

Orally-active, clinically-translatable senolytics restore α -Klotho in mice and humans



Yi Zhu,^{a,b*} Larissa G.P. Langhi Prata,^a Erin O. Wissler Gerdes,^a Jair Machado Espindola Netto,^a Tamar Pirtskhalava,^a Nino Giorgadze,^a Utkarsh Tripathi,^a Christina L. Inman,^a Kurt O. Johnson,^a Ailing Xue,^a Allyson K. Palmer,^a Tingjun Chen,^c Kalli Schaefer,^a Jamie N. Justice,^d Anoop M. Nambiar,^e Nicolas Musi,^f Stephen B. Kritchevsky,^d Jun Chen,^g Sundeep Khosla,^{a,h} Diana Jurk,^{a,b,c} Marissa J. Schafer,^{a,b} Tamar Tchkonja,^{a,b} and James L. Kirkland^{a,b,i*}

^aRobert and Arlene Kogod Center on Aging, Mayo Clinic, 200 First St., S.W., Rochester, MN 55905, USA

^bDepartment of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN 55905, USA

^cDepartment of Neurology, Mayo Clinic, Rochester, MN 55905, USA

^dSticht Center for Healthy Aging and Alzheimer's Prevention, Internal Medicine, Gerontology and Geriatric Medicine, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA

^eDivision of Pulmonary Diseases and Critical Care Medicine, Department of Internal Medicine, University of Texas Health Sciences Center at San Antonio and South Texas Veterans Health Care System, San Antonio, TX 78229, USA

^fBarshop Institute for Longevity and Aging Studies, Center for Healthy Aging, University of Texas Health Sciences Center at San Antonio and Geriatric Research, Education, and Clinical Center, South Texas Veterans Health Care System, San Antonio, TX 78229, USA

^gDivision of Computational Biology, Department of Quantitative Health Sciences, Mayo Clinic, Rochester, MN 55905, USA

^hDivision of Endocrinology, Department of Medicine, Mayo Clinic, Rochester, MN 55905, USA

ⁱDivision of General Internal Medicine, Department of Medicine, Mayo Clinic, Rochester, MN 55905, USA

Summary

Background α -Klotho is a geroprotective protein that can attenuate or alleviate deleterious changes with ageing and disease. Declines in α -Klotho play a role in the pathophysiology of multiple diseases and age-related phenotypes. Pre-clinical evidence suggests that boosting α -Klotho holds therapeutic potential. However, readily clinically-translatable, practical strategies for increasing α -Klotho are not at hand. Here, we report that orally-active, clinically-translatable senolytics can increase α -Klotho in mice and humans.

Methods We examined α -Klotho expression in three different human primary cell types co-cultured with conditioned medium (CM) from senescent or non-senescent cells with or without neutralizing antibodies. We assessed α -Klotho expression in aged, obese, and senescent cell-transplanted mice treated with vehicle or senolytics. We assayed urinary α -Klotho in patients with idiopathic pulmonary fibrosis (IPF) who were treated with the senolytic drug combination, Dasatinib plus Quercetin (D+Q).

Findings We found exposure to the senescent cell secretome reduces α -Klotho in multiple nonsenescent human cell types. This was partially prevented by neutralizing antibodies against the senescence-associated secretory phenotype (SASP) factors, activin A and Interleukin 1α (IL- 1α). Consistent with senescent cells' being a cause of decreased α -Klotho, transplanting senescent cells into younger mice reduced brain and urine α -Klotho. Selectively removing senescent cells genetically or pharmacologically increased α -Klotho in urine, kidney, and brain of mice with increased senescent cell burden, including naturally-aged, diet-induced obese (DIO), or senescent cell-transplanted mice. D+Q increased α -Klotho in urine of patients with IPF, a disease linked to cellular senescence.

Interpretation Senescent cells cause reduced α -Klotho, partially due to their production of activin A and IL- 1α . Targeting senescent cells boosts α -Klotho in mice and humans. Thus, clearing senescent cells restores α -Klotho, potentially opening a novel, translationally-feasible avenue for developing orally-active small molecule, α -Klotho-enhancing clinical interventions. Furthermore, urinary α -Klotho may prove to be a useful test for following treatments in senolytic clinical trials.

Funding This work was supported by National Institute of Health grants AG013925 (J.L.K.), AG062413 (J.L.K., S.K.), AG044271 (N.M.), AG013319 (N.M.), and the Translational Geroscience Network (AG061456: J.L.K., T.T., N.M., S.B.K., S.K.), Robert and Arlene Kogod (J.L.K.), the Connor Group (J.L.K.), Robert J. and Theresa W. Ryan (J.L.K.), and

*Corresponding authors at: Robert and Arlene Kogod Center on Aging, Mayo Clinic, 200 First St., S.W., Rochester, MN 55905, USA.

E-mail addresses: Zhu.Yi@Mayo.edu (Y. Zhu), Kirkland.James@Mayo.edu (J.L. Kirkland).

eBioMedicine 2022;77:
103912
Published online 13
March 2022
<https://doi.org/10.1016/j.ebiom.2022.103912>

the Noaber Foundation (J.L.K.). The previous IPF clinical trial was supported by the Claude D. Pepper Older Americans Independence Centers at WFSM (AG021332: J.N.J., S.B.K.), UTHSCA (AG044271: A.M.N.), and the Translational Geroscience Network.

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Keywords: Cellular senescence; α -Klotho; Senolytics

Research in context

Evidence before this study

α -Klotho levels decline and senescent cells accumulate with ageing in mice and humans. α -Klotho-deficient mice exhibit accelerated ageing-like phenotypes. α -Klotho overexpression increases lifespan and delays or prevents onset of many age-related diseases. Similarly, accumulation of senescent cells is associated with tissue dysfunction in multiple organs and multiple age-related diseases, recapitulating effects of α -Klotho deficiency. Clearance of senescent cells through genetic targeting or senescent cell-targeting (senolytic) drugs alleviates or prevents many age-related diseases, as does overexpressing α -Klotho. Hence, we speculate α -Klotho and cellular senescence are inversely and potentially mechanistically interrelated.

Added value of this study

We demonstrated senescent cell CM reduces α -Klotho in cultured non-senescent human umbilical vein endothelial cells (HUVECs), human primary kidney glomerular endothelial cells (HKEs), and human brain astrocytes (HBAs). Effects of senescent cell CM were partially attenuated by neutralizing antibodies against the SASP factors, activin A and IL-1 α . Transplanting senescent cells into younger mice decreased kidney and brain α -Klotho. Genetically reducing highly *p16^{Ink4a}*-expressing cells in old *INK-ATTAC* mice or administering the senolytics, Dasatinib plus Quercetin (D+Q) or Fisetin, to young mice transplanted with senescent cells, young DIO mice, or naturally-aged mice increased urine, kidney, and/or brain α -Klotho. Treating patients with IPF, a cellular senescence-related disease, with D+Q increased urinary α -Klotho.

Implications of all the available evidence

α -Klotho and cellular senescence are inversely related and causally linked to the genesis and progression of age-related diseases. Targeting senescent cells increases the geroprotective factor α -Klotho, potentially amplifying beneficial effects of senolytic drugs. Our study also opens a novel, translationally-feasible avenue for developing orally-active small molecules to increase α -Klotho, which may also be a useful biomarker for senescent cell burden or senolytic drug activity in clinical trials.

Introduction

α -Klotho is a geroprotective factor that exerts anti-physiological stress effects¹ and protects against oxidative damage, hypoxia, and cytotoxic drugs.^{2,3} Soluble α -Klotho is an endocrine protein that regulates multiple biological processes, including phosphate homeostasis, mineral metabolism,⁴ and signaling by insulin-like growth factor I (IGF-I),^{5,6} mammalian target of rapamycin (mTOR),^{7,8} cyclic adenosine monophosphate (cAMP),^{9,10} p53/ p21^{CIP1,11,12} and Wnt proteins.^{2,13,14} α -Klotho is synthesized and secreted by the distal renal tubule and so is present in urine.^{15–17} It is also highly expressed in the choroid plexus and other regions of the brain and to a lesser extent in adipose tissue. Several preclinical studies have implicated α -Klotho as a molecule that impacts lifespan, health-span, and renal and cognitive function. α -Klotho declines with ageing in mice and humans.^{2,18} Mice deficient in α -Klotho develop premature ageing-like phenotypes, including shortened lifespan, atherosclerosis-like vascular dysfunction, cognitive impairment, sarcopenia, physical dysfunction, cardiac hypertrophy and fibrosis, and osteopenia.^{2,19–26} Conversely, high α -Klotho levels attenuate age-related functional declines. Mice overexpressing α -Klotho have increased lifespan, enhanced cognition, delayed age-related vascular dysfunction, decreased diabetes-related inflammation, and improved skeletal muscle regeneration.^{27–29} As α -Klotho holds therapeutic potential, various strategies to increase α -Klotho have been proposed. However, despite extensive effort there are few approaches for increasing or restoring α -Klotho that are either feasible to advance into clinical trials or that have shown efficacy in trials. This led us to seek a novel approach based on the interrelatedness of fundamental ageing mechanisms.

Along with reductions in α -Klotho, senescent cells accumulate with ageing. Cellular senescence is a cell fate involving essentially permanent replicative arrest, extensive changes in metabolic state, and resistance to apoptosis. Senescent cell abundance is increased in multiple age-related diseases and disorders in experimental animals and humans.^{30–32} Cellular senescence can be associated with a SASP^{33–35} that entails release of pro-inflammatory and tissue-damaging cytokines, chemokines, extracellular matrix-destroying proteases, bioactive lipids, metabolites, nucleotides, exosomes,

pro-apoptotic factors, and factors that impede stem cell, progenitor, and tissue function and can spread senescence to previously normal cells, both locally and systemically.^{33–37} Reducing senescent cell burden with senolytics, a class of drugs that selectively clears senescent cells, extends health-span in mice^{36,38} and has effects similar to those of overexpressing or administering recombinant α -Klotho.^{11,39–43} Conversely, transplanting senescent cells into young mice shortens health-span and causes frailty, early onset of age-related phenotypes and diseases, and premature death from the same causes of death as naturally-aged mice.³⁶ Collectively, these findings suggest a feed-forward cycle, with deficiency of α -Klotho contributing to increased senescent cell burden^{11,44} and hence increased SASP factors that, in turn, might further exacerbate α -Klotho deficiency. Therefore, we speculated that α -Klotho and cellular senescence are inversely and potentially causally interrelated. If so, decreasing senescent cell abundance using orally-active senolytics could be a clinically effective and practical way to increase α -Klotho.

To determine if senolytics increase α -Klotho and explore translational relevance, we performed *post hoc* specimen analyses of urinary α -Klotho in three different mouse models and the first human study of the senolytic drug combination, D+Q, in persons with IPF. IPF is a progressive, fatal age-related disease linked to both cellular senescence and reduced α -Klotho. Bleomycin inhalation induces lung senescent cell accumulation together with an IPF-like syndrome in mice that is alleviated by decreasing senescent cell burden genetically or with senolytics.⁴⁵ Also, lung α -Klotho is lowered by Bleomycin administration. Transgenic mice overexpressing α -Klotho are protected from Bleomycin-induced pulmonary fibrosis.^{46,47} Patients with IPF have decreased plasma α -Klotho⁴⁸ as well as an increased burden of senescent cells.⁴⁵ α -Klotho is lower in lung fibroblasts cultured from IPF patients than control subjects without IPF.⁴⁷ We previously reported that the senolytic drug combination, D+Q, may alleviate physical dysfunction in IPF patients⁴⁹ (a single-arm phase I pilot trial, ClinicalTrials.gov identifier: NCT02874989). Here, we tested the hypothesis that α -Klotho and cellular senescence are causally linked in mice and humans by determining effects of senolytics on urinary α -Klotho levels in naturally aged, diet-induced obese (DIO), or senescent cell-transplanted mice. We then assayed urinary α -Klotho in specimens from the senolytic drug study in patients with IPF to determine if effects of senolytics on α -Klotho in mice are also apparent in humans.

Methods

Study design overview

We conducted a translational study of α -Klotho by first establishing α -Klotho signalling mechanisms in

cultured human primary senescent cells, then we explored the casual links between α -Klotho and senescent cells *in vivo* following genetic clearance of senescent cells and senolytic drug administration in naturally aged, DIO, and senescent cell-transplanted mice administered senolytic drugs vs. vehicle. Finally, we measured urinary α -Klotho before and after senolytic drug treatment in a *post hoc* analysis of a previous single-arm, open-label trial⁴⁹ in older adults with IPF.

Ethics statement

Mice were humanely euthanized at defined end points and all experimental procedures had prior approval from the Institutional Animal Care and Use Committee at Mayo Clinic. All human study procedures complied with the Declaration of Helsinki and the informed consent and study documents were approved by the Institutional Review Boards at Wake Forest School of Medicine (WFMS) and the University of Texas Health Science Center San Antonio (UTHSCSA) and were reviewed by Data and Safety Monitoring Boards at both clinical sites conducting the previous clinical trial of senolytics for IPF.⁴⁹ The nature, benefits, and risks of that study had been explained to the patient volunteers and their written informed consent was obtained prior to participation in that previous study (NCT02874989). Deidentified urine samples from that study collected from the patients with IPF were used here for exploratory analyses of α -Klotho.

Reagents

Human primary cells were obtained from commercial sources, passed quality control procedures, and were certified by the commercial sources. All reagents were validated by the manufacturer and/or has been previously cited in the literature. Detailed information about these reagents is provided in Supplementary Table 1.

Animals

Wild-type C57BL/6 mice (young = 8-month-old, male) were purchased from Charles River and maintained in the Mayo Clinic animal facility. *INK-ATTAC* mice were developed by J.L.K, T.T., J. van Deursen, and D.J. Baker.⁵⁰ In *INK-ATTAC* mice, the *ATTAC* gene devised by Trujillo et al.⁵¹ is driven by a senescence-related *p16^{Ink4a}* promoter fragment devised by Wang et al.⁵² The fusion protein product of the *ATTAC* “suicide” gene contains a mutated FKBP moiety that can be cross-linked by AP20187, thereby activating the *ATTAC* protein caspase-8 moieties, causing apoptosis. *INK-ATTAC* mice were bred, genotyped, and aged at Mayo Clinic’s animal facility, which is pathogen-free and maintained at 23–24 °C under a 12 h. light, 12 h. dark regimen. Mice had free access to water and a 20% protein by weight, 5% fat (13.2% fat by calories), and 6%

fiber diet (Lab Diet). The *INK-ATTAC* mouse strain is patented by Mayo Clinic can be requested under a material transfer agreement. DIO mice were fed a diet in which 60% of calories were from fat (Research Diets) for 7–8 months before experiments.

Human IPF trial

This is a *post hoc* analysis of a previously published observational study.⁴⁹ That study was conducted at the Clinical Research Units of WFSM ($n = 2$) and UTHSCSA ($n = 18$). Intermittent D (100 mg/day) plus Q (1250 mg/day) were orally administered over three consecutive days for three weeks (*i.e.* 9 total participant administered dosing days) at the two outpatient clinical research centers using a single-arm, open-label design, as previously described.⁴⁹

Cell transplantation

Wild-type C57BL/6 mice were randomly assigned to be transplanted with senescent (induced by 10Gy x-ray) or non-senescent adipocyte progenitors or vehicle (phosphate buffered saline [PBS]) and matched for body weight across groups. Mouse preadipocytes were isolated and cultured as previously described.³⁶ Mice were anesthetized using Isoflurane and 2 million cells were transplanted intraperitoneally (*i.p.*) in 150 μ l PBS through a 22G needle, as previously described.

Drug treatments

INK-ATTAC mice were randomly assigned to AP20187 or vehicle groups. AP20187 was purchased from Clontech. Vehicle (10% ethanol, 30% polyethylene glycol, 60% Phosal) or AP20187 in vehicle was injected *i.p.* (10 mg/kg) for 3 consecutive days every 2 weeks for 4 weeks. Wild-type C57BL/6 mice were randomly assigned to D+Q, Fisetin (F), or vehicle treatments. Treatments were started at age 26–27 months for old animals, and for DIO mice after 7–8 months of high fat-feeding beginning at 4 months of age. Mice were treated every 20 days with D+Q, F, or vehicle by oral gavage for 3 consecutive days in each of 3 cycles over 2 months (9 doses in total).

Cell culture

HKEs (ScienCell Research Laboratories, #4000), HUVECs (Lonza, # C2519A), and HBAs (ScienCell Research Laboratories, #1800) were cultured following the suppliers' directions. All cells purchased from commercial sources were validated by the manufacturers (Supplementary Table 1). Briefly, CM from senescent or non-senescent HKEs, HUVECs, or HBAs was filtered (0.2 μ m) before being added to target non-senescent HKEs, HUVECs, or HBAs, respectively, with or without neutralizing antibodies against Interleukin-1 β (IL-1 β ;

Biolegend, #508202, RRID:AB_315514), Interleukin-18 (IL-18; Thermo Fisher Scientific, #PA5-47803, RRID: AB_2606212), CXCL1 (R&D Systems, #MAB275, RRID:AB_2292460), Interleukin-6 (IL-6; R&D Systems, #MAB2061, RRID:AB_354281), activin A (R&D Systems, #MAB3381), Tumour Necrosis Factor α (TNF- α) (Cell Signaling Technology, #7321S), or Plasminogen Activator Inhibitor-1 (PAI-1; R&D Systems, #MAB1786) for 48 h. Cells were then collected for assay by qPCR.

Non-senescent HKEs, HUVECs, and HBAs were cocultured with recombinant human IL-1 α protein (R&D Systems, #00-LA-010) or recombinant human activin A (R&D Systems, #338-AC) for 48 h, when cells were assayed by qPCR.

qPCR

Each cDNA sample was generated by reverse transcription using 1 μ g RNA following the manufacturer's protocol (Thermo Fisher Scientific). Reverse transcription involved incubation for 10 min at 25 $^{\circ}$ C, 120 min at 37 $^{\circ}$ C, 5 min at 85 $^{\circ}$ C, and holding at 4 $^{\circ}$ C using Taqman Fast Advanced Master Mix (Thermo Fisher Scientific). TBP was used as a control. Data were analysed by the $\Delta\Delta$ Ct method.

Human and mouse urinary α -Klotho

α -Klotho was assayed by ELISA in 1:100 diluted mouse urine (IBL America, #27601). Human urine α -Klotho was assayed by ELISA (IBL America, #27998, RRID: AB_2750859). Urine α -Klotho measurements are expressed as a function of urine volume, creatinine (R&D Systems, #KGE005), and/or cystatin C (R&D Systems, #MSCTC0).

Senescent cell quantification by mass cytometry (CyTOF)

CyTOF experiments were performed as described.⁵³

Immunofluorescent staining

Mouse brains were processed for paraffin embedding, cut into 4 μ m-thick sagittal sections, deparaffinized in Histoclear (National Diagnostics), and rehydrated in decreasing percentages of ethanol diluted in water. Prior to staining, antigen retrieval was performed by incubating sections in Tris EDTA pH9 buffer in a steamer for 20 min and cooling to room temperature. Sections were washed with PBS and blocked with 5% Normal Goat Serum and 0.3% Tween20 in PBS-BSA 0.1% for 30 min. Rabbit anti-mouse α -Klotho antibody (Invitrogen, #MA5-32784) was diluted 1:50 in blocking solution, added to sections, and incubated overnight at 4 $^{\circ}$ C. Slides were washed with PBS and incubated with secondary goat anti-rabbit 647 antibody (Invitrogen, A-21244, RRID: AB_2535812) for 1 h at room temperature

in the dark. Slides were washed and mounted in Pro-Long Gold Antifade mountant with DAPI (Invitrogen, P36935).

Imaging and analysis

Stained α -Klotho mouse whole brain sections were scanned using an Axio Scanner Z1 (Zeiss) at 20x magnification. Photoshop CC 19.1 (Adobe, Inc.) was used to virtually dissect the choroid plexus, whole cerebellum, and background samples, avoiding major blood vessels with erythrocytes or tissue folding that could interfere with fluorescence intensity calculations. Using ImageJ FIJI,⁵⁴ the region of interest (ROI) was selected for analysis to exclude blood vessels and folding of tissue that can interfere with intensity measurements. ROI areas were quantified for raw intensity and then corrected with background on the same section. Results are shown as the corrected mean intensity, calculated as corrected intensity divided by area.

Western blotting

Tissue extracts were homogenized using a Bead Mill 24 homogenizer (Waltham) and lysed in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), 5 mM NaF, a protease inhibitor cocktail (Millipore Sigma), and 5 mM nicotinamide. After incubating 30 min at 4 °C, samples were centrifuged at 15,000 rpm for 10 min at 4 °C. Protein concentrations of the supernatants were determined by the Bradford protein assay (BioRad). Lysates were separated by SDS-PAGE and transferred by electrophoresis to PVDF membranes (Millipore Sigma). Membranes were immunoblotted with primary mouse α -Klotho antibody (R&D Systems, AF1819) at 1:1000 dilution and GAPDH antibody (Cell Signaling Technology, 97166) at 1:1000 dilution. Enhanced chemiluminescence detection was performed using SuperSignal West Pico or Femto Chemiluminescence Substrate (Thermo Scientific). All blots were imaged with a GelDoc Go Imaging System (BioRad).

Statistical analyses

At least four independent replicates were studied in all cell culture experiments. *Randomisation*: Mice were randomized using a random number generator in all experiments. *Blinding*: Experiments were conducted with the investigators being blinded as to treatment, group allocation for immunofluorescence (IF) staining, ELISA assays, quantitative PCR (qPCR), and quantification of protein expression. *Sample size determination*: Sample sizes were chosen based on the means and variation of preliminary data to achieve at least 80% power and allow for a 5% type I error. *Inclusion/exclusion*: The animals were matched for age, gender, and body weight. No animals or experimental data were excluded.

Inclusion and exclusion criteria for the clinical trial from which de-identified urine was acquired for *post hoc* analysis have been published in.⁴⁹ All data were plotted and analyzed for statistical significance using Prism 9.0 (GraphPad) for cell culture and animal experiments. We reported R^2 (same as η^2), calculated in Prism, as the effect size. Comparisons between a single treated group and vehicle were made using non-paired T-tests. When comparisons were made between multiple treatment levels, a mixed effects repeated measures model was used, accounting for the repeated measures within each subject. For IPF clinical trial data, the following statistical methods were used and due to the wide range of the values, a log transformation was performed before analysis. Zeros ($n = 3$) were replaced with half of the minimum non-zero value before log transformation. Imputation using half of the minimum was used to impute zeros due to under-detection.⁵⁵ This imputation method⁵⁶ is available in MetaboAnalyst 2.0, a web server for data analysis and interpretation. MetaboAnalyst 2.0 allows selected analyses to avoid divide-by-zero problems to process missing values, which can be replaced by the half of the minimum value found in the dataset by default. Wilcoxon signed rank tests (two-sided) were used to test differences before and after treatment in human trial data since the data were not normally distributed. Both Spearman's rho and Kendall's tau are non-parametric tests for association and test the same null hypothesis and both control for type I error at the nominal level. In our study, we chose to use Spearman's rank correlation to test the association between urinary α -Klotho and SASP factors in urine.

Role of funder

The funders had no role in the study design, data collection, analysis, interpretation, decision to publish, or writing the manuscript.

Results

Senescent cells decrease α -Klotho through paracrine mechanisms

HKEs, HUVECs, and HBAs were radiated (20Gy X-ray). By 25 days after radiation, the cells had become senescent (Supplementary Fig. 1). To determine if senescent cells can directly impact α -Klotho expression, HKEs, HUVECs, and HBA were exposed to CM from cultured senescent or non-senescent human HKEs, HUVECs, or HBAs, respectively. Senescent cell CM exposure resulted in decreased α -Klotho expression in the non-senescent cells (Figure 1A–C, Supplementary Fig. 2). These reductions were attenuated in non-senescent HKEs or HUVECs by neutralizing antibodies against activin A, and in non-senescent HBAs by anti-IL-1 α

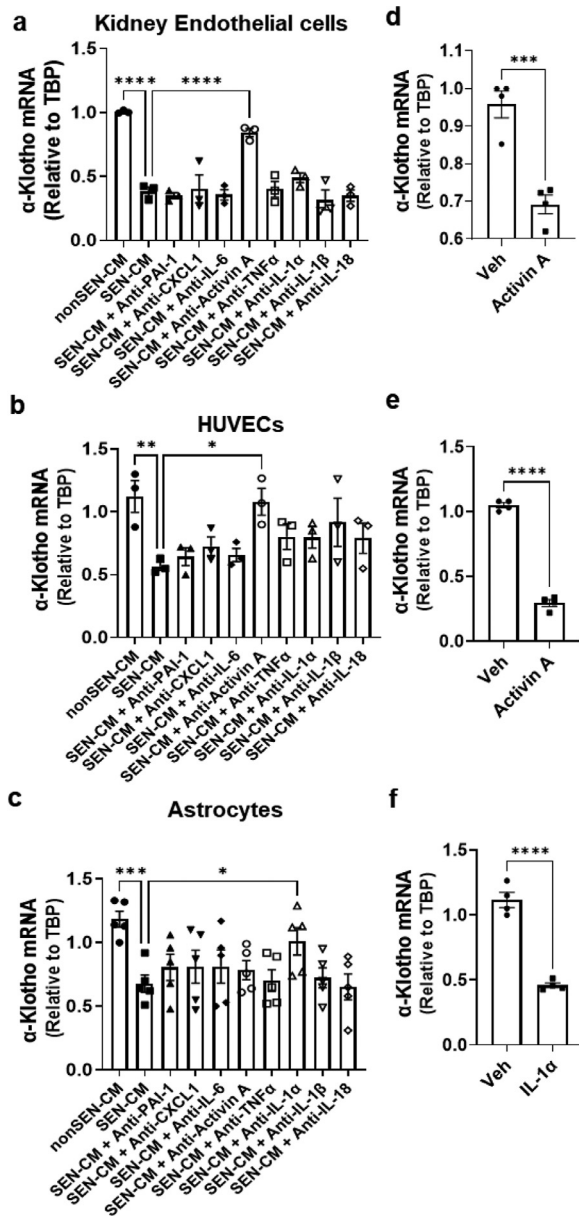


Figure 1. Senescent cell conditioned medium decreases α -Klotho; blocking particular SASP factors with neutralizing antibodies partially restores α -Klotho. (a–c) Conditioned media (CM) collected from senescent or non-senescent (a) human primary kidney endothelial cells (HKEs; one-way ANOVA: $F = 19.10$, $p < 0.0001$, $R^2 = 0.89$; $n = 3$; **** $p < 0.0001$); (b) human umbilical vein endothelial cells (HUVECs; one-way ANOVA: $F = 3.02$, $p = 0.02$, $R^2 = 0.58$, $n = 3$; * $p = 0.02$, ** $p = 0.01$); or (c) human brain astrocytes (HBAs; one-way ANOVA: $F = 7.93$, $p = 0.02$, $R^2 = 0.66$; $n = 5$; *** $p < 0.001$, * $p = 0.03$) were applied to non-senescent HKEs, HUVECs, or HBAs, respectively, with or without antibodies against PAI-1, CXCL1, IL-6, activin A, TNF- α , IL-1 α , IL-1 β , or IL-18 for 48 h. α -Klotho mRNA was assayed by qPCR. Means \pm SEM. (d) Primary HKEs ($n = 4$), (e) HUVECs ($n = 4$), or (f) HBAs ($n = 4$) were treated with recombinant activin A or IL-1 α . α -Klotho was assayed by qPCR 24 h after treatments. Means \pm SEM; unpaired T-tests; *** $p < 0.001$, **** $p < 0.0001$.

(Figure 1A–C). Conversely, treating human non-senescent HKEs, HUVECs, or HBAs with recombinant activin A or IL-1 α caused decreased expression of α -Klotho (Figure 1D). Thus, components of the SASP can decrease α -Klotho in non-senescent cells. Our results are consistent with findings by others that activin A, a transforming growth factor- β (TGF- β) superfamily member, binds to its receptor, ActRIIA, to activate TGF- β signaling. Activated TGF- β /Smad signaling reduces both α -Klotho mRNA and protein by altering α -Klotho gene promoter methylation.^{57–59} However, since targeting the individual SASP factors, activin A or IL-1 α , was not sufficient to fully restore α -Klotho across all cell types, multiple factors might need to be targeted to prevent SASP-induced reductions in α -Klotho *in vivo*. Alternatively, eliminating senescent cells with senolytics, thereby reducing all SASP factors with a single intervention, might be a more encompassing approach.

To test causality further, we ascertained if senescent cells can decrease α -Klotho *in vivo* by transplanting small numbers of senescent or non-senescent adipocyte progenitors vs. vehicle (PBS) i.p. into young mice (8-month-old) (Figure 2A). Transplanting these mice with senescent cells decreased urinary, cerebellar, and choroid plexus α -Klotho protein (Figure 2B–F) compared to control non-senescent cell transplanted- or PBS-treated mice.

Genetic clearance of $p16^{Ink4a}$ -expressing senescent cells increases α -Klotho

Next, we determined if removal of $p16^{Ink4a}$ -expressing cells, many of which are senescent, increases α -Klotho in old mice. To accomplish this, we used transgenic *INK-ATTAC* mice, in which the drug, AP20187, dimerizes the ATTAC “suicide” caspase-8 moiety-containing fusion protein,⁵¹ causing death of cells that highly express $p16^{Ink4a}$. Conditions alleviated by decreasing $p16^{Ink4a+}$ cells in *INK-ATTAC* mice parallel those alleviated by increasing α -Klotho, including age- or high fat diet-induced adipose tissue and metabolic dysfunction, age-related osteoporosis, and bleomycin-induced lung fibrosis.^{45,60–62} Eight- and 26–27-month-old *INK-ATTAC* mice were administered 10 mg/kg AP20187 in cycles of 3 consecutive days every 15 days and euthanized 5 days after the third 3-day course (9 doses in total) of AP20187 (Figure 3A). Kidney, urinary, and brain α -Klotho, which was lower in old than younger *INK-ATTAC* mice, was increased by AP20187 in the old mice (Figure 3B–D,E–G, Supplementary Fig. 4). Consistent with results in our *in vitro* experiment (Figure 1A–F), activin A and IL-1 α were high in kidneys and brains of old *INK-ATTAC* mice, respectively, but decreased after AP20187 treatment (Figure 3H,I), supporting the hypothesis that senescent cells contribute to the age-related decline in α -Klotho in part through the SASP factors, activin A and IL-1 α .

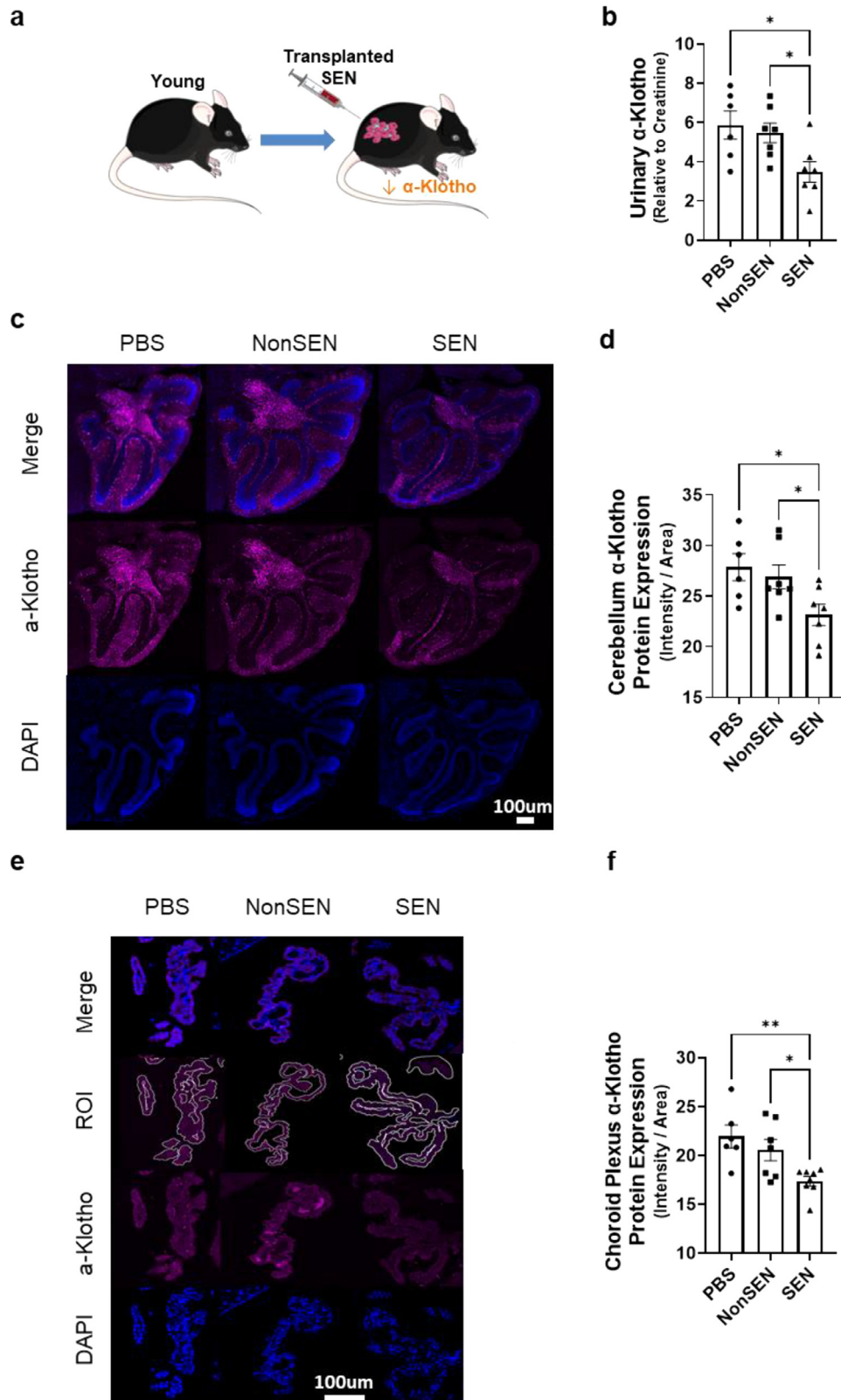


Figure 2. Transplanting senescent cells decreases urinary and brain α -Klotho. (a) 8-month-old male mice were transplanted i.p. with 2 million senescent or non-senescent mouse preadipocytes in 150 μ l PBS through a 22-G needle. (b) Urinary α -Klotho was assayed

Senolytics increase α -Klotho *in vivo*

Senolytics are agents that reduce senescent cell burden.⁶³ Senolytics, which were discovered independently from developing *INK-ATTAC* mice and do not depend on *p16^{Ink4a}* expression to target senescent cells. Indeed, not all senescent cells have high *p16^{Ink4a}* and not all cells that highly express *p16^{Ink4a}*, such as activated macrophages, are senescent.⁶⁴ Rather, senolytic agents act by transiently disabling the Senescent Cell anti-Apoptotic Pathways (SCAPs) that protect those senescent cells with a pro-apoptotic SASP from their own cytotoxic microenvironment.^{31,38,65} Treating mice orally with the senolytics, D+Q or F,^{38,65,66} increased urine α -Klotho in aged mice (Figure 4A). In young wild-type DIO mice, which have an increased burden of senescent cells compared to lean mice^{53,67–69} and a decline in urinary α -Klotho (Supplementary Fig. 3), D+Q was effective in increasing urinary α -Klotho (Figure 4B, Supplementary Fig. 4). Both D+Q and F increased urinary α -Klotho in young mice that had been transplanted with senescent cells (Figure 4C). Kidney α -Klotho, which was lower in old than young mice (Supplementary Fig. 3), was increased by D+Q in the old mice (Figure 4D).

The brain is another primary site of α -Klotho production.^{70–72} We found senolytics increase α -Klotho protein in the cerebellum and choroid plexus (Figure 5A,B) as well as α -Klotho mRNA in whole brains of old mice in which α -Klotho decreases with ageing (Figure 5C). In young obese mice, senolytics increased brain α -Klotho (Figure 5D). Furthermore, α -Klotho was inversely related to adipose tissue senescent cell burden in untreated obese mice (Figure 5E).

To determine if senolytics increase α -Klotho through direct, off-target effects in α -Klotho-expressing non-senescent cells, we exposed human cultured non-senescent preadipocytes or astrocytes to D+Q or F (Supplementary Fig. 5). Treatment with senolytics did not increase α -Klotho in these cultured non-senescent cells. Treating young *INK-ATTAC* mice with AP20187 or D+Q also did not increase kidney α -Klotho (Supplementary Fig. 6A). Furthermore, senolytics did not increase α -Klotho in urine of young mice (Figure 6B). Thus, senescent cell targeting strategies do not appear to increase α -Klotho when senescent cell burden is low, consistent with increases in α -Klotho being due to removal of senescent cells, rather than other mechanisms.

Senolytics increase α -Klotho in humans

We recently reported that treating patients who have IPF with 9 doses of D+Q over 3 weeks alleviated physical dysfunction, with increased gait distance and speed, ability to rise out of a chair, and improved short physical performance battery scores 5 days after the last dose of senolytics.⁴⁹ In these subjects, urinary α -Klotho was increased after (392.79 ± 95.24 , normalized to urinary creatinine), compared to before (293.49 ± 115.48 , normalized to urinary creatinine), D+Q administration (Figure 6A). We also found that urinary SASP factors were inversely correlated with urinary α -Klotho (Figure 6B–D, Supplementary Table 2).

Discussion

Senolytics appear to be an effective small molecule, orally-active interventional strategy for increasing α -Klotho. Senolytics eliminate mouse and human senescent cells *in vitro* and reduce senescent cell burden across a range of animal models as well as in humans.^{31,36,38,66} The senolytic combination, D+Q, improves cardiovascular function in old mice, the first demonstration that reducing senescent cell abundance improves health-span in naturally-aged mice,³⁸ and is effective in alleviating multiple ageing phenotypes and chronic diseases across a number of mouse models.^{36,38,45,49,53,62,73–79} D+Q may also alleviate physical dysfunction in humans with IPF.⁴⁹

Besides senolytics, other small molecules that may increase α -Klotho include PPAR γ agonists,⁸⁰ losartan,⁸¹ statin HMG-CoA reductase inhibitors,⁸² and vitamin D derivatives.⁸³ Whether the latter agents affect α -Klotho through effects on senescent cells or other mechanisms remains to be determined. At least in the case of cultured HUVECs, angiotensin II exposure can promote cells to become senescent.⁸⁴ This angiotensin II-driven senescence was prevented by α -Klotho as well as an angiotensin II-inhibiting peptide that also increased α -Klotho, suggesting links between α -Klotho and senescence in HUVECs. This also implies that α -Klotho could potentially have either senomorphic (suppression of senescence) or senolytic effects, which needs further investigation.

A potential advantage of oral senolytics over other approaches is that senolytics are effective when administered intermittently in a “hit-and-run” fashion. Elimination half-lives of some senolytics are short (<4 h for D, 11 h for Q, and 4 h for F) and senescent cells take

by ELISA and expressed as a function of creatinine (one-way ANOVA: $F = 6.45$, $p = 0.01$, $R^2 = 0.42$; $n = 7$; $**p < 0.01$, $*p = 0.02$). Representative images of immunofluorescent (IF) α -Klotho stains of cerebellum (c) and choroid plexus (e) are shown. ROI (Region of Interest, outlined in white) were quantified by ImageJ (d,f) from mice that had been transplanted with senescent or non-senescent cells. Means \pm SEM; one-way ANOVA and *post hoc* Tukey's tests: $F = 4.94$, $p = 0.02$, $R^2 = 0.38$. $*p = 0.02$, $*p = 0.03$ in (d), $F = 7.84$; $p < 0.01$, $R^2 = 0.38$, $**p < 0.01$, $*p = 0.01$ in (f).

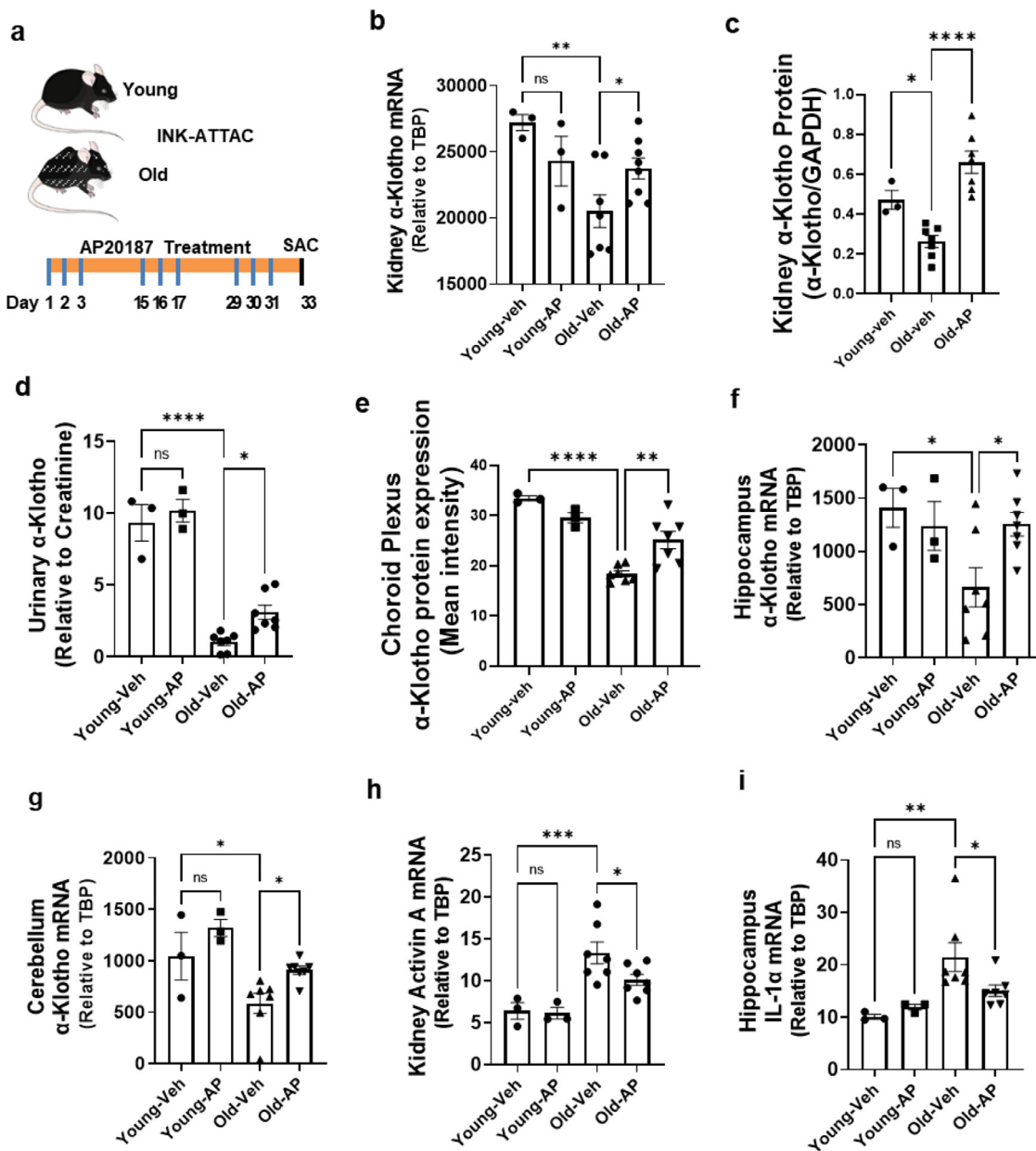


Figure 3. Genetic clearance of highly $p16^{Ink4a}$ -expressing cells increases α -Klotho. Young (8-month-old) or old (27-29-month-old) INK-ATTAC male mice were treated with vehicle or AP20187 ($n = 3$ young+ vehicle; $n = 3$ young+ AP20187; $n = 7$ old + vehicle; $n = 7$ old + AP20187) to dimerize the FKBP-caspase-8 fusion protein expressed in highly $p16^{Ink4a}$ -expressing cells to selectively eliminate $p16^{Ink4a+}$ cells. AP20187 (10 mg/kg) or vehicle was administered i.p. every 2 weeks for 6 weeks. (a) Schematic view of treatments. (b) Kidney α -Klotho mRNA was assayed by qPCR. One-way ANOVA, $F = 4.81$, $p = 0.01$, $R^2 = 0.46$; $*p = 0.03$, $**p < 0.01$. (c) α -Klotho protein was assayed relative to GAPDH (Western blots in Supplementary Fig. 7). One-way ANOVA: $F = 4.81$, $p = 0.01$, $R^2 = 0.46$; $*p < 0.05$, $****p < 0.0001$. (d) Mouse urine was collected 2 days after the last dose of AP20187. Urinary α -Klotho was assayed by ELISA and expressed as a function of creatinine. One-way ANOVA, $F = 61.53$, $p < 0.00001$, $R^2 = 0.90$; $*p = 0.04$, $****p < 0.0001$. Choroid Plexus (e) α -Klotho protein was assayed by immunofluorescence. One-way ANOVA, $F = 20.98$, $R^2 = 0.80$, $**p < 0.01$, $****p < 0.0001$. Hippocampal (f) and cerebellar (g) α -Klotho mRNA was assayed by qPCR. Activin A mRNA in kidney (h) and IL-1 α mRNA in hippocampus (i) were assayed by qPCR. Means \pm SEM; one-way ANOVA and *post hoc* Tukey's tests; $F = 3.84$, $p = 0.038$, $R^2 = 0.40$; $*p = 0.04$ in (f); $*p = 0.04$, $F = 7.87$, $p < 0.01$, $R^2 = 0.60$; $*p = 0.03$ in (g); $F = 10.85$; $p < 0.001$, $R^2 = 0.64$; $*p = 0.03$, $***p < p < 0.0001$ in (h); $F = 5.49$; $p = 0.01$, $R^2 = 0.49$; $*p < 0.05$, $**p = 0.01$ in (i).

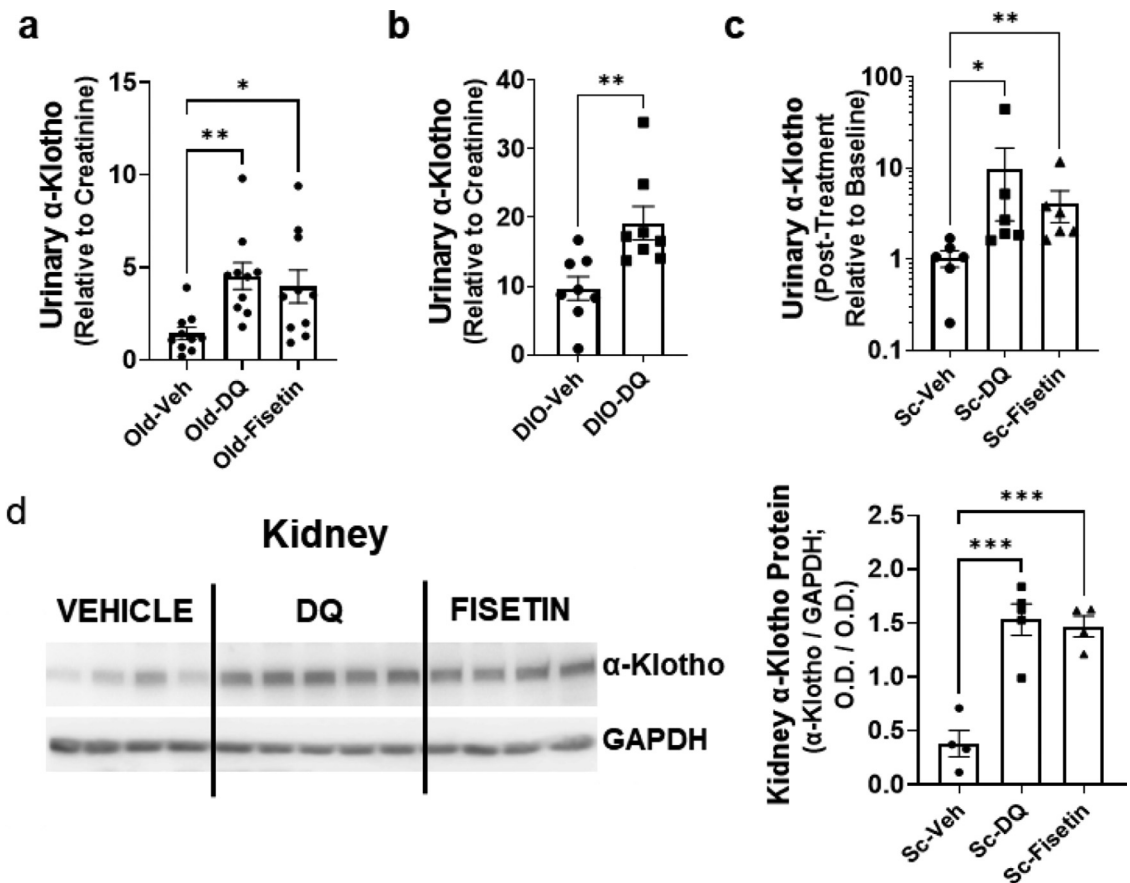


Figure 4. Senolytics increase urinary and kidney α -Klotho in old or obese mice. (a) Naturally-aged male mice (28–29-month-old, $n = 10$ in each group) were treated with vehicle, Dasatinib plus Quercetin (D+Q), or Fisetin. Urinary α -Klotho was measured by ELISA and expressed as a function of creatinine. One-way ANOVA: $F = 6.39$; $p < 0.05$, $R^2 = 0.30$; $*p = 0.02$, $***p < 0.01$. (b) DIO mice (male, 10-month-old, $n = 8$) were treated with vehicle or D+Q and urinary α -Klotho was assayed by ELISA and expressed as a function of creatinine. Unpaired T test; $**p < 0.01$. (c) Six-month-old male mice ($n = 6$ in each group) were transplanted i.p. with senescent preadipocytes. After 3 months, they were treated with vehicle, D+Q, or Fisetin. Urinary α -Klotho was assayed by ELISA and expressed as a function of creatinine. One-way ANOVA and *post hoc* Tukey's tests: Kruskal-Wallis statistic=10.19; $*p = 0.02$, $**p < 0.01$. (d) Kidney α -Klotho protein was also assayed in the same transplanted mice by Western blotting. One-way ANOVA: $F = 24.24$; $p < 0.001$, $R^2 = 0.83$; $***p < 0.001$.

weeks to over a month to re-develop, at least in cell culture,^{31,36,62,85} enabling a “hit-and-run” administration strategy. Additionally, senolytics may be more practical for increasing α -Klotho than recombinant α -Klotho protein or related peptides,^{40,41} since the latter may need to be administered repeatedly and by injection, rather than intermittently and orally. Furthermore, α -Klotho protein or related peptides may not readily act across the blood brain barrier. However, D+Q reduces brain senescent cell burden in mice that are models of Alzheimer's disease⁷⁸ as well as in obese mice.⁷⁵

Our results indicate that assaying urinary α -Klotho could be a useful approach for monitoring effects of senolytics during clinical trials or, eventually, if senolytics are translated into clinical practice, in the course of treating patients. First morning urine assays have the advantage of providing an integrated representation of

α -Klotho production over several hours, as opposed to the instantaneous view from blood tests. In addition, urine collections are less invasive than venipuncture. Furthermore, urine tests are probably less confounded by the more extensive presence of cells of various types in blood than urine and possible effects of other proteins in blood that might bind to α -Klotho. α -Klotho is a complex molecule. Changing its conformation by binding to other proteins might affect availability of α -Klotho epitopes for antibody binding, and hence interfere with reliable detection by immunoassay.

Our study has caveats regarding the precise mechanisms through which senolytics increase α -Klotho: is this by reducing bystander effects of senescent cells or removing α -Klotho deficient senescent cells? We will dissect such potential mechanisms in future studies. Additional future studies are needed to: (i) ascertain if

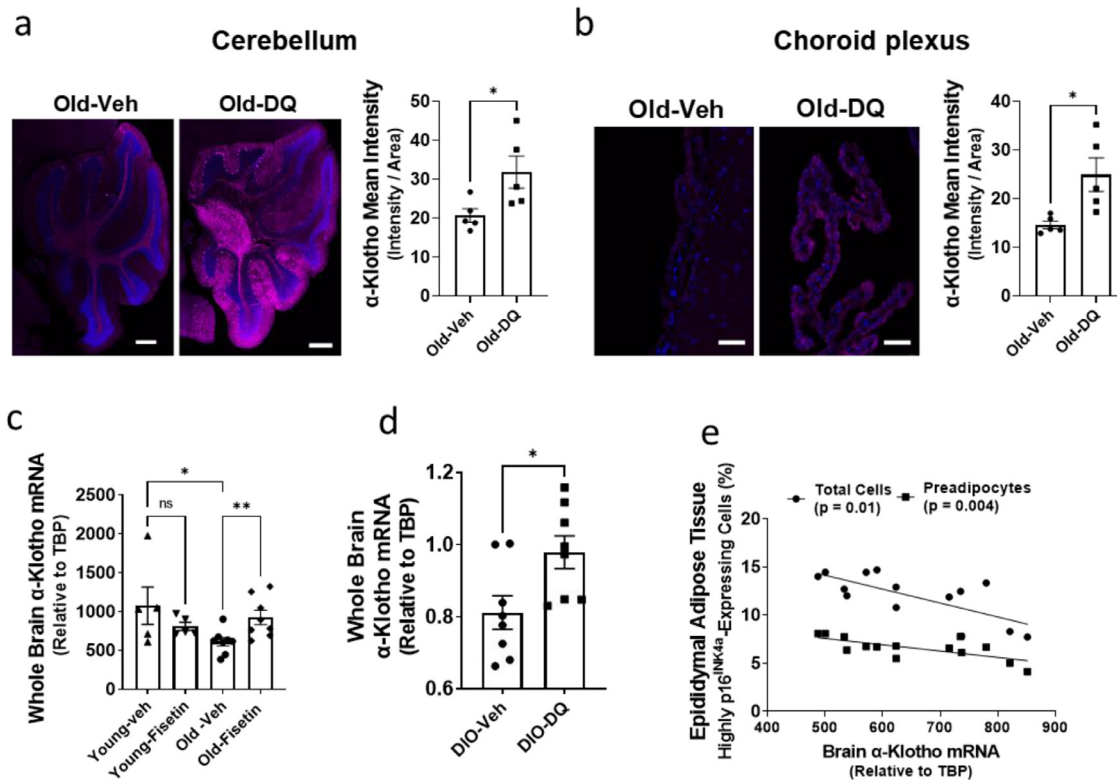


Figure 5. Senolytics increase brain α -Klotho in old mice. Naturally-aged mice (female, 28-month-old) were treated with vehicle ($n = 5$) or D+Q ($n = 5$). Brain α -Klotho was analysed by IF. (a) Representative IF images of α -Klotho expression (red) in the cerebellum are shown. Mean intensities of red fluorescence in the cerebellum were quantified by ImageJ. Unpaired T-tests; $*p = 0.03$. (b) Brain choroid plexus α -Klotho (purple), representative IF images. Mean intensities of fluorescence in the choroid plexus were quantified by ImageJ. Unpaired T-tests; $*p = 0.02$. (c) Young (female, 6-month-old, $n = 5$ in vehicle and treated groups) and naturally-aged mice (female, 22-month-old, $n = 8$ in vehicle and treated groups) were treated with Fisetin or vehicle. α -Klotho in whole brain was assayed by qPCR. One-way ANOVA and *post hoc* Tukey's tests: Kruskal-Wallis statistic=9.49; $*p = 0.02$, $**p < 0.01$. (d) DIO mice (male, 8-9-month-old) were treated with D+Q ($n = 8$) or vehicle ($n = 8$). Brain α -Klotho was assayed by qPCR. Mann-Whitney test; $*p = 0.03$. (e) Correlation between brain α -Klotho mRNA and peripheral p16^{INK4a}-expressing adipose tissue progenitor cells was quantified by CyTOF. Spearman correlation analysis.

CSF α -Klotho increases after administering senolytics to humans with Alzheimer's disease (currently being studied in the clinical trials, NCT04785300 and NCT04685590), (2) compare blood indices of senescent cell burden to urinary α -Klotho in patients across the age spectrum in other current and future clinical studies of senolytics, and (3) ascertain why in pre-eclampsia, a condition that we found is associated with increased senescent cells⁸⁶ and that has been linked to decreased urinary α -Klotho,⁸⁷ plasma α -Klotho is increased.⁸⁸ This discrepancy between α -Klotho in urine and plasma in pre-eclampsia is consistent with the previous finding that in chronic kidney disease (CKD), plasma α -Klotho is not related to kidney function and does not predict adverse outcomes.⁸⁹ These findings in pre-eclampsia suggest that, while cellular senescence appears to be a major regulator of α -Klotho production, it is unlikely to be the only one.

The results here showing interactions between α -Klotho and cellular senescence add to evidence supporting our "Unitary Theory of Targeting Fundamental Ageing Mechanisms"^{90,91}: that targeting any one fundamental ageing mechanism may positively impact most or all of the others. Cellular senescence can contribute to other "pillars of ageing", including inflammation, fibrosis, DNA damage, mitochondrial dysfunction, ROS generation, NAD⁺ depletion, protein dysregulation, failed autophagy, lipotoxicity, and stem/progenitor cell dysfunction. Conversely, other fundamental ageing processes can lead to cellular senescence. This may partly explain why targeting even a single fundamental ageing process, such as senescence, appears to delay, prevent, or alleviate multiple age-related diseases and disorders as a group.^{30,90-92} We speculate that senolytics might impact many, or perhaps even all of the pillars of ageing,⁹³ with increased α -Klotho resulting from

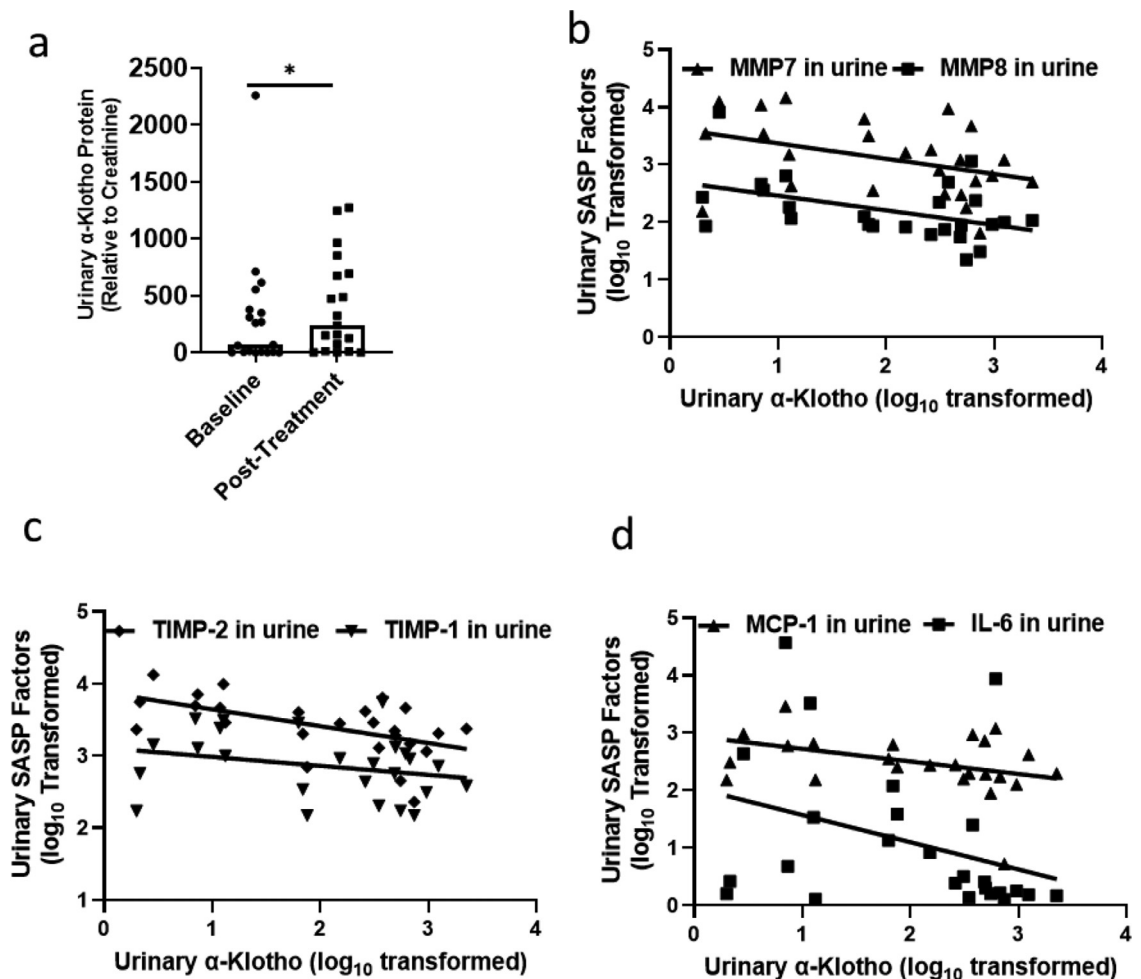


Figure 6. Senolytics increase α -Klotho in human urine; urinary SASP factors are inversely related to urinary α -Klotho. Subjects ($n = 20$) with idiopathic pulmonary fibrosis (IPF) were administered 3 courses of D+Q for 3 sequential days each (total 9 doses over 3 weeks). (a) Urinary α -Klotho and SASP factors were assayed at baseline and 5 days after the last D+Q treatment. Urinary α -Klotho in subjects with IPF was increased (Baseline: 293.49 ± 115.48 , Post-treatment: 392.79 ± 95.24 , normalized to urinary creatinine) by D+Q. Wilcoxon signed rank tests (two-sided) were used to test differences before and after treatment. $*p = 0.04$. (b–d) Spearman correlations of urinary α -Klotho and SASP factors are shown. FDR-corrected R^2 and p values are in Supplementary Table 2.

administering senolytics being an example. Examples of the impact of senolytics on other pillars of ageing include: (1) preventing or reversing age-related decreases in the ecto-enzyme CD38, which metabolizes nicotinamide adenine dinucleotide (NAD^+), leading to restoration of NAD^+ levels,⁹⁴ (2) attenuating age-related inflammation and fibrosis,^{37,45,75,95–98} (3) reducing the extent of age-related declines in mitochondrial function and blunting ROS generation,⁹⁹ (4) decreasing DNA damage,¹⁰⁰ (5) restoring insulin sensitivity,^{53,60} and (6) blunting age-related increases in mTOR (manuscript in preparation). Additionally, targeting senescent cells can alleviate age- and chronic disease-related progenitor cell dysfunction: senolytics enhance adipose progenitor,^{36,53} osteoblast progenitor,⁶² and cardiac progenitor cell

function⁷⁶ and restore neurogenesis^{75,77} in ageing animals or animals with cellular senescence-related conditions. If our Unitary Theory holds true, it will be critical to test whether combining individual interventions targeting interlinked or independent fundamental ageing mechanisms has less than additive, additive, or synergistic effects.

Declaration of interests

Y.Z., T.T., N.G., T.P., A.K.P., and J.L.K. have a financial interest related to this research. Patents on senolytic drugs are held by Mayo Clinic. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and was conducted in compliance with Mayo

Clinic Conflict of Interest policies. No conflicts of interest, financial or otherwise, are declared by the other authors.

Contributors

Y.Z., L.G.P.L.P., J.M.E.N., N.G., T.P., A.X., U.T., E.O.W. G., and T.C. performed experiments and verified the data in this study. K.O.J. and C.L.I. bred, genotyped, and pre-treated mice. L.G.P.L.P. and K.S. conducted immunofluorescence staining and quantification. J.C. performed statistical analyses. J.N.J., A.M.N., N.M., S.B.K., S.K., T.T., and J.L.K. designed or conducted the previous human clinical trial. J.L.K., Y.Z., and T.T. designed the current study and wrote the manuscript. A.K.P., D.J., and M.J.S. reviewed and edited the manuscript. All authors read, edited, and approved the final version of the manuscript.

Acknowledgements

We thank Victoria S. Shea and Jacqueline L. Armstrong for administrative help during manuscript submission. We thank Dr. Darren J. Baker for providing feedback and comments on this work. This work was supported by National Institute of Health grants AG013925 (J.L.K.), AG062413 (J.L.K., S.K.), AG044271 (N.M.), AG013319 (N.M.), and the Translational Geroscience Network (AG061456: J.L.K., T.T., N.M., S.B.K., S.K.), Robert and Arlene Kogod (J.L.K.), the Connor Group (J.L.K.), Robert J. and Theresa W. Ryan (J.L.K.), and the Ted Nash Long Life (J.L.K.) and Noaber Foundations (J.L.K.). The previous IPF clinical trial was supported by the Claude D. Pepper Older Americans Independence Centers at WFSM (AG021332: J.N.J., S.B.K.) and UTHSCA (AG044271: A.M.N.), and the Translational Geroscience Network.

Data sharing statement

All data associated with this study are in the article or the Supplementary Materials. The *INK-ATTAC* mouse strain is patented by Mayo Clinic and should be requested under a material transfer agreement.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2022.103912.

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