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Cancer cell senescence: a new frontier in drug development

Claire J. Cairney[1], Alan E. Bilsland[1], T.R. Jeffry Evans[1], Jon Roffey[2], Dorothy C. Bennett[3,6], Masashi Narita[4,6], Christopher J. Torrance[5,6] and W. Nicol Keith[1,6]

[1] Institute of Cancer Sciences, University of Glasgow, CRUK Beatson Laboratories, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, UK
[2] Cancer Research Technology Limited, Wolfson Institute for Biomedical Research, Gower Street, London WC1E 6BT, UK
[3] Division of Biomedical Sciences, St George's, University of London, Cranmer Terrace, London SW17 0RE, UK
[4] Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre, Cambridge CB2 0RE, UK
[5] Horizon Discovery Ltd, IQ Cambridge, Cambridge CB25 9TL, UK
[6] Senectus Therapeutics Ltd, c/o Cancer Research Technology Ltd, Angel Building, 407 St John Street, London EC1V 4AD, UK

Senescence forms a universal block to tumorigenesis which impacts on all hallmarks of cancer, making it an attractive target for drug discovery. Therefore a strategy must be devised to focus this broad potential into a manageable drug discovery programme. Several issues remain to be addressed including the lack of robust senescence-inducing compounds and causally related biomarkers to measure cellular response. Here, we review the latest progress in translating senescence as a target for cancer therapy and some promising approaches to drug and biomarker discovery. Finally, we discuss the potential application of a senescence-induction therapy in a clinical setting.

Introduction

Cancer has traditionally been described as a multistep disease that evolves progressively with the acquisition of numerous characteristics enabling escape from the normal constraints of cell growth. These characteristics were initially categorised into six and subsequently ten biologically functional groups termed the hallmarks of cancer [1]. The idea that interfering with a key hallmark of cancer would cause tumour regression has made them and the biological processes that define them attractive targets for drug development strategies. However, hallmarks are not discrete entities but consist of diverse and complex interconnected signalling networks and processes. From a drug development point of view an in-depth understanding of the targets, pathways and cellular fate is crucial, because targeting one biological process can simultaneously impact other processes or endpoints.

Cellular senescence, the irreversible arrest of proliferation, can be considered a universal barrier to tumour progression which all cancer cells must overcome during the process of immortalisation. Rather than being a unique signalling event defined by the expression of individual molecules, senescence is a collective phenotype of multiple effector mechanisms including global chromatin and epigenetic modification [2–4], DNA-damage response [5], secretory pathway [6,7] and autophagy [8], which could best be thought of as a biological process in its own right [9]. If we consider senescence signalling as a target in terms of the hallmarks of cancer it would be defined as preventing immortalisation. However, a closer look at the signalling events underlying the senescent phenotype reveals cross-cutting effects on all hallmarks documented to date (Fig. 1); including, but not limited to, invasion and metastasis [10], immortalisation [11], proliferation [12] and immune modulation [6,7,13], all of which make this process an attractive target for drug discovery.

Tumorigenesis relies on a balance between senescence and immortalisation, therefore senescence should be considered in the context of a larger signalling complex consisting of overlapping processes of immortalisation and apoptosis. Although the regulatory mechanisms remain to be fully uncovered, a complex network of interacting signalling pathways underlying senescence

Corresponding author:. Keith, W.N. (nicol.keith@glasgow.ac.uk)

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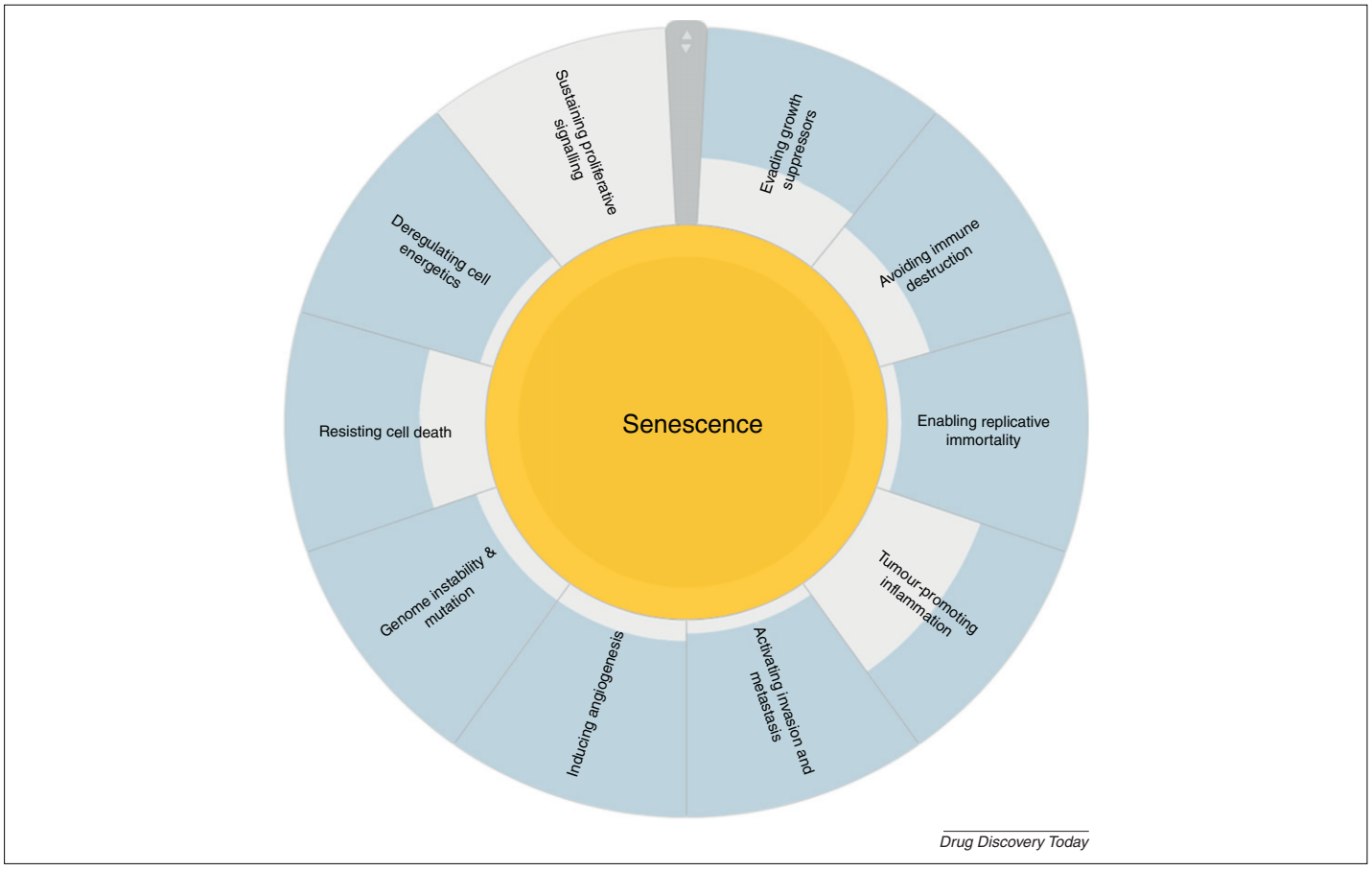

FIGURE 1

Association of senescence signalling with hallmarks of cancer. The defining characteristics of cancer are classified into ten functional groups termed hallmarks [1]; however, a range of biological processes underlie each hallmark and these processes can be shared by several at once. When considered in terms of hallmarks, senescence would be defined as preventing immortalisation yet the various processes underlying the senescence response can fall into multiple hallmarks. To investigate the impact of senescence signalling on all hallmarks of cancer, enrichment analysis of the biological function of genes associated with senescence [9] was performed in MetaCore™ (GeneGo; http://www.genego.com/). The resulting top 50 biological process networks relating to senescence were distributed into the ten hallmarks based on the functional description of the networks and the senescence genes mapping to each one to give a percentage overlap between senescence signalling and each hallmark. The largest proportion of senescence-associated networks came under the heading of sustaining proliferative signalling, therefore this was set to 100% on the chart and all other hallmarks were scaled accordingly.

is emerging offering a wealth of opportunity for therapeutic intervention to tip the balance in favour of tumour suppression. In this review we discuss the current thinking on senescence-targeted drug development and outline the requirements for translating pro-senescence therapies into a clinical setting.

The adoption of senescence for drug discovery

Senescence and immortalisation are currently underserved areas in drug development [14,15]. During recent years interest in the application of a senescence agonist therapy for oncology has gained momentum and this has been fuelled by increasing evidence regarding the importance of senescence as a block to tumour progression. Senescence signalling has been shown in benign lesions of the skin, blood, lung and prostate, and also in advanced disease [9,16] and the senescence response was restored in an established liver carcinoma model [13] confirming the tumour suppressive role for senescence, also suggesting that senescence signalling remains present but not fully engaged or perhaps below a threshold level during tumorigenesis. Consistent with this is the now well documented fact that many conventional chemotherapeutic agents and radiotherapy regimens aimed at inducing cell death through DNA damage and apoptosis can, when analysed with the appropriate endpoints, induce a senescence-like response in treated cells [17,18] showing that the pathways are capable of re-engagement.

The presence of senescence signalling was not considered in clinical trials for these agents, therefore its role in the therapeutic activity remains to be determined. The processes of senescence and apoptosis share many key pathways including the DNA-damage response; therefore it could be that in some instances senescence was the primary target. In fact, a review of the FDA-approved drugs in relation to our knowledge of senescence signalling highlights at least three known drug targets with roles in senescence signalling [B-Raf: target of sorafenib tosylate (Nexavar®) and vemurafenib (Zelboraf®); mammalian target of rapamycin (mTOR): target of temsirolimus (Torisel®) and everolimus (Afinitor®); topoisomerase (TOP)1: target of irinotecan (Camptosar®) and topotecan (Hycamtin®)] (Fig. 2), whereas the histone proteins can be targets of histone deacetylase inhibitors such as vorinostat through their action on histone deacetylases (http://www.cancer.gov/cancertopics/factsheet/Therapy/targeted; http://www.drugbank.ca/), suggesting that re-positioning of existing drugs [19] might be one mechanism

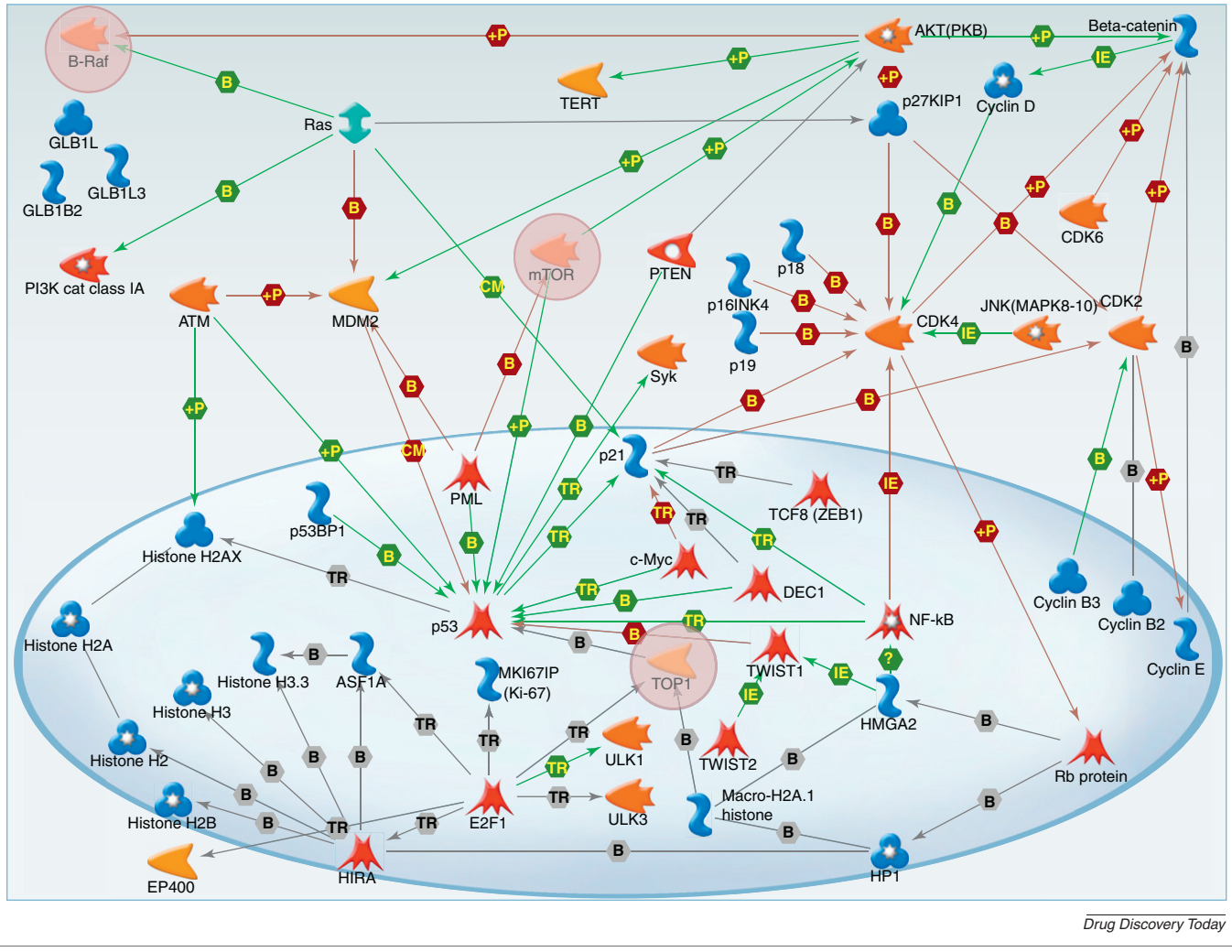

FIGURE 2

Association of known drug targets with senescence signalling. Some of the known signalling pathways and interactions between gene products involved in cellular senescence are visualised in MetaCore™ MapEditor™ (GeneGo; http://www.genego.com/). Red and green arrows represent negative and positive regulation, respectively, whereas grey represents unknown interactions. Mechanism: +P, phosphorylation; B, binding; CM, covalent modification; TR, transcriptional regulation; IE, influence on expression. A list of the 89 gene targets of FDA-approved drugs (http://www.cancer.gov/cancertopics/factsheet/Therapy/targeted) was overlaid on this map. Targets that overlap with known senescence-signalling molecules are highlighted in red. B-Raf is a target of sorafenib tosylate (Nexavar®) and vemurafenib (ZELBORAF), mammalian target of rapamycin (mTOR) is a target of temsirolimus (Torisel®) and everolimus (Afinitor®), topoisomerase (TOP)1 is a target of irinotecan (Camptosar®) and topotecan (Hycamtin®).

for targeting senescence signalling. In agreement with this Ewald *et al.* recently identified several small-molecule senescence agonists, one of which was the quinone diaziquone (AZQ), a DNA alkylating agent identified in the 1980s for its antitumour activity, which later failed in clinical trials owing to its inability to induce tumour regression [20]. Similarly, Labay *et al.* demonstrated the potential for re-positioning of existing drugs as radiosensitisers and enhancers of therapy-induced accelerated senescence in screens of approved and investigational drugs from the National Cancer Institute collections and MicroSource Discovery Systems [21].

Targeting senescence for drug discovery

Detection and measurement

To mount a successful drug discovery programme to target senescence several issues must be addressed, including the ability to detect senescent populations reliably and to quantify the response accurately. A point of contention for many years was whether senescence was an *in vitro* phenomenon accentuated by the stress of tissue culture conditions, however an increasing body of evidence confirms the existence of senescence *in vivo* in animal models and human clinical samples [16,22–25].

Senescence can be induced by multiple biological stimuli including telomere attrition and dysfunction, termed replicative senescence, and also by stress-inducing conditions including oncogene expression and DNA-damaging insults, which induce a senescence-like response termed accelerated or premature senescence [9,15,17]. Although there are some important molecular differences in the response to specific triggers that still remain to be fully elucidated [26], many of the associated characteristics are shared including: a large, flattened morphology; accumulation of lysosomal vacuoles; decreased methylation of DNA and presence of senescence-associated heterochromatin foci (SAHF) [2,3]; upregulation of cell cycle inhibitors; expression of a secretory phenotype [6,7]; and positive staining for senescence-associated beta-galactosidase (SA-βGal)

TABLE 1

Biomarkers associated with the senescent phenotype (Ref. [9])

Component of senescence	Biomarker
Cell cycle	p16
	p21
	Ki67
Chromatin	HMGA1
	HMGA2
	HIRA
	ASF1a
	H2AFY
	H2AFY2
	PML
	53BP1
	HP1γ
Secretome	IL6
	IL8
	PAI1
	MMP1
	MMP3
	IGFBP3
	IGFBP5
	IGFBP7
	IL1A
	CXCR2
	IGF1
	IGF2
	IGF2R
	WNT2
	CAMP
	STMN1
	EEF1A1
	TGFB1

[27]. These characteristics provide several biomarkers with which the senescent phenotype can be defined (Table 1).

A major barrier to the development of senescence agonist therapeutics is the lack of a 'gold standard' marker of senescence to enable efficient detection and measurement *in vivo* and *in vitro*. No single marker for senescence documented to date is specific or unique enough to identify senescence. The most commonly used marker of senescence, SA-βGal, is not suitable for use in formalin-fixed paraffin-embedded clinical samples and might not be specific to senescence *in vivo* making it unsuitable as a marker for clinical applications [25,28,29]. Similarly, it has recently been documented that SAHF formation is not a universal component of the senescent phenotype, but instead is cell-type- and senescence-trigger-specific and does not always accompany other features of senescence. Despite the fact that markers of facultative heterochromatin H3K9Me and HP1γ can be detected in paraffin-embedded clinical specimens of normal human bladder and colon, bladder tumours, colon carcinomas and fresh frozen and paraffin-embedded samples of colorectal adenomas in partly focal patterns, no evidence of SAHF formation was found [30]. However, in a recent study by Di Micco *et al.* SAHF formation was detected *in vivo* associated with oncogene-induced stress in untreated head and neck squamous cell carcinomas (HNSCC), but not in irradiated normal tissue or in normal respiratory epithelium with persistent staining for γ-H2AX indicative of X-ray-induced senescence [31]. As for SA-βGal, the variable nature of SAHF presence *in vivo* will limit its application as a marker of senescence. In the absence of one 'gold standard' marker the definition of senescence currently relies on simultaneous expression of several markers, which gives a more robust and quantitative assessment [32]. The biomarkers identified to date largely fall into three main categories: chromatin, cell cycle and secretome (Table 1). Combinations of markers from each of these groups could be applied depending on the biological context of the sample to provide a senescence index to define the phenotype clearly. This type of strategy was recently applied to analysis of senescence in a retrospective study of metastasised colorectal carcinoma as a predictor of therapeutic outcome [25]. Data generated from analysing a panel of biomarkers in various cell types could also help to identify the most appropriate markers or endpoints for measuring the success of a senescence agonist in a clinical setting. Consistent with this the authors of this review recently reported a senescence scoring method based on the gene expression profiles of multiple markers of senescence, which could provide a measurement of DNA damage and secretory senescence signalling in various human tumours. A proinflammatory signature generated from a subset of secretory markers had prognostic significance in mesothelioma [9].

Cell-based screening

The investigation of a senescence response in human tumours has been hampered by the lack of a therapy specifically designed to induce senescence, in addition to the lack of causally related, clinically relevant biomarkers that can be used to measure the response in clinical samples. A better understanding of the signalling complexities underlying the senescent phenotype will undoubtedly reveal novel targets for pro-senescence therapy; however, in the absence of detailed knowledge, the biology of senescence lends itself to a cell-based screening approach to drug discovery. Identified drug targets could then be fed back into research programmes to assist with the elucidation of mechanisms and signalling pathways downstream of the known key regulators and might also be applicable as tools for analysis of senescence in clinical samples.

Automated microscopy approaches offering high-content imaging can be applied to the detection of parameters relating to senescence induction including proliferative arrest, the distinctive morphological changes and associated biomarkers [33–35]. However, in the case of a morphology screen for senescence the performance of some cell segmentation or detection algorithms could be adversely influenced by the irregular morphology characteristic of senescent cells [36] or indeed the cell density, in which case seeding density should be optimised and closely adhered to and some care should be taken in the analysis steps to ensure reliable assessment of the phenotype. Combining the primary screen with appropriate secondary assays encompassing biomarkers that are causally related to the functional endpoint of senescence will also enable confirmation, as discussed above.

One definitive distinction between senescence and quiescence is irreversible growth arrest; therefore, a proliferation assay would be a suitable screen for a senescence-targeting compound, with the aim of measuring irreversible proliferation arrest after a period of compound-free growth post-treatment. In such an assay Breier *et al.* recently used a combination of 5-bromo-2′-deoxyuridine (BrdU), Hoechst 33258 and propidium iodide staining to detect

effects on cell viability and proliferation in a screen for neurotoxic compounds [37]. Similarly, Ewald *et al.* recently reported on the identification of four novel compounds that induced characteristics of senescence in the prostate cancer cell line DU145 by combining a semi-automated high-throughput method to detect proliferation arrest using Hoechst 33342 fluorescence with visual confirmation based on cellular morphology and SA-βGal staining [20].

In the absence of a robust biomarker for the senescence response SA-βGal staining is frequently used. Although SA-βGal staining is not exclusive to senescence it could still be applied as a first-pass screen or secondary assay [20]. Various chemiluminescent formats have been described [38,39] and single cell measurements could also be carried out in a screening setting using fluorogenic βGal substrates [40]. Alternatively, SAHF detection as punctate heterochromatin foci in DAPI-stained nuclei should be feasible using high-content imaging with the correct detection algorithms applied [2]. However, immunofluorescent methods used to label components of the SAHF assembly can improve detection [21]. Because the presence of SAHF is not a universal component of the senescent phenotype and appears to be cell-type- as well as context-specific [30,31], the robustness of the response should be carefully assessed during assay development.

Several signalling pathways have been implicated in the senescence response (Fig. 2) [41,42]. Transcriptional regulation of many genes including downstream effectors of these pathways is associated with senescence, suggesting that promoter reporter assays could be another valuable screening tool for senescence-inducing targets, for example reporter constructs under the control of promoters for cell cycle inhibitors. We have provided proof-of-concept for the value of promoter assays in a screening application for mechanisms regulating immortalisation by the identification of Jun kinase signalling in telomerase gene regulation [43].

Alternatives to compound screening

Another contributing factor to tumorigenesis is genomic instability. Senescence bypass can be the consequence of duplication or amplification, gain or loss of gene function, therefore inhibition or overexpression studies could be alternative strategies to a compound library screen with the same final endpoint [15]. Overexpression studies, using for example adenovirus expression vectors or plasmid cDNA clone libraries [44,45], aim at replacing or overactivating components of a signalling pathway, mimicking the effects of gain of function *in vitro*. Using high-throughput functional genomics screens Huang *et al.* identified nine novel protein regulators of p53 transcriptional activity by combining transfection of a p53 response element reporter construct with a cDNA expression library [46], whereas Koenig-Hoffmann *et al.* identified putative tumour suppressor genes by morphological detection of cDNA inducers of apoptosis or growth reduction [47].

A contrasting study would use RNA interference (RNAi) to knockdown gene expression to identify gene targets or signalling pathways regulating senescence. Using such an approach in a whole human kinome siRNA screen we identified several kinases with modulatory effects on expression of the telomerase reverse transcriptase (hTERT) promoter in tumour cell lines expressing telomerase and the alternative recombination-based telomere maintenance mechanism – alternative lengthening of telomeres (ALT) [48,49]. In a recent complementary analysis modulatory effects of protein kinases were also demonstrated to affect telomerase activity [50]. Together, these studies provide proof-of-principle for the application of this type of screen to the identification of target genes and pathways regulating senescence and immortalisation. Further validation could also be achieved by combining siRNA with small-molecule screens [48]. A consensus between hits from the two methods would provide confidence in the quality of hits to pursue to secondary assays, to feed into research programmes to elucidate pathways or to progress to animal models and clinical development.

Tumour type and cell line selection

To enable targets from a cell-based screening programme to be transferred to a clinical setting the aim of the screen would be to find targets with the ability to induce senescence in a range of cancer cell types that have overcome the normal upstream regulators of senescence to acquire an immortal phenotype. The theoretical application of a senescence agonist is universal, but this might not be the case in practice and screens might not give the desired results if the cell line used is unable to mount a senescent response. A pilot screen can be run to obtain surrogate controls and to select a panel of cell lines for the primary screen, which could be applied in a high-content approach. Evidence suggests a role for senescence in the evolution of melanoma, lung adenocarcinoma, pancreatic ductal adenocarcinoma and prostate adenocarcinomas [9,23]. Because these tumours might respond, senescence induction screening could be carried out in cell lines corresponding to these lesions.

A battery of cell lines with defined genetic changes in oncogenes and tumour suppressor genes, including RAS, B-Raf, p53, RB1 and PTEN have been derived over the years for the purpose of identifying pathways involved in tumorigenesis, which could be appropriate for a screening assay to recapitulate the genetic backgrounds in some tumour types. These cell lines are available from several sources, including commercial suppliers (http://www.horizondiscovery.com/; http://www.hpacultures.org.uk/collections/ecacc.jsp; http://www.lgcstandards-atcc.org/). The availability of high-quality isogenic cell lines opens up opportunities to explore personalised medicine and could inform patient stratification in clinical trials. Knowledge gained from screening multiple cell lines will enable target profiles to be generated to determine those that work across the board or which ones are specific to certain genetic backgrounds.

Clinical application of a senescence-inducing therapy

Patient selection

The two key components required to test the validity of senescence in clinical trials, a senescence agonist and a measurable readout in the form of a panel of causative biomarkers, can be provided from high-throughput cell-based screens and targeted secondary assays as discussed. Information gained from screening multiple cell lines combined with the availability of banked tumour specimens pre- and post-treatment with standard chemotherapeutic agents will enable the co-development of drug candidates and biomarkers. This will provide unequivocal evidence for the validity of a senescence-directed therapy in multiple tissue types and should direct patient selection towards a specific molecular pathology or

tumour type, in addition to the identification of biomarkers that enable reliable measurement of response to treatment.

Evidence of residual senescence signalling in benign lesions might indicate an association with better prognosis in the presence of senescence [23]. Consistent with this, we recently showed that a proinflammatory signature generated from clustering analysis of genes associated with the secretory component of senescence had prognostic significance in peritoneal mesothelioma [9]. Furthermore, in a recent retrospective study of 30 patients with previously untreated stage IV colorectal cancer Haugstetter *et al.* showed that the presence of senescence at presentation related to longer progression-free survival following treatment with 5-fluorouracil and leucovorin [25]. Examination of a larger range of archival tumour samples for which immune response and patient outcome data exist will enable the prognostic association to be addressed fully and could inform on the patient populations for whom senescence therapies will be most effective.

Addressing the potential risks of senescence-induction therapy

The potential risks of a senescence agonist are difficult to predict. As with traditional cancer therapies, there is a risk that normal cells might be adversely affected. This could lead to dysfunction of tissues in which senescence is a normal aspect of ageing such as the skin and liver. In addition, there is evidence to suggest that the activation of senescence can favour the survival of a subset of tumour cells that need to maintain a quiescent state for continued self-renewal such as leukaemic stem cells [51]. The question of what becomes of a senescent cell *in vivo* also raises some concern. A recent report documented a subpopulation of small-cell lung cancer cells that escaped the accelerated senescence phenotype induced by treatment with Hsp90 inhibitors. These cells had acquired additional genetic mutations and underwent a growth arrest that could be reversed when the inhibitors were withdrawn [52]. Furthermore, p53-null human lung carcinoma cells show an accelerated senescence phenotype in response to the chemotherapeutic camptothecin. A small subset of these cells escaped replication arrest and re-entered the cell cycle through upregulation of Cdc2/Cdk1. These escaping cells were proposed to be a potential mechanism of cancer progression and drug resistance in lung cancer [53].

Some aspects of the senescent phenotype can also pose problems. Proinflammatory immune cytokines secreted by senescent fibroblasts have been shown to induce bystander effects on cell proliferation, invasion and characteristics of epithelial to mesenchymal transition [22], although Ewald *et al.* showed that the paracrine effects of senescent cancer cells have a much smaller effect than senescent fibroblasts on proliferation of bystander cells and no increase in establishment, growth or proliferation of prostate cancer xenografts was shown *in vivo* [54]. The design of a drug discovery programme is such that candidate agents are selected for an optimal balance of activity, selectivity and toxicity; therefore concerns about off-target toxicity should be addressed before clinical testing. Although, interestingly, it appears from experimental evidence of drug-induced senescence that a lower dose of many chemotherapeutic agents is required to induce senescence than is required to induce apoptosis, which suggests that targeting senescence will enable less-toxic doses of an agent to be applied [17].

Clinical trial development and drug re-positioning

The predicted outcome of a senescence agonist therapy is likely to be cytostasis rather than cell death, as induced by standard chemotherapy and/or radiotherapy regimens, however there might be some benefit to treatments during which arrested cells ultimately engage a cell death pathway such as apoptosis or autophagy or are removed by the immune system. Xue *et al.* showed that reactivation of p53 in deficient tumours in a mouse model of liver carcinoma was sufficient to induce tumour regression *in vivo* through activation of the innate immune system [13], which provides a rationale for induction of senescence as a single-agent therapy. However, the effect of chemotherapeutic regimens on senescence signalling should not be overlooked. Trials for agents

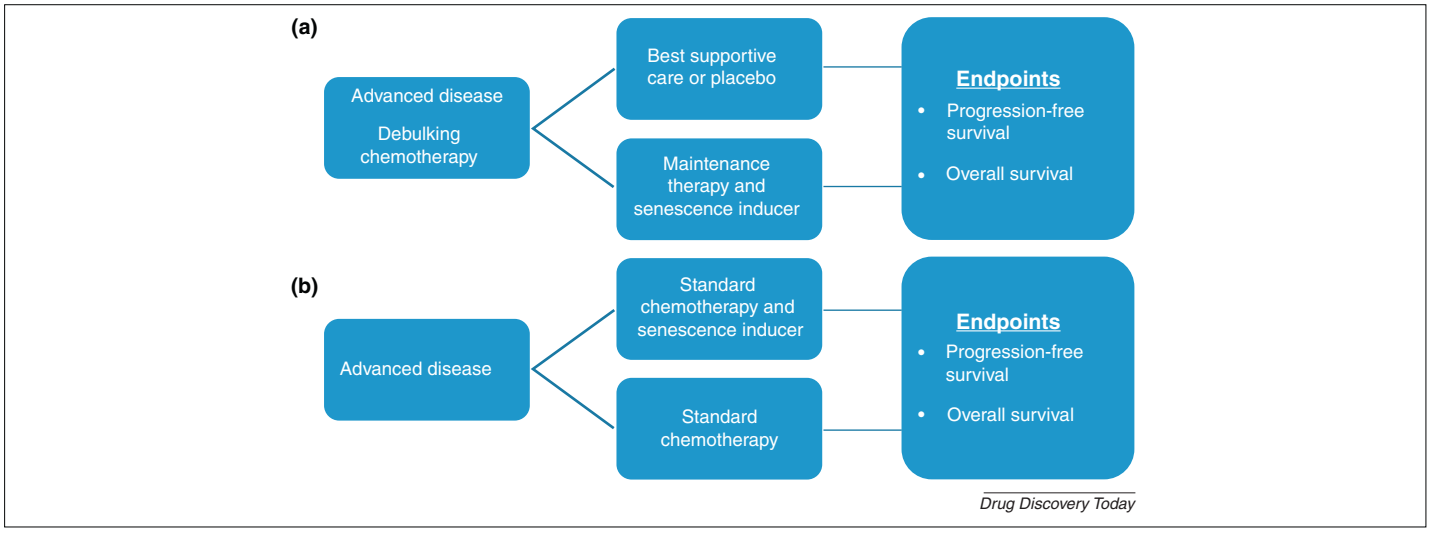

FIGURE 3

Design of a clinical trial for a senescence agonist. **(a)** A proposed Phase III clinical trial design of senescence agonists as maintenance therapy. This situation could be applicable to solid tumours after debulking chemotherapy. **(b)** A proposed Phase III clinical trial design of senescence agonists in combination with cytotoxic chemotherapy. The main endpoints are progression-free survival and overall survival.

developed to target processes such as apoptosis and angiogenesis did not look at the effect of senescence in the response to treatment in these patients, so further investigation is required to determine if a senescence agonist will have additive, synergistic or even antagonistic effects when combined with chemotherapy. Information gathered from co-development of biomarkers and drug candidates will enable rational design of future clinical trials.

At present it is not clear how senescence-inducing therapeutics might best be used and there are several potential applications that could be considered. Senescence agonists could have most impact in minimal disease states such as in maintenance therapy after tumour debulking by cytotoxic chemotherapy or in combination with cytotoxic chemotherapy, and Phase III clinical trial design for these settings is possible (Fig. 3). Phase III trials in advanced disease will address questions of whether senescence is an active target in combination with or after debulking chemotherapy (Fig. 3a), and if there will be an additive or synergistic effect when a senescence agonist is combined with chemotherapy (Fig. 3b). Currently, the most appropriate ways for combining senescence agonists with established therapies are unknown. Emerging data from early-phase clinical trials, demonstrating the desired biological effect of putative senescence agonists, will aid the development and design of these later-phase pivotal studies. Following proof-of-concept in advanced disease, it should be feasible to explore this therapeutic strategy in other minimal disease states such as adjuvant and/or perioperative therapy in addition to 'standard of care' in operable solid tumours.

Where chemotherapy is not the standard of care, other possibilities are combining senescence agonists with non-chemotherapy agents such as inhibitors of cell signalling or chemotherapy or radiotherapy delivered with radical intent. However, again, the effect of combining signalling agents with senescence agonists is currently unknown. The optional combinations should be developed based on preclinical evidence of mechanistic rationale to inform on the best clinical strategy.

Concluding remarks

Senescence could be a universal barrier to tumour evolution, which could theoretically be applied to all tumour types; however, before engaging in a clinical testing programme, clinicians must balance hypothetical effectiveness against potential risks and be convinced of the overall merits of a novel drug therapy. An ideal preclinical data package would show the effect of a pro-senescence therapeutic in tissue culture, a demonstration of senescence in an animal model and justification of a relevant endpoint for human studies so that the hypothesis can be tested. We now have the tools at our disposal to make this a reality. As outlined in Fig. 4, the combination of biomarker development alongside cell-based screening programmes for novel senescence agonists and preclinical studies in relevant tumour samples will provide essential proof-of-concept information to enable progression and realisation of a pro-senescence therapeutic in the clinic.

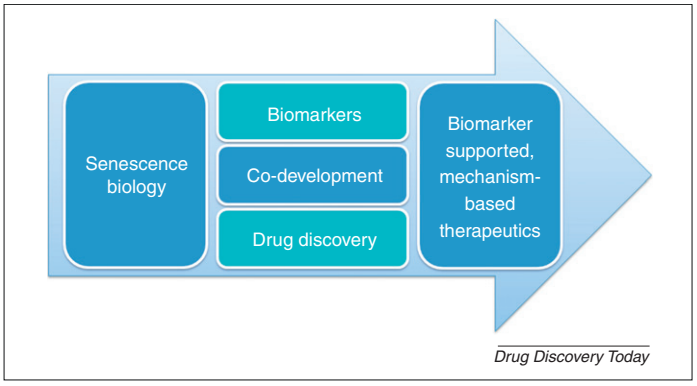

FIGURE 4

The way forward for senescence-induction therapy. There are several unknowns which must be addressed to move a senescence-induction therapy towards the clinic. One major gap is the absence of a 'gold standard' biomarker for detecting and measuring senescence *in vitro* and *in vivo*. The discovery and development of senescence agonists and biomarkers are interdependent.

Conflicts of interest and financial disclosures

WNK, DCB and MN have affiliation to Senectus Therapeutics. CJT has affiliations to Senectus Therapeutics and Horizon Discovery.

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