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Characterization of a role of senescence in the induction and regulation of cancer cell death

Charakterizace vlivu senescence na indukci a regulaci smrti nádorových buněk

Diplomová práce

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Prohlášení:

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Podpis

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Abstract

Senescence is a specific cell state distinquished by cessation of cell division and proliferation and changes in gene expression. Normal cells enter senescence after distinct number of cell divisions or in case of an unrepairable damage. Senescence in cancer cells can be induced by subliminal stress as sublethal treatment with certain drugs. Senescent cancer cells persist in the tissue and may secrete a number of factors and nutrients affecting surrounding cells. Senescence can thus change the response of cancer cells to various apoptogens during cancer therapy. In this study, we focused on the elucidation of presumed differences between normal proliferating and senescent cancer cells in their response to selected apoptogens. Implementing bromodeoxyuridine (BrdU)-mediated replication stress in cancer cells derived from pancreatic (PANC-1) or mesothelioma (H28) tumors, we efficiently forced these cells to acquire senescent phenotype. We document that these senescent cells gain higher resistance to combined TRAIL and homoharringtonine (HHT) treatment and enhance sensitivity to other apoptogens such as FasL, camptothecin and mVES. These cells also showed increased expression of anti-apoptotic protein c-FLIP in senescent cells and changes in the expression of some Bcl-2 family proteins. ShRNA-mediated downregulation of c-FLIP expression sensitized H28 cells to BrdU treatment but it apparently did not affect attenuated sensitivity of senescent H28 shFLIP cells to TRAIL+HHT treatment. We also generated clones of H28 cells inducibly overexpressing two CDK inhibitors, $p21^{CIP1/WAF1}$ and p16. These cells exhibited some markers of senescent cells and became resistant to most apoptogens. However, these cell-cycle arrested cells lack some aspects of senescent phenotype arising probably from DNA damage signaling which precedes senescence. In this work we confirmed and extended some previous findings, gathered new, potentially interesting data on a role of c-FLIP, death receptors, Bcl2 family and cell cycle inhibitors in the communication between cellular senescence and apoptotic signaling and slightly opened door for further discoveries in this so far overlooked field of research.

Keywords: apoptosis, cell death, senescence, death receptors, c-FLIP, $p21^{CIP1/WAF1}$, $p16^{ARF/INK4a}$, cancer cells

Abstrakt

Senescence je stav, kdy se buňka přestane dělit, mění expresi některých genů a je trvale zastavena v určité fázi buněčného cyklu. Nenádorové buňky vystupují z cyklu po určitém počtu buněčných dělení, nebo jsou-li poškozeny natolik, že jejich další dělení již není žádoucí. U nádorových buněk může být senescence indukována podprahovým stresem např. subletálními dávkami chemoterapeutik. Nádorové buňky tak neumírají, ale zůstávají v tkáni a mění svůj fenotyp na fenotyp charakteristický sekrecí faktorů, které tkáň dále ovlivňují. Stav senescence tedy může měnit odpověď nádorových buněk na léčbu pomocí apoptózu indukujících terapeutik. V této práci jsme se soutředili na analýzu rozdílů v apoptotické signalizaci mezi proliferujícími a senescentními nádorovými buňkami indukované různými proapoptotickými látkami. Pomocí bromodeoxyuridinu (BrdU) jsme u nádorových pankreatických (PANC-1) a mesoteliálních (H28) buněk indukovali replikační stres, jehož vlivem se buňky přestaly dělit a získaly fenotyp senescentních buněk. U těchto buněk jsme pozorovali zvýšenou rezistenci vůči apoptóze indukované ligandem TRAILem v kombinaci s homoharringtoninem (HHT) a zvýšenou senzitivitu senescentních buněk vůči Fas ligandu, camptothecinu a mVESu. Tyto buňky take vykazovaly zvýšenou expresi antiapoptotického proteinu c-FLIP a změny v expresi proteinů z rodiny Bcl-2 proteinů. Snížení exprese c-FLIP prostřednictvím shRNA sice neovlivnilo odolnost senescentních nádorových buněk vůči TRAIL +HHT indukované apoptóze,ale zvýšilo ale jejich citlivost k BrdU. V rámci této práce jsme rovněž připravili klony H28 buněk se současnou inducibilní expresí dvou inhibitorů cyklin dependentních kináz (CDK), $p21^{CIP1/WAF1}$ and p16. Tyto buňky svým fenotypem připomínaly buňky senescentní a současně téměř neodpovídaly vůči většině použitých apoptogenů. Tyto výsledky napovídají, že “pouze” zastavené buňky postrádají některé vlastnosti senescentních buněk, které jsou pravděpodobně spjaté s poškozením DNA. Naše práce navazuje na některé již dříve publikované výsledky popisující změny v apoptotických drahách u senescentních buněk a přináší nové zajímavé informace o možné roli proteinu c-FLIP, receptorů smrti, Bcl-2 proteinů a inhibitorů CDK v kontextu buněčné senescence a apoptotické signalizace.

Klíčová slova: apoptóza, buněčná smrt, senescence, receptory smrti, c-FLIP, $p21^{CIP1/WAF1}$, $p16^{ARF/INK4a}$, nádorové buňky

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List of abbreviations

(P)-ChK1/2	- (phospho) checkpoint kinase 1/2
53BP1	- p53-binding protein 1
A	- adenine
ARF	- alternative reading frame
ATB	- antibiotics
ATP	- adenosine triphosphate
ATM	- Ataxia Telangiectasia Mutated
ATR	- Ataxia Telangiectasia and Rad3-related
BCA	- bicinchoninic acid assay
Bcl-2	- B-cell lymphoma 2
BrdU	- 5-bromo-2'-deoxyuridine
CDKs	- cyclin-dependent kinases
c-FLIP	- cellular FLICE-inhibitory protein
Chk1/2	- checkpoint kinase 1/2
CPT	- camptothecin
C	- cytosine
dATP	- deoxyadenosine triphosphate
DcR1/2/3	- decoy receptor 1/2/3
DD	- death domain
DED	- death effector domain
DDR	- DNA damage response
DISC	- death-inducing signaling complex
DMEM	- Dulbecco's Modified Eagle's medium
DNA	- deoxyribonucleic acid
doxy	- doxycycline
DR3/4/5/6	- death receptor 3/4/5/6
EV	- empty vector
FACS	- Fluorescence Activated Cell Sorter
FasL	- Fas ligand
FasR	- Fas receptor
FBS	- fetal bovine serum

FITC	- fluorescein isothiocynate
FSC	- forward scatter
G	- guanine
HHT	- homoharringtonine
H28 N/S	- H28 normal (proliferating)/senescent
H28 wt	- H28 wild type
IL	- interleukin
iPS	- induced pluripotent stem (cells)
K	- lysine
MMP-3	- matrix metalloproteinase-3
MOMP	- mitochondrial outer membrane permeabilization
mVES	- mitochondrially targeted vitamin E succinate (mitoVES)
NFκB	- nuclear factor kappa-light-chain-enhancer of activated B cells
NT	- non-targeting
PANC-1 N/S	- PANC-1 normal (proliferating)/senescent
PARP	- poly (ADP-ribose) polymerase
PBS	- phosphate buffered saline
PML	- promyelocytic leukemia
pRb	- retinoblastoma protein
qRT-PCR	- quantitative reverse transcriptase polymerase chain reaction
RNA	- ribonucleic acid
ROS	- reactive oxygen species
rpm	- rotations per minute
RPMI	- Roswell Park Memorial Institute medium
SAHF	- senescence-associated heterochromatin foci
SASP	- senescence-associated secretory phenotype
SA-β-gal	- senescence-associated beta-galactosidase
SD	- standard deviation
SDS	- sodium dodecylsulphate
SDS-PAGE	- SDS-polyacrylamide gel electrophoresis
shRNA	- short hairpin RNA
SSC	- side scatter
T	- thymine
TNFR-1	- tumor necrosis factor receptor 1

TRAIL	- TNF-related apoptosis-inducing ligand
γH2AX	- phosphorylated form of histone H2AX

1. Introduction

Cellular senescence is a specific cellular state, in which cells cease to divide and proliferate and they alter their expression of many genes. Normal non-transformed cells enter senescence after a certain number of cell divisions or if they suffer in continuous, non-lethal stress conditions and their further proliferaiton could be harmful or protumorigenic. Cellular senescence can be triggered in dividing cells even during embyogenesis by certain stimuli including telomeres detrition, activation of some oncogenes, various drugs and is invariantly connected with persistent, unrepairable lesions in chromosomal DNA. Senescence plays a role during normal physiological processes like aging, embryo development or recovery after fibrosis and it is triggered also in various pathological processes as carcinogenesis and tumour treatments, ischemia or liver pathologies.

Treatment of cancer cells with sublethal stress stimuli such as locally low levels of chemotherapeutic drugs can drive them to senescence. In principle, via intrinsic or extrinsic stimuli triggered stepping out of the cell cycle into cellular senescence appears (together with apoptotic/necrotic elimination of cancer cells) as an efficient anti-tumor response. Although at first glance senescence seems to be beneficial from a cancer patient´s point of view, presence of senescent cells can be also detrimental and disadvantageous. Senescent cancer cells persist in the tissue and secrete a number of factors affecting surrounding (cancer) cells (cytokines, chemokines, growth factors or metalloproteinases). Senescent cells have altered sensitivity to various pro-death stimuli and can also change the response of cancer cells to various apoptogens during cancer therapy.

However, mechanisms altering apoptotic response of senescent cells and the proximal cancer cells have not been clarified yet and only little is known about crosstalk between apoptosis and senescence in cancer cells. Elucidation of these mechanisms would be in addition to better general understanding of these processes also an advantage for increased efficiency of cancer treatment, and could help to eliminate potentially harmful senescent cancer cells.

2. Literature review

2.1. Senescence

Senescence (from latin word *senescere* – to grow old) is defined as an irreversible cell cycle arrest distinquished by a number of morphological and biochemical markers. It is a mechanism, which protects organism from potentially harmful proliferation of old, damaged and pretransformed cells that naturally emerge with age and increasing stress. Senescence also represents one of the barriers against tumorigenesis. In literature senescence is sometimes called cellular ageing, but this term is not entirely correct as all cells do age but not all of them (e.g. stem cells) become senescent.

Senescence was first observed in primary cell cultures. Primary cells such as fibroblasts can be cultivated *in vitro* only for limited time span. They gradually lose their proliferation capacity till they stop dividing definitively and leave the cell cycle. Such cells remain viable but despite sufficient nutrients, space and other factors they do not grow and proliferate. The definite number of cell divisions cell is able to undergo was firstly described by Leonard Hayflick in 1961 (Hayflick and Moorhead, 1961) and later it was named after him as the „Hayflick´s Limit". First evidence of senescence came from *in vitro* cultivations and experiments, but later its presence was observed also *in vivo* (for example Storer et al., 2013; Swarbrick et al., 2008). Also range of cells susceptible to senescence broadened. The ability to trigger senescence was originally ascribed to primary non-transformed cells as a consequence of disrupted telomere stability and accumulated mutations. In contrast cancer cells that are immortalized through the activation of telomerase were considered as senescence-incompetent cells. But nowadays it is evident that even cancer cells can leave the cells cycle and enter senescence-like state.

In general, senescence is mainly caused by DNA damage and activation of stress signaling pathways that switch on the expression of cyclin-dependent kinases (CDKs) inhibitors. As those inhibitors are active in distinct phases of the cell cycle, senescent cells can emerge within the G1, G0 or G2 phase (Mao et al., 2012) – see Figure 1.

Senescence is not the only cell status characterized with the extended or permanent cell cycle arrest – the most typical examples of non-dividing cells are *quiescent* and *terminally differentiated cells*. *Quiescence* (or so called *G0 phase*) is defined as the reversible cells cycle arrest – compared to irreversible and permanent arrest in case of

senescence. Quiescent cells can in contrast to senescent cells re-enter the cell cycle in a response to proper proliferation signals. (Good et al., 1996). *Terminally differentiated cells* (post-mitotic cells) have also lost the ability to proliferate but the process of differentiation is tightly controlled by different pathways and it is not accompanied with DNA damage (Campisi and d'Adda di Fagagna, 2007).

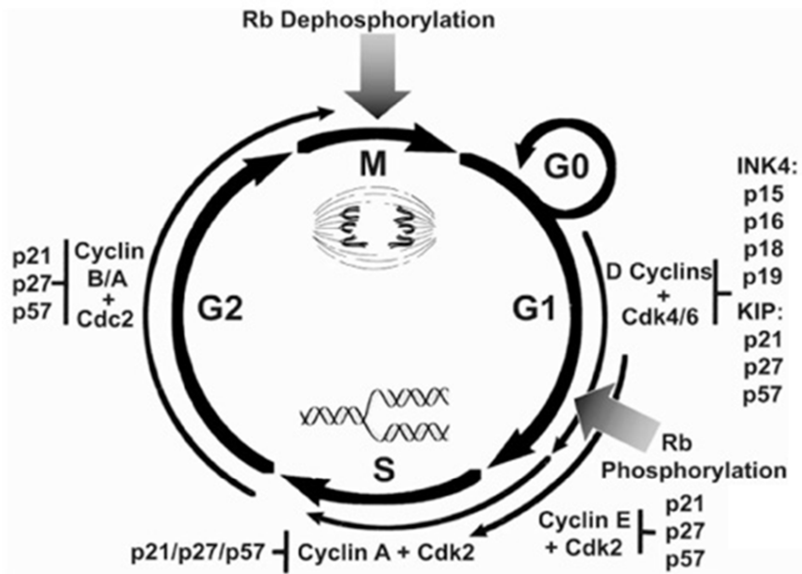


Figure 1. Cell cycle and CDK inhibitors. Progression within the cell cycle is driven by complexes of cyclins and CDKs. Activity of CDKs can be regulated by CDK inhibitors. There are two families of CDK inhibitors – INK4/ARF family ($p15^{INK4b}$, $p16^{ARF/INK4a}$, $p18^{INK4c}$, $p19^{ARF/INK4d}$) and CIP/KIP family ($p21^{CIP1/WAF1}$, $p27^{KIP1}$, $p57^{KIP2}$). Inhibitors of the INK4 family induce only G1 cell cycle arrest, inhibitors of the CIP family can induce cell cycle arrest also within S and G2 phases of the cell cycle (Donovan and Slingerland, 2000).

2.1.1. Induction of senescence

Senescence can be induced by myriads of stimuli, mainly resulting in damage of chromosomal DNA. However, some data suggest that senescence can occur even without detectable DNA damage-triggered signaling (Alimonti et al., 2010; Ramirez et al., 2001; Rodier et al., 2009).

Most cells leave the cell cycle and become senescent in response to some extensive stress and irrepairable DNA damage, especially DNA double-strand breaks. This stress activates various pathways including two prominent pathways of senescent signaling – p53-p21 and p16-pRb pathways.

Double strand breaks in DNA induce recruitment of the DNA damage sensor - Mre11/Rad50/NBS1 (MRN) complex – see Figure 2.

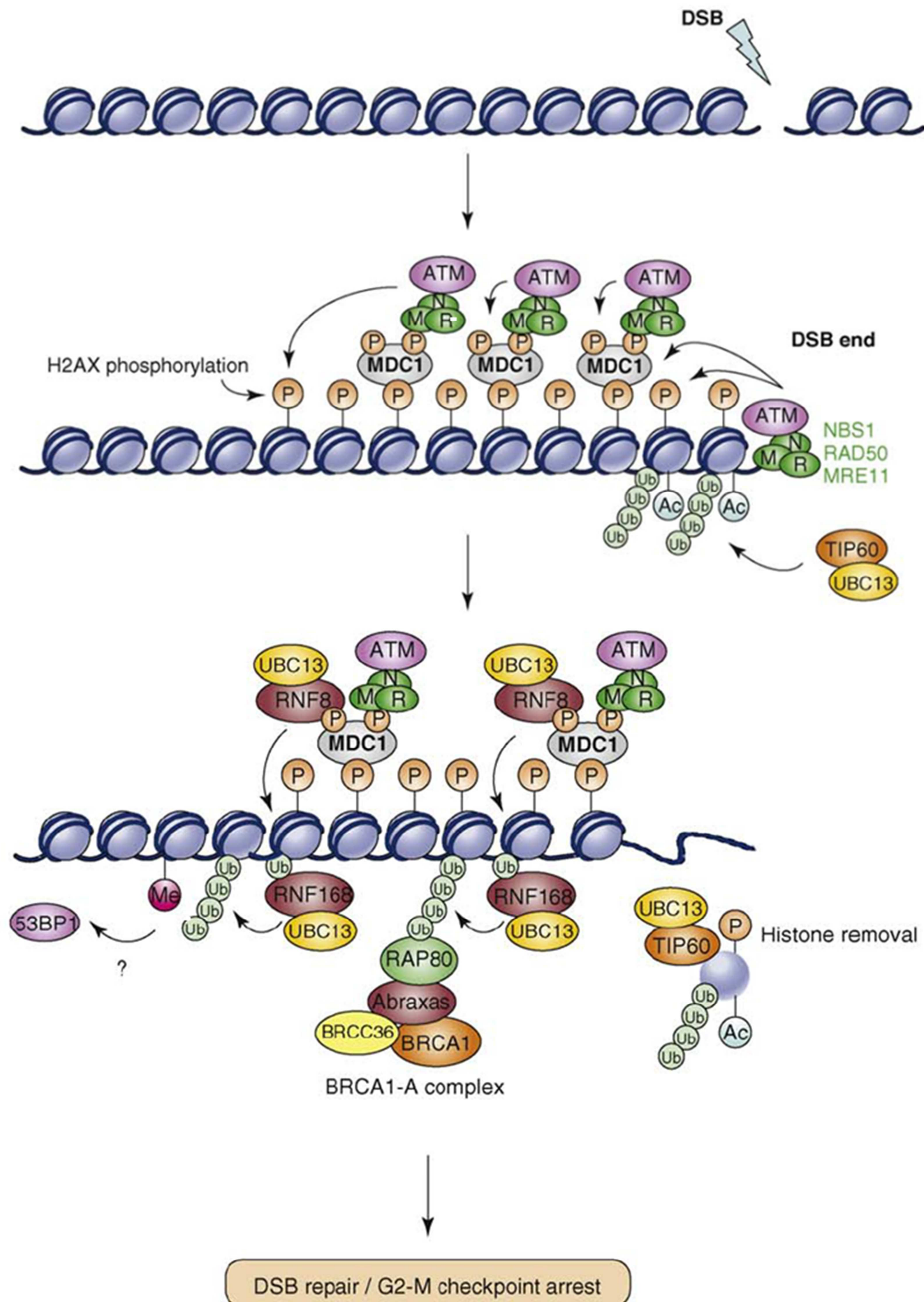


Figure 2. Signaling at double strand breaks. The MRN complex binds to the end of the double strand breaks. ATM is recruited to the site of DNA damage, binds to the MRN complex via its association with NBS1 and phosphorylates histone H2AX. MDC1 binds to the phosphorylated form of H2AX (γH2AX) and subsequently recruites other MRN complexes and ATM kinases forming large and detectable γH2AX foci. Histone γH2AX is then acetylated, ubiquitilated and polyubiquitilated. Polyubiquitilated histone γH2AX acts then as a scaffold for binding of BRCA1-A complex and 53BP1 protein. Usually, this DDR leads to the induction of (transient) cell cycle arrest and then to repaired DNA (van Attikum and Gasser, 2009).

MRN complex is also involved in the recruitment and activation of ATM (Ataxia Telangiectasia Mutated) kinase, respectively ATR (Ataxia Telangiectasia and Rad3-related) kinase in case of single-strand breaks. These kinases have many substrates, but from the perspective of DDR, the most important ones are histone variants H2AX, member of the Mre11/Rad50/NBS1 (MRN) complex NBS1 (Nijmegen breakage syndrome) protein and p53 binding protein 1 (53BP1), that form scaffold for other DDR proteins. ATM/ATR also phosphorylates and activate checkpoint kinase 2/1 (Chk2/Chk1), that in turn phosphorylate other proteins including p53 (Sancar et al., 2004; van Attikum and Gasser, 2009) – see Figure 3.

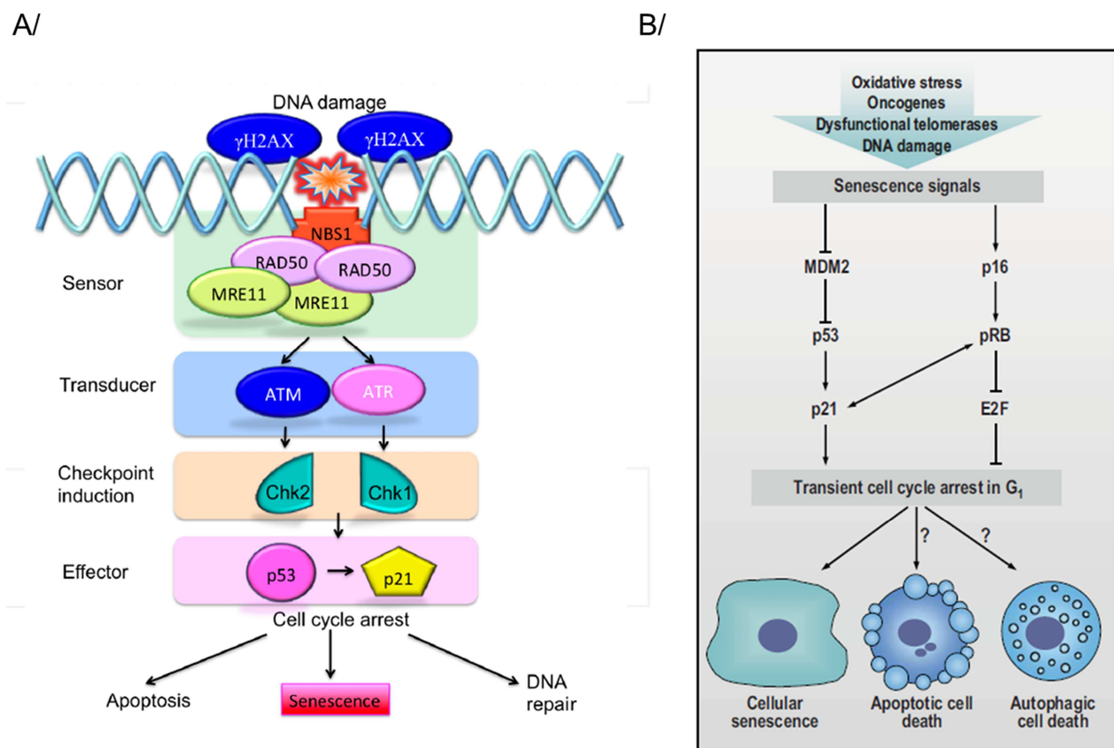


Figure 3. DNA damage pathways and major pathways inducing senescence and apoptosis. A/ ATM and ATR kinases transduce signals to effector kinases Chk2 and Chk1 that phosphorylate and stabilize main tumor suppressor protein and transcription factor p53. p53 then induces expression of proteins driving cells into apoptosis, transient cell-cycle arrest or senescence. B/ p53-p21 and p16-pRb are the main tumor suppressor pathways leading to apoptosis, senescence or either autophagy. Active p53 induces expression of CDK inhibitor p21, which inhibits progression through the cell cycle and allows cells to repair DNA and recover. The second pathway is triggered by CDK inhibitor p16, which keeps pRb in hypophosphorylated state. Hypophosphorylated pRb binds E2F transcription factors and keeps them inactive (Becker and Haferkamp, 2013; Vicencio et al., 2008).

Tumor suppressor protein p53 is a key regulatory protein of apoptotic and senescent signaling. It is often called „guardian of the genome“. Under normal conditions in healthy cells p53 levels are kept low by its ubiquitination with E3 ubiquitin-protein ligases – typically MDM2/HDM2 – and subsequent degradation. There are two major

pathways activating p53 in stressed or damaged cells. One of them is stress signaling-triggered phosphorylation of p53 which prevents p53 from binding to E3 ligases and p53 becomes stabilized. The other pathway is mediated by $p19^{ARF/INK4d}$, which sequesters ubiquitin-protein ligases MDM2/HDM2 and thereby prevents its binding to p53 and consequent p53 degradation (Wahl and Carr, 2001). p53 acts mainly as a transcription factor and regulates expression of its target genes – in case of senescence important role plays induction of expression of CDK inhibitor $p21^{WAF1/CIP1}$ (only p21 in following text) (Lee and Gu, 2010), which is the key mediator of p53-dependent senescence (Brown et al., 1997). p21 binds and inhibits activity of cyclin dependent kinases cdk1 (cdc2), cdk2 and cdk4 via direct binding to CDK-cyclin complexes and by mimicking ATP it inhibits its activity (Pavletich, 1999). p21 was also shown to bind and inhibit PCNA (proliferating cell nuclear antigen) protein, which is essential during DNA replication as a factor enhancing processivity of DNA polymerases δ and ε. p21 seems to have crucial role for the ability of cells to become senescent, as $p21^{-/-}$ cancer cells undergo apoptosis under same conditions that induce senescence in their $p21^{+/+}$ counterparts (Han et al., 2002). DDR and p53 activated pathways provide first but not the only barrier against growth and potential tumorigenic transformation of damaged cells.

The second senescence-inducing pathway is driven by p16-pRb axis, which is the major pathway controlling G1 to S transition (Stein et al., 1999). $p16^{ARF/INK4a}$ (only p16 in following text) acts as a CDK inhibitor, but in contrast to CDK inhibitors from the CIP/KIP family, p16 inhibits specifically G1 kinases CDK4 and CDK6 . p16 contains binding site only for CDK and not for cyclin, and upon binding to CDK it alters its conformation and prevents cyclin from its interaction-mediated activation of CDK (Pavletich, 1999). CDK4/6 inactivate retinoblastoma protein (pRb) by its phosphorylation. p16 then keep pRb in an active, hypophosphorylated state, in which pRb binds transcription factors from the E2F family preventing them from activating transcription of genes necessary for DNA replication and thus for cell cycle progression (Sherr and McCormick, 2002).

These two pathways are not isolated and there are crosstalks between them. Moreover, they usually act together to induce and maintain senescence in damaged cells. Also other CDK inhibitors ($p15^{INK4b}$, $p18^{INK4c}$, $p19^{ARF/INK4d}$, $p27^{KIP1}$ or $p57^{KIP2}$) may participate in inducing cell-cycle arrest and senescence. While inhibitors of INK4

family mediate G1 cell-cycle arrest through specific inhibition of CDK4 or CDK6, CIP/KIP inhibitors have broader range of action.

Depending on triggering signal cellular senescence can be divided into several groups.

- replicative senescence,
- drug-induced senescence,
- oncogene-induced senescence, and
- bacterial toxin-induced senescence.

They all are similar in the basic principle of their mechanism – they all are driven by irrepairable DNA damage which leads cells into senescence. While the replicative senescence can be naturally triggered in aged cells, other types are caused by unnatural or exogenous mechanisms and are sometimes called stress-induced premature senescence or accelerated senescence.

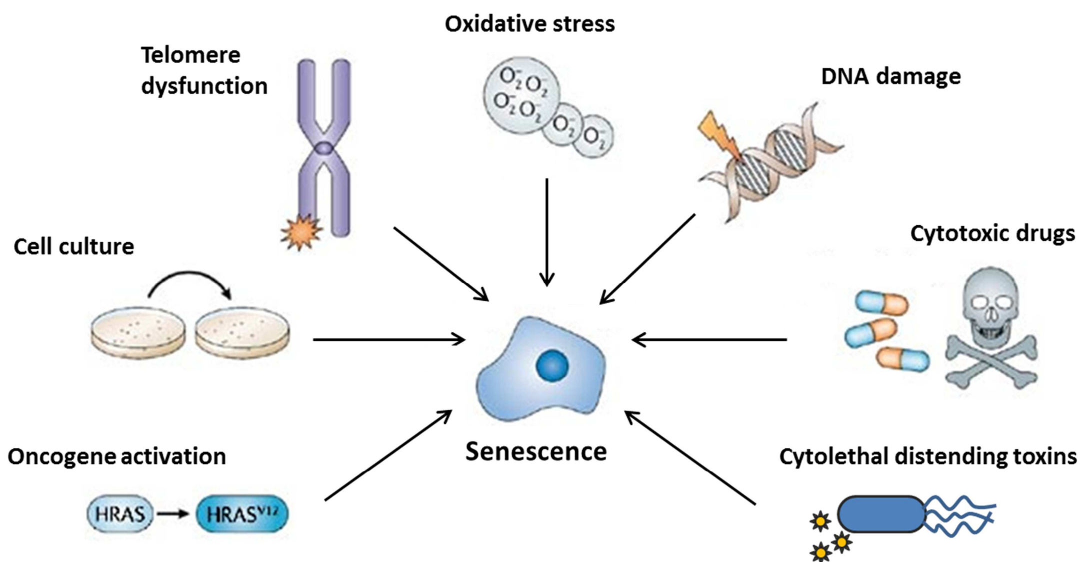


Figure 4. Senescence-inducing signalings. Various signals can drive cells to senescence – telomere attrition, cytotoxic drugs, cytolethal toxins, ROS or oncogene activation. Basis of their effect is the same for all of them – they all cause DNA damage and induce DDR (Collado and Serrano, 2006).

Replicative senescence

The phenomenon of senescence and senescent cells undergoing the replicative senescence were initially observed in the cultures of primary cells such as fibroblasts. Plausible molecular mechanism of triggering replicative senescence was proposed by Calvin B. Harley (Harley et al., 1990). The major senescence triggering signal was connected with ongoing shortening of chromosomes in dividing normal cells. This

phenomenon was initially described by Alexey Olovnikov and named as the „end replication problem“ (Olovnikov, 1971, 1973). Chromosomal DNA has to be replicated prior to each cell division. Due to the linear character of human chromosomes, DNA polymerases are not able to replicate DNA strands to the very end, and chromosomes lose 50-200 base pairs at the respective ends with each round of replication. Inevitably chromosomes are getting shorter after each cell division and specific terminal sequences - telomeres (long sequences of TTAGGG repeats, about 2-30 kb), function as “buffers” protecting essential internal chromosomal DNA.

As shown in Figure 5 telomeres together with various protein complexes form complicated structures – so called T-loops, which protect chromosomal ends from their recognition as double-strand or single-strand breaks by a DNA repair machinery. The very end of the chromosome is folded back and free single-strand 3´ overhang invades into the telomeric DNA double strand forming three-stranded structure (D-loop). Free single strand is then hidden and thus does not induce DDR signaling (Griffith et al., 1999).

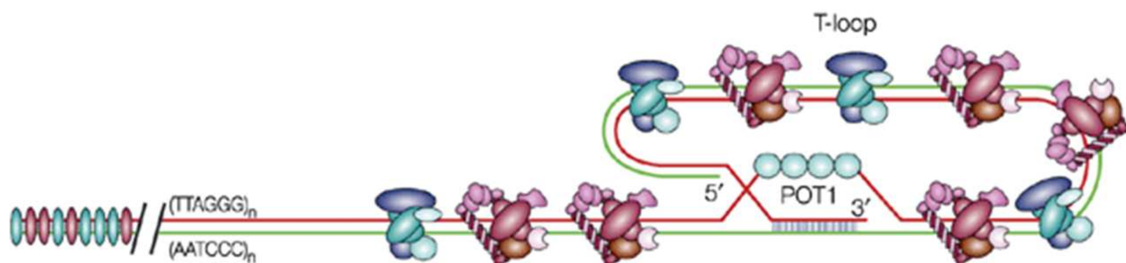


Figure 5. Structure of human telomeres. Human telomeres are long duplex TTAGGG repeats, that are folded back and form a T-loop. The 3´ overhang of telomeres invades into the double-strand telomeric repeats and forms a D-loop. Telomeres are complex structures and many proteins are bound to them. Main proteins binding telomeric repeats are two telomeric repeat-binding factors TRF1 (turquoise) and TRF2 (violet). They form dimers, bind duplex telomeric repeats and recruit other proteins to telomeres. (de Lange, 2004).

As telomeres shorten, the T-loop structures start to collapse. Former hypotheses proposed shortening of telomere per se as the major cause of cellular ageing and loss of replication capacity. However, the recent evidences suggest that the core of replicative senescence is the loss of telomere-associated factors (Herbig et al., 2004) and subsequent DDR, which is activated by absence of those factors (d'Adda di Fagagna et al., 2003; d'Adda di Fagagna et al., 2004).

Drug-induced senescence

Many drugs used in cancer treatment can induce senescence as well as apoptosis, and the final outcome usually depends on the intensity of imposed damage/stress and the intrinsic resistance of affected cells to cell death. Unlike replicative senescence, drug-induced senescence is not limited only to normal mortal cells, but it can be triggered in cancer cells as well. Many drugs and treatments can induce cellular senescence – for example gamma irradiation (Gewirtz et al., 2008), cisplatin (Wang et al., 1998), doxorubicine, taxol, vincristine, etoposide (Chang et al., 1999), camptothecin (Han et al., 2002), 5-azacytidine (Schwarze et al., 2005), hydroxyurea (Yeo et al., 2000), 5-bromodeoxyuridine (Michishita et al., 1999), ROS (Lu and Finkel, 2008), H_2O_2 (Duan et al., 2005) etc. Most of these drugs induce senescence through generation of DNA damage, some drugs (for example DNA methyltransferase inhibitor 5-azacytidine) induce senescence through changes in DNA structure.

Oncogene-induced senescence (OIS)

Oncogenes resp. their gene products can, when mutated or overexpressed, drive normal cells to tumorigenic transformation. Activation of one oncogene is usually not sufficient to transform primary cells into cancer cells and efficient transformation requires cooperation of more oncogenes as well as simultaneous inactivation of tumor suppressor (as p53 and pRb) pathways. Therefore, instead of malignant transformation excessive activation of single oncogenes drives primary cells into permanent cell cycle arrest or induces cell death. H-ras was the first oncogene described as potential activator of senescence in primary human cells. Expression of H-ras alone is not sufficient to trigger malignant transformation of human primary cells (Newbold and Overell, 1983), but in addition to inducing autophagic cell death (Elgendy et al., 2011), it drives cells into cell cycle arrest with typical markers of senescence – increased expression of p21 and p16 proteins, stabilization of p53 protein, decreased levels of hyperphosphorylated pRb and cyclin A, positive SA-β-gal staining or reduced BrdU incorporation (Serrano et al., 1997). The mechanism of inducing senescence by oncogene activation is not fully clear yet, but according to recently published data it is probably result of unrepairable DNA damage caused by combination of replicative and oxidative stress (Bartkova et al., 2006). Oncogene activation induces mitogenic response, which drives cells to undergo unscheduled replication that causes replicative stress and consequently DNA damage response (Di Micco et al., 2006; Kilbey et al., 2008). In addition to the excessive

replication, H-ras activation also increases intracellular levels of ROS, which contribute to oncogene-induced senescence, as some markers of senescence (expression of p21) are suppressed while cultivating H-ras-expressing cells in low oxygen (Lee et al., 1999). H-ras is the most often oncogene studied and mentioned in terms of senescence, but also other oncogenes are capable of inducing senescence – for example B-Raf (Tran and Rizos, 2013), c-mos, cdc6 (Bartkova et al., 2006), STAT5a (Mallette et al., 2007) etc. In relation to tumorigenesis, oncogene-induced senescence is considered as one of the anticancer barriers.

Bacterial toxin-induced senescence

Bacterial toxin-induced senescence is still the least described type of senescence. In 2010 it was shown that some toxins (cytolethal distending toxins) produced by pathogenic gram-negative bacteria *Haemophilus ducreyi* are able to induce not only apoptosis of affected cells but also senescence in cells that have survived primary intoxication. Intoxication of cells with cytolethal destending toxins causes DNA damage (detected by increased number of γH2AX, 53BP1 and P-ChK2 foci), induces expression of CDKs inhibitors (p21 and p16) and consequently makes cells leave the cell cycle. Arrested cells adopt senescence-like phenotype (changed morphology, SA-β-gal positive staining, increased number of PML bodies) including SASP (increased expression of proinflammatory cytokines IL-6, IL-8 and IL-24) (Blazkova et al., 2010). The primary cause of senescence is in this case again similar as for the above mentioned inducers of senescence – toxins cause genotoxic stress and senescence is the consequence of unrepairable DNA damage.

2.1.2. Characteristics and markers of senescence

Detection of senescent cells is not trivial, as there is no universal and fully reliable marker. Usually combination of more markers (for some of them see Figure 6) is required for proving the senescent phenotype.

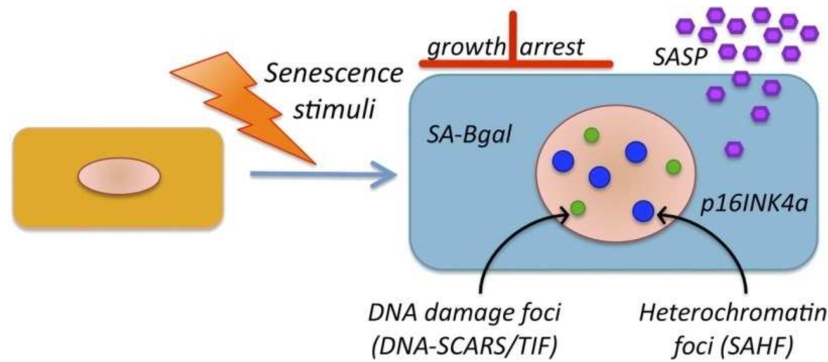


Figure 6. Selected features of senescent cells. Senescent cells differ from their proliferating counterparts in a number of ways. They change their morphology, leave the cell cycle, express inhibitors of CDKs and increase expression of SA-β-galactosidase. Senescent cells express and secrete numerous cytokines, chemokines and growth factors (SASP). In their foci containing heterochromatin (SAHF) and DNA damage-related proteins (DNA-SCARS). (Rodier and Campisi, 2011).

Distinquished features of senescent cells:

a) **Morphology.** The most obvious characteristic of senescent cells is their changed morphology – they become larger, flatter and multinucleated. Some senescent cells show extensive vacuolization caused by the endoplasmatic reticulum-associated unfolded protein response and enhanced macroautophagy (Denoyelle et al., 2006).
b) **SA-β-gal staining.** The first fairly specific staining of senescent cells was introduced in 1995 (Dimri et al., 1995). This staining detects activity of lysosomal senescence-associated β-galactosidase (SA-β-gal). Under optimal pH (pH 4) this enzyme is active and detectable in both senescent and proliferating cells. But in senescent cells likely due to an expansion of lysosomal compartment its activity increases and its activity could be detectable under suboptimal conditions (pH 6) (Kurz et al., 2000) – see later.
c) **Staining of proliferating cells**. Senescent cells cease to proliferate and thus the absence of proliferation markers – such as Ki67 (Scholzen and Gerdes, 2000), PCNA, or quantification of BrdU or ^{3}H-thymidine incorporation can be used to distinquish them from proliferating cells.
d) **Altered expresion of cell-cycle related genes**. Cell cycle arrest is one of the major signs of senescence. Altered expression of cell-cycle activators and inhibitors can be then used as a marker of senescence as well. As already mentioned, senescent cells exhibit increased expression of CDKs inhibitors – mostly p21$^{CIP1/WAF1}$ (Chang et al., 2002) or p16 (Rayess et al., 2012). They also show decrease in the expression of cell cycle activators like PCNA, histones (Pang and Chen, 1994), cyclin A, cyclin B

(Stein et al., 1991), cyclin D1, topoisomerase IIα, MAD2 (Chang et al., 2002) or c-fos (Seshadri and Campisi, 1990).

e) **DNA damage – DNA-SCARS**. Senescence is associated with persistent DNA damage, which is characterized by activated kinases ATM/Chk2, ATR/Chk1, phosphorylated histone H2AX (γH2AX) etc. – that are accumulated nuclear foci called DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence) (d'Adda di Fagagna et al., 2003; Rodier et al., 2011).

f) **Senescence associated heterochromatin foci (SAHF)**. Senescent cells can be also identified by enhanced presence of heterochromatin foci that encompase silenced genes (for example some E2F target genes). Markers of those foci are various proteins typical for heterochromatin (for example heterochromatin protein-1) and histone modifications, typically either present in heterochromatin such as H3K9 trimethylation or absent as H3K4 methylation or H3K9 acetylation (Narita et al., 2003).

g) **PML bodies**. Senescent cells show increased number of PML bodies in their nuclei (Janderova-Rossmeislova et al., 2007). PML bodies are subnuclear structures, that recruit, activate or degrade various proteins related to different processes like transcription, apoptosis, senescence, response to DNA damage, stem cell self-renewal etc. (Lallemand-Breitenbach and de The, 2010).

h) **Increased number of lysosomal structures**. Senescent cells expand their lysosomal compartment and these changes are detectable by flow cytometry (higher side scatter profile) or Acridine Orange staining (Kurz et al., 2000). Increased number of lysosomes could be related to higher demand of senescent cells on chromatin remodelling (Ivanov et al., 2013) and recycling of accumulated non-degradable proteins and organelles by macroautophagy (Brunk and Terman, 2002).

i) **Senescence associated secretory phenotype (SASP)**. Among the overall change in the gene expression senescent cells show considerable increase in the expression of various inflammatory and immune-modulating cytokines and chemokines, growth factors, survival factors etc. This phenomenon is called senescence associated secretory phenotype (SASP).

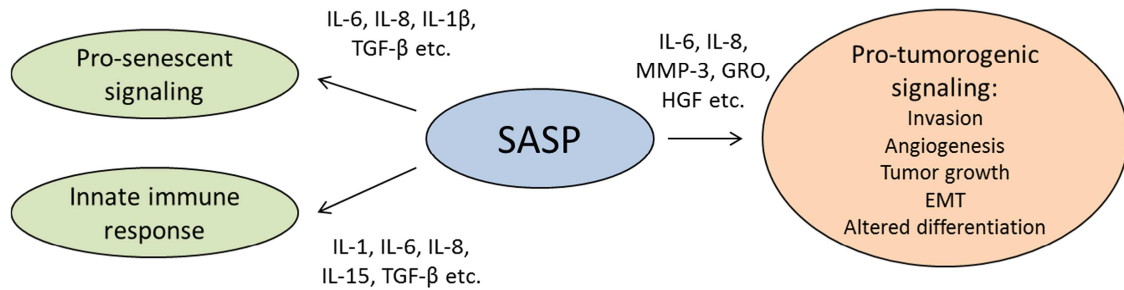


Figure 7. Senescence associated secretory phenotype. Senescent cells reinforce expression of many cytokines, chemokines or growth factors that can both suppress and promote tumor growth. (Acosta et al., 2013; Coppe et al., 2008; Davalos et al., 2010; Fumagalli and d'Adda di Fagagna, 2009; Krtolica et al., 2001; Liu and Hornsby, 2007)

Senescent cells express and secrete molecules that have wide influence both on cells themselves (via autocrine or juxtacrine signaling) and their neighborhood (via paracrine signaling). SASP can suppress tumor progression through the promotion of senescence in neighboring cells (IL-6, IL-8, IL-1β, TGF-β etc) or through the activation of immune response and subsequent clearance of senescent and tumor cells (IL-1, IL-6, IL-8, IL-15, TGF-β etc.). In contrary, these and other factors expressed by senescent cells (IL-6, IL-8, MMP-3, vascular endothelial factor – VEGF, hepatocyte growth factor – HGF and others.) can promote growth and tumorigenic progression of premalignant cells and formation of metastasis (Acosta et al., 2013; Coppe et al., 2006; Coppe et al., 2008; Davalos et al., 2010; Fumagalli and d'Adda di Fagagna, 2009; Hoare and Narita, 2013; Krtolica et al., 2001; Liu and Hornsby, 2007).

2.1.3. Physiological role of senescence

Role of senescence in physiological and non-cancer processes has not been well understood yet. Well-described physiological and also beneficial role of senescent cells is associated with the liver fibrosis (Krizhanovsky et al., 2008). Increased secretion of matrix metalloproteinases by senescent cells stimulates degradation of extracellular matrix and reduces fibrosis. Recently published articles also point to a role of senescence in murine embryonic development. Senescent cells are present through the mouse embryo and their number decreases as they undergo macrophage-dependent clearance (Munoz-Espin et al., 2013; Storer et al., 2013). They also participate in maintaing balance between different cell populations during the development of endolymphatic sac (Munoz-Espin et al., 2013). Disrupted senescent signaling can lead to defective development of limbs (Storer et al., 2013), defective development of vagina and reduced fertility in mice (Munoz-Espin et al., 2013).

2.1.4. Role of senescence in carcinogenesis

In addition to uncovering physiological role of senescence, significant attention is turned to a role of senescence in carcinogenesis and cancer therapy. First evidence showing correlation between senescence and anticancer chemotherapy *in vivo* was documented in 2002 in a study analyzing SA-β-gal positive breast tumors in sets of untreated and treated patients. While in untreated patients only 2 out of 20 samples were SA-β-gal positive, in case of treated patients 42 % (15 out of 36 tumors) of tumors were SA-β-gal positive (te Poele et al., 2002).

In relation to carcinogenesis, senescence is usually characterized as a barrier, which prevents further proliferation of damaged – and so potentionally dangerous – cells. It can play a role of an alternative mechanism of tumor inhibition in case of disrupted apoptotic pathways, as it was shown in murine tumors with Bcl-2-mediated apoptotic block. Mice with cells that could enter senescence-activated cell cycle arrest had better prognosis than the mice with cells defective in pro-senescence signaling (Schmitt et al., 2002). Senescence was thus considered as an alternative outcome of successful cancer treatment. Current cancer therapy is still mainly based on cytotoxic treatment – either using drugs or ionizing radioation. Its aim is complete destruction of tumor mass, but such extensive damage has broad and severe side effects for patients. Ability of tumor cells to enter senescence under lower – and so less toxic – concentrations of drugs has

brought an idea, that some tumors could be treated with lower doses of chemotherapeutics and less severe side effects (Desai and Stadler, 2006; Martin and Schilder, 2006). Such cytostatic treatment would not lead to complete tumor clearance, but it could stop further tumor growth and thus work even on tumors that are not possible to target with established treatment. Moreover, senescent cells produce factors that induce senescence in neighboring cells and inhibit further tumor growth (Hodny et al., 2010) and secrete inflammatory cytokines activating innate immune response leading to tumor clearance.

Recent evidence proposed also a role of oncogene-induced senescence during the induction of pluripotent stem (iPS) cells, as cellular backup programs (particularly $p16^{INK4A/ARF}$ and $p53\text{-}p21^{CIP1/WAF1}$) counteract conversion of primary cells into pluripotent stem cells and so they become a barrier to the cell reprogramming (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009). And as cancer stem cells probably arise from similar processes like iPS cells, cellular senescence can inhibit tumor growth by suppressing generation of cancer stem cells as well (Krizhanovsky and Lowe, 2009) – see Figure 8.

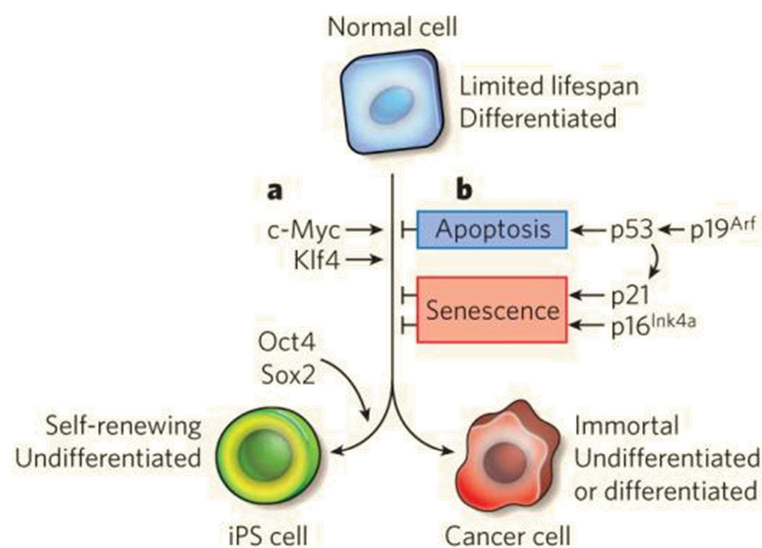


Figure 8. Transformation of normal cells into iPS cells and cancer cells. Normal cells can be reprogrammed into induced pluripotent stem (iPS) cells. Transcription factors c-Myc, Klf4, Oct4 and Sox2 promote iPS cells formation, c-Myc and Klf4 play role also in transforming normal cells into cancer cells. In contrast tumor suppressor p53 function as a barrier which limits cell transformation through inducing apoptosis or senescence (Krizhanovsky and Lowe, 2009).

In contrast, recent data also point to protumorigenic effects of senescent cells, usually related to their senescence-associated secretom producing variety of factors that may promote tumor growth, invasion and angiogenesis as well as inhibit tumor cell

proliferation. Besides inducing senescence in neighboring cells, senescent cells may induce proliferation in bystander cells as well (Ewald et al., 2008). Moreover, senescent cells seem to promote proliferation of premalignant and malignant epithelial cells and so they may enhance formation of tumors in mice (Capparelli et al., 2012; Krtolica et al., 2001). In contrast to apoptotic cells, senescent cells may persist in the tissue for a long time and although they do not seem to be able to reenter the cell cycle, they may have rather detrimental effect both on tumor formation and its further progression.

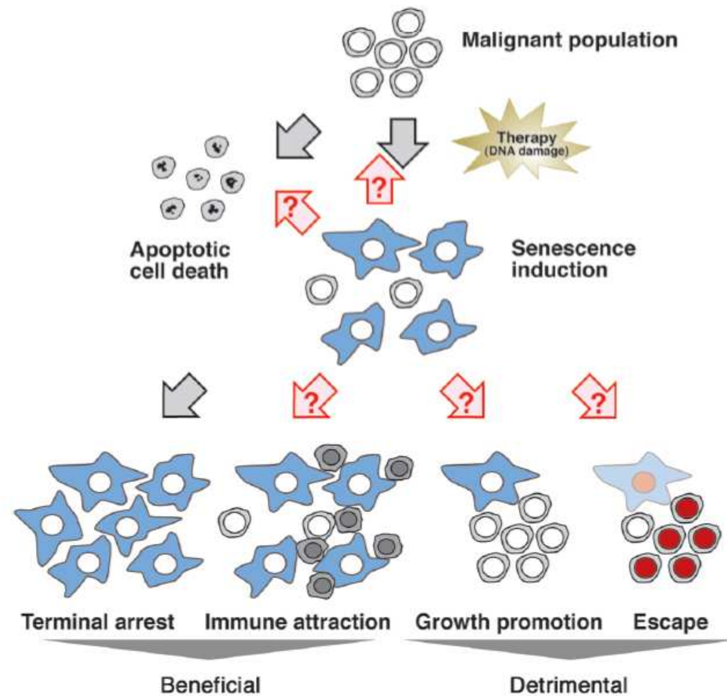


Figure 9. Role of senescence in carcinogenesis. Role of senescence can be both beneficial (growth arrest of damaged cells, activation and attraction of immune system) and detrimental (growth and angiogenesis promotion, possible escape from senescence through mutations) for the final outcome of anti-tumour therapy. (Kahlem et al., 2004).

1.1. Apoptosis

The process of programmed cell death was firstly described by Carl Vogt already in 1842. He observed disappearance of some cells in the vertebrae formation during the development of midwife toad. But the term „apoptosis" (from greek words *apo* – from and *ptosis* – to fall) was firstly introduced by british scientists Kerr, Wyllie and Currie in 1972. They proposed this term for „mechanism of controlled cell deletion, which appears to play a complementary but opposite role to mitosis in the regulation of animal cell populations" (Kerr et al., 1972). Apoptosis then became studied more intensively and in 2002 Nobel Prize was awarded to S. Brenner, H. R. Horvitz and J. E. Sulston for their discoveries on programmed cell death.

Major function of apoptosis is the removal of redundant, damaged and potentially dangerous cells. Apoptosis plays an essential role in embryonic development, regulation and termination of the immune response and elimination of excessive and damaged cells. Thus taking in account its universal importance, each deregulation of apoptosis (either suppression or hyperactivation of apoptosis) can lead to severe diseases – for example cancer, autoimmune diseases or neurological disorders.

Apoptosis can be induced by different stimuli, both external (toxins, cytokines, loss of intercellular contact, loss of contact with extracellular matrix, lack of pro-survival signals, etc.) and internal (stress, DNA damage, reactive oxygene species, activation of oncogenes etc.). Apoptotic cell changes its morphology, detaches from surrounding cells or extracellular matrix and shrinks. Hallmarks of terminal stages of apoptosis are plasma membrane blebbing, appearance of apoptotic bodies, chromatin condensation and DNA fragmentation. Apoptotic bodies consist of cytoplasm, nuclear fragments and mainly intact organelles. Finally, cell is completely desintegrated and removed by phagocytosis by other cells (macrophages, parenchymal cells or neoplastic cells). Whole process of apoptosis is not accompanied by imflammation, which one of major differences between apoptosis and necrosis. In contrast to necrosis, induction and progression of apoptosis depends on activation and activity of specific proteases – caspases.

As already mentioned, apoptosis can be triggered in principle by two main pathways, so called the intrinsic and extrinsic apoptotic pathways – see Figure 10.

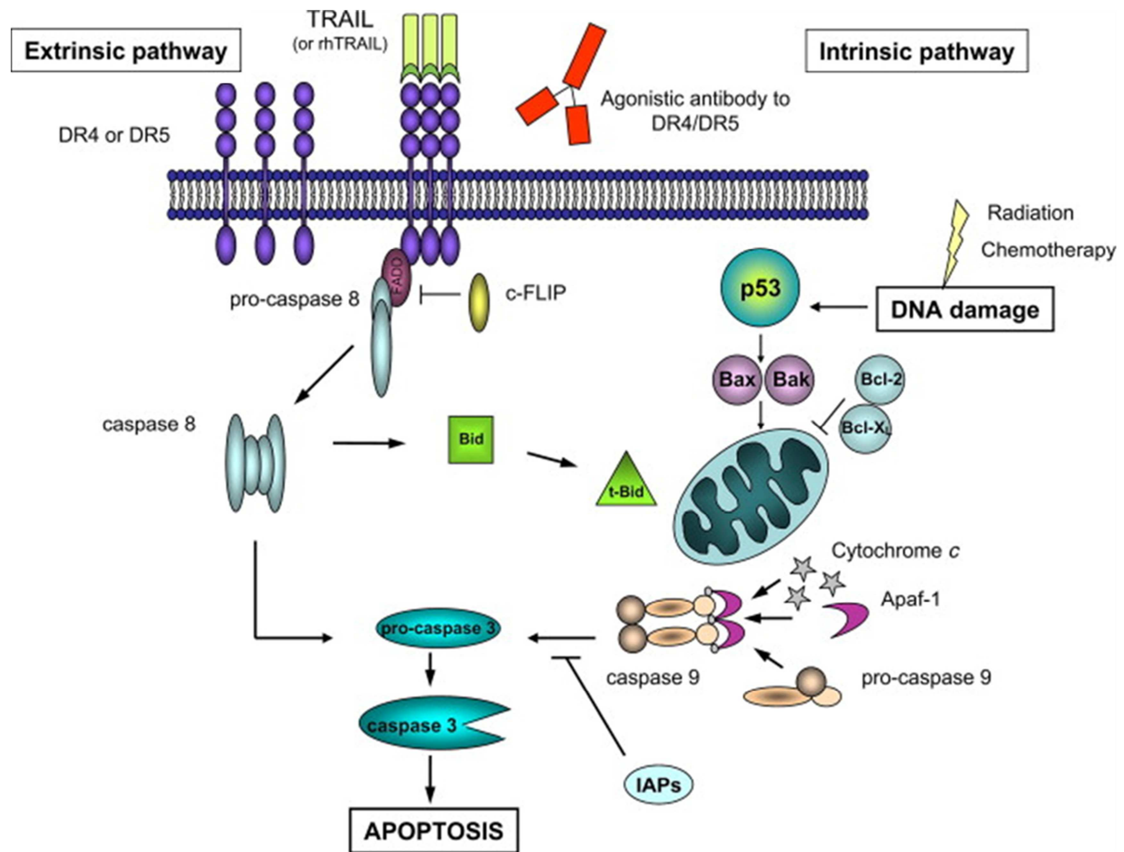


Figure 10. Apoptotic pathways. Extrinsic apoptotic pathway is triggered by binding of the the death ligand to its specific receptor, which leads to its activation, assembly of the DISC and activation of the initiator procaspase-8. Intrinsic apoptotic pathway is controlled by mitochondria. Upon specific signal, proapoptotic BH3-only proteins of the Bcl-2 family are translocated to mitochondria, induce opening of the outer mitochondrial membrane by Bax/Bak channels and subsequent release of the cytochrome c and other proteins. Cyt c then induces formation of the apoptosome – multiprotein complex mediating activation of the initiator procaspase-9. Both pathways converge on activation of the same effector caspases. There is one more upstream crosstalk between extrinsic and intrinsic pathway – initiator caspase-8 cleaves and activates pro-apoptotic protein Bid, which translocates to the mitochondria and it is involved in the release of cytochrome c (Duiker et al., 2006).

2.1.5. Intrinsic apoptotic pathways

Activation of the intrinsic apoptotic pathway is triggered by intracellular sensors of damaged DNA, organelles, oxidative stress, loss of cell-to-cell or cell-to-matrix contact etc. Intrinsic pathway is controlled by anti-apoptotic and pro-apoptotic proteins from the Bcl-2 (B-cell lymphoma 2) family. The final outcome of their action is the mitochondrial outer membrane permeabilization (MOMP), which is executed by two major pro-apoptotic proteins – Bax and Bak – that homomultimerize and form pores in the outer membrane. Under normal conditions Bax and Bak channel-forming activities are controlled by anti-apoptotic proteins of the Bcl-2 family (Bcl-2, Bcl-xL and Mcl-1) and kept in inactive state. Upon activation of apoptosis, function of anti-apoptotic Bcl-2

proteins is blocked by increased expression, mobilization or activity of pro-apoptotic proteins of the Bcl-2 family – so called BH3-only proteins (Bim, t-Bid, Bad, Puma, Noxa etc.) (Youle and Strasser, 2008). Some of these proteins (Bim or tBid) also enhance pores-forming activities of Bax and Bak.

MOMP enables release of cytochrome-c from mitochondrial intermembrane space. Cytochrome-c, Apaf1 and dATP then form complex (apoptosome), which binds procaspase-9 and enables its activation through autocatalytic cleavage. Active caspase-9 then cleaves executionary caspase-3, -6 and -7. Together with cytochrome-c also other proapoptotic proteins are released – among them Smac/DIABLO, which binds to, blocks and inactivates caspase inhibitors from the IAP family (Tait and Green, 2010). In addition to cytochrome c and Smac/DIABLO, some other proteins are released from the mitochondrial intermebrane space – for example endonuclease G or AIF that subsequently translocate to nucleus where they contribute to cleavage of chromosomal DNA (Arnoult et al., 2003).

2.1.6. Extrinsic apoptotic pathways

Typical and best-known activators of the extrinsic apoptotic pathway are death ligands from the Tumor Necrosis Factor α (TNFα) family.

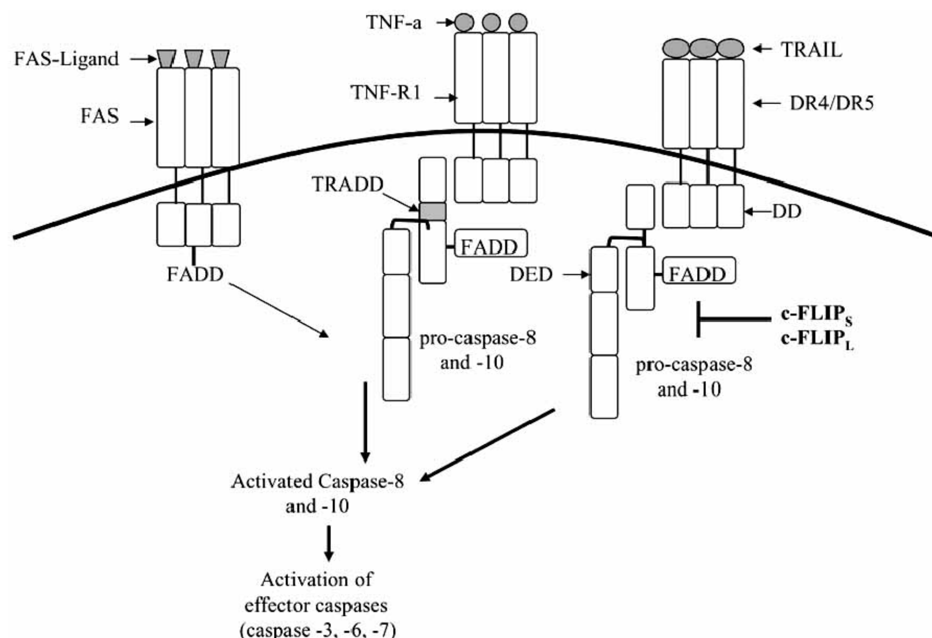


Figure 11. Death receptor pathways. Upon binding of death ligands to their death receptors adaptor proteins TRADD or FADD are recruited to intracellular part of death receptors via their death domains (DD). TRADD/FADD proteins recruit initiator procaspases via their death effector domains (DED) and form DISC. Activated initiator caspases dissociate from DISC and activate effector caspases (Safa et al., 2008).

The apoptotic signal is transduced into the cell via specific transmembrane death receptors from the Tumor Necrosis Factor Receptor (TNFR) family – DR3, DR4/TRAIL-R1, DR5/TRAIL-R2, DR6, FasR/CD95 and TNFR-1. There are also four decoy receptors (DcR1, DcR2, DcR3 and osteoprotegerin), that bind death ligands, but do not activate the apoptotic pathway in target cells (Locksley et al., 2001).

Upon binding of ligands, receptors cluster and rearrange their intracellular parts (Figure 11). Cytoplasmic adaptor proteins (FADD or TRADD) are recruited to the intracellular parts via their death domains (DDs) and initiate formation of death inducing complex (DISC). The major purpose of the DISC formation is clustering-enhanced activation of procaspase-8 through its autocatalytic cleavage. Procaspase-8 is bound to the TRADD/FADD via its death effector domain (DED).

In addition to the receptor, adaptor protein and caspase-8 there is one more important facultative component of the DISC complex – cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (so called c-FLIP/CFLAR), which binds the DISC complex via its DED and predominantly (though not exclusively) inhibits selfprocessing and activation of procaspase-8 (Figure 12).

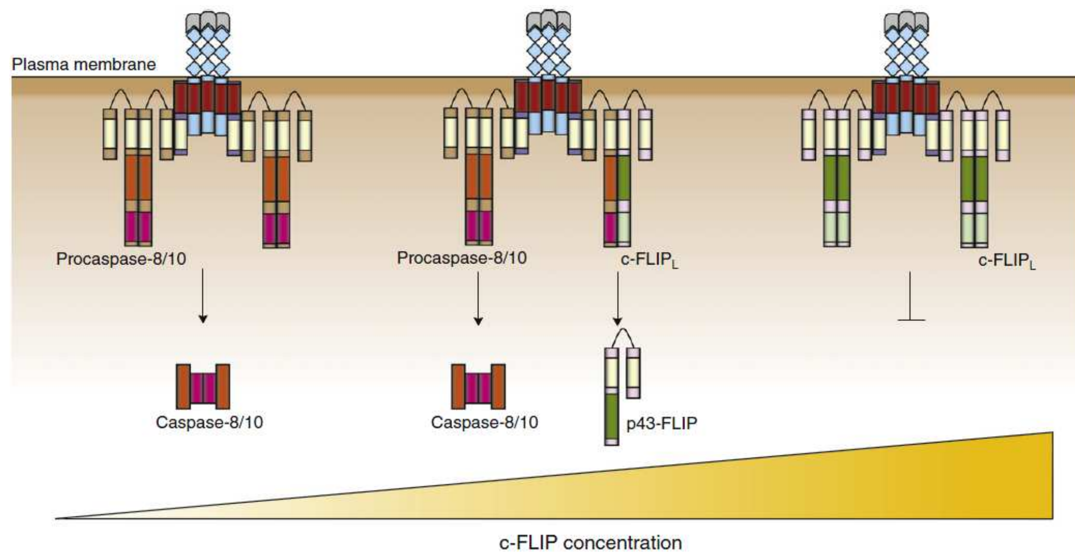


Figure 12. Role of c-FLIP in apoptotic signaling. Participation of c-FLIP in apoptotic signaling depends also on its intracellular concentration. While low concentration of c-FLIP does not prevent procaspase-8 from being cleaved and could enhance its self-processing, high c-FLIP levels prevent procaspase-8 binding to the DISC, blocks its activation and prevents induction of apoptotic pathway. Also intermediate levels of c-FLIP allow procaspase-8 activation, but together with that c-FLIP itself is cleaved and induces non-apoptotic NFκB signaling (Ozturk et al., 2012).

Active caspase-8 then processes and activates effector procaspases-3, -6 or -7. This is the point (but not the only one), where intrinsic and extrinsic pathways meet each other. Effector caspases cleave many proteins that normally maintain structure of the

cytoskeleton and nucleus, as well as proteins involved in the transduction of apoptotic signaling. Major proteins cleaved by effector caspases are structural cytoplasmic and nuclear proteins (actin, gelsolin, β-catenin, spectrin, lamin A/B etc.), and proteins directly involved in apoptotic signaling – other caspases, proteins of the Bcl-2 family (Bid, Bad or Bax), inhibitors of apoptosis (c-FLIP or XIAP), inhibitor of nuclease CAD/DFF40 (ICAD/DFF45), inhibitors of p53 (MDM2 and MDMX), antiapoptotic transcription factors (NFκB, STAT1 etc.). Last but not least caspases cleave proteins involved in DNA repair and metobolism (PARP, DNA-dependent protein kinases, RAD51, DNA topoisomerases etc.), various protein kinases (PKC, MEKK1, Raf1, Akt1, Wee1 etc.), proteins involved in regulation of the cell cycle (p21, pRb or p27) etc. (Earnshaw et al., 1999).

2.2. Crosstalk between senescence and apoptosis in cancer

Both apoptosis and senescence are results of cellular response to stress. They both eliminate, respectively suppress, the growth of damaged and potentially dangerous cells, and so they are part of tumor-suppressive mechanism.

But the crosstalk between senescence and apoptosis remains poorly understood. Final decission if cells tend to undergo senescence or apoptosis depends on a number of factors, but most probably the major role is played by:

- **Level of stress**. One of the most important factors is the level of stress which cells are exposed to. Cells are usually able to respond in both ways – apoptosis and senescence. Under lower stress cells tend to enter senescence, while if the level of stress or damage exceeds certain treshhold cells undergo apoptosis (Han et al., 2002; Rebbaa et al., 2006).
- **Deregulation of senescence or apoptotic pathways**. Some proteins seem to be essential for the induction of senescence – for example CDK inhibitor p21. While majority of human colon cancer cells can enter senescence after exposure to low concentration of camptothecin, same cancer cells lacking p21 protein fail to enter senescence after the same treatment and undergo apoptosis as at higher CPT concentration (Han et al., 2002). Overexpression of some anti- or pro-apoptotic proteins may change cellular response as well – for example cells with Bcl-2 mediated block of apoptotic pathway undergo senescence instead of apoptosis in response to chemotherapy (Schmitt et al., 2002).

In general senescent cells are considered being more resistant to apoptosis than normal proliferating cells. That would be an explanation why the number of senesent cells increases with age. For example senescent human fibroblasts acquire resistence to ceramide-induced apoptosis (Hampel et al., 2004), oxidative stress-induced apoptosis (Sanders et al., 2013) or staurosporine-induced apoptosis (Tepper et al., 2000). But at the same time senescent human fibroblasts are more susceptible to Fas-mediated apoptosis (Tepper et al., 2000). However, we can not generalize, that the response to certain apoptogens is suppressed or enhanced in senescent cells. For example while senescent human fibroblasts become more resistant to ceramide induced apoptosis, senescent human endothelial cells are more prone to ceramides than their proliferating counterparts (Hampel et al., 2004).

Higher resistence or sensitivity of senescent cells to apoptogens has not been fully understood yet, but a most probable cause of different sensitivity of senescent cells is changed expression or modification of proteins, that are somehow related to apoptotic signaling.

- **Changed expression of pro-apoptotic or anti-apoptotic proteins**. Constitutive change in the expression of anti-apoptotic or pro-apoptotic proteins may influence cellular response to apoptotic signals. For example senescent human fibroblasts, that are more resistant to various apoptogens, show increased expression of anti-apoptotic Bcl-2 protein and lower expression of pro-apoptotic Bax protein. Changed ratio of Bcl-2 and Bax expression in favor of the anti-apoptotic protein is epigenetically regulated at the level of changed activity of histone methyltransferases and histone acetyltransferases in senescent fibroblasts (Sanders et al., 2013).
- **Changed expression of death receptors.** In addition to the intracellular anti- and pro-apoptotic proteins expression and posttranslational modification of death receptors could play a role in the sensitivity of senescent cells to apoptosis. Two recent publications document increased expression of Fas receptor in senescent cancer cells (Crescenzi et al., 2011; Jeon and Boo, 2013) and their correspondigly higher sensitivity to FasL-induced apoptosis. One article also described elevated expression of DR4 in senescent cells (Mendoza et al., 2008).

3. Aims of the thesis

The major aim of this thesis was to study effects of senescence and senescence-like phenotype on response of cancer cells to different apoptogens and compare it with the response of their proliferating counterparts.

Specific aims of this thesis are:

1. Induction of drug-induced senescence in different cancer cell lines and preparation of stable population of senescent cells for biochemical analyses.
2. Analysis and comparison of apoptotic response in senescent vs proliferating cancer cell lines triggered with agents inducing apoptosis through distinct mechanisms – activation of death receptor pathways (FasL, TRAIL), ROS production through destabilization of mitochondrial electron transport chain (mVES), induction of DNA damage via inhibition of DNA topoisomerases (camptothecin).
3. Preparation of cancer cell lines with inducible expression of senescence-associated cell cycle inhibitors p21 and p16.
4. Analysis and comparison of apoptotic response in cells with upregulated expression of p21 and p16 and their proliferating counterparts.

4. Materials and methods

4.1. Mammalian cell culture

Human cancer cell lines PANC-1 and H28 were cultured in appropriate medium – DMEM (in case of PANC-1) or RPMI (in case of H28) – supplemented with 10% FBS and antibiotics. Cells were grown under 5% CO_2 atmosphere at 37°C and maintained at low to medium confluency. All cells were obtained from ATCC.

PANC-1 cells are pancreatic epitheliod carcinoma cells. They contain mutated p53 gene in its DNA-binding domain (gene: c.818G>A; protein: p.R273H). H28 cells are mesothelioma cells (stage 4) derived from pleural effusion that express wt p53.

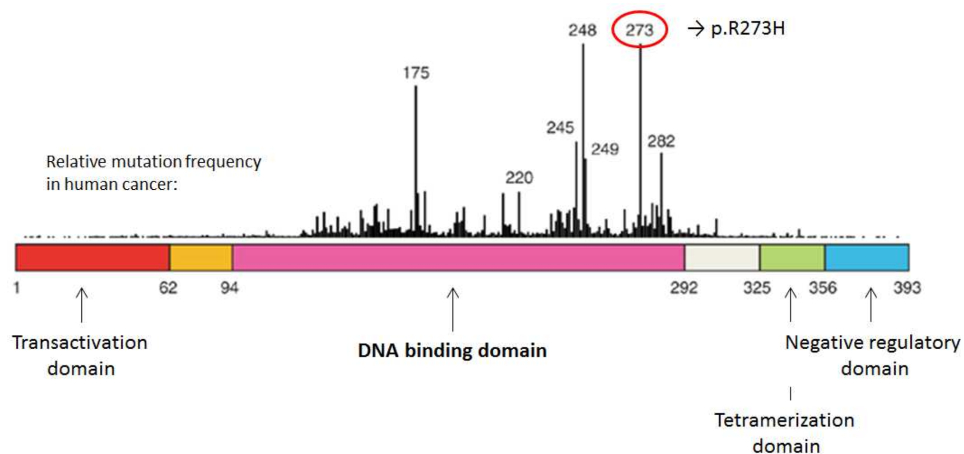


Figure 13. Tumor suppressor protein p53, its structure and mutations. PANC-1 cells have point mutation R273H in the DNA binding domain of p53 gene. Arginine 273 is one of the most frequently mutated aminoacids in p53 in human cancer (Joerger and Fersht, 2007).

4.2. Induction of senescence and detection of senescent cells

Senescence was induced by the DNA replication stress agent 5-bromo-2'-deoxyuridine (BrdU) (Masterson and O'Dea, 2007; Michishita et al., 1999). Cells were seeded on appropriate cell culture dishes at the average confluence ($3x10^3$ cells/cm^2) and subsequently they were treated with 50µM BrdU (H28 cells) or 100µM BrdU (PANC-1) for 8 days.

For the detection of senescence we used set of different morphologic and biochemical markers – change (flattening) of cellular shape by phase contrast microscopy, SA-β-gal staining, Ki67 staining and γH2AX staining using indirect immunofluorescence and semi-quantification of p53, p21 and p16 expression using indirect immunofluorescence and Western Bloting.

4.3. SA-β-gal staining

Cells were cultivated on 12mm round coverslips, washed twice with PBS, fixed with 0,5% glutaraldehyde in PBS for 11 minutes at room temperature and washed twice with PBS+1mM $MgCl_2$ solution (adjusted to pH 6,0) at the room temperature. Cells were then incubated in X-gal staining solution containing 40mM citric acid/Na phosphate buffer, 5mM $K_4[Fe(CN)_6]$ $3H_2O$, 5mM $K_3[Fe(CN)_6]$, 150mM NaCl, 2mM $MgCl_2$ and 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside at 37°C for 48 hours, washed three times with PBS, mounted in Mowiol (Debacq-Chainiaux et al., 2009; Dimri et al., 1995) and viewed by a fluorescence microscope (Leica DMRXA).

4.4. Indirect immunofluorescence

Cells were cultivated on 12mm round coverslips. They were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0,25% Triton X-100 for 10 minutes. Cells were then washed with PBS and incubated in DMEM + 10% FBS for 1 hour – serum (FBS) was used as a blocking agent. After that cells were incubated with primary antibody (diluted in DMEM + 10% FBS) overnight at 4°C, washed with PBS/0,01% Triton X-100, then washed with fresh PBS and incubated with the secondary antibody (diluted in DMEM + 10% FBS) for 1 hour at room temperature. Coverslips with cells were then washed with PBS/0,01% Triton X-100, then with PBS, incubated with 4',6-diamidino- 2-phenylindole (DAPI) and again washed with PBS. Finally coverslips were mounted in Mowiol and viewed by a fluorescence microscope (Leica DMRXA).

List of antibodies used for immunodetection (immunocytofluorescence):

p21 (DCS-60) – Santa Cruz, sc-56335
Ki67 (MM1) – Diagnostic Biosystems, Mob 237

4.5. SDS-PAGE and Western Blotting

Cells on 6- or 12-well culture dishes were washed twice with PBS, lysed using 2x SDS lysis buffer lacking bromphenolblue and reducing agent and transferred into 1,5ml eppendorf tubes. Cell lysates were heated at 96°C for 3 minutes and filtered through 200μl filter tips into new eppendorf tubes. Concentration of total protein was measured by bicinchoninic acid assay (BCA) using BCA Protein Assay Kit (Pierce

Biotechnology) following manufacturer´s instructions. All samples were then diluted in 2x lysis buffer to the same concentration (usually 2 mg/ml). Dithiothreitol and bromophenol blue were added to the lysates to final 0.5% respectively 0.02% concentrations and then these lysates were denaturated at 96°C for 10 minutes.

Equal amounts of proteins (20-40 μg) were loaded into the wells of 12% or 15% acrylamide gel and resolved by SDS-PAGE. Proteins from the gel were transferred to the nitrocellulose membrane (Amersham™ Hybond ECL, GE Healthcare Life Sciences) using Hoefer SemiPhor semi-dry transfer unit (20 V, (0.8 x membrane surface in cm^2) mA, 85 min).

Efficiency of protein transfer was checked via staining the total proteins on the membrane using Ponceau red and the membrane was subsequently blocked in 5% non-fat milk at the room temperature for at least 1 hour. Membrane was then incubated overnight at 4°C with primary antibody diluted in PBS/Tween with 1% non-fat milk. After incubation, the membrane was washed three times in PBS/Tween (10 minutes each wash), incubated with the appropriate HRP-conjugated secondary antibody diluted in PBS/Tween with 1% non-fat milk for one hour and then again washed four times in PBS/Tween for 10 minutes each wash. Proteins were finally detected using enhanced chemiluminescent system (Pierce) according to manufacturer´s instructions.

List of antibodies used for immunodetection (Western blotting):

actin (C-11) – Santa Cruz, sc-1615
α-tubulin – Sanbio, MON4009
Bcl-xL – Cell Signaling, 2764
Bcl-2 (50E3) – Cell Signaling, 2870S
Mcl-1 – BD, 559027
Bim (C34C5) – Cell Signaling, 2933
Bid – Cell Signaling, 2002
Bax – Cell Signaling, 2772
XIAP – BD Trans-Lab, 610762
DR4 – ABCAM, ab8414
DR5 – Sigma, D3938
FLIP (NF-6) – Enzo, ALX-804-428
p16 (C-20) – Santa Cruz, sc-468
p21 (DCS-60) – Santa Cruz, sc-56335
p53 (DO-1) – Santa Cruz, sc-126

4.6. Detection and quantification of apoptosis

Cells were grown in 24-well plates (proliferating cells) and 12-well plates (senescent cells). For the analysis of apoptosis in senescent cells cancer cells were seeded 8 days prior to induction of apoptosis and treated with BrdU as described in the chapter 4.2. Control proliferating cells were seeded 2 days prior to induction of apoptosis to reach approx. 70% confluency before being treated with apoptogens. Apoptotic cells were detected and quantified using Annexin V-FITC/Hoechst 33258 staining and flow cytometry (Koopman et al., 1994). Cells were harvested by trypsinization and divided equally into wells of 96-well plate (WP, 1.5 x 10^5 cells/well). Cells in the 96-WP were centrifuged (5810R centrifuge, 200 x g, 4°C, 4 min), each sample was washed twice in 200 µl of ice-cold PBS and once in 50 µl of 1x Annexin V-binding Buffer (Apronex), centrifuged and resuspended in 100µl of 1x Annexin V-binding Buffer with diluted 1 µl of FITC conjugated Annexin V (Apronex). Cells were incubated on ice in dark for 20 minutes. Finally, for the determination of late apoptotic/dead cells Hoechst 33258 was added to the final concentration of 2 µg/ml. Samples were analyzed by flow cytometry on LSRII (BD Biosiences) and quantified using FlowJo software.

4.7. Cell surface staining of death receptors

Cells were grown on 6cm plates and prepared for analysis as in the chapter 4.6. Cells were trypsinized, harvested and divided equally into 96-well plate (1,5 x 10^5 cells/well). Cells in the 96-WP were centrifuged (5810R centrifuge, 200 x g, 4°C, 4 min), washed in 200 µl of ice-cold PBS, centrifuged, washed in 200 µl of ice-cold PBS with 0,2% gelatine and 0,01% NaN_3 (PBS-GA), centrifuged again. Cells were then resuspended in 25 µl of ice cold PBS-GA with appropriate primary antibodies (10 µg/ml) and incubated on ice for 30 minutes. After that cells were washed twice in 200 µl of PBS-GA and centrifuged. Cells were resuspended in 20 µl of PBS-GA with appropriate fluorochrome-conjugated secondary antibodies and incubated on ice in dark for 20 minutes.

After incubation, cells were washed twice with 200 µl of PBS-GA, centrifuged and pellets were resuspended in 90 µl of ice cold PBS-GA. Finally 10 µl of Hoechst 33258 were added (to the final concentration of 2 µg/ml). Samples were analyzed by flow cytometry on LSRII (BD Biosiences) and the data were processed in FlowJo software.

In each essay samples stained only with the secondary antibody were used as a negative control. Dead cells stained with Hoechst 33258 were excluded from the analysis.
The data were plotted as relative MFI (median fluorescence intensity) and values were then compared betweeen normal proliferating and senescent cells.

$$relative\ MFI\ (X) = \frac{MFI\ (X)_{primary+secondary\ antibody}}{MFI\ (X)_{secondary\ antibody}}$$

$$relative\ expression\ of\ receptor\ X = \frac{relative\ MFI\ (X)_{senescent}}{relative\ MFI\ (X)_{normal}}$$

List of antibodies used for receptor staining by flow cytometry:

DR4 – Exbio, DR-4-02
DR5 – Exbio, DR-5-01-1
DcR1 – Enzo Life Sciences, HS301
DcR2 – Enzo Life Sciences, HS402
FasR-DX2 – Sigma-Aldrich, F4424
TNFR-1 – Enzo Life Sciences, ALX-804-200

4.8. RNA isolation and quantitative real time RT-PCR (qRT-PCR)

RNA was isolated using RNA Blue reagent (Top-Bio) according to the manufacturer´s instructions. Concentration of RNA dissolved in PCR water (RNase-free water) was determined using NanoDrop (Thermo Scientific) and diluted to the same concentration in each sample.
Isolated RNA was then transcribed into the cDNA using RevertAid Reverse Transcriptase (Thermo Scientific) according to the manufacturer´s instructions. Random hexamers and PCR RNase-free water were to the template RNA in the sterile, nuclease-free safelock tubes.

	per 1 reaction:	
template RNA - total RNA (250 ng/μl)	1.0 μg	4.0 μl
primers - random hexamers (0.2mg/ml)	0.2 μg	1.0 μl
PCR RNase-free water		7.0 μl

Samples were mixed, centrifuged, incubated at 65°C for 5 minutes, chilled on ice and centrifuged. Following components were mixed together and added to each sample:

	per 1 reaction
5x reaction buffer	4.0 µl
dNTPs (10mM)	2.0 µl
RiboLock Rnase Inhibitor	0.5 µl
RevertAid Reverse Transcriptase	1.0 µl

Samples were mixed, centrifuged and incubated at the room temperature for 10 minutes, which was followed by incubation at 42°C for 60 minutes. Finally the reverse transcription was terminated by incubating the reaxtion mixture at 70°C for 10 minutes. cDNA was diluted 10 times with the PCR RNase-free water and used for the qRT-PCR. As negative control samples for the qRT-PCR template RNA diluted with the PCR RNase-free water (to the same final concentration as the cDNA) was used.

Samples for the qRT-PCR were prepared in 384-well plate using LightCycler® 480 SYBR Green I Master (Roche). Plate, samples, MasterMix and primers were kept on ice and each well contained following components:

	per 1 reaction
cDNA (or negative control)	2.0 µl
MasterMix (Roche)	2.5 µl
primers (each 5µM)	0.5 µl

qRT-PCR itself was performed and analyzed on LightCycler 480 (Roche). The relative expression of mRNA was estimated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Primers for qRT-PCR:

	Forward primer (5´-> 3´ sequence)	Reverse primer (5´-> 3´ sequence)
DR4	AACATCGTGCCCTTTGACTC	TACCAGCTCTGACCACATCG
DR5	GCACCAGGTGTGATTCAGG	CCCTCTGGGACACCCTGT
FasR	CAATGTCCAAGACACAGCAGA	TGCCAATTACGAAGCAGTTG
TNFR-1	CCTTCAGAAGTGGGAGGACA	GAATTCCTTCCAGCGCAAC
Bim	GAGATATGGATCGCCCAAGA	TCGGCTGCTTGGTAATTATTC
c-FLIP	CGAGGCAAGATAAGCAAGGA	TCTGGGGCAACCAGATTTAG
Bcl-2	TACCTGAACCGGCACCTG	GCCGTACAGTTCCACAAAGG
Bcl-xL	CAGTAAAGCAAGCGCTGAGG	TGGGATGTCAGGTCACTGAA
Mcl-1	CGAGGCTGCTTTTCTTCG	GGGGCTTCCATCTCCTCAA
Bax	GCTGGACATTGGACTTCCTC	GTCTTGGATCCAGCCCAAC
Bak	CCTGCCCTCTGCTTCTGA	CTGCTGATGGCGGTAAAAA
BMF	AGTTCCACCGGCTTCATGT	TCTTCTCCATTCAAAGCAAGG
Bid	TGCAGCTCAGGAACACCA	TCTCCATGTCTCTAGGGTAGGC
Bad	ACCAGCAGCAGCCATCAT	GGTAGGAGCTGTGGCGACT
p21	GACTCTCAGGGTCGAAAACG	GGATTAGGGCTTCCTCTTGG
β-actin	GGCATCCTCACCCTGAAGTA	AGGTGTGGTGCCAGATTTTC

4.9. Lentiviral vector production in HEK293 cells

HEK293 cells were seeded on 10cm tissue dish to reach approximately 60% confluency by the time of transfection. Just before transfection, the medium was replaced with the fresh one containing 25μM chloroquine. Two mixes were prepared in separate tubes: 1. 500 μl of 2x BBS solution (adjusted to pH 6.95) and 2. 500 μl of 0.25M $CaCl_2$. Into the tube with $CaCl_2$ DNA (plasmid of interest and packaging vectors pMD2G and psPAX, Addgene) was added and the tube was mixed gently. $CaCl_2$ containing DNA was mixed with 2x BBS and let stay for 15 minutes at the room temperature. Then the transfection mix was spread all over the tissue dish and cultivated for 6 hours in incubator with 3% CO_2. After incubation cells were washed with warm PBS, which was then replaced with fresh medium containing 10mM sodium butyrate (Sigma, B5887). Cells were then cultivated in the incubator with 5% CO_2 atmosphere and after approximately 24 hours medium was collected and centrifuged (3000 x g for 15 min). Supernatant was transferred into new tube, 1/5 (v/v) of PEG/it Virus Precipitation Solution (SBI, LV810A-1) was added to it and the tube was incubated at 4°C. After 15 hours supernatant/PEG-it mix was centrifuged at 1500 × g at 4 ºC for 30 minutes, lentiviral pellet was resuspended in sterile PBS and the resuspended recombinant lentiviruses were stored in aliquots at -80°C.

4.10. Transduction of cells by shRNA lentiviral vector

Cells were grown in 12-well plate to reach cca 50% confluency by the day of transduction. Then cells were infected with following lentiviruses – all with puromycin selection:

1/ pLKO-puro shFLIP 1 (Open Biosystems, TRCN0000007229)
2/ pLKO-puro shFLIP 2 (Open Biosystems, TRCN0000007230)
3/ pLKO-puro non-targeting (NT) shRNA (Sigma-Aldrich, SHC002)
4/ pLKO-puro empty vector (EV)

Cells were incubated in the virus-containing medium for two days, then the medium was removed and replaced with appropriate medium containing puromycin (2 μg/ml). To obtain stable transduced population, cells were cultured in medium with puromycin for one week. After that efficiency of shRNA-mediated downregulation of c-FLIP was checked on western blot (Figure 14). All transduced cells were cultured under puromycin selection.

Both PANC-1 and H28 cells were transduced with shFLIP/NT/EV lentiviral vectors, but only H28 cells survived. PANC-1 cells with downregulated expression of c-FLIP (verified on western blot) had decreased viability and although they were continuously proliferating, they kept dying and we could not establish stable transduced population.

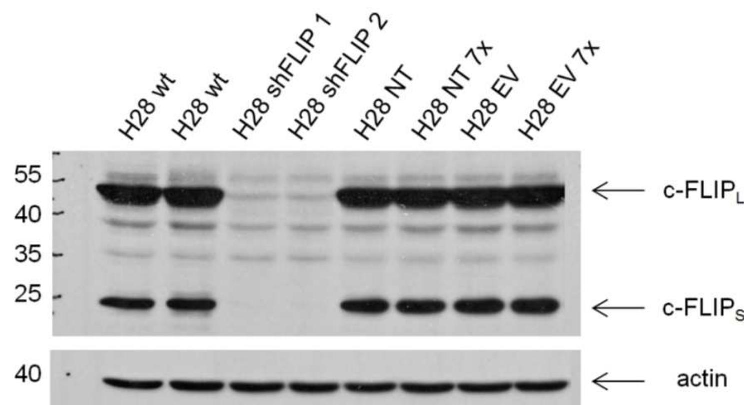


Figure 14. Verification of lentiviral shRNA-mediated downregulation of c-FLIP expression. H28 cells were transduced with two shFLIP hairpins (shFLIP1 and shFLIP2) and different amount of non-targeting (NT) shRNA vector and empty vector (EV). Both hairpins successfully downregulated c-FLIP expression.

4.11. Clonal expansion and selection of tet-on adv p16-T2A-p21 cells

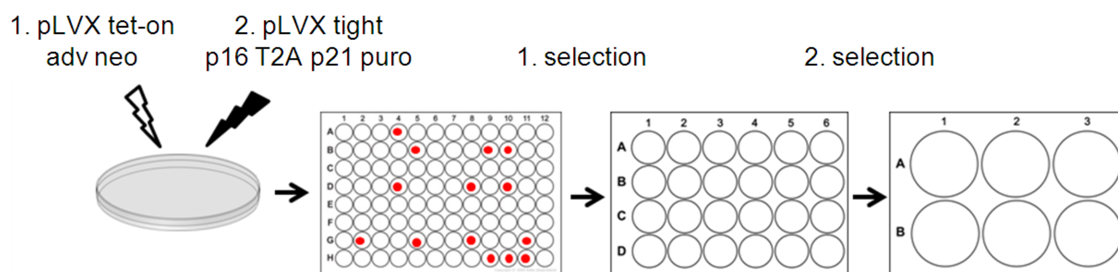
Tet-on system is commonly used system for inducible protein expression which is controlled by tetracycline or its derivatives – in this case by doxycycline (from this reason tet-on cells have to be cultivated in serum without tetracycline). The system contains tetracycline transactivator, which in case of tet-on system requires the presence of doxycycline to induce and activate expression of gene of interest. Without doxycycline, transactivator is not capable of binding to the operator (Shaikh and Nicholson, 2006; Urlinger et al., 2000). 2A peptide-linked multicistronic vector is quite new and efficient system for the expression of multiple proteins from one open reading frame. This small (18-22 aminoacids) self-cleaving peptide was first described in 1991 in picornaviruses (Ryan et al., 1991). 2A peptide results in co-translational cleavage of multiproteins – ribosomes skip synthesis of the glycyl-prolyl peptide bond of the 2A peptide which leads to cleavage between the 2A peptide and its downstream neighboring peptide while proline remains bound to the the downstream peptide. There are 4 variants of 2A peptides (derived from different viruses) used in the research - FMDV 2A (F2A), equine rhinitis A virus 2A (E2A), porcine teschovirus-1 2A (P2A) and Thoseaasigna virus 2A (T2A). They differ in the cleavage efficiency – T2A and

F2A peptides have the highest *in vitro* cleavage efficiency close to 100 % (Donnelly et al., 2001; Kim et al., 2011; Szymczak-Workman et al., 2012; Szymczak et al., 2004; Trichas et al., 2008). 2A peptide lentiviral vectors is very efficient alternative to commonly used IRES (internal ribosomal entry sites) system (Ibrahimi et al., 2009) enabling equimolar expression of transcribed genes.

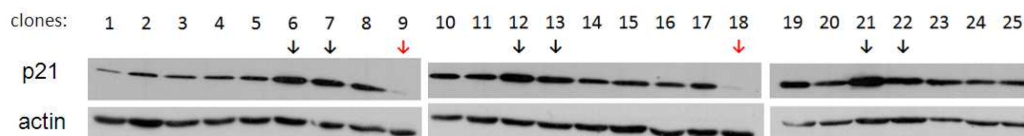
H28 cells were transduced first with pLVX tet-on advanced neo lentiviral vector and cultured under neomycin (G418) selection (in concentration of 1 mg/ml). Selected cells were then transduced with pLVX p16-T2A-p21 puro lentiviral vector and cultured under combined neomycin (1 mg/ml) and puromycin (2 µg/ml) selection.

All transduced cells were cultured in media, that contained serum without tetracycline (RPMI + 10% FBS tet- + ATB + puromycin + G418).

For the clonal selection, the transduced cells were seeded on the 96-well plate – in average one cell per one well using automated cell counter. Only colonies grown from one single cell were taken into account and analyzed (Figure 15). Expression of p21 and p16 was induced with doxycycline (1 µg/ml). Doxycycline concentration was titrated using mixed culture of transduced H28 cells (Figure 16).



1. selection:



2. selection:

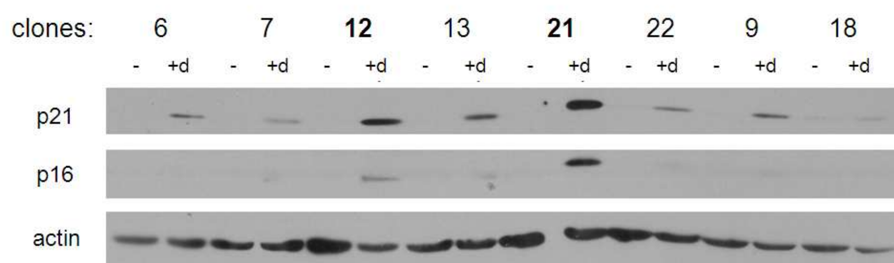


Figure 15. Schematic overview of clonal selection and screening for p21/p16-expressing clones of H28 cells. Clones were selected in two rounds. In the first round all samples were treated with doxycycline (1 µg/ml, for 24 hours) and 5 positive clones and 2 negative clones were selected. In the second round these clones were treated with doxycycline (1 µg/ml, 30 hours) and compared with untreated (-d – untreated, +d – treated) clones. Finally two clones (H28-12 and H28-21) were chosen for further experiments.

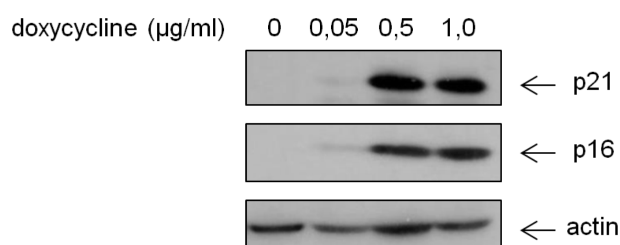


Figure 16. Titration of doxycycline concentration for the inducible expression of p21 and p16. Mixed cultures of H28 p16+21 cells were treated with different concentrations of doxycycline (0 – 0,05 – 0,5 – 1,0 µg/ml) for 24 hours and analyzed using Western blot.

Based on these results we decided to select two clones – H28-12 and H28-21 – for our further experiments.

5. Results

5.1. BrdU induces senescent phenotype in H28 and PANC-1 cells

H28 and PANC-1 cells were treated with BrdU for 8 days and then they were analyzed for the acquired characteristics of senescent cells. Visually, they changed their morphology – they became larger, flatter, often multinucleated and showed increased cellular granulosity (see Figure 17 and Figure 18). Fixed cells were positively stained for SA-β-gal activity (Figure 17) as a marker of senescence and negatively for Ki67 as a marker of proliferating cells (Figure 19).

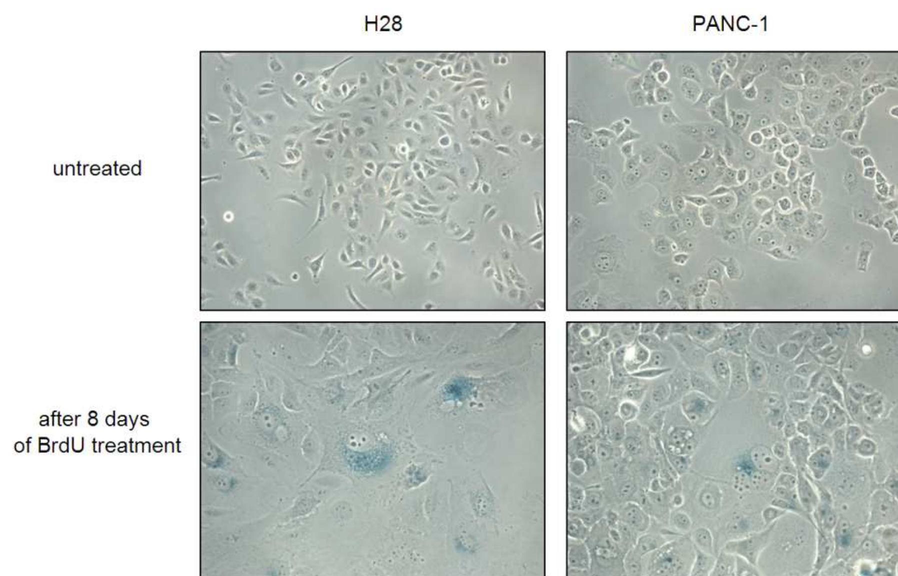


Figure 17. Changes in cellular morphology and SA-β-gal staining. Both cells, H28 and PANC-1, were fixed and stained for SA-β-gal activity. Normal (untreated) and senescent (after BrdU treatment) cells were incubated in the staining solution for the same time until senescent cells turn blue (while normal cells stay unstained). Both BrdU-treated cells showed distinct morphologic features of senescent cells as well as increased occurence of SA-β-gal positive cells.

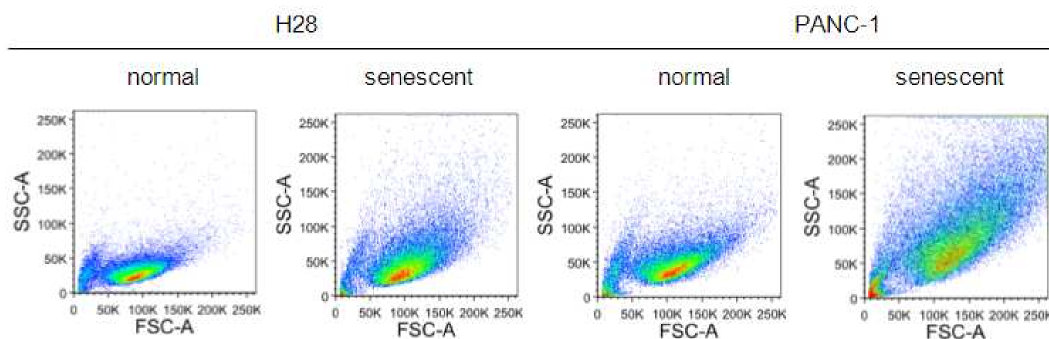


Figure 18. Difference in size and granularity of normal and BrdU treated cells. Treated and untreated cells were analyzed using flow cytometry. BrdU-treated H28 and PANC-1 cells show increase in their size (FSC) and higher granularity (SSC).

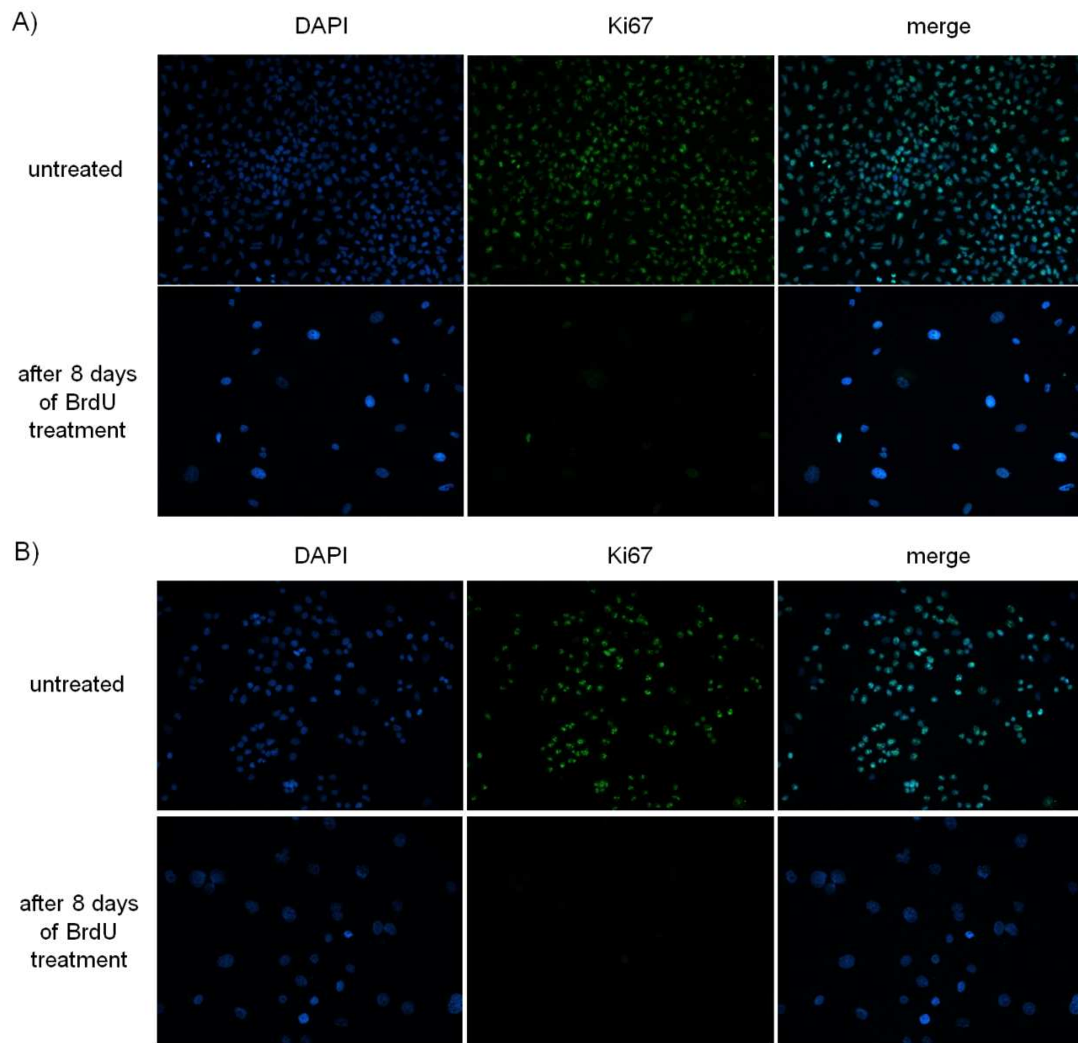


Figure 19. Assessment of cell proliferation by Ki67 staining of normal and senescent cells. Cells were stained with specific antibody against Ki67 (green) and DAPI (blue). Both cells, H28 (A) and PANC-1 (B), showed decrease of Ki67 positive cells after 8 days of BrdU treatment.

Normal proliferating and BrdU treated cells were also analyzed for the stabilization of p53, which marks activation of DNA damage response and also for the expression of CDK inhibitors – p21 and p16 (Figure 20).

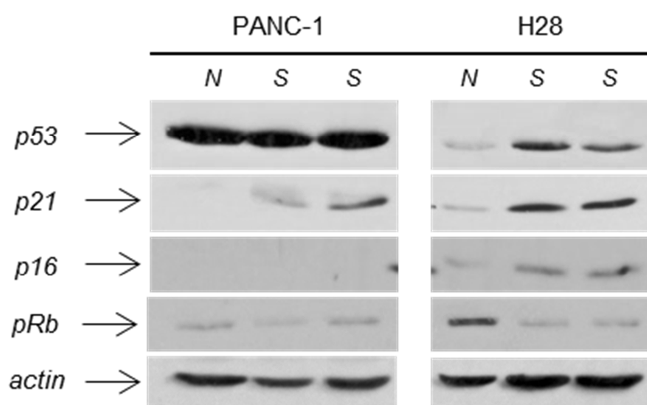


Figure 20. p53 and p21 expression in normal and BrdU treated H28 and PANC-1 cells. Untreated and BrdU treated cells were analyzed by Western blotting. Actin was used as loading control.

Upon BrdU treatment wt p53-containing H28 cells stabilized and accumulated p53 protein. They also showed the increased expression of CDK inhibitors p21 and p16 and attenuated levels of pRb. As PANC-1 cells are mutated in p53, they express and accumulate p53 even under normal, non-senescent conditions. Senescent PANC-1 did increase expression of p21 (though not with the same efficacy in all experiments and not fully reproducibly) but in contrast to H28 cells we did not observe almost any senescence-associated changes in the expression of pRb or p16 proteins.

5.2. Senescent H28 and PANC-1 cells are more resistant to TRAIL+HHT induced apoptosis and prone to FasL, CPT and mVES treatment

To evaluate sensitivity of normal and senescent cancer cells to apoptotic stimuli, these cells were treated with various apoptogens that trigger apoptosis through different mechanisms:

- TNF-related apoptosis-inducing ligand (TRAIL – 100 ng/ml, 4 hours),
- homoharringtonine (HHT – 100nM, 4,5 hours),
- TRAIL (100 ng/ml, 4 hours) in combination with HHT (100nM, 4,5 hours),
- Fas ligand (FasL – 50 ng/ml, 20 hours),
- camptothecin (CPT – 4µM, 20 hours) and
- mitochondrially targeted vitamin E succinate (mVES – 10µM, 20 hours).

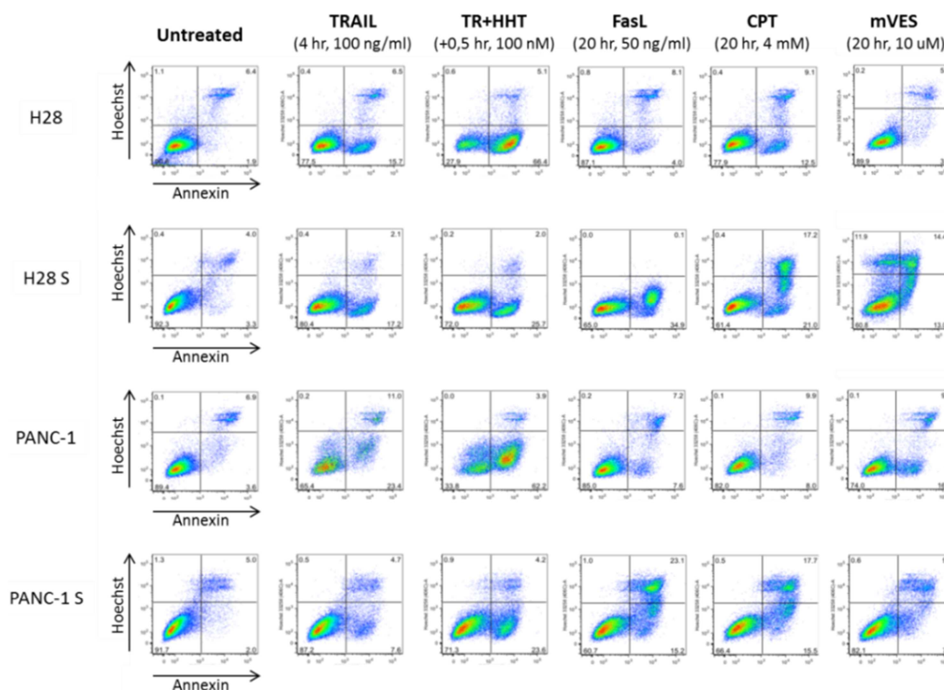


Figure 21. Induction of apoptosis in normal and senescent H28 and PANC-1 cells. Cells were treated with different apoptogens, stained with Annexin V-FITC and Hoechst 33258 and analyzed with flow cytometry (representative dot plots with Annexin V-FITC staining on X-axes and Hoechst 33258 staining at Y-axes).

TRAIL and FasL activate extrinsic apoptotic pathway via binding of death ligands to their specific death receptors (DR4, DR5 and FasR), initiation of DISC assembly and subsequent caspase-8 cleavage. HHT inhibits initiation of translation (Gurel et al., 2009) and sensitizes cells to TRAIL treatment (Beranova et al., 2013). Both CPT – inhibitor of DNA topoisomerase I (Koster et al., 2007) – and mVES – agent destabilizing mitochondria through binding to the mitochondrial complex II, its inhibition and ROS generation (Dong et al., 2011a; Neuzil et al., 2013) – are DNA damaging agents inducing apoptosis via activation of intrinsic apoptotic patways.

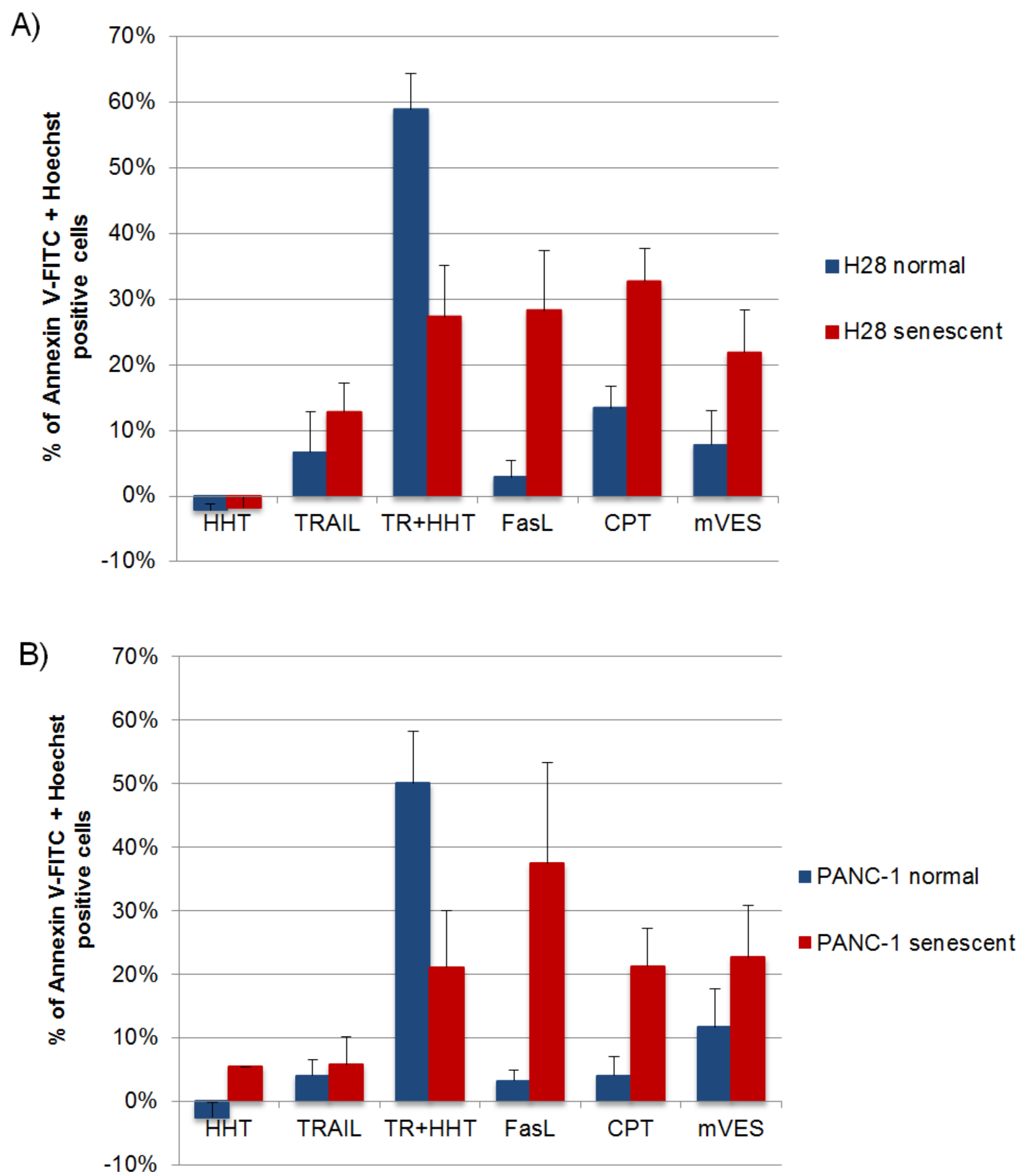


Figure 22. Quantification of apoptosis in normal and senescent H28 (A) and PANC-1 (B) cells. Cells were treated with different apoptogens, then stained with Annexin V-FITC and Hoechst 33258 and analyzed with flow cytometry. Results are average values of 3 independent experiments. Bars indicate average +/-SD.

Apoptosis was detected by flow cytometry using Annexin V-FITC and Hoechst 33258 staining and primary data from three independent experiments (see representative data in Figure 21) were quantified in FlowJo software and statistically analyzed (Figure 22). Both normal non-senescent cell lines were completely resistant to HHT treatment and limitedly sensitive to TRAIL. They stayed resistant to HHT and TRAIL even after BrdU treatment – although H28 cells showed slight increase in TRAIL-induced apoptosis. In contrast to the individual TRAIL or HHT treatment, both cells, H28 and PANC-1, were sensitized to combined TRAIL+HHT treatment (about 50-60 % cells were Annexin V-FITC positive). BrdU-treated, senescent cells showed then suppressed apoptosis in response to combined TRAIL+HHT treatment – we reproducibly detected approx. 50% reduction in Annexin V-FITC positive cells. In contrast to suppressed apoptotic response to TRAIL+HHT treatment, senescent cells were more prone to FasL, CPT and mVES treatment than their proliferating counterparts. Notably, FasL-induced apoptosis was greatly enhanced in senescent cells – fully resistant proliferating cells increased their FasL-triggered apoptotic response to 30-40 % after BrdU treatment. In all these cases (except for FasL-treated H28 cells), we observed mainly increased percentage of late apoptotic cells (Annexin V-FITC and Hoechst 33258 double positive cells) in senescent cells. Senescent cells showed also higher level of medium acidification after mVES treatment (data not shown).

5.3. Senescent H28 and PANC-1 cells increase expression of FasR and suppress cell surface expression of DR4

We observed significant differences in apoptosis of nonsenescent vs. senescent cells triggered by the activation of death receptors. As the expression and availability of these receptors is one of the decisive factors in cellular sensitivity to death ligands-induced apoptosis, we measured and analyzed expression of death receptors in both cell lines. Cell surface expression of death receptors was analyzed using flow cytometry (Figure 23) and evaluated as relative expression in normal versus senescent cells – see Material and methods section – part 4.7.

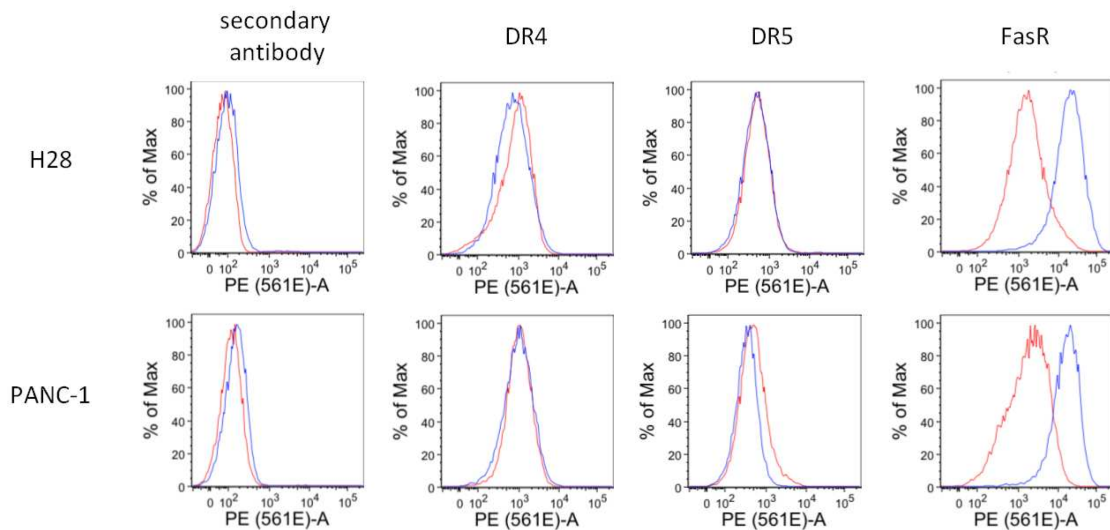


Figure 23. Cell surface expression of death receptors in H28 and PANC-1 cells. Normal (red line) and senescent (blue line) cells were stained with specific primary antibodies followed by corresponding secondary antibody. Cells stained only with secondary antibody were used as negative controls and standards for MFI calculations.

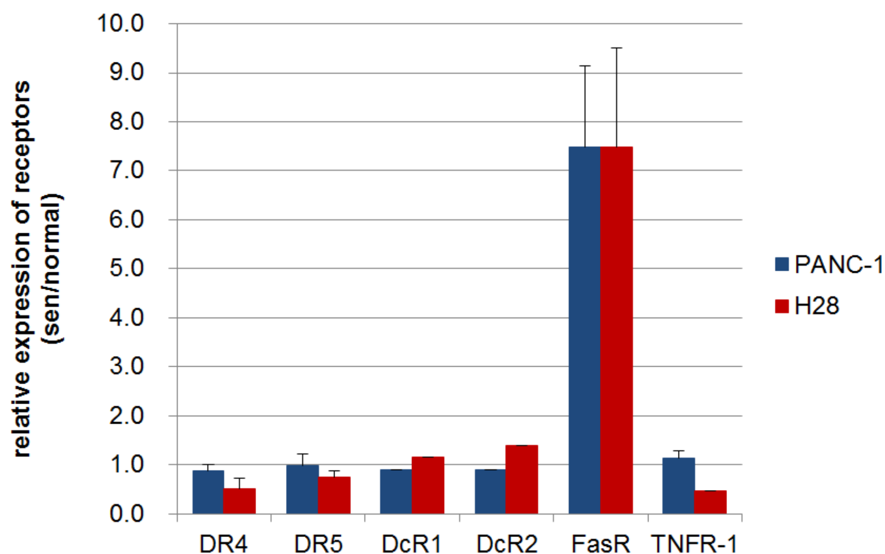


Figure 24. Relative cell surface expression of death receptors in H28 and PANC-1 cells. Results are average values of 5 independent experiments (except from DcR1, DcR2 and TNFR-1, that were measured once). Bars indicate average +/-SD.

Both cell lines showed more than seven-fold increase in FasR surface expression. PANC-1 cells did not exhibit significant change in the expression of other receptors (DR4/5, DcR1/2 or TNFR-1). In contrast, cell surface expression of DR4, DR5 and TNFR-1 decreased – in case of DR4 and TNFR-1 to approx. 50 %, in case of DR5 to 70 % of their expression in non-senescent cells.

Although the cell surface expression is essential for the induction of apoptotic signaling, we also analyzed total protein level of death receptors and their expression on mRNA level.

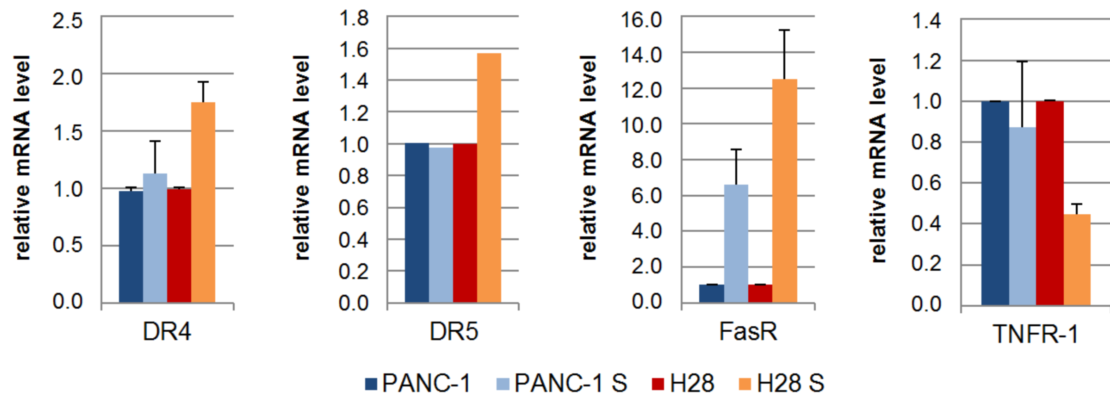


Figure 25. Relative mRNA expression of death receptors in normal vs senescent H28 and PANC-1 cells. Results represent average values of 4 independent experiments (except for DR5, which was measured twice). Columns indicate mRNA expression relatively to normal (proliferative) state. Bars indicate average +/-SD. Values were normalized to β-actin.

Data from qRT-PCR (Figure 25) confirmed upregulation of FasR also at the mRNA level. Correlation between mRNA and cell surface expression was also found for TNFR-1, which is in senescent H28 cells downregulated on both mRNA and cell surface protein levels. Change in the cell surface expression of FasR and TNFR-1 are thus transcriptionally controlled. In contrast, there is discrepancy between cell surface expression of DR4 and DR5 and their mRNA and total protein levels. While mRNA and total protein expression of DR4 increased (Figure 25 and Figure 27), its cell surface expression was either almost unchanged (PANC-1) or suppressed (H28) (Figure 24 and Figure 26). Cell surface expression of DR5 was also almost unchanged (Figure 24 and Figure 26), but in senescent PANC-1 cells its total protein levels were strongly suppressed (Figure 27).

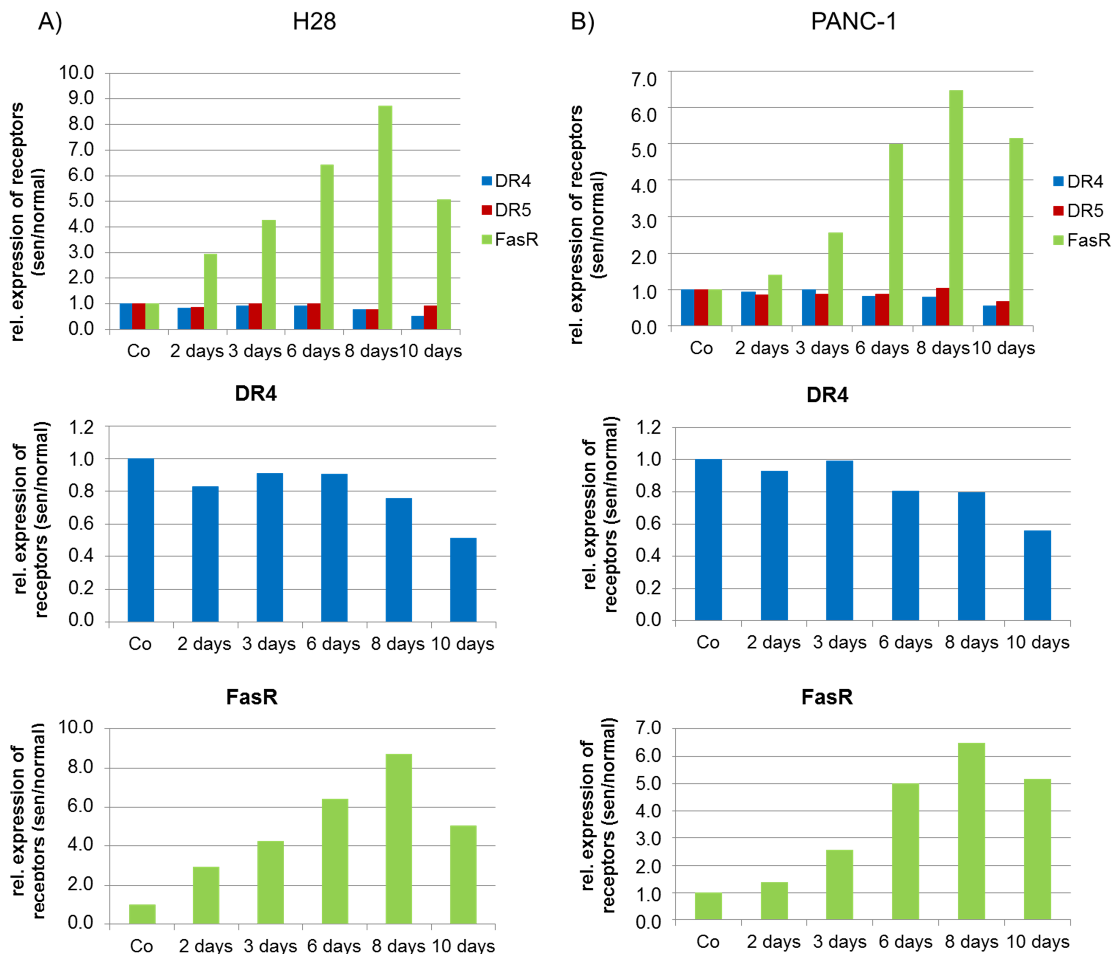


Figure 26. Time-dependent analysis of cell surface expression of death receptors in H28 (A) and PANC-1 (B) cells. Cells were treated with BrdU for indicated time (0 – 10 days) and cells surface expression of death receptors was analyzed by flow cytometry. Results are average values of two independent experiments.

Changes in cell surface expression of death receptors were also analyzed in time-dependent manner. Change in the DR4 expression starts to be detectable after 8 days of BrdU treatment. In contrast, the expression of FasR increased already after 2-3 days of BrdU treatment.

5.4. Senescent cells alter expression of major apoptosis-related proteins

In addition to death receptors, a number of other apoptosis-related or –regulating proteins participates in apoptotic signaling. By Western blotting we examined expression of selected major pro-apoptotic and anti-apoptotic proteins that could play some role in higher or lower resistance of senescent cells to various apoptogens (Figure 27).

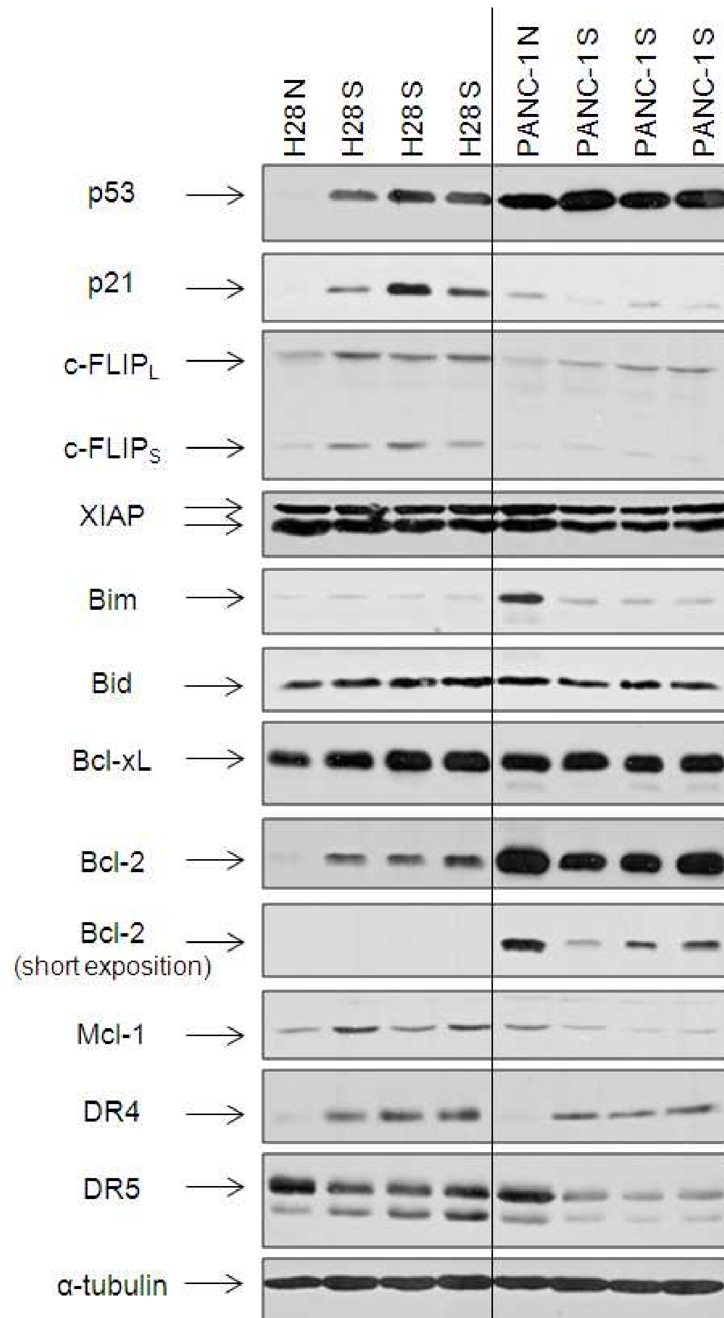


Figure 27. Expression of apoptosis-related proteins in normal vs senescent H28 and PANC-1 cells. Cell lysates from three independent senescent cultures (treated with BrdU for 8 days) of each cell line were by Western blotting compared with the lysates from normal proliferating culture for the expression of indicated proteins.

Pro-senescence BrdU treatment triggered in H28 cells stabilization of wt p53, which correlated with p53-dependent upregulation of CDK inhibitor p21. In contrast PANC-1 cells with mutated p53 show high levels of p53 even in non-stressed state, and they did not increase their expression of p21. Neither in H28, nor in PANC-1 cells, we detected differences in the expression of proteins Bid, Bcl-xL or XIAP. While expression of these proteins remained unchanged in senescent cells, we observed pronounced and

interestingly cell type-dependent changes in the senescence-triggered expression of other, mainly Bcl-2 family proteins. Senescent H28 cells upregulated expression of anti-apoptotic Bcl-2 and Mcl-1 proteins and also pro-apoptotic Bax, but senescent PANC-1 cells behaved quite oppositely – they downregulated expression of both of these proteins (Figure 27). Moreover, senescent PANC-1 cells considerably downregulated expression of the pro-apoptotic protein Bim.

We also quantified expression of mRNAs coding for these proteins (Figure 28). There are notable differences in the mRNA expression between normal proliferating and senescent cells. However, they do not always correlate with differences at the total protein level. While senescence-triggered changes in protein expression of c-FLIP or Bax in both cell lines correlate with the expression of their mRNAs, the increased mRNA levels of Bid or Bim are not reflected in the increase of their protein levels (compare Figure 27 and Figure 28). In contrast, cellular levels of Bim protein are strongly suppressed in senescent PANC-1 cells likely via a transcription-independent mechanism. Similarly, Bcl-2 expression is increased at protein level in senescent H28 cells without any change in its mRNA expression and while its protein levels markedly drop in senescent PANC-1 cells, its mRNA expression exhibits two-fold increase. We also detected two-fold increase in the mRNA level of Bak and BMF, but we were unsuccessful in detecting these proteins by Western blotting.

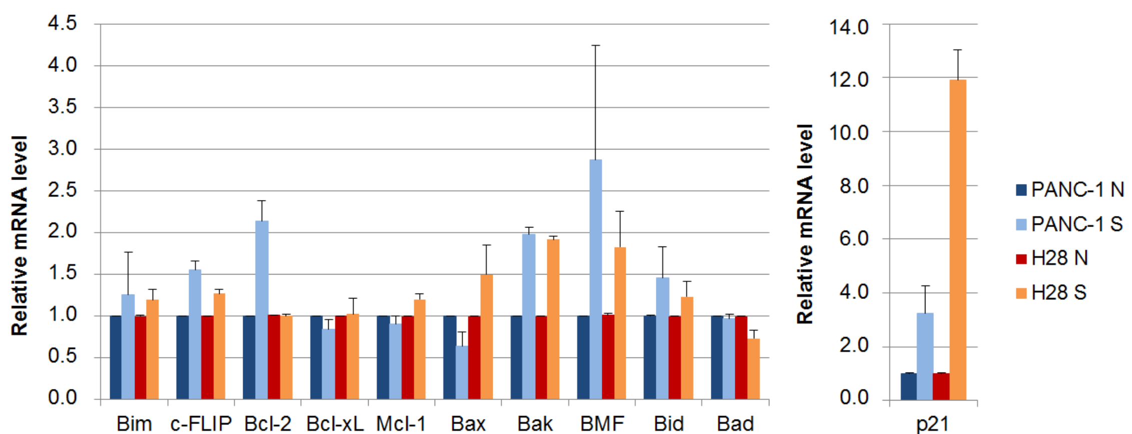


Figure 28. Relative mRNA level of selected pro-apoptotic and anti-apoptotic proteins in normal vs senescent H28 and PANC-1 cells. Data were acquired from five independent experiments. Columns indicate mRNA expression in relation to normal (proliferative) state. Bars indicate average +/-SD. Values were normalized to β-actin.

5.5. Increased expression of c-FLIP does not play a role in higher resistance of senescent H28 cells to TRAIL+HHT treatment

FLIP is a part of the DISC and it inhibits apoptotic signaling through competing with caspase-8 activation and its dysregulation may influence sensitivity of cells to TRAIL- or FasL-induced apoptosis. To address possible role of c-FLIP in senescence-modulated changes in sensitivity to FasL- or TRAIL-induced apoptosis, we prepared H28 cells with shRNA-mediated downregulation of c-FLIP expression.

Interestingly, H28 cells with suppressed expression of c-FLIP were more sensitive to BrdU treatment with a pronounced lethality at 50µM BrdU and thus we had to use lower 20-25 µM concentration of BrdU.

After BrdU treatment all senescent cells (wt, NT, shFLIP1 and shFLIP2) showed increased expression of p21 driven by stabilization of its transcription factor p53. Both wt and NT H28 cells increase their expression of c-FLIP after exposure to BrdU, but in cells with suppressed expression of c-FLIP (shFLIP1 and shFLIP2) we did not observe any increase in its expression after BrdU exposure – see Figure 29.

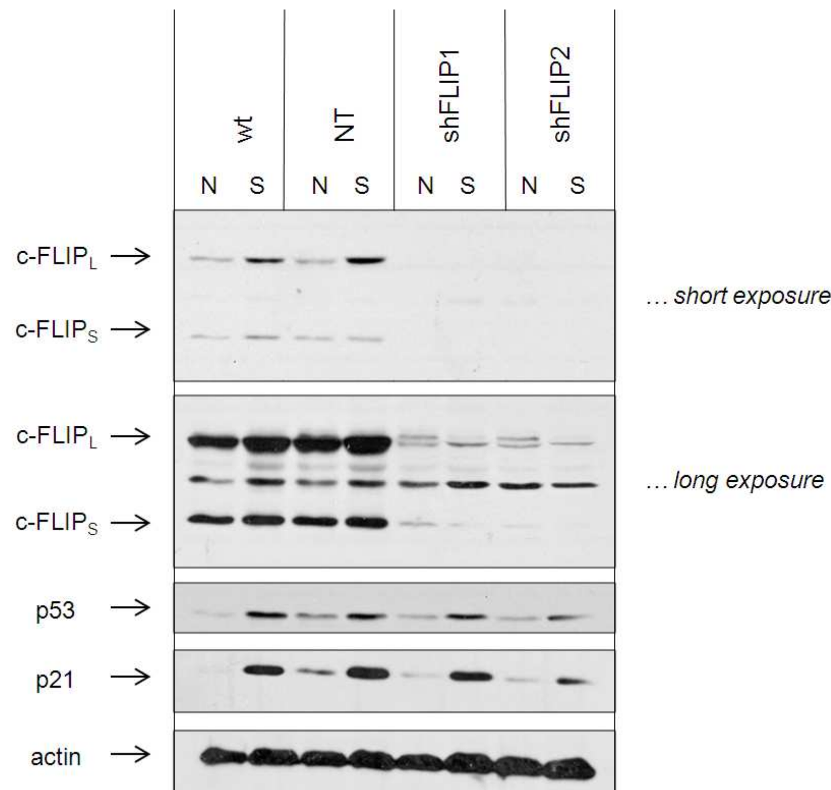


Figure 29. c-FLIP expression in normal vs senescent H28 wt/NT/shFLIP1/shFLIP2 cells. Cells were treated with BrdU (25µM in case of H28 wt and H28 NT, 20µM in case of shFLIP 1/2) for 8 days and analyzed by Western blot.

Downregulation of c-FLIP expression had almost no impact on TRAIL- or TRAIL+HHT-induced apoptosis. Although there was an increase in apoptotic response after TRAIL treatment in shFLIP cells, this increase could be connected to process of transduction itself, because H28 cells transduced with non-targeting vector showed similar response to TRAIL as cells with downregulated c-FLIP expression. Transduced cells were also generally more sensitive to mVES treatment. In contrast both normal and senescent shFLIP cells showed lower viability after FasL treatment, implying that suppressed expression of c-FLIP could enhance FasL-mediated apoptosis. Interestingly downregulation of c-FLIP enhanced sensitivity of proliferating but not sescent H28 cells to CPT (Figure 30).

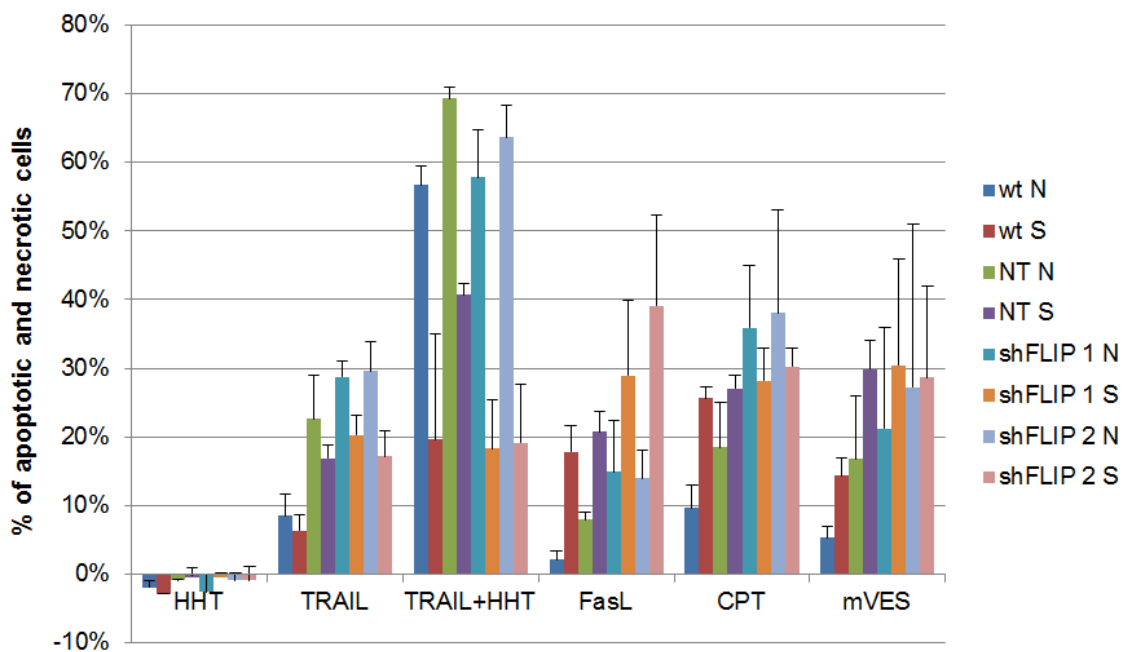


Figure 30. Induction of apoptosis in normal vs senescent H28 wt/NT/shFLIP1/shFLIP2 cells. Senescence was induced with BrdU (20µM BrdU in case of H28 shFLIP 1/2 cells, 25µM BrdU in case of H28 wt and H28 NT cells) treatment for 8 days. Cells were treated with different apoptogens, then stained with Annexin-FITC and analyzed by flow cytometry. Results are average values of three independent experiments. Bars indicate average +/-SD.

5.6. p21 and p16 expressing H28 cells acquire some markers of senescence

In order to induce senescence or senescence-like phenotype without extensive DNA damage, we used system for simultaneous expression of two CDK inhibitors p16 and p21. These CDK inhibitors were already shown to induce senescent phenotype in human breast cancer cells (Capparelli et al., 2012). p16 and p21 expressing cells were prepared using clonal selection and expansion of transduced H28 cells. We were forced to proceed with clonal selection as the mixed populations of tranduced cells exhibited too high heterogeneity, and did not efficiently respond to doxycycline-induced expression of CDK inhibitors with growth arrest, respectively relatively large number of non-expressing cells quickly overgrew the arrested cells. Finally two clones (H28-12 and H28-21) were selected and used for further analyses.

Cells were treated with doxycycline (in concentration of 1 μg/ml) and with combination of doxycycline (in concentration of 1 μg/ml) and BrdU (10μM) for 4 days and compared with untreated proliferating cells. Doxycycline was used to induce expression of p21, lower concentration of BrdU was used to generate DNA damage which is associated with senescent phenotype. In these cells we analyzed changes in the expression of p21 and p16 by Western blot (Figure 31) and in case of p21 also by indirect immunofluorescence (Figure 32).

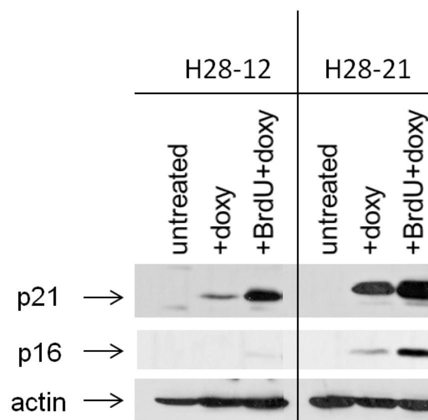


Figure 31. Analysis of p21 and p16 expression in H28-12 and H28-21 cells. H28-12 and H28-21 cells were treated with doxycycline (1 μg/ml), combination of doxycycline (1 μg/ml) + BrdU (10μM) and doxycycline with BrdU pretreatment and p21 and p16 expression was determined by Western blot.

Western blotting showed that doxycycline induces expression of p21 and p16, but BrdU cotreatment even enhances their expression in both clones (Figure 31). Interestingly, BrdU alone had no effect on p21/p16 expression – see Figure 37. We also observed higher expression of both CDK inhibitors in H28-21 cells compared to H28-12 cells.

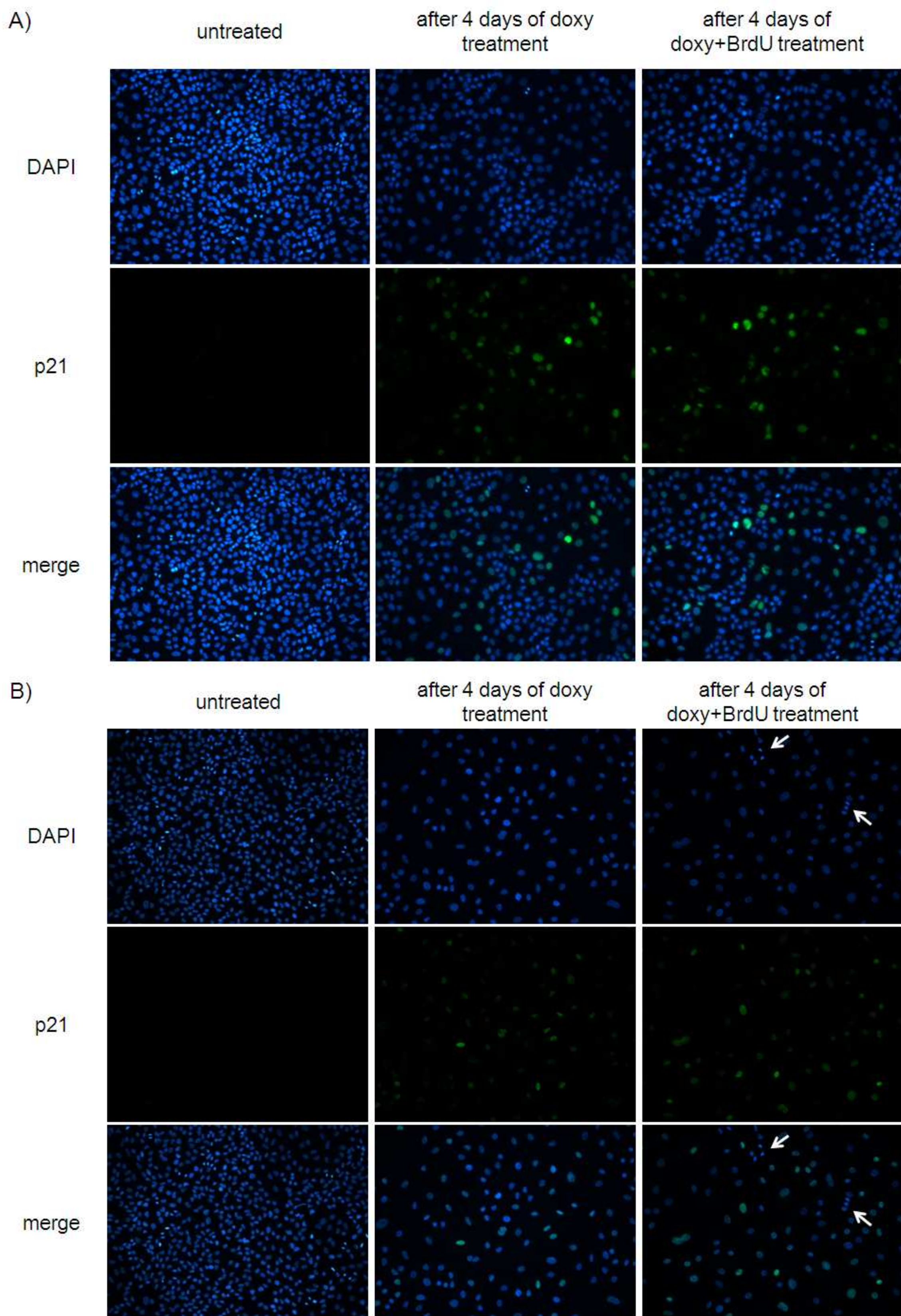


Figure 32. Analysis of p21 expression in H28 clones by immunofluorescence. H28-12 (A) and H28-21 (B) cells were treated with doxycycline or combination of doxycycline and BrdU for 4 days. Together with control untreated cells they were stained with antibody against p21 (green) and DAPI (blue) and analyzed by fluorescence microscopy (arrows show likely dividing, p21-non-expressing cells).

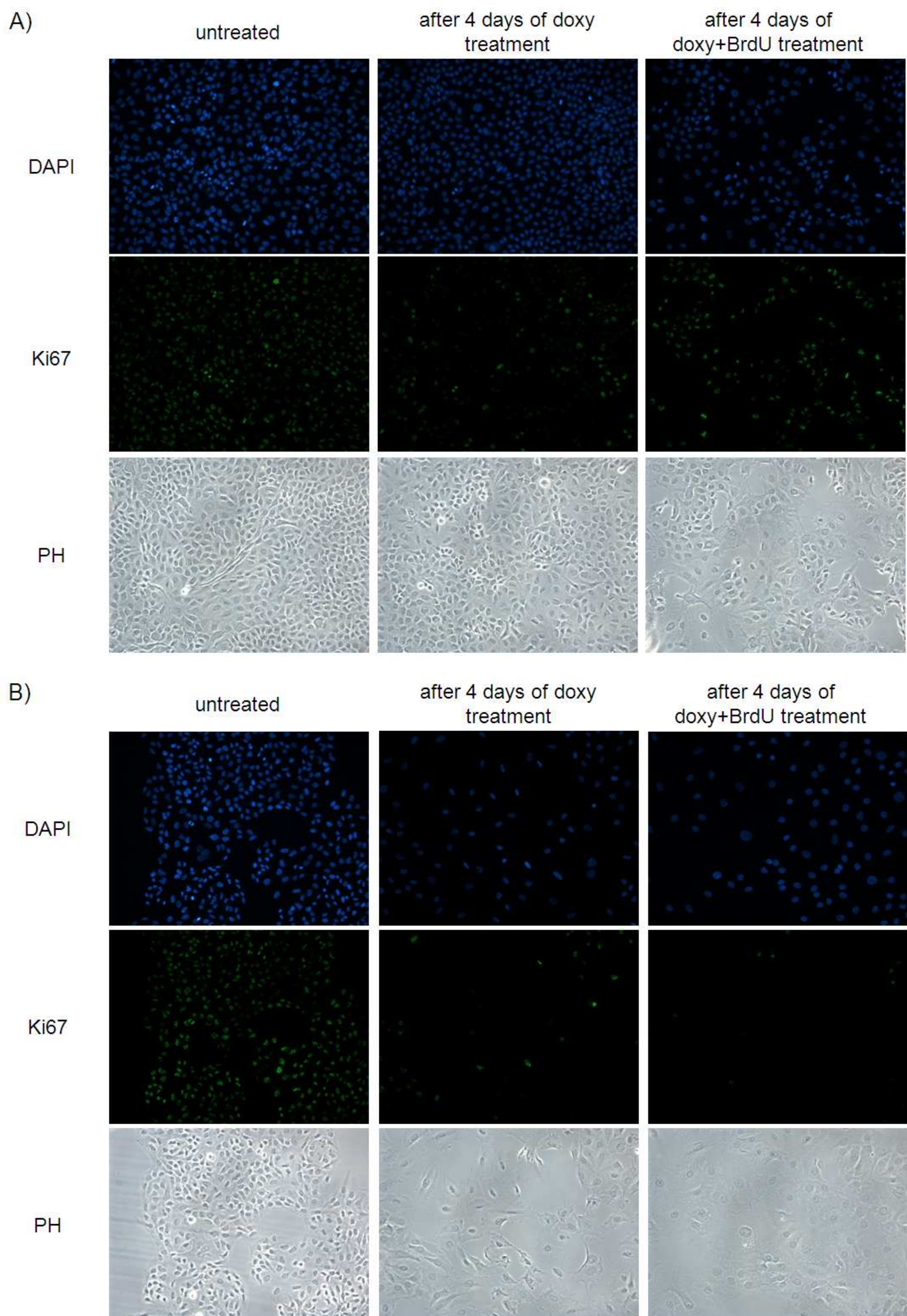


Figure 33. Ki67 staining of H28-12 and H28-21 cells. H28-12 and H28-21 cells were treated with doxycycline or combination of doxycycline and BrdU for 4 days. Together with untreated cells they were stained with specific antibody against Ki67 (green) and DAPI (blue) and analyzed by fluorescence and light (phase contrast/PH) microscopy.

Although cells treated with doxycycline increased their expression of p21, staining of p21 using indirect immunofluorescence (Figure 32) showed that especially H28-12 but also H28-21 cultures are also not fully homogenous in their expression of p21 (and likely also p16), despite they originated from one single cell in clonal selection (see cells marked with arrows in Figure 32).

Both H28 clones showed upon doxyxycline-mediated induction of p21/p16 expression decrease of Ki67 positive cells that was even more pronounced (at least for H28-21 cells) after their co-treatment with BrdU (Figure 31). However, while H28-21 cells exhibited considerable suppression of Ki67 positive cells, in H28-12 cells we still observed high number of Ki67 positive and dividing cells. Doxycycline- and doxycline+BrdU-treated cells also showed change in their morphology and acquired senescent-like phenotype. And again, H28-21 cells were more enlarged, flatter and last but not least also more homogeneous than H28-12 cells.

We also analyzed cellular parameters of untreated (control) and treated cells by flow cytometry. Both clones showed significant increase in cellular size and granularity after their treatment with doxycycline or combination of doxycycline and BrdU (Figure 34).

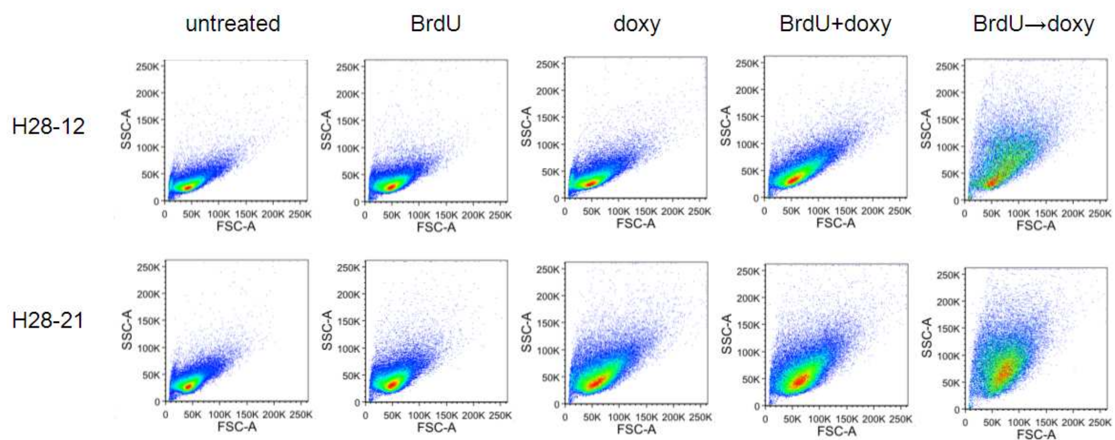


Figure 34. Difference in size and granularity after BrdU or doxycycline treatment in H28-12 and H28-21 cells. Cells were treated with different combinations of BrdU and doxycycline and analyzed using flow cytometry. Both clones show increase in their size (FSC) and higher granularity (SSC).

Taken into account Ki67 staining, changed morphology and p21 staining H28-21 cells seem to be more suitable for further analysis than H28-12 cells as they exhibited several features of senescent cells: – they acquired more senescent-like phenotype which was similar to phenotype acquired after 50μM BrdU treatment; they showed higher increase in the expression of both CDK inhibitors (p21 and p16); and

last but not least they formed more homogenous population, while H28-12 cells exhibited presence of a number of proliferating and obviously non-senescent isles.

Although initially we planned to treat these cells only with doxycycline, we decided to include BrdU in our study, because BrdU pretreatment/cotreatment enhanced and stressed senescent phenotype of doxycycline-treated cells and these cells could be thus more related to senescent cells acquired after 50μM BrdU treatment.

5.7. H28 clones expressing p21/p16 show variable predominantly lower sensitivity to apoptogens

Based on these preliminary results we decided to apply different combinations of BrdU and doxycycline treatment. Both clones were treated according to following scheme and at the time point labeled with * cells were treated with apoptogens or their protein expression was analyzed.

days: 0 1 2 3 4 5 6 7 8

1.	-	-	-	*
2.	BrdU	-	-	*
3.	-	doxy	doxy	*
4.	-	BrdU+doxy	doxy	*
5.	BrdU	doxy	doxy	*

** - induction of apoptosis/analysis of proteins and death receptors*

Cells that were treated with BrdU and/or doxycycline as shown above were treated with different apoptogens, stained with Annexin V-FITC and analyzed using flow cytometry (Figure 35):

- TRAIL (100 ng/ml, 4 hours),
- HHT (100nM, 4,5 hours),
- TRAIL (100 ng/ml, 4 hours) with HHT pretreatment (100nM, 4,5 hours),
- FasL (50 ng/ml, 20 hours) and
- CPT (4μM, 20 hours).

Both clones treated with doxycycline or combination of doxycycline and BrdU showed higher resistance to TRAIL+HHT and CPT treatment than untreated cells or for CPT treatment senescent cells generated using higher concentration of BrdU (Figure 22). In contrast to senescent H28 cells generated using 50 mM BrdU, these clones of H28 cells did not increase their sensitivity to FasL. Notable and reproducible exception were H28-

21 cells sequentially treated with BrdU and then doxycycline. These cells surpricingly became more sensitive to all apoptogens, interestingly even to TRAIL and TRAIL+HHT treatment (Figure 35).

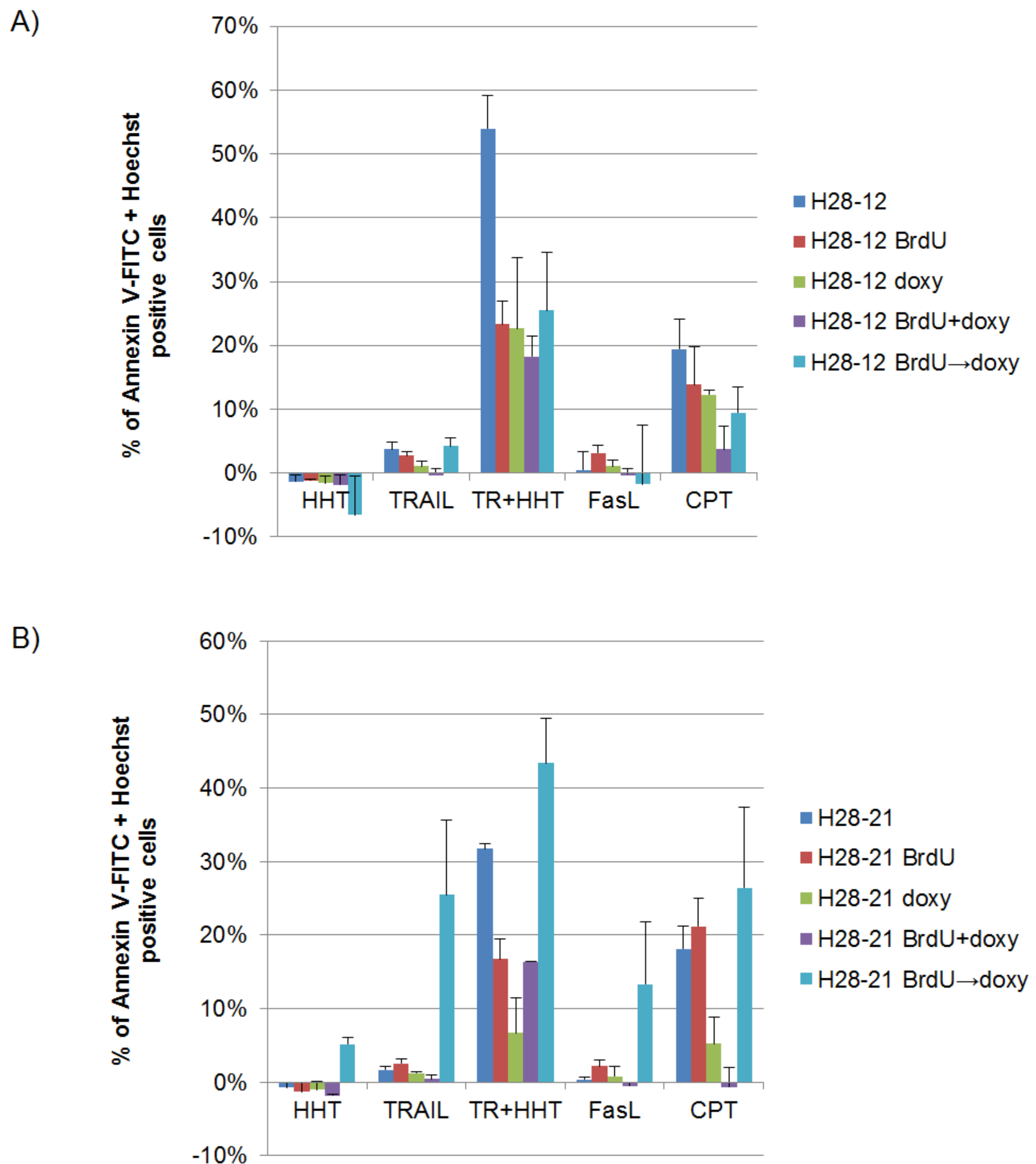


Figure 35. Induction of apoptosis in H28-12 and H28-21 cells. Cells were incubated with different combinations of 10μM BrdU and doxycycline (1 μg/ml). After that they were treated with different apoptogens, stained with Annexin-FITC and analyzed with flow cytometry. Results are average values of 3 independent experiments. Bars indicate average +/-SD.

5.8. BrdU-treated p21/p16-expressing H28 cells enhance expression of death receptors and anti-apoptotic proteins

Variable impact of different BrdU/doxycycline treatment regimes on the response of H28 clones 12 and 21 to selected apoptogens prompted us to examine expression of death receptor and other apoptosis-related proteins in these clones. Doxycycline treatment alone or with BrdU cotreatment did not have effect on the cell surface expression of death receptors (Figure 36). Treatment with 10µM BrdU led to increased expression of FasR in both H28 clones (though significantly more in H28-21), and subsequent induction of p21/p16 expression with doxycycline enhanced FasR expression even further up to approx. 9-fold in H28-21 cells. Same treatment regime in H28-21 cells also elevated expression of DR4 and slightly DR5 (Figure 36). Increased expression of DR4 was detectable also at total protein level (Figure 37).

These results correlate with higher sensitivity of H28-21 cells (after consequent treatment with BrdU and doxycycline) to TRAIL, TRAIL+HHT and FasL treatment.

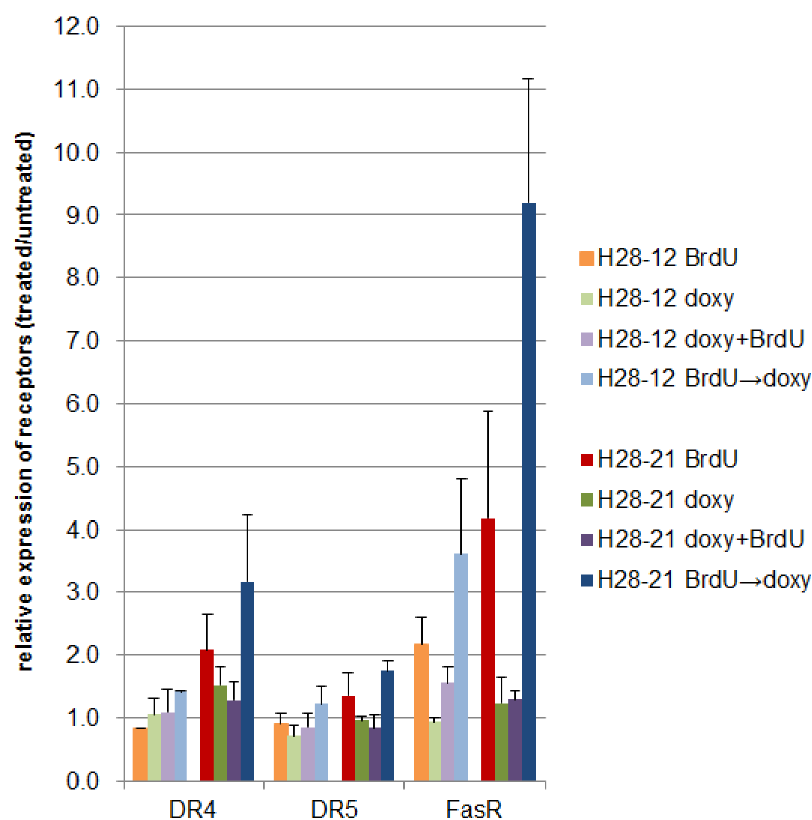


Figure 36. Relative cell surface expression of death receptors in H28-12 and H28-21 cells. H28-12 and H28-21 cells were with different combination of 10µM BrdU and doxycycline (1 µg/ml) and analyzed using flow cytometry. Results represent average values of two independent experiments. Bars indicate average +/-SD.

We also analyzed protein levels of cell cycle inhibitors and apoptosis-related proteins in both H28 clones undergoing the same treatment regimes (Figure 37). At first glance H28-21 cells increased their expression of p21 and p16 more significantly – while in doxycycline-treated cells H28-12 cells we did not detect expression of any of these CDK inhibitors, using same exposure we detected increase in the expression of both of these proteins in H28-21 cells. Interestingly although 10μM BrdU treatment alone did not affect expression of p21 and p16 at all, it markedly enhanced doxycycline-mediated inducible expression of both CDK inhibitors. Doxycycline treatment alone did not increase expression of either c-FLIP or Bcl-2 protein in H28-12 cells – it slightly increased expression of Bcl-2 in H28-21 cells. In contrast BrdU alone or in combination with doxycycline increased expression of Bcl-2 in both clones. We also observed upregulation of FLIP expression after BrdU and doxycycline cotreatment – notably in H28-21 cells, but slightly also in H28-12 cells.

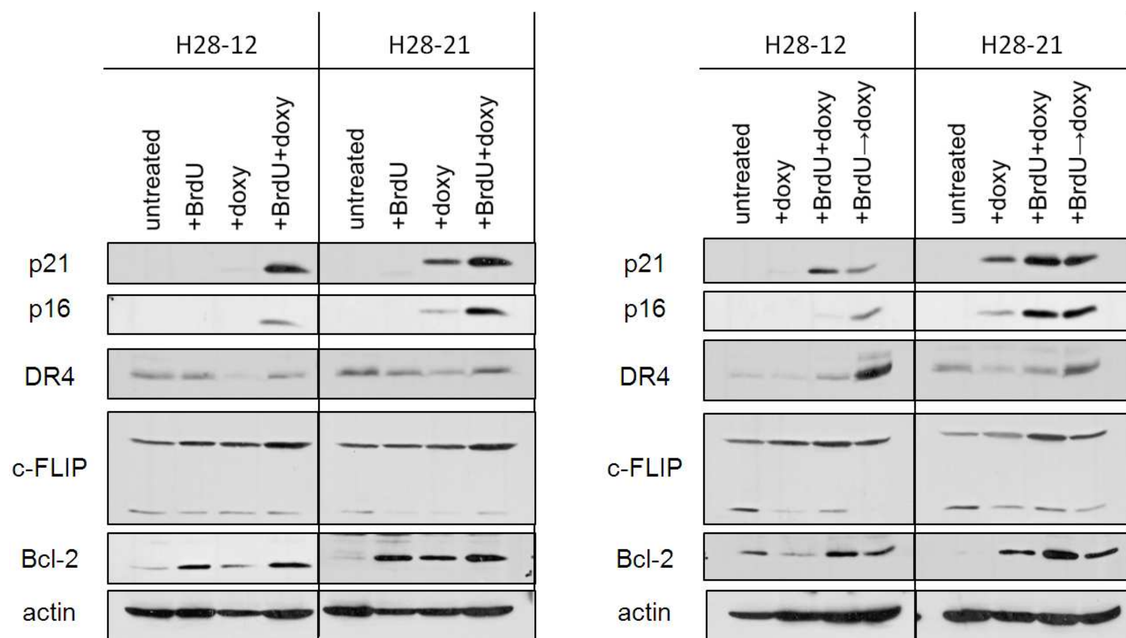


Figure 37. DR4, c-FLIP and Bcl-2 expression in H28-12 and H28-21 cells. H28-12 and H28-21 cells were treated with different combinations of doxycycline (1 μg/ml) and BrdU (10μM) and analyzed using Western blot.

6. Discussion

Senescence and apoptosis are important processes that represent essential barrier against carcinogenesis. Although senescence has become widely studied topic, many aspects of senescent signaling and its role during carcinogenesis are in contrast to apoptotic signaling still poorly understood. Even less is known about a crosstalk between senescence and apoptosis and about influence of senescence-associated changes in gene expression and signaling on the induction and progression of apoptosis. In this work we focused on the analysis and identification of differences in apoptotic signaling between proliferating and senescent cancer cells.

To address this aim, we selected two different cancer cell lines that differ in one of the most important proteins with respect to both senescence and apoptosis – tumor suppressor protein p53. In these cell lines we induced senescence or senescent-like phenotype through two different mechanisms – 1) via using sublethal concentrations of thymidine analogue 5-bromo-2'-deoxyuridine and 2) via inducible expression of two prominent CDK inhibitors p21 and p16 without or with low levels of concurrent BrdU-induced DNA damage. Both of these mechanisms were already described and used for inducing senescence.

BrdU is widely used to induce senescent phenotype in various mammalian cancer cell lines (Levkoff et al., 2008; Masterson and O'Dea, 2007; Michishita et al., 1999). As an analogue of thymidine it is incorporated into DNA and by still not fully understood mechanism it generates DNA damage. For the induction of premature senescence we treated H28 and PANC-1 cells with BrdU treatment for up to eight days and then analyzed them for various markers of senescence. BrdU-treated cells considerably changed their morphology and suppressed their proliferation – even though PANC-1 cells exhibited less homogenous phenotype and we observed isles of proliferating cells persisting in the population. We also stained these cells for SA-β-gal activity, but although we observed increase in number of SA-β-gal-positive cells, not all apparently senescent and cell-cycle arrested cells turned blue and the staining was not fully reproducible. Efficiency of SA-β-gal staining could depend for example on the basal level of SA-β-gal activity. If this level is too low, even its increase may not be sufficient for detection of its activity. To further prove senescent phenotype of BrdU-treated cells, we stained cells with specific antibody against Ki67 protein, which is absent in non-cycling cells (Scholzen and Gerdes, 2000). After BrdU treatment both cell lines

exhibited significant decrease in number of Ki67 positive cells. Together with suppressed Ki67 expression we observed apparent enlargement of the nuclear compartment in BrdU treated cells. Moreover, flow cytometry analysis uncovered higher granularity of BrdU treated cells (increased side scattering) caused probably by expansion of lysosomal and endosomal compartment in these cells, which is related to higher demands of senescent cells on chromatin and protein processing (Brunk and Terman, 2002; Ivanov et al., 2013). Finally we examined expression of some proteins that are typically activated during DNA damage response and senescence. In H28 cells we noticed stabilization of p53 tumor suppressor protein and subsequent upregulation one of its targets including – CDK inhibitor p21. In contrast, PANC-1 cells contain high levels of mutated p53(R273H), which lost its tumor-suppressive functions and fails to activate downstream members of DDR pathways. (Dong et al., 2007; Wang et al., 2013). Thus in addition to higher heterogenity of BrdU-treated PANC-1, presence of mutated p53 could be cause of only mild increase of p21 expression in senescent cells (likely through alternative p53-independent mechanisms). In conclusion, under our experimental conditions BrdU treatment led to the efficient induction of cellular senescence especially in wt p53-expressing H28 cells.

Having established protocol for efficient induction of BrdU-induced senescence we could address the main aim of this thesis and analyze possible and presumed changes in apoptotic signaling in senescent cancer cells. We used TRAIL and FasL as well known and natural inducers of the extrinsic apoptotic signaling (Ashkenazi and Dixit, 1998; Curtin and Cotter, 2003; Duiker et al., 2006). As both cell lines are fairly resistant to TRAIL-induced apoptosis, we used also TRAIL with HHT pretreatment. HHT is a natural alkaloid which binds to the ribosomal A-site and inhibits initiation of translation in the cell (Gurel et al., 2009). In relation to apoptosis it works as an enhancer of TRAIL-induced apoptosis and it sensitizes even TRAIL-resistant cells to TRAIL (Beranova et al., 2013). As other apoptogens we selected DNA damaging agent camptothecin and ROS generating mitochondrially targeted vitamin E succinate – mVES. Camptothecin generates covalent adducts between topoisomerase I and DNA and subsequent replication-triggered generation of double-strand breaks (Koster et al., 2007; Slichenmyer et al., 1993). In contrast to CPT, which is known for decades, mVES represents group of quite new anti-cancer agents, called mitocans. This vitamin E analog destabilizes mitochondrial oxidative phosphorylation via binding to the

mitochondrial complex II (succinate dehydrogenase complex), its inhibition and generation of ROS (Dong et al., 2011a; Dong et al., 2011b; Neuzil et al., 2013).

Compared to the reproducibly strong enhancement of FasL-induced apoptosis in both senescent cancer cell lines, we did not observe any effect of BrdU-induced senescence on sensitivity of H28 or PANC-1 cells to TRAIL. Increased sensitivity of senescent normal and cancer cells to FasL treatment was already reported for senescent human keratinocytes, fibroblasts and MCF-7 breast cancer cells and was at least partly caused by increased expression of Fas receptor in senescent cells (Crescenzi et al., 2011; Tepper et al., 2000; Wang et al., 2004). Unresponsiveness of senescent cancer cells to TRAIL is in contrast to recent publications describing enhanced sensitivity of pretransformed fibroblasts (BJ) treated with conditioned medium from senescent cells (Vjetrovic et al., 2014) and etoposide-treated colorectal carcinoma cells (HT29) (Mendoza et al., 2008) to TRAIL. Discrepancy between their and our data may be connected with different senescence-inducing treatment and partly with different expression of anti-apoptotic protein c-FLIP. While in our study we observed upregulation of c-FLIP expression in senescent (BrdU-treated) cells, Vjetrovic et al. observed c-myc-dependent downregulation of c-FLIP expression.

Although we did not notice any difference between TRAIL-treated proliferating and senescent cancer cells after TRAIL alone, we repeatedly observed increased resistance of senescent cells to TRAIL+HHT treatment, which in nonsenescent, proliferating cancer cells very efficiently triggered their apoptosis. These data support general notion that senescent cells are more resistant to drug treatment and their presence in the tumor may be more detrimental than desirable. Many cancer cell lines are resistant to TRAIL-induced apoptosis and HHT was found as an efficient sensitizer to TRAIL treatment. However, senescence-associated changes apparently negatively influenced this HHT-mediated sensitization.

Similarly as for FasL, senescent cancer cells became more prone to other apoptogens – CPT and mVES. Higher sensitivity of senescent cells to CPT treatment is unexpected as CPT acts mainly during the S-phase of the cell cycle (Liu et al., 2000). Higher susceptibility of senescent cells to mVES treatment extends range of mediators (for example phloretin and cytochalasin B inhibiting glucose transporters, antimycin A disrupting respiratory chain or etomoxir suppressing fatty acid oxidation) disrupting energy-generating pathways that are selectively toxic to senescent cancer cells (Dorr et

al., 2013). According to these data senescent cells seem to be more susceptible to events affecting their metabolic pathways than their proliferating counterparts.

mVES treatment of senescent cells also led to significantly higher acidification of the cultivation medium compared with proliferating cells. Senescent cells have then apparently altered metabolism and mode of glucose utilization. Metabolic pathways in senescent cells seem to be altered through different activity of various enzymes involved in glycolysis or Krebs cycle (Zwerschke et al., 2003) and recently published data suggest that drug-induced senescence enhances glycolytic activity in cancer cells (Dorr et al., 2013). mVES-mediated disruption of mitochondrial respiratory chain may then lead to even enhanced lactate production, its secretion and subsequent medium acidification.

Different apoptotic response of normal and senescent cells could be related to possible differences in the expression of major proteins of apoptotic pathways. As we observed differences in death ligand-induced apoptosis, first of all we naturally analyzed expression of death receptors in normal and senescent cells. In an agreement with already published data (Crescenzi et al., 2011; Curtin and Cotter, 2003; Jeon and Boo, 2013; Liu et al., 2012; Tepper et al., 2000; Wang et al., 2004) we confirmed strong upregulation of FasR in senescent cells correlating with their higher susceptibility to FasL-induced apoptosis. According to Crescenzi et al. (2011), increased expression of FasR in senescent cells is triggered by NFκB pathway-dependent secretion of some SASP cytokines – tumor necrosis factor α (TNFα) and interferon γ (IFNγ). SASP dependence of FasR expression also corresponds to our observation in cells arrested through the inducible expression of CDK inhibitors p21 and p16. Although these cells do not proliferate and even exhibit some markers of senescence, without DNA damage and fully pronounced senescent phenotype (including SASP) they do not increase expression of FasR.

In contrast to the upregulation of FasR we observed low but significant downregulation of the cell surface expression of the TRAIL receptor DR4 in senescent H28 cells (down to about 50 % of normal expression), which could be related to higher resistance of these cells to TRAIL+HHT treatment. However, downregulation of DR4 expression likely could not be the only reason of higher resistance of senescent cancer cells to TRAIL, as PANC-1 cells – that do not show significant change in DR4 or DR5 surface expression – become more resistant to TRAIL+HHT treatment as well. It is also not clear how the expression of TRAIL receptors is regulated. In case of FasR its

upregulation is controlled transcriptionally. In contrast, decreased surface expression of DR4 is probably regulated posttransciptionally because mRNA levels and even total protein levels of DR4 in senescent cells are even higher compared to levels in proliferating cells. Thus we suppose that there may be some obstacles in DR4 protein trafficking to the cytoplasmic membrane.

As changed expression of death receptors did not fully explain altered response of senescent cells to TRAIL+HHT treatment we analyzed expression of major pro- and anti-apoptotic proteins, mostly of the Bcl-2 family. We observed major differences in the expression of pro-apoptotic protein Bim and anti-apoptotic proteins c-FLIP and Bcl-2. Upregulated expression of anti-apoptotic Bcl-2 protein in senescent cells was already described and likely related to increased resistance of senescent fibroblasts to oxidative stress-induced apoptosis (Sanders et al., 2013). We observed increase in Bcl-2 expression only in H28 cells, whereas in PANC-1 cells the trend was just opposite and in addition we also detected downregulated expression of pro-apoptotic Bim protein. While changes in Bim and Bcl-2 expression differed between H28 and PANC-1 cells, upregulation of c-FLIP protein expression was reproducibly observed in both senescent cell lines. c-FLIP binds to DISC and in high concentration it prevents caspase-8 from being cleaved and activated (Ozturk et al., 2012). To address possible role of c-FLIP protein in higher resistance of senescent cells to TRAIL+HHT treatment we prepared H28 cells with downregulated expression of c-FLIP using two different shFLIP-lentiviral vectors (our attempts to prepare also PANC-1 cells with suppressed expression of c-FLIP were unfortunately unsuccessful). Interestingly, H28 cells with downregulated expression of c-FLIP showed higher sensitivity to the senescence-inducing BrdU treatment and BrdU concentration which was inducing senescence in normal or mock-transduced cells was lethal to them. Thus, in order to obtain viable senescent H28 shFLIP cells, we had to decrease BrdU concentration to 20 μM. Although these senescent cells did not increase expression of c-FLIP and maintained its low level as their proliferating counterparts, they did not differ in their response to TRAIL+HHT treatment. Moreover there was no difference in response to TRAIL+HHT treatment even between proliferating H28 wt and H28 shFLIP cells, while in response to FasL treatment H28 shFLIP cells showed lower viability. Thus the increased expression of c-FLIP in senescent H28 cells might not be apparently related to their higher resistance to TRAIL+HHT treatment.

Treatment with sublethal (but stil severe) concentration of BrdU is quite efficient and often way of inducing senescence in both non-cancer and cancer cells. On the other hand it generates relatively extensive permanent stress which could have some side-effects on apoptotic response of such prepaired senescent cells. To eliminate this problem and separate influence of cell cycle arrest alone from the complex senescent phenotype we to prepare cell-cycle arrested cells that would not be too stressed by extensive DNA damage. We used quite new system for simultaneous and equimolar expression of more genes at the same time. We prepared H28 cells inducibly expressing two important CDK inhibitors p21 and p16. Similar system has been already published using immortalized human fibroblasts and breast cancer cells constitutively expressing either p21 or p16 (Capparelli et al., 2012). These cells acquired markers of senescence including increased SA-β-gal activity and flatter morphology. Interestingly human fibroblasts expressing another CDK inhibitor $p19^{ARF/INK4D}$ showed tumor-promoting activity, when coinjected with breast cancer cells into immunodeficient mice (Capparelli et al., 2012). We used this system to examine effect of cell cycle arrest alone or together with minimized BrdU-induced DNA damage. However, mixed culture of transduced H28 cells exhibited high heterogenity and presence of isles of cells with no or silenced expression of p21 and p16, that were overgrowing neighboring cells and hindering further analyses. Therefore in order to obtain more homogenous population expressing high levels of p21 and p16 we had to use clonal selection. Finally we selected two clones with highest inducible expression of p21 and p16 that showed similar characteristics and morphology as original H28 cells. Although expression of these CDK inhibitors alone was able to arrest these cells, they did not exhibit all markers of senescence and did not show pronounced change in their morphology. In order to get closer to more distinquished senescent phenotype we – in addition to the inducible expression of p21 and p16 – treated these cells also with low concentration of BrdU that alone did not lead to senescence in a given time window. In contrast to treatment with 50µM BrdU used previously we did not observe any dying cells after treatment with 10µM BrdU in combination with doxycycline – such treated populations were extremely stable and resistant. Although treatment with low concentration of BrdU alone did not increase expression of p21 or p16, its pretreatment and even cotreatment with doxycycline enhanced doxycycline-inducible expression of both CDK inhibitors. Underlying mechanism remains unclear, but doxycycline-induced cells pretreated or cotreated with BrdU exhibited more senescent markers including changed morphology,

increase in size and granularity or Ki67 negative staining. These markers emerged faster and population of senescent cells remained stable and did not overgrow.

Such prepared cell cycle arrested cells were treated with various apoptogens and analyzed using flow cytometry. In general we observed increased resistance of both clones to all pro-apoptotic treatments. Similarly to 50μM BrdU-induced senescent H28 cells we detected significant upregulation of anti-apoptotic protein Bcl-2, which is often proposed to be one of the main mediators of higher resistance of senescent cells (Sanders et al., 2013). In H28-21 cells pretreated or cotreated with BrdU we also detected increased expression of anti-apoptotic c-FLIP protein as in 50μM BrdU-treated H28 cells. This indicates that c-FLIP could probably increase its expression in response to DNA damaging agents. But according to already mentioned article describing sensitization of pretransformed cells to TRAIL, c-FLIP expression is downregulated after treatment with conditioned medium from senescent cells (Vjetrovic et al., 2014), which contains several factors causing ROS-mediated DNA damage and subsequent DNA damage response (Hubackova et al., 2012). Regulation and role of c-FLIP expression in senescent cells certainly deserve more detail investigation and clarification.

Although we observed predominantly higher resistance of both arrested cells to all apoptogens, there were also differences between these clones, particularly in their response to TRAIL and FasL treatment. While both clones behaved similarly after their treatment with doxycycline alone, or doxycycline cotreated with BrdU, clone H28-21 adopted higher sensitivity to TRAIL, FasL and also CPT, when it was pretreated with BrdU. This unexpectadly increased sensitivity could be caused by significantly increased expression of death receptors, notably DR4 and FasR. Differences in the expression of both receptors between H28-12 and H28-21 cells might be related to clonal selection – H28-12 and H28-21 populations originate from different single maternal cells that could have some differences in their overall gene expression profile.

From the so far published data and also from the results of our experiments, the relation and crosstalks between senescence and apoptosis remain stil very poorly understood topic that certainly merits further investigation. These relations might play a role in tumorigenesis and tumor therapy but can be rather complex as documented by our results presented in this thesis, which raise more questions than answers to mechanisms lying behind these processes.

7. Conclusion

1. We set-up efficient treatment regime for the induction drug-mediated senescence in two cancer cell lines, mesothelial H28 and pancreatic PANC-1. We used replication stress-inducing agent BrdU and prepared stable cultures of senescent cancer cells.
2. We analyzed response of senescent cells to various apoptogens and compared it with the response of non-senescent H28 and PANC-1 cells. We found that these senescent cells acquire resistance to combined death ligand TRAIL+HHT treatment, but manifest markedly increased sensitivity the death ligand FasL as well as to stress-inducing apoptogens camptothecin and mVES. The increased apoptotic response to FasL treatment could be related to strong upregulation of its receptor in senescent cancer cells. Attenuated sensitivity of senescent cells to TRAIL+HHT-induced apoptosis could be in part related to lower expression of DR4 receptor in senescent cells – mainly in case of H28 cells.
3. We also observed significant changes in the expression of apoptosis-related proteins in senescent cells such as an increased expression of anti-apoptotic proteins c-FLIP (PANC- and H28) and Bcl-2 (only H28). However, shRNA-mediated knockdown of c-FLIP in H28 cells did not significantly affect acquired resistance of senescent cells to TRAIL+HHT treatment.
4. We prepared H28 cancer cell line with inducible expression of CDK inhibitors p21 and p16. To ensure high penetration of p21 and p16 expression we used clonal expansion and we selected two promising clones.
5. In these clones the senescent phenotype was mimicked by induced expression of these CDK inhibitors and co- or pre-treatment with low (10 μM) concentration of BrdU.
6. In these cells we induced and quantified apoptotic response to various apoptogens. Cells with induced expression of CDK inhibitors, but without DNA damage became more resistant to all apoptogens. However, depending on the treatment regime (combined BrdU treatment and concurrent or sequential doxycycline-mediated induction of CDK inhibitors) the H28-21 clonal cells can be also sensitized to FasL (likely via increased expression of FasR) and to other apoptogens as well.

7. According to our results we confirmed that senescence may modulate response of cancer cells to various apoptogens. Final outcome (suppressed or enhanced apoptosis) depends on particular apoptosis-inducing, but also senescence-inducing, agent. We identified some proteins probably playing important role in modulating apoptotic response in senescent cells (notably anti-apoptotic protein c-FLIP or death receptor DR4).

8. References

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