Effects of a growth hormone-releasing hormone antagonist on telomerase activity, oxidative stress, longevity, and aging in mice

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Both deficiency and excess of growth hormone (GH) are associated with increased mortality and morbidity. GH replacement in otherwise healthy subjects leads to complications, whereas individuals with isolated GH deficiency such as Laron dwarfs show increased life span. Here, we determined the effects of treatment with the GH-releasing hormone (GHRH) receptor antagonist MZ-5-156 on aging in SAMP8 mice, a strain that develops with aging cognitive deficits and has a shortened life expectancy. Starting at age 10 mo, mice received daily s.c. injections of 10 μg/mouse of MZ-5-156. Mice treated for 4 mo with MZ-5-156 showed increased telomerase activity, improvement in some measures of oxidative stress in brain, and improved pole balance, but no change in muscle strength. MZ-5-156 improved cognition after 2 mo and 4 mo, but not after 7 mo of treatment (ages 12, 14 mo, and 17 mo, respectively). Mean life expectancy increased by 8 wk with no increase in maximal life span, and tumor incidence decreased from 10 to 1.7%. These results show that treatment with a GHRH antagonist has positive effects on some aspects of aging, including an increase in telomerase activity.

Alzheimer's disease | learning | memory | peptide | sarcopenia

The aging process is associated with decreased physical and mental functioning, increased morbidity, and inevitable mortality. Specific neurodegenerative diseases such as Alzheimer's disease lead to decreased learning and memory. The incidence of many benign and malignant tumors clearly increases with aging. Populations show significant variations in the rate at which aging advances and many mechanisms have been proposed to explain aging or were found to be associated with the rate of aging. Oxidative stress, telomerase activity, and variation in hormonal levels are examples of these associations and mechanisms.

Among hormones that are implicated in aging is growth hormone (GH). GH levels decrease dramatically with aging (1) and some of the findings in aging, such as thinning of the skin and bones, are the opposite of those found in acromegaly, a condition in which GH is overproduced (2). This has led to the proposal that replacement of GH will reverse or slow the aging process. However, several clinical trials showed that replacement of GH is associated with increased mortality (3, 4). Acromegaly is also linked to many adverse conditions that are associated with aging, including glucose intolerance, heart disease, and an increased cancer incidence (5). Transgenic mice overexpressing GH have a decreased life span and evidence of accelerated aging in several organs and tissues, including the brain (6).

Whereas GH replacement and acromegaly are associated with adverse outcomes, GH deficiency or resistance to GH are associated with longer life expectancy. Laron dwarfs show resistance to GH at the receptor level and so do not secrete insulin-like growth factor I (IGF-I) and can live to an advanced age (2). Mouse strains with a deficiency in GH activity because of transcription factor mutations (the Ames mouse and the Snell mouse), resistance at the GH receptor (Laron mouse), or heterozygotes for an IGF-I receptor gene deletion (7–9), all live longer than their wild-type counterparts (10, 11).

Given the hypothesis that excess levels of GH can accelerate aging (2, 6, 7), we tested an antagonist of GH-releasing hormone (GHRH) MZ-5-156 for effects on life span, telomerase activity, tumor incidence, muscle strength, oxidative stress in brain, body weights, serum glucose, serum triglycerides, and cognition in aged SAMP8 mice. MZ-5-156 inhibits growth of various cancers (12) and, like other GHRH antagonists, has antioxidant effects (13). The SAMP8 mouse strain has a natural mutation that results in increases in amyloid precursor protein (APP) and amyloid beta peptide (AβP) with aging (14). The SAMP8 mouse also develops age-related cognitive impairments, which are severe by age 12 mo, increased oxidative stress levels in brain, and decreased brain acetylcholine levels, all of which are reversed by treating with antibodies directed against AβP or antisense directed against APP. The SAMP8, therefore, is used to model Alzheimer's disease. The SAMP8 has a median life expectancy of 16–18 mo, but remains healthy when compared with age-matched CD-1 mice until the last months of life.

Results

Survival. In all studies, treatment with the GHRH antagonist MZ-5-156 was begun with 10-mo-old SAMP8 mice. Treatment with MZ-5-156 compared with no treatment increased median survival from 26 wk to 34 wk and mean survival from 30.6 ± 2.6 to 34.2 ± 2.7 wk. The curves for survival proportions (Fig. 1*A*) were not statistically different when compared by either the Mantel-Cox test or the Gehan-Breslow-Wilcoxon test. However, the assumption of proportional hazards was violated after 55 wk. When linear regression was performed on the first 55 wk of data, regressing the number of surviving mice against weeks of treatment, the slopes of the lines were statistically different: $F(1,24) = 10.4$, $P < 0.005$ (Fig. 1*B*). Areas under the curve (AUCs) were computed for all weeks. For MZ-5-156–treated mice, the AUC was 1,612 mouse-weeks and for controls 1,426 mouse-weeks, a 13% increase in AUC with MZ-5-156.

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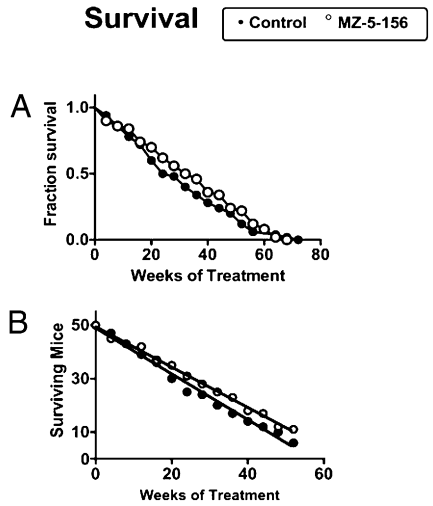

Fig. 1. Effects of MZ-5-156 on life expectancy. (*A*) Effects on fraction survival for all mice. (*B*) Surviving mice before change in proportional hazards at about day 55.

Tumor Incidence. A total of 60 control and 60 MZ-5-156–treated mice were assessed for tumors. Occurrence of tumors was 1/60 in the MZ-5-156 group, representing an incidence of 1.7%, and 6/60 in the control groups, an incidence of 10%. Two of the control group tumors were in mice killed at 14 mo for telomerase assay. Of the other four tumors found in control mice, two were in mice that died at the ages of 13–14 mo, one in a mouse that died at 16 mo, and one in a mouse that died at 20 mo. The single tumor found in the MZ-5-156–treated group was in a mouse that died at 18 mo. Use of the one-tail test is justified as the literature clearly indicates that tumor incidence should decrease with antagonism of GH. We found a statistically significant reduction by the one-tailed χ^2 test ($\chi^2 = 3.79$, $P < 0.05$).

Cognitive Testing. Cognitive assessments were conducted on randomly selected mice at ages 12 mo (active avoidance T-maze, lever press), 14 mo (step-down passive avoidance and object recognition), and 17 mo (object recognition). Active avoidance T-maze performed at 12 mo showed a statistically significant effect on acquisition ($t = 2.22$, $n = 12$ controls, $n = 17$ MZ-5-156, df = 27, $P < 0.05$), but not on retention (Fig. 2*A*). A two-way analysis of variance (ANOVA) for lever press at age 12 mo (Fig. 2*B*) showed a significant effect for treatment [$F(1,54) = 9.07$, $P < 0.01$] and day of testing [$F(2,54) = 11.9$, $P < 0.001$]. The Bonferroni post-test showed a difference between control and MZ-5-156–treated mice on day 3 ($P < 0.05$). In the step-down passive avoidance performed on 14-mo-old mice, a statistically significant improvement was found in mice treated with MZ-5-156: $t = 2.74$, $n = 11$/group, $P < 0.05$ (Fig. 2*C*). In the object recognition test, MZ-5-156 significantly improved memory at 14 mo ($t = 5.61$, $n = 13$, df = 24, $P < 0.001$), but not at 17 mo of age (Fig. 2*D*).

Strength Testing, Feeding, and Body Weights. At 10 mo of age, 20 mice were randomly chosen from the treatment and control groups ($n = 10$/group) after assignment to these groups but before treatment had begun. There was no difference in body weight between the control (30.4 ± 0.8) and treatment-assigned (30.3 ± 0.6) groups. Similarly, body weights did not differ between control vs. treatment at 12 mo (control, 29.8 ± 0.7; MZ-5-156, 31.1 ± 0.6), 14 mo (control, 30.0 ± 0.7; MZ-5-156, 31.0 ± 0.6), or 16 mo (control, 30.6 ± 0.6; MZ-5-156, 30.5 ± 0.6) of age. Two-way ANOVA of food intake over the first 4 mo of treatment (ages 10–14 mo) showed an increase with time but no effect of treatment nor interactions between time and treatment.

Significant effects were found on some measures of strength, but not others (Table 1). At 14 mo of age, MZ-5-156 significantly improved pole balance ($t = 2.19$, $n = 10$/group, df = 18, $P < 0.05$), but had no effect on cage hang, string hang, or strength meter. At 16 mo, MZ-5-156 significantly improved pole balance ($t = 3.33$, $n = 10$/group, df = 18, $P < 0.005$) and string hang values were decreased ($t = 2.47$, $n = 10$/group, df = 18, $P < 0.05$. Cage hang and strength meter were unaffected. There were no differences in body weights either with treatment or between 14 and 16 mo of age in the 10 mice randomly chosen for strength testing (Table 1).

Oxidative Stress and Organ Weights. Oxidative stress was measured in brain samples at 14 mo of age (4 mo of treatment). Glutathione levels (GSH) were increased (Fig. 3*A*, $t = 2.05$, df = 18, $P < 0.05$) whereas levels of glutathione peroxidase (GPx) were reduced (Fig.

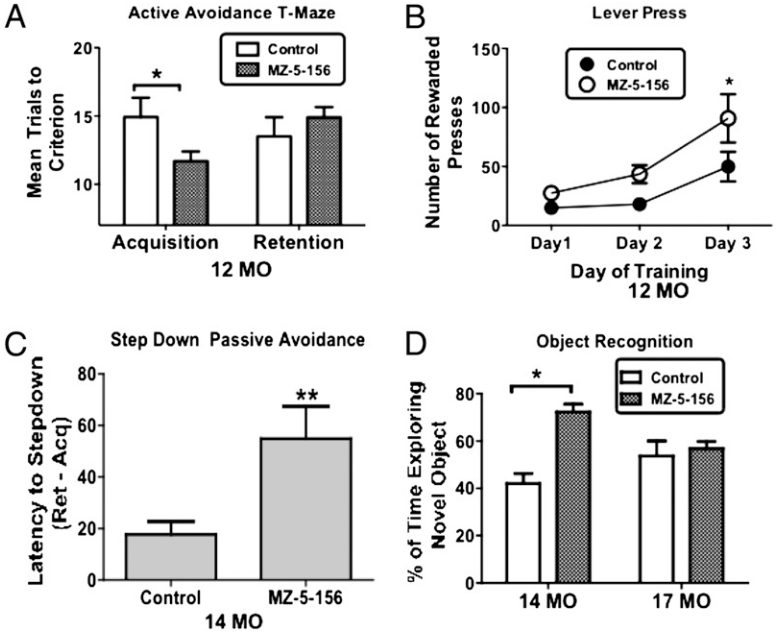

Fig. 2. Effects of MZ-5-156 on cognition. (*A*) Improvement in acquisition (learning) but not retention (memory) at age 12 mo after 2 mo of treatment. (*B*) Improvement in learning as measured by lever press in 12-mo-old mice after 2 mo of treatment. (*C*) Improvement in memory in the step-down passive avoidance in 14-mo-old mice after 4 mo of treatment. (*D*) Improvement in memory as measured by object recognition in 14-mo-old mice (4 mo of treatment) but not at 17 mo (7 mo of treatment).

Table 1. Strength testing and body weight values

	Control 14 mo	MZ 14 mo	Control 16 mo	MZ 16 mo
Cage hang (s)	32.83 ± 4.79	30.83 ± 4.89	11.73 ± 2.26	13.30 ± 2.26
String hang (s)	30.70 ± 6.71	31.03 ± 4.26	22.80 ± 7.44	16.63 ± 2.64*
Pole balance (s)	113.9 ± 18.6	131.7 ± 19.2*	83.63 ± 22.83	120.0 ± 26.0**
Strength meter (kgF)	0.53 ± 0.15	0.62 ± 0.18	0.46 ± 0.02	0.52 ± 0.04
Body weight (g)	29.4 ± 0.8	31.1 ± 0.8	31.8 ± 0.8	31.6 ± 0.9

Means are presented with their SE terms. MZ indicates mice treated with MZ-5-156. $N = 10$ for all groups. *$P < 0.05$ from corresponding control age group; **$P < 0.005$ from corresponding control age group.

3*B*, $t = 2.30$, df = 17, $P < 0.05$) in mice treated with MZ-5-156. In comparison, neither malondialdehyde (MDA) (Fig. 3*C*) nor glutathione reductase (GR) (Fig. 3*D*) was altered. There were no statistical differences in the weights of heart (control, 0.211 ± 0.13 g; MZ-5-156, 0.203 ± 0.007 g) or liver (control, 1.82 ± 0.14 g; MZ-5-156, 1.91 ± 0.10 g) in the 14-mo-old mice.

Blood Chemistries. IGF-I measured 2 h after single s.c. injection of 10 μg/mouse of MZ-5-156 showed a significant decrease of about 12% from 332 ng/mL to 293 ng/mL ($t = 2.31$, df = 23, $P < 0.05$); Table 2. In blood taken 24 h after the last injection from nonfasted mice treated for 4 mo with MZ-5-156, there was an arithmetic decrease in IGF-I of 14% and a nonstatistically significant decrease in GH of about 37%. Glucose and triglycerides were unchanged in mice treated for 4 mo.

Telomerase Activity. A highly significant difference in telomerase activity was detected between the threshold cycle (Ct) measurements (Ct ± SE) of SAMP8 mice treated with GHRH antagonist MZ-5-156 and controls. Values for Ct are inversely related to telomerase gene activity, so that a decrease in Ct corresponds to an increase in telomerase activity (7, 15, 16). The hearts of MZ-5-156–treated mice showed a statistically significant decrease in Ct activity (MZ-5-156, 21.1 ± 0.7; control, 23.3 ± 0.2, $P < 0.005$), corresponding to a 4.7-fold increase in telomerase activity. MZ-5-156 treatment resulted in a 3.9-fold increase in telomerase activity for aorta (Ct values, 20.2 ± 0.7 for MZ-5-156 and 22.2 ± 0.2 for control, $P < 0.01$), a 5.13-fold increase in telomerase activity for liver (Ct values, 20.1 ± 0.5 for MZ-5-156 and 22.4 ± 0.3 for control, $P < 0.05$), and a 2.8-fold increase for stomach (Ct values, 20.2 ± 0.6 for MZ-5-156 and 21.74 ± 0.18 for control, $P < 0.05$). Treatment with MZ-5-156 produced no statistically significant difference for ileum; there was an arithmetic increase of 2.87-fold.

Discussion

We examined the effects of the GHRH antagonist MZ-5-156 on various aspects of aging, including oxidative stress, learning, memory, tumor incidence, metabolic parameters, life expectancy, and telomerase activity. Most indices were affected, including some measures of oxidative stress, telomerase activity, median life expectancy, tumor incidence, and learning and memory. Other parameters were not influenced to a statistically significant degree. These results not only indicate that GHRH antagonists can have positive effects on the aging process, but also reveal some of the mechanisms involved. Here we consider all of the indices, the effects of MZ-5-156 on each, and the implications of these on the mechanisms of aging.

MZ-5-156 had demonstrable effects on survival. Median survival increased from 26 wk in controls to 34 wk in mice treated with MZ-5-156. However, analysis with the Mantel-Cox test or with the Gehan-Breslow-Wilcoxon test did not show a statistically significant change in survival curves. The assumptions of these statistical tests, however, were violated in that proportional hazards ratios were not constant as indicated by the two survival curves' crossing at week 60 (Fig. 1*A*). This change in hazards ratios occurred primarily because of an extended life span of a subgroup of controls and that began at about week 56 (Fig. 1*A*). Linear regression between number of surviving mice vs. weeks of treatment up to week 56 showed a statistically significant difference between the slopes of the resulting lines. The AUCs calculated for all of the data including the period after the change in hazards ratios showed a 13% increase with MZ-5-156 treatment.

GHRH antagonists have potent anticancer properties (12). MZ-5-156 and other GHRH antagonists have inhibitory effects against several human cancers, including prostate, breast, brain, lung, colorectal, and gastric cancers (12). GHRH antagonists exert these effects not only indirectly, by inhibiting the release of pituitary GH and subsequent release of hepatic IGF-I, but also directly by blocking the autocrine/paracrine release and action of

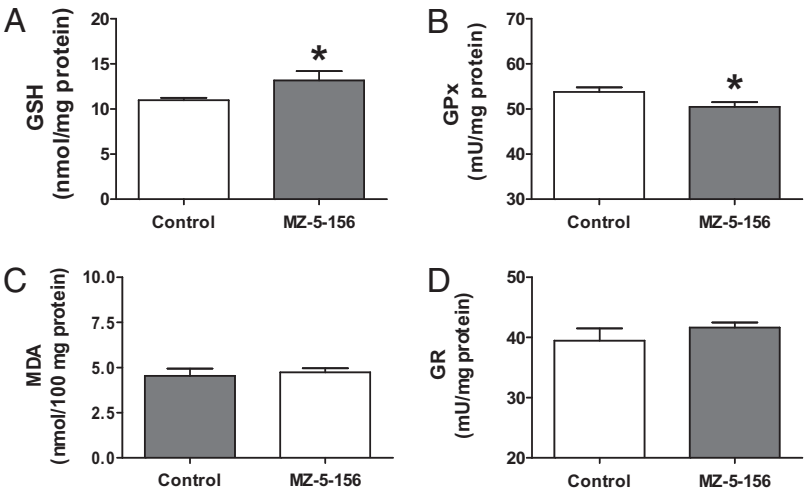

Fig. 3. Effects of MZ-5-156 on measures of brain oxidative stress. Increased GSH (*A*) and decreased GPx (*B*) indicate decreased oxidative stress. MDA (*C*) and GR (*D*) were unchanged.

Table 2. Levels of glucose, triglycerides, GH, and IGF-I in blood of mice

	Control 14 mo (*n*)	MZ 14 mo (*n*)	% change
Glucose (24 h) mg/dL	134 ± 11 (10)	140 ± 6 (10)	4
Triglycerides (24 h) mg/dL	167 ± 18 (10)	183 ± 20 (10)	10
Growth hormone (24 h) ng/mL	19 ± 6 (10)	12 ± 4 (10)	(–)37
IGF-I (24 h) ng/mL	86 ± 36 (10)	74 ± 25 (10)	(–)14
IGF-I (2 h) ng/mL	332 ± 13 (12)	293 ± 11* (13)	(–)12

Means ± SE. MZ indicates mice treated with MZ-5-156. *$P < 0.05$ from corresponding control age group. The 24-h measures were taken 24 h after an injection in 14-mo-old SAMP8 mice that had been treated for 4 mo. The 2-h value was taken 2 h after a single injection in 12-mo-old SAMP8 mice.

GHRH, IGF-I, and IGF-II by the tumors themselves (12). Because the effect of GHRH on tumor incidence was predicted a priori to decrease, we performed a one-tailed comparison using the χ^2 test. Here, the reduction in tumors from 6/60 to 1/60 was statistically significant.

Effects of MZ-5-156 on blood chemistries were measurable, but not dramatic. MZ-5-156 has inhibitory effects on both GH and IGF-I secretions (17, 18). GH elevates both triglycerides and glucose, but patients with GH deficiency can also have metabolic abnormalities. Here, there were no statistically significant changes in glucose or cholesterol in 14-mo-old mice treated with MZ-5-156 for 4 mo. There was no statistically significant change in GH or IGF-I levels 24 h after treatment discontinuation, although there were arithmetic decreases of about 37% in GH and 14% in IGF-I. GH and IGF levels were taken 24 h after the last injection of MZ-5-156 and had coefficients of variation over 100%. We therefore tested the effect of MZ-5-156 on IGF-I levels 2 h after a single injection and found a statistically significant decrease of 12%. We conclude that the long-term effects of MZ-156 on GH and IGF-I were modest and that its effects on aging were unlikely mediated through parameters of glucose or triglyceride metabolism.

Positive effects on cognition were seen with MZ-5-156 at 12 mo and 14 mo of age. At 12 mo of age, MZ-5-156 improved acquisition (learning) but not retention (memory). The lever press, a test of learning, found significant improvement at 12 mo of age. At 14 mo of age, memory was assessed in two tests, the step-down passive avoidance and the novel object recognition. As performed here with a 24-h delay between training and testing (19, 20), the novel object recognition tests hippocampal-dependent memory. Both memory tests showed significant improvement in mice treated with MZ-5-156. However, by 17 mo of age, the effect of MZ-5-156 on object recognition was lost. These results show that MZ-5-156 had a short-term, positive effect on memory, but with advancing age, this effect was lost.

One mechanism by which MZ-5-156 could have exerted its positive effects on cognition is through antioxidant actions. GHRH antagonists have antioxidant effects (12, 13). The brains of aged SAMP8 mice have significant elevations in measures of oxidative stress, which are attributed to the increased levels of amyloid beta protein (21–23). Reduction of oxidative stress by decreasing amyloid beta protein or the use of antioxidants reverses cognitive impairments in the aged SAMP8 mouse (23, 24). We examined levels of GSH, GPx, MDA, and GR levels in the brains of 14-mo-old mice after 4 mo of treatment. We found increases in levels of GSH, consistent with a reduction in oxidative stress. With increased oxidative stress, GSH is converted to its oxidative form, GSSG. This oxidized form is produced by reduction of an organic peroxide by cellular GPx and is recycled to its reduced state by the enzyme GR. Reduction in GPx is therefore indicative of a lower state of oxidative stress. GR levels, however, did not change, nor did MDA levels, a measure of lipid peroxidation. Thus, MZ-5-156 had measurable, positive effects on oxidative stress.

The effects of MZ-5-156 on muscle strength were minimal (Table 1) and body weight was not affected at 12 mo, 14 mo, or 16 mo of age, nor was food intake altered. Decreased muscle strength in control animals between the ages of 14 mo and 16 mo was obvious in the various measures of strength assessment; MZ-5-156 did not ameliorate these. MZ-5-156 did improve pole balance, but that could reflect improvements in coordination rather than strength. Despite the loss in muscle strength, SAMP8 mice did not show any decreases in body weight in this 2-mo period, so it was not possible to determine whether MZ-5-156 had an effect on the anorexia associated with aging (25, 26).

The DNA repair enzyme, telomerase, maintains eukaryotic chromosome stability by ensuring that telomeres regenerate each time the cell divides, protecting chromosome ends and helping to preserve genome integrity and prevent senescence (27). Telomeres protect chromosome ends from damage because damaged chromosomes, lacking telomeres, undergo fusion, rearrangement, and translocation (28), which potentially lead to adverse recombination events. Telomeres may also act as "mitotic clocks," reflecting numbers of past and potential cell divisions (29). Quantitative real-time PCR based on the SYBR green high-sensitivity telomeric repeat amplification protocol (TRAP) was used for rapidly quantifying telomerase activity. Decreases in Ct values correspond to an increase in telomerase activity (15, 16). Results are presented as Ct to allow for comparability with other studies on telomerase activity (15, 16). Ct values are determined from semilog amplification plots (log increase in fluorescence vs. cycle number) compared with standard curves generated from serial dilutions of telomerase TSR8 control template (30). Our quantitative RT-PCR results showed a statistically significant increase in telomerase activity in heart, aorta, liver, and stomach of mice treated with GHRH antagonist MZ-5-156. There may be a causal relation between activity of telomerase and telomere-length stabilization and the extension of the life span of the SAMP8 mice treated with GHRH antagonist MZ-5-156. Previously, it was shown in cancer cells that MZ-5-156 decreased the expression of the hTRT gene encoding for the telomerase catalytic subunit (31). However, it did not affect the mRNA levels for hTR, which encodes for telomerase RNA or for TP1, which encodes for telomerase-associated protein in cancer cells (31). Thus, we saw the opposite effect in normal tissues to that previously reported in cancer cells.

In conclusion, MZ-5-156 had positive effects on aspects of aging, including reducing oxidative stress in brain, improving cognition, increasing mean life span, decreasing tumor incidence, and increasing telomerase activity. Other aspects of aging were not affected, including maximal life span and effects on glucose and triglyceride levels. MZ-5-156 had minimal effects on measures of muscle strength, but improved balance. Collectively, our results are consistent with the hypothesis that antagonists of growth hormone-releasing hormone have beneficial effects on aging.

Materials and Methods

Treatment of Mice and Schedule of Tests. MZ-5-156 was dissolved in 0.1% DMSO and then 10% propylene glycol in sterile water. Male, 10-mo-old SAMP8 mice were randomized to treatment and control groups (n = 60/group) and given daily s.c. injections between the scapulae of 0.1 mL of vehicle (0.1% DMSO + 10% propylene in sterile water) with or without MZ-5-156 (10 μg/mouse). Upon death of the mouse, a postmortem for tumor assessment was performed by an investigator blinded to treatment. Blood

from the retrobulbar plexus was collected under isoflurane anesthesia before and after 2 mo of treatment (age 12 mo) from 10 mice randomly selected from each group. After 4 mo of treatment (age 14 mo), 10 mice from each group were randomly selected, the brains harvested for measurement of oxidative stress, the carcass assessed for the presence of tumors, and liver and heart weighed. The heart, liver, stomach, ileum, and aorta were harvested for measurement of telomerase activity; blood was stored for measures of glucose, triglycerides, GH, and IGF-I. Cognitive assessments were conducted on randomly selected mice (see *Results* for *n*) at ages 12 mo (active avoidance T-maze, lever press), 14 mo (step-down passive avoidance and object recognition), and 17 mo (object recognition). Strength assessments (cage hang, string hang, pole balance, and strength meter measurements) were performed and body weights recorded at ages 14 and 16 mo.

Cognitive Testing. ***Active avoidance T-maze.*** Acquisition and retention were assessed at age 12 mo after 2 mo of treatment. The T-maze is a hippocampal-dependent reference learning task in which the animal integrates multiple cues in a novel environment to learn a new task (32). The T-maze consists of a black plastic alley with a start box at one end, two goal boxes at the other, and a guillotine door separating them. An electrifiable stainless steel rod floor runs throughout the maze to deliver a scrambled foot shock. A block of training trials begins when a naïve mouse is placed in the start box, the guillotine door is raised, a buzzer sounded (55 dB), and a foot shock applied 5 s later. The goal box entered on the first trial was designated "incorrect" and the foot shock was continued until the mouse entered the other goal box, which, in all subsequent trials, was designated as "correct" for that particular mouse. At the end of each trial, the mouse was returned to its home cage until the next trial. The intertrial interval was 30 s with a foot shock intensity of 0.35 mA. Mice were trained until they made one avoidance. The number of trials to make one avoidance was the measure of acquisition. Retention was tested 1 wk later by continuing training until the mice achieved the criterion of making five avoidances in six consecutive trials. The number of trials needed to reach this criterion was the measure of retention.

Lever press. Cognition was assessed at age 12 mo after 2 mo of treatment. The lever press is an appetitive bar-pressing task that challenges response memory. In this task, mice learn to press a lever to receive a reward. Mice were first habituated to the milk (one part evaporated milk and two parts water) by giving them access to it in their home cages for three consecutive nights during which food and water were removed and returned the next morning. To measure learning, mice were placed into a fully automated chamber. Pressing a lever on one wall of the chamber caused a light and dipper containing 100 μL of milk to rise into a reward compartment located on the wall opposite the lever. The reward compartment was 4 cm high and 3.1 cm across with a depth of 3.7 cm. Photo sensors are located in the reward compartment to determine whether the mouse claimed the reward. On day 1, mice had 11 s to run to the reward compartment to claim the reward, after which access was denied. The reward had to be claimed to count as a rewarded lever press. On days 2 and 3, a shorter time period of 6 s was used to avoid a possible ceiling effect in the number of lever presses made. Mice were given one 40-min training session on each of 3 d (Monday, Wednesday, and Friday) with data automatically recorded by computer. The measure of acquisition was the number of rewarded lever presses.

Step-down passive avoidance. Memory was assessed in this test at age 14 mo after 4 mo of treatment. Mice were placed in a box (55 × 55 × 20 cm) with a stainless steel rod floor. A square platform (7.5 × 7.5 × 2.5 cm) was in the center of the box. Mice were placed on the platform and their latency to step down, defined as placing all four paws on the floor, was measured. On stepping down, mice received a 0.28-mA foot shock for 2 s. One trial was given during training. Retention was tested 24 h after training by placing the mouse on the platform and measuring latency to step down onto the grid. No foot shock was given when they stepped off. Retention test scores were expressed as test minus training step-down latency (ceiling 180 s).

Novel object recognition. Memory was assessed at ages 14 mo and 17 mo after 4 and 7 mo of treatment as previously described (33). Mice were habituated to the apparatus, a 58 × 66 × 11-cm white plastic box, for three consecutive days before testing. Each mouse was allowed to freely explore the testing box for 5 min. On the first day of training, mice were placed in the testing apparatus with two identical objects (A and B), both with dimensions of 7 × 6.3 × 5.1 cm. On the second day, one of the original objects was removed and a new object (C) with dimensions of 8.2 × 3.8 × 7.4 cm added. Mice were placed in the testing apparatus for 5 min and the time each mouse spent sniffing or touching the new object was recorded. Results were expressed as the percentage of time spent investigating the new object.

Strength Testing, Food Intake, and Body Weights. Body weights were measured in 20 randomly chosen mice at 10 mo of age (before treatment began) and at 12, 14, and 16 mo of age. Food intake over 24 h was monitored weekly during the first 4 mo of treatment. Strength testing was performed on 10 randomly chosen mice at ages 14 and 16 mo after 4 and 6 mo of treatment, respectively. The following tests comprise the strength testing. String hang: A mouse gripped a string suspended 12 inches above a 3/4 inch foam pad. The time that a mouse was able to hang on the string was recorded with a maximum hang time of 3 min. Cage hang: The mouse was allowed to grip the top of a wire mesh cage, which was then inverted 12 inches over a 3/4 inch foam pad. The time the mouse was able to hang was recorded, the test ending after a maximum of 3 min. Pole balance: A mouse was placed on a pole 1/4 inch in diameter 12 inches over a 3/4 inch foam and the time the mouse balanced on the pole recorded. The test was stopped after 3 min. Strength meter: Forelimb grip strength was measured by allowing the mouse to grasp a wire mesh connected to a grip strength meter (Columbus Instruments). The values were corrected by dividing the values for strength meter by body weight in kilograms.

Blood Chemistries. Whole blood was centrifuged at 4,500 *g* for 10 min at 4 °C and serum removed from blood within 30 min of collection to prevent glycolysis. Serum was immediately placed on ice to prevent hydrolysis of phospholipids and subsequent release of free glycerol, which falsely elevates triglyceride values. Glucose, triglycerides, GH, and IGF-I were measured in blood from mice killed at 14 mo of age (4 mo of treatment). IGF-I and GH were also measured 2 h after an initial dose of MZ-5-156. Triglyceride readings were determined by using a quantitative enzymatic-colorimetric assay (Pointe Scientific). Glucose readings were determined by using a quantitative enzymatic-colorimetric assay (Stanbio Glucose LiquiColor kit). Serum was assayed for GH using the Millipore mouse growth hormone ELISA kit and for IGF-I using the R&D Systems mouse IGF-I ELISA kit.

Measurements of Oxidative Stress in Brain and Organ Weights. These measurements were conducted in 14-mo-old (4 mo of treatment) mice and used an *n* = 10/group. We purchased acetonitrile, acetic acid, water, and phosphoric acid (all HPLC grade) (Fisher Scientific), *N*-(1-pyrenyl)-maleimide (NPM) (Aldrich), and all other chemicals from Sigma. The HPLC system (Thermo Electron Corporation) consists of a Finnigan TM SpectraSYSTEM SCM1000 vacuum membrane degasser, Finnigan TM SpectraSYSTEM P2000 gradient pump, Finnigan SpectraSYSTEM AS3000 autosampler, and Finnigan SpectraSYSTEM FL3000 fluorescence detector (λ_{ex} = 330 nm and λ_{em} = 376 nm). The HPLC column was a Reliasil ODS-1 C_{18} column (Column Engineering).

Glutathione (GSH) concentrations were determined by reverse phase HPLC by the method developed by Winters et al. (1). Brain tissue samples were homogenized in serine-borate buffer [100 mM Tris·HCl, 10 mM boric acid, 5 mM L-serine, 1 mM DETAPAC (diethylenetriaminepentaacetic acid), pH 7.4] on ice, then derivatized with 1.0 mM NPM [*N*-(1-pyrenyl)-maleimide] in acetonitrile. Briefly, sufficient HPLC grade water was added to each sample to make a volume of 250 μL and 750 μL NPM (1 mM in acetonitrile) was added. This mixture was incubated for 5 min at room temperature, then acidified with 2 N HCl. The derivatized samples were filtered through a 0.2-μm acrodisc filter (Advantec MFS) and injected onto a 5-μm C_{18} column (Column Engineering) in a reverse-phase HPLC system. The mobile phase was 70% acetonitrile and 30% water and was adjusted to a pH 2.5 through the addition of 1 mL/L of both acetic and *o*-phosphoric acids. The NPM derivatives were eluted from the column isocratically at a flow rate of 1 mL/min (34, 35). All sample quantitation was determined from standard curves using purified glutathione as described (34).

MDA content was determined according to the method of Draper et al. (36). Briefly, 550 μL of 5% tricholoroacetic acid (TCA) and 550 μL of 500 ppm butylated hydroxytoluene (BHT) in methanol were added to brain homogenates, the samples heated in a boiling water bath for 30 min, cooled, and centrifuged. The supernatant fractions were mixed 1:1 with saturated aqueous thiobarbituric acid (TBA) solution, boiled in a water bath for 30 min, cooled on ice, and 0.50 mL of each sample was extracted with 1 mL l-butanol and centrifuged. The organic layers were filtered through a 0.45 acrodisc and then chromatographed as described above. Concentrations of the TBA–MDA complex in samples was determined by using the calibration curve obtained from a 1,1,3,3-tetraethoxypropane standard solution.

GPx activity was determined by test kit (OxisResearch). A cell sample was added to a solution containing GSSG, GR, and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance for 3 min at 340 nm and provides a spectrophotometric means for monitoring GPx enzyme activity following addition of 350 μL of tert-butyl hydroperoxide as the working substrate.

GR activity was determined using a commercial kit (OxisResearch).

Protein content was determined by the Bradford method using concentrated Coomassie Blue (Bio-Rad) on optical density determinations at 595 nm (37). A standard curve using BSA was constructed. Homogenized brain tissues were subjected to appropriate dilutions before protein was determined.

Quantitative RT-PCR Assay for Telomerase Activity. SAMP8 mouse organs (heart, aorta, liver, stomach, and ileum) were harvested from 10 randomly selected mice from each group at age 14 mo after 4 mo of treatment. Tissues were shipped overnight on dry ice to the Miami, Florida Veterans Administration from Saint Louis University (St. Louis, MO) and stored at −80 °C until protein extraction was performed. Tissue extracts for assays of telomerase activity were prepared following the manufacturer's instructions (Millipore). Briefly, organs were homogenized on ice with a motorized pestle in sterile 1.5-mL microcentrifuge tubes with 200 μL 1× CHAPS lysis buffer (1 U/μL) SUPERase-In Rnase inhibitor (Applied Biosystems) per 100 mg of tissue. The suspension was incubated on ice for 30 min and spun in a microcentrifuge at 12,000 × *g* for 20 min at 4 °C. The supernatant was transferred (190 μL) into a fresh tube and protein concentrations were determined with a NanoDrop ND-1000 spectrophotometer.

Telomerase activity was determined using TRAPeze RT telomerase detection kit (Millipore), an optimized and previously validated SYBR Green real-time PCR method (15). Standards, inactivated samples, and no-template reactions were also assayed for quality control purposes. Serial dilution calibration curves from which experimental values were extrapolated were performed on each plate to further minimize interplate variability and optimize accuracy. All samples except ileum (duplicate) were analyzed in triplicate. A melting curve analysis was performed to verify specificity and identity of the PCR products. Real-time amplifications were performed with a Bio-Rad iCycler iQ5 multicolor real-time PCR detection system (Bio-Rad). Dilutions of TSR8 (control template) instead of the sample extract were prepared by manufacturer's instructions, to generate a standard curve. TSR8 is an oligonucleotide with a sequence identical to the TS primer extended with eight telomeric repeats AG(GGTTAG)7 (38). This standard curve permits the calculation of the amount of TS primers with telomeric repeats extended by telomerase in a given extract. The stock TSR8 control template (20 attomoles/μL) was used undiluted and in serial dilutions (1:10) with CHAPS lysis buffer to obtain concentrations of 20 attomoles/μL, 2 attomoles/μL, 0.2 attomoles/μL, 0.02 attomoles/μL and 0.002 attomoles/μL, using 2 μL of each TSR8 dilution/well. The Ct measurements for experimental samples were extrapolated from the TSR8 standard curve.

Statistics. Results are reported as means with their SE terms and their *n*. Two means were compared by Student's *t* test. More than two means were compared by ANOVA followed by Newman-Keuls multiple comparisons test. Two-way ANOVA was followed by the Bonferroni posttest. *P* values of <0.05 were taken as statistically significant. Two-tailed tests were used unless otherwise indicated. Survival was compared by log-rank (Mantel-Cox) test, Gehan-Breslow-Wilcoxon test, and with linear regression analysis. Linear regression with comparison of the slopes of the resulting curves and AUC were performed with the Prism 5.0 statistical package (GraphPad). The one-tailed χ^2 test was used to compare tumor incidence.

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