EBioMedicine 21 (2017) 21–28

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Review

Cellular Senescence: A Translational Perspective

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ARTICLE INFO

Article history:
Received 13 March 2017
Received in revised form 6 April 2017
Accepted 6 April 2017
Available online 12 April 2017

Keywords:
Senolytics
SASP inhibitors
Dasatinib
Quercetin
Navitoclax
Fisetin
A1331852
A1155463
Senescent Cell Anti-apoptotic Pathways (SCAPs)

ABSTRACT

Cellular senescence entails essentially irreversible replicative arrest, apoptosis resistance, and frequently acquisition of a pro-inflammatory, tissue-destructive senescence-associated secretory phenotype (SASP). Senescent cells accumulate in various tissues with aging and at sites of pathogenesis in many chronic diseases and conditions. The SASP can contribute to senescence-related inflammation, metabolic dysregulation, stem cell dysfunction, aging phenotypes, chronic diseases, geriatric syndromes, and loss of resilience. Delaying senescent cell accumulation or reducing senescent cell burden is associated with delay, prevention, or alleviation of multiple senescence-associated conditions. We used a hypothesis-driven approach to discover pro-survival Senescent Cell Anti-apoptotic Pathways (SCAPs) and, based on these SCAPs, the first senolytic agents, drugs that cause senescent cells to become susceptible to their own pro-apoptotic microenvironment. Several senolytic agents, which appear to alleviate multiple senescence-related phenotypes in pre-clinical models, are beginning the process of being translated into clinical interventions that could be transformative.

Contents

1. Cellular Senescence: Causes and Consequences.

Cellular senescence is a cell fate that involves essentially irreversible replicative arrest, apoptosis resistance, and frequently increased protein synthesis, metabolic shifts with increased glycolysis, decreased fatty acid oxidation, increased reactive oxygen species generation, and acquisition of a senescence-associated secretory phenotype (SASP; Fig. 1)

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http://dx.doi.org/10.1016/j.ebiom.2017.04.013

(Tchkonia et al., 2013; LeBrasseur et al., 2015). The SASP entails secretion of cytokines, bradykines, prostenoids, miRNA's, damage-associated molecular pattern proteins (DAMPs), and other pro-inflammatory mediators, chemokines that attract immune cells, factors that cause stem cell dysfunction such as activin A, hemostatic factors such as PAI-1, pressors, and extracellular matrix-damaging molecules, including proteases (Xu et al., 2015a, Xu et al., 2015b; Coppé et al., 2006). Senescence can occur in response to potentially oncogenic mutations, activated oncogenes, metabolic insults, and damage/danger signals.

2. Aging, Chronic Diseases, and Cellular Senescence

Aging is the major risk factor for most of the chronic diseases that account for the bulk of morbidity, mortality, and health costs in the developed and developing worlds (Kirkland, 2016; Goldman et al., 2013). Chronic diseases, including dementias, atherosclerosis, diabetes, blindness, kidney dysfunction, and osteoarthritis among many others, become more prevalent with increasing age and tend to cluster together within older individuals (St Sauver et al., 2015). Risk for geriatric syndromes, including frailty, immobility, mild cognitive impairment, and incontinence, increases with aging. These conditions also cluster within individuals and are associated with age-related chronic diseases. Additionally, loss of physiological resilience, the capacity to recover following stresses such as surgery or pneumonia, occurs with advancing age and tends to precede onset of chronic diseases and geriatric syndromes (Kirkland et al., 2016). Fundamental aging processes, including chronic "sterile", low-grade inflammation, macromolecular and organelle dysfunction, stem/progenitor cell dysfunction, and cellular senescence, are not only associated with development of age-related phenotypes, but are also frequently apparent at sites of pathogenesis in age-related chronic diseases (Kirkland, 2016). For example, senescent cells accumulate in adipose tissue in diabetes and with age-related metabolic dysfunction (Minamino et al., 2009; Tchkonia et al., 2010; Xu et al., 2015a), osteoarthritic joints (Xu et al., 2016), the aorta in vascular hyporeactivity and atherosclerosis (Roos et al., 2016), and the lung in idiopathic pulmonary fibrosis (Schafer et al., 2017). Indeed, transplantation of small numbers senescent cells around the knee joint can cause osteoarthritis (Xu et al., 2016). Senescent cells can be eliminated from transgenic INK-ATTAC mice by administering a drug, AP20187, that does not affect normal cells. AP20187 activates the "suicide" protein, ATTAC, which is expressed only in senescent cells due to a senescence-induced promoter, $p16^{Ink4a}$ (Baker et al., 2011). Activating ATTAC alleviates multiple phenotypes in progeroid mice, naturally-aged mice, or mice with age-related diseases (Baker et al., 2011; Xu et al., 2015a; Roos et al., 2016; Schafer et al., 2017). These include adipose tissue and metabolic dysfunction, vascular hyporeactivity and calcification, chemotherapy-induced pulmonary fibrosis, and progeria-associated cataracts, lipodystrophy, and muscle dysfunction, among others. Thus, targeting senescent cells is a promising potential approach for delaying, preventing, or alleviating multiple age- and cellular senescence-associated conditions.

3. Senescence-associated Secretory Phenotype (SASP) Inhibitors

The composition of the SASP appears to vary depending on the cell type from which senescent cells originated, how senescence was induced, hormonal *milieu*, and presence of drugs including glucocorticoids, rapamycin, metformin, or JAK1/2 inhibitors (Xu et al., 2015b, Wiley et al., 2016; Laberge et al., 2012; Moiseeva et al., 2013; Laberge et al., 2015). Thus, the SASP is modifiable. At least in the case of rapamycin in senescent cultured fibroblast strains, suppression of the SASP is segmental: not all SASP components are down-regulated.

Metformin alleviates a range of age-related disorders in experimental animals and humans, including insulin resistance, diabetes, metabolic dysfunction, cardiovascular disease, cancer development and spread, and cognitive dysfunction (Huffman et al., 2016). It may even increase 5 year survival in elderly humans (Bannister et al., 2014). Rapamycin and related agents increase lifespan in mice, delay age-related adipose tissue loss, alleviate frailty in old mice, decrease heart failure, cancers, cognitive impairment, and immune dysfunction in mouse models, and enhance antibody response to influenza vaccination in elderly humans, among other effects (Harrison et al., 2009; Li et al., 2014; Majumder et al., 2012; Wilkinson et al., 2012, Zhang et al., 2014, Mannick et al., 2014; Bitto et al., 2016). Ruxolitinib, a JAK1/2 inhibitor in human application, alleviates age-related adipose tissue dysfunction, insulin resistance, and stem cell dysfunction in old mice (Xu et al., 2015a). Importantly, ruxolitinib reduces frailty even in very old mice (Xu et al., 2015b), a circumstance once believed to be impervious to interventions. Ruxolitinib partially corrects reduced strength, body weight, and appetite, which are features of frailty, in older humans with myeloproliferative

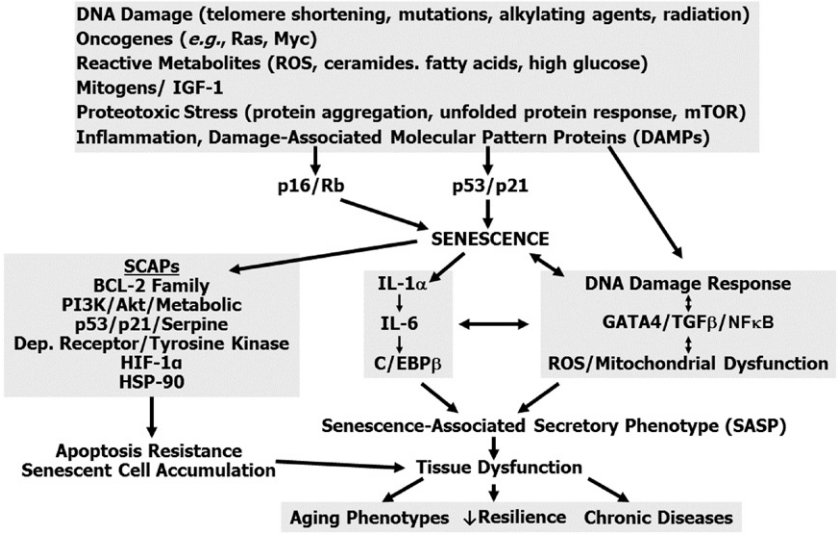

Fig. 1. Inducers, mediators, SCAPs, the SASP, and effects of senescent cells. Cellular senescence is a cell fate that, like replication, differentiation, or apoptosis, is 1) induced by a range of intra- or extracellular stimuli or combinations of them, 2) mediated by a cascade of transcriptional regulators that affect expression of multiple downstream target genes, and 3) associated with widespread changes in chromatin structure. Senescence takes days to weeks to become fully established and changes in quality over time. Senescent Cell Anti-apoptotic Pathways (SCAPs) shield senescent cells from their own pro-apoptotic SASP. These SCAPs constitute the Achilles' heel of senescent cells (Zhu et al., 2015b) that have turned out to be the critical key for developing the senolytic drugs and peptides discovered so far.

syndrome, without affecting their underlying hematological condition as manifested by clonal myeloproliferation assays (Verstovsek et al., 2010).

SASP inhibitors may alleviate conditions related to aging and cellular senescence when administered intermittently (Laberge et al., 2015). However, SASP inhibitors also act through other drug-specific mechanisms, making it difficult to disentangle effects on age-related phenotypes due to modulating the SASP from other "off-target" age-related processes that SASP inhibitors may affect. These other off-target mechanisms can also contribute to side effects that may not be related to suppression of the SASP. For example, rapamycin can induce insulin resistance through acting on the TORC2 complex (Lamming et al., 2012), cataract formation, and other effects. SASP inhibitors might see clinical application, especially in situations where short-term treatment is indicated, such as to help in promoting recovery after myocardial infarction or effectiveness of immunizations.

4. The Achilles' Heel of Senescent Cells

The first senolytic drugs, compounds that selectively eliminate senescent cells by causing apoptosis, were discovered using a hypothesis-driven approach that was reported in early 2015 (Zhu et al., 2015b). This approach was based on the observation that senescent cells are resistant to apoptosis (Wang, 1995), suggesting senescent cells have up-regulated pro-survival pathways that protect them from their own pro-apoptotic SASP (Zhu et al., 2015b). Up-regulation of these Senescent Cell Anti-apoptotic Pathways (SCAPs) might be related to senescence-associated mitochondrial dysfunction (SAMD) (see the accompanying review by T. von Zglinicki). An essential part of SAMD appears to be a decrease in mitochondrial membrane potential related to mitochondrial membrane permeabilization (Passos et al., 2010, Passos et al., 2007). SAMD could explain why senescent cells depend on upregulated pro-survival pathways and why they are more sensitive to drugs that interfere with these SCAP pathways than non-senescent cells.

The first SCAPs were identified through expression profiling of senescent *vs.* non-senescent human cells and confirmed in RNA interference studies (Zhu et al., 2015b). The pathways included networks related to BCL-2/BCL-X_L, PI3K/AKT, p53/p21/serpines, dependence receptors/tyrosine kinases, and HIF-1α. The PI3K/AKT and p53/p21/serpine pathways, which are closely inter-connected, are activated by IGF-1, perhaps explaining increased senescent cell accumulation in cells treated with IGF-1 (Tran et al., 2014) or mice treated with growth hormone, which causes IGF-1 production (Stout et al., 2014). Drugs that target these SCAPs were tested for senolytic activity. The tyrosine kinase inhibitor, dasatinib (D) and the flavonoid, quercetin (Q), were shown to induce apoptosis in senescent, but not non-senescent primary human preadipocytes and HUVECs, respectively. In combination, they caused apoptosis of both cell types. D targets the dependence receptors/tyrosine kinase SCAP and Q targets the BCL-2/BCL-X_L, PI3K/AKT, and p53/p21/serpine SCAPs.

Ten months later, two groups simultaneously reported that navitoclax (N; ABT-263), which targets components of the Bcl 2 pathway, is senolytic (Zhu et al., 2015a; Chang et al., 2016), as later confirmed by another group (Yosef et al., 2016). N is senolytic in HUVECs and IMR-90 cells, a culture-habituated human lung fibroblast cell stain, but not senescent primary human lung fibroblasts or human preadipocytes (Zhu et al., 2015a; Schafer et al., 2017). The related drug, TW-37, does not appear to be senolytic (Zhu et al., 2015a). TW-37 targets BCL-2 and BCL-W, as does N, but not BCL-X_L. N targets BCL-X_L, which had been shown to be required for survival of some types of senescent cells by RNA interference studies reported in the first article about senolytics in early 2015 (Zhu et al., 2015b). Recently, the specific BCL-X_L inhibitors A1331852 and A1155463 (Leverson et al., 2015), were found to be senolytic in human IMR-90 lung fibroblasts and HUVECs (Zhu et al., 2017). Fisetin, related to Q, was discovered to be senolytic (Zhu et al., 2017). Fisetin is an especially promising candidate because of its favorable side-effect profile. Piperlongumine, which is also related to Q, was noted to be senolytic *in vitro* in some senescent cell types (Wang et al., 2016). None of the individual agents reported so far selectively induces apoptosis of all senescent cell types. N, A1155463, and possibly A1331852 appear to be more toxic than D, Q, piperlongumine, or fisetin.

A number of additional senolytic drugs are currently being developed. Many of these were identified based on the strategy of targeting the SCAPs first reported in (Zhu et al., 2015b) as well as additional, as yet unreported SCAPs. For example, using the strategy of targeting SCAPs, a FOXO4-related peptide that inhibits the PI3K/AKT/p53/p21/serpine SCAP originally described in 2015 (Zhu et al., 2015b) was recently noted to be senolytic, at least in certain human fibroblast-like culture-habituated cell strains (Baar et al., 2017). Drawbacks are that peptides are not usually orally-active, unlike the small molecule senolytics discovered so far and *in vitro* senolytic activity of the peptide was only tested in three related cell strains, limiting conclusions about generalizability across senescent cell types, particularly those originating from truly primary human cells. Some of the most promising senolytic agents are already being moved through preclinical studies towards clinical application.

5. Senolytics: Demonstrating Decreased Senescent Cell Burden *In Vivo*

Using several different approaches in preclinical studies, the combination of D + Q as well as N were shown to clear senescent cells *in vivo* (Zhu et al., 2015a; Zhu et al., 2015b; Roos et al., 2016; Schafer et al., 2017; Chang et al., 2016). D and Q were administered together to mice, since each drug targets different types of senescent cells (Zhu et al., 2015b). Combining D + Q did not detract from effects found with each drug individually on susceptible senescent cell types *in vitro*. A single oral dose of D + Q reduced $p16^{Ink4a}$ expression and senescence-associated β-galactosidase (SA-βgal) activity in adipose tissue of 24 month old mice within 5 days compared to vehicle-treated animals. $p16^{Ink4a}$ $mRNA^+$ cells detected by FISH were also decreased by D + Q in old mice. A single treatment with D + Q reduced $p16^{Ink4a}$ mRNA in muscle and SA-βgal^+ cells in adipose tissue in legs of mice after senescence had been induced by localized ionizing radiation to mice 3 months previously. In another study, D + Q substantially reduced abundance of cells with telomere-associated DNA damage foci (TAFs) in aortae of 24 month old mice (Roos et al., 2016). TAFs appear to be a more specific marker for senescent cells than most others (Hewitt et al., 2012). D + Q also reduced TAF^+ cells in the media of younger hypercholesterolemic $ApoE^{-/-}$ high fat-fed mice (Roos et al., 2016). Additionally, D + Q reduced senescent cell burden in lungs of mice with bleomycin-induced senescent cell accumulation and consequent pulmonary fibrosis, as shown by decreases in expression of $p16^{Ink4a}$ and the SASP components, Mcp1, Il-6, Mmp-12, and Tgf-β (Schafer et al., 2017). Like D + Q, N also appears to be senolytic *in vivo*, as indicated by decreases in $p16^{Ink4a}$-driven luminescence in transgenic mice in which senescent cell burden had been induced by whole body radiation (Chang et al., 2016). mRNA levels of the SASP components Il-1a, Tnf-α, Ccl-5, and Cxcl-10 were decreased in lungs of these mice.

Almost any drug is eventually found to have off-target effects, beyond effects anticipated based on knowledge about their presumed targets. To prove that putative senolytic drugs actually cause alleviation of phenotypes through eliminating senescent cells *in vivo* over and above any off-target effects, a number of criteria need to be met. Merely showing that a candidate drug has effects that parallel those of genetic clearance, for example use of AP20187 in INK-ATTAC mice, is not sufficient to prove causation. Parallel effects of senolytics to genetic clearance can only suggest associations among administering the drug, reducing senescent cells, and alleviating phenotypes. Reasons for this include: 1) not all senescent cells necessarily have increased $p16^{Ink4a}$ expression and consequent susceptibility to clearance by AP20187. 2) Not every cell with substantial $p16^{Ink4a}$ expression is senescent. AP20187 might

clear non-senescent cells that have high $p16^{Ink4a}$ levels and therefore increased ATTAC, such as activated macrophages (Hall et al., 2016). 3) Targeting aging mechanisms other than cellular senescence can phenocopy effects of genetic or pharmacological senescent cell clearance without actually affecting senescent cells. For example, 17α-estradiol, which promotes considerable extension of mouse median and maximum lifespan but does not appear to profoundly affect characteristics of senescent cells, shares effects with those due to senescent cell clearance, including decreased adipose tissue inflammation and enhanced insulin responsiveness (Stout et al., 2016; Strong et al., 2016; Xu et al., 2015a; Xu et al., 2015b). 4) Hypothetically at least, genetic clearance of $p16^{Ink4a+}$ cells could have the same effects on a particular downstream phenotype as a drug that affects that same downstream phenotype directly, without acting through effects on truly senescent $p16^{Ink4a+}$ cells. For example, foam cells, derived from activated macrophages, accumulate in atherosclerotic lesions. Activated macrophages have increased $p16^{Ink4a}$ expression (Hall et al., 2016). In 2007 it was demonstrated that ablating macrophages reduces plaque development and promotes plaque stabilization (Stoneman et al., 2007). Thus, genetically clearing $p16^{Ink4a}$-expressing cells may alleviate atherosclerosis through reducing macrophage-foam cells, rather than an effect principally through classically senescent cells. These points imply that showing genetic clearance of senescent cells has phenotypic effects resembling those of a potentially senolytic drug is not sufficient to prove that the drug causes those effects because it is senolytic. Parallels between effects of genetic clearance of senescent cells and those of a particular drug are only consistent with the possibility that effects of the drug *in vivo* could be due to its being senolytic. Furthermore, showing that a drug causes apoptosis of senescent cells *in vivo*, while consistent with the drug's being senolytic, does not prove it affects phenotypes because it is senolytic.

The ideal way to prove that a drug causes a phenotype by acting through a particular pathway to is to include control experiments in which the target of the drugs has been disabled, for example by knocking out the drug target in mutant mice through an inducible shRNA. This approach also allows determination of the contribution of "off-target" effects of the drug. However, with respect to senolytics, so far this approach is not feasible. The targets of current senolytic drugs, such as BCL-2 family members, cannot be knocked out without severe consequences, such as disrupting basic cellular function or causing cancer. Short of knocking out the target of a drug, another approach for showing if a drug actually causes alleviation of senescence-associated phenotypes due to senescent cell clearance is to follow a modified set of Koch's postulates. These are: 1) senescent cells must be present in the individuals in whom the phenotype occurs, 2) clearing these senescent cells genetically or pharmacologically must be associated with alleviation of the phenotype, 3) introducing senescent cells into individuals without the phenotype (*e.g.*, by transplantation) must cause the phenotype, 4) clearing these transplanted cells genetically or pharmacologically should prevent or reverse that phenotype. Whether any senolytics reported so far meet all of these criteria needs to be tested, and 5) effects on the phenotype should persist long after the drug is no longer present (since senolytics act by altering cellular composition rather than needing to be present continuously to act on a receptor, enzyme, or other target).

6. Senolytics: Pre-clinical Studies Demonstrating Phenotype Alleviation

In mice, senolytics alleviate a range of conditions that have been associated with effects of senescent cells. So far, these include effects on cardiac, vascular, metabolic, neurological, radiation-induced, chemotherapy-induced, renal, and pulmonary function as well as mobility and frailty in several animal models (Zhu et al., 2015b; Xu et al., 2015a; Roos et al., 2016; Schafer et al., 2017; Chang et al., 2016; Baar et al., 2017 #4270).

The first demonstration that clearing senescent cells has any effects on phenotypes in naturally-aged animals, as opposed to the progeroid INK-ATTAC;$BubR1^{H/H}$ mice from which $p16^{Ink4a+}$ senescent cells had been reduced by AP20187 (Baker et al., 2011), was reported in 24 month old mice treated with D + Q (Zhu et al., 2015b). In these mice, cardiac ejection fraction and fractional shortening, measured by echocardiography, were improved within 4 days after a single course of D + Q, which was sufficient to clear senescent cells from multiple tissues in the same animals. In these old mice, D + Q also enhanced aortic vascular reactivity, with modest improvement in endothelium-dependent relaxation elicited by acetylcholine and substantially-improved vascular smooth muscle cell relaxation in response to nitroprusside. This alleviation of vascular hypo-reactivity in old mice was confirmed in old mice as well as hypercholesterolemic high fat-fed $ApoE^{-/-}$ mice that develop an atherosclerosis-like state in another study (Roos et al., 2016). Genetic removal of senescent cells in INK-ATTAC mice paralleled the effects of D + Q. Furthermore, vascular calcification was reduced in these mice.

Importantly, there are no current treatments available for the many atherosclerotic or elderly subjects with hypo-reactivity and calcification of major blood vessels. These vascular changes are associated with and may contribute to systolic hypertension (and therefore stroke risk), atrial fibrillation, and congestive heart failure in elderly or atherosclerotic humans (Chaikriangkrai et al., 2017; Lanzer et al., 2014). Perhaps senolytics will become the first effective treatment for this common condition. Subsequent to the studies showing that decreasing senescent cell abundance improves cardiac function and alleviation of vascular hypo-reactivity, reducing macrophages with senescent-like properties by genetic targeting of $p16^{Ink4A+}$ cells or administering N was suggested to be an approach for stabilizing plaques, at least in the atherosclerosis-like state in LDL-$R^{-/-}$ mice fed a high fat diet (Childs et al., 2016). This adds another option for slowing plaque development or stabilizing plaques to the treatments that are already available for this indication, such as statins. This study confirmed an earlier one demonstrating that suppressing activated macrophages stabilizes atherosclerotic plaques in CD11b diphtheria toxin receptor transgenic mice (Stoneman et al., 2007). Thus, senolytics may have multiple applications in cardiovascular disease.

Intermittent administration of D + Q alleviated frailty, neurological dysfunction, osteoporosis, and vertebral disk degeneration related to loss of glycosaminoglycans in $Ercc^{-/\Delta}$ mice, which have an accelerated aging-like state (Zhu et al., 2015b). Furthermore, in mice with impaired mobility due to radiation of one of their legs 3 months previously, treadmill endurance improved within 4 days after completing a single course of D + Q. This improvement persisted for at least 7 months. D + Q has an elimination half-life of a few hours. These outcomes following intermittent or single courses of agents with short elimination half-lives are consistent with the long-lasting type of effect expected from reducing senescent cell abundance, as opposed to what would be expected if D + Q had to be continuously present to suppress or activate cellular processes by occupying a receptor or acting on an enzyme. Thus, intermittent rather than continuous treatment with senolytics may be effective in alleviating senescence-related diseases or disorders, allowing these agents to be administered during periods of good health and potentially decreasing risk of side-effects.

Idiopathic pulmonary fibrosis is frequently a fatal condition for which treatment options are limited. Telomeres are shortened and senescence increased in lung fibroblasts from patients with this disease, with increased SASP factor expression in the lungs (Schafer et al., 2017). The SASP appears to contribute to the progressive fibrosis that contributes to lung dysfunction in idiopathic pulmonary fibrosis. Pulmonary fibrosis can also develop in patients treated with bleomycin for cancers. D + Q selectively causes apoptosis in human primary lung fibroblasts *in vitro*, while N is less effective. In mice with pulmonary fibrosis caused by bleomycin, D + Q decreased lung senescent cell abundance, reduced lung inflammation and fibrosis, and alleviated

respiratory dysfunction (Schafer et al., 2017). Thus, senolytics hold promise for treating pulmonary fibrosis.

Although the main impetus behind developing senolytics and other interventions that target fundamental aging mechanisms is to enhance healthspan, some evidence suggests that reducing senescent cell burden might increase lifespan as well. In a key study, caloric restriction, an invention that increases maximum lifespan in mice, was associated with decreased $p16^{Ink4a+}$ and SA-βgal$^+$ cell abundance in mice (Krishnamurthy et al., 2004). In that and another study, single gene mutations that increase maximum lifespan in mice were associated with decreased senescent cell burden (Krishnamurthy et al., 2004; Stout et al., 2014). Furthermore, a linear association between senescent cell burden and mean and maximum lifespan over a wide range of ages has been demonstrated in mice (Jurk et al., 2014). An increase in maximum lifespan, as opposed to median lifespan, is perhaps the best indication that an aging mechanism has been targeted (Harrison et al., 2009). In a later study, an increase in median lifespan was suggested in INK-ATTAC mice in which $p16^{Ink4a+}$ cells had been targeted by repeated intraperitoneal (ip) injections of AP20187 beginning in mid-adulthood at the equivalent of 40 years of human age, compared to vehicle-treated controls (Baker et al., 2016). However, some of the groups of mice were short-lived, with the treated animals having a median lifespan about the same as that of control animals in other colonies of mice of the same background strain. Thus, AP20187 could have affected the processes that caused the short lifespan of the mice in that colony, protected against effects of repeated ip injections, or targeted cancer cells, in addition to any effects on aging itself. No increase in maximum lifespan was demonstrated. Definitive studies are still needed to prove if clearing senescent cells genetically or with senolytics increases lifespan. It will be especially interesting to determine if clearing senescent cells enhances survival in already old mice, a more readily translatable situation than one in which clearance would have to be initiated in young, asymptomatic individuals to have an effect much later in life. It will also be important to do so using senolytics, a translatable approach, instead of genetically-modified mice in which $p16^{Ink4a+}$ cells are targeted.

There is emerging evidence that senolytics may alleviate a number of other conditions in as yet unpublished pre-clinical studies, including diabetes in obesity, age-related lipodystrophy, side effects of chemotherapy, cognitive dysfunction, renal dysfunction, osteoarthritis, chronic obstructive pulmonary disease, tobacco-related lung dysfunction, cataracts, macular degeneration, glaucoma, skin disorders, and others.

7. Biomarkers of Senescent Cell Burden

To conduct clinical trials with senolytics, it will be important to have ways to track changes in senescent cell burden (Huffman et al., 2016). It might be feasible to do so using biopsies, blood assays, other body fluids, and imaging, but more research on developing and optimizing assays needs to be done and reported. Complicating matters, the definition of cellular senescence is somewhat vague, particularly since several potentially pro-inflammatory cell types, such as macrophages or osteoclasts as well as pre-cancerous or cancer cells share many characteristics of senescent cells and could arguably be the same as what are currently regarded as being senescent cells (Hall et al., 2016). Few tissue assays are very sensitive or specific for senescent cells. For example, SA-βgal can be increased in activated macrophages and $p16^{INK4A}$ is high in a number of differentiated cell types and pro-inflammatory cells, such as macrophages, and conversely might not be high in all cells that could be considered to be senescent. Generally, a combination of assays may be needed to estimate senescent cell burden in tissue samples, such as SA-βgal$^+$ cell numbers, tissue levels or proportions of cells expressing $p16^{INK4A+}$, $p21^{Cip1}$, or SASP factors (*e.g.*, IL-6, PAI-1, MMP's, *etc.*), DNA damage foci (γH2.AX, *etc.*), DAMPs (such as HMGB-1 localization), and cells with senescent-associated distension of satellites (SADS) or TAFs. It is also unknown if senescent cell abundance in biopsies of skin, adipose tissue, or other tissues, cheek swabs, or cells in blood reliably reflect senescent cell abundance overall or in the tissues affected by particular senescence-associated diseases being investigated. Similarly, whether levels of SASP factors or senescence-associated microRNA's in plasma or blood cells reflect senescent cell burden is not clear. Work needs to be done to establish, optimize, and validate these assays. Novel assays, such as of the microvesicles shed into blood or urine by senescent cells, need to be developed and optimized for use in clinical trials of senolytic drugs. Imaging methods need to be developed to identify and follow localized collections of senescent cells in clinical trials of senolytics for such conditions as idiopathic pulmonary fibrosis (Schafer et al., 2017) or osteoarthritis (Xu et al., 2016). To begin with, a constellation of tissue and blood tests will need to be done in clinical trials to establish if candidate senolytics decrease senescent cell numbers *in vivo* in humans.

8. Translating Senolytics into Clinical Treatments

Healthspan, lifespan, or other very long-term potential endpoints for clinical trials of interventions that target basic aging processes, including SASP-inhibitors or senolytics, would be difficult or next to impossible to study for reasons that are obvious, as would endpoints occurring in old age as a consequence of beginning to administer a drug in adulthood or middle-age (Burd et al., 2016; Justice et al., 2016; Kirkland, 2013; Kirkland, 2016; Kirkland and Tchkonia, 2015; Kirkland and Peterson, 2009; Newman et al., 2016; Tchkonia et al., 2013; Zhu et al., 2014). Initial trials of senolytics or other agents that target fundamental aging processes will need to test effects on endpoints that can be measured weeks to a couple of years after initiating treatment. Furthermore, because the risk:benefit ratio must favor benefits for the ethical conduct of clinical trials, new interventions would have to be tested in situations in which side-effects would be considered to be acceptable. In diseases for which no effective treatment is available, some side effects may be acceptable in individuals who are already symptomatic or who are almost certain to become symptomatic within a short time. If any consequential side effects are anticipated, the treatment would also need to address a problem that would cause serious harm if left untreated.

Based on these premises, a group of possible clinical trials scenarios were devised for testing senolytics and other agents that target basic aging processes by the Geroscience Network, a consortium of aging centers funded by the NIH to map procedures for translating these interventions from bench to the bedside (Burd et al., 2016; Justice et al., 2016; Kirkland, 2016; Newman et al., 2016). These clinical trials scenarios include the following:

1. Simultaneous Alleviation of Co-morbidities. For example, in elderly subjects with multi-morbidity, could a candidate senolytic alleviate 3 or more of: mild diabetes, atherosclerosis, hypertension, mild cognitive impairment, sarcopenia, osteoarthritis, mild renal insufficiency, *etc.* occurring within a subject? In such a scenario, short-term outcomes such as glucose tolerance tests, carotid flow velocity, blood pressure, timed walking ability, joint pain inventories, and creatinine clearance could be measured following administration of the senolytic or placebo. Combinations of skin biopsy $p16^{INK4A}$ and other biomarkers of senescent cell burden would need to be assayed before and at various times after administering the drug, as discussed above.
2. Delaying Accelerated Aging-like Conditions. In this scenario, effects of a candidate senolytic agent on frailty and endurance measures, metabolic parameters, assays of cardiovascular and cognitive function, and other outcomes could be determined in: childhood cancer survivors, bone marrow transplant survivors, subjects with progeroid syndromes, patients with diabetes due to obesity, or in conditions related to latent viruses, such as HIV. Again, as with the other clinical trials scenarios, assays of senescent cell burden would be needed.
3. Treating Conditions with Localized Cellular Senescence. Particularly for potentially toxic senolytic drugs, like some BCL-2 family

inhibitors, that could be relatively risky at systemic concentrations sufficient to kill senescent cells selectively, high local concentrations could be achieved by injections, drops, aerosols, or topical solutions. Such conditions include: osteoarthritis, fracture non-union, glaucoma and macular degeneration, sites subjected to therapeutic radiation, the lungs in idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, or damage due to tobacco, or atherosclerotic plaques (by catheterization), among others.

4. Intervening for Otherwise Fatal Conditions. A higher degree of risk due to a new, untested therapy is acceptable if the intervention could benefit those with fatal conditions for which safer effective treatments are not available. Such conditions include: idiopathic pulmonary fibrosis, primary sclerosing cholangitis, some cancers, or HIV dementia, among others.
5. Increasing Resilience or Clinical Stresses in Pre-frail Subjects. Capacity to recover following a medical or physiological stress generally declines with aging, as reviewed in (Kirkland et al., 2016). Potentially, senolytics may increase resilience, providing an opportunity for testing candidate drugs in short-term clinical trials. An example might include the use of such agents before chemotherapy in an effort to improve recovery and to allow providing higher, more effective doses of chemotherapy to elderly frail subjects with high initial burdens of senescent cells. Other scenarios might include use of senolytic agents or other interventions that target basic aging processes to accelerate recovery after elective surgery, bone marrow transplantation, therapeutic radiation, pneumonia, or myocardial infarction or to enhance immune response to influenza vaccination. Interestingly, brief administration of lower doses of a drug related to rapamycin, which inhibits the SASP among other effects, two weeks before influenza vaccination resulted in better influenza antibody generation in elderly subjects (Mannick et al., 2014). Perhaps a single course of a senolytic agent before vaccination might do the same.
6. Frailty. Since senolytics are effective in reducing frailty, at least in progeroid mice (Zhu et al., 2015b), and the JAK1/2 SASP inhibitor, ruxolitinib, reduced frailty in naturally-aged, already frail mice (Xu et al., 2015b), senolytics and SASP inhibitors could be tested in clinical trials in frail older subjects to determine if they alleviate slow gait, decreased strength, or sarcopenia or delay loss of independence (Table 1).

Table 1
Senolytic agents and the Senescent Cell Anti-apoptotic Pathways (SCAPs) and cell types they target.

Senolytic	SCAP	Target senescent cell types
Dasatinib (D)	Dependence receptor/Src kinase/tyrosine kinase	Primary human and mouse preadipocytes (adipose-derived stem cells)
Quercetin (Q)	Bcl-2 family, p53/p21/serpine, & PI3K/AKT	HUVECs, mouse bone marrow-derived mesenchymal stem cells
D + Q	Dependence receptor/Src kinase/tyrosine kinase, Bcl-2 Family, p53/p21/serpine, & PI3K/AKT	As for D + Q plus primary human lung fibroblasts and mouse embryonic fibroblasts
Navitoclax (ABT263)	Bcl-2 family (Bcl-2, Bcl-xL, Bcl-w)	IMR-90 Cells, HUVECs
Piperlongumine	p53/p21 & Bcl-2 family (Bcl-2 binding component 3, also known as PUMA)	WI-38 Cells
A1331852	Bcl-2 family (Bcl-xL)	IMR-90 Cells, HUVECs
A1155463	Bcl-2 family (Bcl-xL)	IMR-90 Cells, HUVECs
Fisetin	PI3K/AKT	HUVECs
FOXO4-related peptide	Bcl-2 family & p53/p21/serpine	IMR-90, WI-38, BJ cells

9. Conclusions

There is a possibility that senolytics and SASP inhibitors could be transformative, substantially benefiting the large numbers on patients with chronic diseases and enhancing healthspan. That said, as this is a very new treatment paradigm, there are many obstacles to overcome. Treatments that appear to be highly promising in mice frequently fail once clinical trials start, with lack of effectiveness in humans compared to mice related to the unique aspects of human biology, unforeseen side-effects, and a host of other issues. At least one reassuring advantage of targeting cellular senescence is the conservation of fundamental aging mechanisms such as senescence across mammalian species. In diseases like Alzheimer's dementia, atherosclerosis, or non-injury-related osteoarthritis, which do not occur naturally in mice, translation from genetically- or surgically-induced mouse models of these conditions to humans is more likely to fail than conditions that are more evolutionarily conserved, such as aging or obesity-related diabetes. Furthermore, unlike the situation for developing drugs to eliminate infectious agents or cancer cells, not every senescent cell needs to be eliminated to have beneficial effects. Unlike microbes or cancer cells, senescent cells do not divide, decreasing risk of developing drug resistance and, possibly, speed of recurrence. With respect to risk of side-effects, single or intermittent doses of senolytics appear to alleviate at least some age- or senescence-related conditions in mice. This suggests that intermittent treatment may eventually be feasible in humans, perhaps given during periods of good health. If so, this would reduce risk of side-effects, such as delayed wound healing, since the drugs could be held until skin wounds have healed.

Progression from the discovery of the first senolytics to being at the point of initiating proof-of-concept clinical trials has been remarkably fast. With sustained effort and a lot of luck, these agents could be transformative.

Outstanding Questions

A number of important issues remain to be investigated, including the following:

What are the side-effects of senolytics? Apart from potentially delaying wound healing, as suggested from studies of genetic clearance of senescent cells from mice (Demaria et al., 2014), little information is available about possible side-effects of senolytics besides known side-effects of repurposed senolytic compounds when used in other contexts. Potential side-effects of senolytics as a class need to be defined.

How quickly do senescent cells re-accumulate after removal in various disease states, tissues, or with aging? Senolytic agents are effective when administered intermittently in pre-clinical models. Information about rates of senescent cell generation, re-accumulation, and removal mediated by the immune system could help in optimizing frequency of treatments with senolytic drugs.

What are the best senolytic agents or combinations for treating particular conditions? Do senolytics have additive effectiveness when combined with other agents that affect fundamental aging mechanisms, such as 17α-estradiol (Stout et al., 2016), or lifestyle modifications, such as exercise (Schafer et al., 2016)?

More remains to be learned about the potential optimal times for initiation of senolytic treatments for each disorder, identification of all conditions that can be alleviated by senolytics, and improving bioavailability and other pharmacological characteristics of senolytics.

To what extent does each senolytic agent alleviate phenotypes by reducing senescent cells as opposed to off-target effects? Formal proof is needed that drugs that have been shown to induce apoptosis of senescent cells and reduce senescent cell abundance actually cause phenotype alleviation partly or completely through their senolytic effects.

Does senescent cell clearance by genetic approaches or senolytics increase median or maximum lifespan? Although there are suggestions that genetic clearance of senescent cells beginning from mid-adulthood

might increase median lifespan in mice, this is not certain, as discussed above. A definitive study excluding potential artifacts and alternative explanations is needed to show if genetic clearance or senolytics truly affect median or maximum lifespan.

Can cells harboring latent viruses, such as HIV, be eliminated by senolytics? Host reservoir cells containing integrated viral DNA and consequently with transcription of viral DNA into RNA's, have activation of processes, including increased interferon, which should cause apoptosis of the host cell. However, like senescent cells, these host cells resist apoptosis, suggesting that drugs such as D + Q, BCL-2 inhibitors including N, A1331852, or A1155463, and related drugs that target SCAPs could kill these cells, much as they kill pro-inflammatory senescent cells by temporarily disabling pro-survival pathways. This could be a way to eradicate HIV, which current treatments only suppresses.

Are beneficial effects of senolytics due to their causing apoptosis not only of classically senescent cells, but also cells harboring HIV, CMV, or other latent viruses, precancerous cells, cancer stem cells, or early stage cancers? Consistent with these possibilities, the senolytic drugs published so far have shown anti-cancer effects *in vitro* or *in vivo*.

As many interventions that target fundamental aging processes vary in their effectiveness between sexes in mice (Austad and Bartke, 2015), effects of sex on responses to senolytic agents and SASP inhibitors needs to be determined.

Does targeting FOXO4/p53 cause apoptosis of all types of senescent cells or only the subset of senescent cells that depend on the Bcl-2 family- and p53-related SCAPs originally described in (Zhu et al., 2015b)? Is FOXO4 increased in senescent cell types beyond the 3 culture-habituated fibroblast-like cell strains tested so far (Baar et al., 2017), such as senescent primary human preadipocytes or other truly primary cells that do not depend principally on the BCL-2 family SCAP to resist apoptosis (Zhu et al., 2015a; Zhu et al., 2015b)?

Search Strategy and Selection Criteria

Articles referred to were selected based on searching PubMed, MEDLINE, and the internet for peer-reviewed publications about "senolytics", "cellular senescence", and related search terms as well as by searching based on names of investigators in the field.

Conflicts of Interest

J.L.K., T.T., and Mayo Clinic have a financial interest related to this research. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and is being conducted in compliance with Mayo Clinic conflict of interest policies.

Funding

This work was supported by NIH grant R37 AG013925 (J.L.K.), the Connor Group, and the Noaber and Ted Nash Foundations (J.L.K.).

Acknowledgments

The authors are grateful for the assistance of Jacqueline L. Armstrong.

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