Actionable heterogeneity of hepatocellular carcinoma therapy-induced senescence

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In loving memory of my dad, Dr. Rolf Engels

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Summary

Senescence is a cellular process defined by a stable cell cycle arrest, substantial morphological changes, and an elevated resistance to apoptosis. Cellular senescence can be induced through various mechanisms, including oncogene activation (oncogene-induced senescence), telomere shortening (replicative senescence), or treatments/therapies such as radiation or chemotherapy (therapy-induced senescence). In many instances, the emergence of a potent inflammatory senescence-associated secretory phenotype (SASP) has been recognized as major hallmark of cellular senescence.

Notably, senescence is considered a critical cellular state in hepatocellular carcinoma (HCC) with relevance for both, progression as well as treatment. HCC is the most common form of liver cancer and the fourth-most leading cause of cancer-associated mortality worldwide. In recent years, several approaches have been conducted to utilize HCC therapy-induced senescence for treatment purposes. However, due to a lack of systematic studies, distinctions among TIS inducing treatments and potentially actionable targets arising from HCC TIS remain to be determined.

In this study, we systematic compared the three TIS inducers etoposide, CX5461 and alisertib in two representative human HCC cell lines. We evaluated their effects on intracellular innate immune signaling, SASP factors, and surfaceome changes. Despite of strong heterogeneities for SASP composition, innate immune clearance and surfaceome profiles, all three compounds induced the expression of both metastasis-related surface antigens and immunotherapeutically targetable antigens such as CD95 (Fas), CD340 (HER2) and CD276 (B7-H3). Furthermore, alisertib, CX5461, and etoposide made HCC cells more amenable to targeting with death receptor activating antibodies and chimeric antigen receptor (CAR) NK cells. This study suggests that the distinct and selective characteristics of senescence in HCC could be utilized for various therapeutic strategies, including death receptor activation by agonistic antibodies or ligands, conventional monoclonal antibody immunotherapy, bi-and tri-specific antibody therapy, and cell therapy using CAR-NK or CAR-T cells.

Zusammenfassung

Seneszenz ist ein zellulärer Prozess, der durch einen Stillstand des Zellzyklus, erhebliche morphologische Veränderungen und eine erhöhte Apoptoseresistenz gekennzeichnet ist. Zelluläre Seneszenz kann durch verschiedene Mechanismen, wie z. B. durch Telomerverkürzung (replikative Seneszenz), Onkogenaktivierung (Onkogen-induzierte Seneszenz) oder Behandlungen/Therapien wie Bestrahlung oder Chemotherapie (Therapie-induzierte Seneszenz) ausgelöst werden. Zusätzlich ist das Auftreten des Seneszenz-assoziierten sekretorischen Phänotyps (SASP) eines der Hauptmerkmale der zellulären Seneszenz. Insbesondere bei hepatozellulären Karzinomen (hepatocellular carcinoma, HCC) gilt die Seneszenz als ein wichtiger Zellzustand, der sowohl für das Fortschreiten als auch für die Behandlung von HCC von Bedeutung ist. HCC ist die häufigste Form von Leberkrebs und die vierthäufigste Ursache für krebsbedingte Todesfälle weltweit. In den letzten Jahren wurden verschiedene Ansätze verfolgt, um eine durch Therapie induzierte Seneszenz (TIS) für die Behandlung von HCC zu nutzen. Da es jedoch an systematischen Studien mangelt, müssen Unterschiede zwischen TIS-induzierenden Behandlungen und deren therapeutisches Potential noch ermittelt werden.

In dieser Studie haben wir die drei TIS-Induktoren Alisertib, CX5461, und Etoposid in zwei repräsentativen menschlichen HCC-Zelllinien systematisch verglichen. Zusätzlich wurden ihre Auswirkungen auf die intrazelluläre angeborene Immunsignalisierung, SASP-Faktoren und die Veränderungen der Expression von Oberflächenmarkern ermittelt. Trotz starker Heterogenität in Bezug auf die SASP-Zusammensetzung, die angeborene Immunabwehr und die Oberflächenprofile, induzierten alle drei Behandlungen die Expression von Metastasen-assoziierte Oberflächenantigene, sowie auch immuntherapeutisch angreifbarer Antigene wie CD95 (Fas), CD340 (HER2) und CD276 (B7-H3). Darüber hinaus machten Alisertib, CX5461, und Etoposid die HCC-Zellen für eine gezielte Behandlung mit Apoptose-aktivierenden Antikörpern oder chimären Antigenrezeptor (CAR)-NK-Zellen zugänglicher. Diese Studie legt nahe, dass die ausgeprägten und selektiven Merkmale der Seneszenz bei HCC für verschiedene therapeutische Strategien genutzt werden könnten, einschließlich der Aktivierung von Apoptose-induzierenden Rezeptoren durch agonistische Antikörper oder Liganden, der konventionellen Immuntherapie mit monoklonalen Antikörpern, der bi- und trispezifischen Antikörpertherapie und der Zelltherapie mit CAR-NK- oder CAR-T-Zellen.

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Abbreviations

AURKA	Aurora kinase A
AURKB	Aurora kinase B
AURKC	Aurora kinase C
B7-H3	B7 homolog 3
Bcl	B-cell lymphoma
bFGF	Basic fibroblast growth factor
BiKE	Bi-specific killer engager
Calcein-AM	Calcein acetoxymethyl esther
CAR	Chimeric antigen receptor
casp-8	Caspase-8
Cck-8	Cell counting Kit-8
CDK	Cyclin-dependent kinase
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
cGAS	Cyclic GMP–AMP synthase
DDR	DNA damage response
DMSO	Dimethylsulfoxid
E2F	E2 transcription factor
ECM	Extracellular matrix
EGF	Endothelial growth factor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
FACS	Fluorescence activated cell sorting
Fas	FS-7-associated surface antigen
G4	G-quadruplex
HCC	Hepatocellular carcinoma
HER2	Human epidermal growth factor receptor 2
ICAM-1	Intercellular adhesion molecule 1
IF	Immunofluorescence
IFN	Interferon
IL	Interleukin
KIR	Killer Ig-like receptor
LDH	Lactate dehydrogenase
LFA-3	Lymphocyte function-associated antigen 3
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex

MMP	Matrix-metalloproteinase
MPO	Myeloperoxidase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKG2	Natural killer group 2
OIS	Oncogene-induced senescence
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PMN	Polymorphonuclear neutrophil
RFA	Radiofrequency ablation
RB	Retinoblastoma
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
SA-β-Gal	Senescence-associated β-galactosidase
sFAS	Soluble Fas
STING	Stimulator of interferon genes
TIS	Therapy-induced senescence
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRACE	Trans-arterial chemoembolization
TriKE	Tri-specific killer engager
VEGF	Vascular endothelial growth factor

1. Introduction

1.1.Cellular senescence

Senescence was first described by Leonard Hayflick and Paul Moorhead in 1961 as a cell-intrinsic process that limits proliferation of human diploid fibroblasts to a definite cell passage number, termed the Hayflick limit (Hayflick and Moorhead, 1961). This mechanism involves shortening of repetitive DNA sequences at the end of linear chromosomes, so called telomeres, leading to an irreversible growth arrest. Nowadays, senescence is known as a cellular process triggered by various mechanisms but generally characterized by a stable proliferation arrest. In many conditions, the onset of a strongly inflammatory senescence-associated secretory phenotype (SASP) (Hernandez-Segura et al., 2018; Huang et al., 2022) has been described as another hallmark of cellular senescence. Besides an irreversible growth arrest, senescent cells exhibit strong resistance to apoptosis and undergo considerable morphological changes such as multinucleation and a substantial cell size increase. Cellular senescence is primarily associated with natural ageing, due to an age-related accumulation of senescent cells that is driven by genomic instability and constitutive telomere shortening (McHugh and Gil, 2018). However senescence can also be induced by other cell intrinsic and extrinsic stresses, such as oncogene activation, oxidative stress and cytokine signaling (Huang et al., 2022) (Fig 1.1). Therefore, senescence is classified into different sub-types based on the underlying initiation process. Therapy-induced senescence (TIS) for instance, is a type of senescence that is initiated by therapeutic interventions such as chemotherapy or radiation therapy (Saleh et al., 2020). On the other hand, oncogene-induced senescence (OIS) only occurs in malignant and pre-malignant cells and is mediated by activation of certain oncogenes (Chandeck and Mooi, 2010; Zhu et al., 2020). Moreover, cytokine-induced senescence is triggered by stimulation with distinct cytokines, namely tumor necrosis factor (TNF) and Interferon-γ (IFN-γ) (Hashimoto et al., 2022; Rentschler et al., 2022).

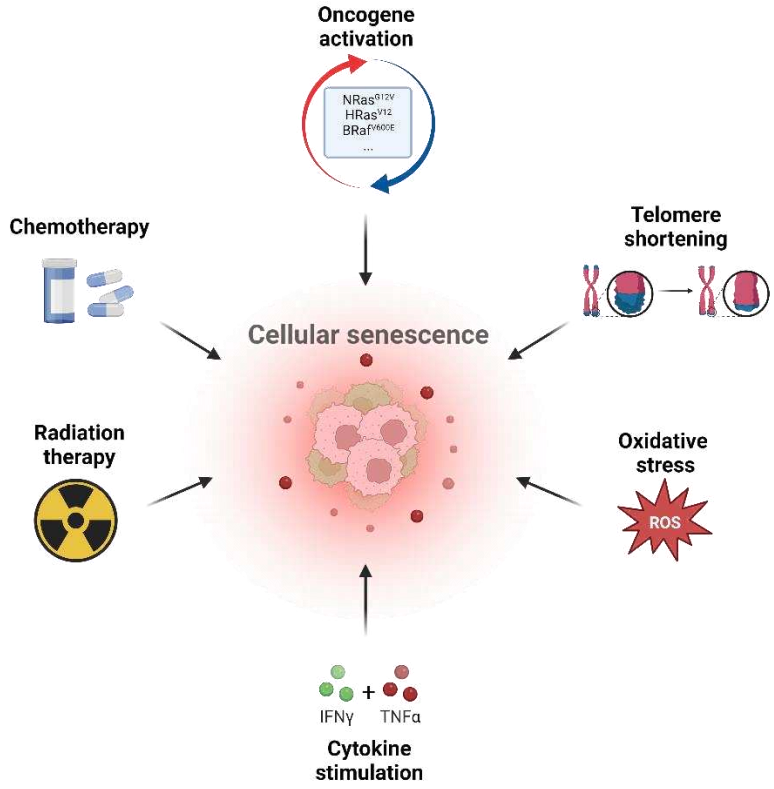

Figure 1.1: Senescence inducing stresses and treatments.

Various extrinsic and intrinsic stress signals as well as different cancer therapies can induce cellular senescence. These include oncogene activation, telomere shortening, oxidative stress, cytokine stimulation, chemotherapy, and radiation therapy.

Research on cellular homeostasis has revealed that senescence is a complex cellular phenotype that drives and modulates a variety of cellular mechanisms and pathophysiological processes, such as tissue remodeling, wound healing and anti-tumor response (Di Micco et al., 2021; Marin et al., 2022; Wilkinson and Hardman, 2020). Additionally, several disease models have been developed to investigate the contribution of senescence responses to disease development, progression, and protection. For instance, a murine disease model of lung fibrosis has indicated that senescent fibroblasts are crucial for development of fibrotic pulmonary disease (Schafer et al., 2017). Other findings have demonstrated that senescent cells are critical players in the progression of neurodegenerative disorders, such as Alzheimer's and Parkinson disease. For examples, senescent astrocytes accumulate during Alzheimer's disease, driving disease progression (Bhat et al., 2012). Additionally, inflammatory factors secreted by senescent cells have shown to promote the development of Parkinson disease (Russo and Riessland, 2022). Although senescence plays a pivotal role in the development and progression of several diseases, it also promotes cellular reprogramming and immune-

mediated elimination of dysfunctional cells. Correspondingly, previous studies have reported that senescence represents a highly heterogenous phenotype that varies between cell types, types of induction, and underlying cell-intrinsic pathways (Kirschner et al., 2020). In general, it has to be borne in mind that the term “senescence” is not a precise definition that always includes a defined set of molecular or cellular hallmarks but may be applied in the literature to different phenomena on an imprecise manner. Despite of these ambiguities and functional heterogeneities several senescence biomarkers have been selectively characterized for efficient identification of senescent cells.

1.1.1. Senescence biomarkers

Cellular senescence can be identified on multiple layers, including altered metabolic activity, secretory responses, transcriptional changes, or morphological alterations in different entities and upon different inducer pathways (Huang et al., 2022). Despite of cell-type and inducer-dependent heterogeneities, a set of biomarkers has been proposed for efficient identification of senescent cells. A summary of such reported senescence biomarkers and associated cell responses is given in table 1.1.

Table 1.1: Senescence biomarkers and associated cell intrinsic pathways.

Cellular mechanism	Senescence biomarkers and associated pathways/mechanism	Description
Cell cycle inhibition	**p16-pRb pathway:** $p16^{INK4a}$ CDK4/6 pRb	Increased expression and phosphorylation of p16-pRb- and p53-p21-associated proteins. Downregulation of proliferation markers such as Ki67 or PCNA. Alternatively, DNA incorporation assays such as BrdU can be conducted to confirm proliferation arrest.
	p53-p21 pathway: p53 CDK2 $p21^{WAF/CIP1}$	
	Proliferation arrest: Ki67 PCNA	
Morphological changes	Enlarged and flattened cell morphology	Strong increase in cell size and irregular shaped nuclei. Some cells display multinucleation. Increased vacuolization and granularity.
	Multinucleation	
	Vacuolization	
Lysosomal activity	Senescence-associated β-galactosidase (SA-β-Gal)	Elevated expression of lysosomal β-galactosidase.
Secretory response	Senescence-associated secretory phenotype (SASP)	Strong secretion of inflammatory, cytokines, chemokines, and growth factors.
Histone modifications / epigenetic regulation	Senescence-associated heterochromatin foci (SAHF), H3K9me3 H3K27me3 γ-H2AX	Presence of senescence and DNA-damage associated histone modifications.
Nuclear membrane remodeling	Lamin B1	Decrease in expression of nuclear envelop protein Lamin B1.
Apoptosis resistance	**Anti-apoptotic proteins:** Bcl-2 Bcl-w Bcl-xl	Elevated expression of anti-apoptotic proteins Bcl-2, Bcl-w and Bcl-xl.
Metabolic activity	Loss of NAD^+ Autophagic vesicles Glycolytic activity	Senescence-associated increase in autophagy, increased glycolytic activity and altered NAD metabolism.

(Hernandez-Segura et al., 2018, Kudlova et al., 2022)

As previously mentioned, one hallmark of senescent cells is an irreversible proliferation arrest. Depending on the cell type and senescence inducer this arrest can be initiated via different cell cycle regulators. Two crucial pathways involved in senescence-associated growth arrest are the p16-pRb and p53-p21 pathways (Kumari and Jat, 2021). Activation of p53-p21 ultimately results in an increased expression and activation of the cyclin-dependent kinase (CDK) inhibitor $p21^{Waf/CIP1}$, blocking CDK-complex formation and subsequently inducing an irreversible proliferation arrest (Kumari and Jat, 2021). Similar to $p21^{Waf/CIP1}$, the CDK inhibitor

$p16^{INK4a}$ exerts its anti-proliferative function via blocking CDK-complexes, thereby inhibiting phosphorylation of retinoblastoma protein (RB). Notably, cancer cells often display strong functional dysregulations in p16-pRb and p53-p21 pathways (Liggett and Sidransky, 1998; Soussi and Wiman, 2007). Additionally, recent studies have reported a p53-independent activation of p21 during TIS (Fleury et al., 2019) as well as OIS (Storer et al., 2013). This has raised questions regarding the existence of a reversible senescence phenotype in cancer cells, what has already been demonstrated for various models of senescence (Beauséjour et al., 2003; Chitikova et al., 2014). However, further investigations are essential to explore the reversibility of senescence in cancer cells, particularly in relation to cell cycle regulation and the involvement of p16-pRb and p53-p21 pathways.

Besides a stable proliferation arrest, senescent cells also display strong morphological changes, accompanied by an increased vacuolization and multinucleation (Hernandez-Segura et al., 2018; Huang et al., 2022). Although they typically maintain their cell shape *in vivo*, senescent cells exhibit an enlarged cell size and flattened morphology when cultured *in vitro* (Biran et al., 2017; Herranz and Gil, 2018). Additionally, nuclear envelop remodeling, in particular dysregulation of nuclear membrane proteins, is a biomarker of senescence. For instance, loss of Lamin B1 has been associated to senescence *in vitro* and *in vivo* (Freund et al., 2012). Due to a gradual decline that correlates with the magnitude of senescence, Lamin B1 has been suggested as a diagnostic marker for neoplastic diseases and age-related disorders (Saleh et al., 2022; Wang et al., 2017).

One of the most commonly used senescence biomarkers is senescence-associated β-galactosidase (SA-β-Gal). SA-β-Gal was first described in 1995 (Dimri et al., 1995), and has ever since been characterized as a reliable biomarker of senescence. Consequently, several SA-β-Gal biotracers have been developed for identification of senescent cells *in vitro* and *in vivo* (Krueger et al., 2019; Yang and Hu, 2004). Although the physiological role and underlying mechanisms behind the elevated expression of β-galactosidase remain unclear, SA-β-Gal has often been associated with increased lysosomal activity due to its cellular localization in the lysosome (Lee et al., 2006).

Furthermore, senescent cells display a resistance to programmed cell death, namely apoptosis (Huang et al., 2022). This resistance is mediated by several anti-apoptotic proteins, mostly of the B-cell lymphoma 2 (Bcl-2) family, including Bcl-xl, Bcl-2 and Bcl-w. Due to their strong and

consistent overexpression Bcl-2 proteins have shown to be the most promising targets for selective clearance of senescent cells (Martin et al., 2023). Hence, several small molecule inhibitors have recently been developed targeting Bcl-2-associated proteins.

Moreover, senescence also affects the epigenetic profile of cells. Specific heterochromatin foci and distinct histone modifications, such as methylation of different lysine motifs on histone H3, have shown to occur exclusively during senescence (Aird and Zhang, 2013). Many markers of these so-called senescence-associated heterochromatin foci (SAHF) are found at promoter sides of E2F target genes (Schulz and Tyler, 2005). E2F is a family of transcription factors that are essential for maintaining proliferative capacities. Hence, SAHF are crucial for transcriptional silencing of genes that regulate cell cycle progression and thus, promote stable proliferation arrest of senescent cells (Aird and Zhang, 2013; Schulz and Tyler, 2005). Notably, SAHF have also been associated with regulating the secretory response of senescent cells (Strzyz, 2019).

1.1.2. Senescence-associated secretory phenotype (SASP)

One of the main hallmarks of senescent cells is their inflammatory senescence-associated secretory phenotype (SASP). Despite of cell type and senescence inducer-dependent heterogeneities, the SASP has shown to be essential for senescence surveillance by initiating a signaling cascade that culminates in immune-mediated clearance of senescent cells (Coppé et al., 2010; Huang et al., 2022). Nevertheless, in case of pre-existing disease, the SASP can promote disease progression and provides the basis for the development of inflammatory disorders (Huang et al., 2022). Due to the high complexity and cell type-specificity, characterization and modulation of the SASP have been a major focus of senescence-associated research in past years (Cuollo et al., 2020). Although the composition of secreted SASP factors appears to vary greatly between cell types and senescence inducers, some SASP factors are more prominent than others. One cytokine that has recently been associated to being one of the main drivers of senescence and SASP is Interleukin-1α (IL-1α) (Frisch, 2022; Wiggins et al., 2019). However, mechanisms on how IL-1α regulates senescence responses remain unclear. Two studies demonstrated that IL-1α is an essential activator of the transcription factor Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which in turn transcriptionally regulates the expression of other SASP cytokines and chemokines (C. Kang et al., 2015; Laberge et al., 2015). Nonetheless, both studies present

conflicting results on upstream mechanisms that are responsible for IL-1α overexpression and secretion.

Besides IL-1α, other well characterized SASP cytokines and chemokines are IL-8, IL-6, IL-1β, C-X-C motif chemokine ligand 1 (CXCL1), CXCL10 or IFN-γ (Coppé et al., 2010; Cuollo et al., 2020). Most SASP factors initiate distinct downstream signaling pathways. Therefore, the SASP can have distinct physiological outcomes depending on its composition and may act on senescent cells themselves, non-senescent bystanders or tissue-infiltrating cells. Additionally, not only cytokines and chemokines but also various growth factors can constitute this secretory phenotype, e.g. endothelial growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and matrix-metalloproteinases (MMPs) namely, MMP-1, MMP-3 and MMP-10 (Coppé et al., 2010; Cuollo et al., 2020).

Due to the various cytokines, chemokines and growth factors that constitute the SASP, this secretory phenotype is tightly regulated and controlled by various innate pathways. One cell intrinsic mechanism that plays a crucial role during senescence and SAPS responses is DNA damage (Glück et al., 2017). The role of cytosolic DNA sensing in SASP regulation has been a major focus of senescence-associated research in past years. A crucial DNA sensing pathway that has been determined as a main driver of SASP is mediated by cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS) and its adaptor stimulator of interferon genes (STING) (Glück et al., 2017). This so called cGAS-STING pathway is activated via cGAS-mediated sensing of double-stranded DNA (Decout et al., 2021). Activated cGAS initiates a signaling cascade that results in the formation of the second-messenger molecule 2´3´-cyclic GMP-AMP (cGAMP). cGAMP in turn, activates STING leading to the expression of type I interferons (Decout et al., 2021; Ou et al., 2021). Strikingly, cGAS-STING has shown to control senescence responses by initiating STING-mediated SASP factor secretion and promoting paracrine or secondary senescence (Glück et al., 2017). This was demonstrated for various senescence inducing stimuli including OIS and irradiation (Glück et al., 2017).

Conclusively, the SASP presents a tightly regulated senescence-associated process that is essential for senescence surveillance but may also drive disease progression due to its highly inflammatory character. Although the SASP has distinct effects depending on the tissue and cell status its role in immune-mediated clearance is well studied and has been a major focus

of senescence-associated research due to it physiological importance (Faget et al., 2019; Yang et al., 2021).

1.1.3. Innate immune-mediated senescence surveillance

Tissue maintenance and repair requires immune-mediated elimination of aging and damaged cells (Kuehnemann and Wiley, 2023). As aforementioned, one mechanism initiating immune cell attraction and activation against dysfunctional cells is cellular senescence. Immune cells employ various mechanisms to detect and remove senescent cells, including SASP-mediated chemotaxis and activating surface molecules (Huang et al., 2022). Innate immune cells, particularly natural killer (NK) cells, have emerged as pivotal players in the recognition and removal of senescent cells.

1.1.4. NK cell-mediated clearance

NK cells are cytotoxic innate immune cells of the lymphoid lineage. They account for 5 – 20 % of all circulating lymphocytes and serve as a first line of defense against tumor cells, virus-infected cells and other abnormal cells (Murphy and Weaver, 2018). NK cell activation is a tightly regulated process and controlled by various receptor and cytokine interactions (Fig. 1.2). An indispensable mechanism involved in this process is the concept of "altered-self". This concept refers to a skewed balance between activating and inhibitory molecules on the surface of target cells (Abel et al., 2018; Paul and Lal, 2017). In particular, a lack of inhibitory major histocompatibility complex class I (MHC-I) molecules leads to insufficient inhibitory signals, thereby activating NK cells. Besides MHC-I binding proteins, several inhibitory and activating NK cell receptors have been identified including proteins of the natural killer group 2 (NKG2) family and killer-cell immunoglubolin-like receptors (KIRs) (Abel et al., 2018) (Fig. 1.2). In addition to direct activation, NK cells can also be activated by soluble factors secreted by other immune cells such as macrophages or dendritic cells. Cytokines like IL-2, IL-12, IL-15 or IL-18 can activate NK cells and promote their proliferation (Abel et al., 2018).

NK cells eliminate target cells primarily through the secretion of granzymes and perforins (Murphy and Weaver, 2018). Upon activation, they release granules that contain these proteolytic enzymes, which subsequently induce target cell death (Malhotra and Shanker, 2011). NK cell clearance can also be mediated via stimulation with distinct TNF receptor ligands. These ligands directly interact with death receptors expressed on the surface of target

cells, thereby initiating intrinsic signaling pathways that induce target cell apoptosis (Abel et al., 2018; Paul and Lal, 2017). Besides direct interactions, TNF receptor ligands can also be secreted via exosomes and initiate apoptosis independently of cell-to-cell contact (Paul and Lal, 2017). Additionally, activated NK cells do not only exert cytotoxic effects themselves, but also recruit other immune cells by secretion of inflammatory cytokines and chemokines such as IFN-γ.

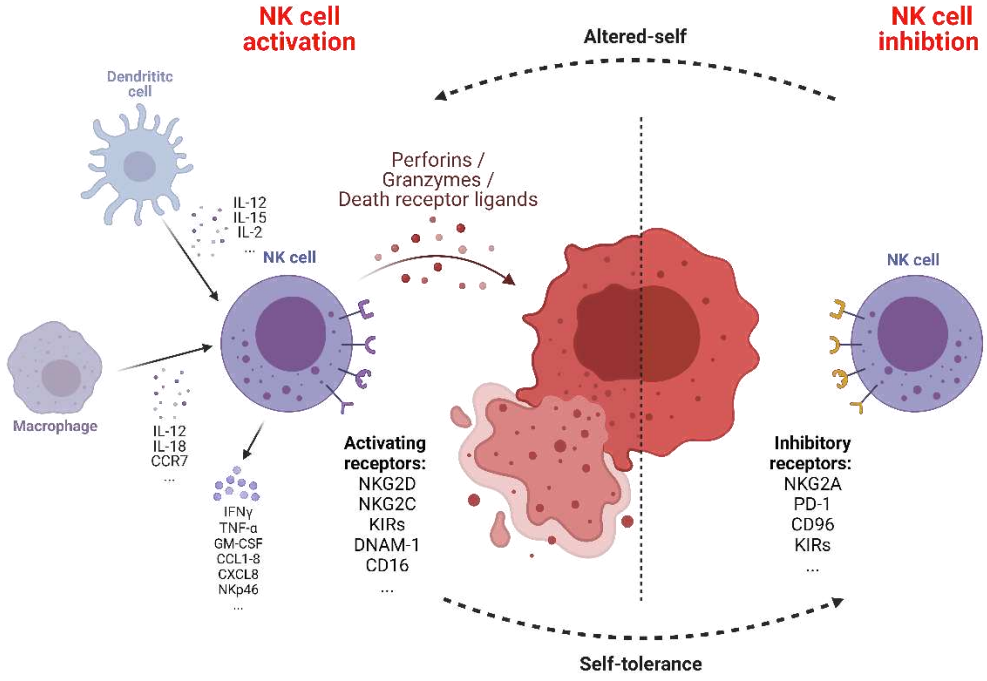

Figure 1.2: **Schematic overview of natural killer cell activation and inhibition.**

Several surface molecules, cytokines, and chemokines regulate NK cell activation. A crucial mechanism involved in this process is the concept of "altered-self", which describes a skewed balance between activating and inhibitory molecules on the surface of target cells. NK cells cytotoxicity is primarily mediated by release of perforins and granzymes that kill target cells. Additionally, NK cells can release death receptor ligands that, upon binding to respective receptors, induce target cell apoptosis.

NK cells are crucial for the clearance of senescent cells (Antonangeli et al., 2019). The secretome of senescent HSCs, fibroblasts, and endometrial stromal cells has shown to be strongly 'NK cell engaging', due to a presence of IL-6, CXCL8 (IL-8), IL-15 and CXCL10 (Antonangeli et al., 2019). Additionally, therapy-induced senescence in multiple myeloma and breast cancer cell lines resulted in increased expression of NK cell recruiting cytokines and activating ligands, thereby promoting NK cell-mediated clearance (Antonangeli et al., 2019; Soriani et al., 2009). Furthermore, restoration of p53 in a murine liver cancer model has shown to promote NK cell infiltration and activation via senescence-associated IL-15 secretion and ICAM-1 expression (Xue et al., 2007). Conversely, recent studies have suggested that

fibroblasts can evade NK cell clearance upon initiation of OIS by increasing expression of HLA-E, which interacts with inhibiting NK cell receptors (Kale et al., 2020; Pereira et al., 2019). Altogether, NK cells are essential for senescence surveillance during tumor progression and tissue homeostasis. Despite of functional differences between senescence inducers and tissue types, various senescence-associated processes have shown to promote NK cell recruitment as well as activation.

1.1.5. Neutrophils in senescence

In addition to NK cells, also neutrophils are crucial first responders and play an essential role in preserving tissue health and recruiting other immune cells. Neutrophils are the most abundant leukocytes and belong to the group of granulocytes (Murphy and Weaver, 2018). They account for 50 – 70% of all circulating leukocytes and are essential for clearance of bacteria and other pathogens (Liew and Kubes, 2019). As rapid responders to danger signals neutrophils are essential for initiating inflammatory responses by secretion of antimicrobial compounds, reactive oxygen species (ROS) and extrusion of DNA, RNA and other intracellular material in form of neutrophil-extracellular traps (NETs) (Németh et al., 2020). Despite playing an essential role in anti-microbial immune responses neutrophils have been associated with modulating pathogenesis of different diseases such as cancer, sepsis, and various autoimmune diseases (Németh et al., 2020).

Although neutrophils have shown to be crucial for tissue homeostasis, their role in senescence is not well characterized (Shim et al., 2022). It is evident that various SASP factors cause neutrophil attraction suggesting that senescence may drive neutrophil recruitment to an inflamed tissue. However, it is not entirely clear whether neutrophils monitor senescence surveillance or rather promote senescence propagation. Notably, increased neutrophil levels were observed for several senescence-associated pathologies, such as age-related pulmonary inflammation. Strikingly, increased level as well as dysregulated signaling was associated with worse clinical outcome (Kulkarni et al., 2019; Nomellini et al., 2012; Van Avondt et al., 2023). Furthermore, some studies have shown that neutrophils can induce paracrine senescence in the liver by causing telomere dysfunction and removal of senescent cells from aged livers reduced neutrophil infiltration (Lagnado et al., 2021). Although neutrophils are mainly recognized for their function in rapid immune responses to infection, they can thus also have an impact on senescence-associated processes, indirectly by promoting chronic inflammation

or directly by inducing paracrine senescence. Nevertheless, further research is required to uncover the functional interplay between neutrophil activity and cellular senescence.

1.2.Senescence in cancer

Senescence has major pathophysiological impacts on various neoplastic diseases. While OIS affects the onset and progression of different malignancies, TIS can be crucial in the context of therapeutic intervention, namely anti-cancer treatment (Yang et al., 2021). Notably, cancer senescence has both, beneficial and detrimental effects (Huang et al., 2022; Yang et al., 2021). On one hand, senescence promotes tumor growth by secretion of growth factors and development of an inflammatory environment. On the other hand, it is associated with growth arrest and initiates anti-tumor responses by promoting immune cell infiltration and activation (Huang et al., 2022).

For example, recent studies have demonstrated that cancer senescence increases tumor immunogenicity (Marin et al., 2022). Moreover, senescent cells not only release inflammatory signals in form of SASP factors but also sense environmental triggers as evidenced by the enhanced response to IFN-γ stimulation (Chen et al., 2023). Furthermore, increased MHC-I expression suggests that senescent cells have an elevated capacity for antigen presentation and thus, trigger immunosurveillance (Chen et al., 2023).

Other studies have demonstrated that senescent cells promote cancer relapse and increase side effects of chemotherapy due to systemic inflammation (Demaria et al., 2017). Additionally, many SASP cytokines and chemokines can induce a pro-tumorigenic environment. Strikingly, inhibition of SASP regulator polypyrimidine tract-binding protein 1 (PTBP1) reduces inflammation-driven cancer progression, while inhibiting tumor immune surveillance (Georgilis et al., 2018).

Altogether, cancer senescence can thus have distinct effects depending on the type of cancer, senescence trigger and disease state (Kirschner et al., 2020). These controversial effects emphasize the complexity of cancer senescence, which warrants further investigations. Particularly, therapy-induced senescence can have diverse outcomes due to beneficial immune cell activating and adverse tumor promoting effects. Hence, an overview of TIS-inducing compounds and their associated mechanisms will be given in the following.

1.2.1. Therapy-induced senescence (TIS)

As previously mentioned, TIS describes a type of senescence induced by chemotherapeutic drugs, radiation therapy, or other therapeutic agents. Depending on the inducing trigger different underlying mechanisms are involved which highlights the complexity of the senescent phenotype. TIS-inducing mechanisms include CDK4/6 inhibition, DNA-damage, aurora kinase inhibition, ribosomal stress, and telomerase inhibition (Fig 1.3) (Ewald et al., 2010; Wang et al., 2022). Selected TIS regimens are briefly summarized in the following:

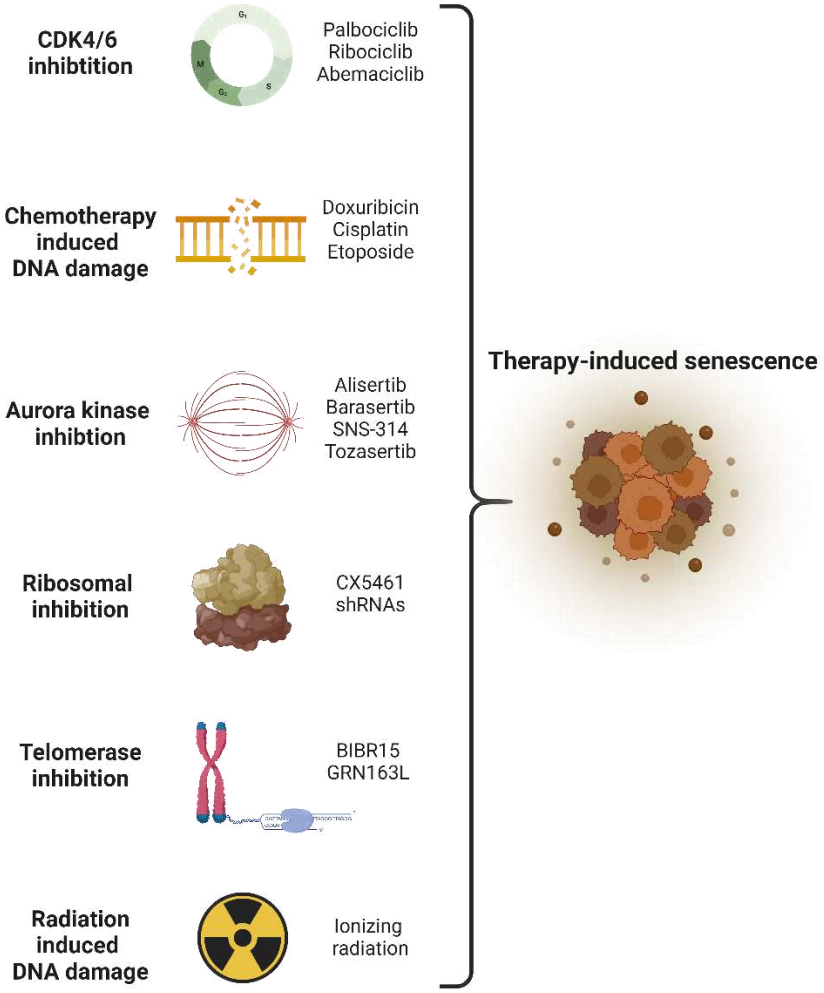

Figure 1.3: TIS-inducing therapy and associated cellular pathways

TIS can be initiated by various therapies that activate distinct cell intrinsic pathways. Therapeutic approaches that induce TIS are CDK4/6 inhibition, chemotherapy- or radiation therapy-induced DNA damage, aurora kinase inhibition, ribosomal inhibition, and telomerase inhibition.

1.2.2. DNA damage-induced senescence

Chemotherapeutic compounds can cause genomic instability through various mechanisms, such as, inducing double-strand DNA breaks, targeting DNA repair mechanism, or interfering

with DNA synthesis. DNA damage represents a cellular danger signal that leads to the activation of distinct cell intrinsic pathways. These, so called DNA damage responses (DDR) initiate signaling cascades that result in the induction of apoptosis or cellular senescence (Chen et al., 2007; Wang et al., 2022). Both cell fates are associated with DDR, while commitment to one of them is often a matter of the initial extend of DNA damage (d'Adda di Fagagna, 2008). However, what exactly drives the selective choice between senescence and apoptosis remains to be determined.

DNA damage is sensed by different adaptor proteins that initiate two distinct signaling pathways, Ataxia-telangiectasia and Rad3 related - checkpoint kinase 1 (ATR-CHK1) and Ataxia-telangiectasia mutated – checkpoint kinase 2 (ATM-CHK2) (d'Adda di Fagagna, 2008; Wang et al., 2022). Activation of these DNA-damage-associated pathways may trigger the interconnected p53-p21 pathway by phosphorylation and subsequent activation of p53, thereby initiating senescence responses.

The chemotherapeutic agents doxorubicin and etoposide are used for the treatment of various solid tumors and hematologic malignancies (Nitiss, 2009; Reyhanoglu and Tadi, 2023; Wang et al., 2022). Both are topoisomerase II inhibitors and thus, prevent the repair of double stranded DNA, resulting in DDR and subsequent apoptosis or senescence induction (Nitiss, 2009). While moderate concentrations of etoposide and doxorubicin have shown to induce senescence, higher concentrations initiate apoptosis (Chang et al., 1999; Childs et al., 2014; Jochems et al., 2021). Together with the purine binding chemotherapeutic agent cisplatin, doxorubicin and etoposide are some of the most commonly used DNA damage-mediated senescence inducers (Chang et al., 1999). Besides chemotherapeutic compounds, ionizing radiation can also induce senescence in different types of cancer (Kim et al., 2023). Here, single- and double-stranded DNA breaks induce a strong DNA damage response, thereby initiating apoptosis or senescence. However, radiation-induced senescence carries an increased risk of spreading to surrounding non-cancer tissue, promoting a highly inflammatory and persistent senescence phenotype that accelerates neoplastic growth (Li et al., 2018; Tabasso et al., 2019).

1.2.3. CDK4/6 inhibition-induced senescence

Dysregulated cell cycle progression is one of the main hallmarks of senescence. Hence, targeting key regulators of this process has been a promising strategy to induce cancer senescence. CDK4 and CDK6 are protein kinases that are essential for controlling and

regulating various stages of the cell cycle (Vermeulen et al., 2003). A tightly regulated interplay between $p16^{INK4a}$, CDK4/6, cyclin D1 and pRb mediates the activation of E2F transcription factors, subsequently initiating DNA replication and cell division (Kumari and Jat, 2021). In particular, active cyclin D1-CDK4/6 complexes phosphorylate RB and thus, disassociate pRB from the transcription factor E2F promoting transcription of E2F-associated genes (Kumari and Jat, 2021). CDK4/6 inhibitors are designed to specifically bind CDK4 and CDK6 and thus inhibit their cell cycle promoting activity by preventing formation of active cyclin D1-CDK4/6 complexes. The most commonly used CDK4/6 inhibitors are palbociclib, ribociclib and ademaciclib (Braal et al., 2021). Besides being ideal for *in vitro* investigations of cancer senescence, all three compounds have significantly improved treatment options for patients with HER2-negative breast cancer (Wu et al., 2020). Additionally, CDK4/6 inhibitors have shown promising results in pre-clinical studies and clinical trials for treatment of pancreatic cancer (Rencuzogulları et al., 2020), non-small lung cancer (Ahn et al., 2020) and sarcoma (Hsu et al., 2022). Furthermore, when used in combination with other chemotherapeutic agents CDK4/6 inhibitors have strong anti-proliferative effects in human colorectal cancer cells (Huang et al., 2023; Lee et al., 2023; Lin et al., 2020).

Another actionable target for senescence induction is CDK2. As a crucial cell cycle regulator, CDK2 is essential for G1 to S phase transition (Tsai et al., 1993). Pharmacological inhibition as well as transcriptional repression of CDK2 have shown to induce senescence in different types of cancer (Bazzar et al., n.d.; Zalzali et al., 2015). However, so far, no selective CDK2 inhibitor has been clinically tested for anti-tumor therapy. Nevertheless, recently developed multikinase inhibitors that inhibit CDK2, CDK4 and CDK6 are currently being tested in clinical trials for treatment of HER2-negative breast cancer (Freeman-Cook et al., 2021). Additionally, CDK2 inhibition has shown to overcome resistance to selective CDK4/6 inhibitors, indicating that CDK2 is a suitable target for cancer therapy (Al-Qasem et al., 2022; Pandey et al., 2020).

Altogether CDK inhibition represents an ideal strategy to study senescence-associated mechanisms *in vitro*. Additionally, selective CDK inhibitors are intriguing prospects for cancer therapy and have already shown great success in the treatment of HER2-negative breast cancer.

1.2.4. Ribosomal stress-induced senescence

A striking similarity between CDK-inhibitor induced senescence and ribosomal-stress induced senescence is their influence on cell cycle progression. While CDK inhibitors halt cell proliferation by inhibiting crucial cell cycle regulators, ribosomal stress triggers a cell cycle arrest response as part of cellular defense mechanisms (Donati et al., 2012). Both pathways involve the activation of tumor suppressors that initiate senescence responses.

Ribosomal stress represents a critical cellular response to ribosome-related dysfunctions, with implications for both cancer biology and therapeutic strategies (Zisi et al., 2022). However, only few ribosomal inhibitors have been developed in the past years. The most therapeutic ribosomal inhibitor is CX5461. CX5461 interferes with ribosomal biogenesis by inhibiting RNA-polymerase I, thereby blocking transcription of ribosomal genes (Mars et al., 2020). The reduced synthesis of ribosome-associated proteins triggers a cellular response that involves the activation of tumor suppressor proteins and DNA-damage responses (Bywater et al., 2012; Negi and Brown, 2015). Besides being associated to ribosomal biogenesis, CX5461 has also drawn attention for its ability to stabilize DNA G-quadruplex (G4) structures (Xu et al., 2017). G4 structures are unique secondary structures that play an important role in DNA-damage responses (Linke et al., 2021).

CX5461 has shown to induce senescence in a variety of cancers (Drygin et al., 2011; Mars et al., 2020). Additionally, anti-cancer effects of CX5461 have been reported for neuroblastoma (Taylor et al., 2019), prostate cancer (Lawrence et al., 2018), ovarian cancer (Yan et al., 2017), multiple myeloma (Lee et al., 2017) and acute lymphoblastic leukemia (Negi and Brown, 2015b). Hence, several clinical trials on CX5461 have been conducted in patients with advanced solid tumors and haematologic malignancies (Hilton et al., 2022; Khot et al., 2019).

Besides pharmacological inhibition of ribosome biogenesis, transcriptional repression of ribosomal genes has also shown to efficiently induce senescence. Knockdown of genes that are essential for ribosomal maturation induced robust senescence responses in a p53-dependent manner (Pantazi et al., 2019). Notably, this gave rise to a secretory phenotype that was lacking most of the commonly observed SASP features, while still inducing SA-β-Gal, $p16^{INK4a}$ and p53 expression (Pantazi et al., 2019).

Altogether, disruption of ribosome biogenesis and associated ribosomal stress can induce secondary responses in cancer cells, such as DDR or activation of tumor suppressor proteins. This may result in initiation of cellular senescence. The ribosomal inhibitor CX5461 has shown promising results in pre-clinical studies and is currently under investigation in several clinical trials. However, to fully understand the connection between senescence responses and dysfunctional ribosome activity other ribosomal-inhibitors have to be generated and tested for their senescence-inducing capacities.

1.2.5. Aurora kinase inhibition-induced senescence

Aurora kinases are a group of highly conserved serine/threonine kinases that are crucial regulators of mitosis, meiosis, and cytokinesis (Willems et al., 2018). In mammals, they are categorized into three sub-classes, namely Aurora kinase A (AURKA), B (AURKB) and C (AURKC). AURKB and AURKC share many functional characteristics and are essential for chromosome alignment and regulation of cytokinesis. Conversely, AURKA has a pivotal role during early phase mitosis by regulating centrosome assembly and spindle formation (Ewald et al., 2010; Willems et al., 2018). Aurora kinases also display strong heterogeneities in their cellular localization. While AURKA and AURKB are globally expressed in all actively proliferating cells, AURKC is primarily expressed in germ cells (Carmena and Earnshaw, 2003; Willems et al., 2018).

Dysregulation or hampered activity of aurora kinases has shown to induce senescence in various cancers *in vitro* and *in vivo* (Borah and Reddy, 2021; Huck et al., 2010; Liu et al., 2013). Additionally, some studies have reported functional interplays between aurora kinase activity and DDR, linking aurora kinase inhibition to DNA damage-mediated senescence (Liu et al., 2013; Ma and Poon, 2020). Other non-mitotic functions of aurora kinases have also been identified, that may play a role during aurora kinase-associated senescence, such as maintenance of telomeres (Ma and Poon, 2020).

Due to their enhanced proliferative capacities, cancer cells often display increased expression as well as hyperactivation of aurora kinases (Tang et al., 2017). Additionally, aurora kinases have shown to activate metastasis-promoting signaling pathways (Tang et al., 2017). Hence, targeting these highly conserved kinases has become of great interest for cancer therapy. The AURKA inhibitor Alisertib and the AURKB inhibitor Barasertib have indicated good safety profiles in clinical trials for treatment of various solid tumors and hematologic malignancies

(Löwenberg et al., 2011; Martinelli et al., 2012; Melichar et al., 2015). Additionally, Alisertib has shown great success in the treatment of acute myeloid leukemia when administered in combination with induction chemotherapy, with 51% of patients achieving complete remission (Brunner et al., 2020).

Conclusively, interference of mitotic regulation presents a great strategy for induction of senescence. Due to their functional characteristics, aurora kinases are an ideal target for inducing mitotic dysregulation and thus, senescence. AURKA and AURKB inhibitors have shown great efficiency in inducing senescence responses and anti-cancer effects in various cancer settings.

1.3.Targeting senescent cancer cells

As previously described, TIS represents an efficient therapeutic strategy to terminate rapid proliferation of cancer cells. Hence, several TIS agonists have been tested in clinical trials, with some showing promising therapeutic outcomes. However, while TIS has the short-term benefit of stably arresting cancer cell proliferation, persistent tumor senescence has shown to promote tumor growth rather than exert anti-tumor effects (Huang et al., 2022). Thus, several therapeutic approaches have been developed targeting tumor senescence, thereby eliminating cells that fuel the inflammatory and pro-tumorigenic environment (Saleh et al., 2022). Due to their anti-apoptotic character senescent cells display strong resistance to chemotherapy (Jo et al., 2023). In recent years, numerous small molecule inhibitors targeting anti-apoptotic proteins have been developed to specifically target senescent cells. These, so called senolytics are drugs that selectively eliminate senescent cells by targeting pathways, cellular mechanisms or antigens that are exclusively upregulated in senescent cells (Kudlova et al., 2022; Zhang et al., 2023). However, in past years the term “senolytic” has also been associated with immunotherapies that selectively target senescent cells. In the following an overview of different senolytic approaches will be given.

The most widely used senolytics are the small molecule inhibitors navitoclax, quercetin, dasatinib and fisetin (table 1.2). While navitoclax targets anti-apoptotic Bcl-2 proteins, dasatinib is a potent SRC and ABL kinase inhibitor (Kudlova et al., 2022; Zhang et al., 2023). Conversely, the naturally occurring flavonoids quercetin and fisetin exert senolytic activities through various mechanism, such as targeting p53, NF-κB, PI3K and Bcl-2 signaling (Zhang et al., 2023). However, their main mechanism of action is still not fully understood.

Table 1.2: Most commonly used senolytic drugs

Senolytic drug	Mechanism of action
Navitoclax	Bcl-2, Bcl-w and Bcl-xl inhibitor
Dasatinib	SRC- and ABL-family kinase inhibitor
Quercetin	Targets Bcl-2, PI3K and p53 signaling, other unknown mechanism
Fisetin	Bcl-2 and PI3K/AKT inhibitor, targets p53 and NF-κB signaling, other unknown mechanism

Senolytic activity has been demonstrated for navitoclax in various pre-clinical cancer models (Wang et al., 2022; Zhang et al., 2023). However, clinical trials indicated that navitoclax can cause side effects such as thrombocytopenia and neutropenia (Chaib et al., 2022; Wang et al., 2022; Wilson et al., 2010; Zhu et al., 2016). The combination of dasatinib and quercetin has shown more success regarding clinical translation. Phase I clinical trials of dasatinib and quercetin in patients with idiopathic pulmonary fibrosis (IPF) indicated a good safety profile and are currently ongoing (Nambiar et al., 2023).

Besides small molecule inhibitors also senolytic immunotherapy has shown high efficiency in eliminating senescent cancer cells. Particularly, senescence-specific chimeric antigen receptor (CAR) T cells have emerged as promising therapeutic tools (Amor et al., 2020). CAR-T cells are genetically modified T cells that carry a chimeric antigen receptor consisting of an extracellular antigen recognition domain and an intracellular signaling domain (Alnefaie et al., 2022). CAR-T cells are activated upon binding to their target antigen resulting in the release of cytotoxic molecules and subsequent death of target cells. Various types of CAR-T cells have shown great success in treatment of distinct hematologic malignancies (Sterner and Sterner, 2021). Notably, recent studies have uncovered urokinase-type plasminogen activator receptor (uPAR) as a actionable senescence-associated antigen (Amor et al., 2020). Hence, uPAR has emerged as a promising senolytic target. Strikingly uPAR-specific CAR-T cells exerted strong cytotoxicity against senescent cell *in vitro* and *in vitro.* Treatment with uPAR CAR-T cells extended survival of mice with lung adenocarcinoma when pre-treated with TIS inducers (Amor et al., 2020). Additionally, administration of CAR-T cells targeting uPAR improved tissue homeostasis in mice with liver fibrosis (Amor et al., 2020). However, further investigations in clinical trials are crucial to determine safety and efficiency of uPAR CAR-T cell therapy in humans.

Another putative approach for immunotherapeutic targeting of senescent cancer cells is senolytic peptide vaccination. Despite of promising preliminary findings, this approach has not been a major focus of senescence-associated research. Recent studies have indicated that senescent cells have an altered immunopeptidome (Marin et al., 2022). The immunopeptidome describes the repertoire of peptides that are presented on MHC molecules (Yewdell, 2022). MHC-peptide complexes are displayed on the surface of various cells and are essential for T-cell activation. The altered immunopeptidome of senescent cells allows for novel therapeutic approaches on the basis senescence-associated peptide vaccines. Immunization with senescence-associated peptides could result in immune priming and increased senescence surveillance. Strikingly, recent studies have indicated that splenocytes from mice that were vaccinated with senescent fibroblasts were strongly activated upon stimulation with senescence-associated peptides (Marin et al., 2022). However, whether vaccination with immunogenic senescence peptides can promote anti-cancer responses remains to be determined.

Altogether, senescent cancer cells can be eliminated through various therapeutic approaches (Fig 1.4). These include senolytic small molecule inhibitors, immunotherapeutic targeting with senolytic effector cells, or peptide vaccines that result in increased immune-mediated clearance of senescent cells. However, further research is necessary to determine if these methods can be applied to cancer patients. Additionally, the outcomes of such investigations will provide a greater understanding of the potential benefits and risks associated with tumor senescence.

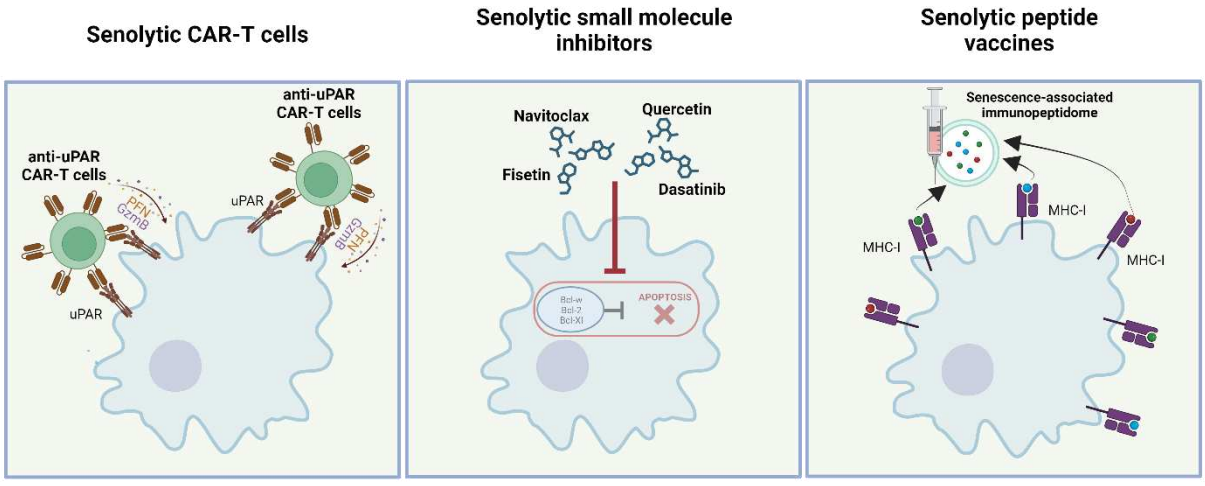

Figure 1.4: Targeting cancer senescence with different senolytic approaches

Senescent cancer cells can be cleared using various therapeutic approaches. These include CAR-T cell therapy, senolytic drug treatment, and peptide vaccines. Various senolytic drugs are currently being tested in clinical trials. Addtionally, senolytic CAR-T cell therapy and senescence-targeting peptide vaccines have shown great success in preclinical studies (Amor et al., 2020; Marin et al., 2022; Wang et al., 2022).

1.4. Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and the fourth-most leading cause of cancer-associated mortality worldwide (Llovet et al., 2021; Vogel et al., 2022). When diagnosed at early stages HCC has a relative survival rate of 70% at 5 years, while predicted survival decreases to 20% when diagnosed at advanced stages (Bray et al., 2018; Calderon-Martinez et al., 2023). More than 800.000 new cases are reported annually with the highest incidence observed in Asia and Africa (Llovet et al., 2021). Especially in these global areas HCC development and progression is strongly associated to viral infection. Strikingly, chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection account for more than 50% of HCC cases (Llovet et al., 2021; Stella et al., 2022). However, rising incidence and mortality rates are also observed in Europe and North America (Llovet et al., 2021; Stella et al., 2022). The most common causative factor for HCC in Europe and North America apart from HCV infection, is alcohol intake (Llovet et al., 2021). Other risk factors include non-alcoholic steatohepatitis (NASH), pre-existing metabolic diseases, such as diabetes, or immune-related disorders. A schematic illustration of global etiological factors of liver cancer is given in figure 1.5. To show local differences global areas with the strongest incidence of each etiological factors are depicted as well.

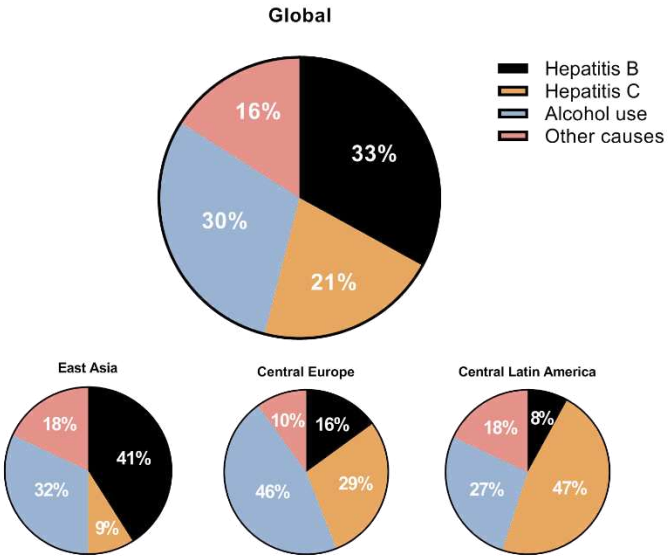

Figure 1.5: Etiological factors of liver cancer.

The most common causative factor for liver cancer worldwide is hepatitis B infection, accounting for 33% of all cases. Similarly, 41% of all cases in east asia arise from preexisting hepatitis B infection. Additionally, in central Europe 46% of all liver cancer cases occur due to alcohol use, while hepatitis C is the most common etiological factor in central latin America (47%). Other causes included NASH, aflatoxins and liver flukes (Akinyemiju et al., 2017).

In past years, a step-wise model of canonical HCC development has been proposed that implies an underlying cascade of constitutive liver damage leading from initial liver fibrosis through cirrhosis to development of HCC (Fig 1.6). Strikingly, liver cirrhosis, precedes HCC development in 90% of all cases, making it an ideal predictor for disease development (Llovet et al., 2021).

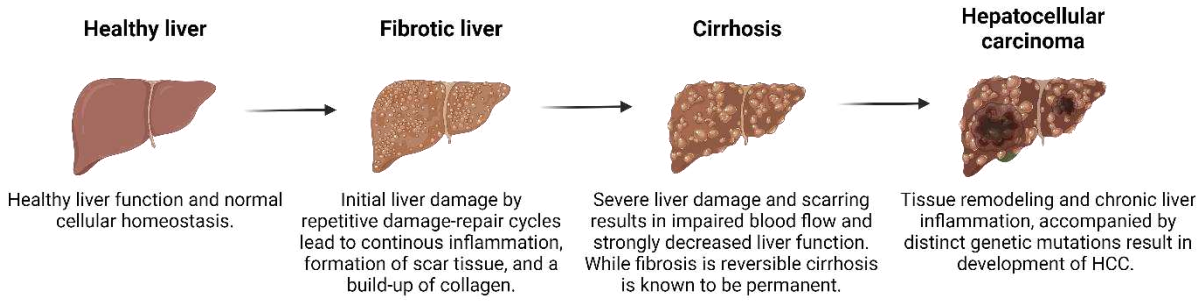

Figure 1.6: Liver damage cascade cumulating in development hepatocellular carcinoma.

In most cases, HCC develops due to repetitive damage-repair cycles of liver tissue that cause a continuous liver inflammation, called liver fibrosis. Liver fibrosis often results in the development of cirrhosis, which is characterized by severe liver scarring and a strongly impaired liver function. Liver fibrosis and cirrhosis precede HCC development in 90% of all cases.

1.4.1. HCC therapy

Early detection of HCC has led to better treatment outcomes and higher survival rates (Llovet et al., 2021). Strikingly, a study on primary HCC patients diagnosed between 1988 and 2015 indicated that one-year overall survival of patients with localized HCC increased from 32% in 1988-1992 to 70.4% in 2013-2015 (Ding and Wen, 2021). This increase is attributed to the improvements in early diagnosis and the advances in HCC therapy.

HCC treatment options differ between early- and late-stage HCC. Early treatment options of HCC are liver transplantation and surgical resection. While liver transplantation is only recommended when neoplastic lesions are not larger than 5 cm, surgical resection is exclusively performed in patients where a post-surgical preserved liver function is guaranteed (Raza and Sood, 2014). Nevertheless, surgical resection has high recurrence rates due to the risk of incomplete resection or vascular invasion. Patients that undergo surgical resection have

a 5-year survival rate of 70%, while predicted survival for transplantation is 75% (Raza and Sood, 2014). Another early-stage therapeutic intervention that has proven to be highly efficient for elimination of cancer cells is local thermal ablation. In particular, radiofrequency ablation (RFA) is one of the most commonly used types of thermal ablation (Facciorusso et al., 2016; Gallage et al., 2021). RFA is used to locally apply electrical currents to tumor cells. These electric currents generate strong heat, destroying cells that are in close proximity to the injected electrode. Similarly to RFA, microwave ablation (MWA) also displays a thermal ablation method used for treatment of early-stage HCC (Facciorusso et al., 2016; Gallage et al., 2021).

Besides thermal ablation, trans-arterial chemoembolization (TACE) presents another locally limited, minimally invasive therapeutic approach for HCC. TACE describes the injection of chemotherapeutic agents into hepatic arteries with subsequent embolization, resulting in tumor ischemia (Gallage et al., 2021). Additionally, TACE has shown to increase the concentration of chemotherapeutic agents at tumor sites and induce longer retention periods (Gallage et al., 2021; Tsurusaki and Murakami, 2015). Other than for thermal ablation, TACE is mostly applied for treatment of intermediate-stage HCC. Another therapeutic measure primarily used for inoperable intermediate-stage HCC is selective internal radiotherapy (SIRT) (Raza and Sood, 2014). By administration of radioactive particles into hepatic arteries, SIRT is used to directly deliver lethal radiation doses to tumor sides .

While early- and intermediate HCC treatments include surgical interventions and tumor-selective treatments, therapeutic options for advanced HCC are more systemic. The most commonly used systemic drugs for the treatment of advanced HCC are the multi-kinase inhibitors (MKIs) sorafenib and levantinib (Gallage et al., 2021; Llovet et al., 2021). Sorafenib was clinically approved in 2007 and thus the first systemic treatment of advanced HCC (Luo et al., 2021). Besides inhibition of several RAF kinases, sorafenib targets multiple growth factor receptors such as platelet-derived growth factor receptor (PDGFR) or vascular endothelial growth factor receptor 2 (VEGFR2) (Gallage et al., 2021). Similarly, levantinib targets several VEFGRs, PDGFR and fibroblast growth factor receptor (FGFR) (Gallage et al., 2021). Hence, by blocking growth factor responses and tumor-promoting signaling pathways, sorafenib and levantinib inhibit angiogenesis as well as tumor growth.

Apart from MKIs and growth factor inhibitors, immunotherapy has emerged as a promising strategy for treatment of advanced HCC. Immunotherapy describes the therapeutic approach of utilizing or enhancing endogenous anti-tumor immune responses for efficient clearance of cancer cells. This includes adoptive transfer of patient-derived, genetically modified immune cells or administration of monoclonal antibodies directed against tumor-associated antigens(C. Liu et al., 2022). Among the various immunotherapeutic drugs, checkpoint inhibitors have shown the greatest promise in treating HCC (Gallage et al., 2021). Cancer cells can downregulate anti-tumor immune responses by overexpressing immune-regulating surface antigens. Blocking these pathways with checkpoint inhibitors has shown great success for the treatment of several neoplastic diseases (Shiravand et al., 2022). In HCC, checkpoint inhibitors are typically given in combination therapy or as a post-sorafenib treatment option with nivolumab and atezolizumab being the most commonly used checkpoint inhibitors (Gallage et al., 2021). Both target the programmed cell death protein 1 (PD-1)/PD-L1 axis that is a major contributor to cancer immune evasion (C. Liu et al., 2022). Besides PD-1 and PD-L1 inhibitors also cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) blockers are currently being tested in several clinical trials (Gallage et al., 2021). An overview of therapeutic measures and most commonly used anti-cancer drugs for the treatment of HCC is given in table 1.3.

Table 1.3: Therapeutic options for HCC.

Therapeutic intervention / drug	Disease stage	Description / Mechanism of action
Liver transplantation	Early stage	Full liver transplantation
Liver resection	Early stage	Surgical removal of neoplastic tissue
Ablation (Mostly thermal ablation)	Early / Intermediate stage	Local tissue destruction by induction of hyperthermia
Transarterial chemoembolization (TACE)	Intermediate stage	Embolization of hepatic arteries leading to tumor ischemia
Selective internal radiotherapy (SIRT)	Intermediate stage	Delivery of radioactive microspheres through hepatic arteries
Sorafenib	Intermediate / advanced stage	Inhibition of tumor growth and angiogenesis by targeting, VEGR2, PDGFRs and RAF kinases
Lenvantinib	Intermediate / advanced stage	Inhibition of tumor growth and angiogenesis by targeting multiple VEGRs, PDGFR, FGFR and RET signaling
Atezolizumab	Intermediate / advanced stage	Checkpoint inhibitor, monoclonal antibody targeting PD-L1
Nivolumab	Intermediate / advanced stage	Checkpoint inhibitor, monoclonal antibody targeting PD-1

(Gallage et al., 2021; Llovet et al., 2021; Raza and Sood, 2014)

Due to its frequency and high mortality, HCC presents a major threat to global health. Additionally, the increasing prevalence of underlying liver diseases such as non-alcoholic fatty liver disease (NAFLD) will lead to a drastic rise in HCC incidence (Rawla et al., 2018). Therefore, improving current treatment options and developing novel therapeutic approaches are crucial to combat HCC.

1.4.2. Models to study HCC

HCC cell lines are established from patient-derived tumor samples and represent ideal cellular models to study cancer-associated processes and develop new treatment strategies. They provide a robust and controlled system for conducting a wide range of experiments, ranging from mechanistic studies to clinical screening approaches. The most commonly used HCC cell lines include HepG2, Hep3B, HUH6, HUH7 and C3A (Blidisel et al., 2021). In the following the HCC cell lines HUH7 and HepG2, both used in this study, will be described and compared.

HepG2 cells are a well characterized cell line derived from liver tissue of a 15-year-old male HCC patient. They are the most commonly used experimental model of HCC and exhibit many well-described characteristic of liver cancer cells (Blidisel et al., 2021). Besides having distinct hepatic functions such as bile acid synthesis, insulin signaling, or cholesterol and lipoprotein metabolism, one major advantage of HepG2 cells is their reactivity to pharmacological drugs such as sorafenib, despite the lack of some crucial drug-metabolizing enzymes (Blidisel et al., 2021; Donato et al., 2015). Additionally, HepG2 cells have been used to develop 3D models of hepatic cancer such as tumor spheroids (Blidisel et al., 2021). Unlike most HCC cell lines, HepG2 cells express functional wild-type p53 making them suitable for investigation of p53-dependent processes. Nevertheless, the use of HepG2 cells as an HCC cell line has raised controversy in the past decades as some studies suggest that they are derived from an epithelial hepatoblastoma-like tumor rather than hepatocellular carcinoma (Blidisel et al., 2021; López-Terrada et al., 2009; Molina-Sánchez and Lujambio, 2019).

Since p53 plays a crucial role in senescence regulation and tumor progression a second, p53-mutated HCC cell line was investigated in this study. HUH7 cells are a human liver cancer cell line derived from a 57-year old male with well-differentiated HCC (Blidisel et al., 2021; Molina-Sánchez and Lujambio, 2019). After HepG2 and Hep3B cells, HUH7 cells are the third most frequently used HCC cell line (Blidisel et al., 2021). While primarily being used for drug metabolism studies and 3D modeling, HUH7 cells and respective subclones, such as HUH7.5 or HUH7-Lunet, are highly permissive for HCV infection and thus, often being used for viral infection studies (Blidisel et al., 2021; Dächert et al., 2019).

Conclusively, HCC cell lines represent ideal tools for *in vitro* investigation of cancer-associated processes, hepatic function and drug metabolism. Although responses might differ in comparison to primary liver cancer, HCC cell lines can be used to functionally characterize cellular processes such as senescence.

Alternatives to human cell lines are various *in vivo* models of HCC, such as xenograft mouse models. Xenograft models of HCC can be generated by orthotopic injection of human HCC cell lines or transplantation of patient-derived tumor tissue (Romualdo et al., 2021). They are ideal for investigation of various tumor-associated mechanisms, such as tumor development or treatment response. However, due to the use of immunocompromised mice xenograft models are often limited and do not reflect the complexity of human immune responses. Besides

orthotopic injection of human HCC cells, also intrahepatic oncogene delivery has shown to induce HCC. Hydrodynamic tail vein injection of transposable elements has been a commonly used model to intraheptically deliver HCC promoting oncogenes, such as $NRas^{G12V}$ (Kang et al., 2011). Notably, these $NRas^{G12V}$-derived HCC models have been widely used to investigate senescence-associated processes in HCC (Amor et al., 2020; Kang et al., 2011)

1.5.Senescence in HCC

Senescence has a pivotal role in HCC progression and anti-tumor response, highlighting the conflicting effects of HCC senescence (Fig 1.7). Several studies have indicated that senescent cells accumulate in the liver, not only during HCC but also in pre-existing diseases such as fibrosis and cirrhosis (Cai et al., 2022; Ferreira-Gonzalez et al., 2021). Furthermore, senescence has also been associated with other pathologies that drive HCC development, such as HCV and HBV infection (Ikeda et al., 2009; Paradis et al., 2001), NAFLD (Papatheodoridi et al., 2020) and alcoholic liver damage (Meng et al., 2017). Nevertheless, the precise role that senescence plays in the pathophysiology of HCC development remains unclear. Some studies suggest that activated hepatic stellate cells (HSCs) may restrict their own excessive proliferation through induction of senescence, thereby exhibiting anti-fibrotic effects and lowering the risk for development of cirrhosis and HCC (Cai et al., 2022; Krizhanovsky et al., 2008). Conversely, other studies reported that senescent HSCs are crucial for the transition from cirrhosis to HCC due to secretion of SASP factors that fuel the tumor-promoting inflammatory environment (B. Liu et al., 2022). Further conflicting findings include the role of senescence immune surveillance in HCC development. While senescence of pre-malignant hepatocytes has shown to be crucial for immune surveillance and tumor suppression (Kang et al., 2011) other studies indicated that senescence promotes macrophages infiltration, what changes the tumor microenvironment, thereby promoting HCC development (Huang et al., 2021).

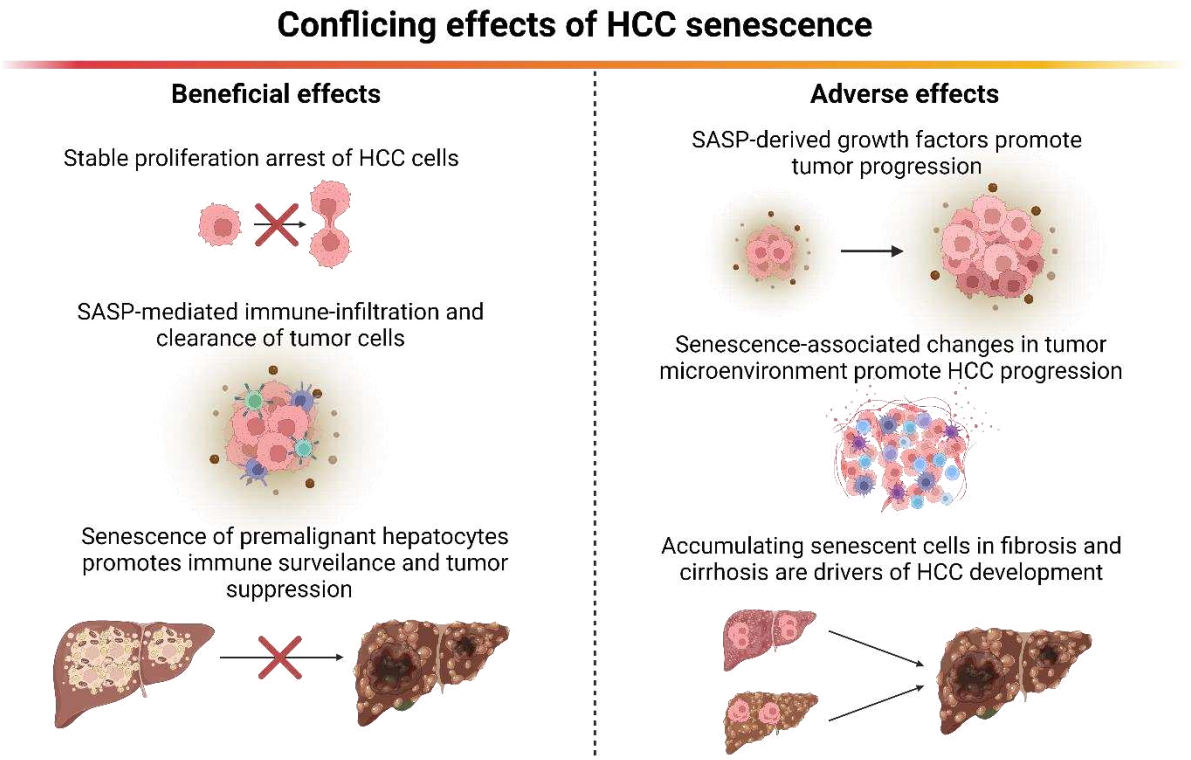

Figure 1.7: Conflicting effects of HCC senescence

Beneficial effects of HCC senescence include a stable growth arrest of rapidly proliferating HCC cells, a SASP-mediated immune infiltration and clearance of tumor cells and the delayed onset of HCC due to senescence of premalignant hepatocytes. Conversely, adverse outcomes of HCC senescence are tumor promoting effects of SASP-derived growth factors and changes in tumor microenvironment. Furthermore, accumulation of senescent cells during fibrosis and cirrhosis drive HCC development.

Although the role of senescence in HCC development is not fully understood, several findings have indicated that senescence induction in progressing HCC can have beneficial anti-tumor effects. This was shown for a p53-restoration model of murine liver cancer (Xue et al., 2007) as well as CDK4/6 inhibitor-mediated TIS in human HCC cell lines, primary HCC samples and human HCC xenografts (Bollard et al., 2017). Thus, several clinical trials using CDK4/6 inhibitors for the treatment of advanced or inoperable HCC have been conducted in the past years. Strikingly, palbociclib exhibited anti-tumor activity in patients with inoperable and advanced HCC who failed first-line treatment with sorafenib (Littman et al., 2015). Additionally, other clinical trials on abemaciclib in combination with nivolumab and ribociclib together with chemoembolization were conducted. However these were either terminated or did not obtain promising results (Cai et al., 2022).

As previously mentioned, senescence can have beneficial as well as adverse effects in HCC (Fig 1.7). Therefore, induction of senescence, followed by senolytic clearance may provide a promising therapeutic strategy for treatment of HCC. Several studies have investigated the effects of therapeutically targeting senescent cells in liver tumors by senolytic intervention (Cai et al., 2022; Li et al., 2020; Thadathil et al., 2022). Among the many types of senolytics, navitoclax, dasatinib and quercetin have shown the greatest success in promoting anti-tumor responses. Hence, several clinical trials have been conducted using navitoclax or dasatinib for treatment of relapsed, unresectable, or metastatic advanced HCC (Cai et al., 2022). While some studies are currently ongoing, others were terminated early due to severe side effects.

Conclusively, HCC senescence represents a highly complex cellular process that is already detectable early before the onset of HCC. Accumulating senescent cells in fibrosis and cirrhosis have shown to be main drivers of HCC development. However, senescence has also shown to protect from extensive proliferation and early onset of HCC. Although its role in disease progression and development is still not fully understood, several approaches have been conducted to utilize HCC senescence for therapeutic purposes. Hence, characterizing this highly clinically relevant process could pave new ways towards development of novel senescence-inducing and targeting HCC therapies.

1.6. Aims of this study

Numerous studies have investigated the therapeutic applicability of therapy-induced senescence in HCC (Bollard et al., 2017; Littman et al., 2015; Yoshino et al., 1989). Despite these efforts, a comprehensive understanding of how TIS can be utilized for the treatment of HCC remains incomplete. This study aims to contribute to the current understanding of therapy-induced senescence in hepatocellular carcinoma by comparing several TIS inducers in human HCC cell lines. It was hypothesized that senescence-associated phenotypes vary, depending on the underlying induction mechanisms, what influences senescence surveillance and inflammatory capacities. Furthermore, characterization of the senescence-associated surfaceome was conducted to identify actionable targets of HCC TIS that were shared among TIS inducers and thus, could be utilized for therapeutic targeting. Overall, this study aims to determine the potential of HCC therapy-induced senescence for therapeutic implication.

2. Material and Methods

2.1.Materials

2.1.1. Reagents and special consumables

Table 2.1: **List of reagents and special consumables.**

Reagent / special consumable	Company	Product number
96-well black plates	SPL Life Sciences	33396
Ammonium persulphate $(NH_4)_2S_2O_8$	Sigma Aldrich	A3678
Bovine Serum Albumin (BSA)	Biomol	01400.100
Bradford reagent	Sigma Aldrich	B6916-500ML
Cell Staining Buffer	BioLegend	420201
Cell Strainer Snap Cap	Thermo Fisher Scientific	08-771-23
DMEM	Sigma	D5796-24X500ML
DPBS	Thermo Fisher	14190-169
EMEM	ATCC	30-2003
FCS (heat inactivated)	Th. Geyer	11682258
Fixation buffer	BioLegend	420801
Hoechst 33342	Thermo Fisher Scientific	H1399
IMDM	Lonza	12-722F
Human IL-2	Miltenyi Biotech	130-097-742
DMSO	Carl Roth	A994.2
Isopropanol	VWR	1.09634.1011P
L-glutamine	Gibco	25030081
Live Cell Imaging Solution	Invitrogen	12363603
MEM Non-Essential Amino Acids Solution	Gibco	11140050
MEMα GlutaMax	Gibco	32561037
Methanol	Honeywell	32213-2.5L
Nunc Polycarbonate Cell Culture Inserts	Thermo Fisher Scientific	140629
NuPAGE LDS sample buffer	Invitrogen	NP0007
NuPAGE sample reducing agent	Invitrogen	NP0009
NuPAGE transfer buffer	Invitrogen	NP00061
Opti-MEM reduced serum medium	Gibco	31985062
Penicillin/Streptomycin	Gibco	15140122
Pierce ECL Western Blotting Substrate	Thermo Fisher Scientific	32109
Pooled human serum	Internal supplier	
Powdered milk, 500 g	Carl Roth	T145.1
ProLong Diamond Antifade Mountant, 815	Thermo Fisher Scientific	P36961

Protease inhibitor	Sigma Aldrich	11836153001
ROTIPHORESE Gel 30, 30% Acrylamide	Carl Roth	3029.1
RPMI-1640	Sigma	R8758-500ML
TEMED	Carl Roth	2367.1
Triton X-100	Applichem	A1388
Tween20	Sigma Aldrich	P7949
UltraPure DNase/RNase-Free Distilled Water	Invitrogen	10977049
Trypan Blue Solution (0.4%)	Gibco	15250061
Trypsin (0.05% EDTA)	Gibco	25300054
UltraPure 0.5 M EDTA	Invitrogen	15575-038
Empty gel cassettes, mini, 1.5 mm	Invitrogen	NC2015
0.45 µm nitrocellulose membrane	Amersham cytiva	GE10600002

2.1.2. Senescence inducers

Table 2.2: **List of senescence inducers.**

Inducer	Company	Product number	Stock concentration
Alisertib	Selleckchem	S1133	10 mM in DMSO
CX5461	Selleckchem	S2684	1 mM in DMSO
Etoposide	Sigma-Aldrich	E1383	50 mM in DMSO

2.1.3. Cell culture media

Table 2.3: **List of cell culture media.**

Cell line / primary cell	Basal medium and supplements for complete media	Source
HepG2	RPMI 10% FCS 1% Pen./Strep.	ADCC, HB-8065
HUH7	DMEM 10% FCS 1% Pen./Strep. 1% L-glutamine 1% Non-essential amino acids	Gift from Daniel Dauch, AG Dauch, University Hospital Tübingen
IMR90	EMEM 10% FCS 1% Pen./Strep.	ADCC, CCL-186
NK92 and anti-CD276 CAR-NK92	MEMα GlutaMax 20% FCS 1% Pen./Strep.	Gift from Guillermo Urena Baillen, AG Mezger, University Hospital Tübingen

	1000 U/µl IL-2	
NK92 MI	IMDM 10% FCS 1% Pen./Strep.	Gift from Melanie Märklin, AG Salih, University Hospital Tübingen
Primary human polymorphonuclear neutrophils (purified from freshly drawn venous blood)	RPMI 10% FCS	Freshly isolated from registered donors

2.1.4. Buffers and solutions

Table 2.4: **List of buffers and solutions.**

Buffer / solution	Components
Blocking buffer for Western Blot	5% BSA / 5% milk powder 0.1% Tween20 in TBS
Blocking/permeabilization buffer for immunofluorescence microscopy	0.1% Triton X-100 0.1% Tween20 5% BSA in PBS
Erythrocyte lysis buffer (ACK, 10x)	1.54 M NH4Cl 100 mM KHCO3 1 mM EDTA, pH=8 dissolved in Ampuwa water, pH adjusted to 7.3
Flow cytometry blocking buffer	10% heat-inactivated pooled human serum in Cell Staining Buffer (Biolegend, 420201)
RIPA lysis buffer (2x), 500 ml	2.4 g Tris base 8.8 g NaCl 2 ml 500 mM EDTA 10 ml Triton X-100 5 g Sodium deoxycholate 1 g SDS 100 ml glycerol fill up with ddH_2O
SDS running buffer, 5x	25 mM Tris 250 mM Glycine 0.5% SDS in ddH_2O

Solution for preparing 10% resolving gel, 5 ml	4.0 ml ddH_2O 3.3 ml 1 M Tris 2.5 ml (pH 6.8) 100 µl 10% SDS 100 µl 10% ammonium persulfate 4 µl TEMED
Solution for preparing 5% stacking gel, 10 ml	3.4 ml ddH_2O 830 µl 1 M Tris 630 µl (pH 6.8) 50 µl 10% SDS 50 µl 10% ammonium persulfate 5 µl TEMED
TBS (20x)	2.73 M NaCl 0.4 M Tris base in ddH2O
WB transfer buffer	1 x NuPAGE TransferBuffer 20% Methanol in ddH_2O

2.1.5. Agonists, ligands, and inhibitors

Table 2.5: **List of agonists, ligands, and inhibitors.**

Stimulant / ligand / inhibitor	Company	Product number	Stock concentrations
2'3'-cGAMP (STING agonist)	Invivogen	tlrl-nacga23-02	1 mg/ml in ddH_2O
Anti-Human sFAS Ligand	Peprotech	310-03H	10 µg/ml in ddH_2O
H151 (STING inhibitor)	Invivogen	inh-h151	10 mg/ml in DMSO

2.1.6. Antibodies

Table 2.6: **List of antibodies.**

Antibody	Company	Product number
Caspase-8 (1C12), mouse anti-human mAb	Cell Signaling Technology	9746
CD13, mouse anti-human, Brilliant Violet 605	BioLegend	301727
CD340, mouse anti-human, PE/Dazzle 594	BioLegend	305641
CD54, mouse anti-human, Alexa Fluor 700	BioLegend	353125
CD66a/c/e, mouse anti-human, APC	BioLegend	342307
CD73, mouse anti-human, Brilliant Violet 421	BioLegend	344007
CD95, mouse anti-human, Brilliant Violet 711	BioLegend	305643
EphA2, mouse anti-human, PE	BioLegend	356803
Fas Recombinant, rabbit anti-human mAb	Thermo Fisher Scientific	MA5-35308
Fas, mouse anti-human (activating)	Sigma-Aldrich	05-201
HER2/ErbB2, rabbit anti-human	Cell Signaling Technology	2242
HLA-E, mouse anti-human, PE	Thermo Fisher Scientific	12-9953-42

IgG Antibody (H+L), Peroxidase, Goat Anti-Rabbit	Vector Laboratories	PI-1000-1
IgG, Anti-Mouse (H+L), HRP Conjugate	Promega	W4021
IgG1 κ Isotype Ctrl, mouse, Brilliant Violet 605	BioLegend	400161
IgG1, goat anti-rabbit secondary antibody, Alexa Fluor 488	Thermo Fisher Scientific	A11034
IgG1, κ Isotype Ctrl, mouse PE/Dazzle 594	BioLegend	400175
IgG1, κ Isotype Ctrl, mouse, Alexa Fluor 700	BioLegend	400143
IgG1, κ Isotype Ctrl, Mouse, Brilliant Violet 421	BioLegend	400157
IgG1, κ Isotype Ctrl, mouse, Brilliant Violet 711	BioLegend	400167
IgG2b, κ Isotype Ctrl, mouse, APC	BioLegend	401209
IgG2b, κ Isotype Ctrl, mouse, PE	BioLegend	400313
Ki67, rabbit anti-human	Abcam	Ab16667
p16 INK4A (D7C1M), rabbit anti-human	Cell Signaling Technology	80772
p21, mouse anti-human	BD Bioscience	556431
STING (D2P2F), rabbit anti-human mAb	Cell Signaling Technology	13647
β-Actin, mouse anti-human mAb	Sigma Aldrich	A5441

2.1.7. Kits

Table 2.7: **List of Kits.**

Kit	Company	Product number
Aqua LIVE/DEAD viability dye	Thermo Fisher Scientific	L34957
Calcein-AM	BioLegend	425201
Cell counting kit-8 (cck8)	Dojindo	CK04-13
CellEvent Senescence Green Flow Cytometry Assay Kit	Thermo Fisher Scientific	C10840
Cytotoxicity Detection Kit (LDH)	Roche	11644793001
ELISA MAX Deluxe Set Human CXCL10	BioLegend	439904
ELISA MAX Deluxe Set Human IL-8	BioLegend	431504
ELISA MAX Deluxe Set Human VEGF	BioLegend	446504
Human MMP-9 DuoSet ELISA	R&D Systems	DY911-05
Human Myeloperoxidase DuoSet ELISA	R&D Systems	DY3174
LegendScreen, lyophilized antibody array, human PE kit	BioLegend	700007
RNeasy Mini Kit	QIAGEN	74104
β-galactosidase Detection Kit (Fluorometric)	Abcam	ab176721

2.1.8. Instruments and special devices

Table 2.8: **List of instruments and special devices.**

Instrument / special device	Purpose of usage	Company
BD LSRFortessa	FACS experiments with 6 or more than 6 colors	BD Bioscience
BDFACS Canto II,	FACS experiments with less than 6 colors	BD Bioscience
BioTek Synergy Neo2	Microplate reader for various colorimetric and fluorometric assays	Agilent
Centrifuge 5810 R	Centrifugation	Eppendorf
FLUOstar OPTIMA	Microplate reader for Bradford assay	BMG Labtech
MACSQuant VYB	LegendScreen experiments	Miltenyi Biotech
Microstar 17R	Centrifugation	VWR
Odyssey XF Imaging System	Imaging of western blots	LI-COR BIOSCIENCES
Trans-Blot Turbo	Membrane blotting	Bio-Rad
ZEISS LSM800	Confocal microscopy	Zeiss

2.1.9. Software for data analysis

Table 2.9: **List of software for data analysis.**

Software	Supplier	Version
Excel	Microsoft	2019
FACSDiva	BD Bioscience	Version 6
Fiji	National Institutes of Health	Win64
FlowJo	FlowJo LLC	Version 10.8.1
GraphPad PRISM	GraphPad Software	Version 8
Image Studio Lite	LI-COR BIOSCIENCES	Version 5.2
PowerPoint	Microsoft	2019
Word	Microsoft	2019
ZenBlue	Zeiss	Version 3

2.1.10. Flow cytometer configurations

2.1.11. BD LSRFortessa

Table 2.10: **BD LSRFortessa configuration.**

Lasers	Filters (nm)
Violet 405nm	450/50 560/40 605/15 660/20 710/50 780/60
Blue 488 nm	510/20 670/30
Green 532 nm	582/15 610/20 710/50 780/60
Red 640 nm	670/30 730/45 780/60

2.1.12. BDFACS Canto II

Table 2.11: **BDFACS Canto II configuration.**

Lasers	Filters (nm)
Violet 405nm	450/50 510/50
Blue 488 nm	530/30 585/42 670/LP 780/60
Red 633 nm	660/20 780/60

2.1.13. MACSQuant VYB

Table 2.12: **MACSQuant VYB configuration.**

Lasers	Filters (nm)
Violet 405nm	450/50 525/50

Blue 488 nm	525/50 614/50
Yellow 561 nm	586/15 615/20 661/20 750/LP

2.2.Cell biology methods

2.2.1. Culturing of HCC cell lines

While HUH7 cells (kindly provided by Daniel Dauch, University Hospital Tübingen) were cultured and stimulated in complete DMEM medium (table 2.3), HepG2 cells were grown and treated in complete RPMI medium (table 2.3). For maintenance, both cell lines were cultured in T75 flasks and passaged twice a week (split 1:10), never exceeding a confluency of 80 – 90%, up to 20 passages before switching to a new batch. Passaging was performed by washing cells with PBS prior to incubation with trypsin, 0.05% EDTA for 5 -10 min. After cell detachment, culture media was added and cells were centrifuged for 5 min at 400 x g. Finally, cell pellets were resuspended in 12 ml culture media and transferred to a new T75 flasks.

2.2.1. IMR90 fibroblast cell culture and stimulation

IMR90 fibroblasts were cultured and stimulated in complete EMEM medium (see table 2.3). For maintenance cells were grown in T75 flasks and passaged twice a week. Additionally, cells were always used in cultures lower than passage 15. Passaging was performed as described above (“Culturing of HCC cell lines”).

2.2.2. HCC cell lines and IMR90 fibroblasts senescence induction

Senescence was induced using 1 µM Alisertib, 500 nM CX5461 or 10 µM Etoposide diluted in respective cell culture medium from the appropriate stock solutions (table 2.2). Cells treated with Alisertib or Etoposide were stimulated for 48/72 h at 37°C and 5% CO_2. CX5461-treated cells were treated for 24 h and rested in fresh cell culture media for 48/72 h at 37°C and 5% CO_2. After stimulation, senescence induction was confirmed by detection of general senescence markers.

2.2.1. cGAS-STING modulation

For cGAS-STING modulation HUH7 cells were seeded in 6-well plates and senescence was induced as described above. After senescence induction cell culture media containing the

following dilutions of STING agonist 2´3´cGAMP or STING inhibitor H151 was added to respective wells:

Table 2.13: Final concentrations of 2´3´cGAMP and H151

2´3´cGAMP	H151
2 µg/ml	2 µM
1 µg/ml	1 µM
0.5 µg/ml	0.5 µM

Subsequently, cells were incubated for 24 h at 37°C and 5% CO_2. Finally, media were centrifuged at 500 x g for 5 min and supernatants were transferred to fresh tubes. cGAS-STING response was assessed by CXCL10 ELISA.

2.2.1. NK92 MI cell culture and killing assay

NK92 MI cells (kindly provided by Melanie Märklin, University Hospital Tübingen) were cultured in complete IMDM medium (table 2.3) and passaged three times a week (1:3), up to 15 passages before switching to a new batch. To investigate NK cell-mediated cytotoxicity 2 x 10^4 - 4 x 10^4 target cells were seeded in a 24-well plate and senescence was induced as described above. Afterwards, target cells were washed and NK92 MI cells were counted and centrifuged at 500 x g for 5 min. To investigate NK cell-mediated cytotoxicity NK92 MI cells were resuspended in 500 µl Opti-MEM and added to target cells in different effector to target (E:T) ratios. Furthermore, standard and maximum release were analyzed for normalization to respective target cell numbers and NK cell independent cell death. While for standard release only Opti-MEM without NK92 MI cells was added, maximum release was determined by adding 500 µl Opti-MEM containing 0.5% Triton X-100. After 3 h incubation at 37 °C and 5% CO_2 supernatants were harvested and centrifuged for 5 min at 1500 rpm to remove cell debris. Subsequently, cell death was determined using a cytotoxicity detection kit (see below).

2.2.2. Cytotoxicity detection - Lactate dehydrogenase (LDH) release assay

For quantification of NK92 MI cell-mediated killing a LDH release-based cytotoxicity detection kit was used (table 2.7) according to manufacturer's instructions. Hence, 100 µl supernatant of NK cell treated samples, and of maximum/standard controls were pipetted in flat bottom 96-well plates. Standard and maximum release are defined in section 2.2.1. To prevent saturation, maximum release values were diluted 1:3 - 1:5 in Opti-MEM. Afterwards, 100 µL reaction buffer, consisting of a 1:45 mixture of reconstituted catalyst solution and dye solution,

was added to respective wells. After 20 min incubation in the dark absorbance was measured at 490 nm using the BioTek Synergy Neo2 microplate reader. To quantify relative cell death, the following formula was used:

$$\text{Cell death (\%)} = \frac{\text{Sample value} - \text{Standard release}}{(\text{Maximum release x dilution factor}) - \text{Standard release}} \times 100$$

2.2.3. NK92 and CAR NK92 cell culture

NK92 and CAR NK92 cells (kindly provided by Guillermo Urena Bailen, University Hospital Tübingen) were cultured in complete MEMα GlutaMax medium (table 2.3) and passaged three times a week (1:3), up to 15 passages before switching to a new batch. For passaging, cells were transferred to a new T75 flasks at a concentration of 0.3 – 0.5 x 10^6 cells per ml. Additionally, fresh IL-2 (1000 U/µl) was added at every passaging step.

2.2.4. NK92 and CAR NK92 killing assay (Calcein AM assay)

For investigation of NK cell-mediated cytotoxicity a calcein acetoxymethyl esther (calcein AM) assay was performed. Prior to the calcein AM assay, 2 x 10^4 - 4 x 10^4 target cells were seeded in a 24-well plate and senescence was induced as described above. After senescence induction, calcein was reconstituted by dissolving 50 µg of the lyophilized compound in 50 µl anhydrous DMSO, resulting in a stock concentration of 1 µg/µl. Reconstituted calcein was then added to live cell imaging solution (table 2.1) at a ratio of 10 µl per ml (1:100 dilution), and cells were incubated with calcein AM for 1 h at 37°C and 5% CO_2 for the dye to be taken up. Meanwhile, effector cells were prepared as described above (see "NK92 MI cell culture and killing assay") with the only difference being the resuspension media (RPMI with 2% FCS instead of Opti-MEM). Following calcein AM incubation, target cells were washed three times with live cell imaging solution for 5 min each. Afterwards effector cells were added to target cells in different E:T ratios. Furthermore, standard/spontaneous, and maximum release were analyzed for normalization to respective target cell numbers and NK cell independent cell death. While for standard/spontaneous release medium without NK92 MI cells was added to target cells, maximum release was determined by adding 500 µl assay medium containing 0.5% Triton X-100. After 3 h incubation at 37 °C and 5% CO_2 supernatants were harvested and centrifuged for 5 min at 500 g to remove cell debris. Subsequently, supernatants (100 µl each, including controls) were transferred to black 96-well plates and measured in triplicates using

the GFP filter of the BioTek Synergy Neo2 microplate reader. To quantify relative cell death, the following formula was used:

$$\text{Cell death (\%)} = \frac{\text{Sample value} - \text{Standard release}}{(\text{Maximum release x dilution factor}) - \text{Standard release}} \times 100$$

2.2.5. Polymorphonuclear neutrophil (PMN) isolation

Polymorphonuclear neutrophils were isolated by Francesca Bork and Vinicius Nunes Cordeiro Leal (working group Alexander Weber, Department of Immunology, University of Tübingen) as previously described (Herster et al., 2020). In brief, 9 mL of whole blood was collected in EDTA blood collection tubes and diluted with 20 mL PBS. This mixture was then layered onto 20 mL of Ficoll (Sigma-Aldrich, 10771) in a 50 mL Falcon tube for density gradient separation. After centrifugation for 25 min at 509 x g all layers,except the erythrocyte.granulocyte layer were removed. Next, erythrocyte lysis was performed by incubation with ACK buffer (table 2.4) for 20 min at 4C° and cells were centrifuged at 509 x g for 10 min. This erythrocyte lysis step was subsequently repeated, and cells were finally centrifuged and resuspended in RPMI with 10% FCS.

2.2.6. Neutrophil co-culture and transwell assay

Primary human neutrophil isolation and HCC cell senescence induction were performed as described above. One day prior to isolation 1 x 10^5 senescent and non-senescent target cells were seeded in a 24-well plate for transwell migration assays. On the next day, 1 x 10^5 freshly isolated neutrophils were added to cell culture inserts (table 2.1) and transferred into respective target cell-containing wells. To investigate SASP-mediated migration, media of the lower compartment was exchanged to conditioned or fresh cell culture media prior to incubation. Finally, migrated primary human neutrophil were manually counted after 6 h of incubation at 37 °C and 5% CO_2.

To determine neutrophil activation after target cell co-culture, 2 x 10^5 - 4 x 10^5 target cells were seeded in a 6-well plate and senescence was induced as described above. Subsequently, target cells were washed once with cell culture media, and 1.5 x 10^5 freshly isolated neutrophils were added in complete RPMI media. After 3 h incubation at 37 °C and 5% CO_2, supernatants were harvested, centrifuged for 5 min at 500 x g and transferred to fresh tubes. Neutrophil activation was determined by MPO and MMP9 ELISA. For normalization to respective cell number target cells were trypsinized and counted after removal of supernatant.

2.2.7. LegendScreen of HCC cell lines

To characterize the surfaceome of senescent HCC cell lines, a previous protocol (Herster, Bittner et al. 2020) was adapted. Hence, 1.5 x 10^6 - 2.5 x 10^6 HUH7 or HepG2 cells were seeded on four T175 flasks per condition. After senescence induction as described above, cells were detached using PBS, 5 mM EDTA and filtered through a cell strainer (table 2.1). LegendScreen was performed according to manufacturer's instruction using the LegendScreen human PE kit (table 2.7.). Lyophilized antibodies were resuspended in 25 µl ddH_2O and distributed onto 4 plates (5 µl antibody per well). Measurements were performed on a MACSQuant Analyzer 10 (Miltenyi Biotec) and data was analyzed using FlowJo V10 analysis software. For analysis the median fluorescent intensity (MFI) of each surface antigen was normalized against the baseline of the corresponding isotype control's MFI. For large (n>40) isotype sets on a plate, in order to minimize the effect of the isotype control's MFI variability propagating to a large number of markers, the baseline MFI was determined as the mode of a Gaussian kernel density estimator (bandwidth=0.05). Differential expression was defined as the fold change of the normalized marker MFIs between the treated and untreated conditions. Analysis of LegendScreen data was performed by Dr. Andras Szolek, a PostDoc from AG Weber, Department of Immunology, University hospital Tübingen.

2.2.8. Fas-mediated cytotoxicity assays

To initiate Fs-7-associated surface antigen (Fas)-mediated apoptosis in senescent HCC cell lines, 2 x 10^4 - 4 x 10^4 target cells were seeded in 24-well plates and senescence was induced as described above. After senescence induction lyophilized Fas ligand was resuspended in ddH_2O to a final concentration of 10 µg/ml. Soluble Fas ligand was then diluted in cell culture media and cells were treated with 150 ng/ml soluble Fas ligand (table 2.5) for 6 or 24 hours. For antibody-mediated Fas activation target cells were treated with 10 ng/ml, 100 ng/ml or 1000 ng/ml activating Fas antibody (table 2.6). After incubation at 37 °C and 5% CO_2, cytotoxicity was determined using the cell counting kit-8 (table 2.7) according to manufacturer's instructions and relative cell death was determined by normalizing absorbance values of Fas ligand- and antibody-treated conditions to untreated controls according to the following formular:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance value Fas ligand or antibody treated sample}}{\text{Absorbance value untreated sample}}\ x\ 100$$

$$\text{Cell death (\%)} = 100 - \text{cell viability (\%)}$$

Furthermore, whole cell lysates were generated as described above and initiation of apoptosis was verified by caspase-8 immunoblotting.

2.3.Immunochemical methods

2.3.1. Flow cytometry of HCC cell lines

For flow cytometry analysis, senescent and non-senescent cells were detached using PBS, 5 mM EDTA and a single cell suspension generated by passing through a cell strainer (table 2.1). Single cell suspensions of 1 x 10^6 cells/ml were generated and 200 µl of cells were transferred into a 96-well plate (U-bottom). After centrifugation for 5 min at 500 x g, blocking was performed with 1:10 diluted pooled human serum in cell staining buffer (table 2.4) for 20 min. If necessary, LIVE/DEAD staining was performed according to manufacturer's instructions (table 2.7). Afterwards, cells were stained with respective antibodies for 30 min at RT in the dark. To remove residual antibodies cells were washed twice with cell staining buffer and each washing step was followed by respective centrifugation for 5 min at 500 x *g*. Finally, cells were fixed with 100 µl fixation buffer (table 2.1) for 10 min at RT in the dark and washed twice with PBS. Cell pellets were resuspended in 100 µl PBS and measurements were performed on a FACS Canto II or LSR Fortessa (BD Bioscience, Diva software). For analysis FlowJo analysis software was used. Typical results and gating strategy are shown in figure 2.1.

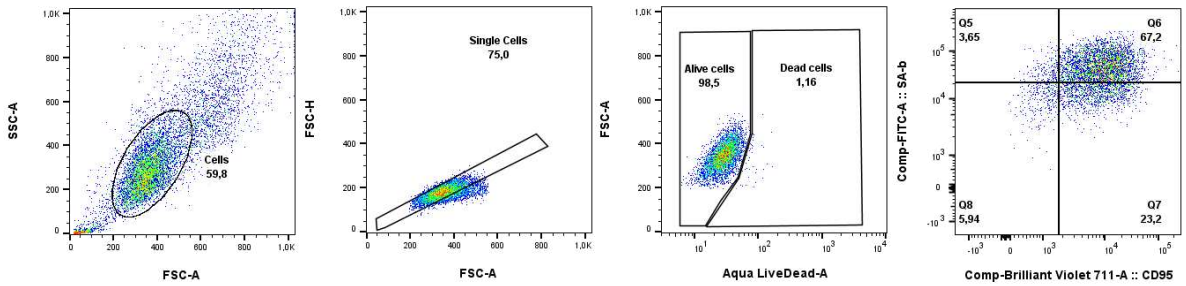

Figure 2.1: **Typical results and gating strategy of FACS experiments**

2.3.2. Detection of β-galactosidase hydrolysis via flow cytometry

Fluorescent detection of β-galactosidase hydrolysis was performed according to the manufacturers protocol (table 2.7) after respective cell surface antigen staining (see "Flow cytometry of HCC cell lines"). Hence, fixed cells were resuspended in 100 µl of working solution containing 1:1000 diluted senescence-green-probe. Cells were then incubated for 1 h at 37°C in the dark. In order to prevent pH-related unspecific signals, samples were incubated in the absence of CO_2. After incubation cells were washed twice followed by respective centrifugation for 5 min at 500 x g and final resuspension in PBS. For flow cytometric detection the 488 nm laser and FITC filters were used.

2.3.3. Ki67 fluorescence microscopy of fixed human HCC cell lines

For fluorescence microscopy of fixed human HCC cell lines 500 µl of 4 x 10^4 HCC cells were seeded in a 24-well plate containing sterile uncoated 12 mm coverslips. After senescence induction (as described above) cells were washed with PBS for 5 min on a plate shaker and fixed with fixation buffer (table 2.4) for 10 min at RT in the dark. Afterwards cells were washed twice as described above and blocked/permeabilized with permeabilization buffer containing 0.1% Triton X-100, 0.1% Tween20 and 5% bovine serum albumin (BSA) in PBS. Cells were then incubated with Ki67 antibody (table 2.6, 1:250 diluted) for 1 h in staining buffer containing 0.1% Tween 20 and 5% BSA. Afterwards cells were repeatedly washed on a plate shaker for 5 min with staining buffer and subsequently incubated with Alexa Fluor 488-conjugated secondary antibody (table 2.6, 1:500 diluted). After two additional washing steps, cells were incubated with 1:10000 diluted Hoechst 33342 in PBS for 7 min to stain nuclear DNA. Finally, coverslips were mounted with ProLong Diamond Antifade Mountant on glass slides and left to dry overnight at RT in the dark. Microscopy was performed using the Zeiss LSM800 Confocal microscope (AiryScan mode) and images were analyzed using ImageJ-Win64 and 818 Zen Blue3 software.

2.3.4. Enzyme-linked Immunosorbent Assay (ELISA)

To measure cytokine release, 1 x 10^5 – 3 x 10^5 cells were seeded in 6-well plates and senescence was induced as described above. After senescence induction supernatants were replaced by fresh cell culture media and cells were incubated for 24 h at 37°C and 5% CO_2. Afterwards supernatants were removed, centrifuged for 5 min at 500 x g and transferred to fresh microcentrifuge tubes. For normalization to respective cell numbers cells were

trypsinized and counted after removal of supernatant. ELISA Kits for IL-8, CXCL10, VEGF-A, MMP9 and MPO (table 2.7) were used according to the manufacturer's instructions. Usual dilution factors of supernatants are given in table 2.14. Samples as well as standard curve dilutions were assessed in triplicates. Additionally, to save supernatant for further experiments half-area 96-well plates were used.

Table 2.14: Usual dilution factors of supernatants for respective ELISA experiments.

ELISA Kit	Dilution factors
ELISA MAX Deluxe Set Human CXCL10	Undiluted
ELISA MAX Deluxe Set Human IL-8	1:10 for HUH7 and undiluted for HepG2
ELISA MAX Deluxe Set Human VEGF	1:5 for HUH7 and HepG2
Human MMP-9 DuoSet ELISA	1:50
Human Myeloperoxidase DuoSet ELISA	1:50

2.3.5. Fluorometric senescence-associated β-galactosidase (SA-β-Gal) detection

Fluorometric detection of SA-β-Gal was performed according to manufacturer's instructions using a β-galactosidase detection kit (table 2.7). Target cells were seeded on a 6-well plate and senescence was induced as described above. Afterwards, cells were washed with PBS and trypsinized, followed by centrifugation for 5 min at 400 x g. After another washing step cells were counted and 1.5 x 10^5 cells were centrifuged and resuspended in 150 µl lysis buffer containing 0.1% β-mercapto-ethanol. Each lysate was distributed into three wells of a 96-well black plate (50 µl/well), and 50 µl working solution was added per well containing the fluorescent β-galactosidase substrate fluorescein-digalactoside (FDG). Finally, 96-well plates were incubated at 37 °C for 1 h. Fluorescence intensity was measured using the BioTek Synergy Neo2 microplate reader with a GFP filter set.

2.3.6. Generation of whole cell lysates and Bradford assay

Whole cell lysates were generated in RIPA buffer containing protease inhibitor. Hence, 2x RIPA buffer (table 2.4) was diluted 1:1 in UltraPure Distilled Water and one protease inhibitor tablet was added per 10 ml RIPA buffer. Afterwards cells were lysed in a ratio of 50 µl RIPA buffer / 5 x 10^5 cells. Whole cell lysates were then transferred to microcentrifuge tubes and incubated for 30 min at 4 °C. Finally, lysates were spun down in a pre-cooled centrifuge for 15 min at maximum speed. Supernatants were transferred to fresh tubes and protein concentrations were measured by Bradford assay.

For Bradford assay standard curve dilutions of bovine serum albumin (BSA) were generated. Afterwards 5 µl of 1:4 diluted whole cell lysates and standard curve dilutions were transferred to 96-well flat bottom plates. Finally, 250 µl Bradford reagent (table 2.1) was added to each well. After 5 min incubation at RT absorbance was measured at 595 nm using the the BioTek Synergy Neo2 or FLUOstar OPTIMA microplate reader. Samples as well as standard curve dilutions were measured in triplicates.

2.3.7. Immunoblotting

For analysis of protein expression by immunoblotting whole cell lysates were mixed with LDS sample buffer (table 2.1) as well as reducing agent (table 2.1). Subsequently, proteins were denatured by boiling for 5 min at 95 °C and briefly centrifuged in a microcentrifuge. Samples were then subjected to SDS-PAGE on prepared 8-12% acrylamide gels. Recipes for respective acrylamide gels can be found in table 2.4. A maximum of 40 µg protein was loaded onto prepared gels for each whole cell lysate. After running for 120 min at 120 V, gels were removed from chambers and samples were transferred to a 0.45 µm nitrocellulose membrane in a semi-dry transfer for 40 – 45 min. Transfers were performed using the Trans-Blot Turbo blotting machine (table 2.8). Subsequently, membranes were blocked with 5 % BSA in TBS (table 2.4) with 0.1 % Tween-20 (TBS-T) for 1 h at RT. Afterwards membranes were cut according to molecular weights of investigated protein and incubated overnight at 4°C in 5 ml TBS-T containing diluted primary antibodies (table 2.15, product numbers in table 2.6). On the next day, membranes were washed three times with TBS-T for 5 min each and incubated with secondary, HRP-conjugated antibodies (table 2.16, product numbers in table 2.6). Secondary antibodies were diluted in TBS-T containing 5% BSA or milk powder. After 1 h incubation, membranes were washed three times with TBS-T for 5 min each. For chemiluminescence detection, ECL substrate (table 2.1) was prepared according to the manufacturer's instructions (1:1 dilution of detection reagent 1 and 2). Subsequently substrate was slowly pipetted onto membranes (250 µl/membrane) and imaging was performed using the chemiluminescence channel of the LI-COR Odyssey XF Imaging System. While house keepers were exposed for 30 seconds other investigated proteins were exposed for a minimum of 2 min. Pictures were analyzed and edited using the Image Studio Lite software.

Table 2.15: List of primary antibodies and respective dilutions, blocking buffers and incubation conditions

Antibody	Dilution	Blocking buffer	Incubation
Caspase-8 (1C12), mouse anti-human mAb	1:1000	TBS-T, 5% BSA	4°C over-night
FAS Recombinant, rabbit anti-human mAb	1:1000	TBS-T, 5% BSA	4°C over-night
HER2/ErbB2, rabbit anti-human	1:1000	TBS-T, 5% BSA	4°C over-night
p16 INK4A (D7C1M), rabbit anti-human	1:1000	TBS-T, 5% BSA	4°C over-night
p21, mouse anti-human	1:1000	TBS-T, 5% BSA	4°C over-night
STING (D2P2F), rabbit anti-human mAb	1:1000	TBS-T, 5% BSA	4°C over-night
β-Actin, mouse anti-human mAb	1:7000	TBS-T, 5% BSA	4°C over-night

Table 2.16: **List of secondary antibodies and respective dilutions and blocking buffers and incubation conditions**

Antibody	Dilution	Blocking buffer	Incubation
IgG Antibody (H+L), Peroxidase, Goat Anti-Rabbit	1:10000	TBS-T, 5% BSA or milk powder	RT, 1h
IgG, Anti-Mouse (H+L), HRP Conjugate	1:10000	TBS-T, 5% BSA or milk powder	RT, 1h

2.4.Statistical analysis

Normal distribution of data was assessed using the Shapiro–Wilk test. Parametric tests were performed using ANOVA or Student's t-test for normally distributed data while non-parametric analysis was conducted using a Kruskal-Wallis test. Multiple testing was accounted for using the indicated post-hoc tests (see figure legends). For statistical analysis and graph design GraphPad Prism 8 was used, while calculation templates were generated with Excel 2019. All microscopy data was processed using the ZenBlue3 software and ImageJ-Win64. Furthermore, flow cytometry data was analyzed with FlowJo V10. Finally, p-values were determined via statistical analysis in GraphPad PRISM and are indicated in figure legends.

3. Results

This study provides a comprehensive overview and characterization of hepatocellular carcinoma therapy-induced senescence (Fig 3.1). By establishing various *in vitro* models of HCC TIS, distinct and shared characteristics between different TIS-inducers and cell lines were identified. Furthermore, SASP heterogeneity, innate signaling pathways, senescence-associated surfaceome and innate immune responses were characterized, and the respective findings were used to identify actionable targets of HCC therapy-induced senescence. Furthermore, these targets were utilized for development of various therapeutic approaches for elimination of senescent HCC cells.

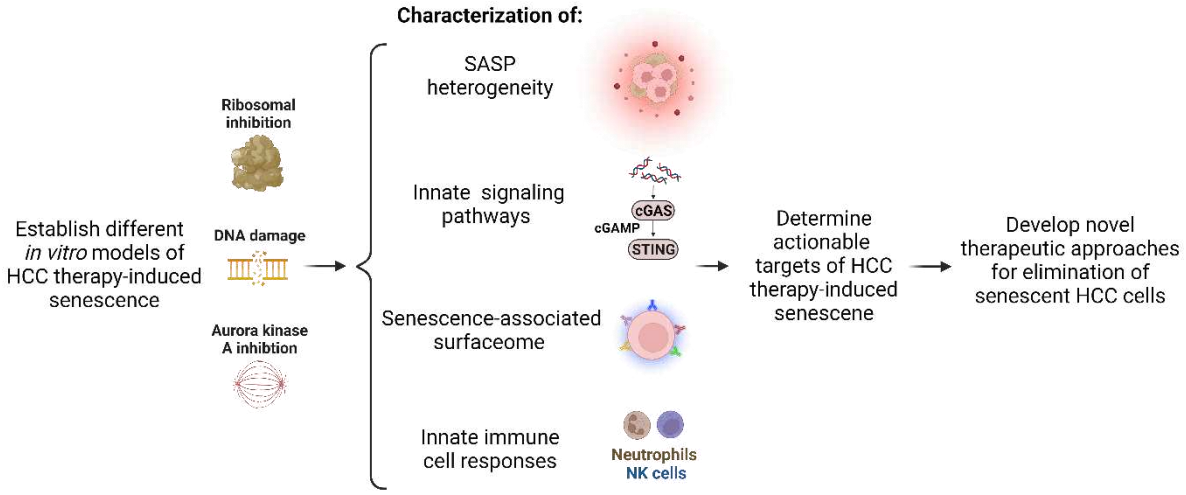

Figure 3.1: Flow chart illustrating the investigations carried out in this study.

Several *in vitro* models of HCC therapy-induced senescence were established. Furthermore, SASP heterogeneity, innate signaling pathways, senescence-associated surfaceome changes and innate immune cell responses were characterized. Subsequently, actionable targets of HCC TIS were identified and novel therapeutic approaches for elimination of senescent HCC cells were tested.

3.1. Establishing different models of HCC therapy-induced senescence

In order to characterize treatment-specific differences of HCC therapy-induced senescence and explore the functional heterogeneity between TIS-inducers, different *in vitro* models of cellular senescence were established and compared. First, proliferation arrest as well as expression and activity of some of the most commonly shared biomarkers of senescence, namely SA-β-Gal, $p16^{INK4a}$, $p21^{WAF1/CIP1}$ (Hernandez-Segura et al., 2018; Kudlova et al., 2022), were assessed and quantified. To cover a broad spectrum of distinct senescence-inducing mechanisms, DNA damage- (using etoposide), ribosomal stress- (using CX5461), and aurora kinase A (using alisertib)-induced senescence were functionally characterized. Besides treatment-specificity,

cell line-specific effects were also investigated by comparing the p53-mutated HCC cell line HUH7 with the wild-type cell line HepG2.

3.1.1. Alisertib, CX5461 and etoposide induce proliferation arrest in HCC cell lines

One of the general hallmarks of senescence *in vitro* and *in vivo* is the induction of a stable proliferation arrest (Hernandez-Segura et al., 2018; Kudlova et al., 2022) that can be assessed by growth quantification and detection of proliferation markers. Hence, cell numbers of alisertib, CX5461- and etoposide- treated HCC cell lines were determined at different timepoints after treatment, indicating a proliferation arrest upon treatment with all three TIS inducers (Fig. 3.2).

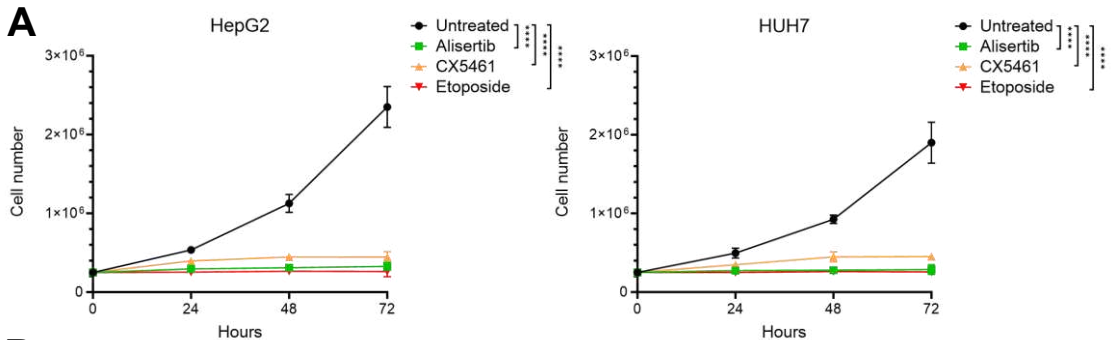

Figure 3.2: **Induction of proliferation arrest in HCC cell lines upon treatment with alisertib, CX5461 and etoposide.**

HCC cell numbers quantified in technical triplicates by cell counting at the indicated treatment time points. Alisertib and Etoposide treatments were conducted for 72 hours. CX5461 was added for 24 hours, followed by 48 h recovery in fresh cell culture media. (n=3, combined data, mean+SD, statistical analysis was performed at 48 h timepoint, ****p<0.0001 according to two-way ANOVA test).

Furthermore, proliferative capacities of treated HCC cells were assessed by Ki67 immunofluorescence microscopy (fig. 3.3). Ki67 is a well-studied and prognostically relevant proliferation marker of cancer cells that was first discovered in Hodgkin lymphoma (Gerdes et al., 1983). Respective quantification of Ki67 positive cells revealed a significant decrease in TIS agonist-treated HCC cell lines. Thus, cell number quantification, as well as Ki67 staining confirmed a strong proliferation arrest upon treatment with alisertib, CX5461 and etoposide, indicating initiation of senescence. For all following experiments described in this study alisertib and etoposide treatments were conducted constitutively for 48 h or 72 h, and treatment with CX5461 was performed for 24 h, followed by recovery in fresh cell culture media for either 48 h or 72 h, as indicated respectively.

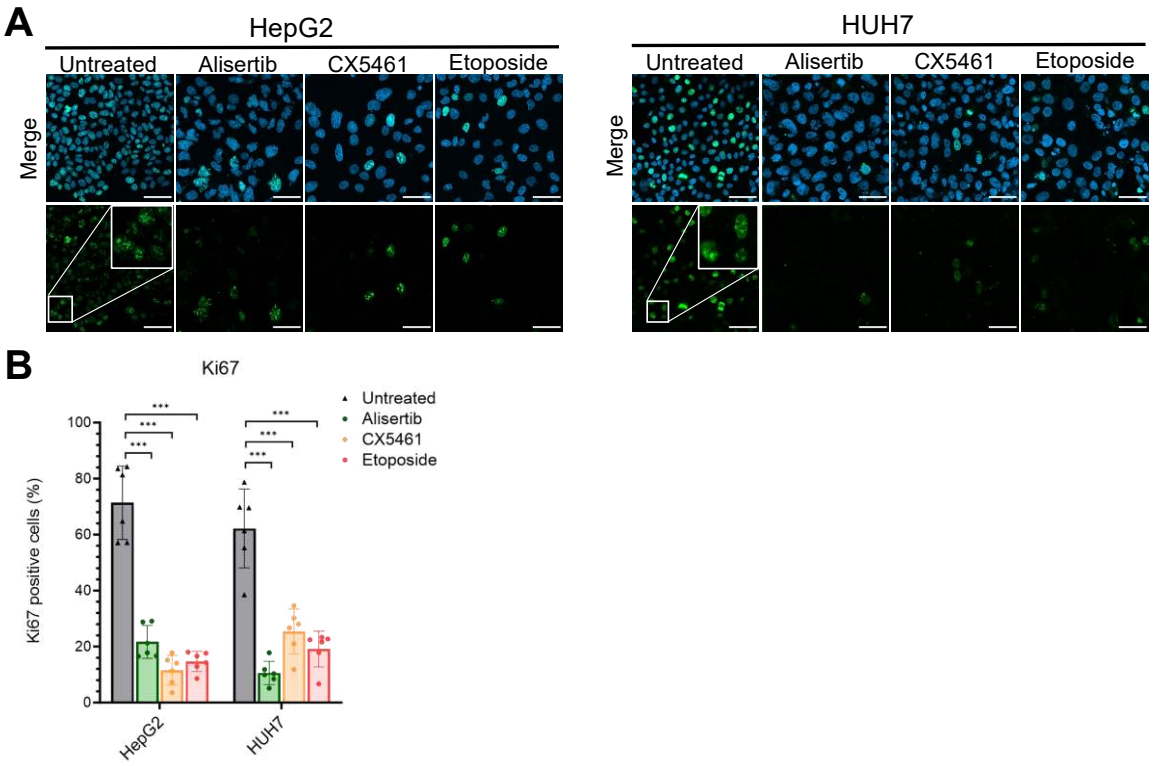

Figure 3.3: **Ki67 staining of HCC cell lines after treatment with alisertib, CX5461 and etoposide.**

(A) Ki67 immunofluorescence after treatment with TIS-inducers (scale bar, 50 µm). Alisertib and Etoposide treatments were conducted for 48 hours. CX5461 was added for 24 hours, followed by 48 h recovery in fresh cell culture media. DNA staining was performed using Hoechst 33342. Ki67 positive cells were quantified in **(B)**. N=3, each dot represents one 3x3 tile, 2 tiles per experiment, combined data, mean+SD, ***p<0.001 according to two-way ANOVA test.

3.1.2. Alisertib, CX5461 and etoposide induce expression of senescence-associated biomarkers

Therapy-induced senescence initiates a stable growth arrest of cancer cells, accompanied by expression of senescence biomarkers. Thus, several senescence-associated proteins were investigated in order to further confirm an initiating senescence response and find comparable timepoints of similar senescence magnitude between TIS-inducers. One of the most commonly used senescence biomarkers is senescence-associated β-galactosidase (SA-β-Gal), an enzyme that accumulates in the lysosome upon senescence induction (Lee et al., 2006). Therefore, expression and enzymatic activity of SA-β-Gal was investigated using a fluorometric β-galactosidase detection kit (table 2.7). Notably, elevated SA-β-Gal activity was observed after treatment with TIS inducers, indicating induction of senescence. Furthermore, the similar levels obtained from all treatments imply a highly comparable state of senescence between TIS inducers.

Besides SA-β-Gal, the cyclin-dependent kinase inhibitors $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ are well characterized senescence-associated biomarkers. Both are essential cell cycle regulators that typically exhibit an upregulation and elevated activity upon senescence induction (Kumari and Jat, 2021). Hence, immunoblotting of $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ was performed to determine expression changes after treatment with TIS inducers. Evidently, TIS inducer treated HepG2 cells displayed an elevated expression of $p21^{WAF1/CIP1}$ compared to untreated cells, while HUH7 cells exhibited an upregulated $p16^{INK4a}$ expression in comparison to their untreated counterparts. Although $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ are highly interconnected cell cycle regulators, this indicates that senescence may be initiated through different routes depending on the cell type.

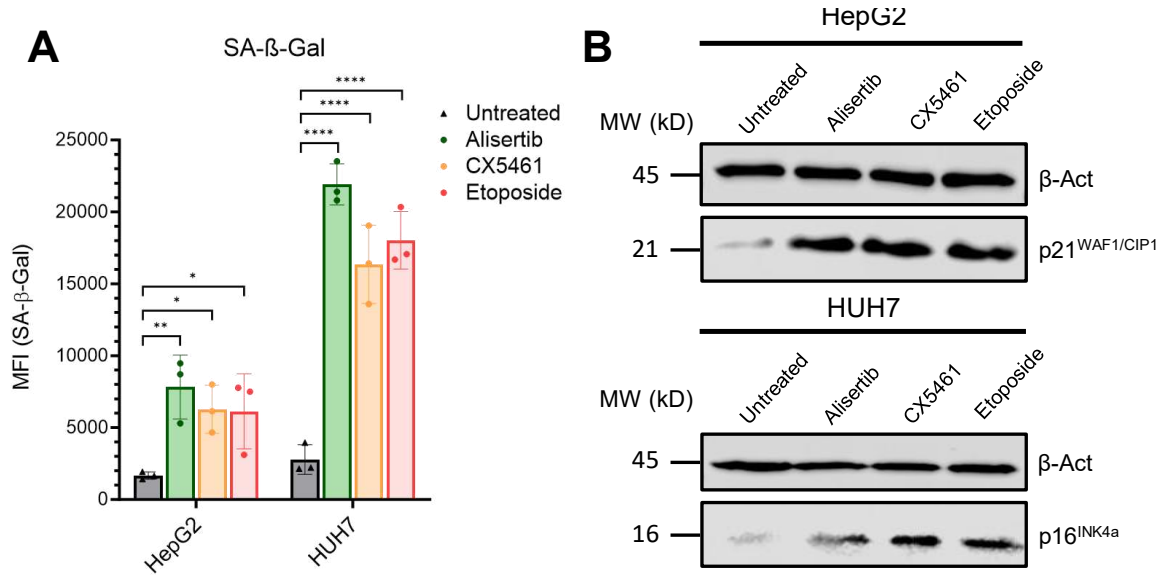

Figure 3.4: **Expression of senescence biomarkers upon treatment with alisertib, CX5461 and etoposide**

(A) Fluorometric detection of SA-β-Gal activity in alisertib-, CX5461- and etoposide-treated HCC cell line (n=3 biological replicates, combined data, *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001 according to two-way ANOVA test) **(E)** $p21^{WAF/CIP1}$ and $p16^{INK4a}$ immunoblot analysis after treatment with TIS inducers (n=3 biological replicates, representative data). Alisertib and Etoposide treatments were conducted for 48 hours. CX5461 was added for 24 hours, followed by 48 h recovery in fresh cell culture media.

3.1.3. Senescence-inducing regiments do not initiate apoptosis response

One further characteristic of senescent cells is their strong resistance to apoptosis, mediated by several anti-apoptotic proteins including Bcl-2, Bcl-xl and Bcl-w (Martin et al., 2023; Wang, 1995). For many chemotherapeutic agents the administered concentration determines whether cancer cells become senescent or undergo apoptosis. Nevertheless, both cell intrinsic

pathways share many regulatory mechanisms that are treatment and cell type specific (Childs et al., 2014). Hence, testing if cells undergo apoptosis upon treatment with TIS inducers is essential to confirm initiation of cellular senescence. In order to investigate cell death responses HCC cells were stained with a fixable viability dye (table 2.7) after treatment with alisertib, CX5461, and etoposide, and subjected to flow cytometry. Notably, the fraction of HUH7 cells positively stained by the viability dye remained under 3 % upon treatment with TIS-inducers (Fig 3.5). Similar results were obtained for HepG2 cells, confirming that the administered concentrations induce senescence rather than apoptosis. Only etoposide treated HepG2 cells displayed a cell death rate of approximately 7%, suggesting an apoptosis response in a small subset of cells.

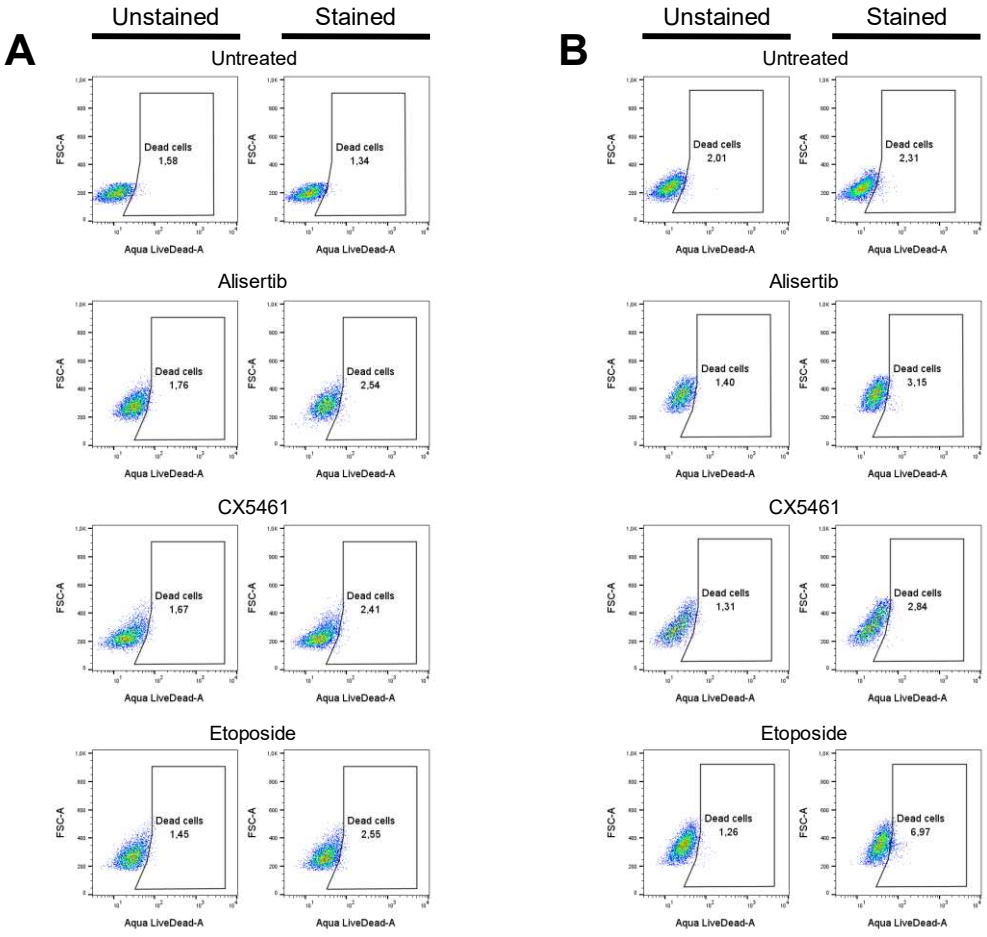

Figure 3.5: **Viability staining of senescent HCC cell lines.**

(A, B) Viability staining of senescent HUH7 (A) and HepG2 cells (B) cells (n=3 biological replicates, representative data). Staining was performed using a flow cytometry-based Aqua LIVE/DEAD viability dye. Alisertib and Etoposide treatments were conducted for 48 hours. CX5461 was added for 24 hours, followed by 48 h recovery in fresh cell culture media.

3.2. The senescence-associated secretory phenotype (SASP) displays strong inducer-dependent heterogeneities in HCC therapy-induced senescence

Besides a stable proliferation arrest and expression of typical senescence biomarkers, one additional characteristic of senescent cells is induction of a strong secretory phenotype that promotes senolytic immune responses. This, so-called senescence-associated secretory phenotype (SASP) describes a transcriptional reprogramming of senescent cells, resulting in an increased expression and secretion of inflammatory cytokines, chemokines, and growth factors. To gain an insight into whether the three inducers give rise to a differentially characterized SASP, secretion of several SASP factors were next analyzed.

3.2.1. Secretion of SASP-associated cytokines, chemokines, and growth factors

Previous studies indicated that the composition of SASP factors strongly differs between cell types and types of senescence (Hernandez-Segura et al., 2017). This heterogeneity impedes unified senescence-based treatment efficiency and predictability. Due to a lack of systematic studies focusing on the secretory phenotype of senescent HCC cells, SASP characteristics were determined by ELISA. Since senescent cell undergo a stable proliferation arrest, secretion was quantified relative to the respective cell numbers (Fig. 3.6). Notably, TIS inducers enhanced the secretion of the chemokines IL-8 and CXCL10, as well as of the growth factor VEGF-A compared to untreated cells. While alisertib was the strongest VEGF-A inducer, CX5461 induced the strongest IL-8 and CXCL10 secretion. No CXCL10 secretion was observed for TIS-treated HepG2 cells. Furthermore, HUH7 exhibited a stronger IL-8 induction than HepG2 cells after treatment with TIS inducers. Other investigated cytokines were IL-6 and TNF-α. However, no secretion was observed upon TIS induction. Taken together, these findings indicate a strong heterogeneity between TIS inducers and cell lines regarding SASP characteristics. This is notable since striking differences in the active secretion of SASP factors might influence innate immune responses and immune clearance of senescent HCC cells respectively.

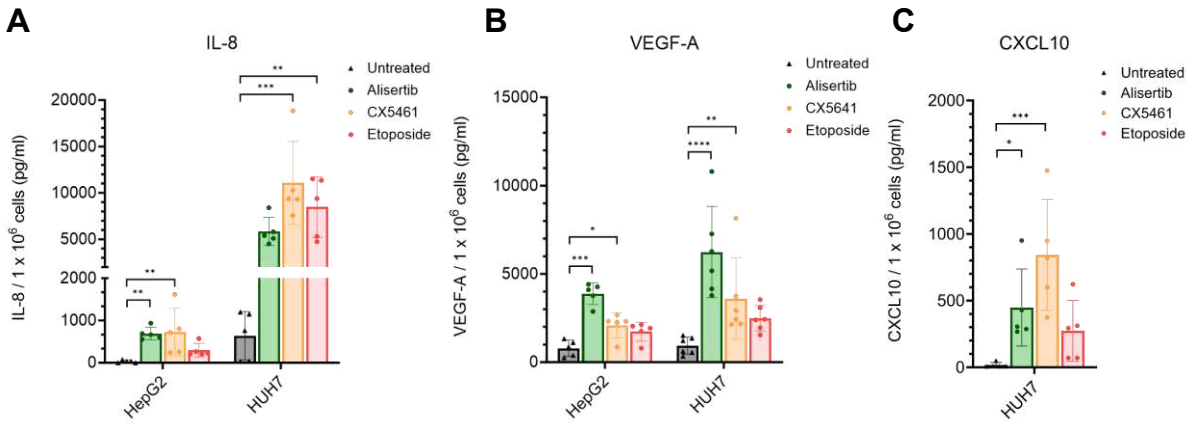

Figure 3.6: Characterization of the senescence-associated secretory phenotype in HCC therapy-induced senescence.

(A) Secretion of IL-8, and VEGF-A **(B)** and CXCL10 **(C)** of senescent HCC cell lines analyzed by triplicate ELISA (n=5 biological replicates, combined data, *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001 according to Kruskal-Wallis test). Alisertib and Etoposide treatments were conducted for 48 hours. CX5461 was added for 24 hours, followed by 48 h recovery in fresh cell culture media.

3.2.1. cGAS-STING modulation effects secretion of CXCL10 in an inducer-dependent manner

The SASP represents a tightly regulated cellular process that is modulated by many innate signaling pathways, such as the cGAS-STING pathway. Previous studies indicated that the cGAS and its adaptor STING play an essential role in senescence responses and SASP initiation (Glück et al., 2017). cGAS-STING is a crucial sensor of DNA damage and detects e.g., leakage of DNA into the cytosol, initiating the transcription of several pro-inflammatory chemokines, such as CXCL10.

Since all TIS-inducers displayed a considerable SASP heterogeneity, we sought to investigate the cell intrinsic SASP regulation and examine the sensitivity to cGAS-STING modulators. Hence, senescent HUH7 cells were stimulated with the STING ligand 2´3´-cGAMP or the STING antagonist H151 for 24 h post-senescence induction, and sensitivity was determined by CXCL10 ELISA. To ensure that differences in STING expression did not affect responses, STING levels were monitored by immunoblotting. Notably, STING expression was slightly increased for alisertib (Fig. 3.7, A). Moreover, only CX5461-treated HUH7 cells displayed a concentration dependent sensitivity to 2´3´-cGAMP, indicated by an enhanced CXCL10 secretion (Fig. 3.7, B). Similarly, H151-mediated STING inhibition resulted in a significant decrease in CXCL10 secretion, exclusively observed for CX5461-treated HUH7 cells (Fig. 3.7, C). Taken together, this data suggests that SASP heterogeneity could arise from a differential regulation via cGAS-

STING, indicated by a distinct sensitivity to cGAS-STING activation and inhibition. Nevertheless, to further explore differences in cell intrinsic SASP regulation other innate pathways have to be investigated.

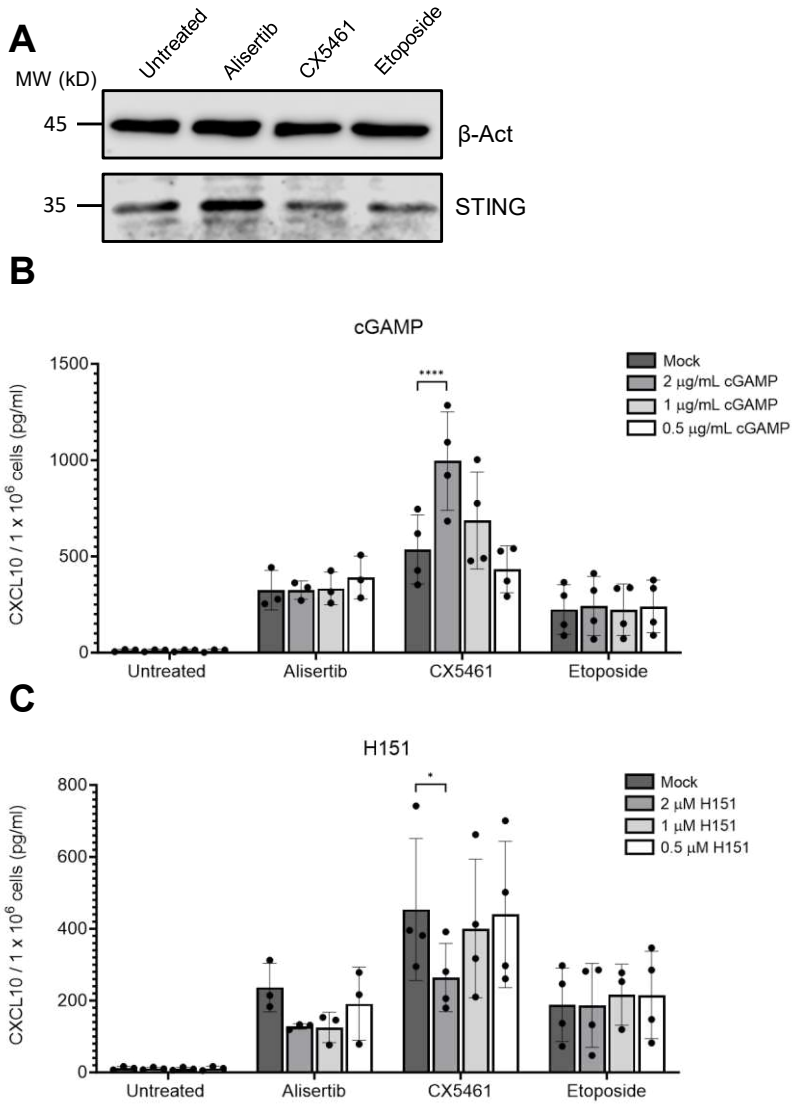

Figure 3.7: **cGAS-STING modulation in HCC therapy-induced senescence.**

(A) STING immunoblot analysis after treatment with TIS inducers (n=3 biological replicates, representative data). **(B)** Plotted CXCL10 secretion after 24 h stimulation of senescent HUH7 cells with the STING ligand 2´3´-cGAMP (n=3-4 biological replicates, combined data, mean+SD, *p<0.05, ****p<0.0001 according to two-way ANOVA test) **(C)** CXCL10 secretion of senescent HUH7 cells after 24 h inhibition of cGAS-STING with the STING antagonist H151 (n=3-4 biological replicates, combined data, mean+SD, *p<0.05, ****p<0.0001 according to two-way ANOVA test). Human PMNs, were freshly isolated from healthy donors by Dr. Vinicius Nunes Cordeiro Leal and Dr. Francesca Bork, PostDocs of AG Weber, Department of Immunology, University of Tübingen

3.3. SASP heterogeneity influences innate immune responses

Apoptosis resistance of senescent cells favors a persistent, chronically senescent phenotype due to a lack of cell intrinsic clearance responses. Hence, other clearance mechanisms such as immune-mediated clearance are indispensable for eliminating senescent cells. Notably, neutrophils play an essential role in senescence surveillance by not only being recruited and activated by SASP factors and cell-to-cell contact, but also by promoting paracrine senescence through the production of reactive oxygen species (Lagnado et al., 2021). Furthermore, recent studies have indicated that NK-cell responses are crucial for the clearance of senescent cells, which are driven by SASP-mediated recruitment and upregulated expression of NK-cell activating ligands of the surface of senescent cells (Antonangeli et al., 2019; Ruscetti et al., 2018). Conversely, other studies have suggested that an elevated HLA-E expression on senescent fibroblasts hampers NK cell activation (Pereira et al., 2019).

These well-characterized functional differences of innate immune cell responses, accompanied by the strong SASP heterogeneity in HCC therapy-induced senescence led us to investigate innate immune responses against senescent HCC cell lines.

3.3.1. Neutrophil migration and activation display strong TIS-inducer dependency

Neutrophils are attracted and activated by distinct soluble and surface-associated factors. One main chemoattractant inducing neutrophil chemotaxis is IL-8. Since all TIS inducers displayed a considerable heterogeneity in secretion of IL-8 (*cf.* Fig. 3.6), we sought to determine the migratory capacities of human polymorphonuclear neutrophils (PMN) during HCC therapy-induced senescence. Hence, a transwell co-culture assay was established. The upper transwell insert was filled with primary human PMN, freshly isolated from healthy donors by Dr. Vinicius Nunes Cordeiro Leal and Dr. Francesca Bork, PostDocs of AG Weber, Department of Immunology, University of Tübingen. The insert was then placed into a 24-well plate with senescent or non-senescent HUH7 cells at the bottom (Fig 3.8, A). PMNs of different healthy human donors were isolated according to previously established lab protocols (Herster et al., 2020). Furthermore, migration through the porous membrane of the insert was quantified by manual counting after 6 h and the fraction of migrated PMN determined. As controls neutrophil migration was investigated in the absence of HCC cells. Moreover, in order to determine the effect of SASP factors on the migratory potential of HCC cells, fresh cell culture media was compared to SASP conditioned media taken from senescent HCC cells 24 h after

senescence induction. Notably, significant increases in neutrophil migration were observed for CX5461 as well as etoposide compared to untreated cells, whereas alisertib did not induce significant increases (Fig. 3.8, B). Furthermore, observed increases were stronger in the presence of SASP conditioned media, emphasizing the essential role of secretory factors for innate immune recruitment. These SASP-associated increases were predominantly observed for CX5461 as well as etoposide, indicating that neutrophil-attraction is promoted by SASP-associated secretory factors.

To determine if an enhanced migration coincided with an elevated neutrophil activation, myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9) release were assessed. Both are well-characterized neutrophil activation markers and thus, were used to determine activation status of PMNs (Boettcher et al., 2020; Sarr et al., 2021). Freshly isolated PMNs were co-cultured with senescent or non-senescent HCC cells for 3 h and media was collected for respective ELISAs. To prevent neutrophil activation by residual TIS-inducers, cells were extensively washed with cell culture media before performing the transwell and co-culture assays. As controls, neutrophils were incubated without HCC cells and media were subjected to MPO and MMP9 ELISA.

Notably, co-cultures with etoposide- and CX5461-treated HUH7 cells induced significant MPO and MMP9 increases compared to untreated HUH7 cells (Fig. 3.8, C). Conversely, for HepG2 cells neutrophils only showed significant increases in the presence of etoposide-treated cells, whereas alisertib did not induce any notable changes in MPO and MMP9 secretion. In line with results from the transwell assay this indicates that CX5461 and etoposide induce the strongest neutrophil activation. Collectively, these findings suggest that SASP heterogeneity reflects on neutrophil attraction and activation. At the same time, cell-cell interaction could also alter neutrophil responses and affect activation profiles.

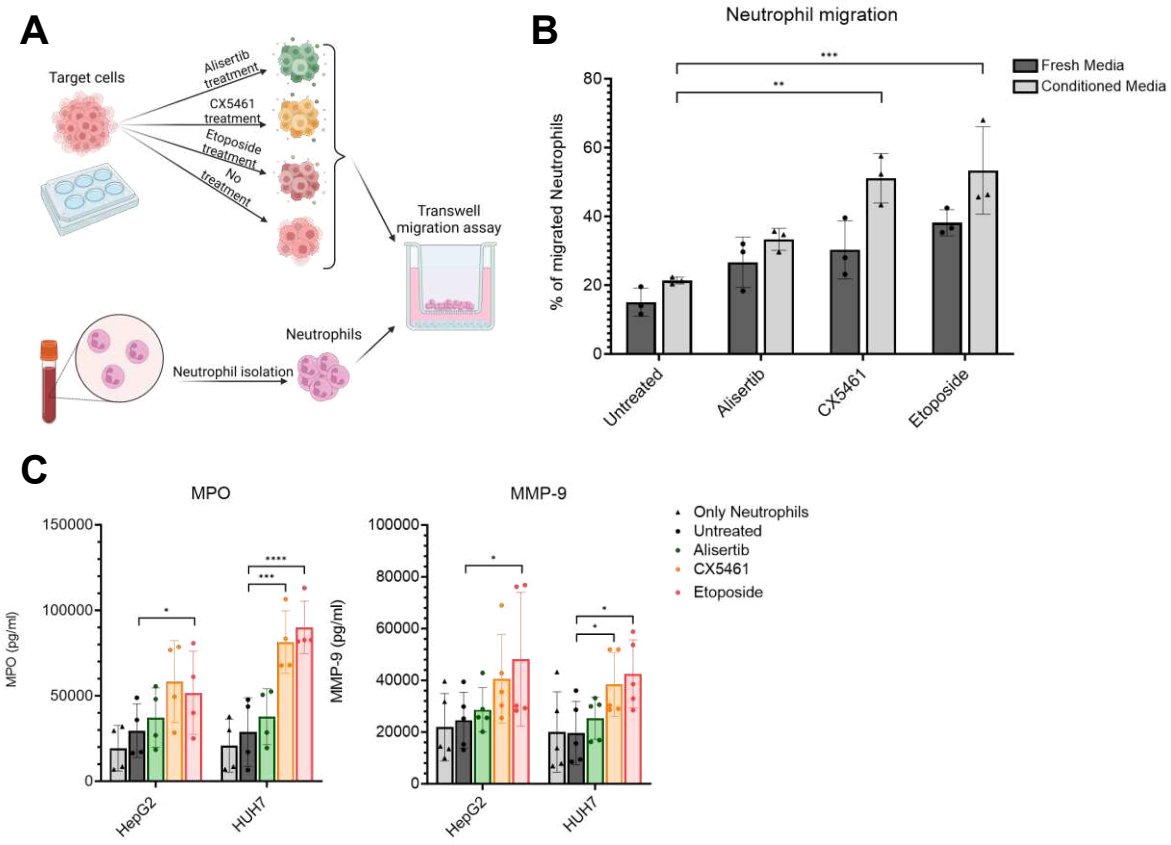

Figure 3.8: **Neutrophil migration and activation in HCC therapy-induced senescence**

(A) Neutrophil migration assessed in a transwell setup and **(B)** quantified by manual counting (n=3 biological replicates, combined data, mean+SD, **p<0.01, ***p<0.001 according to two-way ANOVA test). **(C)** MPO and MMP-9 secretion of co-cultured neutrophils with senescent HCC cells analyzed by triplicate ELISA (n=3 biological replicates, combined data, *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001 according to Kruskal-Wallis test). Human PMNs were freshly isolated from healthy donors by Dr. Vinicius Nunes Cordeiro Leal and Dr. Francesca Bork, PostDocs of AG Weber, Department of Immunology, University of Tübingen.

3.3.2. NK cell clearance of senescent HCC cells is TIS-inducer dependent

As previously described, NK cells play an essential role in the recognition and clearance of senescent tumor cells. Their activation is governed by the integration of signals from NK cell receptors sensing surface molecules on target cells (‘altered self’) (Abel et al., 2018; Paul and Lal, 2017). In order to characterize NK cell responses more thoroughly, a second co-culture model of senescent HCC cells with NK92 MI cells was generated (Fig. 3.9, A). NK92 cells are an immortalized NK cell line derived from a 50-year-old male patient with non-Hodgkin´s lymphoma (Gong et al., 1994). By endogenously expressing human IL-2, the modified NK92 MI cells are an immensely robust cell line, ideal for comparative killing assays, although they lack ADCC-mediated cytotoxic responses (Tam et al., 1999). To investigate differences in NK cell-mediated killing between TIS inducers, NK92 MI cells were co-cultured with senescent or

untreated HCC cells for 3 h and killing efficiency was determined by LDH-release assay. As controls for NK cell independent cell death and spontaneous LDH release, NK92 MI cells were cultured for 3 h in the absence of HCC cells (standard release). Additionally, maximum release was determined by inducing target cell death with Triton X-100.

Notably, a strong heterogeneity between TIS-inducers was observed (Fig. 3.9, B). For instance, NK cells exerted an increased cytotoxicity against CX5461-treated HUH7 cells compared to untreated cells, whereas alisertib treatment induced a decrease in killing efficiency. Furthermore, NK cell-mediated clearance of etoposide- and CX5461-treated HepG2 cells was significantly elevated compared to untreated cells, while no difference was observed for alisertib. Consistent with previous observations on neutrophil migration and activation, CX5461 induced a strong NK cell response, increasing LDH release of HepG2 cells from 25% (untreated) to 70% (CX5461-treated).

Collectively, these findings demonstrate that, although NK cell responses were comparable between HCC cell lines, striking differences were observed between TIS inducers. Furthermore, combining the finding on neutrophil migration and NK cell responses, reveals that CX5461 gives rise to a more advantageous senescence profile compared to other TIS inducers.

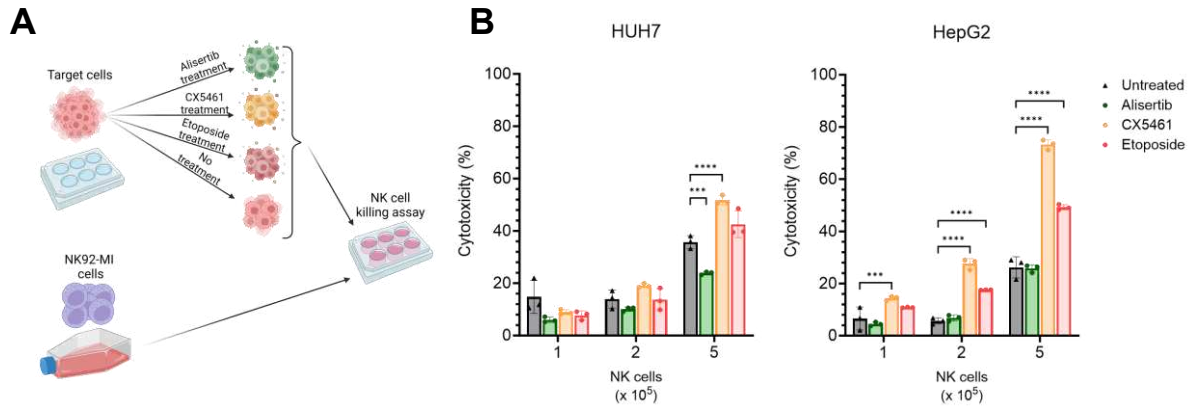

Figure 3.9: **NK cell killing of senescent HCC cell lines**

NK92 MI cell killing was assessed as shown **(A)** and quantified by triplicate LDH release assay **(B)** (n=3 biological replicates, representative data, mean+SD, ***p<0.001, ****p<0.0001 according to two-way ANOVA test). After respective senescence induction as described above, senescent or untreated HCC cells were co-cultured with NK92 MI cells for 3 h.

3.3.3. Differential NK cell responses against senescent, non-tumorigenic IMR90 fibroblasts

Fibroblasts are one of the most abundant cell types of the tumor microenvironment (TME) and have previously been described to modulate tumor growth and anti-tumor immune responses (Maia and Wiemann, 2021). Furthermore, targeting cancer cells with senescence inducing compounds always carries the risk of also effecting surrounding cells of the TME, such as primary fibroblasts (H. Liu et al., 2022; Saleh et al., 2020). In order to investigate if heterogenous NK cell responses are also observed for non-HCC/non-tumorigenic cells of the TME, senescent IMR90 fibroblasts were co-cultured with NK92 MI cells. IMR90 fibroblasts are human fetal lung cells most frequently used as an *in vitro* model of Ras-mediated oncogene-induced senescence (Nichols et al., 1977).

To explore NK cell responses against senescent IMR90 fibroblasts, senescence was induced as previously described and validated by fluorogenic SA-β-Gal detection (Fig 3.10, A) and $p21^{WAF1/CIP1}$ immunoblot (Fig 3.10, B). Senescent fibroblasts were then co-cultured with NK92-MI cells for 3 h and NK cell killing was determined by LDH release. Strikingly, a decreased cytotoxicity was observed for all TIS inducers (Fig 3.10, C), indicating that the previously described heterogeneity in NK cell response is HCC specific. In line with other studies on senescent fibroblasts and lung cancer cells (Pereira et al., 2019; Ruscetti et al., 2018), these findings suggest that senescence-associated NK cell responses display a cell type specificity, potentially due to a differential secretome or distinct expression of NK cell activating and inhibiting surface antigens.

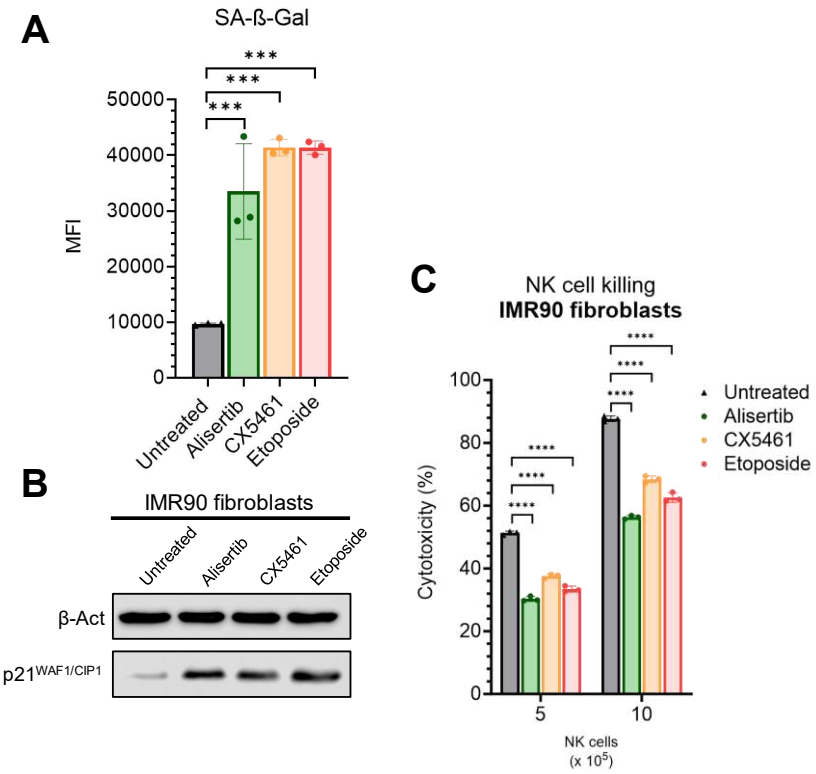

Figure 3.10: **NK cell killing of senescent IMR90 fibroblasts**

(A) Fluorometric SA-β-Gal detection after treatment of IMR90 fibroblasts with different TIS inducers (n=3 biological replicates, representative data, mean+SD, ***p<0.001 according to two-way ANOVA test) **(B)** $p21^{WAF/CIP1}$ immunoblot analysis after treatment with TIS inducers. Alisertib and Etoposide treatments were conducted for 48 hours. CX5461 was added for 24 hours, followed by 48 h recovery in fresh cell culture media. **(C)** NK92 MI cell co-culture with senescent and non-senescent IMR90 fibroblasts. NK-cell cytotoxicity was determined by triplicate LDH release assay (n=3 biological replicates, representative data, mean+SD, ***p<0.001, ****p<0.0001 according to two-way ANOVA test). After respective senescence induction, senescent or untreated IMR90 fibroblasts were co-cultured with NK92 MI cells for 3 h.

3.4.Senescence-associated surfaceome in HCC therapy-induced senescence

As previously indicated, the senescent secretome displays a cell- and treatment-specific heterogeneity that strongly affects immune signaling. Besides soluble factors, surface-associated immune activation also plays an essential role in immune-mediated clearance of senescent and malignant cells. As aforementioned, NK cell responses are tightly regulated by receptor-ligand interactions. These include the interaction of activating receptor NKG2D with surface ligands, such as MIC-A, MIC-B, and ULBP-1, or the inhibitory receptor NKG2A with non-classical MHC-I molecule HLA-E (Tremblay-McLean et al., 2019). Other NK cell inducing surface antigens include a set of globally expressed cell adhesion molecules such as ICAM-1/CD54 or LFA-3/CD58 (Robertson et al., 1990). Interestingly, ICAM-1 has previously been reported to play a role in NK-cell mediated clearance of senescent lung cancer cells (Ruscetti et al., 2018).

Therefore, we sought to investigate ICAM-1 and HLA-E expression during HCC therapy-induced senescence. Hence, flow cytometry analysis was performed, revealing an upregulation of both markers for all TIS-inducer. Notably, out of the three TIS inducers CX5461 cells displayed the strongest increase in ICAM-1 expression compared to untreated cells (Fig. 3.11, A). Furthermore, HUH7 cells exhibited the highest HLA-E expression upon treatment with alisertib (Fig. 3.11, B).

Combining these findings with previously addressed literature may explain the effects that were observed for NK cell-mediated killing of senescent HCC cells. While alisertib displayed strong expression of the NK-cell inhibiting ligand HLA-E, CX5461 induced the strongest expression of ICAM-1, which is a crucial driver for NK cell-mediated cytotoxicity. This illustrates the complexities of senescence-inducing therapy, namely the differential induction of signals activating or deactivating cytotoxic responses. To gain a more comprehensive impression, a thorough surface antigen screening was performed to give insights into differential immune cell regulation and uncover shared senescence-associated surface antigens between TIS inducers.

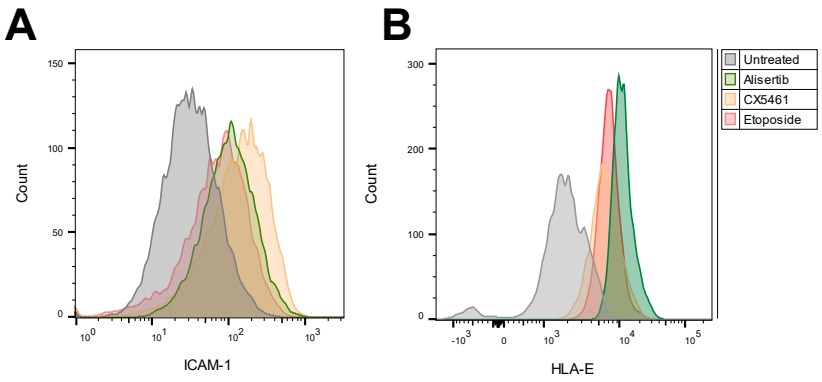

Figure 3.11: **ICAM-1 and HLA-E expression during HCC therapy-induced senescence**

(A) ICAM-1 expression histograms of untreated and senescent HUH7 cells (n=3, biological replicates, representative data). Expression was determined by flow-cytometry analysis after senescence induction. Untreated cells were used a control. **(B)** Expression histograms of HLA-E from senescent and untreated HUH7 cells (n=2, biological replicates, representative data). Untreated cells were used a control.

3.4.1. Surface antigen screening of senescent HCC cell lines

3.4.1.1. Experimental setup of surface antigen screening (LegendScreen)

Antigen screening of HCC cell lines was performed according to a bulk flow cytometry setup using the LegendScreen Kit (BioLegend, 70007). This kit enables expression analysis of altogether 360 distinct surface antigens arrayed on four different 96-well plates. Each well contains one PE-conjugated antibody directed against one of 360 surface antigens or isotype controls. TIS-inducer-treated and untreated HUH7 or HepG2 cells were aliquoted to each well (Fig 3.12) and subjected to flow cytometry analysis. After measurements, median-fluorescence intensities (MFI) of all events was determined and compared to those of untreated controls.

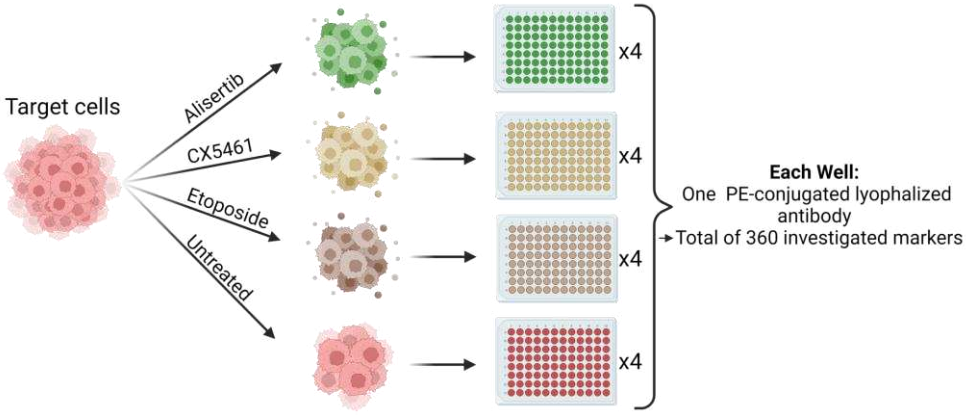

Figure 3.12: **Schematic overview of surface antigen screening**

(A) Surface antigen screening was performed using the LegendScreen Kit, according to manufacturer's instruction. Expression of 360 surface antigens, arrayed on four different 96-well plates per treatment, was determined by flow-cytometry analysis. Staining and acquisition between same markers for each treatment were performed simultaneously.

3.4.1.2. Senescent HCC cells display a heterogenous surfaceome

In order to determine shared senescence markers between TIS inducers and identify pathologically and therapeutically relevant surface antigens, expression profiles were normalized to the untreated controls and plotted as logarithmic fold expression differences. Furthermore, generated heat maps were organized according to globally up- and down regulated markers (Fig. 3.12 and 3.13). Notably, out of the 360 surface antigens, 224 (62.2%) were expressed under basal conditions for HUH7 cells and 245 (68.1%) for HepG2 cells, which was determined by comparing expression levels of surface antigens to those of respective isotype controls. Hence, for all following analysis and visualizations in this study only markers with expression levels above those of isotype controls are considered. Furthermore, for HUH7

cells only 43 markers (11.9%) displayed a up-or down-regulation for all TIS inducers cells and 32 (8.9%) for HepG2 cells when comparing TIS inducers. Surface antigens that showed upregulation for all TIS inducer in both cell lines included CD73, CD13, CD54, and CD146, whereas downregulated antigens were e.g., E-cadherin and CD138 for HUH7 cells and CD97 and Sialyl Lewis X for HepG2 cells. While most of the markers did not exhibit strong expression changes upon TIS induction, some antigens showed up- or down-regulation only for single TIS inducers. Globally upregulated markers will be further addressed in the following section.

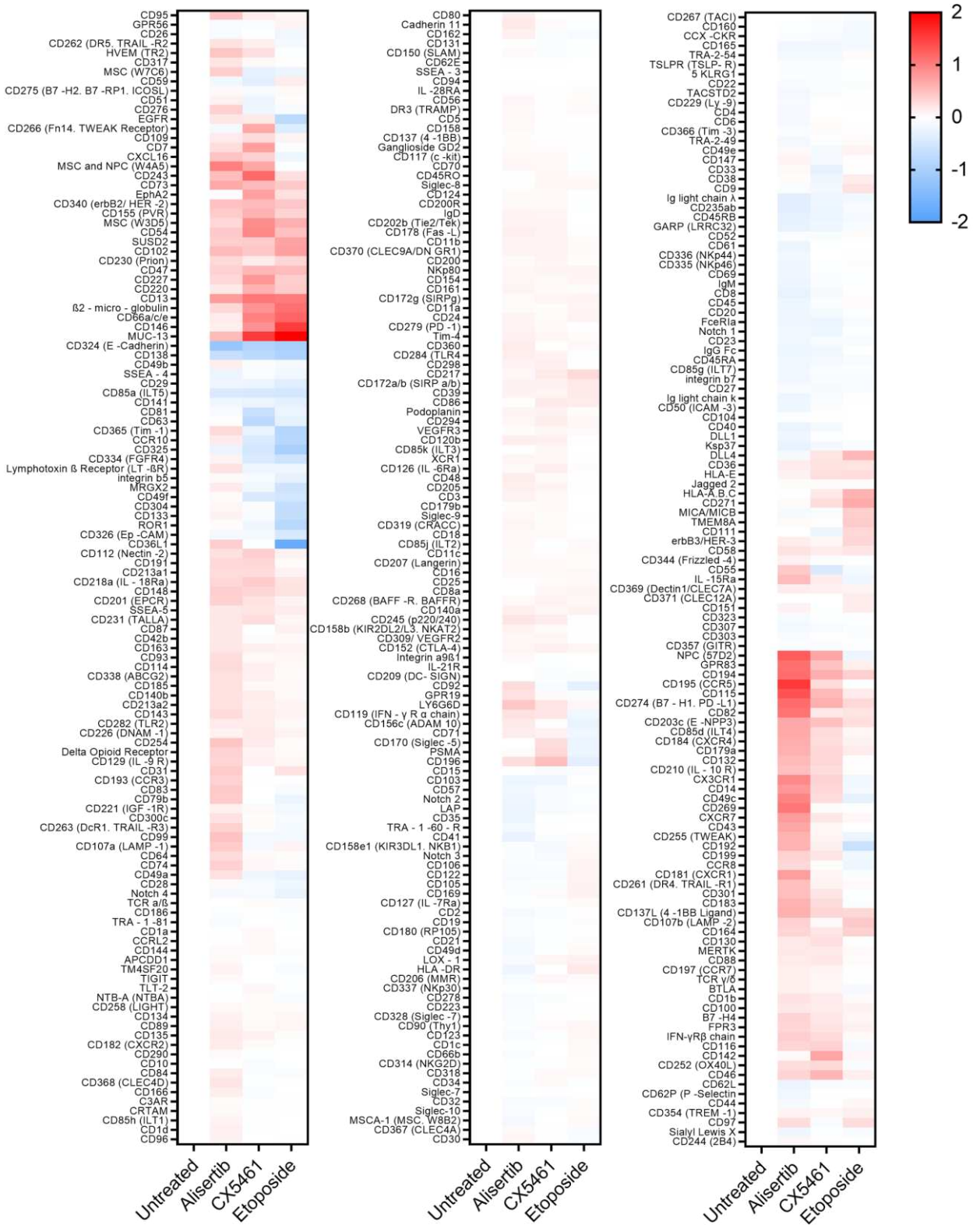

Figure 3.13: **LegendScreen analysis of senescent HUH7 cells.**

Expression of 360 surface antigens was explored by flow cytometry. Data is represented as a heat-map of logarithmic median expression normalized to untreated expression levels (n=1). Flow cytometry setup was generated together with Georgios Vavouras, a PhD student of AG Schindler, University hospital Tübingen.

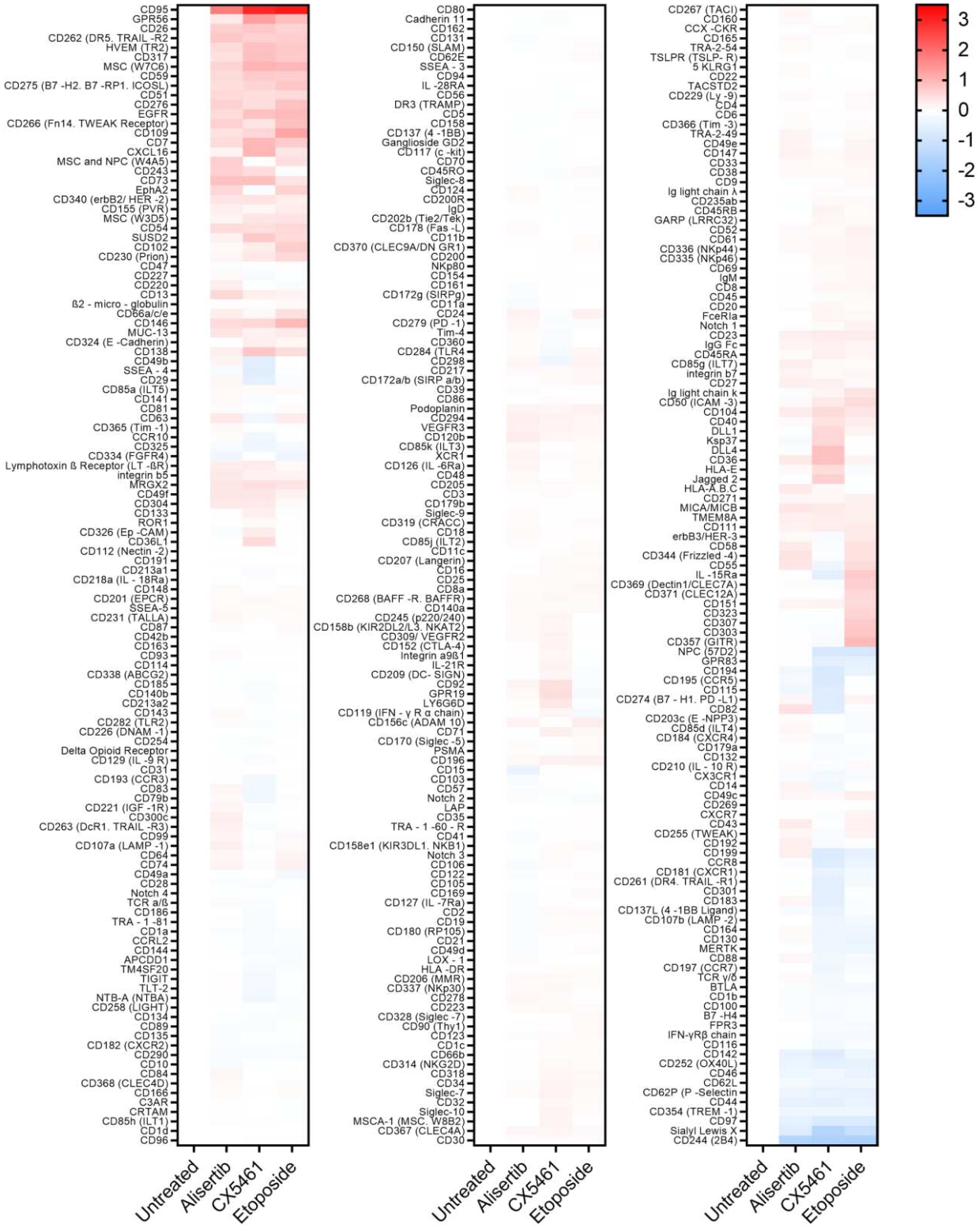

Figure 3.14: **LegendScreen analysis of senescent HepG2 cells.**

Expression of 360 surface antigens was explored by flow cytometry. Data is represented as a heat-map of logarithmic median expression normalized to untreated expression levels (n=1). Flow cytometry setup was generated together with Georgios Vavouras, a PhD student of AG Schindler, University hospital Tübingen.

Collectively, LegendScreen analysis indicated a strong surfaceome heterogeneity between TIS inducers and HCC cell lines. Despite of heterogeneities, few shared TIS-associated markers were identified and will be further addressed in the following section. Hence, this dataset was subsequently analyzed for

a) Strongly upregulated markers that could be targeted either intrinsically by induction of cell death, or via immunotherapeutic approaches, such as therapeutic antibodies or CAR-NK cells.

b) Hallmarks of subsequent tumorigenesis, e.g. metastasis.

3.4.2. Identification of globally upregulated, senescence-associated surface antigens

As previously indicated, HCC therapy-induced senescence is characterized by a strong heterogeneity regarding secreted factors and the surfaceome. These variations arise from differences of the underlying initiation pathways and effect innate immune clearance by NK cells and neutrophils. Although senescent tumor cells undergo a proliferation arrest, they give rise to a highly inflammatory milieu that can promote further tumor growth. Hence, finding novel surfaceome-associated targets to deplete senescent tumor cells after TIS induction can be an efficient strategy to avoid the onset of highly inflammatory and persisting tumor senescence. To determine surface antigens that were globally upregulated (≥1.5 fold change) in all types of TIS, a Venn diagram was generated (Fig. 3.15, A). Notably, a total of 6 surface antigens showed a shared expression increase between TIS inducers of at least 1.5 fold in HUH7 cells, while 13 markers were upregulated for all TIS inducers in HepG2 cells. Interestingly, several of the globally upregulated markers showed an elevated expression in both cell lines, indicating cell line independent effects. To determine the most promising markers for therapeutic targeting a ternary plot was generated by Dr. Andras Szolek, a PostDoc from AG Weber (Department of Immunology, University hospital Tübingen), showing the magnitude of upregulation under the three senescence inducing treatments (Fig 3.15, B). Strikingly, the cell death receptor CD95 displayed a strong expression increase in HepG2 cells for all TIS inducers. However, only a slight upregulation was observed for HUH7 cells. Other mentionable therapeutic markers were CD276 (B7-H3) in HepG2 cells as well as CD340 (Her2) in HUH7 cells. These were specifically investigated further in section 3.5. First however, TIS-associated phenotypic changes were analyzed in the next session.

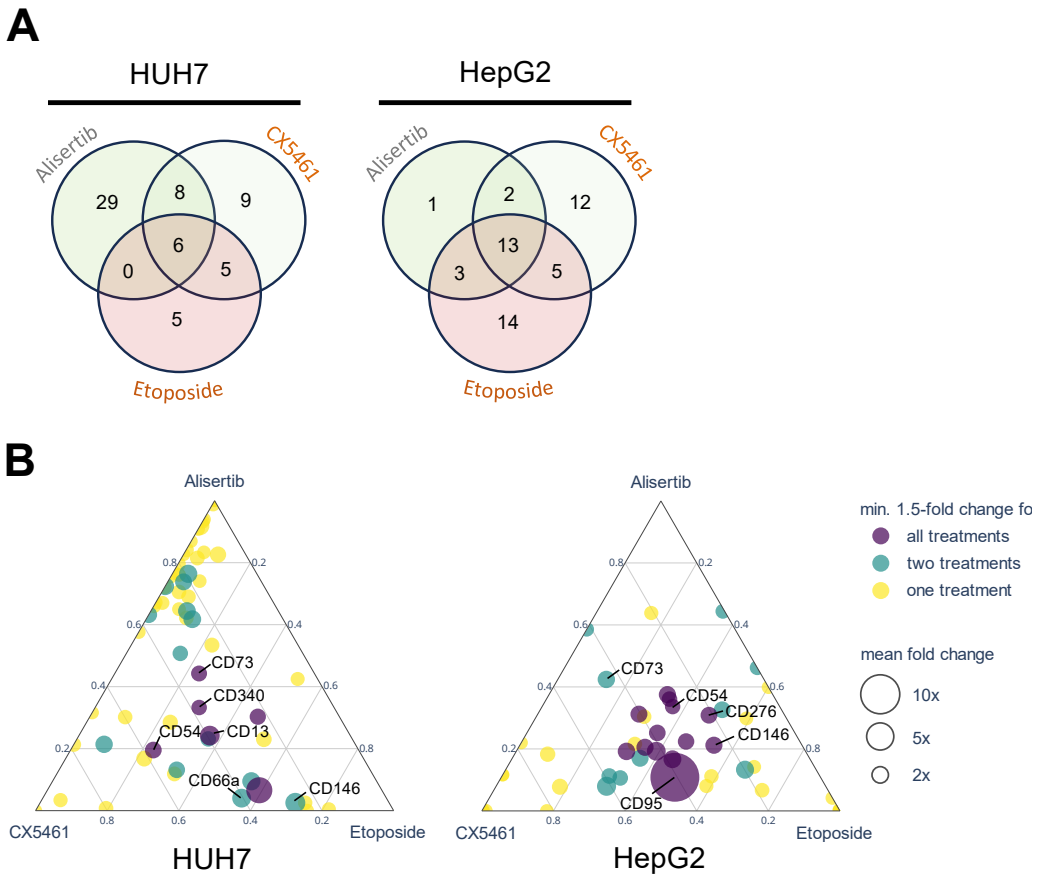

Figure 3.15: **Identification of shared senescence markers.**

(A) Venn diagram indicating upregulated markers, with an inclusion threshold of ≥1.5-fold change under at least one treatment compared to untreated surface expression. **(B)** Ternary plot showing the magnitude of upregulation of the same surface antigens under the three senescence inducing treatments. Marker size is proportional to average fold change, with marker position reflecting the relative contribution of each treatment to said average. Figure generated by Dr. Andras Szolek, a PostDoc of AG Weber, Department of Immunology, University of Tübingen.

3.4.3. HCC TIS induces expression of metastasis-associated surface markers

Besides focusing on markers with therapeutic relevance also surface-associated phenotypic changes that potentially correlate with senescence were investigated. While examining globally upregulated markers, striking expression increases of several known metastasis-associated surface antigens were observed (table 3.1), (Fig. 3.16, A). Notably, ecto-5'-nucleotidase (CD73), recently described to be a novel target for immune checkpoint therapy (Roh et al., 2020) displayed considerable expression increases for all TIS-inducing treatments in both HCC cell lines. CD73 plays an important role in metastasis formation and tumor invasiveness (Zhang, 2012). Besides CD73, also other metastasis-promoting surface antigens exhibited strong expression increases during HCC therapy-induced senescence. For instance, melanoma cell adhesion molecule (CD146) that has been demonstrated to induce HCC

metastasis by promoting epithelial-mesenchymal transition (Jiang et al., 2016). Furthermore, the previously described cell adhesion marker ICAM-1/CD54 is a crucial regulator during different stages of metastasis formation (Benedicto et al., 2017). Another metastasis-promoting cell adhesion molecule is CD66a that was recently described as a novel therapeutic cancer target (Dankner et al., 2017; Wicklein et al., 2018). One of the most thoroughly characterized proteins associated to metastasis formation is the cell-cell adhesion protein E-cadherin. Its transcriptional downregulation is associated to a strong increase in metastatic capacities of tumor cells due to weakened cell-cell contacts (Na et al., 2020; Onder et al., 2008). Interestingly, HUH7 cells displayed a strong decrease in E-cadherin expression indicating that the induced metastatic profile is also mediated by changes in cell adhesion capacities. Finally, the alanyl aminopeptidase CD13 displayed a strong upregulation upon TIS induction in both HCC cell lines. CD13 has been associated to metastasis formation in human melanoma cells via degradation of ECM (Fujii et al., 1995). Although this has yet to be confirmed for HCC, CD13 has been demonstrated to drive tumor reoccurrence and negatively affect survival of HCC patients (Yamanaka et al., 2018).

Table 3.1: **Metastasis-associated surface markers that showed differential expression during HCC therapy-induced senescence.**

Name	Fold-change (given in x-fold expression difference)		Role in metastasis	Reference
	HUH7	HepG2		
CD73	Alisertib: 1.95 CX5461: 1.69 Etoposide: 1.51	Alisertib: 2.32 CX5461: 2.37 Etoposide: 1.42	Contributes to metastasis by inducing cell migration and invasion.	(Roh et al., 2020; Zhang, 2012)
CD146	Alisertib: 1.12 CX5461: 2.32 Etoposide: 4.59	Alisertib: 1.63 CX5461: 1.73 Etoposide: 2.60	Induces HCC metastasis by promoting EMT.	(Jiang et al., 2016; Wang et al., 2020)
CD54	Alisertib: 1.52 CX5461: 2.53 Etoposide: 1.62	Alisertib: 1.61 CX5461: 1.54 Etoposide: 1.66	Regulates cell migration during various stages of metastasis.	(Benedicto et al., 2017)
CD66a	Alisertib: 1.17 CX5461: 2.69 Etoposide: 3.31	Alisertib: 1.26 CX5461: 1.10 Etoposide: 1.52	Cell adhesion marker that promotes metastasis by regulating EMT.	(Dankner et al., 2017; Wicklein et al., 2018)

E-cadherin	Alisertib: 0.29 CX5461: 0.40 Etoposide: 0.36	Alisertib: 0.97 CX5461: 1.24 Etoposide: 1.12	Cell adhesion marker that is an essential regulator of cell migration.	(Na et al., 2020; Onder et al., 2008)
CD13	Alisertib: 2.18 CX5461: 2.90 Etoposide: 2.78	Alisertib: 1.69 CX5461: 1.62 Etoposide: 1.17	Important for degradation of ECM.	(Fujii et al., 1995; Yamanaka et al., 2018)

To confirm the correlation between HCC therapy-induced senescence and expression of metastasis-associated surface markers a separate flow cytometry experiment was conducted. Therefore, stainings for the abovementioned markers were combined with a functional SA-β-Gal stain (Fig. 3.16, B), so that upregulation could be measured simultaneously with senescence properties. Different senescence timepoints were investigated in order to determine if extended senescence treatments coincided with enhance expression of the examined markers. Notably, isotype control stainings of TIS-inducer treated cells displayed an increased signal intensity, arising from the strong cell enlargement induced by cellular senescence. Nevertheless, expression of all investigated markers increased considerably beyond changes in isotype. Additionally, CD73, CD66a and CD54 showed a substantial expression increase at later senescence timepoints. As expected, CD95 expression was exceedingly elevated upon TIS induction in HepG2 cells making it a suitable target for ligand-mediated clearance of senescent HepG2 cells. Single cell correlation analysis of CD95 and SA-β-Gal will be addressed in section 3.5.2.

Conclusively, this data suggests that the heterogeneity of HCC therapy-induced senescence is not only observed for soluble factors but also at the level of surface antigen expression. These distinct expression patterns hamper development of novel senescence-targeting therapies. Characterizing novel therapeutic surface antigens associated to senescence could give rise to new treatment approaches for efficient elimination of highly inflammatory and metastatic senescent tumor cells. Hence, actionable targets of HCC therapy-induced senescence were investigated next.

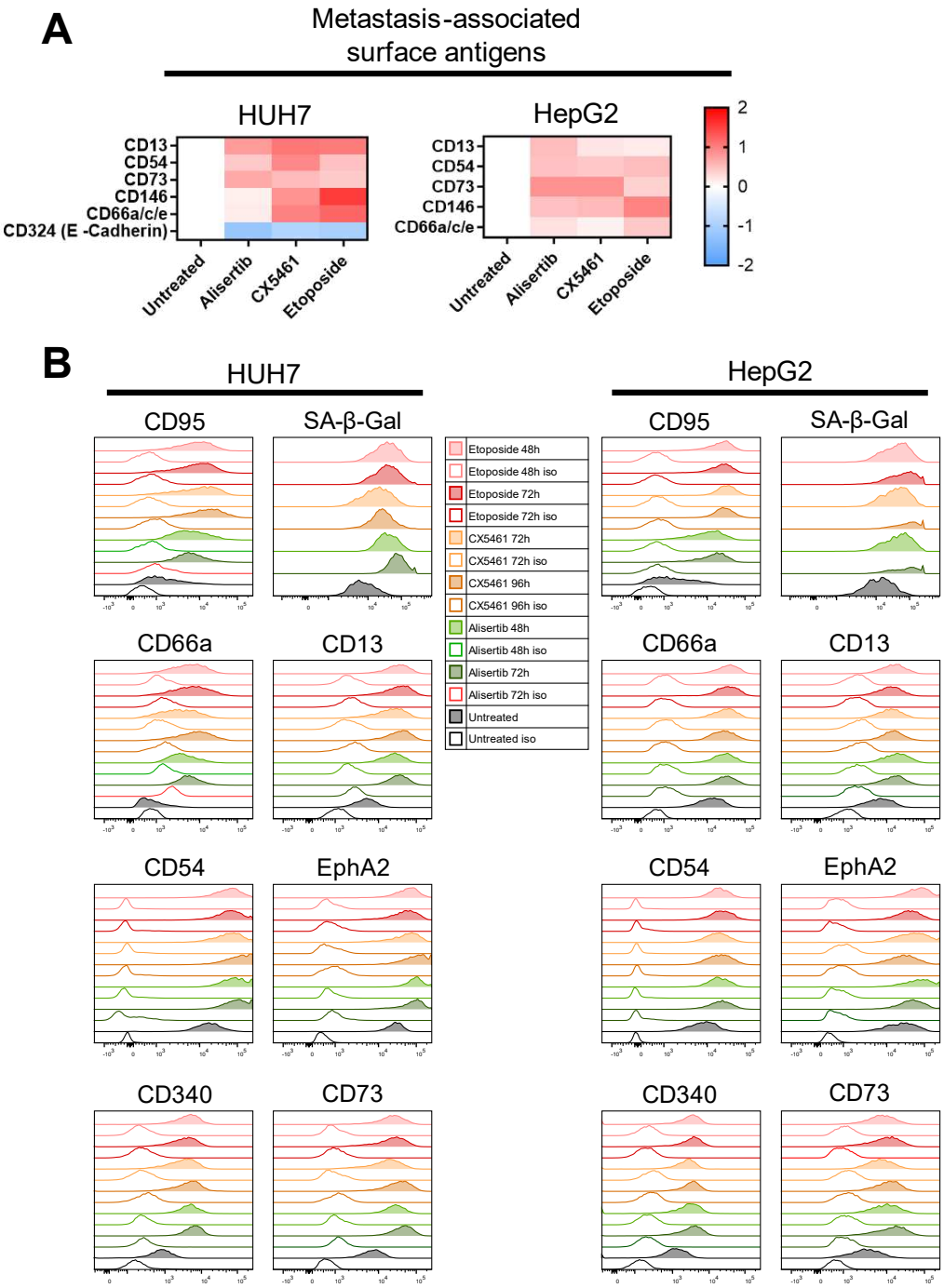

Figure 3.16: **HCC senescence induces a metastasis-associated surfaceome.**

(A) Expression of metastasis-associated surface antigens upon TIS induction in HCC cell lines. Expression was determined by LegendScreen analysis (see 3.13 and 3.14) **(B)** Validation of relevant therapeutic and metastasis-associated markers in separate flow-cytometry screening. Additionally, a functional SA-β-Gal staining was performed using the CellEvent senescence kit (n=3 biological replicates, representative data)

3.5. Actionable targets of HCC therapy-induced senescence

3.5.1. Immunoblotting of TIS-regulated targets CD95 and CD340

As previously mentioned, senescent cells exhibit strong morphological changes manifested by a considerable cell size increase and an enlarged cell surface. In order to confirm that the obtained expression profiles arise from modulated protein levels rather than a cell size increase, Bradford analysis and a normalization of total protein content with subsequent immunoblotting of CD95 and CD340 were performed (Fig. 3.17). While the cell death receptor CD95 displayed the most promising expression increase for HepG2 cells, the epidermal growth factor receptor CD340 presented a favorable target in HUH7 cells. As expected, immunoblotting confirmed that CD95 expression was elevated upon senescence induction in HepG2 cell. Furthermore, HUH7 cells displayed a strong CD340 expression increase after treatment with TIS-inducers, especially for CX5461.

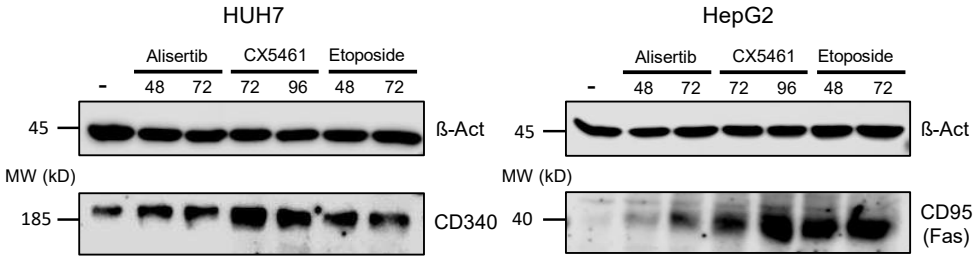

Figure 3.17: Immunoblot analysis of therapeutic, senescence-associated surface antigens.

Immunoblots of CD340 in HUH7 cells and CD95 in HepG2 cells were performed at different senescence timepoints. As a loading control, the housekeeper protein, β-actin was investigated (n=3 biological replicates, representative data). Alisertib and Etoposide treatments were conducted for 48/72 hours. CX5461 was added for 24 hours, followed by 48/72 h recovery in fresh cell culture media.

3.5.2. Senescence-associated surface markers correlate with SA-β-Gal expression

In order to investigate the direct association between surface antigen expression and the senescence phenotype a single cell correlation analysis between SA-β-Gal and therapeutic senescence markers was performed (Fig. 3.18). While 5.56% of untreated HepG2 cells displayed positivity for SA-β-Gal and CD95, 53.5% of alisertib-, 63% of CX5461-, and 60.4% of etoposide-treated cells were positive for both markers respectively. Furthermore, HUH7 cells showed a simultaneous expression of CD340 and SA-β-Gal for 6.02% of untreated, 78.3% of alisertib-, 58.5% of CX5461-, and 68.1% of etoposide-treated cells. These findings suggest a strong association between the TIS-associated markers CD95 and CD340 and SA-β-Gal activity, indicated by a reciprocal upregulation.

Taken together, the TIS-mediated upregulation of CD95 and CD340 was validated in HCC cell lines. Not only did they display strong expression increases, confirmed by flow-cytometry and immunoblotting, but their elevated expression also correlated with an increased SA-β-Gal activity, indicating an association with the senescence phenotype. To investigate if TIS-associated markers are therapeutically actionable, several targeting approaches were tested next.

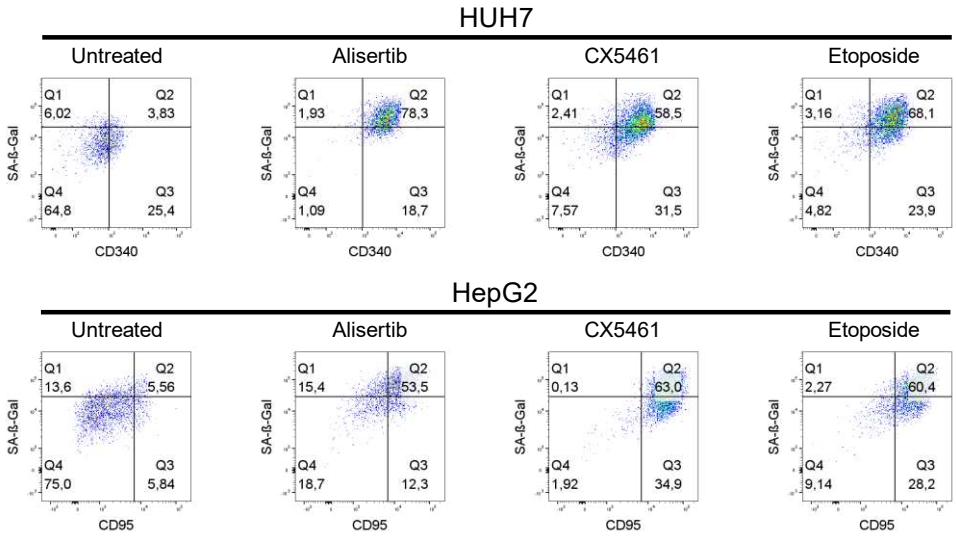

Figure 3.18: **Correlation analysis between SA-β-Gal activity and senescence marker expression.**

Correlation analysis was performed for CD95 expression in HepG2 cells and CD340 expression in HUH7 cells. CD95 and CD340 stainings were combined with a functional SA-β-Gal staining and measured by flow cytometry (n=3 biological replicates, representative data).

3.6. Targeting senescence-associated antigens in HCC therapy-induced senescence *in vitro*

3.6.1. Soluble Fas ligand induces apoptosis response in senescent HepG2 cells

As previously described, CD95 and CD340 were identified as novel therapeutic senescence markers for HCC therapy-induced senescence. The cell death receptor CD95 or Fas is a well characterized regulator of programmed cell death, namely apoptosis (Peter et al., 2015). Upon ligand binding a caspase-8-mediated signaling cascade is initiated that culminates in apoptotic cell death. Our findings indicate that therapy-induced senescence can induce a considerable expression increase of CD95 in HepG2 cells, suggesting that CD95 could be an ideal target for ligand-mediated clearance of senescent HepG2 cells. Conversely, HUH7 cells displayed a low CD95 expression and only slight upregulation upon senescence induction. To determine

whether CD95 targeting, in combination with TIS induction presents a way to eliminate senescent HCC cells, *in vitro* assays of CD95-ligand mediated cytotoxicity were generated. For all following experiments described in this study CD95 is referred to its more commonly used name Fas.

In order to investigate correlation between TIS-induced Fas expression and sensitivtiy to ligand-induced apopstosis, senescent HCC cells were treated with 150 ng/ml soluble Fas ligand (sFas-Ligand) for 6 h or 24 h (Fig. 3.19). Subsequently, relative cell death was quantified via Cell Counting Kit 8 (CCK8) assay, which is a widely used assay to determine cell viability and proliferation by assesing their metabolic acitvity. As expected, HUH7 cells did not display a strong sensitivity to sFas-Ligand, indicated by a cell death rate of less than 10% for all conditions. Only CX5461 induced a slightly higher sensitivity (9%, afer 24 h treatment) to sFas-Ligand compared to untreated HUH7 cells (6% after 24 h treatment). Conversely, senescence induction in HepG2 cells considerably increased cell death response to sFas-Ligand, with CX5461 inducing the strongest sensitivity. An increase in cell death from 5% for untreated HepG2 cells to more than 20% for CX5461-treated cells was observed after 24 h treatment with sFas-Ligand. Moreover, alisertib and etoposide enhanced ligand-mediated cytoxicity as well, resulting in 8% and 13% cell death, respectively. Previous studies demonstrated that besides sFas-Ligand, also activating anti-Fas antibody CH11 initiates a strong apoptosis response (Yonehara et al., 1989). Since then, several studies have reported that CH11 can induce apoptosis in different types of cancer (Odoux et al., 2002; Seta et al., 2000; Sun et al., 2000). Hence, antibody-mediated Fas activation was investigated next.

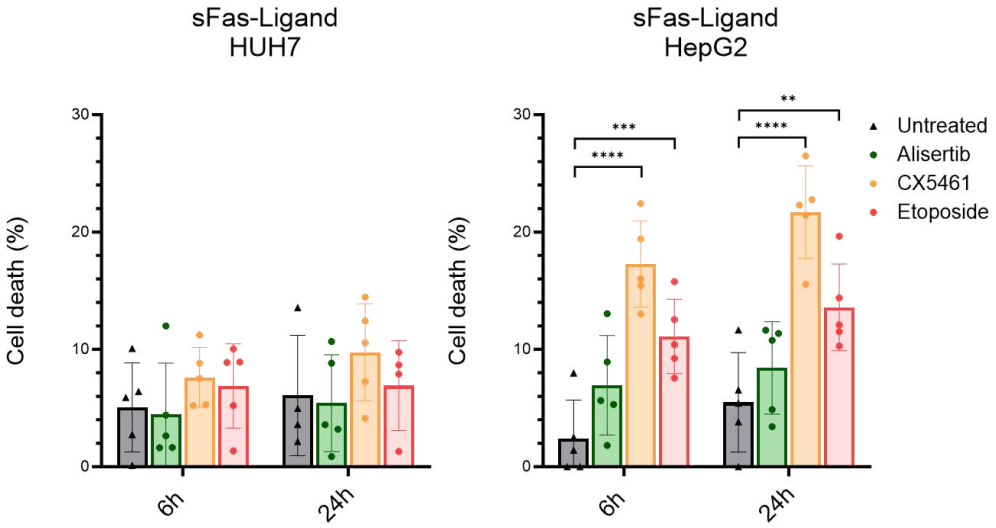

Figure 3.19: **Ligand-mediated Fas targeting in HCC therapy-induced senescence.**

Senescent and non-senescent HCC cell lines were treated with 150 ng/ml soluble Fas ligand for 6 h or 24 h and relative cell death (compared to those of untreated cells) was subsequently determined by CCK8 assay (n=5 biological replicates, combined data, **p<0.01, ***p<0.001 ****p<0.0001 according to two-way ANOVA test).

3.6.2. Activating Fas antibody CH11 initiates caspase-8-mediated apoptosis cascade in senescent HepG2 cells

To investigate antibody-mediated Fas activation in HCC therapy-induced senescence, HepG2 cells were treated with different concentrations of CH11 for 6 h or 24 h and cell death measured via CCK8 assay (Fig. 3.20, A). Strikingly, senescent HepG2 cells displayed a strong, concentration-dependent sensitivity to CH11 with cell death of over 30% for the highest antibody concentration. Conversely, non-senescent HepG2 cells showed a modest apoptosis response, only reaching a maximum of 5% cell death. Compared to ligand-mediated Fas-activation, CH11 induced stronger cell death of alisertib- and etoposide-treated HepG2 cells, demonstrating that antibody-mediated Fas activation is more suitbale for inducing cell death of senescent HCC cells.

As previously mentioned, caspase-8 (casp-8) is an essential mediator of Fas-induced apoptosis. Upon Fas activation, full-lenght casp-8 is cleaved into its mature, enzymatically active form, inititating the apoptosis cascade (Tummers and Green, 2017). To determine wether Fas-mediated casp-8 cleavage varies during HCC therapy-induced senescence, immunoblotting of CH11-treated HepG2 cell lysates was performed (Fig. 3.20, B). Strikingly, strong cleavage was observed in all lysates with TIS induction after 24 h treatment with 100 ng/ml CH11, whereas untreated HepG2 cell lysatess only showed a faint band. Conversely, after 6 h treatment only CX5461- and etoposide-treated HepG2 cells showed casp-8 cleavage. Taken together, senescent HCC cells display an increased sensitivity to ligand- and antibody-mediated Fas activation that results in stronger casp-8 cleavage and elevated apoptosis responses, respectively. Hence, Fas presents a promising target to elimintate senescent HCC cells that carry the risk of inducing a persistent and strongly inflammatory senescence phenotype. Besides Fas, also other theraptically actionable targets were identified and will be adressed in the following section.

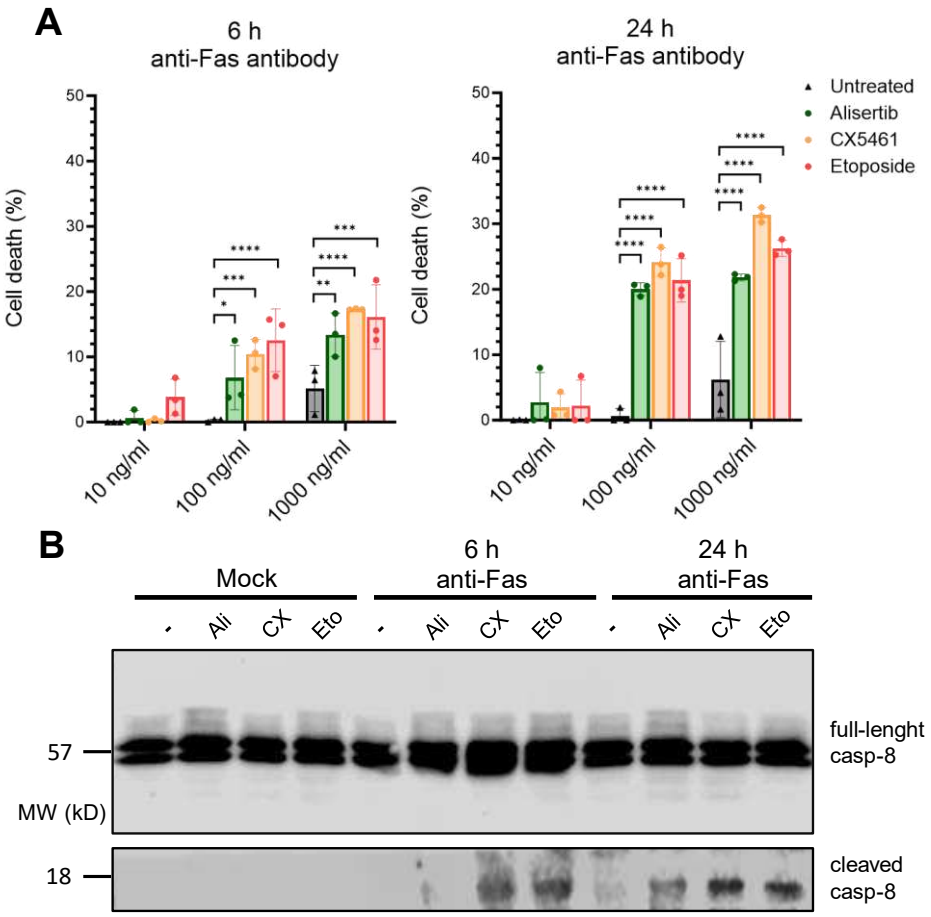

Figure 3.20: **Antibody-mediated Fas targeting in HCC therapy-induced senescence**

(A) Senescent and non-senescent HepG2 cells were treated with different concentrations of anti-Fas antibody CH11 for 6 h or 24 h. Relative cell death (compared to Fas-untreated cells) was determined by CCK8 assay (n=3 biological replicates, combined data, *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001 according to two-way ANOVA test). **(B)** Caspase-8 immunoblot analysis after CH11 treatment. Senescent and non-senescent HepG2 cells were treated with 100 ng/ml CH11 for 6 h or 24 h. Subsequently, lysates were generated, separated by SDS-Page (12% gel) and caspase-8 immunoblot was performed (n=3 biological replicates, representative data).

3.6.3. Anti-CD276 CAR-NK92 cells exert strong cytotoxic effects against senescent HCC cells

Another noticeable therapeutic target associated to HCC senescence was CD276 (also known as B7-H3). Besides having regulatory roles in innate immune cells, CD276 is strongly overexpressed in different types of cancer and has been associated to promote metastasis and tumor growth (Dong et al., 2018; Nygren et al., 2011). In previous years, CD276 has been in the focus of antibody- and cell-based therapies, mostly centering around CAR-T cell therapies (Li et al., 2022). Nevertheless, correlation between CD276 and cellular senescence remains to be determined. Strikingly, our flow cytometric analysis indicated a strong upregulation of CD276 for senescent HepG2 cells (Fig. 3.14). Although HUH7 cells did not display a senescence-

specific upregulation, CD276 is a strongly expressed surface antigen in both investigated HCC cell lines at steady state.

Due to the crucial role of NK cells in the immune-mediated clearance of senescent HCC cells, we sought to explore cytotoxic effects of anti-CD276 CAR-NK92 cells against senescent cells and determine if CAR-NK cell therapy is suitable for clearance of senescent HCC cells via CD276. Of note, using NK92 cell line-based CARs has been explored for different types of cancer (Zhang et al., 2017) and in different phase I clinical trials (Arai et al., 2008; Tonn et al., 2013).. Hence, anti-CD276 CAR-NK92 cells (kindly provided by Guillermo Urena Baillen, a PhD student of AG Mezger, University of Tübingen) were co-cultured with senescent HCC cells for 3 h. To determine cytotoxicity, the target cells were labeled with the fluorescent dye calcein AM which is released upon cell death (Neri et al., 2001). Calcein release was determined by quantification using a microplate reader. As a comparison, conventional NK92 cells lacking a CD276-specific CAR were investigated. Strikingly, anti-CD276 CAR-NK92 cells exerted strong cytotoxic effects against senescent HepG2 cells (Fig 3.21). For all TIS inducers CAR NK92-cell killing was considerably (1.40- to 1.85-fold) elevated compared to conventional NK92 cells. Conversely, clearance of untreated HepG2 cells was similar for CAR and non-CAR NK92 cells, indicating a senescence-specific increase in killing efficiency. Nonetheless, HUH7 cells did not display senescence-associated increases in CAR-mediated clearance, indicated by strong cytotoxic effects against senescent as well as non-senescent HUH7 cells.

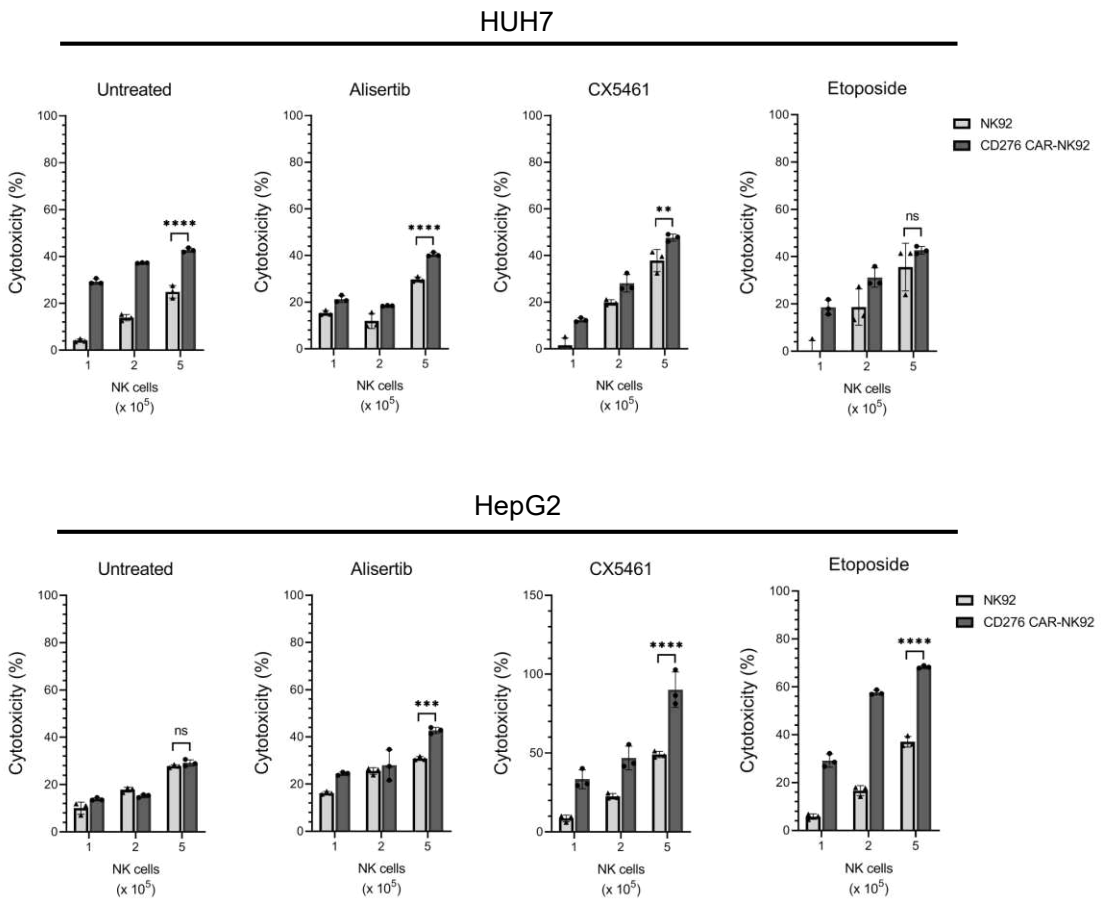

Figure 3.21: **Anti-CD276 CAR-NK92 cells exert strong cytotoxic effects against senescent HCC cells**

(A) Prior to the killing assay, HCC cell lines were labeled with the permeable fluorescent dye Calcein-AM. Anti CD276-CAR NK92 were then co-cultured with senescent or non-senescent HCC cells for 3 h. Cytotoxicity was determined by calcein-AM release assay (n=2 biological replicates, representative data, ns $p \geq 0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$ ****$p<0.0001$ according to two-way ANOVA test).

To investigate if CAR-mediated effects correlate with CD276 expression profiles of senescent HCC cell lines, fold expression changes were compared to CD276 CAR-NK92 cell killing (table 3.2). Notably, etoposide-treated HepG2 cells displayed the strongest increase in CD276 expression (2.3-fold), while also showing the strongest CD276 CAR-NK92 cell response (1.85-fold increase compared to NK92 cells). Similar observations were made for HUH7 cells. Here, alisertib was the only treatment that induced an increase in CD276 expression compared to untreated cells (1.42-fold increase). Notably, alisertib also displayed the strongest increase in killing when comparing CD276 CAR-NK92 cells to NK92 cells (1.35-fold increase). Strikingly, these findings indicate that CD276 expression correlates with CD276 CAR-NK92 responses. Hence, testing other actionable markers for CAR-mediated targeting presents a promising approach to further investigate therapeutic opportunities arising from HCC therapy-induced senescence.

Table 3.2: Comparison of CD276 fold expression changes and CD276 CAR-NK92 killing.

Cell line	CD276 fold expression changes (given in x-fold expression difference)	CAR-mediated fold increase in killing efficiency (CD276 CAR-NK92 vs. NK92)
HUH7	Alisertib: 1.43 CX5461: 0.82 Etoposide: 0.91	Alisertib: 1.35 CX5461: 1.26 Etoposide: 1.20
HepG2	Alisertib: 1.83 CX5461: 1.57 Etoposide: 2.30	Alisertib: 1.40 CX5461: 1.79 Etoposide: 1.85

Conclusively, CD276 presents a promising target for CAR-NK cell clearance of senescent HCC cells. Although TIS-mediated increases in CD276 expression varied among cell lines, anti-CD276 CAR-NK92 cells exerted strong cytotoxic effects against both investigated HCC cell lines. While elevated killing efficiency was observed against senescent HepG2 cells, increased clearance of HUH7 cells was independent of senescence.

In summary, the results presented in this study led to the identification of novel actionable targets of HCC therapy-induced senescence. Furthermore, several senescence-associated mechanisms of HCC TIS were characterized and a comprehensive overview of SASP heterogeneity, innate immune responses, and senescence-associated surfaceome changes was given. The respective findings led to the identification of novel actionable targets of HCC therapy-induced senescence, namely Fas, CD340 and CD276. Furthermore, therapeutic applicability was confirmed *in vitro* for Fas and CD276. While antibody-mediated Fas activation caused apoptosis of senescent HepG2 cells, CD276 CAR-NK92 cells exerted strong cytotoxic effects against senescent HCC cells. Furthermore, experiments on targeting of CD340 using the clinically approved therapeutic antibody trastuzumab are currently ongoing and will determine if this marker can be utilized as another actionable target of HCC therapy-induced senescence.

4. Discussion

In this study novel features of HCC therapy-induced senescence were identified that highlighted new senescence-associated phenotypes and gave rise to potential therapeutic opportunities (Fig. 4.1). These results will here be discussed together with the many open questions that remain regarding pathophysiological relevance of SASP and surfaceome heterogeneity, metastatic capacities of senescent HCC cells, and therapeutic applicability of TIS-associated markers. Moreover, limitations and opportunities arising from this study will be summarized and discussed.

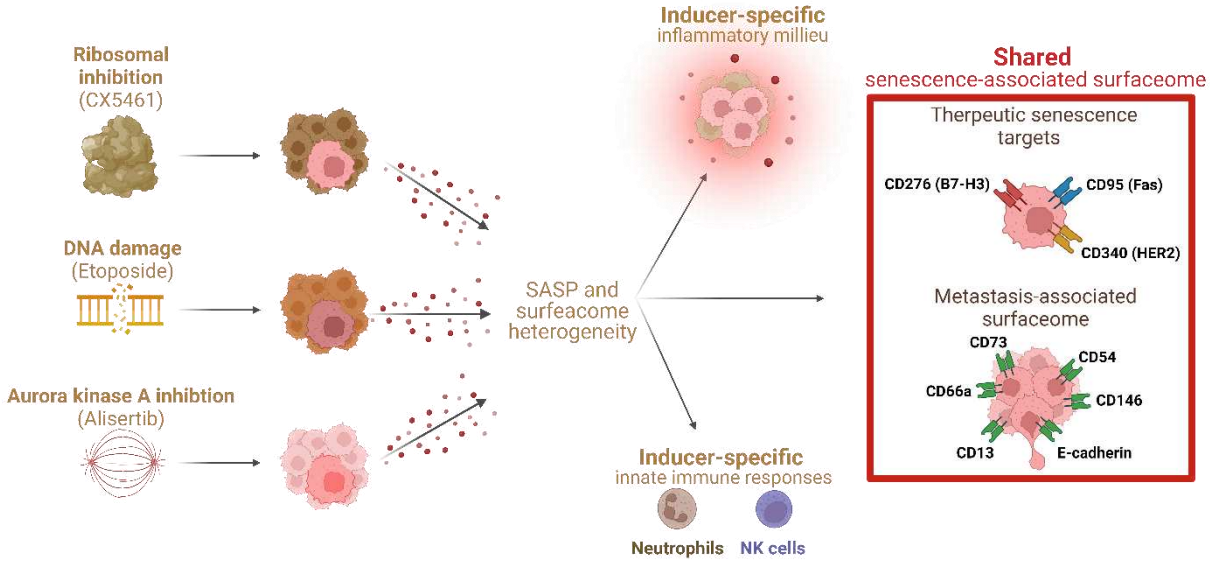

Figure 4.1: **Novel senescence-associated mechanisms and potential therapeutic opportunitites identified in this study.**

Different inducers lead to inducer-specific intrinsic (SASP/surfaceome) and extrinsic (innate immune responses) heterogeneity of HCC therapy-induced senescence. General effects shared by multiple inducer regimens are metastasis-associated changes in the surfaceome as well as overexpression of actionable surface antigens, the targeting of which was explored here and might lead to new approaches in exploiting TIS.

4.1. Observed TIS-induced heterogeneity as a challenge for therapeutic applicability

Our findings reveal that HCC therapy-induced senescence exhibits an unexpected level of treatment- and cell-line specific heterogeneity regarding selected secretory factors, innate signaling pathways and the expression of surface antigens. While the phenomenon of senescence heterogeneity has recently gained recognition in the field of cellular senescence (Cohn et al., 2023; Kirschner et al., 2020; Sun, 2023), we show explicit layers of heterogeneity

(table 4.1) which have implications for senescence surveillance, pathophysiological mechanisms, and therapeutic targeting. Given the critical role that senescence plays in both, the pathogenesis and treatment of HCC, the observed heterogeneity may also impact on and be reflected at the heterogeneity observed for clinical responses and differential treatment success in HCC therapy. Hence, aspects of TIS heterogeneity arising from this study and their respective phenotypic and pathophysiological consequences are summarized in table 4.1 and will be characterized and discussed in the following.

Table 4.1: Aspects of TIS heterogeneity and respective phenotypic effects reported in the literature and observed in this study.

Aspects of TIS heterogeneity	Phenotypic effects and physiological consequences
Inducer-dependent heterogeneity	SASP, surfaceome and innate signaling (intrinsic)
	NK cell and neutrophil-mediated senescence surveillance (extrinsic)
Cell type-specific heterogeneity	Cell-specific senescence initiation (p16/pRb vs. p21/p53), SASP composition Surfaceome changes
Clinical and pathophysiological impact	Response to therapy
	Patient survival
	TIS-mediated prognostic factors

4.1.1. Inducer-related heterogeneity of HCC TIS

Heterogeneity of HCC therapy-induced senescence related to different TIS inducer pathways (e.g. alisertib vs. etoposide vs. CX5461) was observed for

- Secretory factors (SASP composition)
- Innate immune signaling (cGAS-STING activation/inhibition)
- Surface antigen expression and respective senescence-associated changes
- NK cell and neutrophil-mediated senescence surveillance

Furthermore, cell type-specific differences between HCC cell lines (HUH7 vs. HepG2) were observed for surfaceome changes, including actionable targets like Fas, CD340, and CD276, which will be addressed in section 4.1.2.

Inducer-dependent heterogeneity can result from differences in intensity and magnitude of senescence inducing stimuli. Notably, magnitude of cellular senescence has shown to strongly depend on the level of senescence-associated pathway activation (Kirschner et al., 2020). Recent studies have indicated that biomarkers of senescence display a graded rather than a binary profile, indicating that senescence magnitude can be quantified by biomarker intensity (Ashraf et al., 2023). Hence, to prevent magnitude-dependent differences between senescence inducers various senescence biomarkers were investigated for their expression and activity. Whereas the level of SA-beta-gal and $p16^{INK4a}$/$p21^{WAF/CIP1}$ as a major hallmark of senescence were comparable between TIS-inducers other factors diverged. For example, strong inducer-dependent differences were observed for SASP composition. Notably, CX5461 induced the strongest IL-8 and CXCL10 secretion in HUH7 and HepG2 cells, while alisertib was the strongest VEGF-A inducer. SASP heterogeneity is a well described phenomenon and has been extensively studied in previous years (Giroud et al., 2023; Hernandez-Segura et al., 2017; Kirschner et al., 2020; Sun, 2023). Hence, we aimed to also focus on the impact of TIS heterogeneity on surfaceome profiles and senescence surveillance.

Besides inducer-dependent differences in SASP composition, also distinct surfaceome profiles were observed between senescence inducers. Although senescence research has mainly focused on secretome-associated processes in past year (SASP characterization and targeting), surfaceome changes have recently gained importance due to the discovery of clinically actionable targets (Amor et al., 2020; Rossi and Abdelmohsen, 2021). However, strong heterogeneity of surface antigen expression limits standardization of senescence targeting approaches due to the lack of shared senescence markers. A recently developed data base of gene expression profiles, the so called cancer SENESCopedia, can be queried regarding TIS-associated changes in membrane proteins (Jochems et al., 2021). This data base comprises a transcriptome archive of 13 cancer cell lines treated with different TIS inducers, namely alisertib and etoposide. These transcriptomic changes are informative but since the analysis is mRNA based, it does not directly correlate to protein, let alone, surface expression. The LegendScreen analysis thus provides the advantage of allowing for a comprehensive characterization of the actual surface protein expression of various, clinically relevant markers that can also be performed for a broader spectrum of senescence inducing stimuli and cancer types. Being antibody based, further validation is straightforward.

Not only SASP composition and surfaceome profiles displayed heterogeneities between TIS inducer, but also extrinsic processes such as innate immune recognition and senescence surveillance showed strong treatment-specific differences. Several studies have indicated that NK cell-mediated senescence surveillance highly depends on NK cell activating ligands expressed on the surface of senescent cells (Antonangeli et al., 2019; Ruscetti et al., 2018; Soriani et al., 2009). This strong interconnection between intrinsic surfaceome modulation and innate immune surveillance has been demonstrated for various models of therapy-induced senescence (Antonangeli et al., 2019; Ruscetti et al., 2018; Soriani et al., 2009). In a lung cancer mouse model of MEK and CDK4/6 inhibitor-induced TIS, a strong upregulation of NK cell activating ligands MICA/B, ULBP2/5/6 and ICAM-1 was observed. However, expression of these surface antigens differed, when only CDK4/6 inhibitors were used, emphasizing on the inducer-dependent heterogeneity (Ruscetti et al., 2018). Similar results were obtained in a model of DNA-damage mediated TIS in multiple myeloma, where distinct expression profiles of MICA/B were observed for the two cytostatic drugs doxorubicin and melphalan (Soriani et al., 2009). These findings indicate that expression of NK cell activating surface antigens strongly depends on the underlying senescence initiation mechanism. Strikingly, this was also observed for our models of HCC therapy-induced senescence. Despite of a general upregulation, expression levels of ICAM-1 and HLA-E varied between TIS agonists, with CX5461 inducing the strongest expression increase (Fig. 3.11). Additionally, small expression differences of MICA/B were observed between TIS inducers in HUH7 cells (Fig. 3.13 and 3.14). Besides surface antigens also distinct SASP factors have shown to promote innate immune-mediated clearance of senescent cells (Antonangeli et al., 2019; Ruscetti et al., 2018; Soriani et al., 2009). The role of the SASP in senescence surveillance has been characterized in more detail in the previously described model of lunger cancer TIS by transfecting tumor cells with a NF-κB targeting short hairpin RNA (shRNA). Despite of a stable proliferation arrest tumor cells exhibited a strong reduction in many SASP factors that resulted in decreased NK cell activation. Strikingly, antibody-mediated blocking of single SASP factors showed that this decrease is due to an interplay of multiple SASP factors rather than the effect of a single one (Ruscetti et al., 2018). This emphasizes on the detrimental role of the SASP for immune-mediated clearance of senescent cells and thus, reveals that SASP heterogeneity has immense impacts on anti-tumor immune responses and may drive extrinsic senescence heterogeneity. Besides NK cell activation, also neutrophil responses displayed strong heterogeneities between senescence

inducers. Notably, the neutrophil recruiting chemokine IL-8 was strongly secreted by CX5461 and etoposide-treated cells, which also reflected on respective neutrophil migration (transwell migration assay, Fig. 3.8, B) and activation (MPO and MMP9 secretion, Fig. 3.8, C). Combining the previously described literature with our own investigations, reveals that inducer-dependent SASP and surfaceome heterogeneity cause differences in downstream innate immune recognition and activation.

To utilize the inflammatory potential of HCC therapy-induced senescence, its complexity has to be further characterized on the level of intrinsic factors (SASP and surfaceome) as well as extrinsic factors (innate immune response). Not only TIS-inducer-specificity but also cell line- and patient-specificity have to be investigated more thoroughly to guarantee safety of senescence-inducing anti-cancer drugs and promote the development of novel senescence-inducing compounds. These findings reveal that the complexity of HCC therapy-induced senescence may also influence clinical outcome and patient survival, which will be addressed in the following section.

4.1.2. Heterogeneity of HCC TIS may affect clinical outcome and patient survival

Several studies have utilized cellular senescence markers for HCC prognosis and identified a subset of senescence-associated genes that proved to be robust indicators for therapeutic responses in HCC (Sun et al., 2022; Tang et al., 2022; Xiang et al., 2019). However, all of these studies have exclusively investigated untreated tumor settings and none of them has taken into account senescence induced by therapeutic intervention, namely TIS.

Inducer-dependent heterogeneities, as shown for our cellular models of HCC therapy-induced senescence, may cause strong differences in immune-mediated clearance, tumor promoting effects and ultimately, patient survival. For instance, IL-8, which displayed notable inducer-dependent heterogeneity in HCC TIS, has shown to correlate with tumor size and stage, as indicated by IL-8 serum ELISAs from HCC patients (Ren et al., 2003). Additionally, IL-8 and IL-6 have shown to predict sorafenib treatment outcomes and patient survival (Öcal et al., 2022). Other SASP factors that exhibited strong inducer-dependent heterogeneities were VEGF-A and CXCL10. While VEGF-A has been suggested as a prognostic marker in HCC (Qi et al., 2023), CXLC10 has shown to modulate the HCC tumor microenvironment (Brandt et al., 2022). These findings demonstrate that different TIS inducers can give rise to distinct pathophysiological outcomes, thereby impacting tumor development and patient survival.

Additionally, senescence responses in HCC can be strongly influenced by disease stage and timing of TIS inducing treatments. In most cases, HCC develops due to a cascade of constitutive liver damage that starts with initial liver fibrosis and progresses through cirrhosis to the development of HCC. Notably, senescent hepatic stellate cells (HSCs) have shown to limit fibrosis and cirrhosis by inhibiting fibrotic scarring, thereby delaying HCC development (Krizhanovsky et al., 2008). Conversely, other studies indicated that senescent HSCs promote rather than block tumor growth in a setting of already developed HCC, by induction of a highly inflammatory and tumor promoting SASP (Cai et al., 2022). These findings indicate that not only the type of senescence inducing mechanism, but also the timing of senescence induction plays an essential role for pathophysiological outcomes of HCC senescence.

Furthermore, senescence response may vary depending on their p53 mutational status, as shown for HUH7 and HepG2 cells. This may also influence clinical outcome of senescence inducing therapies. Cell type-dependent differences were investigated for the expression of the actionable, TIS-associated targets Fas, CD340 and CD276. Although these therapeutically relevant surface antigens showed a general upregulation for all TIS inducers, strong differences were observed between HCC cell lines. While Fas and CD276 displayed a shared, TIS-associated upregulation in HepG2 cells, CD340 expression was exclusively elevated in senescent HUH7 cells. Hence, surfaceome-based therapeutic strategies may be hindered by heterogeneities among HCC cells, impeding standardized targeting approaches. To further explore this cell type-dependent heterogeneity, transcriptome data from 24 HCC cell lines in the Human Protein Atlas were investigated and compared for expression of the previously described actionable surface antigens CD340, Fas and CD276 (Fig. 4.2). Notably, HUH7 cells exhibited very low expression of Fas, while HepG2 cells were among the most highly Fas-expressing HCC cell lines. This difference in basal expression may explain why Fas is strongly upregulated upon senescence induction in HepG2 cells but not in HUH7 cells. Nevertheless, although CD340 and CD276 displayed moderate basal expression in both cell lines, senescence-associated upregulation was only observed for one of the cell lines for both markers (CD276 in HepG2 cells and CD340 in HUH7 cells), indicating that senescence-associated increases do not always correlate with respective basal expression. Conclusively, the Human Protein Atlas data provide an impression of heterogeneity in expression levels that may also be observed for primary tumors/patients. This cautions against quick extrapolation from one cell line to HCC in general.

Based on the TIS-associated expression changes of Fas, CD340 and CD276 several targeting approaches were conducted, which will be further addressed in section 6.3.

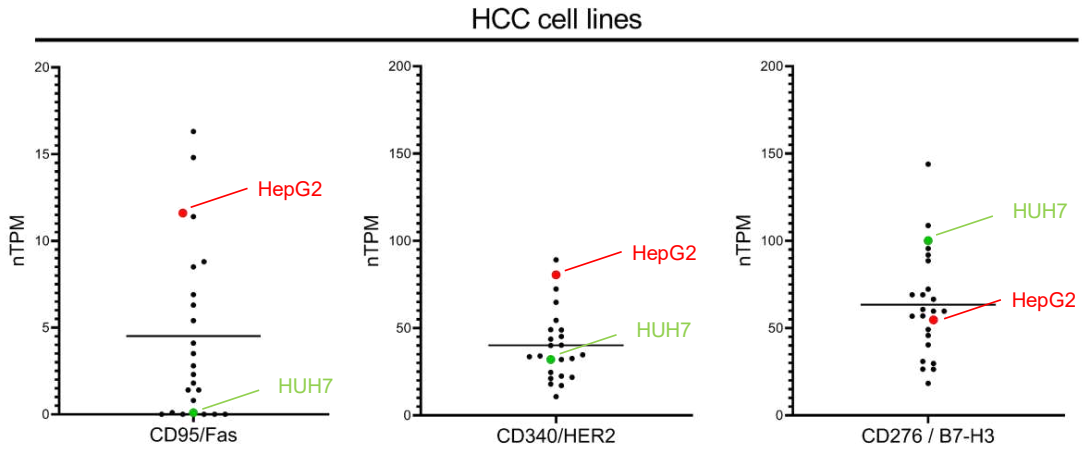

Figure 4.2: **Expression of Fas, CD340 and CD276 in different human HCC cell lines.**

Data was obtained from publicly available RNAseq datasets derived from "The Human Protein Atlas".

Combining the previously described literature with our own investigations reveals that differences in senescence inducing mechanisms, timing of senescence induction as well as cell-type dependent differences cause strong senescence heterogeneities that may impact senescence surveillance, clinical outcomes, HCC patient survival, and expression of actionable targets. Prior to the implementation of new senescence-inducing therapies extensive testing has to be conducted to investigate the associated senescence profiles as well as their respective pathological properties. Especially effects shared across different TIS regimens and cell lines may be valuable to follow up. One shared TIS-associated phenotype that was observed for all TIS inducers as well as both HCC cell line was a metastasis-promoting surfaceome, which will be addressed in the following.

4.2. Metastasis as a shared, adverse feature of HCC therapy-induced senescence

4.2.1. Metastasis-associated surfaceome and ECM degradation

Surface antigens play an essential role during various stages of metastasis formation. Besides facilitating the adhesion to stromal cells during metastatic dissemination, they are also crucial for invasion into the blood stream and directing organ specific homing (Brooks et al., 2010; Karhemo et al., 2012). Recent studies have indicated that doxorubicin can promote metastasis in a murine breast cancer model by induction of senescence in tumor and surrounding healthy

cells (Demaria et al., 2017). Nevertheless, the underlying effects behind increased metastatic capacities were not elucidated. Other studies have demonstrated that therapy-induced senescence can increase tumor invasiveness by induction of a metastasis promoting SASP (Ohanna et al., 2011; Schmitt et al., 2022). However, surfaceome-mediated metastatic effects have not been described for therapy-induced senescence.

As previously addressed, LegendScreen analysis revealed strong heterogeneities in surface antigen expression between TIS inducers and HCC cell lines. Nevertheless, several surface antigens displayed a shared upregulation for all TIS inducing regiments, including CD73, CD66a, CD146, CD54 and CD13. Notably, expression of these membrane proteins is associated with increased metastatic capacities and elevated tumor invasiveness (Benedicto et al., 2017; Fujii et al., 1995; Jiang et al., 2016; Wicklein et al., 2018; Zhang, 2012). Furthermore, the cell-cell adhesion protein E-cadherin exhibited strong downregulation in senescent HUH7 cells, what is a predisposing factor for metastasis formation (Na et al., 2020; Onder et al., 2008). These expression changes may indicate that therapy-induced senescence promote tumor invasiveness and migratory capacities of HCC cells. Hence, characterization of the surfaceome for a broader spectrum of senescence inducing stimuli and cancer types, may uncover other novel senescence-associated pathological features.

In addition to surfaceome-associated metastatic features, degradation, and remodeling of the extracellular matrix (ECM) plays an essential role in metastasis formation. Notably, a co-culture assay of primary neutrophils with senescent HCC cells revealed strong activation of neutrophils resulting in an elevated secretion of MMP9. Strikingly, MMP9 has shown to be a pivotal player in ECM degradation, thereby promoting metastasis (Jabłońska-Trypuć et al., 2016). This finding suggests that metastatic features of HCC therapy-induced senescence are not only mediated by senescent cells themselves, but also indirectly by activation of immune cells such as infiltrating neutrophils that secrete the ECM degrading metalloproteinase MMP9. Besides surfaceome- and neutrophil-mediated metastatic effects, also the SASP has shown to promote migratory capacities of tumor cells, what will be addressed in the following.

4.2.2. Metastasis and angiogenesis promoting SASP

HCC therapy-induced senescence gives rise to a strongly inflammatory SASP that mediates a complex immune cascade. Despite of strong heterogeneities, as described in previous sections, our data indicates that alisertib, CX5461 and etoposide induce secretion of IL-8 in

both investigated HCC cell lines. IL-8 is associated with being a potent immune cell attractant and essential mediator of neutrophil migration and activation. However, studies have suggested that its secretion can also promote HCC metastasis and is associated with poor clinical prognosis (Yu et al., 2013). Additionally, IL-8 has been shown to favor epithelial-mesenchymal transition in prostate cancer (Araki et al., 2007) as well as melanoma (Rofstad and Halsør, 2000). In the latter, neutralizing IL-8 antibodies displayed strong anti-metastatic effects and inhibited tumor progression (Huang et al., 2002). Besides IL-8 also VEGF-A was strongly secreted by TIS-inducer treated HCC cell lines. VEGF-A has strong angiogenic functions and promotes metastatic dissemination of cancer cells as well as growth of metastatic tumors (Yang and Cao, 2022). Notably, commonly used first-line and second-line therapeutics against HCC (e.g. Levatinib or Sorafenib) act on VEGF receptors and induce strong anti-angiogenic functions impeding metastasis formation (Gallage et al., 2021). These findings suggest that the HCC-SASP may give rise to a strongly pro-tumorigenic environment that favors metastasis formation and tumor progression. This indicates that metastatic features of senescent HCC cells are not only mediated by a modulated surfaceome but also by its metastasis and angiogenesis promoting SASP. Although HCC therapy-induced senescence presents a promising therapeutic intervention to induce proliferation arrest and make tumor cells more susceptible to immune-mediated clearance, increased metastatic capacities of senescent HCC cells have to be considered. Hence, a second-line treatment, e.g combining TIS with senolytics such as navitoclax or a combination of dasatinib and querticin could be a promising strategy to overcome non-beneficial features of HCC therapy-induced senescence. Strikingly, elimination of senescent cells by administration of navitoclax has shown to result in a substantially lower metastasis formation rate and delayed tumor reoccurrence in breast cancer (Demaria et al., 2017).

Altogether, our findings reveal that HCC therapy-induced senescence may promote pro-tumorigenic features what should be considered for TIS-driven approaches. On the other hand, TIS generates therapeutic opportunities, that might even be uncoupled from TIS-mediated tumor promoting effects, if its regulatory pathways are better understood. The induced targeting opportunities observed here are discussed in the following section.

4.3. Potential targeting opportunities induced by HCC TIS

Several targeting opportunities arising from HCC therapy-induced senescence were identified in this study. Targeting approaches include

1) Circumventing apoptosis resistance by re-instatement of death receptor pathways
2) Targeting HER2 for antibody-dependent cell-mediated cytotoxicity (ADCC)
3) CAR-NK cells as senolytic effectors
4) Senolytic bispecific antibodies

These approaches will be addressed and discussed in following sections.

4.3.1. Circumventing apoptosis resistance of senescent HCC cells by re-instatement of death receptor pathways via TIS

One main characteristic of senescent cells is their resistance to intrinsically regulated cell death, namely apoptosis. Besides SASP initiation and induction of a stable cell cycle arrest, senescent cells display an anti-apoptotic phenotype that is mainly regulated by Bcl-2 proteins (Hu et al., 2022). Despite their immune activating functions senescent cells can accumulate in tumors causing a persistent, chronically inflamed phenotype that promotes tumor growth and metastasis. Hence, finding ways to circumvent apoptosis resistance and make senescent cells susceptible to apoptosis-inducing compounds presents a promising strategy to eliminate senescent cancer cells. One way of inducing programmed cell death is by activation of distinct death receptors such as TRAIL-R1 (DR4), TRAIL-R2 (DR5) or Fas. However, only few studies have characterized the correlation between therapy-induced senescence and expression of death receptors (Crescenzi et al., 2011; Eren et al., 2021; Soto-Gamez et al., 2022), revealing a lack of systematic studies on cell death responses in HCC therapy-induced senescence.

Our findings suggest that expression of death receptors is elevated during HCC therapy-induced senescence. Besides Fas also TRAIL-R2 (DR5) displayed upregulations during HCC TIS (Fig 13.3 and 13.4). Strikingly, sFAS-Ligand as well as anti-Fas antibody induced apoptosis of senescent HepG2 cells, revealing mechanisms through which senescent HCC cells can circumvent apoptosis resistance. These findings suggest that also other death receptors could be suitable targets for induction of ligand- or antibody-mediated apoptosis. Hence, treatment of senescent HCC cells with DR4- and DR5-activating ligand TRAIL could be a promising approach to further investigate apoptosis responses in HCC therapy-induced senescence.

Since ligand- and antibody-mediated Fas targeting can be utilized to circumvent senescence-associated apoptosis resistance, combining TIS with Fas targeting presents a promising strategy to eliminate highly inflammatory and metastasis-promoting senescent HCC cells by initiating an intrinsic cell death response. Notably, several studies indicated that the anti-apoptotic protein Bcl-2 and other Bcl proteins block Fas-mediated apoptosis in different types of cancer and T-cells (Jäättelä et al., 1995; Kawahara et al., 1998; Poulaki et al., 2001). Strikingly, this negative regulation was also demonstrated for HepG2 cells after Bcl-2 transduction (Takahashi et al., 1999). Since Bcl-2 is also a crucial regulator of senescence-associated apoptosis resistance we suggest that the transcriptional upregulation of Fas outweighs the Bcl-2-mediated anti-apoptotic phenotype (Fig 4.3). However, to confirm this, Bcl-2 expression has to be investigated in senescent HUH7 and HepG2 cells.

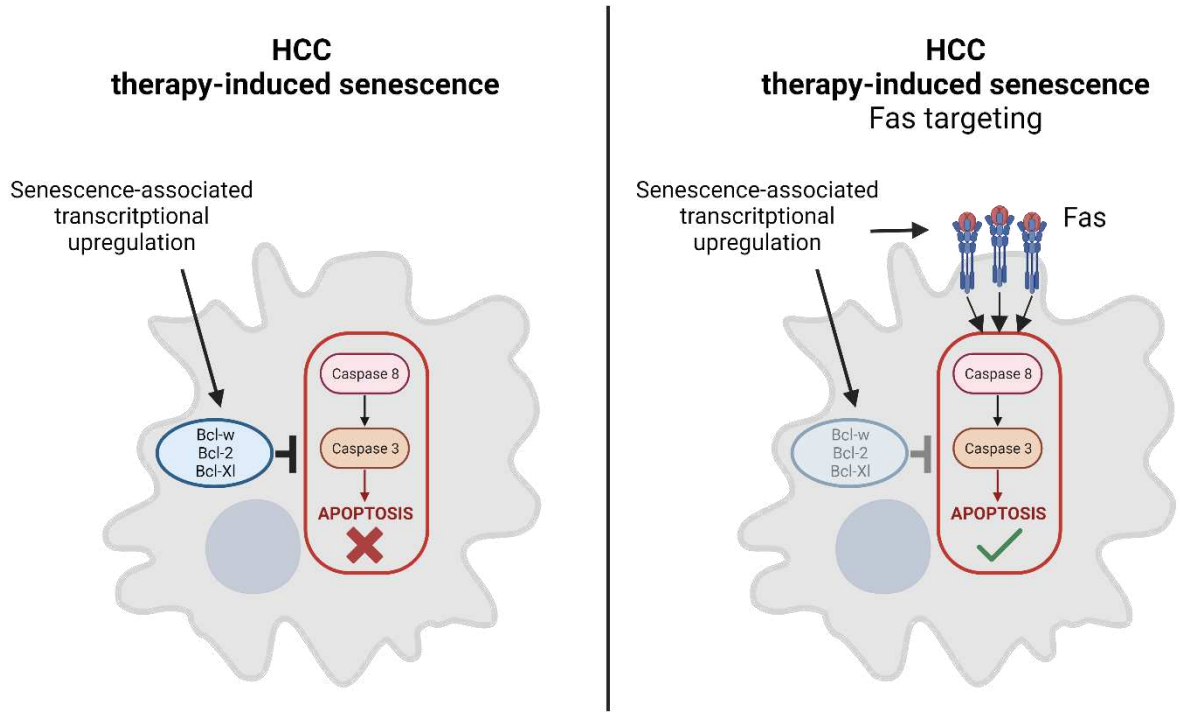

Figure 4.3: **Schematic illustration of apoptosis responses in HCC therapy-induced senescence.**

Senescence-associated apoptosis resistance is mediated by anti-apoptotic proteins such as Bcl-2, Bcl-w and Bcl-Xl. Despite initiation of an anti-apoptotic phenotype upon senescence induction, Fas targeting can circumvent apoptosis resistance and initiate intrinsic cell death responses.

Fas expression in a variety of immune cells, epithelial cells as well as healthy hepatocytes is a major obstacle for Fas-mediated elimination of senescent cells. Previous studies indicated that administration of anti-Fas antibodies in healthy mice resulted in strong apoptosis of healthy hepatocytes causing severe liver damage and acute hepatic failure (Ogasawara et al., 1993).

Other findings suggested that Fas has a pivotal role in progression of liver diseases such as hepatitis B (Kondo et al., 1997). Additionally, Fas is critical for anti-tumor T cell responses in HCC (Fukuzawa et al., 2001) and its expression is a prognostic marker for HCC recurrence (Ito et al., 2000). These findings suggest that Fas is an essential regulator of apoptosis in healthy liver tissue as well as a crucial player during disease progression and immune responses against acute liver diseases. Hence, targeting Fas carries the risk of causing non-tumorigenic tissue damage and a dysregulated anti-tumor response. These limitations have to be considered when developing novel, senescence-targeting anti-Fas therapies. One way of circumventing healthy tissue damage is by generating a senescence-associated bispecific antibody that on one hand binds and activates Fas and on the other hand recognizes a tumor- and senescence-specific antigen. This approach will be further discussed in section 4.3.4.

Many cancer therapies target tumor-associated proteins or cellular processes that are more prevalent in cancer cells than in normal tissue. Consequently, therapeutic interventions such as chemo- or radiation-therapy also exert cytotoxic effects on healthy tissue causing unwanted side effects. Notably, HepG2 cells displayed a striking Fas expression increase upon senescence induction. This strong transcriptional upregulation might allow for a lower anti-Fas dosing regimen, avoiding unwanted side effects on normal tissue. In order to determine if low dose anti-Fas treatments affect healthy liver tissue, different concentrations of sFas-Ligand and CH11 need to be tested on primary hepatocytes. This was planned in collaboration with Prof. Andreas Nüssler but could unfortunately not be carried out due to the non-availability of tissue. Furthermore, Fas has been demonstrated to be an essential regulator of anti-tumor T cell responses. To investigate if administration of sFas-Ligand or CH11 negatively affect T cell responses, a co-culture assay of senescent HCC cells and primary T cells has to be developed.

Another actionable target that displays higher tumor-specificity than Fas, is the well-characterized breast cancer-associated antigen HER2.

4.3.2. Targeting HER2 for antibody-dependent cell-mediated cytotoxicity (ADCC)

Discovery of HER2 as an actionable breast cancer target and subsequent development of the fully humanized anti-HER2 antibody trastuzumab have been one of the major breakthroughs in cancer research in past years (Nahta and Esteva, 2007; Wang and Xu, 2019). Since then, several measures have been taken to understand the mechanistic of trastuzumab-mediated antineoplastic effects and identify HER2 as a target for other solid tumors. Besides inducing

cell cycle arrest and a strong ADCC response also other mechanisms such as inhibition of downstream signaling pathways have been proposed to be the main underlying effect of trastuzumab's anti-cancer properties (Maadi et al., 2021).

Notably, several studies have suggested that HER2 could present an actionable target and prognostic marker of HCC, indicated by an elevated HER2 expression and altered expression profiles in different disease stages (Ito et al., 2001; Shi et al., 2019). While results obtained by RNAseq were contradictory between analyzed cohorts and studies, immunohistochemistry and immunoblotting revealed that HER2 was strongly overexpressed in HCC compared to normal liver tissue. However, other studies indicated that HER2 is low or not expressed in tumorigenic hepatic tissue (Vlasoff et al., 2002; Xian et al., 2005). These contradictions imply that further investigations are necessary to determine whether HER2 is an actionable target for HCC.

Despite the conflicting findings strong expression was observed for both investigated HCC cell lines. Additionally, HUH7 cells displayed an increased HER2 expression upon senescence induction suggesting that HCC therapy-induced senescence combined with HER2 targeting presents another promising therapeutic approach for clearance of senescent HCC cells. Notably, HER2 has also been associated to promote breast cancer metastasis by enhancing migratory and invasive capacities of cancer cells (Freudenberg et al., 2009). This in turn, suggests that HER2 overexpression during HCC therapy-induced senescence may also favor HCC metastasis formation, what corresponds to the previously described senescence-associated metastatic phenotype. To investigate whether HER2 is a suitable target for clearance of senescent HCC cells, trastuzumab-mediated ADCC assays can be established. Since NK92 cells do not express the Fc receptors CD16a and CD16b, ADCC assays have to be conducted with either PBMCs or isolated primary NK cells. These experiments are currently ongoing and will be tested with different concentrations of trastuzumab, as well as different effector to target ratios. Although complement-dependent cellular cytotoxicity (CDC/CDCC) is not a major mechanism of action for trastuzumab, some studies have shown minor induction of CDCC responses after antibody treatment. Additionally, combination with other HER2 targeted therapeutic antibodies, such as pertuzumab, increased cytotoxicity by induction of CDCC (Petricevic et al., 2013; Tsao et al., 2022).

Altogether, induction of ADCC and CDCC by therapeutic monoclonal antibody therapy, presents a promising strategy to increase senescence surveillance and eliminate senescent cancer cells. This approach is not only limited to HER2 but can also be applied to other actionable senescence-associated surface antigens.

4.3.3. CD276 CAR-NK and CAR-T cells as potential senolytic effectors

Besides Fas and HER2 a third actionable target of HCC senescence was identified in this study. The checkpoint inhibitor CD276, also referred to as B7-H3, has shown to be an essential player for tumor immune evasion (Liu et al., 2021). Furthermore, previous studies demonstrated that CD276 is critical for metastasis formation and a crucial regulator of epithelial-mesenchymal transition in HCC (Dong et al., 2018; F.-B. Kang et al., 2015). Consequently, by promoting metastasis and immune evasion CD276 favors tumor progression, thus revealing an ideal actionable target.

Our findings indicated that CD276 is strongly expressed in both investigated HCC cell lines and considerably elevated upon senescence induction in HepG2 cells. Furthermore, NK cell killing assays revealed that anti-CD276 CAR-NK92 cells exert strong cytotoxic activities against both HCC cell lines. Interestingly, upon TIS induction CAR-NK92 cells exhibited even greater cytotoxicity, suggesting that CD276 targeting could elevate immune-mediated clearance of senescent HCC cells. Another previously addressed actionable target that has been associated with CAR-NK cell therapy is HER2. Strikingly, anti-HER2 CAR-NK92 cells have recently been demonstrated to efficiently kill breast cancer, gastric cancer and rhabdomyosarcoma cells (Heim et al., 2023; Schönfeld et al., 2015; Wu and Huang, 2019). Hence, anti-HER2 CAR-NK cells may represent another promising tool for enhanced clearance of senescent HCC cells.

Besides CAR-NK cells, also anti-CD276 CAR-T cells have shown to efficiently eliminate CD276-positive cancer cells. Recent studies have reported that anti-CD276 CAR-T cells are highly effective in clearing a variety of solid tumors, such as glioblastoma and melanoma (Nehama et al., 2019; Tang et al., 2019; Zhang et al., 2020). Additionally, anti-CD276 CAR-T cells exert strong cytotoxic effects against hematologic cancers like acute myeloid leukemia (Zhang et al., 2020). Consequently, several CD276-targeting cell therapies are currently being tested in clinical trials.

Conclusively, CD276 presents an actionable anti-tumor target for several types of cancer. We propose that CD276 is the most promising therapeutic target of HCC therapy-induced senescence, due to its high targeting efficacy *in vivo* and an already existing broad range of immunotherapeutic approaches (table 4.2). This is supported by our data, indicating an elevated expression of CD276 upon senescence induction in HepG2 cells, accompanied by strong cytotoxic effects of anti-CD276 CAR-NK92 cells against senescent HCC cells. Furthermore, using CAR-NK cells to target senescent HCC cell would combine two beneficial aspects related to immune-mediated clearance of these cells: first, the increased expression of actionable targets upon senescence induction and second, the previously mentioned crucial role of NK cells in clearance of senescent tumor cells (Antonangeli et al., 2019).

However, one limitation of CD276 targeting is its expression in healthy tissue. Although cancer cells strongly upregulate CD276, some studies have suggested that CD276 is also expressed in prostate tissue, adipose tissue and other healthy tissue (Getu et al., 2023). One way to increase the tumor specificity of targeted therapies is by introducing a second cancer-associated antigen. This can be achieved by generating dual-targeting bispecific antibodies.

4.3.4. Senolytic bispecific antibodies – tumor-specific death receptor targeting

The development of bispecific antibodies has given rise to many novel anti-cancer immunotherapies. One of the most successful clinically approved bispecific antibodies is blinatomumab, that serves as a linker between CD19 expressing B-cells and cytotoxic CD3 positive T cells (Burt et al., 2019). Blinatomumab belongs to the group of bispecific T cell engagers (BiTE) due to its T-cell binding properties. Besides engaging immune cells, dual-targeted bispecific antibodies can also be utilized to block immune checkpoints or target two tumor-associated antigens (Ma et al., 2021). One tumor-dual bispecific antibody currently being tested in clinical trials is dilpacimab that binds and inhbits VEGF and DLL4, thereby blocking tumor angiogenesis (Gordon et al., 2021). Based on this study, in collaboration with Prof. Helmut Salih, a bispecific anti-CD95-anti-CD340 construct is in preparation, based on the sequences of CH11 (for CD95) as well as trastuzumab, pertuzumab and margituzimab (for CD340). Strikingly, previous approaches to generate anti-CD95 bispecific antibodies have indicated that such antibodies only exert cytotoxic effects when the second recognized antigen exceeds a certain expression level (Jung et al., 2001). This suggests that Fas activation

mediated by a bispecific antibody that recognizes a second senescence-associated tumor antigen, such as CD276 or uPAR, could allow for Fas targeting of solely senescent tumor cells.

If promising, additional combinations of death receptor agonist antibody sequences with targets conferring specificity for the senescent cell could be envisaged. Besides Fas, also other death receptors displayed elevated expression upon senescence induction. Although the expression increases were not as striking, DR5, also referred to as TRAIL-R2, displayed a considerable expression increase for all TIS inducers in HepG2 cells and a slight upregulation for alisertib- and CX5461-treated HUH7 cells (Fig. 3.13 and 3.14). Hence, death receptor-targeted bispecific antibodies may not only be limited to Fas but could also target other death receptors such as DR4 or DR5.

Besides previously discussed BiTEs and dual-tumor bispecific antibodies, also NK cell activating bispecific killer engangers (BiKE) have been in the focus of immunotherapy research. Strikingly, anti-HER2 BiKE antibodies carrying a CD16a binding domain induced strong anti-cancer affects against breast cancer and ovarian cancer cells (Nikkhoi et al., 2023). To further utilize the advantages of multiple antigen recognition anti-HER2 tri-specific NK cell engagers (TriKE) were recently developed that carry an additional human IL-15 as a cross-linker between CD16a and HER2 recognizing variable domains (Vallera et al., 2021). IL-15 is critical for NK cell expansion and activation and thus a crucial stimulator of NK cell-mediated tumor clearance (Kobayashi et al., 2005). Hence, IL-15 containing anti-HER2 TriKEs exerted potent anti-tumor effects against HER2-positive ovarian cancer cells and promoted strong ADCC responses (Vallera et al., 2021). These findings reveal further advantage of bispecific antibody targeting compared to conventional monoclonal-based immunotherapies, considering that NK cells play an essential role in immune-mediated clearance of senescent cells.

Notably, also CD276 has been associated with being a potential target for bispecific antibody therapy. Recently developed bi- and trispecific antibodies targeting CD276 induced strong ADCC responses and T cell activation against various gastrointestinal cancers, prostate cancer, and other solid tumors (Vallera et al., 2020; Yuwen et al., 2022; Zekri et al., 2023).

Altogether, bispecific antibodies directed against senescence-associated antigens may exert strong senolytic activity either by direct cytotoxic effects (targeting of death receptor) or by indirect immune activation (ADCC or CDCC). Generating a senescence-associated bispecific antibody that on one hand binds and activates Fas and on the other hand recognizes a tumor-

and senescence-specific antigen presents a promising strategy to enhance tumor specificity of Fas-targeting therapies. Alternatively, demise of the senescent cells could also be induced via NK cell-mediated ADCC or complement-mediated CDCC. For example, the efficacy of the clinically approved monoclonal antibody rituximab depends on both aspects (Cerny et al., 2002) .

Conclusively, HCC TIS gives rise to several surfaceome-based targeting opportunities including re-instatement of death receptor pathways, monoclonal antibody targeting for antibody-dependent cell-mediated cytotoxicity (ADCC), using CAR-NK cells as senolytic effectors and senolytic bispecific antibodies. To determine, whether these opportunities can also be utilized *in vivo*, therapeutic applicability will be addressed in the following.

4.3.5. Therapeutic applicability of targeting approaches

As aforementioned, Fas, CD340 and CD276 were identified as therapeutically actionable targets of HCC therapy-induced senescence. Furthermore, these actionable surface markers were proposed for various targeting opportunities. However, to determine whether the identified therapeutic markers are also actionable *in vivo,* 365 primary HCC patient samples were selectively compared for their expression of Fas, CD276 and HER2. (Fig 4.4). As previously shown for HCC cell lines, strong heterogeneity between expression of therapeutic markers was observed for primary HCC patients, suggesting that targeting efficiency may exhibit strong patient specificity. However, despite of the strong heterogeneity, expression of therapeutic markers was detected in all examined HCC patient samples. Only Fas showed low to no expression in a subset of patients. Hence, basal expression of identified therapeutic markers was confirmed in primary human HCC, suggesting that senescence-associated therapeutic targets may also be actionable *in vivo*. However, further expression analyses are necessary to determine whether senescence induction also increases expression of the identified therapeutic markers *in vivo.*

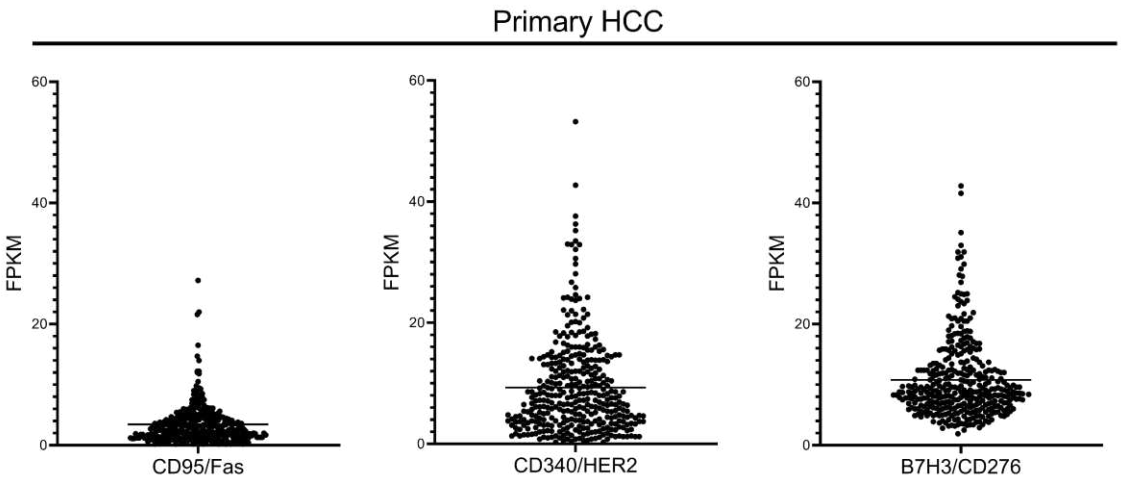

Figure 4.4: **Expression of Fas, CD340 and CD276 in** primary HCC patient samples**.**

Data was obtained from publicly available RNAseq datasets derived from "The Human Protein Atlas".

Furthermore, the implementation of therapeutic approaches against senescent HCC cells is supported by already existing clinical trials targeting the identified senescence-associated surface antigens. A list of the most promising trials and approved therapeutics utilizing the previously discussed therapeutic approaches (Bi-/tri-specific antibody therapy, CAR-based cell therapy, monoclonal antibody therapy) is given in table 4.2.

Table 4.2: **Clinical trials and approved therapeutics targeting senescence-associated therapeutic surface antigens.**

Therapeutic senescence markers	Clinical trial number/ Date of FDA approval	Trial stage	Description (Mechanism of action + cancer type)
CD95 / Fas	No currently recruiting clinical trials		
CD276 / B7-H3	NCT02923180	Phase II	**Monoclonal anti-B7-H3 antibody (Enoblituzumab)** Tested for intermediate and high-risk prostate cancer
	NCT03214666	Phase I/II	**Tri-specific killer engager (TriKE) targeting CD16, IL15 and CD33** Tested for high risk haematologic malignancies
	NCT04077866	Phase I/II	**Anti-B7-H3 CAR-T cells** Tested for recurrent or refractory glioblastoma
CD340 / HER2	September 25th, 1998 (breast cancer) October 20th, 2010 (gastric cancer)	-	**Monoclonal anti-HER2 antibody (Trastuzumab)** For treatment of HER2-positive metastatic breast cancer and gastric cancer
	NCT04521179	Phase II	**Bispecific antibody against two distinct HER2 epitopes**

			Tested in combination with PD-L1 / CTLA-4 targeting bispecific antibody for treatment of HER2-positive solid tumors
	NCT05681650	Phase I/II	**Hypoxia-stimulated anti-HER2 CAR-T cell** Tested for HER2-positive advanced solid tumor
	NCT03383978	Phase I	**Anit-HER2 CAR-NK92 cells** Tested for reccurent HER2-postitive glioblastoma

Altogether, expression of the actionable surface markers in primary human HCC suggests that these antigens may serve as suitable targets for therapeutic intervention *in vivo*. This is reinforced by the ongoing clinical trials that are directed against the identified TIS-associated surface markers. Additionally, these trials offer promising insights into the potential effectiveness of targeting senescence-associated surface antigens as a means of managing HCC. However, comprehensive functional analyses are imperative to confirm that the identified surface antigens can be effectively targeted in the complex *in vivo* environment, which is critical for translation into clinically applicable treatment.

4.4. Surfaceome screening as an approach to identify individual TIS-associated targets

Our findings indicate that induction of senescence, followed by senolytic clearance may provide a promising therapeutic strategy for treatment of HCC. We propose four ways for efficient clearance of senescent HCC cells after TIS induction *in-vitro*: Death receptor activation by agonistic antibodies or ligands, conventional monoclonal antibody immunotherapy, bi-and tri-specific antibody therapy, and cell therapy using CAR-NK or CAR-T cells (Fig 4.5). While death receptor targeting and CAR-NK cell therapy were successfully verified as ways to efficiently eliminate senescent HCC cells *in vitro*, efficacy of monoclonal and bi-/tri-specific antibody-mediated clearance remains to be determined. Notably a bispecific anti-CD95-anti-CD340 construct is in preparation (in collaboration with Prof. Helmut Salih) and will determine if bispecific antibodies can be utilized to reinstate death receptor pathways in HCC therapy-induced senescence. Furthermore, trastuzumab-based ADCC assays are currently ongoing to confirm that CD340 is a therapeutically actionable target of HCC therapy-induced senescence.

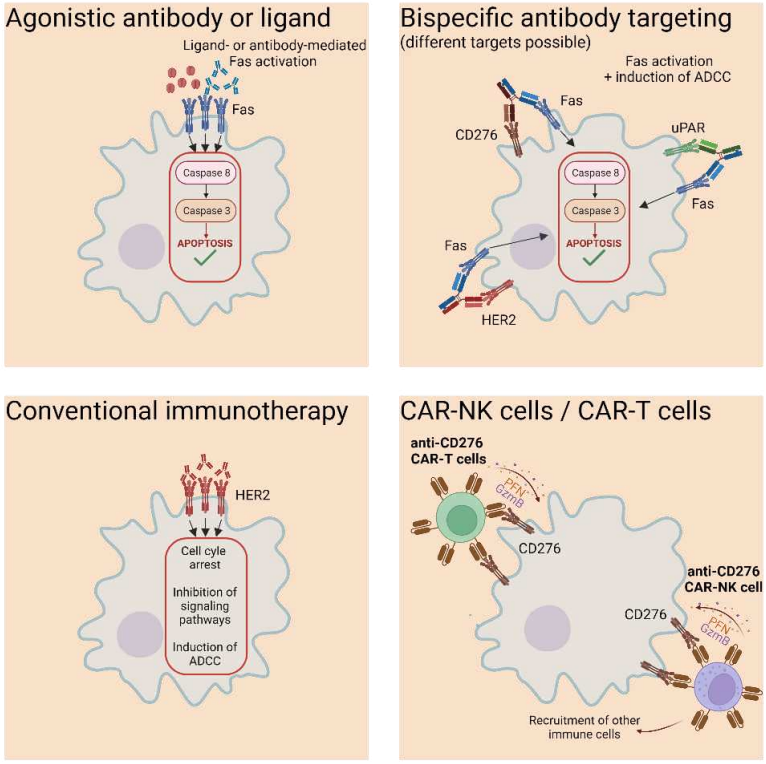

Figure 4.5: **Schematic overview depicting ways for immune- and ligand-mediated clearance of senescent HCC cells.**

Death receptor activation, conventional monoclonal antibody immunotherapy, bi-and tri-specifc antibody therapy, and cell therapy using CAR-NK or CAR-T cells were proposed as different approaches for clearance of senescent HCC cells.

As described, the identified senescence-associated targets may also be actionable *in vivo.* However, the previously addressed inducer-, cell type- and patient specificities of HCC therapy-induced senescence prevent standardized targeting approaches and emphasize on the need for individualized therapy. Hence, we propose a patient-specific individualized targeting approach based on the senescence-associated actionable surfaceome that not only circumvents senescence heterogeneity but also promotes treatment predictability and efficiency (Fig 4.6). Patient-specific surfaceome screening of a selective set of actionable markers may determine the most promising second-line therapies that could be conducted in an individualized manner. However, several limitations arise from this approach. Firstly, safety of senescence-inducing compounds and second-line treatments has to be validated *in vivo.* Additionally, before conducting second-line therapy, TIS induction has to be confirmed by administration of senescence tracers, such as FPyGal (Schwenck et al., 2019), that have not been approved for clinical use yet. Furthermore, other senescence-associated targets have to

be determined and verified *in vivo*. We suggest performing initial testing in xenograft models of human HCC to evaluate safety profiles and therapeutic potential *in vivo.*

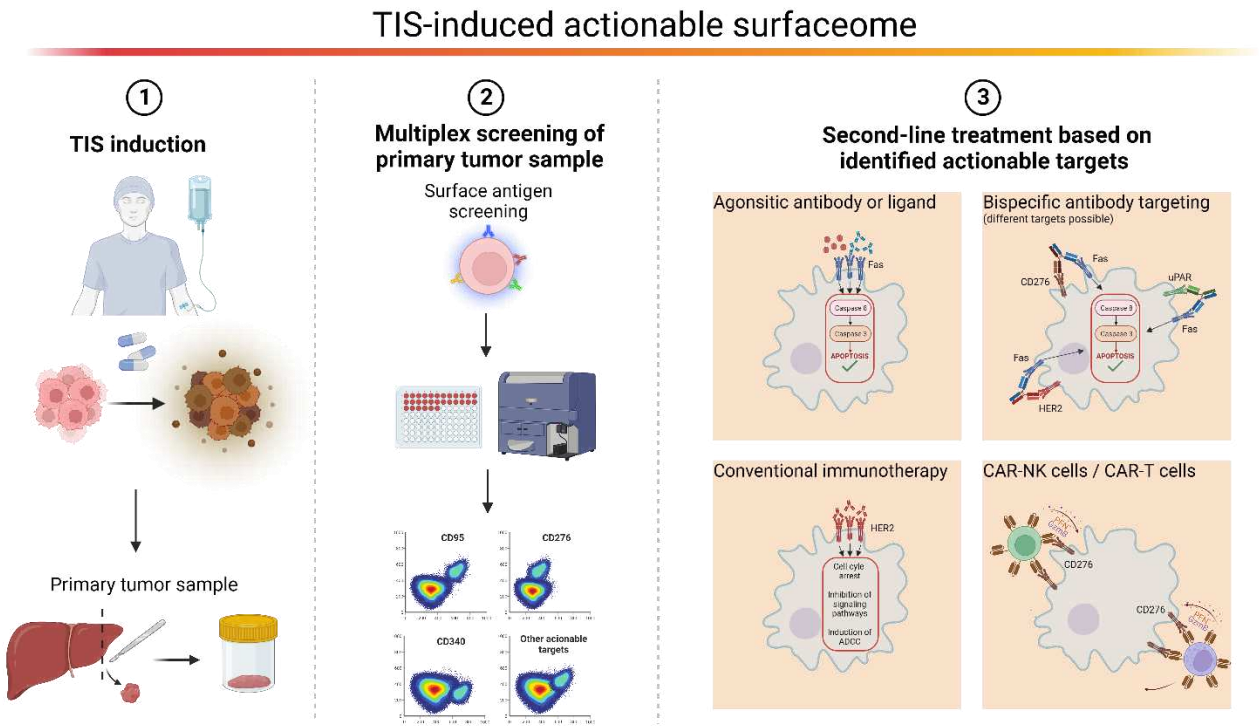

Figure 4.6: **Utilizing the TIS-induced, actionable surfaceome.**

Chronological illustration of therapeutic targeting approaches. TIS induction and subsequent confirmation by administration of senescence tracers, could be followed by multiplex sreening of a primary tumor sample do identify actionable targets. Based on the identified targets, second-line treatments including agonistic antibody or ligands, bispecific antibody targeting, conventional immunotherapy or CAR-NK cell/CAR-T cell therapy, could be conducted.

4.5.Conclusion

The findings presented in this study led to the identification of several new pathological and actionable features of HCC senescence, including a metastasis-promoting surfaceome and therapeutically targetable surface antigens. By screening the senescent secretome a broad characterization of senescence-associated surface markers was performed that uncovered novel features of HCC therapy-induced senescence.

Furthermore, our findings indicate that several metastasis-associated surface markers were overexpressed on senescent HCC cells. Additionally, the HCC-SASP may promote tumor angiogenesis and metastasis formation, further suggesting that senescence favors tumor invasiveness and metastatic capacities. Conclusively, analysis of secretory factors, senescent surfaceome and investigation of innate immune responses revealed that HCC therapy-induced

senescence displays a strong cell line and TIS-inducer specificity. This specificity may also be present in HCC patients.

Altogether we propose that therapy-induced senescence should rather be considered as a short-term intervention to stop rapidly proliferating tumor cells, whereas a persistent senescent phenotype must be avoided. Therefore, we suggest implementation of a dual-treatment, such as TIS-induction combined with subsequent second-line treatment that includes death receptor activation with agonistic ligands or antibodies, conventional monoclonal antibody therapy, bi-and tri-specifc antibody therapy, or cell therapy using CAR-NK or CAR-T cells.

5. Statutory Declaration

Hereby I affirm that I wrote this Doctoral thesis with the topic

'Actionable heterogeneity of hepatocellular carcinoma therapy-induced senescence'

independently and that I used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken paraphrased from other works. Contributions by others have been marked as such in the text and are additionally listed in the following table of contributing scientists. Moreover, I affirm that I performed the scientific studies according to the principles of good scientific practice.

Pujan Engels

_____

Tübingen, Germany

List of contributing scientists

Scientist	Affiliation	Data created	Data shown in this thesis (figure)
Dr. Andras Szolek	Group of Prof. Alexander N.R. Weber, University of Tübingen	Bioinformatic analysis of LegendScreen data	Figure 3.15
Dr. Francesca Bork		Isolation of PMNs	Figure 3.8
Dr. Vinicius Nunes Cordeiro Leal		Isolation of PMNs	Figure 3.8
Kim Hebbel		Repetitions of $p16^{INK4a}$ and $p21^{WAF/CIP1}$ immunoblot experiments	Figure 3.4
Georgios Vavouras	Group of Prof. Michael Schindler	Help with the setup of the MACSquant VYB for LegendScreen experiments	Figure 3.13 Figure 3.14

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