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

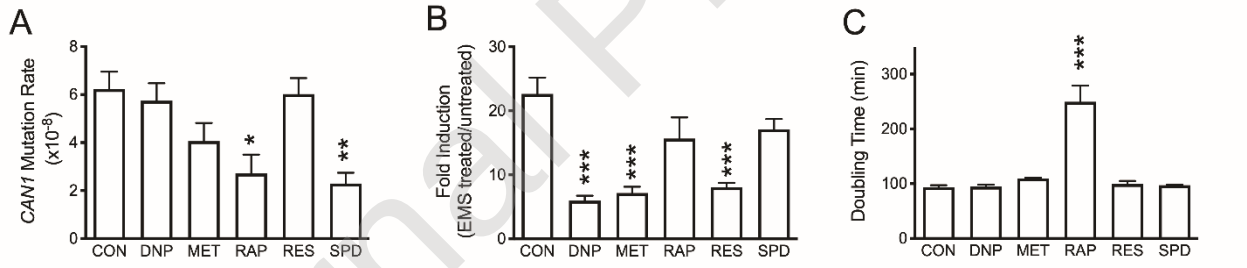

Fig 2. The ability of lifespan-extending chemicals to protect against **(B)** ethyl methansulfonate, **(C)** hydrogen peroxide, and **(D)** ultraviolet light. Unchallenged control or drug supplied yeast spotting is shown in **(A)**. CON, untreated control;

DNP, dinitrophenol; MET, metformin; RAP, rapamycin; RES, resveratrol; SPD, spermidine. Cell numbers spotted are indicated at the top of each panel.

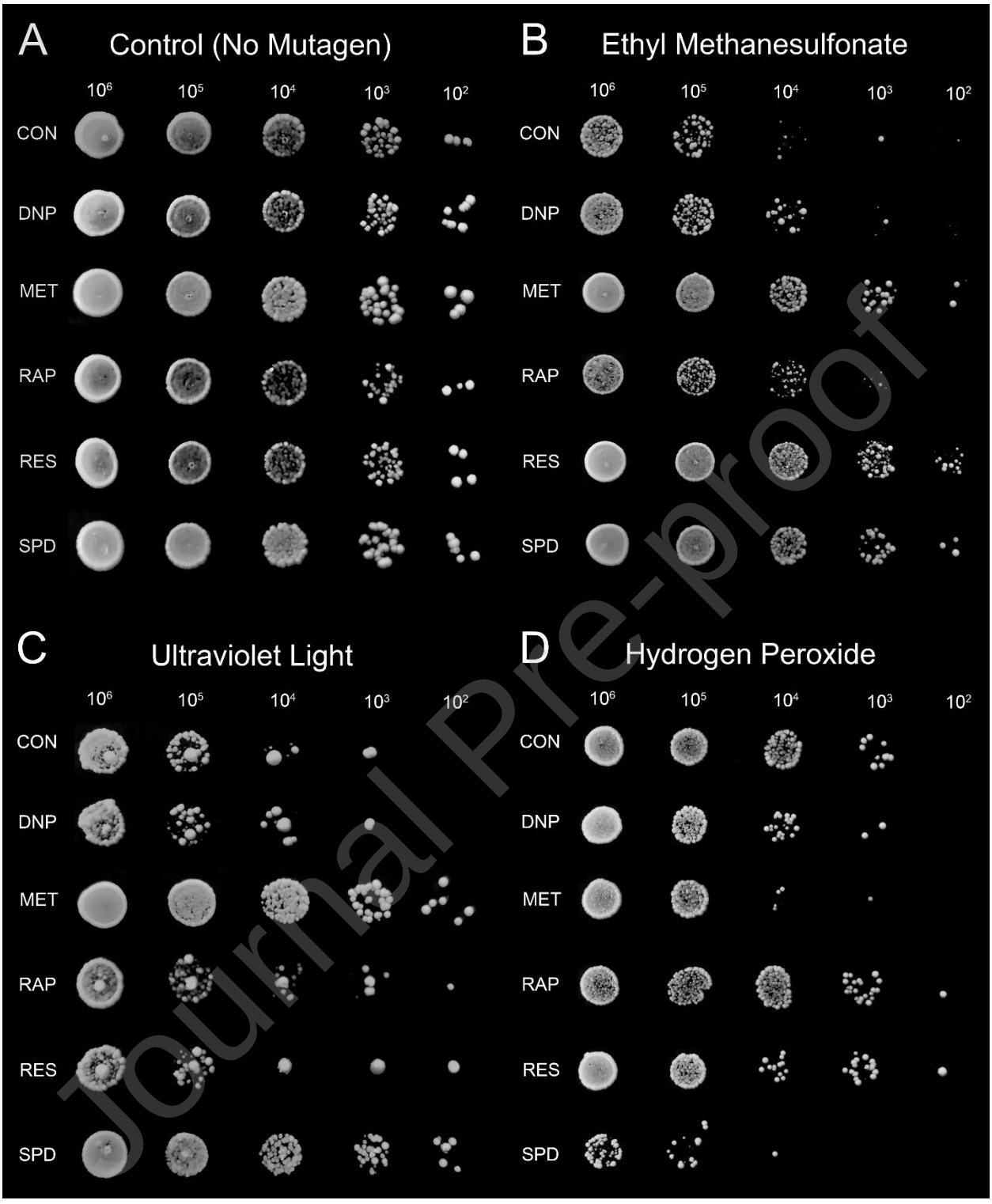

Fig 3. Hydroxyurea significantly decreases survival relative to the untreated control **(A)**. The effect on survival of the lifespan-extending chemicals in the presence of hydroxyurea relative to hydroxyurea alone is shown in **(B)**. Maximum lifespans are shown in **(C)**. The effect of each chemical on cell growth rates in the presence of hydroxyurea is shown in **(D)**, where rapamycin completely arrested cell growth. HU, hydroxyurea-treated control; the following are also treated with hydroxyurea: DNP, dinitrophenol; MET, metformin; RAP, rapamycin; RES, resveratrol; SPD, spermidine. *P*-values were calculated by either (A) the Mann-Whitney test (n = 9), or (B, C, and D) the Dunn's multiple comparison test compared to hydroxyurea treated control (n = 6). $*P < 0.05$, $**P < 0.01$.

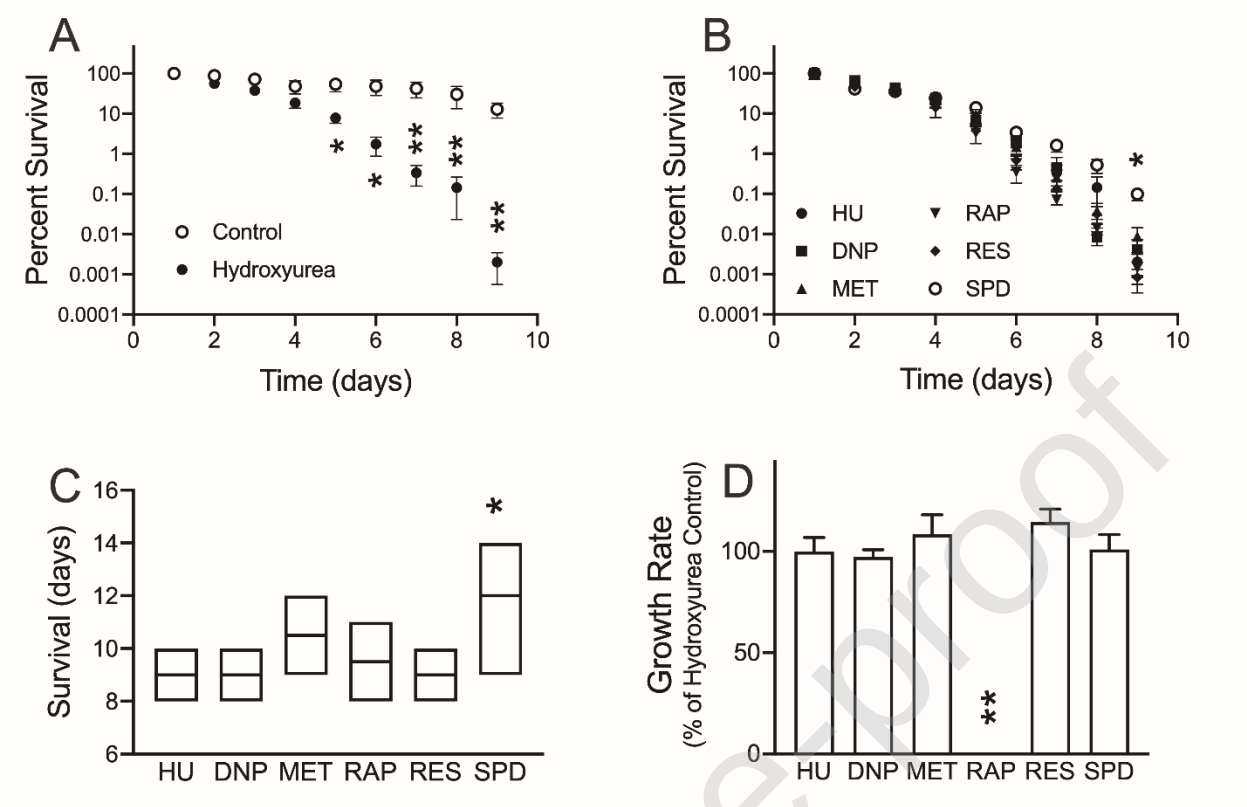

Fig. S1. Hydroxyurea increases yeast doubling time. CON, untreated control; HU, hydroxyurea (HU) control; HU + DNP, dinitrophenol + HU; HU + MET, metformin + HU; HU + RES, resveratrol + HU; HU + SPD, spermidine + HU. *P*-values were calculated by Holm-Sidak' s multiple comparisons test vs. the untreated control; ****$P < 0.0001$.