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Role of 5-azacytidine in therapy of myelodysplastic syndrome

Úloha 5-azacytidinu v terapii myelodysplastického syndromu

Diplomová práce

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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ABSTRACT

The myelodysplastic syndrome (MDS) is a group of hematopoietic clonal disorders resulting in the inefficient production of myeloid lineage blood cells, with the prevalence of patients older than 65 years. One of the possible treatment options for MDS is 5-azacytidine and 5-aza-2‘-deoxycytidine therapy. These compounds have been shown to cause the induction of cell-cycle arrest, cell differentiation and/or apoptosis. The *in vitro* experiments with 5-aza-2‘-deoxycytidine indicated that this compound causes the premature cellular senescence, a state of the irreversible cell-cycle arrest. We have asked, whether 5-azacytidine, as a molecule with similar structure, is capable of causing the same effect. This treatment strategy could be beneficial in case that the negative pro-inflammatory effect of senescent cells on their surroundings can be nullified. In this thesis we have shown that 5-azacytidine induces DNA damage response, which is described as a fundamental event for the onset of the cell senescence. We tested 5-azacytidine treated HeLa cells for several markers of the cell senescence – the increase of the β-galactosidase activity, the PML and PML nuclear bodies and the formation of persistent DNA damage signaling lesions – albeit all these markers were positive, it was the very low increase in values that lead us to the conclusion that 5-azacytidine does not cause the onset of senescence in HeLa cell line under the conditions comparable to the standard treatment protocol that has been used for the MDS patients. However, according to our results, 5-azacytidine does induce the increase in the secretion of interleukin IL6 and TGFβ. These results, if further confirmed on additional cell lines and *in vivo*, could provide valuable help for therapy modification.

Key words: 5-azacytidine, Myelodysplastic syndromes, Cytokines, Cellular Senescence, DNA damage response

ABSTRAKT

Myelodysplastický syndrom (MDS) představuje skupinu klonálních poruch krvetvorby krevních buněk myeloidní řady postihující především pacienty starší 65 let. Jednu z možností léčby MDS představuje terapie pomocí chemoterapeutik 5-azacytidine a 5-aza-2'-deoxycytidine. Tyto látky jsou schopny vyvolat zástavu buněčného cyklu, buněčné diferenciace a/nebo apoptózy. *In vitro* experimenty naznačují, že 5-aza-2'-deoxycytidine způsobuje předčasnou buněčnou senescenci projevující se ireverzibilní zástavou buněčného cyklu, což nastolilo otázku, zda je tohoto efektu schopen i 5-azacytidine, strukturně blízká molekula. Za předpokladu absence pro-zánětlivého efektu senescentních buněk na okolní prostředí by tato léčebná strategie mohla být přínosná. V této práci jsme dokázali, že 5-azacytidine vyvolává odpověď na DNA poškození, která je popsaná jako zásadní pro vznik senescence. Detekovali jsme některé znaky senescence – zvýšení β-galactosidázové aktivity, nárůst PML a PML jaderných tělísek a vznik dlouhodobých lézí signalizujících DNA poškození – změny jejich hladiny však byly nízké, což nás vedlo k závěru, že 5-azacytidine nevyvolává senescenci u HeLa linie při použití standardního léčebného protokolu pro pacienty s MDS. Prokazatelně však podněcuje sekreci interleukinů IL6 a TGFβ. Výsledky, pokud se potvrdí na dalších buněčných liniích a v *in vivo* experimentech, mohou výrazně napomoci při následné modifikaci této terapie.

Klíčová slova: 5-azacytidin, Myelodysplastický syndrom, Cytokiny, Buněčná senescence, Odpověď na poškození DNA

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CONTENT

LIST OF ABBREVIATIONS

5-AzaC	5-azacytidine
AID	Activation-induced Deaminase
AML	Acute myeloid leukaemia
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR-interacting protein
BCA	Bicinchoninic Acad Assay
β-gal	β-galactosidase
BMT	bone marrow transplantation
BRCA1	Breast Cancer Type 1 Susceptibility protein
BrdU	Bromo-2′-deoxyuridine
CDK	Cyclin-dependent kinase
CDKN2B	gene coding CDK inhibitor $p15^{Ink4B}$
CLP	Common Lymphoid Progenitor Cell
CMP	Common Myeloid Progenitor Cell
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNA-SCARS	DNA Segments with Chromatin Alterations Reinforcing Senescence
DNMT	DNA methyltransferase
dNTPs	deoxynucleotide triphosphates
DSBs	double strand breaks
EDTA	ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2'-deoxy-uridine
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-mesenchymal Transition
EVI-1	Ectopic Viral integration Site 1
EZH2	Histone-lysine N-methyltransferase
FACS	Fluorescence-Activated Cell Sorting

FBS	fetal bovine serum
G-CSF	granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage-colony-stimulating factor
HR	homologous recombination
HSC	Hematopoietic Stem Cell
HPC	Hematopoietic Progenitor Cell
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
IFN	interferon
IKK	Inhibitor of IκB kinase
IL	interleukin
IRF	Interferon Regulatory factor
Jak	Janus kinase
MAPK	Mitogen-ativated Protein kinase
MDC1	Mediator of DNA Damage Checkpoint protein 1
MDS	Myelodysplastic syndromes
MDS-U	Myelodysplastic syndromes, unclassified
miRNA	micro RNA
MM	multiple-myeloma derived cell line
MUG	4-methylumbelliferyl-beta-D-galactopyranoside
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBs	NBs nuclear bodies
NEMO	NF-kapa-B essential modulator
NF-kB	Nuclear Factor κB
NHEJ	non-homologous end joining
NK cells	Natural killer cells
PBS	phosphate buffered saline
PBS+T	phosphate buffered saline + Tween 20
PCR	Polymerase Chain Reaction
PD	population dubling
PML	promyelocytic leukemia protein
RAEB-1	Refractory Anemia with excess blasts-1
RAEB-2	Refractory Anemia with excess blasts-2

RARS	Refractory Anemia with ringed sideroblast
Rb	Retinoblastoma protein
RCMD	Refractory cytopenia with multilineage dysplasia
RCUD	Refractory cytopenia with unilineage dysplasia
RNA	ribonucleic acid
ROS	reactive oxygene species
RPA	Replication protein A
rRNA	ribosomal ribonucleic acid
SA β-gal	senescence-associated β-galactosidase
SAM	S-adenosylmethionin
SASP	Senescence-associated Secretory Phenotype
SSBs	single stranded breaks
STAT	Signal Transducer and Activator of Transcription
TNF	Tumor Necrosis Factor
TG	tris glycine
TGF	Transforming Growth factor
TGS	tris glycine SDS
TIS	therapy induced senescence
UV	ultraviolet
WHO	World Health Organisation

1. INTRODUCTION

Myelodysplastic syndromes (MDS) are a group of blood disseases originating from abberrant differentiation of myeloid lineage of blood cells. MDS affects mostly people of advanced age and thereby belongs among age-associated dieseases (MA *et al.* 2007). With the population growing older in general, the incidence rate of MDS increases and it is becoming of pressing need to re-evaluate therapeutic strategies in light of the new discoveries.

Currently, the only cure for MDS is the bone marrow transplantation, an invasive method not suitable for most of MDS patients because of its high demands on their physical condition. Among other therapeutic approaches in MDS treatment belongs the demethylation therapy. In MDS, there is frequent occurence of transcriptional silencing of genes involved in the cell cycle regulation via hypermethylation of their promoter regions. 5-azacytidine and 5-aza-2'-deoxycytidine are inhibitors of DNA methyltransferases (in case of 5-azacytidine of RNA methyltransferases as well), which allows for re-expression of the genes silenced by abberant hypermethylation. However, the exact mechanism of 5-azacytidine therapeutic action remains to be elucidated.

The 5-aza-2'-deoxycytidine has been shown to induce therapy-induced senescence (TIS), a state of permanent cell-cycle arrest associated with specific phenotype of cell (SCHNEKENBURGER *et al.* 2011, VENTURELLI *et al.* 2013, GRANDJENETTE 2014). This thesis aims to explore the possibility of 5-azacytidine inducing the same effect, given its similarity to 5-aza-2'-deoxycytidine. It is crucial to investigate the 5-azacytidine-mediated secretory phenotype into more depth and in exact context of 5-azacytidine treatment - to provide an insight into possibility of employing TIS in MDS treatment.

The experimental part of this thesis investigates whether 5-azacytidine induces the senescence, with the emphasis on the presence of senescence-associated secretory phenotype.

2. LITERATURE REVIEW

2.1. Myelodysplastic syndromes

Myelodysplastic syndromes (MDS) are a wide collection of blood diseases grouped together based on the clinical outcome – deficiency in one or more cell types derived from the Common Myeloid Precursor Cell (see Fig. 1 for cell types likely affected in MDS). Hofmann and Koeffler defined MDS as "a clonal abnormality of the hematopoietic stem cell characterized by defective maturation and in advanced stages of uncontrolled proliferation" (reviewed in GREENBERG *et al.* 2006). MDS is associated with advanced age – the median age of MDS patients diagnosed between years 2001 and 2003 in USA was 76 years, while 86 % of them were 60 years old or above. MDS significantly prevails among men. Studies of population in Netherlands and Germany in the same period as mentioned above showed results corresponding to the statistical data obtained in the USA (MA *et al.* 2007, NEUKIRCHEN *et al.* 2011, DINMOHAMED *et al.* 2014). In the USA, the annual age-adjusted incidence rate of MDS is about 3.4 newly diagnosed patients per 100,000 habitants. In the Czech republic, up to 100 patients are newly diagnosed with MDS every year (MA *et al.* 2007, JONÁŠOVÁ & KAČMÁŘOVÁ 2012).

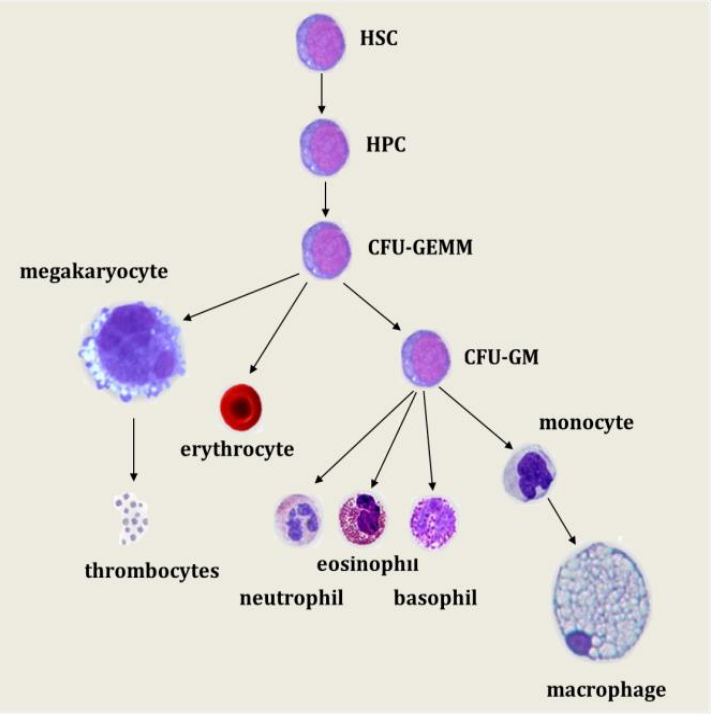


Fig. 1. Differentiation of myeloid lineage of blood cells.

This scheme represents differentiation of myeloid lineage of blood cell types derived from the Human Multipotential Progenitor Cells (CFU-GEMM) which originates by differentiation of the Common Myeloid Progenitor Cell (not shown in the Fig. 1). Hematopoietic Stem Cell (HSC) replicates by asymmetric cell division in which one daughter cell remains multipotential HSC and the other one differentiates into Hematopoietic Progenitor Cell (HPC) and then further into Common Myeloid Progenitor Cell (CMP) or Common Lymphoid Progenitor Cell (CLP). Granulocytes and monocytes share a common precursor - Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) (modified from NIENHUIS 2008).

The clinical manifestation of MDS in patients differs according to the type of cytopenia (deficiency in blood cells) and severity of the disease. The cytopenia of MDS patients might be either anemia (deficiency in erythrocytes), leukocytopenia (deficiency in leukocytes) or thrombocytopenia (deficiency in thrombocytes).

Patients might experience symptoms associated with anemia; such as fatigue, pallor (unusual paleness), shortness of breath, cardiovascular difficulties, etc. Deficiency in platelets may result in easy bruising, epistaxis (nosebleeds), petechiae (red spots from bleeding beneath skin) but also gastrointestinal bleeding, etc. Patients with the deficient production of white blood cells are also sensitive to the infections.

There are several types of MDS fitting different classification systems based on manifestation of the disease; for example French-American-British system and WHO Prognostic Scoring System introduced by World Health Organization or by expected prognosis, i. e. International Prognostic Scoring System. The survival rates vary greatly with types of MDS and risk for AML development; their median ranges from several years to months (GREENBERG et al 1997).

Subtype	Blood	Bone marrow
Refractory cytopenia with unilineage dysplasia (RCUD)	Single or bicytopenia	Dysplasia in ≥ 10% of one cell line, < 5% blasts
Refractory anemia with ringed sideroblast (RARS)	Anemia, no blasts	≥ 15% of erythroid precursors with ring sideroblasts, erythroid dysplasia only, <5% blasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenia(s), < 1 x 10^9/L monocytes	Dysplasia in ≥ 10% of cells ≥ 2 hematopoietic lineages, ±15% ring sideroblasts, <5% blasts
Refractory anemia with excess blasts-1 (RAEB-1)	Cytopenia(s), ≤2% - 4% blasts, < 1 x 10^9/L monocytes	Unilineage or multilineage dysplasia, No Auer rods, 5% - 9% blasts,
Refractory anemia with excess blasts-2 (RAEB-2)	Cytopenia(s), ≤5% - 19% blasts, < 1x 10^9/L monocytes	Unilineage or multilineage dysplasia, No Auer rods, ±10% - 19% blasts
Myelodysplastic syndrome, unclassified (MDS-U)	Cytopenias	Unilineage dysplasia or no dysplasia, but characteristic MDS cytogenetics, <5% blasts
MDS associated with isolated del(5q)	Anemia, platelets normal or increased	Unilineage erythroid dysplasia, isolated del(5q) <5% blasts

Table 1. WHO classification system for de novo MDS (updated 2008).

Classification od MDS patients into subtypes based on blood and bone marrow findings according to WHO Prognostic Scoring System (modified from SWERDLOW *et al.* 2008).

MDS was first characterized as a stage preceding acute myeloid leukemia (AML) and thus was labeled as the pre-leukemic phase (BLOCK *et al.* 1953). Indeed, about third of the patients develop acute myeloid leukemia in time, but the term "preleukemic phase" is nowadays considered to be inaccurate. The line between MDS and AML was set to by FAB system to be 20% of blasts in bone marrow and 30% of blasts by WHO system of classification. In MDS, the cells fail to reach the terminal differentiation step in bone marrow and preferentially undergo apoptosis, whereas in AML are cells more resistant to apoptosis and un-differentiated blasts are more likely to be released into the bloodstream (ALBITAR *et al.* 2002).

The afflicted clonal cells may derive from aneuploidy, the most common chromosome aberration is the deletion of 5q chromosome arm. This aneuploidy is present among more than 10% of patients and has its own class in the scoring systems (SOLE *et al.* 2001). Besides, the loss of 7 chromosome or 8 chromosome trisomy is of frequent occurrence in the aberrant cells (JOHNSON *et al.* 1996, MA *et al.* 2010). Chromosomal translocations may lead to the promotion of MDS clone as well (WLODARSKA *et al.* 1995).

Frequently, MDS patients have altered gene expression due to mutations, deletions or aberrant methylation of gene promoters. The most frequent alterations are in genes coding for $p15^{Ink4B}$ (p15), Neuroblastoma RAS Viral Oncogene Homolog (N-RAS; oncogene), Ectopic Viral Integration site 1 (EVI-1; oncogene), Interferon Regulatory Factor 1 (IRF-1; tumor suppressor), p53 (tumor suppressor) and others. Over-expression of N-RAS, EVI-1 and decreased expression of p15, IRF-1 or p53 are associated with the increased risk of development into AML (PAQUETTE *et al.* 1993, SUGIMOTO *et al.* 1993, QUESNEL *et al.* 1998, XU *et al.* 1999, WILLMAN *et al.* 1993).

Secondary MDS is a specific, therapy-related type of MDS, which may arise after previously administered high-dose chemotherapy followed by autologous hematopoietic cell transplantation. Patients with secondary MDS have worse prognosis and are classified as a higher risk types. Moreover, the people exposed to toxic chemicals, such as benzene or tobacco smoke, have a higher risk of developing MDS (CIOC *et al.* 2007, ZIPURSKI *et al.* 1993, LV *et al.* 2011).

2.1.1. Therapy of MDS

As of today, the only cure for MDS is the bone marrow transplantation (BMT), all other approaches in MDS treatment serve for tempering the disease symptoms and slowing down progression of the disease. BMT is a method used preferably on younger patients diagnosed with high-risk MDS and is not typically considered first-line treatment for low-risk MDS types. As it was addressed above, the vast majority of patients is of advanced age and because of the age-related co-morbidities, they are not suitable for this type of risky intervention. However, several novel studies indicate that the age should not be the deciding factor for the eligibility for BMT and each case should be considered on the individual basis. With the increase of older patients, there are novel protocols with reduced-intensity conditioning prior to BMT treatment emerging (McCLUNE *et al.* 2010, SORROR *et al.* 2011).

The supportive therapy for both low and high-risk MDS patients consists of red blood cells or platelet transfusion, possibly combined with the chelation therapy to protect the patient from iron overload (excess accumulation of iron can arise from the numerous red blood cells transfusions). Pharmaceutically produced forms of growth factors, such as G-CSF, GM-CSF, erythropoietin, IL-3 and IL11, may be employed in treating the symptoms of insufficient erythropoiesis (MUSTO *et al.* 2001, TSIMBERIDOU *et al.* 2005).

Depending on MDS type and its progress, the patients might be presented with an option of drug therapy. One of the treatment strategies is the immunosuppressive therapy based on the observation that in some MDS patients, autologous T cells inhibit erythropoiesis and possibly have also negative impact on the granulopoiesis. For these purposes is employed the use anti-thymocyte globulin, either alone or in combination with cyclosporine, which is primary being used for the treatment of autoimmune diseases such as Reynaud's syndrome, rheumatoid arthritis and aplastic anemia. This powerful combination is used for abolishing T-cells-mediated cytotoxity towards normal progenitors of blood cells (reviewed in BARRETT & SLOAND 2009; PASSWEG *et al.* 2010).

Yet another drug used in MDS therapy is lenalidomide. This drug is derived from thalidomide and has a wide spectrum of anti-tumorigenic effects. It supports NK cells and T lymphocytes-driven immune response to transformed cells and

suppresses the inflammatory environment. Lenalidomide inhibits angiogenesis, induces cell-cycle arrest via $p21^{Waf1}$ (p21) expression and initiates apoptosis (possibly through increased sensitivity to Fas ligand). Moreover, Lenalidomide is especially effective in the treatment of MDS patients with 5q chromosome arm deletion and is also used in low-risk MDS types (reviewed in KOTLA *et al.* 2009).

Finally, MDS can be also treated by hypomethylating agents, Vidaza® and Decitabine® (trade marks for 5-azacytidine and 5-aza-2'-deoxycytidine, respectively). The chemical structure of these compounds is very similar with the only difference being in hydroxyl group at nucleoside sugar backbone (Vidaza® contains ribose and Decitabine® contains deoxyribose). Vidaza® is used as the first-line hypomethylating agent because it prolongs the time until the transformation into AML and increases the overall survival of treated patients (XIE *et al.* 2014).

2.2. 5-azacytidine

5-azacytidine is a ribonucleoside analog of cytidine, which contains additional nitrogen heteroatom in the pyrimidine ring, instead of carbon at fifth position (see Figure 1). Unlike the other structurally very similar analog, 5-aza-2'-deoxycytidine, 5-azacytidine is able to be incorporated into both nucleic acids.

In L1210 cell line (derived from mouse lymphatic leukemia), 10 - 20% of 5-azacytidine diphosphate is reduced to its deoxyribonucleotide form by ribonucleotide reductase. The reduction rate of 5-azacytidine correlates with 10 to 20% of 5-azacytidine being incorporated into DNA (LI *et al.* 1970). It is noteworthy to mention that 5-azacytidine is not being phosphorylated by the same kinase as cytidine is, i.e. by deoxycitidine kinase, but probably by uridine kinase (LI *et al.* 1970).

5-azacytidine was synthesized for the first time by Píškala and Šorm in Czechoslovakia more than 50 years ago and it was firstly used as a cancerostatics. In 2004, 5-azacytidine became the first drug for treatment of MDS that was approved by U. S. Food and Drug Administration (under trademark Vidaza®) (PISKALA & SORM 1964). 5-azacytidine is rather unstable and temperature-sensitive substance in both alkaline and acidic solutions. In neutral aqueous solutions at 37°C, it appears to have a half-time of degradation about 7 hours (ISRAILI *et al.* 1976).

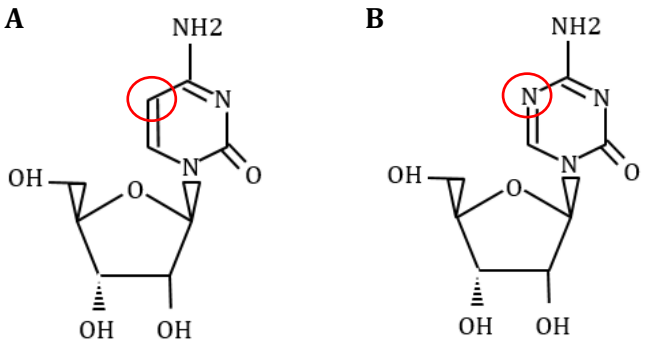


Fig. 2. Chemical structure of cytidine and 5-azacytidine. Chemical structure of cytidine (A) and 5-azacytidine (B). The only difference between both compounds is the addition of nitrogen heteroatom into pyrimidine ring instead of carbon at position 5.

2.2.1. The role of 5-azacytidine in DNA and histone methyltransferases inhibition

5-azacytidine inhibits methylation of cytosine at its fifth carbon in DNA; it is the only base being physiologically methylated in DNA of mammals. This methylation is being executed by DNA methyltransferases (DNMT) and has a crucial role in mammalian development and overall regulation of the transcription. Altered DNA methylation pattern is generally associated with the cell transformation. In MDS, there has been evidence of many genes inhibiting cellular growth being silenced by the hypermethylation in promoter region and these changes were associated with worse prognosis in the patients. Genes like *CDKN2B* (gene coding CDK inhibitor p15), *CTNNA1* (gene coding Cadherine-Associated Protein Alpha 1), *DAPK1* (gene coding Death-Associated Protein Kinase 1) and *SOCS1* (gene coding Suppressor Of Cytokine Signaling 1) are frequently down-regulated in early MDS patients, but are more likely to be hypermethylated in high-risk MDS. The changes in the expression of these genes are also associated with progression to AML (YE *et al.* 2009, WU *et al.* 2006, QIAN *et al.* 2010, CHRISTIANSEN *et al.* 2003).

5-azacytidine treatment has an effect on transcription pattern of more than a thousand genes. The majority of them is however not being altered in transcription via promoter demethylation - 5-azacytidine affects mainly the transcription of genes within active chromatin domains. Still, since these genes belong to the specific ontological families, it is probable that significant number of them is regulated by 5-azacytidine-dependent changes in their upstream regulator sequences (KOMASHKO & FARNHAM 2010). 5-azacytidine also affects transcription of miRNAs (YANG *et al.* 2012).

The mechanism of 5-azacytidine-dependent depletion of DNA methyltransferases is following: Sixth carbon atom of 5-azacytosine (5-azacytidine base) is very reactive and forms the covalent bond with methylation site of DNMT. These adducts are very stable and can last for several days. The complex between 5-azacytosine and DNMT may exist in two forms depending on the presence of S-adenosylmethionin (SAM), i.e. DNA methyltransferase bound to methylated 5-azacytosine (in presence of SAM) or bound to protonated 5-azacytosine (in absence of SAM) (SANTI *et al.* 1984, GABBARA & BHAGWAT 1995).

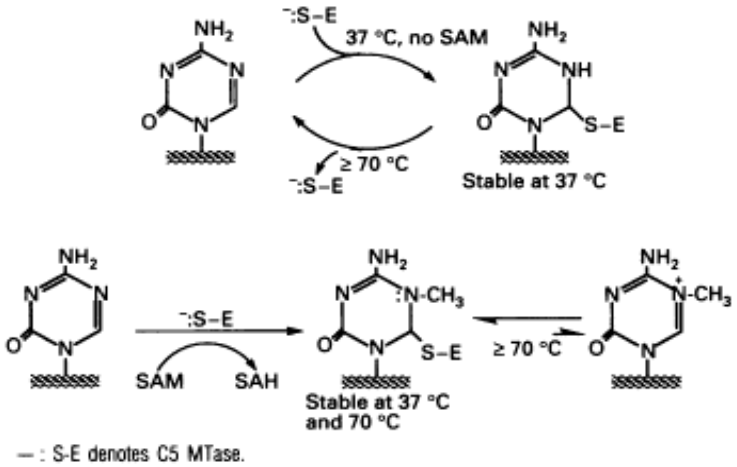


Fig. 3 Mechanism of formation of covalent adduct between 5-azacytosine andDNA methyltransferase.

5-azacytosine is bound to methylation site of DNA methyltransferases (DNMT) at its sixth carbon. This complex is very stable at 37°C and needs high temperature to be dissagregated. In the absence of S-adnosylmethionin (SAM), 5-azacytosine is protonated whereby in the presence of S-adenosylmethionin (SAM), 5-azacytosine is methylated. In vivo, the former option is more likely to occure (adopted from GABBARA & BHAGWAT 1995).

Also, since several histone methyltransferases (for example EZH2 or G9a) are associated with the complexes of DNA methyltranferases, it would be expected that the histone methyltransferases might be inhibited in their function as well. There was a difference observed in status of H3K9 and H3K27 trimethylation between 5-azacytidine treated and untreated HEK 293 cells. While the promoters exhibiting the above mentioned histone modifications lost histone methylation in general after 5-azacytidine treatment, other promoters have gained histone methylation, although in the disorganized manner – some genes known for H3K9 methylation were methylated on lysine 27 and vice versa (KOMASHKO & FARNHAM 2010).

Correspondingly, with overall loss of histone methylation, 5-azacytidine induces acetylation on histone 4 (YANG *et al.* 2010).

2.2.2. The role of 5-azacytidine in RNA methyltransferases inhibition

5-azacytidine also affects the proper function of RNA by depletion of RNA methyltransferases and subsequent demethylation of RNA - RNA modifications plays an important role in its secondary and tertiary structure and stability. 5-azacytidine alters secondary and tertiary structure of pre-ribosomal 45S rRNA and possibly even inhibits 45S rRNA synthesis via DNA demethylation. Demethylation of rDNA CpG sites (genes coding rRNA) inhibits synthesis of rRNA and processing of rRNA precursor (reviewed in MOSS 2011). Moreover, the formation of polyribosomes is affected by 5-azacytidine as well – it decouples ribosomes in the chain. Also, after 5-azacytidine distribution, there is a decrease in cytoplasmic 18S and 28S rRNAs (REUVENI & ROSENTHAL 1979). tRNA methylation is also affected by 5-azacytidine since it inhibits activity of tRNA cytosine-5-methyltransferase and causes delay in the increase in other tRNA methyltransferases (LU & RANDERATH 1979, 1980). The lack of m5C methylation can, in context, induce rapid tRNA degradation and hypomethylated tRNA from 5-azacytidine treated cells significantly down-regulates the protein synthesis (MOMPARLER *et al.* 1976, ALEXANDROV *et al.* 2006).

2.2.3. The role of 5-azacytidine in DNA damage induction

DNA damage induced by 5-azacytidine may emerge in several ways. The covalent bond between 5-azacytosine and DNMT1 forms an adduct preventing passage of the replication machinery and leads to the collapse of the replication fork that subsequently results in the formation of double strand breaks (DSBs). The reparation of DSB is mediated by homologous recombination (HR) and when unrepaired, the covalent bond between 5-azacytosine and DNMT1 may last for three or more days. Subsequently, when the cell cannot use HR, non-homologous end joining (NHEJ) takes its place and fuse both ends non-specifically which may generate chromosomal rearrangements (JÜTTERMANN *et al.* 1994, ORTA *et al.* 2013). In mice and embryonic stem cells, 5-azacytosine-DNMT1 adducts formation,

rather than DNA demethylation, is the cause of 5-azacytidine cytotoxicity (JÜTTERMANN *et al.* 1994). Mouse embryonic fibroblasts with DNMT1-inhibiting siRNA showed significant decrease in amount of DSBs and chromosomal rearrangements in comparison to non-coding siRNA (MASLOV *et al.* 2012).

The DNMT1-independent process of 5-azacytidine DNA damaging action consists of introducing point mutations into DNA by interaction of 5-azacytidine ring with cytosine or by the deamination to 5-azauridine. Due to 5-azacytidine instability in the physiological conditions, it can decay when already incorporated DNA and create a basic site paired with adenosine (ZIELINSKI & SPRINZL 1984). There is another aspect of 5-azacytidine role in DNA damage induction.

5-azacytidine forms adducts with Activation-induced deaminase (AID), a protein involved in the somatic hypermutation and class switch recombination in lymphocytes, and prevents it from introducing mutations into DNA of lymphocytes (TSAI *et al.* 2014).

2.3. Cellular senescence

As was previously presented, 5-azacytidine is potent cancerostatics which inhibits the cell cycle progression. One of the possible responses to the cell-cycle arrest in mitogen stimulated cells is the development of senescence.

Cellular senescence was discovered by Hayflick and Moorhead during the search for the mechanisms of live virus vaccines production. They have distinguished cell strains from cell lines by the use of eleven markers, among them limitation of the cell multiplication, which was limited in cell strains, and comparison of cell morphology to the corresponding primary tissue. They have noticed an arrest in the proliferation of the cells that were beyond certain number of doublings. The "passage potential" of fibroblasts derived from different tissues was around 50 population doublings, independently on continuity of the cultivation (HAYFLICK & MOOREHEAD 1961). Such phenomenon was observed in many cell strains derived for example from T-lymphocytes, mesenchymal stem cells, etc., and also *in vivo*; and was named the replicative senescence (ESTRADA *et al.* 2013, SPAULDING *et al.* 1999, HARRISON et al, 1973). The maximum of population doublings is therefore often termed "Hayflick limit".

Senescence can evolve prior to the exhaustion of replicative potential as well. When the cell acquires unrepairable damage or experiences severe stress that might lead to such, one of its possible fates may lie in entering of the senescence. This way, the senescent cell prevents the propagation of the aberration on the next generations and maintains the integrity of the organism. For this reason, senescence is often described as a barrier against the tumorigenesis (reviewed in CAMPISI 2013). Indeed, *in vivo*, it has been noticed that senescent cells are present in pre-neoplastic lesions and they diminish with the tumor progression (BARTKOVA *et al.* 2006). Also, the senescence participates in wound healing, where it supports the full recovery of the tissue and suppresses the development of fibrotic scar (KANG *et al.* 2011).

However, the role of senescence in organism is complex and not entirely anti-tumorigenic. Due to specific, pro-inflammatory senescence-associated phenotype (SASP), senescent cells can 'spread' senescent phenotype on other surrounding cells in paracrine manner. This phenomenon is called secondary or "bystander senescence" (HUBACKOVA *et al.* 2010).

If senescent cells are not efficiently cleared from the organism, their accumulation might lead to the disrupted function of the respective body organs and tissues. Their increase in numbers is associated with the neurodegenerative diseases and other age-related pathologies, such as osteoarthritis or cardiovascular diseases (reviewed in VASTO *et al.* 2006 , reviewed in CAMPISI 2013). Further, senescence has been described to support the tumorigenic growth. The inflammation induced by SASP is known to strengthen the transformation and invasivity of cancer cells. Also defective mitochondria of senescent cells produce ROS and may result in DNA damage in by-standing cells (GOSSELINK *et al.* 2009, reviewed in CAMPISI 2005, 2013). Nonetheless, cleared senescent cells must be compensated for, otherwise the body organ dysfunction or damage may occur (KRIZHANOVSKY *et al.* 2008).

The role of senescence in the organism is complex and based on context, it might be both beneficial (tumor suppression) and deleterious (tumor growth support, age related diseases). Novel therapeutic strategies employing therapy-induced senescence in cancer therapy emerge and they show promise of overcoming drug-resistance and entailing less severe side effects associated with therapy (reviewed in

NARDELLA *et al.* 2011). Further, the senescent cells attract cells of the immune system, namely monocytes, macrophages, Th1 lymphocytes and NK-cells by which they are cleared and may aid in the elimination of tumor cells as well (KANG *et al.* 2011). Nonetheless, as it has been mentioned, there are negative aspects to senescence. In order to prevent their occurrence, the efficient strategies to eliminate senescent cells must be developed. For example, Weiland and his colleagues introduced rather specific method of targeting viral replication in the tumor and senescent cells (WEILAND *et al.* 2013, reviewed in NARDELLA *et al.* 2011). Also, the existence of senescence lacking pro-inflammatory secretome observed after the ectopic induction of p16 and p21 indicates that the investigation of senescence for the anti-tumor therapeutic strategies might be of a great benefit (COPPÉ *et al.* 2008).

The premature senescence may be induced by countless stimuli, internal (ROS production, mutagenesis, collapse of replication fork, oncogene activation) or by external (genotoxic drugs, bacterial toxins, or physical stress like ionizing and UV radiation) resulting in the oxidative stress, DNA damage or telomere dysfunction (reviewed in RODIER & CAMPISI 2011). The determining factor in the onset of the senescence may be the extent of the damage since lower doses of cytotoxic insults seem to invoke senescence rather than apoptosis. However, the outcome is very context-dependent (HAN *et al.* 2002, TSAI *et al.* 2009).

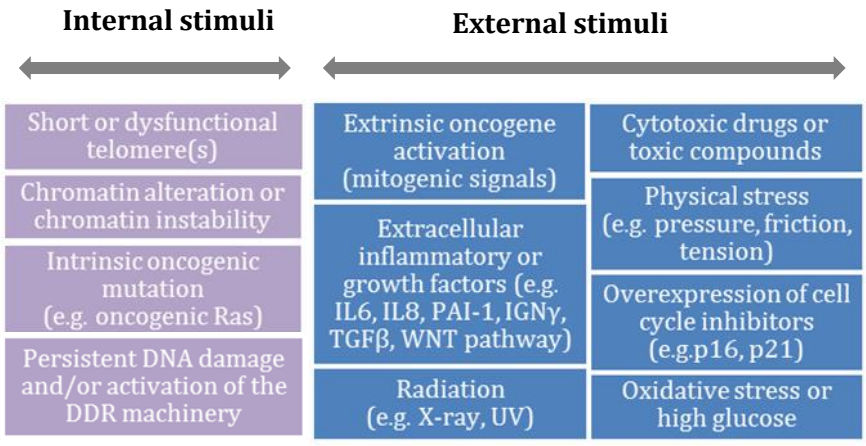


Fig. 4 Internal (pink) and external (blue) stimuli leading to senescence.

If the cell exprerience an irreparable damage resulting in severe stress, it will cease proliferation and may undergo apoptosis or initiate permanent cell-cycle arrest with developing typical senescent phenotype. The origin of stress may arise inside or outside of the cell and may be of both physiological or artificial nature (modified from DAVALOS *et al.* 2010).

2.3.1. Hallmarks of the cellular senescence

There are several causes leading to senescence and they typically result in the specific cell phenotype. Note however, there is no specific marker common to all types of senescence or cell type. The senescent phenotype is therefore characterized as a combination of markers discussed below. The senescent cells do not necessarily express all of the associated markers and these markers are not specific only to the senescence. Since the cellular senescence is of a growing interest, new hallmarks of senescence are emerging in time.

2.3.1.1. Permanent arrest in cell-cycle

Hallmark common to all senescent cells is a permanent arrest in the cell-cycle. While there have been some documented cases of the escape from the senescence, some external interventions have been needed (BEAUSÉJOUR *et al.* 2003, ROBERSON *et al.* 2005).

The cell cycle is an ordered sequence of independent but tightly coordinated events, which enables cells to duplicate. the correct progression trough the cell cycle is monitored by various checkpoints and is driven by cyclin-dependent kinases (CDK) and its corresponding cyclins fluctuating during the cell cycle. The activity of CDK kinases is regulated by their respective cyclins and dephosphorylation at threonine 14 and tyrosine 15 on CDK 1 and 2 and tyrosine 17 on CDK4 (TERADA *et al.* 1995, SEBASTIAN *et al.* 1993).

The activated cyclin-CDK complex phosphorylates its downstream substrates and through them orchestrates the passage through another phase of the cell cycle. The mechanisms, how to arrest the cell cycle then include the inhibition of the activating dephosphorylation of CDKs or the association of cyclin-CDK complex with the CDK inhibitors of INK4 and Cip/Kip protein family (HALL *et al.* 1995, WOHLSCHLEGEL *et al.* 2001). The cell cycle checkpoint preserving the genome integrity is induced in the presence of DNA damage and may arrest the proliferating cell in G1, S and G2 phases. DSBs and SSBs signalizing trough Ataxia telangiectasia mutated kinase (ATM) and Ataxia telangiectasia and Rad3-related protein kinase (ATR) activate Checkpoint kinases 1 and 2 (Chk1, Chk2), which inhibit Cdc25 phosphatase

(activator of CDKs) and in p53-dependent manner increase the expression of p21 (FALCK *et al.* 2005, SHREERAM *et al.* 2008).

The mechanism of the DNA damage response pathway involving ATM is following: MRN complex, composed of Mre11, Rad50 and Nbs1, detects the DSBs and binds to the exposed DNA. MRN complex then attracts the inactive ATM-Tip60 complex and initiates the autophosphorylation of ATM at S367, 1893, S1981 and possibly at other residues (BAKKENIST & KASTAN 2003, KOZLOV *et al.* 2006, FALCK *et al.* 2005, SUN *et al.* 2007). ATM dissociates into the active monomers and phosphorylates histone H2A at S139 in the area of DSBs, 53BP1 at S1219, BRCA1 at S1152 and many more other proteins (LEE *et al.* 2009, BURMA *et al.* 2001, CORTEZ *et al.* 1999). γH2AX phosphorylation creates the positive feed-back loop for ATM because CK2-dependently phosphorylated MDC1 bound to MRN complex recognizes H2AX and binds ATM (STUCKI *et al.* 2005, SPYCHER at al. 2008). It allows ATM to spread the phosphorylation on other histone H2A in the proximity and in turn, to attract MDC1 with MRN complex and ATM kinase. ATM activates Chk2 via the phosphorylation at T68 (AHN *et al.* 2000).

ATR signalization is activated via formation of SSBs. RPA binds single strand DNA and RPA attracts ATRIP in complex with ATR. The complex 9-1-1 consisting of Rad 9, Hus 1 and Rad 1 recruits TopBP1 to the site of SSB which potentiates activity of ATR and enables the activation of Chk1 via the phosphorylation on serine 317 and 345 (WOLD 1997, ZOU & ELLEDGE 2003, DELACROIX *et al.* 2007, WANG *et al.* 2006, ZHAO *et al.* 2001).

The pivotal role in the induction and maintaining of the senescent cell-cycle arrest is played by CDK inhibitors. They are represented by two families, INK4 and Cip/Kip family of CDK inhibitors. Among INK4 protein family, there are $p15^{INK4B}$, 16^{INK4A}, $p18^{INK4C}$ and $p19^{INK4D}$ CDK inhibitors and they inhibit CDK4 and CDK6. Kip/Cip family consists of $^{p21Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ and they are able to inhibit CDK1 and CDK2 (reviewed in DONOVAN & SLINGERLAND 2000).

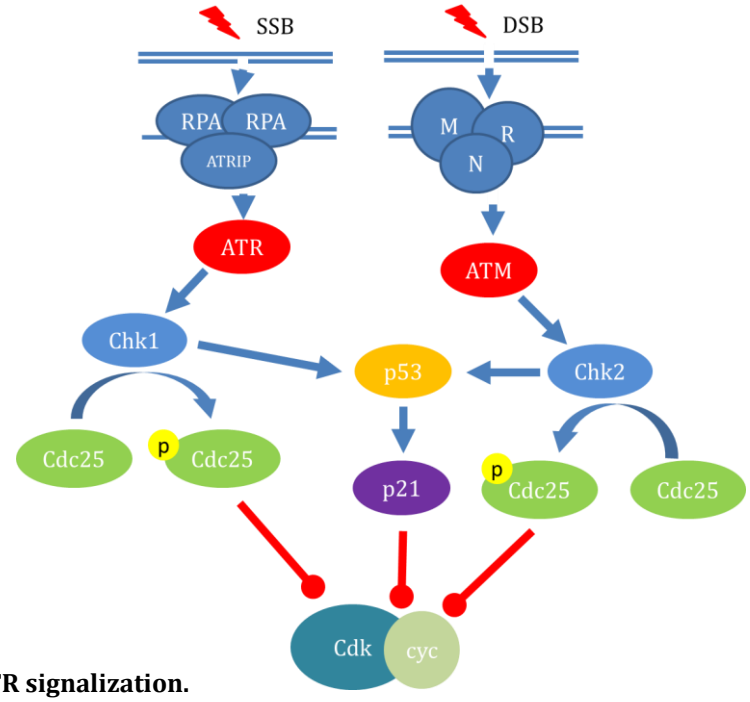


Fig. 5 ATM and ATR signalization.

DSBs activate ATM kinase which phosphorylates p53 and Chk2. SSBs activate Chk1 via ATR. Both Chk1 and Chk2 helps to stabilize tetramer of TSp53 in two ways: by phosphorylation at S15, S20, S35, T18 within C-terminus of TSp53 and by phosphorylation and subsequent degradation of Mdm2 and MdmX (reviewed in MEULMEESTER *et al.* 2005). Furthermore, Chk1 and Chk2 phosphorylates Cdc25A on S123 and thus prevents activating dephosphorylation of *CDK*2 and CDK1 and subsequent DNA synthesis or M phase entry (FALCK *et al.* 2005, SHREERAM *et al.* 2008). p53 acts mainly as a transcription factor and initiates expression of p21 which inhibits CDK kinases and arrest cell cycle.

The expression of p21 is indispensable in the induction of senescence (ROMANOV *et al.* 2012). The cell responds to the senescence-inducing stimuli by activating p53 which is transcription factor of p21. p21 creates the necessary self-sustaining positive feed-back loop through the ROS production (ROS attack DNA and positively stimulate p53 to enhance p21) (PASSOS *et al.* 2010). Other than inhibiting CDK kinases, p21 acts as an inhibitor of wide variety of proteins and complexes so it has an active role for example in inhibition of DNA synthesis and its repair and in avoidance of apoptosis (reviewed in ROMANOV *et al.* 2012)

Another CDK inhibitors involved in senescence are p15 and p16 which prevent the formation of cycD1-CDK4/6 complex and thereby prevent phosphorylation of Rb, the release of E2F transcription factor and the initiation of the proliferation (HALL *et al.* 1995). Furthermore, p16 is partially responsible for ROS production in the senescence which is important component in the establishment and maintenance of the senescence (PASSOS *et al.* 2010). p15 is upregulated by TGFβ

signalization trough Nox4-dependent ROS production (FUXE *et al.* 2000, SENTURK *et al.* 2010). Moreover, Ras oncogene activation can promote p16-regulated senescence by transcription of Ets1 and Ets2 (OHTANI *et al.* 2001).

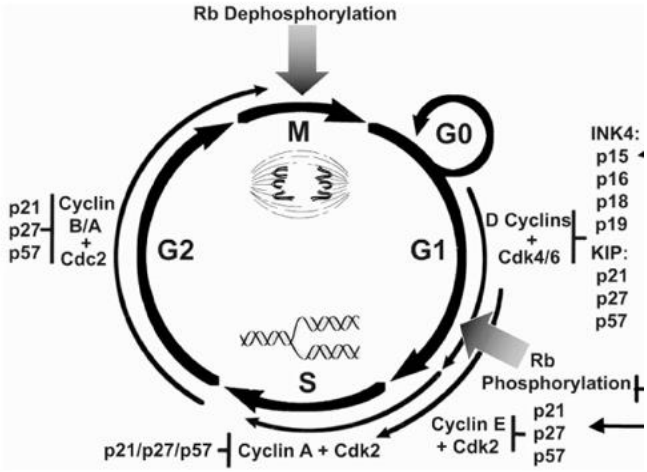


Fig. 6 Regulation of cell proliferation.

Cyclin-dependent kinases in complexes with their respective cyclins regulates progression through cell cycle. Complexes cyclinD1-CDK4/6 and cyclinE-CDK2 phosphorylate Rb tumor suppressor and initiate S phase. CyclinA-CDK2 drives cell through S phase and Cdc2 associated with cyclin A or cyclin B regulates progression of the cell through M phase. INK4 family inhibits activity of CDK4/6 kinases and CDK1 and CDK2 may be inhibited by Kip family of CDK inhibitors (reviewed in DONOVAN & SLINGERLAND 2000).

2.3.1.2. Promyelocytic leukaemia nuclear bodies

Another feature accompanying senescence is the increase in Promyelocytic leukaemia nuclear bodies (PML NBs). PML is a tumor suppressor protein which is associated with several DNA damage signaling proteins and and it is able to activate Rb and p53 tumor suppressor pathways (FERBEYRE *et al.* 2000, reviewed in DELLAIRE & BAZZETT-JONES 2004). Elevated PML NBs were found in all types of senescence and strikingly, only PML isoform IV is able to induce the senescence (FERBEYRE *et al.* 2000, JANDEROVA-ROSSMEISLOVA *et al.* 2007, BISCHOF *et al.* 2002). PML NBs are induced by Jak/STAT signaling pathway, which is activated by IL6 or IFNγ, cytokines present in secretome of the senescent cells (HUBACKOVA *et al.* 2010, KUILMAN *et al.* 2008). Since IFNγ participates in the reaction to the viral infection, PML protects cells against tumorigenesis with origin in viral oncogene activation (REGAD *et al.* 2001, FERBEYRE *et al.* 2000, HUBACKOVA *et al.* 2010, BISCHOF *et al.* 2000).

2.3.1.3. Senescent morphology and SA-beta-galactosidase activity

The morphology of senescent cell is distinct for its increase in size, flatness and frequent occurrence of multiple nuclei in comparison to non-senescent cell (reviewed in CAMPISI 2013). The shape of senescent cells is a result of the overexpression of vimentin, a cytoskeletal protein. While the proliferating cells show network of rather shorter filaments around nuclei, the senescent cells form thick, long fibers across the whole cell (NISHIO *et al.* 2001).

Further, the increase in lysosomes in numbers and size was observed in the senescent cells. This is linked to another marker of senescence - increased activity of ß-galactosidase (measured at suboptimal conditions of pH 6.0) (DIMRI *et al.* 1995, KURZ *et al.* 2000, LEE *et al.* 2006). ß-galactosidase is an enzyme catalyzing cleavage of β-glycosidic bond in galactosides and is localized in autophagosomes and lysosomes of both regular and senescent cells. Its increase in the activity reflects the increased lysosomal mass and amount of the enzyme (LEE *et al.* 2006, KURZ *et al.* 2000). The exact reason for the lysosomal increase is to be elucidated, but it has been proposed that the oxidative stress associated with the senescence results in the oxidative-modification of macromolecules and the formation of lipofuscin, an undegradable material. The aggravated function of lysosomes with accumulated lipofuscin would, according to authors, lead to the lower mitochondrial recycling and further oxidative damage (reviewed in TERMAN & BRUNK 2002).

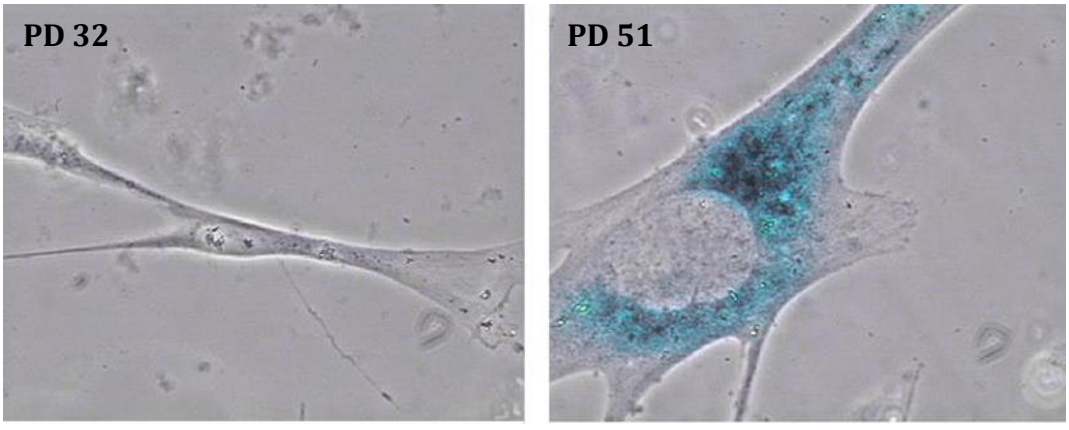


Fig. 7 S-A β-galactosidase activity detected by S-A-β-Gal.

HFFF2 cell strain (derived from human fibroblasts) at PD 32 (young) and PD 51 (senescent). The senescent cell shows increaze in size and nuclei, flatness and high S-A β-galactodidase activity represented by blue staining; magnification x1000 (adopted from GORGOULIS *et al.* 2005)

2.3.1.4. Senescence-associated secretory phenotype

Tumor growth, infection, tissue injury and other damaging events are known to induce inflammation. The immune system reacts to such harmful stimuli by attracting cells of the immune system, mainly macrophages, neutrophils or mast cells to the site of damage and these leukocytes communicate among themselves and other cells present in the site of inflammation by myriad of mediators, among them histamine and serotonin, eicosanoids, chemokines and pro-inflammatory cytokines. While destroying the cause of the immune response, this environment is favorable for the transformed growth and was described as the cancer hallmark (reviewed in ASHLEY at al. 2012 and GRIVENNIKOV *et al.* 2010). The first line of the protection against the inflammation-mediated tumorigenesis is the initiation of the cell cycle arrest and the senescence via pro-inflammatory cytokines, mainly IL1 and TGF-β. IL1 and TGFβ-dependent induction of the senescence is mediated via the ROS production and via the subsequent DNA damage (HUBACKOVA *et al.* 2012,reviewed in MULTHOFF *et al.* 2012).

Both TGFβ/SMAD and IL1/NF-κB signalization pathways increase Nox4 nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, en enzyme producing ROS - the key mediators in the DNA damage induction and the onset of the senescence (HUBACKOVA *et al.* 2012). In turn, the ROS production activates the p38/MAPK kinase that positively regulates NF-κB-dependent transcription and further, NF-κB is positively regulated by ATM, a kinase activated in response to DNA damage, as well (GOETTSCH *et al.*2009, reviewed in MORGAN & LIU 2010). NF-κB signaling pathway is responsible for the expression of many cytokines, besides IL1 and TGFβ also TNFα and IFNγ, which were described to induce senescence in the surrounding cells in paracrine manner as well (reviewed in MULTHOFF *et al.* 2012).

NF-κB initiates the expression of IL6 as well. IL6 activates Jak/STAT pathway and upregulates itself in the autocrine manner. Moreover, IL6-dependent STAT3 is a transcription factor of IL8. Both IL6 and IL8 are required for the development of the senescence and for its maintenance (KUILMAN *et al.* 2008, ACOSTA *et al.* 2008). IL6 and IL8 signalization is later on downregulated by miRNA 146a/b induced probably by the IL1 signalization (BHAUMIK *et al.* 2009). Nonetheless, it is important to note that IL1, IL6, TNFα, TGFβ and IFNγ produced by the cell to spread the senescence

are pro-inflammatory cytokines with the ability to support the transformed growth (ACOSTA *et al.* 2008, MIKULA-PIETRASIK *et al.* 2013, KUILMAN *et al.* 2008, BRAUMULLER *et al.* 2013., HUBACKOVA *et al.* 2012) Fig. 9 shows various detrimental effects of SASP.

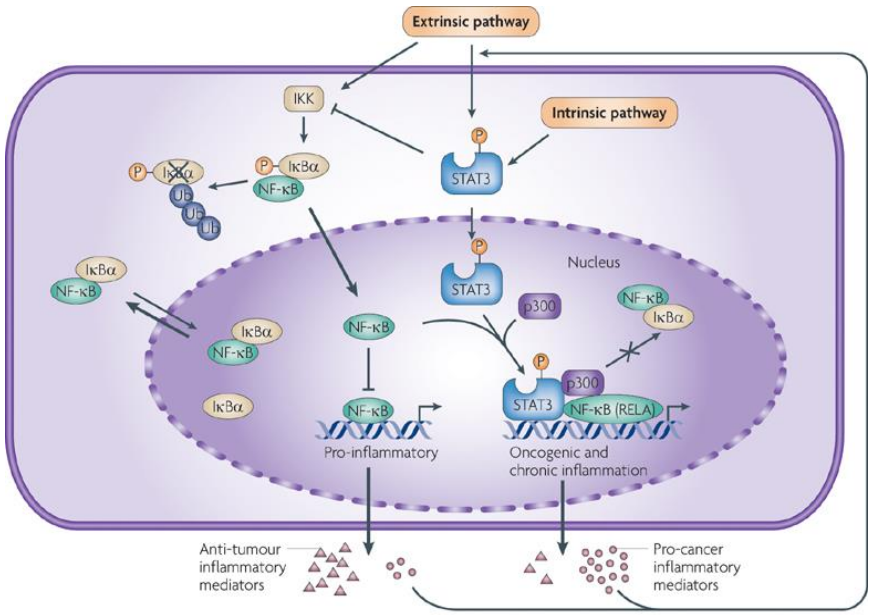


Fig. 8 Induction of senescence-associated secretory phenotype.

NF-κB transcription factors are in inactive form localized in cytoplasm, bound to inhibitor of κB (IκB). Activating stimuli activates regulatory subunit of Inhibitor of IκB kinase (IKK), IKKγ/NEMO, which forms complex with IKKα and IKKβ and phosphorylates IκB. Phosphorylated IκB is degraded and released NF-κB translocate to nucleus where it acts as transcription factor. Further, NF-κB partcipates in expression of IL6 via Jak/STAT pathway (adopted from YU *et al.* 2009) .

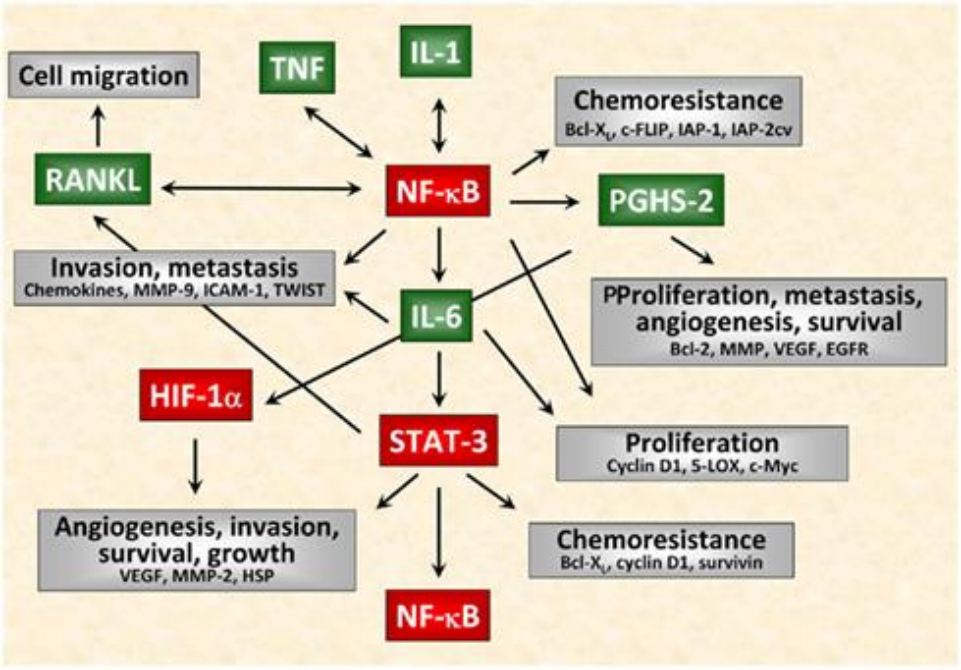


Fig. 9 Schematic visualisation of complex SASP signaling network.

IL1, TNF ad IL6 are involved in complex, selfpotentiating signaling network not only enhancing senescence development but supporting transformed growth as well via many pathways (adopted from MULTHOFF *et al.* 2012).

2.3.1.5. Persistent DNA damage response

Persistent DNA damage response is of frequent occurrence in the senescent cells and its constitutive signaling has a role in the development and maintenance of the senescent phenotype. Unlike the transient foci, senescence-associated chronic DNA damage (also termed DNA-SCARS - DNA Segments with Chromatin Alterations Reinforcing Senescence) are associated with PML NBs with the lack of DNA repair proteins, like RPA or RAD51. These DNA SCARS, detected by 53BP1 and γH2AX, contain the activated forms of p53 (phosphorylated at serine 15) and Chk2 (phosphorylated at threonine 68), and their formation is both p53 and Rb-independent. DNA-SCARS formation is accelerated during the deficiency in DNA repair mechanisms (RODIER *et al.* 2011).

3. AIMS OF THESIS

Since 5-aza-2‘-deoxycytidine is able to induce senescence, we have asked, whether 5-azacytidine, as a molecule with similar structure, is capable of such effect as well. Given it is, our aim is to investigate secretory phenotype of cell with 5-azacytidine-induced senescence in order to contribute to deeper understanding of effect this compound has on cells and their environment. Such knowledge could lead to improvement of 5-azacytidine facilitated therapy of MDS patients. Our aims in particular were:

1. To verify 5-azacytidine possesses cytotoxic effects and induces DNA damage response

2. To investigate whether 5-azacytidine induces irreparable DNA damage

3. To investigate whether 5-azacytidine induces senescence

4. To investigate whether 5-azacytidine induces DDR-activated senescence-associated secretory phenotype

4. MATERIAL AND METHODS

4.1. List of used chemicals and other material

Chemicals etc.	Manufacturer, Country
10 mM dNTPs (deoxynucleotide triphosphates)	Fermentas International Inc., USA
2-Buthanol	Penta, CR
5-Azacytidine (≥98% HPLC)	Sigma-Aldrich, USA
Acetic acid (99%)	Penta, CR
Acrylamide / Bis 30%	Serva Electrophoresis GmbH, Germany
APS (ammonium persulfate, 98%)	Sigma-Aldrich, USA
Aqua pro injection	B. Braun, Germany
BrdU (Bromo-2′-deoxyuridine; 99%)	Sigma-Aldrich, USA
Trisodium citrate dihydrate	Sigma-Aldrich, USA
DAPI, 4',6-diamidino-2-phenylindole	Sigma-Aldrich, USA
Dithiothreitol	Sigma-Aldrich, USA
DMEM (Dulbecco's Modified Eagle's Medium)	IMG ASCR, v.v.i.; CR
DMSO (Dimethyl sulphoxide)	Sigma-Aldrich, USA
Double-distilled sterile H2O	IMG ASCR, v.v.i.; CR
EDTA (Ethylenediaminetetraacetic acid) solution in PBS	IMG ASCR, v.v.i.; CR
Ethanol 96%	Penta, CR
Fetal Bovine Serum	Life Technologies, USA
Glutaraldehyde grade I, ultra-pure for el. microscopy 25%	Sigma-Aldrich, USA
Glycerol (99%)	Sigma-Aldrich, USA
Hoechst 33258 (Bis-benzimide)	Sigma-Aldrich, USA
K3Fe(CN6) (potassium ferricyanide)	Sigma-Aldrich, USA
K4Fe(CN6) x 3 H2O (potassium hexacyanoferrate trihydrate)	Sigma-Aldrich, USA
Medical X-ray film Blue	AGFA HealthCare, Belgium
Methanol	Penta, CR
MgCl2 (magnesium chloride anhydrous)	Fluka, Switzerland
MUG (4-methylumbelliferyl-beta-D-galactopyranoside	Sigma-Aldrich, USA
Na2HPO4 (Sodium phosphate anhydrous)	Sigma-Aldrich, USA
NaCl (Sodium chloride, 99%)	Erba Lachema s.r.o., CR
Nonfat dry milk	Novako, CR
PageRuler prestained protein ladder # 26616	Fermentas
PBS (Phosphate buffered saline)	IMG ASCR, v.v.i.; CR
Ponceau S	Fluka, Switzerland
RNase Inhibitor	Fermentas International Inc., USA
SDS (sodium dodecyl sulfate)	Serva Electrophoresis GmbH, Germany
SYBR Select Master mix	Life technologies, USA

TaqMan reverse transcription reagent	Life technologies, USA
TEMED (N,N,N',N'-etramethylethylendiamine)	Fluka, Switzerland
Trypsin/EDTA (Ethylenediaminetetraacetic acid)	IMG ASCR, v.v.i.; CR
Tween20	Sigma-Aldrich, USA
X-gal (5-Bromo-4-Chloro-3-Indolyl beta-D-galactopyranoside, 98%)	Sigma-Aldrich, USA
β-mercaptoethanol (2-Mercaptoethanol)	Sigma-Aldrich, USA
NaN3(sodium azide)	Koch-Light Laboratories Ldt.
Pure Nitrocellulose Blotting Membrane	Pall Corporation, USA
Penicillin-Streptomycin solution 100x	Sigma-Aldrich, USA
Triton X-100 (polyethylene glycol tertoctylphenyl ether)	Fluka, Switzerland
Mowiol 4-88	Sigma-Aldrich, USA
Tris (Trishydroxymethylaminomethane, 99.9%)	Serva Electrophoresis GmbH, Germany
Benzamidine (N-[4-[(6,7-Dimethoxy-4-quinazolinyl)amino]phenyl]benzamide	Sigma-Aldrich, USA
Para-formaldehyde 4% (m/v) pH 7.2	BDH, UK
10x TGS buffer (192mM glycine, 25 mM Tris, 0.1% (w/v) SDS, pH 8.3)	Bio Rad, USA
10x TG buffer (192mM glycine, 25 mM Tris, pH 8.3)	Bio Rad, USA
Bromophenol Blue	Lachema, CR
Agarose	Serva Electrophoresis GmbH, Germany

4.2. List of used kits and pre-designed systems

Pre-designed system, Country
ECL Western Blotting System, Amersham, USA
BCA Protein Assay, Thermo Scientific, USA
Human Common Cytokines RT PCR assay, Quiagen, USA Click-iT EdU Alexa Fluor 488 Imaging Kit, Life technologies, USA Basic Kit Human Flow, eBioscience, USA Rneasy Micro Kit, Quiagen, USA Annexin V: FITC Apoptosis Dection Kit I, BD Pharmingen, Germany
High Capacity cDNA Reverse Transcription kit, Life technologies / Applied Biosystems, USA The Human Common Cytokines RT^2 Profiler™ PCR Array, Qiagen, USA

4.3. List of used primary antibodies

Epitope	Host	Manufacturer	Catalog number	Working dilution for IF	Working dilution for WB
53BP1	Rb/poly	Santa Cruz	sc-22760	500x	- X-
GAPDH	M/mono	GeneTEX	GTX30666	- X-	2000x
Chk2	M/mono	Millipore	05-649	- X-	1000x
Chk2pThr68	Rb/poly	Cell Signaling	#2661	- X-	1000x
p15	Rb/poly	Santa Cruz	sc-612	- X-	1000x
p16	Rb/poly	Santa Cruz	sc-759	- X-	- X-
p21	M/mono	Santa Cruz	sc-56335	- X-	500x
p53	M/mono	Santa Cruz	sc-126	- X-	- X-
p53pSer15	Rb/poly	Cell Signaling	#9284	- X-	- X-
PML	M/mono	Santa Cruz	sc-966	100x	1000x
Rb	M/mono	BD Pharmingen	554136	- X-	1000x
Smad2	G/poly	Cell Signaling	sc-966	- X-	1000x
Smad2pS465/467	Rb/poly	Santa Cruz	#9284	- X-	1000x
Stat3	M/mono	Santa Cruz	sc-482	- X-	1000x
Stat3pY705	Rb/poly	Cell Signaling	#9138	- X-	1000x
γ-H2AX(pSer139)	M/mono	Millipore	05-636	500x	- X-

4.4. List of used secondary antibodies

Conjugation	Epitope	Host	Manufacturer	Catalog number	Working dilution
Cy3 568	Mouse	Donkey	Jackson Immunosearch	715-165-150	300 - 400x
Alexa Fluor 488	Rabbit	Donkey	Invitrogen	A21206	1,000x
IgG-HRP	Rabbit	Goat	Bio-Rad	170-6515	10, 000x
IgG-HRP	Mouse	Goat	Bio-Rad	170-6516	10,000x
IgG-HRP	Goat	Rabbit	Santa Cruz	sc-2922	10,000x

4.5. List of used beads recognizing cytokines

Epitope	Catalog number	Sensitivity	Standards
IL1β	BMS8224FF	4.2 pg/ml	20 000-27 pg/ml
IL6	BMS8213FF	1.2 pg/ml	20 000-27 pg/ml
IFNγ	BMS8228FF	1.6 pg/ml	20 000-27 pg/ml
TNFα	BMS8223FF	3.2 pg/ml	20 000-27 pg/ml
TGFβ1	BMS8249FF	10 pg/ml	10 000-13.7 pg/ml

4.6. List of used primers for RT-qPCR

Epitope	Forward primers	Reversed primers
IL1B	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
IL6	AGCCCTGAGAAAGGAGACATGTA	TCTGCCAGTGCCTCTTTGC
IL8	TTGGCAGCCTTCCTGATTTC	TCTTTAGCACTCCTTGGCAAAAC
TNFA	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
TGFB	TACCTGAACCCGTGTTGCTCTC	TACCTGAACCCGTGTTGCTCTC
IFNG	AGGTCATTCAGATGTAGCGGATAAT	TTCTGTCACTCTCCTCTTTCCAATT
ACTB	CCAACCGCGAGAAGATGA	CCAACCGCGAGAAGATGA

4.7. List of used equipment

Manufacturer, Country
7300 Real-Time PCR System, Life Technologies / Applied Biosystems, USA
Analytical weights AE 240, Mettler, USA
BD LSRII FACS Flow Cytometer, BD, USA
Modulus™ Microplate Multimode reader, Turner BioSystems, USA
Mini PROTEAN® 3 Cell wet tank system, Bio Rad, USA
BioSafety Cabinet Bio-II-A Telstar, Spain
Bürker counting chamber, Laboroptik, Germany
Centrifuge 5415R, Eppendorf, Germany
Centrifuge 5424 Eppendorf, Germany
Centrifuge NF400 Nüve Inc.,Turkey
CO2 Incubator FORMA Series II Water Jacket, Thermo Fisher Scientific Inc., USA
Countess® Automated Cell Counter, Life Technologies / Invitrogen, USA
Microplate photometer Multiskan® EX, Thermo Fisher Scientific Inc., Waltham, USA
Microscope DMIL, Leica Microsystems GmbH, Germany
Microscope Eclipse T100, Nikon Instruments Europe B.V, The Netherlands
Minicentrifuge Z 100, Hermle LaborTechnik GmbH, Germany
MJ Mini personal thermal cycler, BioRad, USA
NanoDrop® ND-1000 Spectrophotometer, Thermo Fisher Scientific Inc., USA
PIPETMANs Neo® Set, Gilson Inc., Middleton, USA
SDS-PAGE Apparatus Mini-PROTEAN Tetra Cell, Bio Rad, USA
Sonicator SONIPREP MSE 150 AL.BRA. Srl, Italy
The LightCycler® 480 System, Roche Diagnostics GmbH, Germany
Thermomixer comfort Eppendorf, Germany
Vortex Lab dancer VWR, Germany
Water bath BM402, Nüve Inc., Turkey
UV Transilluminator East Port, Prague, Czech Republic

4.8. Cell cultivation

HeLa cell line (derived from human cervical cancer, obtained from prof. Jiri Bartek) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic component consisting of Penicillin (100 U/ml) and Streptomycin (100 ng/ml). Cells were kept at 37 °C under 5% CO_2 atmosphere. Further manipulation with cells was in accordance with the mammalian cell culture protocol (FRESHNEY 2005).

4.9. Thawing cells

Cells in cryovials, previously stored in liquid nitrogen, were during their transfer and manipulation on dry ice and then were rapidly thawed in water bath tempered to 37°C. Cells were resuspended in DMEM and centrifuged at 100x g for 5 minutes. Supernatant containing cryopreservation medium (90% [v/v] FBS and 10% [v/v] DMSO) was removed and cells were resuspended in fresh DMEM. Cells were cryopreserved in concentration of approximately 2x 10^6 cells per 1 ml of cryopreserving medium and were subsequently planted into culturing flask in concentration of approximately 8x 10^4 cells per 1 cm^2.

4.10. Counting cells

Bürker counting chamber was used for estimating cell counts, accordingly to the mammalian cell culture protocol (FRESHNEY 2005). For assessment of cytokines in medium by Bead-based Immunoassay, cells were counted by Countess® Automated Cell Counter accordingly to the manufacturer instructions.

4.11. Treating cells

Cells were seeded in concentrations of approximately 3.4x10^4 cells per 1 cm^2 and rested overnight. They were given fresh medium along with respective doses of 10mM 5-azacytidine, 10mM BrdU or DMSO. In order to preserve cytokine signaling of cells, only half of medium from 5-azacytidine treated and mock treated cells was replaced by fresh medium every 24 hours during experiment. Cells treated by BrdU

were given fresh medium every 48 hours, along with treatment. Otherwise, cells were manipulated accordingly to the mamalian cell culture protocol (FRESHNEY 2005).

4.12. BCA assay

For even loading of protein samples for Western blot and Quantitative assay of SA-ß-galactosidase activity in mammalian cell extracts, protein concentration was measured by BCA assay accordingly to the manufacturer protocol. Microplate photometer Multiskan® EX was used for evaluation of protein concentration.

4.13. SDS-Polyacrylamide Gel Electrophoresis and Western blotting

Cells were washed with PBS thoroughly and all of remaining PBS was removed. Cells were harvested in 1x Sample buffer (250 mM Tris-HCl, 40% [v/v] Glycerol, 8% [w/v] SDS, pH 6.8) and lysate was sonicated two to three times at 3 microns for 15 seconds with 15 s pause. Lysates were stored at -20°C. SDS-PAGE was prepared. Assembled glass cassettes were partially filled with gel of desired Acrylamide-Bis concentration (components listed in Table 2) and layered with butanol. After polymerization, butanol layer was removed and the rest of cassette was filled with stacking gel (Table 3) with its height doubling the height of used combs, then the gel polymerized. Bio-Rad Mini-PROTEAN Tetra Cell apparatus was used to run samples, along with 1x TGS buffer (192mM glycine, 25 mM Tris, 0.1% [w/v] SDS, pH 8.3) as a running buffer. Loaded samples were diluted to the same exact concentration keeping between 25–40 ng of total protein per sample and had added 1M dithiothreitol in amount of one tenth of final sample. Samples were boiled at 96.5 °C for three minutes and centrifuged at maximum speed for 30 s to accumulate condensed water. Samples were run at constant current 50 mA. Proteins were transferred on Pure Nitrocellulose Blotting Membrane from Pall Corporation by Mini PROTEAN® 3 Cell wet tank system from Bio-Rad. 1x TG blotting buffer (192mM glycine, 25 mM Tris, pH 8.3) with addition of 20% [v/v] of methanol was used. Transfer was carried out under constant voltage of 100 V for 90 minutes. To verify quality of transfer, proteins on membrane were stained by 0.1% [w/v] Ponceau S with 5% [v/v] acetic acid. When Ponceau S staining was removed by PBS with

addition of 0.1% [v/v] Tween 20(PBS+T), membranes were blocked by 5% [w/v] non-fat dry milk dissolved in PBS+T for 45 minutes at room temperature. After PBS+T wash, membranes were incubated with corresponding antibody in 3% [w/v] non-fat dry milk dissolved in PBS+T with addition of 0,01% [v/v] sodium azide for 8 – 15 hours. Membranes were washed in PBS+T and incubated with corresponding secondary antibody conjugated with polymers of horseradish peroxidase in 3% [w/v] non-fat dry milk dissolved in PBS+T for 45 minutes. After PBS+T wash, substrate for the horseradish peroxidase was added in ECL mix prepared of solutions ECL 1 and ECL 2 (1:1) from Amersham or in need of higher sensitivity, SuperSignal West Femto Chemiluminescent Substrate was used. X-ray films were developed in hand with developer and stabilizer solutions.

Components	**8%***	**10%***	**12%***	**Stacking****
ddH2O	2.4 ml	2.1 ml	1.75 ml	1.2 ml
Acrylamide / Bis 30%	1.3 ml	1.7 ml	2.0 ml	0.3 ml
Buffer	1.3 ml	1.25 ml	1.25 ml	0.5 ml
APS 10%	35 µl	35 µl	35 µl	16 µl
TEMED	8 µl	8 µl	8 µl	4 µl

Tab. 2 Components for 0.1x6x8cm SDS-PAGE gels of various percentages.

*Buffer 1 (1.5 mM Tris, 0.4% SDS, pH 8.8)
**Buffer 2 (0.5 mM Tris, 0.4% SDS, pH 6.8)

4.14. Direct Fluorescence

Cells planted on coverslips were in the same medium cultivated with the 1 µM EdU nucleotide analog for six hours and then were fixed by 4% para-formaldehyde during 15 minutes of its actuation. They were either washed in PBS and permeabilized by 0.1% Triton X or firstly stored in cold in solution of PBS and 0.01% sodium azide and then permeabilized by 0.1% Triton X. The background signal was decreased by wash in 10% FBS diluted in PBS which lasted 30 minutes. Cells on coverslips were incubated with Click-iT® reaction mix according to the manufacturer protocol (for 30 minutes in dark). The reaction mix contained Click-

iT® Alexa Fluor® azide that bound to incorporated EdU based on click reaction between an azide and an alkyne. The bond is covalent and catalyzed by copper. Cells were washed in PBS and then mounted by Mowiol-4-88 with supplemented by DAPI (0.1 μg/ml) . The microscopic slides were stored in dark and cold. For the estimation of EdU incorporation, only cell exhibiting diffusive pattern of EdU incorporation were taken into account in order to eliminate error of including cells only repairing their DNA lesions. Mounted cells were analyzed by Microscope Leica DMIL.

4.15. Indirect Immunofluorescence

Cells planted on coverslips were, after wash in PBS, fixed by 4% para-formaldehyde for 15 minutes. Subsequently, they were either stored in cold, covered by PBS with addition of 0.01% [v/v] sodium azide, and later permeabilized by 0.1% Triton X or permeabilized immediately. No surface proteins were visualized so permeabilization was a step occurring in each procedure. To reduce unspecifity of primary antibodies, cells were incubated in 10% [v/v] FBS for 30 minutes. After PBS wash, fixed cells were incubated with primary antibodies under humid conditions. Unbounded primary antibody was washed out by PBS+T and PBS and primary antibodies were labeled with according secondary antibodies with fluorescent dye for 60 minutes in dark under humid conditions. Cells were washed with PBS+T, PBS and then mounted on clean microscope slide by Mowiol-488 with DAPI. The microscopic slides were stored in dark and cold. Mounted cells were analyzed by Microscope Leica DMIL.

4.16. S-A β-galactosidase assay

This cytochemical method is based on visualizing B-galactosidase activity by hydrolysis of chromogenic substrate in suboptimal conditions of pH 6.0. B-galactosidase hydrolyzes glycosidic linkage between galactose and 5-bromo-4-chloro-3-hydroxyindole. 5-bromo-4-chloro-3-hydroxyindoles dimerizes by oxidation and thus form blue precipitate of 4-chloro-3-brom-indigo. Cells planted on coverslips were washed in PBS and fixed by 0.25% glutaraldehyde diluted in PBS for 11 minutes. In this state, they were stored in cold and dark, overlaid by PBS with addition of 0.01% [v/v] sodium azide. Later, they were washed in PBS with addition

of 1mM magnesium chloride adjusted to pH 6.0. Reaction mix (Table 4) was added and fixed cells were incubated with this mix at 37°C for the time needed for positive controls to stain heavily while mock treated controls remaining unstained, approximately 2 days. Fixed cells were washed in PBS and mounted on clean microscope slide by Mowiol-488 with DABCO. The microscopic slides were stored in dark and cold. Mounted cells were analyzed by Microscope Leica DMIL.

Composition of 10 ml of reaction mix	**[µl]**
PBS + 1 mM MgCl2, pH 6.0	9.3
{0.12 mM K3Fe(CN)6 + 0.12 mM K4Fe(CN)6 x 3 H2O} in PBS	0.5
X-gal	0.25

Table 4. Constitution of reaction mix for S-A β-galactodise assay.

4.17. Quantitative assay of Senescence-associated ß-galactosidase activity in mammalian cell extracts

This method operates on similar principle as SA- ß -galactosidase assay. It uses pH 6.0 to create suboptimal conditions for ß-galactosidase activity and the substrate, 4-methylumbelliferyl-beta-D-galactopyranoside (MUG), becomes fluorescent after hydrolysis. Cells were washed in PBS and after removal of remaining PBS, they were harvested in 1x Lysis buffer M (40 mM Na2HPO4, 40 mM Trisodium citrate dehydrate, 0.5 mM Benzamidine, pH 6.0) with volume depending on density of cells . Lysates were stored at -20°C. Lysates obtained by Lysis solution M were measured for protein concentration by BCA assay (chapter 1.2). Lysates were diluted to concentration 0.55 ng per µl and 90 µl of lysate with matching amount of 2x Reaction buffer (40 mM citric acid, 40mM Na_2HPO_4, 300 mM NaCl, 10 mM β-mercaptoethanol, 4 mM $MgCl_2$) were mixed in Eppendorf tube and incubated at 37°C. Samples were taken in time points 0 hours, 1 hour, 2 hours and 3 hours and 400 µl of 400mM sodium carbonate stop buffer was immediately added before cooling samples down to 4°C in dark. Collected samples from 4 timepoints were divided into 96-well plate by 205 µl per well in duplicate and had fluorescence measured by UV light at wavelength 360 nm for excitation and 465 nm for emission by Modulus™ Microplate Multimode reader. β-galactosidase activity per 1 µg of cellular lysate corresponded to measured absorbance.

4.18. RNA isolation, reverse transcription and quantitative PCR

Cells were harvested by Rneasy Micro Kit according to manufacturer instructions. The volume of samples was doubled by 70% ethanol of room temperature and resuspended samples were transferred to columns in centrifuge tubes. Samples were centrifuged at 10 000 rpm for 15 s. Samples were washed by 700 µl of RW1 buffer and 500 µl of RPE buffer with centrifugation at 10 000 rpm for 15 s after both steps. Samples were rewashed by 500 µl RPE buffer and centrifugation at 10 000 rpm for 2 minutes. Columns were transferred to clean tubes and centrifuged at 14 500 rpm for 90 s. Columns were transferred to elution tubes and RNA was eluted by 25 µl of RNase-free water and centrifugation at 10 000 rpm for 1 minute and then re-eluted by the supernatant and centrifugation at 10 000 rpm for 1 minute. The contrations of samples and their purity were (ratio of absorbance measuread at 280 and 260 nm and at 260 and 230 nm) measured on Nanodropells were harvested by Rneasy Micro Kit according to manufacturer instructions and isolated by Rneasy Micro Kit accordingly to manufacturer instructions. The contrations of samples and their purity were (ratio of absorbance measuread at 280 and 260 nm and at 260 and 230 nm) measured on Nanodrop® ND-1000 Spectrophotometer. The iIsolated RNA was diluted by RNase-free water to concentration 100 ng per µl. The reaction mix was prepared according to Table 5 and was run on MJ Mini personal thermal cycler. The program is described in Table 6. The product of reverse transcription was diluted four times by RNase-free water and the reaction mix for quantitative PCR was prepared (see Table 7). The reaction was analyzed by 7300 Real-Time PCR System and quantified by ddCt method for qRT-PCR data analysis (ZHANG *et al.* 2014).

Futher, cDNA prepared according to the protocol was used for Common Cytokines RT^2 Profiler PCR array in accordance to the manufacturer instructions. The reaction was analyed by 7300 Real-Time PCR System and the data were quantified by ddCt method.

Composition of one reaction	[µl]
10x RT Buffer	2
RNase-free H2O	11.2
25x dNTP mix (100mM)	0.8
10x RT Random Primers	2
RNase Inhibitor	1
Reverse Transcriptase MultiScribe	1
RNA at conc. 100 ng / µl	2

Table 5 Components for one RT-PCR reaction.

Step	Temperature	Duration
Step 1	25 °C	10 min
Step2	37°C	120 min
Step 3	85°C	5 min
Step 4	4°C	forever

Table 6 Program for RT-PCR.

RNase-free water	2.25
2x Master mix	6.25
Primers (2.5 µM)	1.5
cDNA template	2.5

Table 7 Components for one qPCR reaction.

4.19. Fluorescence-activated Cell Sorting

MMedium with non-adhering cells was collected into a tube and cells were washed with PBS and removed from the surface of dish by thin layer of 0.25% [w/v] Trypsin dissolved in EDTA (T/E). In the meantime, medium containing unattached cells was centrifuged at 375x g for 3 minutes. Cells removed from dish by T/E were resuspended in PBS and transferred to the tube with previously non-adherent cells. Two fractions of cells were mixed by resuspending and centrifuged at 375x g at for 3 minutes. Cells were washed with 300 µl of 1x Annexin V binding buffer and then incubated with Annexin V-FITC diluted in Annexin V binding buffer (1 µL of Annexin V-FITC per 100 µl of Annexin binding buffer) for 20 minutes. Finally, Hoechst 33258 was added to detect dead or necrotic cells (in final concentration of 2 µg/ml). Data were measured by BD LSRII FACS Flow Cytometer and quantified by FlowJo software.

4.20. Bead-based assay

Beads-based assay is based principles of ELISA. There are several types of beads of different emission spectrum and size. Beads are coated in specific antibody against the cytokine of interest and another specific biotinylated antibody recognizing the same cytokine of interest, but different epitope, is added. Secondary streptavidin-linked antibody with conjugated phycoerythrin is added. Beads with bound cytokine of interest and corresponding antibodies are recognized by FACS for type of bead and magnitude of signal for secondary antibody. The last day of treatment, cells were cultivated in half of previously used volume of medium to enhance concentration of cytokines of interest in medium. The medium was centrifuged at 3000x g for 3 minutes to remove non-adherent fraction of culture and remaining adherent cells were removed from culture dish by 0.25% [w/v] T/E, resuspended in 1 ml of fresh medium and counted by Countess® Automated Cell Counter (see chapter 1.7). Assay was carried out correspondingly to the manufacturer instructions. Amount and type of beads were measured by BD LSRII FACS Flow Cytometer and quantified by FlowJo software. Data were calculated to pg of cytokine in 1 ml of media secreted by 10^5 cells.

4.21. DNA electrophoresis.

For horizontal electrophoresis, 3% [w/v] agarose gel disolved in TAE (Tris-acetate-EDTA; pH 8.0) buffer was used. Samples mixed with 1x Loading dye were separated on gel by constant voltage 100 V. DNA vizualized by ethidium bromide was detected by UV transilluminator.

5. RESULTS

5.1 5-azacytidine induces morphologic changes in HeLa cells.

According to pharmacokinetic study of 5-azacytidine (MARCUCCI *et al.* 2005), achievable 5-azacytidine plasma concentration in patients is 3-11 μM (standard protocol of 5-azacytidine administration to patients in a dose of 75 mg/m^2 IV infusion administered daily over 10 minutes for the duration of 7 days). To elucidate the effects of 5-azacytidine in administration relevant to the clinical use, concentrations of 0.0, 0.125, 0.25, 0.5, 1, 2, 4 and 10 μM dissolved in DMSO were tested. We used the same clinical protocol and the HeLa cells were treated every 24 hours with appropriate concentration of 5-azacytidine for 7 days and in further experiments as well. Although concentrations lower than 2 μM did not have significant effect on proliferation and morphology of treated HeLa cells (see Fig. 10), higher concentrations (2 and 4 μM) of 5-azacytidine induced morphological changes in cells – cells became rounded and with enlarged nuclei. Strikingly, a partial to complete loss of adherence in large fraction of cells was observed. While 0 – 1 μM 5-azacytidine treated cells proliferated during the experiment, 2 and 4 μM 5-azacytidine treated cells ceased the proliferation at third day of experiment (as can be seen in Fig. 1, the lower density of 2 and 4 μM cultures indicated affected proliferation). 10 μM 5-azacytidine treatment was cytotoxic. To follow the impact of 5-azacytidine on selected parameters of cell physiology in surviving cells, concentration 2 and 4 treatment was chosen for further experiments. Nucleotide analog with pyrimidine base, Bromodeoxyuridine (BrdU) was chosen as a sort of control as BrdU induces DNA damage response and onset of senescence (MASTERSON *et al.* 2007, MICGISHITA *et al.* 1999).

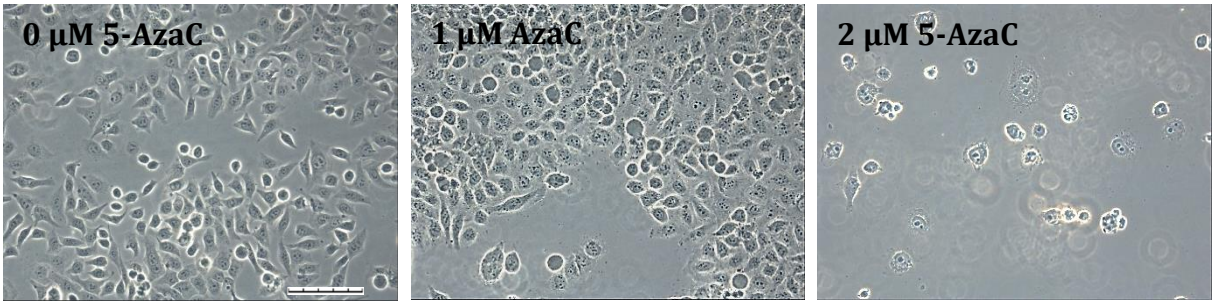


Fig. 10 Effect of 5-azacytidine on morphology of HeLa cells.
HeLa cells were treated 7 days with 1 μM or 2 μM, respectively. HeLa cells treated with 0.04% DMSO were used as a control. Bar 50 μM.

Based on previous experiments with BrdU treatment of HeLa cells, 100 μM BrdU was used (NOVAKOVA *et al.* 2010).

5.2 5-azacytidine induces cell death.

Non-adherent blebbing cells were observed after 2 and 4 μM 5-azacytidine treatment. To assess the number of apoptotic cells, the levels of Annexin V (probe detecting phosphatidylserine) (VERMES *et al.* 1995) and Hoechst 33258 (fluorescent DNA-specific dye staining especially apoptotic cells) (WOO 1995) were measured by FACS. The percentage of apoptotic cells in 2 and 4 μM 5-azacytidine treated cultures were 48.50% (±7.8) and 52.50% (±0.7), respectively (see Fig. 11) (determined as Annexin positive and Hoechst positive cells). Mock treatment as well as BrdU treatment resulted in mild apoptosis (3.00% (±1.3) and 4.35% (±1.4) of apoptotic cells, respectively). Approximately 15-fold increase of apoptotic cells in 5-azacytidine-treated cells compared to mock treated controls shows that 5-azacytidine is cytotoxic and induces apoptosis in HeLa cells.

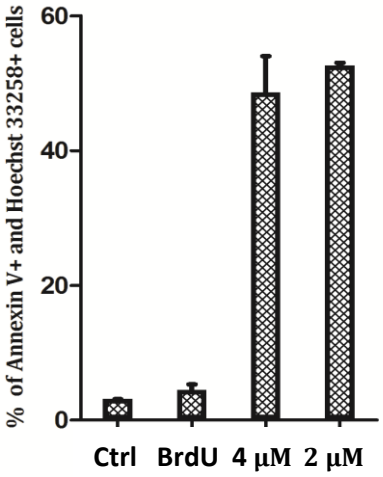


Fig. 11 Percentage of Annexin V and Hoechst 33258 positive cells

HeLa cells were treated 7 days with 2 μM or 4 μM, respectively. HeLa cells treated with 0.04% DMSO were used as a control. HeLa cells treated with 100 μm BrdU were used as a positive control of senescence. Apoptotic cells were determined as an Annexin+ Hoechst+ fraction detected by FACS. T test, $p \geq 0.10$; two independent experiments.

5.3. 5-azacytidine induces DNA damage response in Hela cells.

To test whether 5-azacytidine can induce DNA damage and activate DNA damage response, immunofluorescence staining of γH2AX (WARD *et al.* 2001, BURMA *et al.* 2001) and 53BP1 (CANMAN *et al.* 1998) and activation of Chk2 (AHN *et al.* 2000) and p53/p21 (NELSON & KASTAN 1994) and p16/Rb (ROBLES & ADAMI 1998) pathways by immunoblotting was used in 5-azacytidine treated HeLa cells. All of 5-

azacytidine treated cells and approximately one fifth of cells in positive control showed 53BP1 and γH2AX positive foci (see Fig. 12) in comparison with mock treated cells, where no significant DNA damage foci were detected. One or two 53BP1 nuclear bodies observed in control cells represent G1 phase-associated structures resulting from incomplete DNA synthesis in S phase, also called Opt bodies (LUKAS *et al.* 2010). It should be noted that after 5-azacytidine treatment, part of 53BP1 signal was localized outside of nucleus while no cytoplasmic 53BP1 localization was observed in BrdU treated cells. 53BP1 is solely a nuclear protein and its localization outside nucleus indicates possible aberration in its transport to nucleus after mitosis (MOUDRY *et al.* 2012).

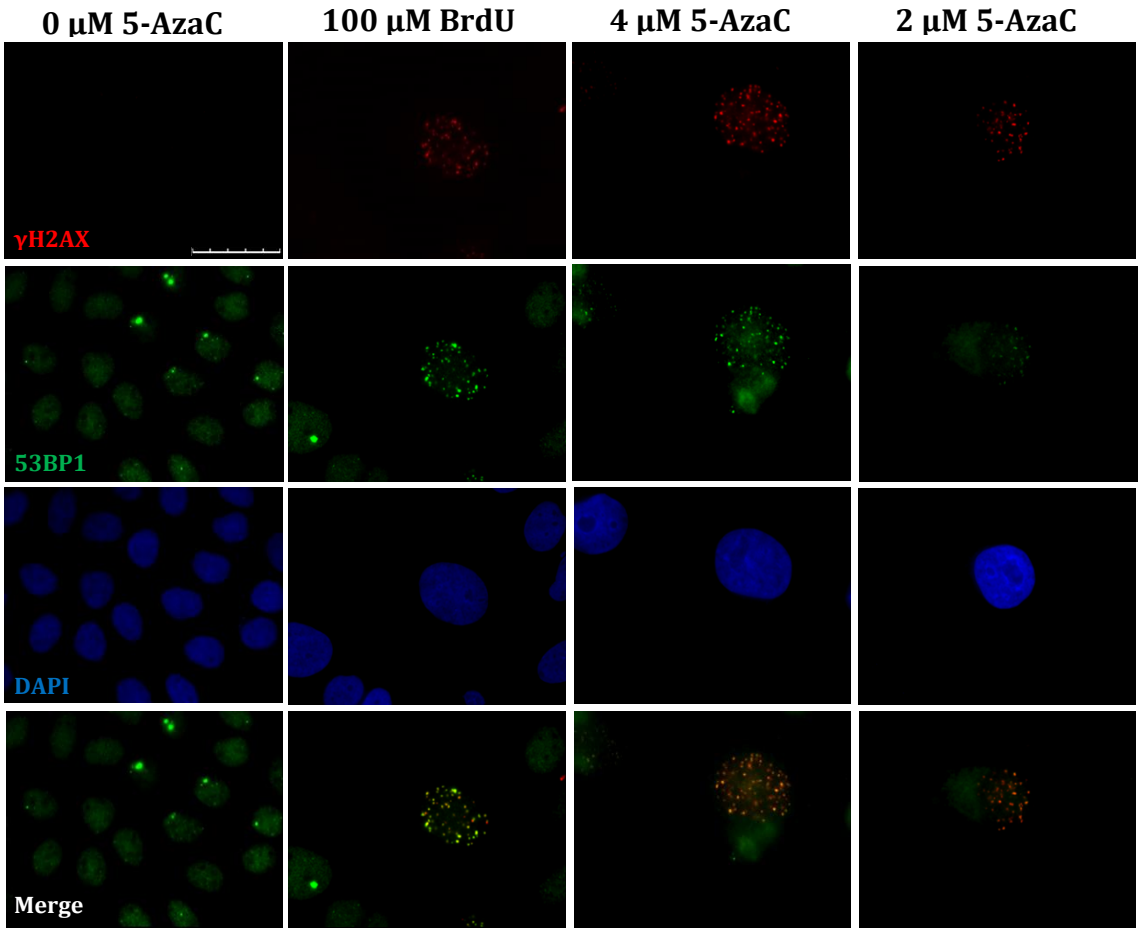


Fig. 12 Presence of γH2AX and 53BP1 foci after 5-azacytidine treatment in HeLa cells.

Immunofluorescent detection of γH2AX and 53BP1 in HeLa cells with 2 µM or 4 µM, respectively. HeLa cells treated with 0.04% DMSO were used as a control. HeLa cells treated with 100 µM BrdU was used as a positive control. Bar 25 µM.

Furthermore, we observed that 5-azacytidine induces activation of p53 (phosphorylated on serine 15) and Chk2 (phosphorylated on threonine 68) (see Fig.13). Phosphorylation of p53 was more expressed in 4 μM 5-azacytidine treatment while Chk2 phosphorylation was stimulated with the same intensity after both doses of 5-azacytidine. Total levels of p53 and Chk2 after 5-azacytidine treatment did not significantly change. p53 and Chk2 activation imply induction of cell-cycle checkpoints. Increased level of p21 protein responds to activation of p53 in 5-azacytidine treated cells. Fig. 13 shows that 5-azacytidine induced level of p15, but not p16 protein. Rb protein level and phosphorylation remained unchanged after 5-azacytidine treatment which corresponds with p16 level. Taken together, these results indicate that 5-azacytidine possibly induces DNA damage and activates DNA damage response and cell-cycle checkpoints. Enhanced levels of CDK inhibitors p15 and p21 could lead to cell cycle arrest.

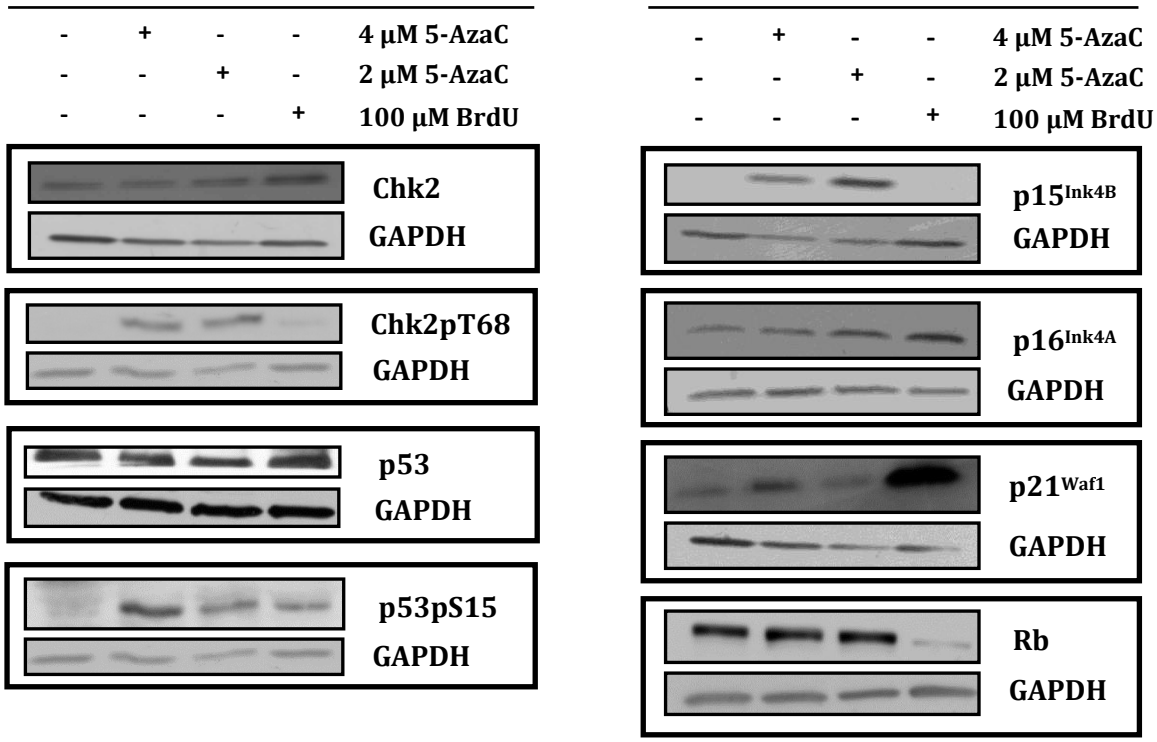


Fig. 13 Immunoblot detection of DNA damage response activation and cell-cycle arrest in 5-azacytidine treated HeLa cells.

Immunoblotting detection of total p53 and Chk2, serine 15 phosphorylated p53, threonine 68 phosphorylated Chk2, p16INK4A, p15INK4B and Rb in HeLa cells treated with 5-azacytidine for 7 days. 100 μM BrdU was used as a positive control. GAPDH was used as a loading control.

5.4 5-azacytidine induces cell-cycle arrest.

To evaluate an effect of 5-azacytidine on cell proliferation, incorporation of EdU - a thymidine analog - was used (CHEHREHASA *et al.* 2009). Cells were treated according to previous treatment regime. During the experiment, coverslips with cells were collected at day 2, 4 and 7, incubated with 1 μM EdU for 6 hours and together stained for EdU by fluorescence. The ratio of EdU positive vs. EdU negative cells was estimated. Fig. 14, shows decrease of EdU incorporation in cells treated with 5-azacytidine which indicates that cell proliferation was slowed down during treatment. As EdU and BrdU are analogues competing for the identical site in DNA, incorporation in BrdU treated cells was not assessed to avoid false results. These data are in agreement with previous results and conclude that 5-azacytidine inhibits proliferation of HeLa cells and possibly induces cell-cycle arrest via checkpoint activation.

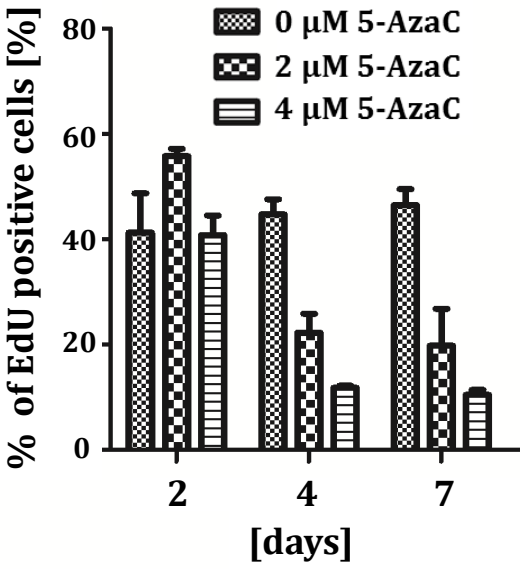


Fig. 14 Percentage of EdU positive cells at day 2, 4 and 7 of 5-azacytidine treatment.

Statistical analysis of EdU incorporation in HeLa cells treated with 5-azacytidine as indicated. T test, $p \geq 0.10$; two independent experiments.

5.5 There is a fraction of S-A ß-galactosidase positive cells after 5-azacytidine treatment.

Since we observed the expression of CDK inhibitors in 5-azacytidine treated cells, we tried to determine whether cells respond to their presence by induction of senescence. One of senescence hallmarks, increased activity of ß-galactosidase, was determined by S-A ß-galactosidase assay (DIMRI *et al.* 1995) and Quantitative assay of S-A ß-galactosidase activity (GARY & KINDELL 2005) in HeLa cells treated 5-

azacytidine. Cells treated with 100 µM BrdU were used as a positive control. As you can see on Fig. 6, 4 µM and 2 µM 5-azacytidine treated cells were not uniform in positivity for S-A ß-galactosidase staining and intensity of signal in comparison with BrdU treated cells. Only small fraction of cells showed weak activity of this enzyme. The mock treated cells were negative for S-A ß-galactosidase activity (see Fig. 15). The quantitative assay confirmed that there was no significant difference in activation of S-A ß-galactosidase activity between control cells and cells treated with either 2 µM or 4 µM 5-azacytidine (see Fig. 16). In conclusion, despite morphological changes, activation of DNA damage and cell cycle arrest 5-azacytidine did not increase S-A ß-galactosidase activity in HeLa cells.

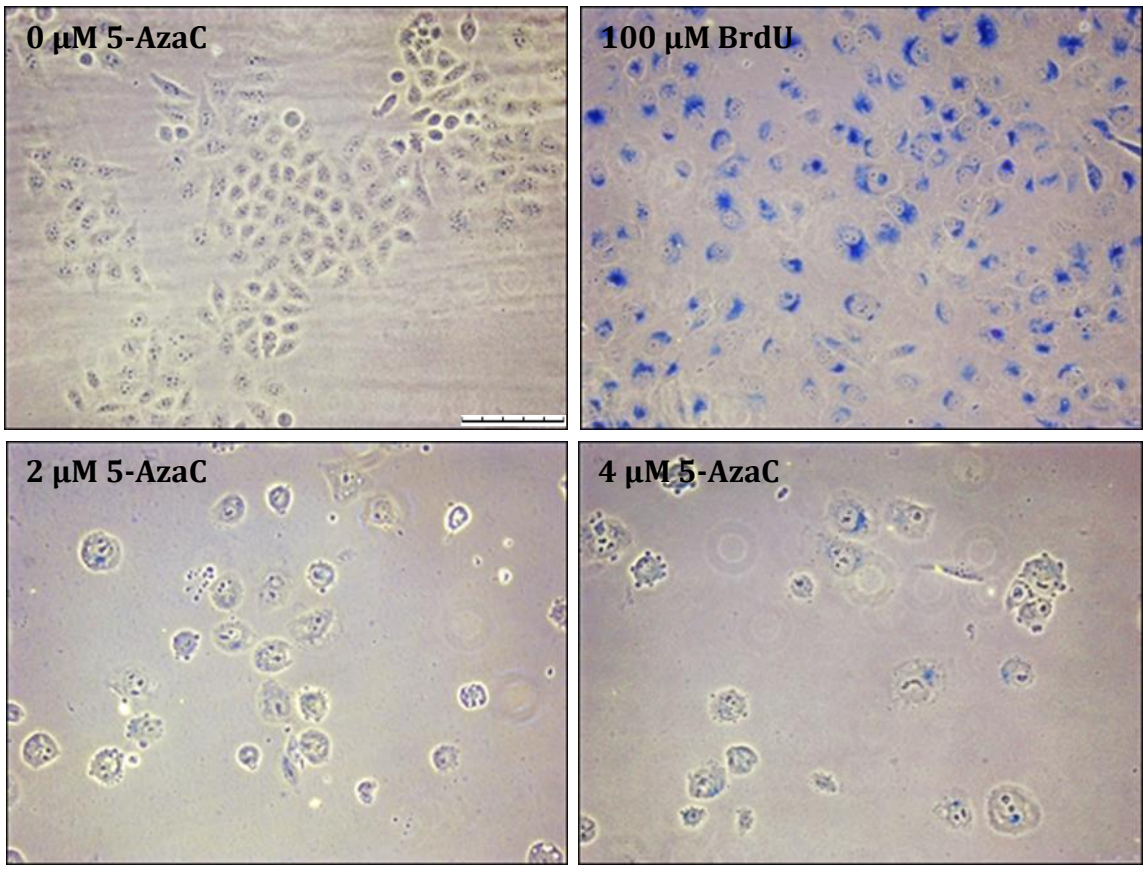


Fig. 15 Senescence-associated ß-galactosidase staining in HeLa cells after 5-azacytidine treatment.

Detection of senescence-associated ß-galactosidase activity in HeLa cells treated with 2 and 4 µM 5-azacytidine for 7 days. Cells treated with DMSO were used as a control (mock). 100 µM BrdU was used as a positive control. Bar 50 µM.

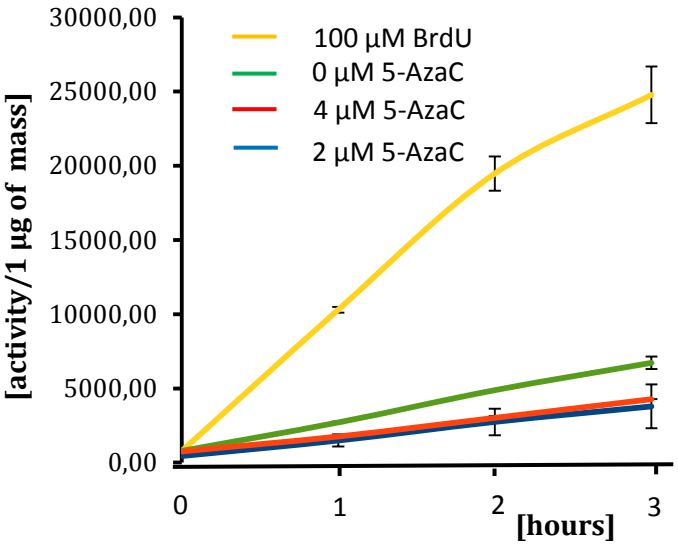


Fig. 16 Quantification of SA-ß-galactosidase activity in 5-azacytidine treated HeLa cells.

Quantitative assay of S-A β-galactosidase activity in HeLa cells treated 7 days with 2 µM and 4 µM 5-azacytidine, respectively. 100 µM BrdU was used as a positive control. The values represent average of two independent experiments and are shown as activity of β-galactosidase in 1 µg of sample.

5.6 5-azacytidine induces PML expression.

As was discussed above, senescent phenotype is characterized as a combination of several markers. Another senescence marker, PML and PML NBs increase, was therefore investigated by immunoblotting and immunofluorescence (HUBACKOVA *et al.* 2010). 2 and 4 µM 5-azacytidine induced mild increase in numbers and intensity of PML bodies (see Fig. 18) which correspond to increase of PML protein level detected by immunoblot (see Fig. 17). Increased level of PML isoforms, localized between 55 and 130 kDa (with unspecific band at 85 kDa, marked with asterisk), is shown on right side (shorter exposition). Increased modification of PML protein (corresponding with formation of PML NBs) was observed after long exposition and is localized in area of up to 130 kDa. 100 µM BrdU was used as a positive control. Taken together, while 5-azacytidine induces PML level and PML NBs, the increase is not strong enough to indicate senescence in 5-azacytidine treated cells.

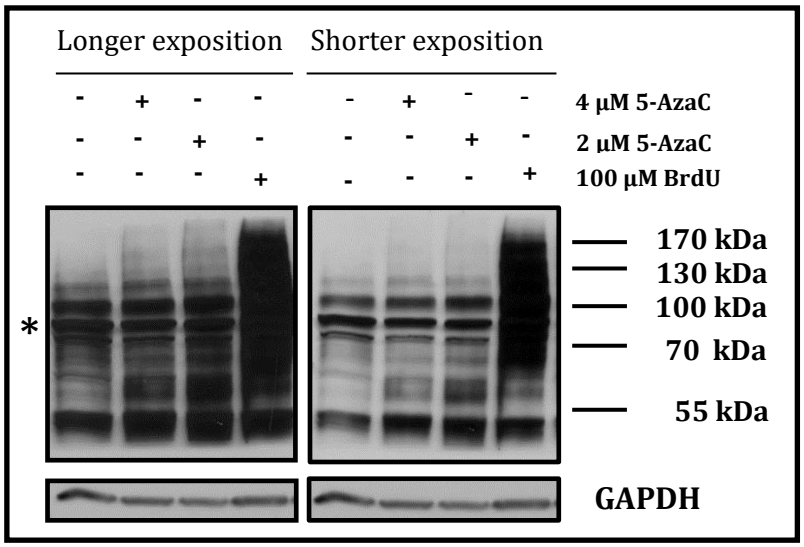


Fig. 17 Detection of PML protein level in 5-azacytidine treated cells.

Immunoblotting detection of PML in HeLa cells treated with 2 µM or 4 µM 5-azacytidine for 7 days. HeLa cells treated with 100 µM BrdU was used as a positive control. GAPDH was used for loading control.

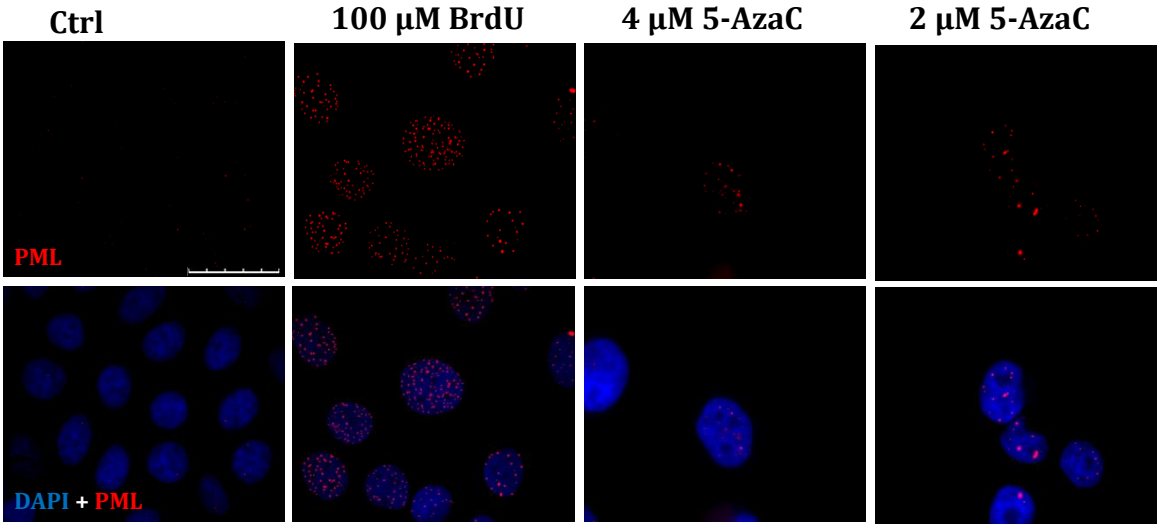


Fig. 18 Presence of PML NBs in HeLa cells treated with 5-azacytidine.

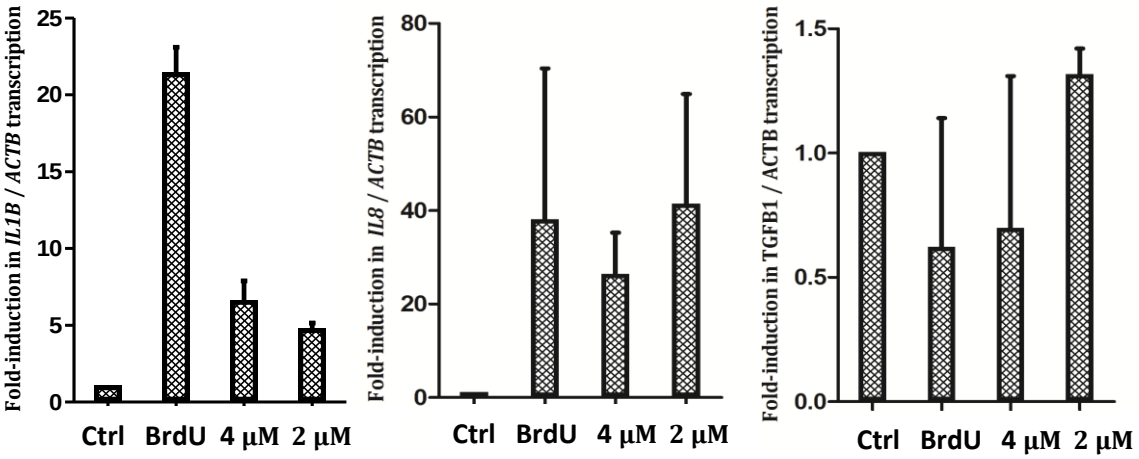
Immunofluorescence detection of PML in HeLa cells treated 2 µM and 4 µM 5-azacytidine for 24 hours for 7 days. Hela cells treated with 100 µM BrdU was used as a positive control; bar 25 µm.

5.7 5-azacytidine induces expression of Senescence-associated secretory phenotype

Since senescence is often linked with changes in secretory phenotype of senescent cells termed Senescence-Associated Secretory Phenotype, we were interested to know whether 5-azacytidine induces expression and secretion of SASP in HeLa cells. We first analyzed the transcription of common cytokines and cytokines-related genes by pre-designed Human Common Cytokines PCR Array (the data from Human Common Cytokines PCR Array were obtained by Sona Hubackova and are presented here with her kind consent). . Genes with transcriptions higher or lesser than 2-fold

compared to mock treated cells can be seen in Fig. 20. From 84 genes, 19 genes were elevated higher than 2-fold. Among these, there were genes supporting hematopoiesis (*IL1A, IL11, IL20, CSF1, CSF2*) and majority of these genes are involved in inflammatory or in T cell-mediated immune response. Genes with transcription lower than 2-fold after 5-azacytidine treatment in comparison with mock treated controls encoded in mostly forms of antiviral IFNα and γ and Bone Morphogenetic Proteins. The most prominent among 5-azacytidine regulated genes were the TGFβ and TNF superfamilies. We focused on IL1β, IL6, IL8, TNFα, TGFβ and IGNγ since they have been described to induce and enhance senescence and SASP production (ACOSTA *et al.* 2008, MIKULA-PIETRASIK *et al.* 2013, KUILMAN *et al.* 2008, BRAUMULLER *et al.* 2013) and also induce secondary, "bystander" senescence (HUBACKOVA *et al.* 2012). Increased transcription of IL1β, IL6, IL8 and TNFα was detected in HeLa cells treated with 5-azacytidine and verified on independent, validated primers (see Fig. 19, 20). Since TNFA transcript isolated from mock treated cells could not be quantitatively analyzed by qRT-PCR for its low yield , RT-quantitative PCR products corresponding to TNF transcript region were separated and detected on agarose gel. On Fig. X you can see that PCR amplicon corresponding to TNFA region from 5-azacytidine treated and BrdU treated cells was elevated, while the amplification of this region from mock treated cells cDNA did not result in any detectable product. TGFB1 did not express significant change in transcription after 5-azacytidine treatment but it is known to be regulated on protein level (SHI *et al.* 2011). Since IFNG was observed to be downregulated after 5-azacytidine using Human Common Cytokines PCR Array, the verification of this gene expression by independent, validated primers showed no transcription of this gene in both control and 5-azacytidine treated samples. To evaluate whether these transcripts were translated and secreted, FACS-Bead Cytokine Assay was used. Increased production of IL6 after 2 μM and 4 μM 5-azacytidine treatment (see Fig. 21) correlates with its increased transcription as well as increased production of TGFβ into medium was detected (see Fig. 22). To prove biological activity of these cytokines, activation of their downstream targets was measured. Phosphorylation of both STAT3 on tyrosine 705 (activated by IL6 (SCHURINGA *et al.* 2000) and SMAD2 on serine 465/467 (activated by TGFβ, (MORI *et al.* 2004) was detected (see Fig. 23). Discrepancy between levels of TGFβ in medium and subsequent SMAD2 activation in

cells treated with BrdU could be explained by oscillation of SMAD7, an SMAD2/3 inhibitor, which is upregulated by SMAD2 activation and thereby downregulates SMAD2 activity in loop. It is possible that the samples were harvested in time of high SMAD7 activity and low SMAD2 activity, both representing activation of TGFβ/SMAD pathway (ZHAO *et al.* 2000). IL1, TNFα and IFNγ were not detected in cellular supernatant , even though transcripts of *IL1* and *TNFA* were strongly increased by 5-azacytidine treatment because protein levels of these cytokines were under detection limit of this method.



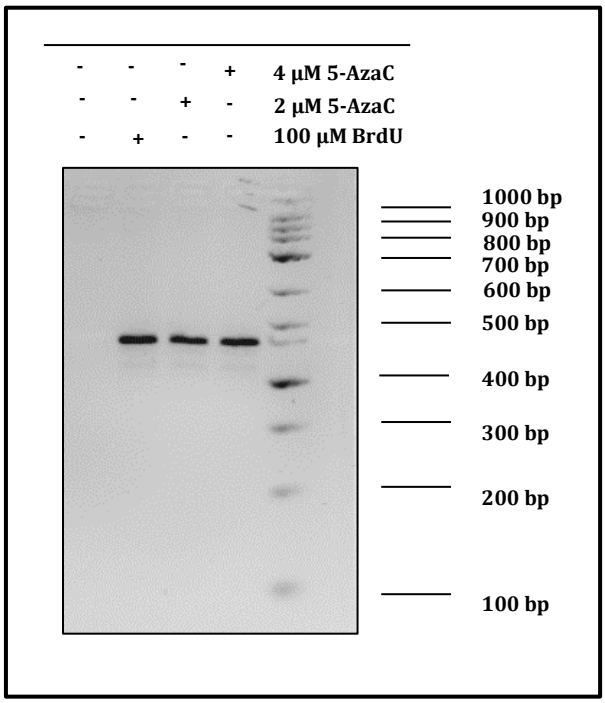


Fig. 20 5-azacytidine–dependent regulation of TNFA transcriptional levels

HeLa cells were mock treated or treated with 2 µM and 4 µM 5-azacytidine every 24 hours for 7 days. 100 µM BrdU was used as a positive control.

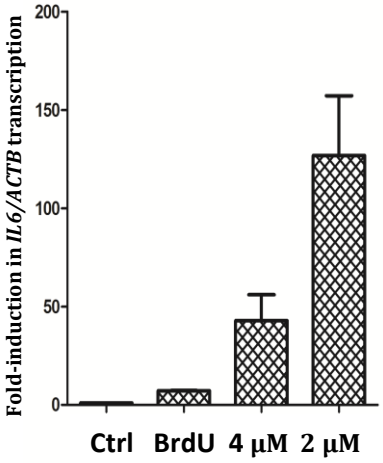


Fig. 19 (above) 5-azacytidine–dependent regulation of transcriptional levels of IL8, IL1B and TGFB1 in relative amounts correlated to actin.

HeLa cells were mock treated or treated with 2 µMb and 4 µM 5-azacytidine every 24 hours for 7 days. 100 µM BrdU was used as a positive control.

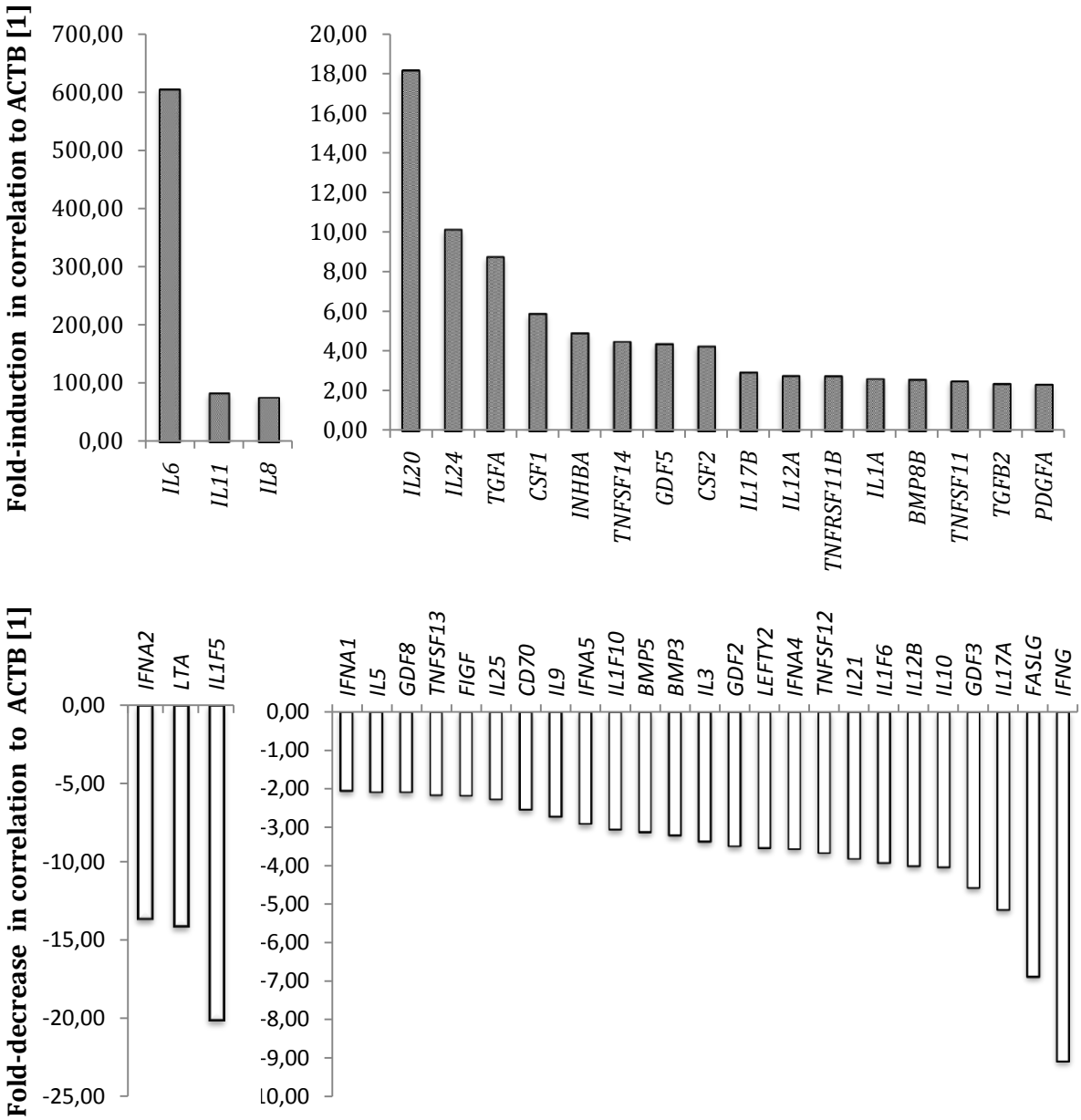


Fig. 21 5-azacytidine–dependent induction or decrease in transcriptional levels of cytokines and cytokines-related genes (higher than 2-fold) detected by Human Common Cytokines PCR Array compared to mock treated cells in 4 μM 5-azacytidine treated HeLa cells in relative amounts correlated to actin.

HeLa cells were mock treated or treated with 4 μM 5-azacytidine every 24 hours for 7 days.

Sample	pg/ml/ 105 cells
0 μM 5-AzaC	41.7
100 μM BrdU	351.3
4 μM 5-AzaC	> 20,000
2 μM 5-AzaC	> 20,000

Fig. 22 Levels of IL6 secreted after 5-azacytidine treatment in HeLa cells.

Amount of IL6 in medium of HeLa cells treated with 2 μM 5-azacytidine for 7 days. 100 μM BrdU was used as a positive control. Mean of two independent experiments.

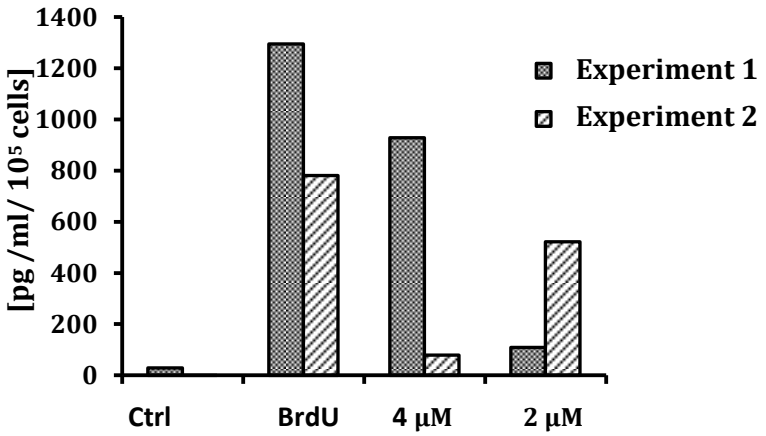


Fig. 23 Levels of TGFβ secreted after 5-azacytidine treatment in HeLa cells.

Amount of TGFβ in medium of HeLa cells treated with 2 μM 5-azacytidine for 7 days. 100 μM BrdU was used as a positive control. Two independent experiments.

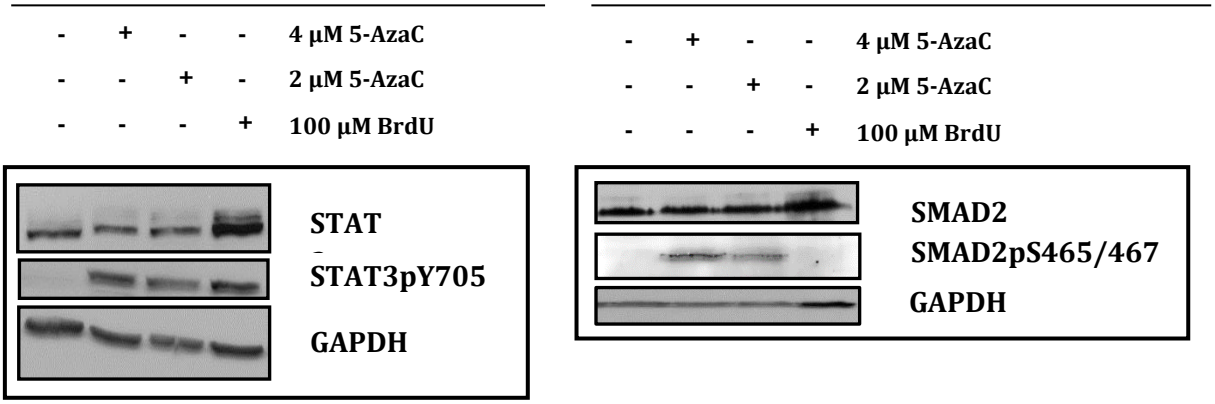


Fig. 24 Activation of Jak/STAT and TGFβ/SMAD pathways in 5-azacytidine treated HeLa cells. Immunoblotting detection of total STAT3 and SMAD2, phosphorylation of STAT3 on tyrosine 705 and phosphorylation of SMAD2 on serine 465/467 in HeLa cells treated with 5-azacytidine for 7 days. 100 μM BrdU was used as a positive control. GAPDH was used as a loading control.

5.8 5-azacytidine induced 53BP1 and γH2AX foci are transient.

To address question whether 53BP1 and γH2AX DNA damage foci developed during 2 and 4 μM 5-azacytidine treatment are persistent, cells were subsequently after termination of 7 days long 5-azacytidine treatment, cultivated in treatment free medium for another 7 days and stained for 53BP1 and γH2AX. In positive control cells treated with BrdU for 7 days and then cultivated for 7 days without treatment, 53BP1 and γH2AX foci remained unchanged in comparison with sample treated 7 days with BrdU which indicate persistent DNA damage in these cells (see Fig. 24).

Cultures previously treatedwith 2 and 4 μM 5-azacytidine and then cultivated for 7 more days without treatment were heterogeneous in nuclei size and positivity for 53BP1 and γH2AX foci. Mock treated cells were negative for significant DNA damage since no 53BP1 and γH2AX foci were detected. This observation indicates that 5-azacytidine induced DNA damage response is not persistent

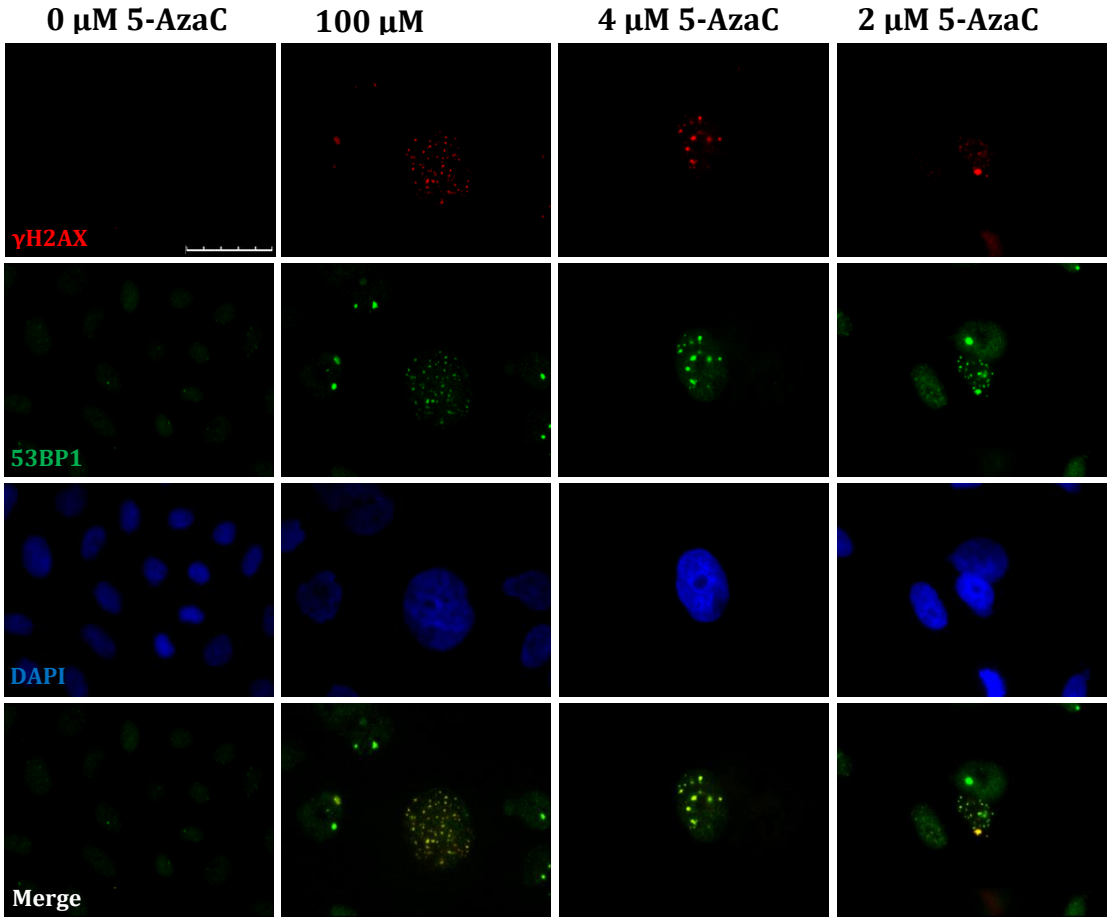


Fig. 25 Presence of γH2AX and 53BP1 foci in HeLa cells 7 days after termination of 5-azacytidine treatment.

Immunofluorescence detection of γH2AX and 53BP1 in HeLa cells treated 7 days with 2 μM and 4 μM 5-azacytidine and then cultivated 7 days in medium without treatment. HeLa cells treated 100 μM BrdU in the same regime were used as a positive control. Bar 25 μm.

5.9 5-azacytidine induced cell-cycle arrest is not permanent.

Following the termination of 7 days long 2 and 4 μM 5-azacytidine treatment, cells regained their ability to proliferate, which was assessed by EdU incorporation of cells collected at the last day of 5-azacytidine treatment (day 7) and second and fourth day of cultivation in treatment free medium (day 9 and 11) (see Fig. 24). EdU incorporation protocol was identical to the previous one (cultivation with 1 μM EdU

for 6 hours) and cells were stained for EdU together with cells collected at day 2 and 4 of 5-azacytidine treatment. After termination of 5-azacytidine treatment, cells formed colonies of various sizes with few cells appearing to have senescent-like morphology, mostly located on colony borders or independently outside the colonies. To investigate whether they are senescent, cells were tested for S-A ß-galactosidase activity by S-A ß-galactosidase assay (see Fig. 25). Cells presenting senescent-like morphology were mostly positive for SA-ß-galactosidase activity, unlike the rest of surrounding colonies. Cells treated with 100 µM BrdU were used as a positive control. Results show that only fraction of cells possibly became senescent after 5-azacytidine treatment, the rest were likely in 5-azacytidine induced transient cell-cycle arrest.

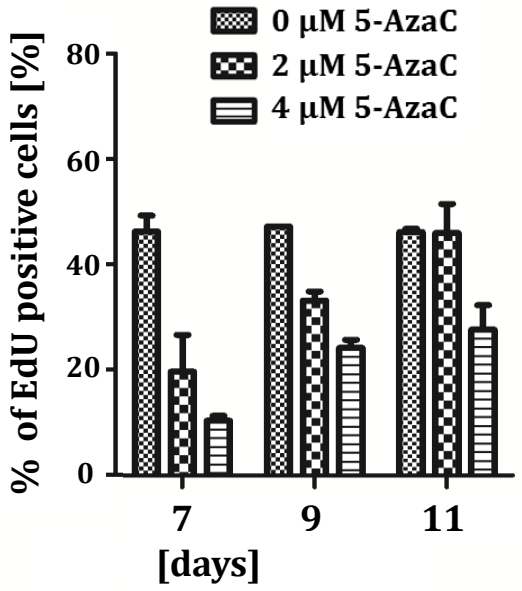


Fig. 26 Percentage of EdU positive cells after termination of 5-azacytidine treatment.

Statistical analysis of EdU incorporation in HeLa cells treated with 5-azacytidine for 7 days as indicated and followingly in treatment free medium for 2 (day 9) and 4 (day 11) days.

T test, $p \geq 0.10$; two independent experiments.

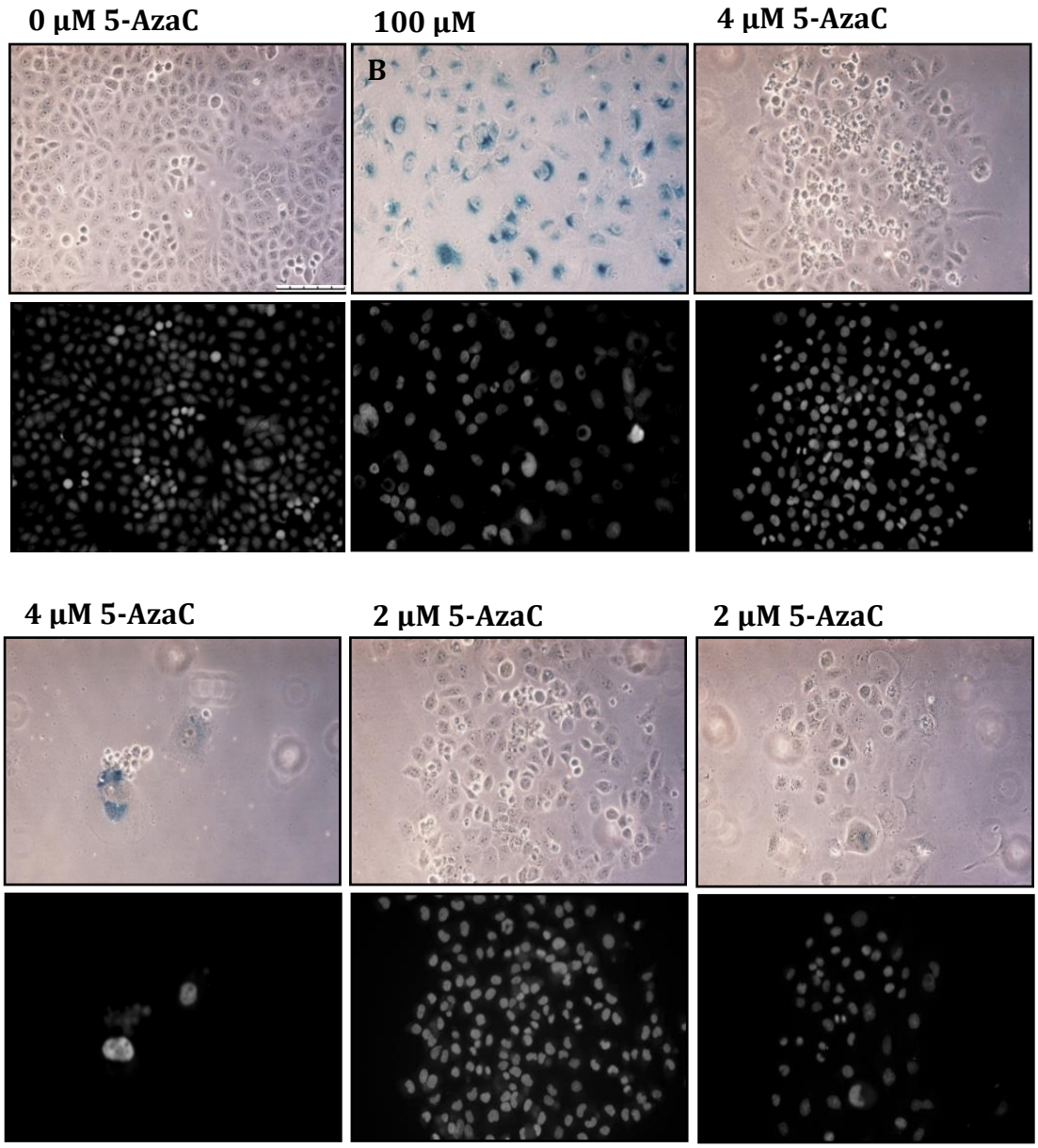


Fig. 27 Senescence-associated ß-galactosidase staining in HeLa cells 7 days after termination of 5-azacytidine treatment.
S-A β-galactosidase detection in HeLa cells treated 7 days with 2 µM and 4µM 5-azacytidine and then cultivated 7 days in medium without treatment. HeLa cells treated with 100 µM BrdU in the same regimewere used as a positive control. Bar 50 µM.

5.10 5-azacytidine induced PML NBs are transient.

To determine whether levels of PML NBs induced by 2 µM and 4 µM 5-azacytidine remain stable, 5-azacytidine was removed and cells were cultivated for next 7 days in treatment free medium. Level of PML NBs were detected by immunofluorescence. After incubation without treatment, in cells previously treated with 2 µM or 4 µM 5-azacytidine, the level of PML NBs decreased to the control level (see Fig. 26)

cultivated in TF medium for 7 days. PML NBs were stained by immunofluorescence. PML NBs numbers and intensity of previously 5-azacytidine treated cells returned to normal in large fraction of cells. Note, BrdU treated cells maintained approximate amount of PML bodies but they had reduced intensity in majority of cells.

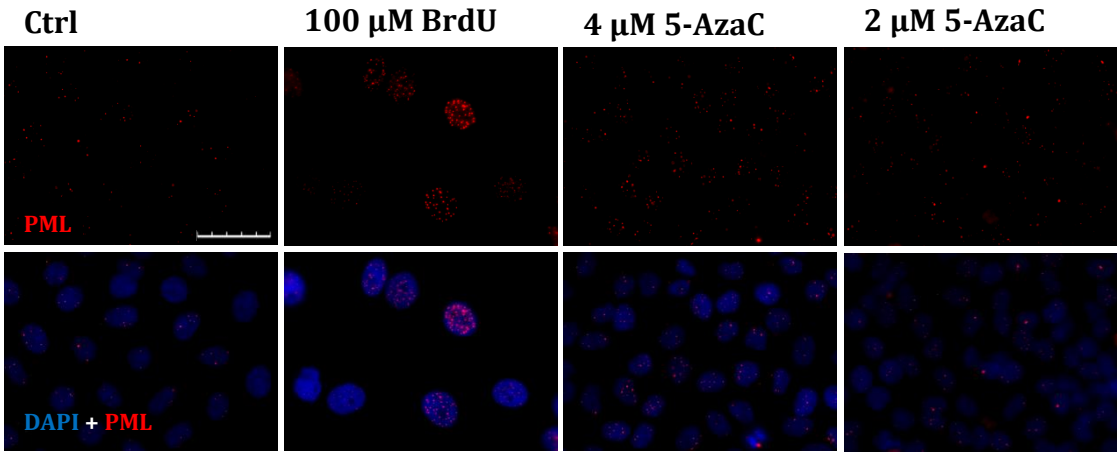


Fig. 28 Presence of PML NBs in HeLa cells 7 days after termination of 5-azacytidine treatment.

Immunofluorescence detection of PML NBs in HeLa cells treated 7 days with 2uM and 4uM 5-azacytidine and then cultivated 7 days in medium without treatment. HeLa cells treated with 100uM BrdU in the same regime were used as a positive control. Bar 50 µm

6. DISCUSSION

5-azacytidine is a cancerostatics approved of by U. S. Food and Drug Administration as an official drug for treatment of Myelodysplastic syndomes (MDS). MDS are a group of clonal diseases of myeloid lineage blood cells diagnosed in up to one hundred people in the Czech republic every year and affect mostly people older than 65 years (MA *et al.* 2007).

5-azacytidine is structuraly very similar to 2′-deoxy-5-azacytidine, which incorporates strictly to DNA and like 5-azacytidine, inhibits DNA methyltransferases and subsequently DNA methylation. *In vitro*, it has been described 2′-deoxy-5-azacytidine induces cellular senescence (SCHNEKENBURGER *et al.* 2011, VENTURELLI *et al.* 2013, GRANDJENETTE 2014). Given the similarity of 5-azacytidine structure to 2′-deoxy-5-azacytidine and its ability to incorporate into DNA as well (while having high preference for RNA), our hypothesis was that 5-azacytidine may induce senescence as well. In that case, it would be crucial to characterize phenotyp of 5-azacytidine mediated senescence, along with its secretoryp henotype, for the possibility of employing TIS in MDS treatment (reviewed in NARDELLA *et al.* 2011).

In accordance with previously published data, 5-azacytidine proved to be cytotoxic since its 2 and 4 μM treatment with duration of 7 days resulted in high level of apoptosis (see Fig. 11) (MURAKAMI *et al.* 1995, KIZILTEPE et a. 2007). Induction of apoptosis in majority of cells may be initiated by DNA damage response observed in culture (see Fig. 12,13) or by inhibition of protein synthesis which was described as an effect of 5-azacytidine treatment (REICHAN & PENMAN 1973). The work of Maslov *et al.* showed that demethylation of gene promotors is likely not the primary cause of apoptosis since inhibition of DNMT1 translation by siRNA resulted in decrease in both γH2AX induction and apoptosis (MASLOV *et al.* 2012). Murakami *et al.* state that mechanism of cytotoxic 5-azacytidine effect is dependent on used concentration and cell type of treated cells (MURAKAMI *et al.* 1995).

We showed presence of γH2AX and 53BP1 positive foci in 7 days long 5-azacytidine treated HeLa cells which were visible in all of cells. Together, with activation of Chk2 and p53/p21 patways (phosphorylated on threonine 68 and serine 15, respectively) (see Fig. 13), results strongly imply that 5-azacytidine

treatment induces DNA damage response of which all of mentioned proteins are participants (WARD *et al.* 2001, BURMA *et al.* 2001, CANMAN *et al.* 1998, AHN *et al.* 2000, NELSON & KASTAN 1994). While 5-azacytidine treatment resulted in high level of apoptosis (see Fig. 11), it is not likely that DNA damage is solely a consequence of apoptotic DNA fragmentation. Percentage of apoptotic cells in 5-azacytidine culture treated for 7 days by 5-azacytidine of 2 and 4 μM doses were approximately 48.50% (±7.8) and 52.50% (±0.7), respectively, which suggests a significant fraction of cells was not apoptotic in time of harvest. 5-azacytidine ability to induce DNA damage was documented before. Orta *et al.* proposed 5-azacytosin incorporated into DNA covalently binds DNMT1 and such adducts prevents processing of replication machinery and thereby cause collaps of relication fork (ORTA *et al.* 2013). Validity of the claim is supported by work of Kiziltepe *et al.* which shows DNA damage response is mediated predominantly via ATR pathway inducing Chk2 activating phosphorylation. DNA damage response appeared before caspase-3 activation (marker of apoptosis) (KIZILTEPE *et al.* 2007). While Chk2 is mostly regulated by ATM, it hase been reported before that ATR can activate Chk2 (WANG *et al.* 2006). Moreover, Hollenbach *et al.* proved association between depletion of DNMT1 and γH2AX signalization (HOLLENBACH *et al.* 2010) which is supported by mentioned work of Maslov *et al.* which. showed significant decrease in γH2AX after inhibition of DNMT1 expression by siRNA. Further, the persistance of γH2AX and 53BP1 positive foci for 7 days after termination of 5-azacytidine treatment in fraction of cells is in accordance with hypothesis that replication stress is at least party responsible for 5-azacytidine induced DNA damage. Jüttermann *et al.* state that 2′-deoxy-5-azacytidine-induced DNA lesions with origin in 2′-deoxy-5-azacytidine-DNMT1 adducts last unrepaired for 3 and more days (JUTTERMANN *et al.* 2007). In conclussion, 7 days long daily treatment of 2 and 4 μM 5-azacytidine induces DNA damage response, possibly via replicative stress, and results in activation of cell-cycle checkpoints.

As senescence was introduced previously, it is a permanent arrest in cell cycle accompanied by specific changes in phenotype of cell. There is no definite marker for detection of senescent cells. Cells are tested for presence of senescence hallmarks which are associated with development of senescence, however cell does not need to express all of senescence hallmarks to be qualified as senescent. Such

markers are spread morphology with enlarged nuclei, SA-β-galactosidase activity, expression of CDK inhibitors and cell cycle arrest, presence of DNA-SCARS and SASP etc. We tested cells for proliferation rate, presence of CDK inhibitors, SA-β-galactosidase activity, PML increase and SASP.

Assesment of the DNA synthesis extent revealed that 5-azacytidine downregulates S phase entry and possibly proliferation in HeLa cells, however only on short term basis. 4 μM 5-azacytidine treatment was more effective in decreasing EdU incorporation than 2 μM 5-azacytidine, as can be seen in Fig. 14, and while decrease in EdU incorporation appeared at day 2 of treatment, the full effect of EdU incorporation was detected at day 4 and lasted until last day of treatment. This trend was apparent at both doses of 5-azacytidine. After termination of 7 days long treatment, cells were cultivated in medium without treatment. Second day after termination of treatment, cells slowly regained their ability to incorporate EdU and the incorporation rate rose further. These data are in concordance with the fact, that after termination of treatment, cells formed growing colonies (see Fig. 27). The method reflects overall growth of culture but it is possible that several cells were arrested in long-termed cell cycle. DNA damage response persisting in fraction of cells 7 days after termination of treatment; presence of cells with senescent-like phenotype and their ability to stain for SA-β-galactosidase activity after 5-azacytidine treatment and increased PML NBs permits such reasoning.

It is a question whether cell cycle arrest arised from DNA damage response, DNA hypomethylation or different stimuli. Weller *et al.* present arrest of proliferation of 5-azacytidine treated cells in second and third cell cycle which corresponds with our findings (WELLER *et al.*1993), It is possible that TGFβ is responsible for the induction of cell cycle arrest by expression of p15 and p21 (DATTO *et al.*, RICH *et al.* 1999). The cell cycle arrest was more effective in 4 μM 5-azacytidine treatment than in 2 μM which corresponds with higher p53 activation and p21 level in 4 μM 5-azacytidine treated cells compared to 2 μM 5-azacytidine (see Fig. 14). Moreover, Venturelli *et al.* showed that 5-azacytidine treated cells seem to arrest preferentialy in G_0/G_1 phase of cell cycle while 2′-deoxy-5-azacytidine in G_2/M phase (VENTURELLI *et al.* 2013). This would imply that cell cycle arrest is induced trough both drugs by different mechanism and cell cycle arrest in 5-azacytidine cells may be dependent more on inhibitors of CycD1-CDK4/6 activity rather than on DNA damage

response. Nonethless, kinetics of cell cycle arrest and dose-dependent effect on cellular proliferation offers possibility of both factors playing a role in cell cycle arrest. More experiments would be needed to decide on mechanism of proliferation inhibition.

Only fraction of cells after 7 days long daily 5-azacytidine treatment was positive for SA-β-galactosidase and the intensity of staining was lower than in BrdU treated controls. While S-A β-galactosidase assay showed fraction of cells positive for S-A β-galactosidase activity (see Fig. 15), Quantitative assay of S-A ß-galactosidase activity in mammalian cell extracts showed higher activity of SA-β-galactosidase in mock treated controls (Fig 16). The difference between SA-β-galactosidase activity per 1 μg of lysates of 5-azacytidine treated and mock treated cell was relatively low . Gary *et al.* (2007) observed fully senescent cells show more than 3-fold increase in β-galactosidase activity in comparison to nonsenescent controls, which was not the case in our experiment . Furthermore, mock treated HeLa cells, while having the density of about 60% when harvested, had higher density in comparison to 5-azacytidine treated cells. It has been observed that higher density of cells increases SA-β-galactosidase positivity, however obtaining density of mock treated HeLa cells that would correspond to the density of 5-azacytidine treated cells would lead to a stress in the culture and deviation in data obtained from mock-treated control. In the 7th day of cultivation in medium without treatment, subsequently to the termination of 7 days long 5-azacytidine treatment, a small fraction of cells, usually outside the colonies or at its border, was significantly stained for SA-β-galactosidase. It is therefore possible that 5-azacytidine treatment induces senescence in minor fraction of cells (see Fig. 25). The induction in S-A β-galactosidase could result from active TGFβ-signaling pathway which was showed by activating phosphorylation of SMAD2 (see Fig. 23). Untergasser *et al.* showed that treatment with TGFβ induced S-A β-galactosidase activity in prostate basal cells while the cells did not develop senescence (UNTERGASSER *et al.* 2003).

Increase in PML NBs and overall PML level after 7 days long 5-azacytidine treatment was not as strong as in BrdU treated controls and after termination of treatment, it diminished in large fraction of cells (see Fig. 17, 18, 26). The increase of PML might be connected to elevated levels of IL-6 since IL-6-activated Jak/STAT pathway is its regulator (see Fig. 22). It is noteworthy that the level of PML increase

was in 5-azacytidine treated cells lower than in BrdU treated cells while the activating phosphorylation of STAT3 (phosphorylated on tyrosine 705) was approximately of the same level as in BrdU treated cells (see Fig. 23). It has been observed that cytoplasmic PML (cPML) is induced by TGFβ signalization and, in turn, cPML potentiates TGFβ signalization (see Fig. 17) (LIN *et al.* 2004). It is possible that abundant PML level in BrdU cells was partly result of TGFβ signalization – TGFβ secretion was higher in BrdU treated cells. Furthermore, while IFNγ was not detected at either mRNA or protein level in 5-azacytidine treated cells or controls, it is possible that BrdU treated cells secreted higher amount of IFNγ which would potentiate PML NBs expression (ref). This option is also suggested by mRNA array which shows 9-fold decrease of IFNG mRNA level after 5-azacytidine treatment. Altogether with the fact that BrdU-mediated senescence was described to induce IFNγ transcription it is plausible that IFNγ is elevated in BrdU treated cells (ref). In addition to that, we show that numbers of 5-azacytidine induced PML NBs were not stable over the time and their amount and intensity was heterogenous. The intensity of PML NBs was reduced after termination of BrdU treatment as well, however the numbers of PML NBs remained approximately the same (see Fig. 28).

SASP is one of senescence hallmarks and its presence affects not only the fate of the individual cell but also modulates responses of its environment. SASP is described to be regulated via NF-κB signalization and may be induced and potentiated by variety of cytokines and antigens, among them are all cytokines we studied, i.e. IL-1β, IL-6, IL-8, TNFα, TGFβ and IFNγ (ACOSTA *et al.* 2008, MIKULA-PIETRASIK *et al.* 2013, KUILMAN *et al.* 2008, BRAUMULLER *et al.* 2013, HUBACKOVA *et al.* 2012). We investigated 5-azacytidine-dependent regulation of pro-inflammatory cytokines expressed in SASP, known for their immunomodulation abilities and possibility of aggravating patient's response to the therapy. IL-1, TNFα, TGFβ and IFNγ regulates IL-6 and IL-8 expression via NF-κB-dependent transcription and, through several pathways, NF-κB activation is positively autoregulated (reviewed in MULTHOFF *et al.* 2012). IL-6 and IL-8 regulate expression of senescence phenotype, such as PML induction, ROS elevation and DNA damage response. Both IL-6 and IL-8, senescence effectors, were elevated on transcriptional level (see Fig. 19) and the increase of IL-6 was above detection limit of Beads based assay (see Fig. 20). Furthermore, the activation of Jak/STAT

pathway, detected by activating phosphorylation of STAT3 (pY705) (see Fig. 23), implies that IL-6 is able to induce signalization through this pathway in 5-azacytidine treated cells. Protein levels of IL-8 after 5-azacytidine treatment were not possible to be evaluated due to technical difficulties, however induction of IL-8 by 5-azacytidine was documented by Ventureli at al. Notably, they also observed decrease of IL-1 antagonist which may enhance IL-1 signalization. (VENTURELLI *et al.* 2013). Conversely, a study done on MM cell line (derived from human multiple myeloma) states that IL-6 level and NF-κB signalization decreased after 5-azacytidine treatment but authors propose that the decrease in IL-6 and NF-κB activation is rather an effect of protein synthesis inhibition than any DNA-dependent effect of 5-azacytidine due to the kinetics of effect. It is possible that 5-azacytidine effect on IL-6 level is dependent on cell-type (KIZILTEPE at al. 2007).

5-azacytidine also induced the secretion of TGFβ (Fig, 22). The induction of TGFB transcript was not significantly elevated after 5-azacytidine, it is however known that TGFβ is regulated on protein level (SHI *et al.* 2011). Moreover, it is probable that TGFβ signalization pathway is activated in 5-azacytidine treated cells since SMAD2 was phosphorylated on its activation site (S465/467) (see Fig. 23). There is discrepancy in secretion of TGFβ and activation of TGFβ signaling pathway in BrdU treated cells. The level of TGFβ is elevated while S465/467 phosphorylation is not present in BrdU cells and the p15 phosporylation, which has been described to be regulated by TGFβ, is not increased either (RICH *et al.* 1999). However this was the case in two out of three independent experiment. One time, we observed p15 upregulation and SMAD2 activating phosphorylation in BrdU treated cells. There is negative loop (trough SMAD7 activation) in TGFβ signalization and it is possible the cells were harvested in the moment of high SMAD7 activation and low SMAD2 activation (ZHAO *et al.* 2000).

Transcriptional IL1B and TNFA levels were elevated in 7 days long daily 5-azacytidine treated HeLa cells (see Fig. 19, 20) but its protein level was under detection limit of FACS-Beads Assay and thus could not be measured. IFNγ was not detected on transcriptional level upon either dose of 5-azacytidine 7 days long treatment.

Taken together, minor elevation in PML compared to BrdU treated cells, low intensity of staining for SA-β-galactosidase, transient effect on cell cycle and no

senescent morphology in majority of cells indicate that 7 days long daily 5-azacytidine treatment of doses 2 and 4 μM does not induce senescence in HeLa cells. These results are supported by work of Venturelli *et al.*, which was published during the work on this thesis in 2013. Authors have claimed that 5-azacytidine does not induce senescence in HepG2 and HepB3 cells (VENTURELLI *et al.* 2013).

Our conclussion is that 5-azacytidine is not capable of inducing senescence in HeLa cells treated by *in vivo* obtainable doses but it is possible that 5-azacytidine treatment may induce an epithelial-to-mesenchymal transition (EMT) in HeLa cells. 5-azacytidine elevated IL-6, TGFβ and possibly IL-8 and it has been described that these cytokines are able to initiate EMT (VENTURELLI *et al.* 2008). TGFβ regulates IL-6 and IL-8 which in turn upregulates SNAI1, SNAI2, TWIST1, etc. – effectors of EMT (YR *et al.* 2013, SULLIVAN *et al.* 2009). Moreover, it has been proposed that induction of EMT via Twist1/2 inhibits the onset of senescence and it was proved that 5-azacytidine induces anoikis in epithelial MCF-7 cell-line which is in corelation with our observations obtained by life-cell imaging (data not presented here) (ANSIEAU *et al.* 2008). While majority of unattached cells underwent anoikis (data from life-cell imaging), our unpublished work shows a fraction of cells survives the loss of attachement and subsequently gain the ability to re-adhere and to continue in proliferation. Furthermore, we noticed change in morphology in HeLa cells after 5-azacytidine treatment – cells are large and rounded (see Fig. 5). It is possible that this change is the result of upregulation of vimentin, intermediate filament protein considered to be a marker for EMT, which was described to be upregulated by IL-6 (SULLIVAN *et al.* 2009).

It has been proposed that Snai1 and Snai2 play important role in HSC and HPC survival and self-renewal which could be beneficial in MDS since one of symptoms is known to be high apoptosis in bone marrow. However, the role of these transcription factors might be more complex. Mice overexpressing Snai1 or Snai2 did not express any visible abnormalities, however mice of 2 separates transgenic lines overexpressing Snai1 started to develop various cancers of both epithelial and mesenchymal origin, that of the mesenchymal origin being acute leukaemia and lymphomas. Similarly, significant number of mice overexpressing Snai2 died of cardiac problems (20%), the majority of survivors developed acute leukemia in later time (B-cell acute leukemia – 60% - and acute myeloid leukemia – 40%). Down-

regulation of both Snai1 and Snai2 did not slow down cancer progression in Snai1/2 overexpressing mice. While 5-azacytidine induces DNA damage response in several cell lines, it has been observed that doses of 5-azacytidine comparable to plasma concentration of MDS patients' after 5-azacytidine treatment do not induce DNA damage in HSC and HPC (VENTURELLI *et al.* 2013).

Considering these observations, it is possible that 5-azacytidine-dependent increase of Snai1 or Snai2 would result in protection of HSC and HPS, however patients could later develop AML or other malignant cancers. However, it is unlikely that 5-azacytidine effect on Snai1 and 2 expression is the sole mechanism of its action (PEREZ-MANCERA *et al.*, 2005 a,b). Furthermore, IL6, has been decribed to be a prognostic factor for increased risk of MDS transmission into AML but also to be beneficial in small doses (GORDON *et al.* 1995, PARDANI *et al.* 2012). Bachegowda *et al.*

The exact therapeutic effect of 5-azacytidine remains to be elucidated. Our findings need to be further investigated on MDS-derived cell lines, so far they however imply that the action of 5-azacytidine might be beneficial and deleterious at the same time. The therapeutic strategy therefore needs to be reconsidered in the light of new findings obtained from MDS-derived cell-lines. Due to EMT induction, the possibillity of 5-azacytidine treatment of epithelial solid tumors needs to be explored further.

7. CONCLUSSION

1. We observed that 7 days long 5-azacytidine treatment of doses comparable to patients' plasma concentration induces apoptosis in HeLa cell line.
2. We detected γH2AX and 53BP1 positive foci in HeLa cells, along with activation of Chk2 and p53/p21 pathways, after 5-azacytidine treatment of doses corresponding to MDS patients' administration protocol and thereby we conclude 5-azacytidine induces DNA damage response and activation of cell-cycle checkpoints.
3. We verified that 5-azacydine inhibited proliferation of Hela cells in doses relevant to MDS patients' treatment protocol.
4. We analyzed 5-azacytidine effect on markers of cellular senescence, i.e. activation of DNA damage response and its persistence, induction of cell-cycle arrest, increase of S-A β-galactosidase activity, increase of PML and PML NBs and presence of senescence-associated secretory phenotype. Albeit we showed mild expression of mentioned markers of senescence, the low increase in values of these markers imply that 5-azacytidine does not induce senescence in HeLa cell line with drug concentration and treatment protocol.
5. We showed 5-azacytidine induces secretion of pro-inflammatory cytokines IL6 and TGFβ which are frequently present in secretory phenotype of senescent cells however we failed to detect IL1β and TNFα on protein level while their transcription was elevated after 5-azacytidine treatment.

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