MAJOR MOUSE TESTING PROGRAM: SENOLYTICS PHASE 1

Abstract

The health and lifespan of mice have been demonstrated to improve by the removal of senescent cells using a transgenic suicide gene (Darren et al., 2011) and later experiments showed the same could be achieved using small molecules. Senolytics are a relatively new class of drugs that focuses on the removal of senescent cells. These senescent cells comprise a small number of total cells in the body but they secrete pro-inflammatory cytokines, chemokines, and extracellular matrix proteases, which together form the senescenceassociated secretory phenotype or SASP. The resulting SASP is thought to significantly contribute to aging (Freund, Campisi, et al, 2010) and cancer (Coppé, Campisi, et al, 2010) and thus Senolytics and removal of SASP is a potential strategy for promoting health and longevity.

It was discovered through transcript analysis that senescent cells have increased expression of prosurvival genes, consistent with their resistance to apoptosis (Zuh et al., 2015). Drugs targeting these prosurvival factors selectively killed senescent cells. Two such drugs were Dasatinib and Quercetin which were both able to remove senescent cells but were better in differing tissue types. However it was discovered that a combination of the two drugs formed a synergy that was significantly more effective at removing some senescent cell types (Zuh et al., 2015).

In other studies whilst only removing thirty percent of senescent cells there were improvements to age related decline. These results suggest the feasibility of selectively ablating senescent cells and the efficacy of senolytics for alleviating symptoms of aging and promoting healthy longevity (Tchkonia et al., 2013; Kirkland et al., 2014; Kirkland and Tchkonia, 2015).

However to date the combination of Dasatinib and Quercetin has yet to be tested in relation to its potential to increase healthy lifespan. Current Senolytic studies have focused only on health improvements rather than the long term effects (either bad or good) of this type of approach. The MMTP aims to address this missing and vitally important question, can Senolytics promote healthy longevity?

Venetoclax (ABT199) is another potential Senolytic drug that targets BCL2 selectively with fewer off target side effects compared to Senolytics such as Navitoclax (ABT263). Our aim is to target different tissuespecific senescent cells and thereby acquire a greater synergetic effect. No other group is currently testing this drug combination. Some initial data for Venetoclax is available showing it has an additive effect when used with other senolytics by targeting the BCL2 family (*R eut Yosef, Noam Pilpel, Ronit TokarskyAmiel, Anat Biran, et al, 2016)*

Project Overview

Summary: This is an exploratory experiment designed to further build on the work of other research undertaken by previous experimenters and apply it to high quality lifespan studies.
We are testing these compounds to measure their effect on lifespan. This work will help us to develop hypotheses into the mechanisms of aging, rejuvenation and intervention strategies for aging decline.

Goals of the MMTP project:

1) Demonstrate that our team has the capacity, knowledge and technical expertise to deliver lifespan studies

To be able to attract greater funding support for even larger scale mouse lifespan tests, it is necessary to show that the team is capable of doing mouse lifespan tests to a high standard. For this reason we have included caloric restriction which is known to improve mouse lifespan (Weindruch et al 1986). This will show that our team and the lab involved have good quality expertise. In addition, the CR control will show that if in the future we reproduce experiments (and this is planned) we will be able to show that our interventions are reproducible. Our control mice group will show that the animal facilities are of an excellent standard and that the mice live their normal lifespan and that the caloric restriction group reproduces the reported lifespan extension.

2) To show that certain substances can increase mouse lifespan.

We have selected a compound combination with a good chance of being able to extend mouse lifespan to excite our donors and produce new results. These are senolytics and have been shown to reduce the amount of senolytic cells in tissues, but have not been tested for lifespan extension.

Senolytics has the potential to modulate lifespan and healthspan by reduction of SASP (senescence associated secretory phenotype) and we will demonstrate this via robust lifespan studies.

Experiment Impact: Our results could have wide implications for the regenerative medicine field, and are of great importance for the long term effects of interventions not previously tested for their impact on lifespan modulation. It could also help guide and focus further research if benefits to health and lifespan in the long term are demonstrated.
Ultimately our aim is not only to explore single interventions, but combinations again in a rapid mass testing approach to find the best synergies for increasing healthy longevity. Single compounds (or compounds manipulating one pathway) are not likely to extend the lifespan by more than 30% and we hope that tackling several known pathways involved in aging could lead to a more significant effect. Please visit our website for more information on the longterm strategy also including gene and stem cell therapy approaches (http://www.majormouse.org).

Experiment Duration: S tarts when a nimal trial begins and until all mice have died.

Mouse type: The Black 6 or the C57BL/6 mouse is often used in life extension studies (MartinMontalvo et al., 2013) and the lifespan of this strain is well documented (Yuan et al., 2009).

This type of mouse also has the distinction of being the second mammalian species after Humans to have its entire DNA sequenced (Chinwalla et al., 2002). If there was a standard mouse that researchers use the Black 6 would be that mouse, a wealth of tests and studies using this strain give us a great deal of data to use. It is the top selling mouse strain at Charles River and many other breeders due to its relative robustness, ease of breeding and is easy to care for.

Mouse numbers: G roup sizes are shown below with a 50/50 mix of Male/Female. Additional mice could potentially be added to test more combinations and doses should more funds be available.

Mouse starting age: A ged mice are sourced from Charles River/Janvier and kept on site until they are 18 months old. Calculations are made for three groups with an estimation that the mice will survive for 36 months.

GROUP 1	GROUP 2	GROUP 3
CONTROL	SENOLYTICS	CR POS CONTROL
40	40	40

Mouse Groups:

- Group one: Control group which will not be treated
- Group two: Dasatinib & Quercetin & ABT199 (Venetoclax)
- Group three: Caloric restriction as a positive control group

Metrics and Assays:

- Animal weight will be measured periodically to give a general metric for health.
- Blood samples will be taken once a month to assess changes to circulating factors
- Postmortem analysis: This will include the analysis of adipose derived mesenchymal stem cell numbers. So far these senolytics have only been proven to reduce the number senescent somatic cells and effects on stem cells have not been studied. With the expertise of Dr. Stolzing in aging of stem cells this makes it an interesting scientific target.

Outreach - Optional Metrics and assays

It is noted that should additional funding be available then more intense metrics can be taken for additional health and aging biomarkers. A gross analysis will be performed straight after death. We will collect tissue for histological analysis and biochemical analysis using volunteers but this will be done beyond this project as it might take time. Reduction of the amount of senescent cells in the most important tissues will be quantified by:

- TGFbeta levels will be assessed via ELISA
- Senescence associated βgalactosidase activity level in adipose, muscle and bone marrow tissue

In addition we would be happy to collaborate with research groups interested in senolytics and provide tissue for further analysis. If you are interested please contact us to organise sample taking early during the project. We do want to get as much out of this experiment and store as much tissue material as possible for later experiments.

Experimental Design

Agents to be tested

In experiments with genetically modified animals, it has been found that removing senescent cells from middleaged mice has substantial benefits for health and life expectancy (Baker, Van Deursen Kirkland et al 2011, Baker, Kirkland et al 2016).

The genetic modification made it easy and straightforward to poison senescent cells with minimal collateral damage to healthy cells. If this can be accomplished in mice that are not genetically modified, we would have a promising treatment that might be applied to aging humans.

Hence we propose to test Dasatinib and Quercetin to see if they can extend the lifespan of normal (not genetically modified) mice. High doses for short duration were used in the preliminary senolytic trials. Dasatinib and Quercetin were found in these preliminary experiments to be able to selectively kill senescent cells and improve health in the animals tested (Zhu et al., 2015).

Interactions

Many treatments are known that extend mouse life span marginally. There are reasons to believe that in scaling up to humans, the percentage life extension will be smaller yet. These interventions of small benefit are of limited interest in themselves, but they become extremely interesting to the extent that they can be combined to offer a longevity benefit of 30%, 40% or more. But it is unlikely that the benefits simply add up. Multiple interventions are known to work via a few metabolic pathways. Once the pathway becomes saturated, adding more agents of a similar type is expected to give no further benefit.

There is almost nothing known about the way in which longevity interventions combine. We propose to begin addressing this situation by looking at interactions at the same that we characterize new treatments.

Caloric restriction and exercise are known to robustly increase mean lifespan in many animals including mice (Weindruch, R., Walford 1982, 1986) and primates (Colman et al 2014). The interventions that we test become far more interesting if their benefits improve upon the known benefits of exercise and CR.

Numbers and significance

We propose an innovative protocol which we believe will enable us to extract more useful information from a given number of mice. In particular, we would like to be able to gather information about dosage and treatment interactions at the same time that we test a the new concept of senolytic cell removal.

There is always scatter in life spans of individual mice. Typically, the standard deviation of life spans amongst mice subject to identical treatment is about 17% of mean life span.
Standard practice in mouse life span experiments is to assign about 40 mice to an identical treatment protocol, to be compared to another 40 mice in a control group.

The significance of this number is that, given the 17% standard deviation, there is a 95% chance of being able to detect a 10% lifespan effect with confidence $p<.05$.

But we think it is not affordable nor is it necessary to assign 40 mice to each of the 3 cage groups (40 test mice, in addition to controls making 120 mice in total). With innovative experimental design, and assuming reasonable assumptions about interactions of treatments, we should be able to extract information both about treatments and their interactions from as few as 40 test mice.

The key will be to apply a parametrized model of longevity response, then use multiple regression techniques in the data analysis to tease apart the effects of the separate treatments, and to estimate their interactions. We are preparing a publication in which the details of this statistical methodology are laid out.

In order to validate the new methodology, we propose to include a positive control group of 40 mice using 30% caloric restriction, to be analyzed in the standard way, generating the familiar form of a smooth survival curve.

Detailed protocol

120 mice will be used in total = 40 test mice, 40 control, and 40 positive controls. All mice will be 18 months old at the beginning of treatment.

Half the mice will be male and half female. The positive control mice will be calorically restricted at 30% via a specialised diet that reduces calories by the desired amount.

40 mice in the senolytic test group will be divided into cage groups of 10, 5 male and 5 female as per the above rationale for maximizing data and reducing wasteful animal testing.

Negative control group
40 mice: *a d libitum*

Positive control group
40 mice: 30% CR

Senescent cell removal group

We are using a unique per cage testing system that allows us to maximize data collection and serves as an exploratory test to lead onto more ambitious testing once we have established optimal doses and combination. We will be testing the following compounds in various cage group combinations:

- Dasatinib
- Quercetin 1 00mg/kg quercetin for first 3 weeks only, repeated with a second 3-week course 6 months later
- ABT199 (Venetoclax)
- 30%CR

Note: A more detailed dosage regime is to be published at a later date once we have a final working budget. This will also be made publicly available.

Literature:

● Anisimov VN., Berstein LM., Egormin PA., Piskunova TS., Popovich IG., Zabezhinski MA., Tyndyk ML., Yurova MV., Kovalenko IG., Poroshina TE., Semenchenko AV. (2008) Metformin slows down aging and extends life span of female SHR mice. Cell Cycle. 2008 Sep 1;7(17):276973.
● Chinwalla AT., Cook LL. (2002) Initial sequencing and comparative analysis of the mouse genome. Nature 420, 520562.
● Harrison DE., Strong R., Sharp ZD., Nelson JF., Astle CM., Flurkey K., Nadon NL., Wilkinson JE., Frenkel K., Carter CS., Pahor M., Javors MA., Fernandez E., Miller RA. (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature;460:392–395.
● MartinMontalvo A., Mercken EM., Mitchell SJ., Palacios HH., Mote PL., ScheibyeKnudsen M., Gomes AP., Ward TM., Minor RK., Blouin MJ., Schwab M., Pollak M., Zhang Y., Yu Y., Becker KG., Bohr VA., Ingram DK., Sinclair DA., Wolf NS., Spindler SR., Bernier M., de Cabo R. (2013) Metformin improves healthspan and lifespan in mice. Nat Commun. 2013 Jul 31; 4: 2192.
● Tchkonia T, Zhu Y, van Deursen J, Campisi J, Kirkland JL. (2013) Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. J Clin Invest. 2013 Mar;123(3):96672.
● Yuan R., Tsaih SW., Petkova SB., de Evsikova CM., Xing S., Marion MA., Bogue MA., Mills KD., Peters LL., Bult CJ., Rosen CJ, Sundberg JP., Harrison DE., Churchill GA., Paigen B. (2009) Aging in inbred strains of mice: study design and interim report on median lifespans and circulating IGF1 levels. Aging Cell. 2009 Jun; 8(3): 277–287.
● Zhu Y, Tchkonia T, Pirtskhalava T, Gower AC, Ding H, Giorgadze N, Palmer AK, Ikeno Y, Hubbard GB, Lenburg M, O'Hara SP, LaRusso NF, Miller JD, Roos CM, Verzosa GC, LeBrasseur NK, Wren JD, Farr JN, Khosla S, Stout MB, McGowan SJ, FuhrmannStroissnig H, Gurkar AU, Zhao J, Colangelo D, Dorronsoro A,

Ling YY, Barghouthy AS, Navarro DC, Sano T, Robbins PD, Niedernhofer LJ, Kirkland JL. (2015) The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. Aging Cell. Aug;14(4):64458.
- Coppé, J.P ., Desprez, P .Y ., Krtolica, A., & Campisi, J. (2010). The SenescenceAssociated Secretory Phenotype: The Dark Side of Tumor Suppression. *A nnual Review of Pathology*, *5* , 99–118.
- Freund, A., Orjalo, A. V., Desprez, P.Y., & Campisi, J. (2010). Inflammatory Networks during Cellular Senescence: Causes and Consequences. *T rends in Molecular Medicine*, *1 6*(5), 238–246.
- Weindruch et al (1986) The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. JNutr. 1986 *Apr;116 (4): 64154.*

Ink4a
- Baker,van Deursen Kirkland et al (2011) C learance of p16 positive senescent cells delays ageingassociated disorders, Nature 479, 232–236
- Baker, van Deursen et al (2016) Naturally occurring p16Ink4apositive cells shorten healthy lifespan nature16932
- Colman, R. J. et al. Caloric restriction reduces agerelated and allcause mortality in rhesus monkeys. Nat. Commun. 5:3557 doi: 10.1038/ncomms4557 (2014)
- Weindruch, R., Walford, R. L., Fligiel, S. & Guthrie, D. The retardation of ageing in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. J. Nutr. 116,641–654 (1986).
- Weindruch, R. & Walford, R. L. Dietary restriction in mice beginning at 1 year of age: effect on lifespan and spontaneous cancer incidence. Science 215, 1415–1418 (1982).
- Reut Yosef, Noam Pilpel, Ronit TokarskyAmiel, Anat Biran, et al Directed elimination of senescent cells by inhibition of BCLW and BCLXL Nature Communications 7, Article number: 11190 doi:10.1038/ncomms11190