Review

Gerontogen as the Toxicology of Aging

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Posted Date: 10 April 2024

doi: 10.20944/preprints202404.0659.v1

Keywords: aging; senescence; toxicology; gerontogen; senotherapy

 doi:10.20944/preprints202404.0659.v1

Review

Gerontogen as the Toxicology of Aging

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Abstract: Aging causes various degenerative diseases in the older adult population. Senescence, a state of permanent cell-cycle arrest accompanied by the production of various pro-inflammatory factors known as senescence-associated secretory phenotype (SASP), is considered a significant contributor to the aging process and its chronic diseases. Ample evidence showed that various stressors could induce senescence, including DNA damage, telomere shortening and damage, activation of oncogenes, and mitochondrial dysfunction. Numerous credible findings indicate that environmental agents can induce senescence, including UV radiation, a high-fat diet, heavy metal exposure, and cigarette smoke. These findings posed the possibility that many more environmental agents may induce senescence and accelerate aging but remain unidentified. Senescence also becomes more intriguing due to its promising future as a pharmacological target to blunt the detrimental effects of aging and prevent aging-related diseases, either by eliminating the senescent cells or by controlling the SASP. On the other hand, investigating senescence has become more intricate due to the need for a multi-marker approach and translation in vivo analysis. This review will discuss senescence and its biomarkers, how to identify gerontogens in vivo, and also the development of senotherapy that targets senescent cells.

Keywords: aging; senescence; toxicology; gerontogen; senotherapy

1. Introduction

Age constitutes a non-modifiable risk factor for a multitude of diseases, including Alzheimer's disease and associated dementias [1], hypertension [2], type 2 diabetes mellitus [3], and cancer [4]. A systematic review reveals that multimorbidity affects a significant portion of the global population, particularly individuals aged 65 and older [5]. Medical research was previously focused on developing distinct treatments for these diseases. Understanding how aging can facilitate the development of chronic diseases, on the other hand, will enable the development of novel therapeutic approaches that target the aging process in order to treat numerous chronic diseases [6].

Aging itself is a complex process and there is no single molecular pathogenic pathway that can account for all features of aging [7]. However, accumulating evidences have shown that cellular senescence plays a significant role in many aging-related diseases [8,9]. Cellular senescence is a state of permanent cell-cycle arrest in response to various stressors such as oxidative stress, telomere shortening, DNA damage, and oncogene activation, or as part of physiological processes [10,11]. In senescent cells, cyclin-dependent kinase inhibitors (CDKI) p21 and p16 are found in higher concentration, which hinders the progression of the cell cycle from the G1 to the S phase [11]. Although senescence is supposed to limit tumor formation, the presence of senescent cells is now known to promote cancers and other clinical diseases [12,13]. This is due to the release of pro-inflammatory cytokines, chemokines, and growth factors such as IL-6, IL-8, MIP-1, VEGF-1 and many others, collectively known as the senescence-associated secretory phenotype (SASP) [7,14]. Barinda et al. found that parabiosis between wild-type mice and mice with senescent endothelium cells reduces the insulin sensitivity of the wild-type mice. As a blood-soluble factor, SASP mediates the systemic metabolic anomalies in wild-type mice [15]. Through the paracrine pathway, SASP can promote senescence in neighboring cells, creating a positive feedback loop [16].

 doi:10.20944/preprints202404.0659.v1

Senescent cells are known to accumulate with aging. Senescence is hypothesized to influence aging via at least two separate mechanisms. First, homeostasis and regeneration capability are hampered by reduced cell proliferation brought on by increased production of anti-proliferative proteins such as cyclin-dependent kinase inhibitors (CDKI). Second, the pro-inflammatory SASP will cause chronic inflammation and carcinogenesis, both of which are age-related symptoms [7].

Individual variations in the rate of aging may be accounted for in part by environmental agents known as gerontogens, which have the capacity to accelerate the molecular aging process. However, the current body of research on gerontogen remains scarce [6,7]. One of the challenges in identifying gerontogens is the lack of biomarkers to examine aging at the molecular level [7]. The measurement of senescence-related events, such as β-galactosidase staining, SASP levels in tissues, leukocyte telomere length, and p16 and p21 expression, is currently being investigated as a potential biomarker of aging [7,11]. To date, however, there has been no universal marker specifically expressed only by senescent cells. Even p16, which is considered more specific as a marker for senescence, was known to be expressed by certain non-senescent cells and also not expressed by all cells that undergo senescence [17]. Other markers also have their shortcomings. Therefore, the selection of the right marker to detect senescence is also still a challenge.

Growing evidences showed that cellular senescence can be exploited as a therapeutic target to slow or prevent aging-related tissue dysfunction [9,18]. In mice, eliminating p16-expressing senescent cells slowed the emergence of aging-related clinical diseases such as cataracts [19]. The removal of senescent cells by prolonged administration of senolytic drugs can also alleviate vasomotor dysfunction in naturally aged and atherosclerotic mice. Elimination can also diminish osteogenesis markers in plaque, hence reducing plaque calcification in the mice tunica intima [20]. These investigations may help establish a link between senescence, aging, and the pathophysiology of chronic diseases that are common in older people and serve as a foundation for medication development to prevent or reverse aging.

This paper will discuss senescence and its biomarkers, how to identify gerontogens, and also the development of senotherapy that targets senescent cells induced by gerontogens.

2. Senescence Inducers

2.1. DNA Damage

DNA damage, particularly DNA double-strand break (DSB), can cause senescence. This damage triggers the DNA damage response (DDR), a checkpoint that prevents cell cycle progression and genetic information loss in daughter cells. Proteins involved in DDR accumulate at the damage site and form foci due to chromatin modifications. These include phosphorylated histone H2AX and related proteins such as MDC1, 53BP1, and activated ataxia kinase mutated telangiectasia (ATM). These foci mark areas with DNA damage, leading to check-point and cell-cycle arrest until repair is achieved. If DNA damage persists, DDR signaling and cell cycle arrest continue, leading to senescence. At the end of the DDR signaling pathway, ATM activates p53, triggering the expression of CDKI p21, resulting in cell cycle termination [21].

2.2. Telomere Shortening and Damage

As cells replicate, the repeating regions at the end of linear chromosomes, the telomere, become shorter. When it is too short, the loss of protective structure in telomeres will be detected as DNA damage that is capable of activating DDR. Just like DNA damage, DDR activation activates p53 and causes the cell cycle to stop [21,22].

The mechanism by which telomere shortening can be identified as DNA damage is because the shortened telomere may no longer bind TRF2, one of the protective proteins that are present in telomeres, in adequate amounts. Van Steensel et al. found that TRF2 gene mutations cause senescence. This is supported by in-vitro studies that showed TRF2 depletion in shortened telomeres can cause senescence [23]. The protective effect of TRF2 to prevent senescence was proved by

 doi:10.20944/preprints202404.0659.v1

Karlseder et al. that found overexpression of TRF2 can protect short telomeres and lower the senescence setpoint from 7kb to 4kb [24].

In contrast, the telomeres of non-dividing cells, such as cardiomyocytes, adipocytes, neurons, osteocytes, and osteoblasts, do not shorten. Nevertheless, chronic activation of DDR can also occur when cells encounter DNA damage in the telomeres area, for instance, due to exposure to genotoxic substances [21].

2.3. Oncogene Activation

Oncogene activation and tumor suppressor gene inactivation will activate the P53/P21 and P16 pathways [25]. Initial activation of oncogenes induces a hyperproliferative phase that is fundamentally linked to the interruption of DNA replication that triggers DDR activation [21]. Bartkova et al. demonstrates that replicative stress in oncogene-induced senescence induces premature termination of the replication fork, resulting in DNA double-strand breaks [26].

2.4. Mitochondrial Dysfunction

Mitochondrial dysfunction can lead to the occurrence of senescence through the activation of AMPK and the p53 pathway. Mitochondria converts NADH to NAD+, so that a decrease in NAD+/NADH ratio indicates mitochondrial dysfunction. NAD+/NADH ratio regulates energy production because NAD+ is necessary for glycolysis while NADH inhibits it. When the ratio of NAD+ to NADH falls, as an energy sensor, AMPK is activated [27]. Senescence caused by mitochondrial dysfunction is referred to as mitochondrial dysfunction-induced senescence (MiDAS), and exhibits a distinct set of SASP compared to other types of senescence [13].

3. Biomarkers of Senescence

Senescence biomarkers can be used to identify aging due to the close relationship between aging and senescence. Two or more senescent cell markers are recommended for detecting senescence [28–30]. Gonzalez-Gualda et al. advocate utilizing three types of markers to indicate three different processes: cell-cycle arrest, structural changes, and markers unique to the subtypes of senescence to be researched, such as DNA damage, SASP factors, or ROS levels [28]. Kohli et al. suggest two-phase senescence marker measurement. Senescence is validated using universal markers in the first step, whereas subtypes are identified in the second phase [30]. To date, however, the selection of markers has been left to the discretion of the researcher [29].

Senescence markers are observable both in vitro and in vivo/ex vivo. However, there are significant limits to in-vivo detections. In contrast to cell culture, the majority of organismal cells experience quiescence or are already terminally differentiated. Quiescence is a state of transient cell cycle arrest. In contrast to senescence, quiescent cells are capable of re-entering the cell cycle when triggered by the appropriate mitogen [7]. Meanwhile, cells that have reached terminal differentiation will perform specified activities and cease to proliferate [11]. Therefore, markers related to cell growth and DNA replication become invalid for the in-vivo detection of senescence. Moreover, changes in morphology or an increase in cell size that can be noticed in vitro are typically not preserved in vivo due to structural and architectural constraints. Cell cycle-associated proteins can also be expressed by non-senescent cells and certain processes such as inflammation, so the reliability of these markers is also limited [28].

3.1. Markers for Cell-Cycle Arrest, Cell Proliferation, and DNA Replication

The cell-cycle arrest is a key hallmark of senescence, marked by increased p16, p21, and p53 proteins and decreased phosphorylated Retinoblastoma protein (pRb). In senescence, the p16/Rb and p53/p21 axes cause cell-cycle arrest. After both processes, hypophosphorylated Rb interacts with the E2F transcription factor. E2F induces cell cycle progression and S phase. When attached to Rb, E2F cannot interact with its target gene's promoter, preventing gene transcription needed for replication and stopping the cell cycle [28].

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On the p16/Rb axis, senescence-inducing stimuli will activate the INK4a/ARF locus, hence increasing p16 levels. The p16 protein, which is a cyclin-dependent kinase inhibitor (CDKI), inhibits the development of the CDK4-Cyclin D complex, resulting in the dephosphorylation and stability of the Rb-E2F complex and the cessation of the cell cycle. Typically, the p16/Rb axis is activated during senescence produced by replicative stress, reactive oxygen species, and oncogene activation. It is believed that the p16/Rb pathway has a more critical role in sustaining senescence. Activation of the p53/p21 axis begins with the phosphorylation of p53 (p-p53), which induces the overexpression of the CDKI p21. p21 inhibits the formation of the CDK2-Cyclin E complex, which is responsible for Rb dephosphorylation and E2F sequestration, hence halting the cell cycle. In senescence triggered by replicative stress, DNA damage, reactive oxygen species, and oncogene activation, the p53/p21 axis becomes activated. This route is hypothesized to be activated during the early stages of senescence [28].

Positive ex-vivo results should be interpreted with caution because p16 is also expressed by aged lymphocytes, cells that undergo Rb inactivation (e.g., tumor cells), and certain physiological circumstances such as inflammation, clastogen exposure, and wound healing. Currently available antibodies for histochemical detection of p16 are likewise unreliable. To address this, researchers have designed experimental mice containing a reporter gene on the p16 promoter. In addition to p16, the use of the marker p53/p21 ex vivo is limited since transient damage can activate p53 for the DNA repair process in quiescent cells and also plays a role in the apoptosis process [28].

The cell-cycle arrest also needs to be evaluated through examination of cell proliferation and/or DNA replication. Cell proliferation can be measured by performing cell counting over time to obtain cell growth profiles in vitro [28]. Another proliferative marker is the Ki-67 protein which can be examined through immunostaining. Ki-67 is a nuclear protein that can be found in all active phases of the cell cycle or as cells proliferate [28,31]. However, the use of proliferative markers such as Ki-67 in living organisms has limitations because most cells are in a state of quiescence or have reached final differentiation [28].

DNA synthesis examination can be done by incorporating modified nucleotides, such as 5-ethynyl-2′-deoxyuridine (EdU) or 5-bromo-2-deoxyuridine (BrdU), into the DNA of replicating cells. The cell cycle arrest is defined by a reduction of replicative ability, which means that fewer nucleotides will be integrated throughout the replication process. Fluorescent imaging or flow cytometry analyses are utilized to detect these nucleotides [28].

3.2. Senescence-Associated β-Galactosidase (SA-β-Gal)

β-Gal is a hydrolase enzyme found in lysosomes that catalyzes the hydrolysis of β-galactosidase into monosaccharides. The majority of cells have endogenous β-galactosidase, which is active at pH 4.0. When the pH of lysosomes is elevated to 6, the activity of β-galactosidase expressed by non-senescent cells is reduced, whilst the activity of β-Gal expressed by senescent cells is increased. To discriminate senescent cells from non-senescent cells, the analysis of β-Gal associated with senescence (SA-β-Gal) is conducted at a suboptimal pH (6.0) [28,32].

SA-β-Gal is one of the most commonly employed biomarkers for histological or cytological detection of senescence. SA-β-Gal can be examined in vitro by adding the chromogenic substrate 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (XGal) which forms a blue precipitate. Additionally, SA-β-Gal testing can be performed on living organisms by using galactose coupled to a fluorescence probe, such as SPiDER-BGal. This probe will be “activated” when the sugar it contains is degraded by B-galactosidase, allowing it to be used to mark senescent cells with elevated B-gal activity, which may subsequently be detected using flow cytometry techniques. However, just like other markers, β-Gal is not only expressed by senescent cells in living organisms, but is also found in secretory cells such as tissue macrophages and osteoclasts, cells that experience increased lysosomal activity during the autophagy process, and some cancer cells [28].

3.3. Nuclear Alteration

 doi:10.20944/preprints202404.0659.v1

Heterochromatin is sparsely distributed at the periphery of the nucleus in non-senescent dividing cells. However, in senescent cells, the chromatin condensed and is known as senescence-associated heterochromatin foci (SAHF). Chromatin condensation occurs to silence the genes needed for the proliferation process. SAHF examination can be performed by staining using 40,6-diamidino-2-phenylindole (DAPI) and confocal microscopy imaging techniques. In addition, SAHF also contains heterochromatin-forming proteins, such as HP1, H3K9me, and H2A macro histones that can be examined by immunostaining [28].

Another more global marker to detect senescence is DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence). DNA-SCARS is a nuclear structure that contains various proteins related to DDR [28].

Telomere length has also been used to detect senescence. Telomere length can be measured by qPCR technique and quantitative fluorescent in situ hybridization (Q-FISH). In addition to telomere length, the formation of telomere dysfunction-induced foci (TIFs) can also be used as a senescence marker. TIFs are DNA-SCARS formed in telomeres. In other words, DNA damage markers, such as γ-H2AX, ATM, and 53Bp1 are found in telomere regions. TIF can be found on telomeres that do not experience shortening, so TIF can also be found in various types of senescence, not just those caused by replicative stress. Detection method for TIF is generally the same as DNA-SCARS because the components are similar [28].

Lamin B1 protein is downregulated in senescent cells. Lamin B1, a significant nuclear lamina component, maintains nuclear structural integrity. In senescent cells, downregulation of lamin B1 allows chromatin fragments to escape from the nucleus. Immunoassay or immunoblotting can be used to detect lamin B1 depletion [28].

3.4. Senescence-Associated Secretory Phenotype (SASP)

SASP is a set of factors, including cytokines, chemokines, growth factors, metalloproteinases, and extracellular vesicles secreted by senescent. SASP can be examined through RT-qPCR, proteomic techniques (LC-MS/MS), and ELISA [28].

The majority of research that correlates senescence with age-related diseases in human tissues does not employ SASP as a marker [29]. Many of these substances are also released by other cells, including immune cells, endothelium, and cancer cells, therefore SASP cannot be used alone as a reliable indicator of senescence. In addition, its expression fluctuated and evolved. The SASP profile depends on the duration, type of stressor, and cell type [28]. SASP from distinct cells within the same tissue exhibit distinct characteristics. SASP is also known to trigger senescence in neighboring cells, therefore the senescence marker can change as senescence occurs in the network of neighboring cells [29].

To use SASP as a more specific marker, it is necessary to first determine the SASP profile of the tissue/organ under research. Chatsirisupachai's transcriptome studies revealed that gene patterns in various senescent tissues are distinct. The outcomes of these investigations can serve as a basis for selecting the appropriate SASP markers for specific tissues [29]. In addition, the SASP Atlas database provides several details regarding dissolved protein components and exosome contents from fibroblast cells and endothelial cells that undergo senescence following oncogene activation, radiation, and specific treatment. The SASP Atlas demonstrates that each stressor has a unique SASP profile and that the overlap is extremely limited, making it difficult to identify a universal SASP marker [28].

3.5. Mitochondria, Reactive Oxygen Species (ROS) and Pro-Survival Pathways

In senescent cells, mitochondria enlarge, and their number increases due to the decreased mitophagy process. Mitochondria also play a role in causing an increase in ROS which is also a marker of senescence. Mitochondrial ROS released by senescent cells may cause senescence by autocrine and paracrine mechanisms. ROS can be detected by various stains, e.g., Dihydroethidium (DHE). DHE that is blue will undergo oxidation by intracellular superoxide and turn red [28].

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Another hallmark of senescence is apoptosis resistance. Apoptosis resistance is caused by Bcl-2 overexpression in mitochondrial membranes and endoplasmic reticulum, which activates pro-survival signaling pathways. Bcl-2 inhibits the release of cytochrome c and the subsequent activation of caspase signaling pathway that triggers intrinsic apoptosis. A more common method employed in the detection of apoptosis resistance is by excluding apoptosis, marked by the absence of annexin V, cleaved caspase 3, and PARP [28].

4. Gerontogen

The rate of physiological aging is influenced not only by genetic, but also by environmental factors [7]. Environmental factors that can accelerate the aging process molecularly are called gerontogen. Currently, there are already several gerontogen that have been identified, such as UV exposure, high-fat diet, heavy metal exposure and cigarette smoke.

4.1. UV Radiation

The sun emits UVA (320-400nm), UVB (280-320), and UVC rays (100-280 nm). UVC is absorbed by the stratosphere, allowing only ~95% of UVA and ~5% of UVB to reach Earth's surface. Reactive oxygen species generated by UVA and UVB can cause indirect damage to the DNA. In addition, UVB can directly damage the DNA through the formation of a cyclobutene pyrimidine dimer (CPD) [33]. Sorrentino et al. found that continuous exposure to UVB can lead to an increase in DNA damage markers, including phosphorylated-H2AX, p16, Cxcl1, and IL-6.15 According to Ma et al., in-vitro UVA radiation causes senescence in human fibroblast cells through telomeres shortening [34].

4.2. High-Fat Diet

Kim et al. show that administration of a high-fat diet (60% fat) for 6 months in mice can cause impaired kidney function and elevated senescence markers in the kidneys, which include SA-β-Gal, p16, p19, and p53 as well as IL-1α, MCP1 and TNF-α. Meanwhile, p21 did not experience a significant increase. Senescent cell elimination with quercetin improved kidney function. High-fat diets can promote dyslipidemia and obesity, which may contribute to senescence [35]. Obesity is associated with chronic inflammatory conditions and increased oxidative stress. Chronic inflammation is known to accelerate the aging process. In the absence of any other genetic or environmental factor, Jurk et al. discovered that systemic chronic inflammation can accelerate aging via ROS-mediated exacerbation of telomere dysfunction and cellular senescence [36]. In addition, obese individuals have shorter telomeres [37–39] and a diminished ability to counteract oxidative damage [40]. Another study conducted by Sorrentino et al. using p16-reporter mice showed that a high-fat diet (42% fat) did not increase the expression of p16 assessed by Total-Body Luciferase Imaging (TBLI). This may be due to senescence that occurs on a scale that cannot yet be detected by TBLI measurements [41].

4.3. Heavy Metal

Currently, there has been a review of heavy metals that have been shown to induce senescence, both in vitro and in vivo, including arsenic and iron [42]. Arsenic (As) is a toxicant commonly found in air, water, and soil and can enter the body through various routes. Exposure to arsenic can increase ROS production which causes DNA damage and affects the DNA repair process [7,41,42]. Okamura et al. showed that exposure to sodium arsenite 5 and 7.5 μg for 144 hours caused the occurrence of senescence in the human stellate cell line, which is characterized by changes in cell morphology, increased SA-β-Gal, increased expression of p21, increased marker of DNA damage (γ-H2AX), and decreased expression of lamin B1. In addition, there is upregulation of various SASP mRNAs, including MMP1, MMP3, IL-8, IL-1β, and Cxcl1. The increase in DNA damage markers indicates the role of DNA damage in causing senescence as a result of arsenic exposure [43]. In-vivo research by Sorrentino et al. showed that chronic exposure to arsenic as much as 50 ppm through drinking water led to increased p16 expression in mice detected at week 24 [41].

 doi:10.20944/preprints202404.0659.v1

Meanwhile, iron (Fe), in the form of iron oxide, is found as air pollutants from exhaust emissions [44]. Excess iron in the body can lead to the formation of ROS through the Fenton and Haber-Weiss reactions [45]. Noh et al. show that iron overload may cause senescence in cerebral endothelial cells derived from aging mice, especially in cells derived from female mice. In that research, senescence is characterized by a decrease in cell proliferation that is not accompanied by cell death, an increase in DNA damage markers (γ-H2AX) and the expression of p16, p21, and IL-6. There is a reciprocal relationship between iron and senescence in which senescent cells showed iron accumulation, and iron accumulation causes senescence [46].

4.4. Cigarette Smoke

Senescence is related to the occurance of several lung pathologies, such as fibrotic pulmonary disease [47] and chronic obstructive pulmonary disease (COPD) [48]. One of the factors that cause senescence in the lungs is cigarette smoke [49]. Cigarette smoke contains various mutagens, such as formaldehyde, carbon monoxide, and nicotine that can cause DNA damage [7]. Nicotine exposure, according to Bodas et al., causes bronchial epithelial cell senescence via ROS-mediated autophagy impairment [50]. Meanwhile, in an experiment conducted by Sorrentino et al., mice exposed to cigarette smoke showed a significant increase in p16 after a few weeks and remained persistent even after exposure was stopped [41]. Table 2 summarizes current research regarding gerontogen.

Table 1. Biomarkers of Senescence [28].

No.	Hallmark	Marker	Estimated Changes
1.	Cell-cycle arrest		
	Decreased DNA synthesis	BrdU, EdU	↓
	Decreased proliferation	Ki67	↓
		Cell-counting	↓ (without decreased viability)
	P16/RB activation	p16	↑
		pRb	↑
		phospho-pRb	↓
	p53/p21 activation	p21	↑
		p53	↑
		phospho-p53	↑
2	Structural changes		
	Morphology, cell size	Cell shape and size	Enlarged and flattened
	Increased lysosomal compartment and activity	SA-β-Gal	↑
	DNA damage	γH2AX	↑
		53BPI	↑
		TIF	↑
		ATM	↑
		MDC1	↑
	Telomere shortening	Telomere length	↓
	SAHF	DAPI	↑
		HP1	↑
		Methylation H3K9	↑
	Nuclear membrane	Lamin B1	↓
3.	Exclusion of apoptosis	Annexin V	None
		Cleaved PARP	None
		Cleaved caspase 2/3/9	None
4	SASP	Cytokine secretion (IL-6, IL-8, CXCR2, IGF2, etc.)	↑

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Table 2. Gerontogen and the corresponding alterations in senescence biomarkers.

No	Gerontogen	Type	Inducers and Dosages	Duration of Exposure	Markers	Detection Method
1.	UV exposure	p16-reporter MICE (whole-body) [41]	UVB 350J/m2, 3x/week	6 months	↑p16	RT-PCR
						Total body luciferase imaging
					↑Cxcl	RT-PCR
					↑IL-6	RT-PCR
					↑phospo-H2AX	Staining
					↑SA-β-Gal	Staining
		Primary culture of human skin fibroblast cells [34]	UVA 500, 1000 or 10 000 mJ/cm2 of UVA	Single dose	Telomere shortening	PCR
2.	High-fat diet	Mice C57BL/6J [35]	High-fat diet (60%)	6 months	↑p16	RT-PCR
					↑p19	RT-PCR
					↑p53	RT-PCR
					↑TNF-α	RT-PCR
					↑IL-1α	RT-PCR
					↑MCP1	RT-PCR
					↑SA-β-Gal	Staining
		P16-reporter MICE (whole-body) [41]	High-fat feed (42%)	18 months	p16 (not significant)	RT-PCR
						Total body luciferase imaging
3.	Heavy metal exposure	Human hepatic stellate cell line [43]	Sodium arsenite 5 and 7.5 µg	144 hours	Morphology	Crystal violet assay (microscope)
					Proliferation	Proliferation assay
					↑SA-β-Gal	Staining
					↑p21	RT-PCR
					↓Lamin B1	RT-PCR
					↑MMP1	RT-PCR
					↑MMP3	RT-PCR
					↑IL-8	RT-PCR
					↑IL-1β	RT-PCR
					↑CXCL1	RT-PCR
					↑γH2AX	WB
		Female mice and primary culture of cerebral endothelial cells of female mice [46]	Mice: 200µL ferric citrate 5% (induction in mice), 3days/week	6 weeks	↑P21	qPCR
					↑IL-6	qPCR
					↑γH2AX	qPCR
					↑SA-β-Gal	immunohistochemistry
			Cell culture: ferric citrate 150µM	7 days	↑IL-6	qPCR
					↑γH2AX	Immunocytochemistry
4	Cigarette smoke	Human fetal lung fibroblasts	Cigarette smoke extract 0.5%	15 days	↑SA-β-Gal	Immunofluorescence
					↑P16	Immunofluorescence
					↑P21	Immunofluorescence

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	(HFLs-1)[48] [49]		↑γH2AX ↑ROS ATP	Immunofluorescence DAPI staining Bioluminescence assay
	P16-reporter MICE (whole-body) [41]	Cigarette smoke	1 hour per day, 5 days/week for 6 months p16	qRT-PCR Total body luciferase imaging

5. Senescence as a Drug Target

5.1. Senolytic and Senomorphic Agents

The involvement of senescence in the pathophysiology of numerous diseases has sparked interest in using senescent cells as therapeutic targets. Currently, medicines known as senolytic agents have been developed to eliminate senescent cells in various ways. For example, navitoclax drugs (ABT-737 and ABT-263) work by inhibiting the activity of Bcl-2 so that senescent cells can be removed through apoptosis. Other drugs use the overexpression of SA-β-gal as a target. Due to the enhanced activity of SA-β-gal in senescent cells, nanoparticles coated with galacto-oligosaccharides can target senescent cells more precisely [21].

The combination of Dasatinib with quercetin (D+Q) constitutes another senolytic agent. Dasatinib works by targeting the senescence cell antiapoptotic pathway through tyrosine kinase inhibition, while quercetin is a natural flavonoid that works by inhibiting BCL-2/BCL-XL, PI3K/AKT, and p53/p21 [51,52]. In experimental animal models, D+Q was shown to reduce the incidence of intervertebral disc degeneration associated with the aging process accompanied by a decrease in senescence markers, including p16, p19, and SASP (IL-6 and MMP13) in mice [51].

In clinical trials, a pilot study in phase I clinical trials showed that oral administration of D+Q combination for 3 days could eliminate senescent cells in adipose tissue of diabetic kidney injury patients on the 11th day after therapy. In the study, there was a decrease in the number of cells expressing p16 and p21, cells with increased SA-β-Gal, and SASP (IL-1α, IL-6, MMP9, and MMP12) [53]. The use of DQ in idiophatic pulmonary fibrosis (IPF) patients has also been evaluated in the first in-human clinical trial in the United States. In the study, oral DQ (Dasatinib 100mg/day and Quercetin 5x150mg/day) was given intermittently for 3 weeks, where each week, DQ was given 3 days in a row, followed by a 4-day no-drug period (total 9 doses). The results showed that intermittent DQ administration for 3 weeks produced good adherence and could improve the physical function of IPF patients. From the aspect of safety and tolerability, in general, adverse events that occur in the administration of the regimen are classified as mild-moderate, reversible and do not cause clinically relevant sequelae [54].

Senomorphic agents serve as an alternative to senolytic agents. The mode of action of a senomorphic agent is to alter various properties of senescent cells, particularly those associated with the generation and secretion of SASP while preserving the viability of the cell. This method will diminish the pro-inflammatory effects caused by senescent cells. Compounds that affect NF-B signaling, including metformin, apigenin, and kaemferol, are known to reduce SASP synthesis. In addition, other neutralizing antibodies for SASP or its receptors, such as IL-6, IL-1, IL-1, and TNF, had senomorphic effects [21].

Currently, there is also a review regarding the anti-senescence properties of various Indonesian natural ingredients [55]. Turmeric (Curcuma longa) containing curcumin has antioxidant effects and exhibits senolytic effect in mice. The content of piperlongumine in Cabe Jawa (Piper retrofractum Vahl.) is known to have a senolytic effect by inducing apoptosis in senescent fibroblast cells. Pegagan (Centella asiatica) can prevent senescence in fibroblast cells through its antioxidant effects [55].

The use of senolytic and senomorphic agents has its own advantages and disadvantages. For senomorphic agents that work by inhibiting SASP, continuous treatment is needed to maintain SASP

 doi:10.20944/preprints202404.0659.v1

suppression and this can cause side effects and off-target effects due to suppression of cytokine secretion by non-senescent cells, such as innate and adaptive immune cells. Meanwhile, senolytic agents that work by targeting the underlying cause of SASP by eliminating senescent cells, can be administered intermittently and their effectiveness is equivalent to continuous administration. This "hit-and-run" strategy in the administration of senolytic agents can reduce side effects [56]. Nevertheless, complete eradication of senescent cells can also be dangerous since senescence is also needed in some important processes such as wound healing. This is also applicable to the inhibition of regulatory pathways, including NF-κB, which controls inflammation and the immune response in addition to being involved in SASP secretion. Therefore, the development of senotherapy must also consider other beneficial functions of the system to be targeted [56,57].

6. Conclusion

The relationship between aging, senescence, and the occurrence of chronic diseases increases interest in using senescent cells as a therapeutic target for the prevention or treatment of numerous diseases. However, the lack of a common marker for senescence makes identification challenging, thus limiting gerontogen research. The challenge lies in developing a detectable marker for identifying unlimited gerontogens to enhance the understanding of aging and chronic diseases induced by environmental factors. Even though this limitation also has an impact to the senotherapy research, numerous studies remain ongoing and have yielded a number of promising agents. While the preliminary findings appear favorable, the advancement of senotherapy, particularly senolytics, still necessitates the investigation and validation of a number of critical factors, including the potential for off-target effects and specificity, which must be addressed in order to avoid possible hazardous side effects.

Author Contributions: All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) guidelines.

Ethical Approval: This study does not involve experiments on animals or human subjects.

Data Availability: All data generated and analyzed are included in this research article.

Conflict of Interest: The authors declare no conflict of interest.

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