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ANALYSIS OF BASE EXCISION REPAIR PROTEINS IN A549 LUNG CANCER CELL LINE UPON INDUCTION OF CANCER CELL SENESCENCE BY GENOTOXICANTS TREATMENTS

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ABSTRACT

Senescence is a cellular process characterized by an irreversible arrest of the cell cycle following various stressors potentially harmful to cells, such as DNA damage, oxidative stress, or oncogene expression. The p53-$p21^{CIP1}$ and $p16^{INK4a}$-Rb pathways were activated in response to these stimuli and promote cell cycle arrest. One of the most important hallmarks of senescence is the "senescence-associated secretory phenotype" (SASP), in which chemokines, cytokines, and proteases are secreted in an autocrine and paracrine manner. Among cytokines, IL-6 and IL-8 are mainly involved in regulating the tissue microenvironment. Several studies have shown that cellular senescence is related to tumor progression and chemoresistance. When damaged DNA is not properly repaired, cell clearance leads to a chronic pro-inflammatory state. The Base Excision Repair pathway (BER), specifically the protein Apurinic/Apyrimidinic endodeoxyribonuclease 1 (APE1), has been shown to be involved in the induction of intrinsic cell senescence. Currently, involvement of BER in extrinsic cancer cell senescence is still unknown. In recent years, a new pharmacological strategy has been developed to selectively eliminate senescent cells by using senolytic compounds. In the present work, we investigated a possible involvement of BER enzymes in the onset of extrinsic cell senescence of A549 cancer cells. To this end, we first established a model of extrinsic cancer cell senescence by using cisplatin (CDDP) and doxorubicin (DOXO) as genotoxic agents. We analysed the expression of the majority of the BER enzymes: APE1, XRCC-1, POL-β, POL-δ. We found that APE1 was downregulated in a proteasomal-dependent manner during the onset of cancer cell senescence, whereas no significant changes were detected for the other BER enzymes. Of interest, APE1 controls the expression of miRNAs involved in cell senescence. We observed that miR regulated by APE1, i.e., miR-130b, which regulates the expression of $p21^{CIP1}$, is downregulated in line with APE1 expression, leading to upregulation of its target $p21^{CIP1}$ and promotion of the senescence process. Moreover, APE1 contributed to the onset of senescence by inducing SASP factors (IL-6 and IL-8) in the early phase of the process. Interestingly, treatment of senescent cells with APE1 inhibitors sensitizes cancer cells to CDDP treatment. These data suggest that APE1 is involved in the early stages of senescence through induction of SASP factors and deregulation of miRNAs. We also demonstrated the senolytic activity of APE1 inhibitors. Overall, this work suggests that

APE1 plays a role in the development of extrinsic cell senescence through several mechanisms and that inhibition of APE1 activity could be a promising target for the development of new senolytic agents.

INTRODUCTION

1 Cellular senescence: friend or foe?

Senescence is a cellular process defined by irreversible cell cycle arrest and loss of proliferative capacity, but at the same time characterized by normal metabolic activity and viability. It occurs as a stress response mechanism against DNA damage, oxidative stress, chemotherapeutic stimuli, radiation, and oncogene expression [1][2][3][4][5]. To prevent the proliferation and spread of the damage, injured cells may respond by inducing: activation of repair, cell death, or entry into senescence. In a context in which cells are capable of repair, the cell cycle resumes, and cells continue to replicate. When the intensity of stress is too high and repair is no more possible, cells enter in apoptosis. If the cells are unable to repair the injury, the cell cycle is prematurely arrested in G1 phase, promoting premature cellular senescence [6] [7]. In the absence of specific markers to define the senescent state, some characteristics of senescent cells can be identified. From a morphological point of view, senescent cells appear enlarged and flattened, and this cell morphology represents an important biomarker. During senescence, the activity of the enzyme β-galactosidase, a lysosomal hydrolase, increases and it is a widely used marker to detect senescence through the activity of SA-β-Gal [8]. Since senescent cells have been shown to accumulate in tissues with aging, this feature makes senescence a valid *in vitro* model for conducting ageing studies [9]. Senescence has also been observed to be involved in various physiological responses such as tissue repair, tumor suppression, wound healing, and age-related degeneration [10][11][12][13][14][15][16]. Senescence has been shown to play an important role in several age-related diseases, particularly atherosclerosis, lung disease, and diabetes. In fact, the accumulation of cells may cause organ dysfunction and becomes an incidental factor of ageing, but this is still under debate [17][18][19][20][21] (***Fig.1***).

Cellular senescence contributes to four distinct processes: tumor suppression, tumor promotion, aging, and tissue repair. In response to stress, cells can choose senescence, entering in an irreversible cell cycle arrest.

The main event is the expression of IL-1α, which activates the transcription factors NF-κB and C/EBPβ (CCAAT/enhancer-binding protein beta) by binding to the IL-1 receptor on the cell surface [22]

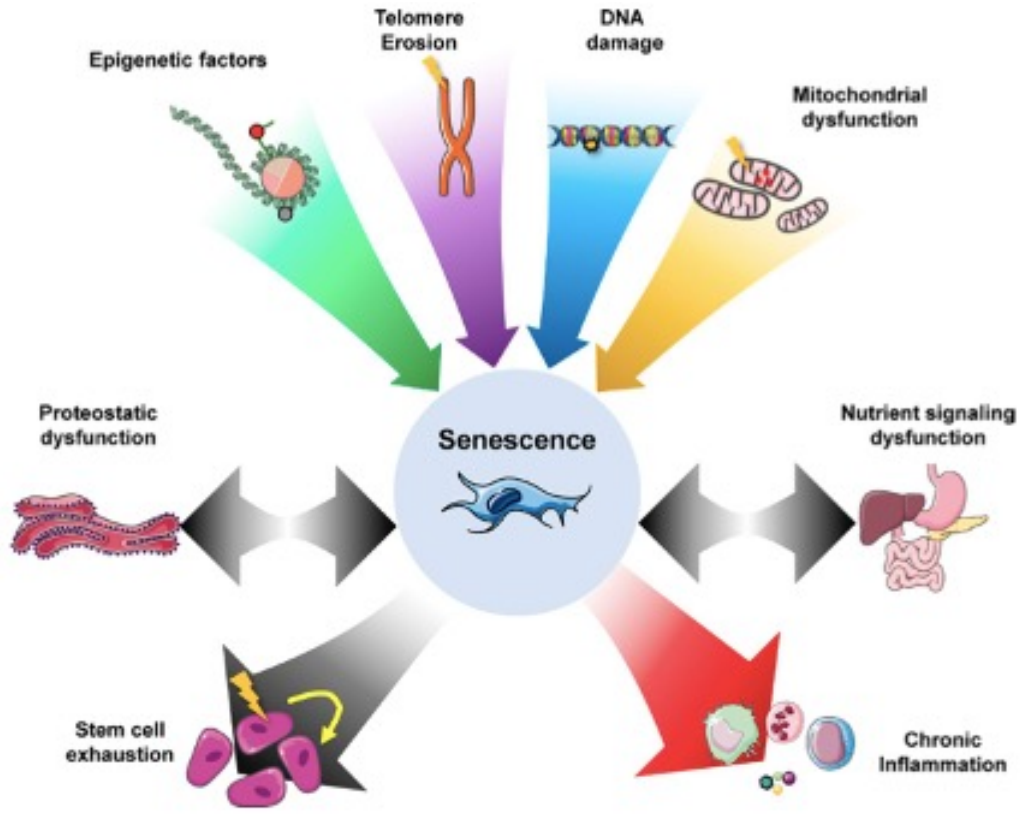


Figure 1. ***Representation of cell senescence features***. State of irreversible cell cycle arrest in which cells remain metabolically active, no longer divide, fail to respond to growth stimuli, shortening their telomeres. Epigenetic dysfunction, mitochondrial dysfunction, and DNA damage (oxidative stress) are the causes that trigger senescence. Senescence can cause stem cell exhaustion, chronic inflammation, metabolic reprogramming, phenotypic changes, chromatin remodeling, increase in autophagy, and activation of the proinflammatory protein complex known as SASP (secretory phenotype associated with senescence) [140].

This activation leads to the initiation of the SASP, characterized by an increased expression of the inflammatory cytokines IL-6, IL-8 and MMPs, which can promote both tissue repair and cancer progression (***Fig.2***). SASP factors such as proteases, cytokines and chemokines can act in an autocrine or paracrine manner to attract immune cells that kill senescent cells. In the advanced phase of senescence, some miRNAs are expressed, including miR-146a and miR-146b, which are both involved in the SASP pathway [23].

Particularly, these miRNAs promote the downregulation of their targets IL-6 and IL-8, causing a reduction of the sustained acute inflammatory response. If senescent cells are not properly removed, some of them may escape the immune response and cause a state of chronic inflammation.

Cellular senescence can be viewed as a process with two effects: tumor suppression and tissue repair, which are associated with a beneficial effect, this aspect can be considered as an important anticancer mechanism to reduce the proliferation of damaged and premalignant cells [24] whereas tumor progression and the promotion of aging are harmful to the organism [25].

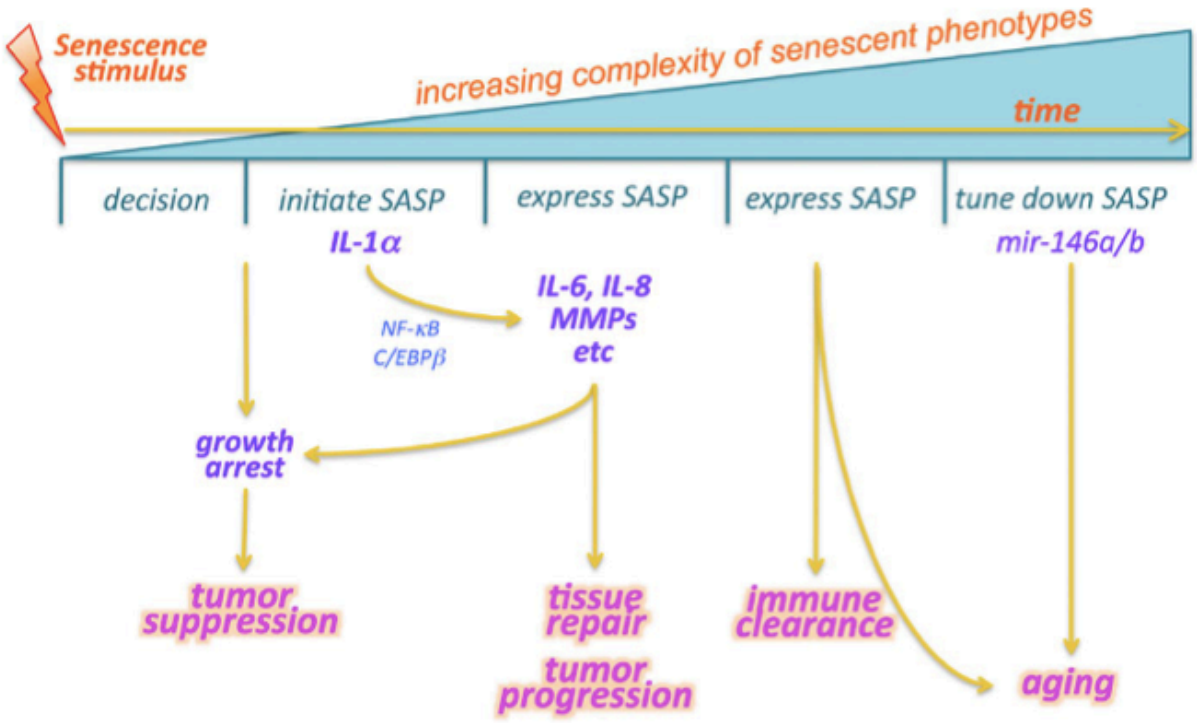


Figure 2. ***Representation of the multiple steps characterizing the complexity of the senescence phenotype.*** Cell growth is rapid (24-48 h), IL-1 activates transcription factors NF-kB and C/EBPβ, which are necessary for SASP factor expression. SASP proteins enhance growth arrest, facilitate tissue repair, and control cancer progression. SASP proteins attract immune cells that kill senescent cells [105].

1.1 Replicative Senescence

In 1961, Hayflick and Moorhead first described the occurrence of cellular senescence, in which diploid primary cells from various human embryonic tissues can proliferate in culture for a limited number of population doublings. This phenomenon of growth arrest, in which cells were metabolically active and viable, was termed the Hayflick limit or, more recently, replicative senescence (RS) [26]. In 1990, a paper published by Harley *et al.* showed that replicative senescence is related to telomere length. Indeed, at the end of the chromosome there are short repetitive sequences called telomeres, which are involved in maintaining genomic stability [27] [28]. The enzyme involved in compensating telomere shortening by adding DNA repeat fragments is called telomerase. It is a ribonucleoprotein with the function of reverse transcriptase, which uses its own RNA as template to add DNA repeats. hTERT is the catalytic subunit that functions as a human telomerase reverse transcriptase [29] [30]. Germ and stem cells express the enzyme telomerase, which is essential for telomere maintenance and also for genomic integrity and viability [31]. In some cases, when cells with telomerase are unable to reach replicative senescence, they become immortal. This aspect is considered more important because expression of telomerase can be used as an anti-aging therapy due to its ability to immortalize human cells [32] [33] [34] [35]. When DNA polymerases are not working properly, telomeres cannot reach the critical length, causing the activation of the DNA damage response (DDR). Through the DDR signal, cells are able to detect double-strand breaks (DSBs), which trigger cell cycle arrest and initiation of senescence.

1.2 Oxidative, genotoxic, oncogene stresses are able to induce premature senescence

Cellular senescence can also be induced by internal or external stimuli, such as exposure to acute or chronic stresses that trigger cell premature senescence. This condition is termed "stress-induced premature senescence" (SIPS) and may occur before the maximum number of population doublings [36] [37] [38] [39].

To ensure normal physiological conditions, it is important to preserve the balance between the generation and elimination of reactive oxygen species (ROS), which include superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) [40] [41] [42]. It is well known that the mitochondrial respiratory chain produces ROS, and these are able to chemically modify lipids, proteins and also DNA, leading to cell damage [43] [44]. In particular, high concentrations of ROS lead to oxidation or peroxide formation with destruction of cell membrane structure, permeability changes and DNA damage. The accumulation of oxidative damage contributes to ageing and various degenerative diseases, including SIPS, when cells are exposed to ultraviolet light (UV) [45] [46] [47]. There are several strategies that the organism can use to cope with oxidative stress. The enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase, prevent oxidative stress and ROS-induced cell damage [48] [49].

Premature senescence can also be triggered by genotoxic substances, as compounds currently used in chemotherapy, which are able to hit the DNA structure by interacting with it [50]. During treatment with chemotherapeutic agents, such as CDDP, DOXO, etoposide, and many others, genomic integrity is compromised by genotoxic stress. For example, CDDP, also known as cis-diamminedichloroplatinum, is one of the most commonly used drugs in chemotherapy. It causes cross-links with purine bases of DNA, disrupts DNA repair mechanisms, causes DNA damage, and subsequently induces apoptosis in cancer cells [51]. Platinum-based compounds are used as anticancer drugs for the treatment of many human cancers such as lung, ovarian, breast, kidney, brain and testicular cancers [51].The anthracycline doxorubicin (DOXO) is used as an antineoplastic agent with a broad spectrum of activity for the treatment of solid tumors, such as hematologic malignancies, breast, prostate, lung, ovarian, and gastric cancers [52] [53]. DOXO is able to intercalate deoxyribonucleic acid (DNA) and covalently binding proteins involved in DNA replication and transcription, thus inhibiting DNA, RNA and protein synthesis, and eventually leading to cell death [54].

Premature senescence can also be triggered by oncogenic activation, which was first described by Serrano *et al.* in a paper published in 1997, where it was defined as oncogene-induced senescence (OIS) [55]. It was observed that overexpression of the

oncogenic version of RAS promotes OIS. An oncogene is defined as a mutated gene whose mutations can cause carcinogenesis and cell proliferation, leading to inactivation of tumor suppressor genes such as p53, PTEN, and NF1 and induction of oncogenes such as BRAF, AKT, E2F1 [56] [57] (***Fig.3***). Senescence may act in response to oncogenes to prevent oncogenic transformation [58] [59].

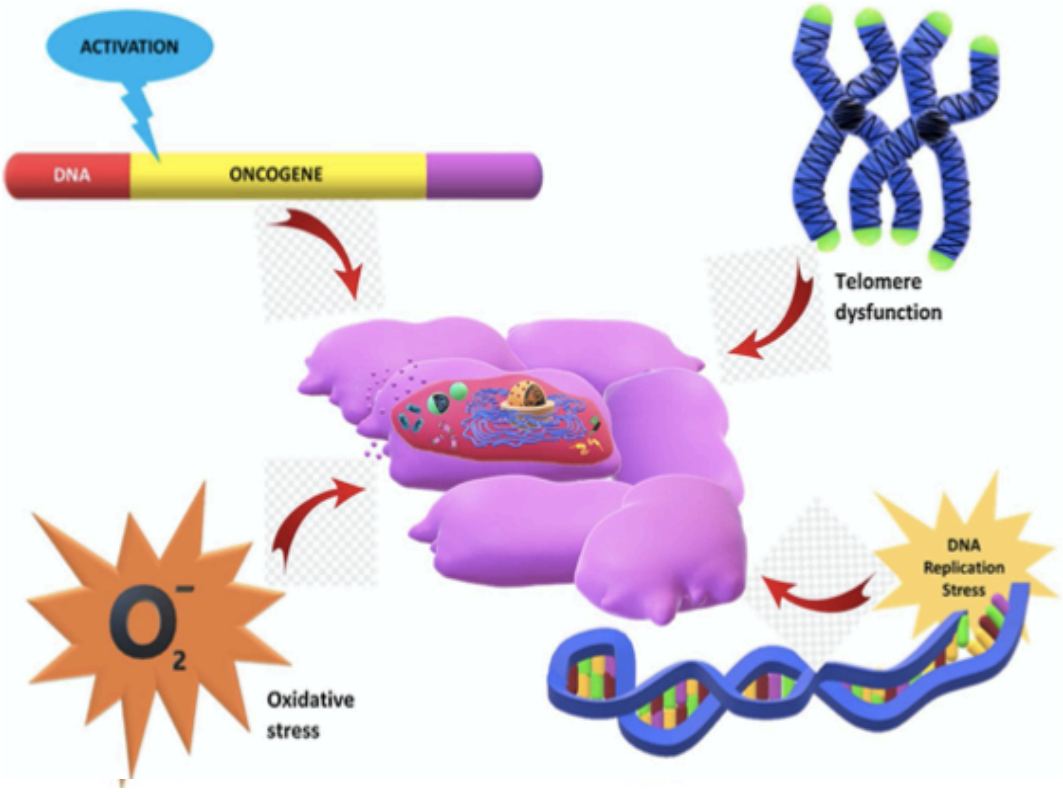


Figure 3. ***Cellular senescence induced by internal or external stimuli*** Oncogene-induced senescence and premature senescence can be triggered by oncogenes and genotoxic stress. On the contrary, telomere dysfunction and oxidative stress are involved in the induction of replicative senescence and stress-induced premature senescence [62].

1.3 Hallmarks of cellular senescence

Cells undergoing senescence show morphological changes: they appear enlarged, with multiple nuclei, the Golgi apparatus and vacuolar compartment increase in size [60] [61] [62] [63]. Due to the reduction in RNA turnover and protein degradation by the proteasome, there is an accumulation of RNA and proteins. In this cellular state, the cell cycle is stalled in late G1 [64] [65] [66]. This growth arrest is due to the activation of the tumor suppressor pathways as p53/ $p21^{CIP1}$ and $p16^{INK4a}$/Rb [67] [68]. Transcription of cyclin-dependent kinase inhibitor (CDKi) $p21^{CIP1}$, which blocks CDK2 activity and ensures that Rb is in its hypophorylated and functional form, is induced by p53 in response to DNA damage, but prolonged stress also activates $p16^{INK4a}$ (known CDK4/CDK6 inhibitor), which maintains cell cycle arrest [69] [70]. Since $p21^{CIP1}$ is induced during cell cycle arrest, it is considered a marker of cellular senescence [71] [72] [73] [74]. The most common biomarker assay to detect senescent cells *in vivo* and *in vitro* is the well-known SA β-Gal (***Fig.4***).

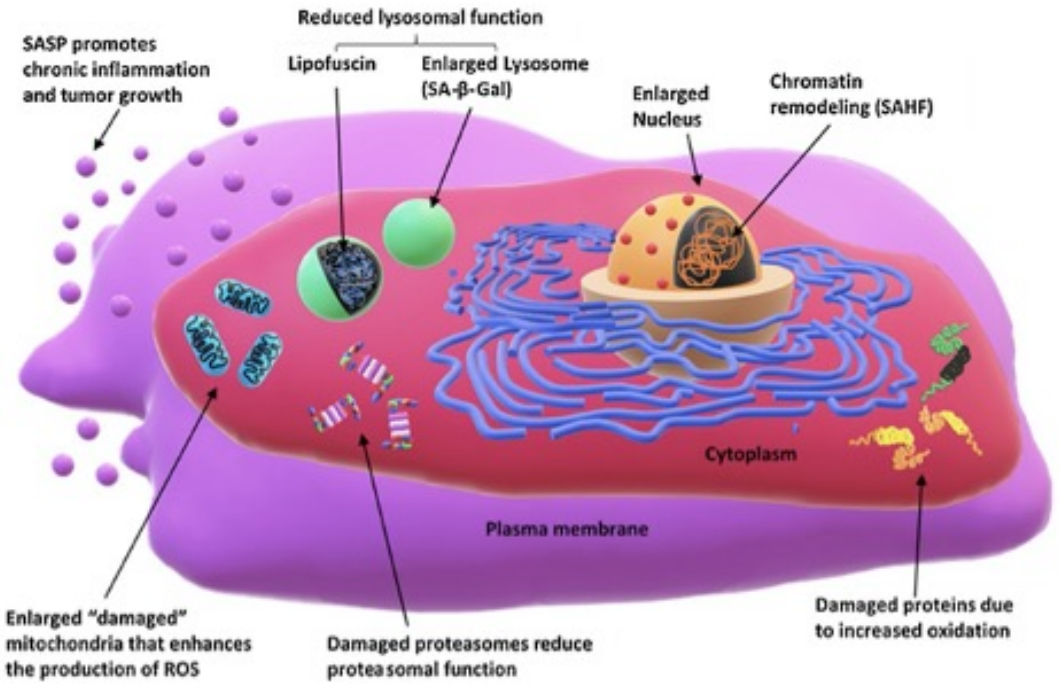


Figure 4. ***The hallmarks of cellular senescence.*** The morphological changes through which cells appear enlarged, have an irregular shape, the integrity of the nucleus is compromised, lysosomal content is increased, and β-Galactosidase activity is high. Mitochondria are impaired and produce high levels of ROS [62].

1.3.1 Current methods used to detect cellular senescence

In 1995, Dimiri *et al.* discovered that senescent cells, like their normal counterparts, express the enzyme β-Galactosidase, which is detectable at pH 6, as opposed to pH 4 in normal cells. The increased expression of this enzyme is from the lysosomal β-D-Galactosidase encoded by the GLB1 gene and is due to the increased size and number of lysosomes and their relative content in senescent cells [75] [76] [77] [78] [79]. The enzyme hydrolyzes the chromogenic substrate 5-bromo-4-chloro-3-indoyl β-D-Galactopyranoside (x-GAL) [80], producing galactose 5-bromo-4-chloro-3-hydroxyndol, which is dimerized and oxidized, producing a blue-colored indigo pigment [80]. The high activity of SA β-Gal only in senescent cells allows them to be distinguished from quiescent or differentiated cells [81].

A second valid method currently used among senescence markers is 5-Bromo-2'-deoxyuridine (BrdU) incorporation, which is used particularly to assess cell proliferation. BrdU is able to insert into DNA and compete with thymidine during replication, and BrdU incorporation is a direct measure for quantifying cell proliferation. This incorporation can be detected by various methods, such as determining the intensity of absorption or by immunohistochemical assays [82][83]. Sasaki and colleagues observed that when cells were treated with H_2O_2, senescent cells were more resistant to death than young cells and that the level of anti-apoptotic Bcl-2 gene expression was significantly reduced in aged cells [84]. Suh *et al.* examined the apoptotic response in aged rats treated with a genotoxic agent such as methyl methanesulfonate (MMS). In response to this stimulation, apoptotic potential was greatly reduced in aged rats compared to younger ones [85].

1.3.2 Senescence Associated Secretory Phenotype (SASP)

During cellular senescence, various proteins are secreted during growth arrest: pro-inflammatory cytokines (IL-6 and IL-8), chemokines (monocyte chemoattractant proteins), proteases, growth factors (transforming growth factor-β, TGF-β, and granulocyte-macrophage colony-stimulating factor, GM-CSF), degradative enzymes such as matrix metalloproteases (MMPs), soluble protein/extracellular matrix (ECM)

Components [86] [87] [88] damage-associated molecular patterns and extracellular vesicles that contain enzymes, miRNAs and DNA fragments [89] [97] [90] [91] [92] [93]. The composition of these secretory factors is termed SASP, which is also known as the senescence-messaging secretome (SMS), a relevant phenotypic program in senescent cells [89]. SASP can be regulated at epigenetic, transcriptional, and post-transcriptional levels, resulting in a complex signaling cascade. Under stress conditions, NF-κB and C/EBP β are involved in the regulation of IL-6 and IL-8 [90], while IL-1α is able to regulate the transcriptional activity of NF-κB, leading to a decrease in the secretion of inflammatory cytokines [94] [94] [95]. DDR signaling also plays an important role as a SASP mediator. Ataxia-Telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) can be activated in response to DNA damage and inhibit the degradation of GATA4, at the same time NF-κB triggers and maintains the SASP network [96]. Several papers have reported that SASP can be induced independently of DNA damage, with the activation of NF-κB mediated by p38 MAPK [97] [98] [99] [100]. There are two mechanisms by which SASP can be regulated at the post-transcriptional level: i) by mTOR, which induces NF-κB and C/EBP β to promote IL -1A transcription; ii) by the phosphorylation of the RNA-binding protein ZFP36L1 by MAPKAPK2, which causes its inhibition, prevents the degradation of transcripts of several SASP components [94] [95] [101].

Regarding the epigenetic regulation, there are several mechanisms that control SASP. For example, the histone variants macroH2A1 and H2AJ are involved in the positive and negative modulation of SASP during senescence [102] [103]. High mobility group B 1 and 2 proteins (HMGB1 and HMGB2) also play role in the expression of SASP factors that protect against heterochromatin spreading [104], as well as the histone deacetylase sirtuin 1 (SIRT1), whose downregulation leads to increased expression of IL-6 and IL-8 through histone acetylation of promoter regions [105]. 3D chromatin remodeling occurs, particularly in the nuclear lamina with the loss of laminin B1 [106], and the formation of Senescence heterochromatin foci (SAHF) complexes.

SAHF can be detected microscopically using DNA dye and appear as condensed regions of DNA/chromatin [107] [108].

SAHF comprises repressive chromatin regulators and markers, including H3K9me3 and H3K27me3, which are able to condense chromosome structure and repress proliferation genes [109]. To ensure senescence stability and tumor suppression, the chromatin of senescent cells is processed via the autophagy/lysosomal pathway, as described in the 2013 paper by Ivanov *et al*. [110]. The functions attributed to SASP could have a positive or negative effect on senescent cells. The immune system is reactivated by SASP to eliminate senescent cells and acts as a tumor suppressor in the context of tumorigenesis [111] [112]. Moreover, SASP can promote tumorigenesis by supporting angiogenesis and tumor growth [113] [114] [115]. From a physiological perspective, SASP may also contribute to fibrotic tissue remodeling [13] stem cell reprogramming [86], and during embryogenesis acting as a complementing mechanism to apoptosis [116] [117] [118].

Senescent cells can communicate with neighboring cells and the extracellular matrix via the paracrine action of SASPs, which has been reported to exert tumor suppressive effects. To enhance the senescence phenotype, secreted factors can also act on senescent cells in an autocrine manner [116] (***Fig. 5***).

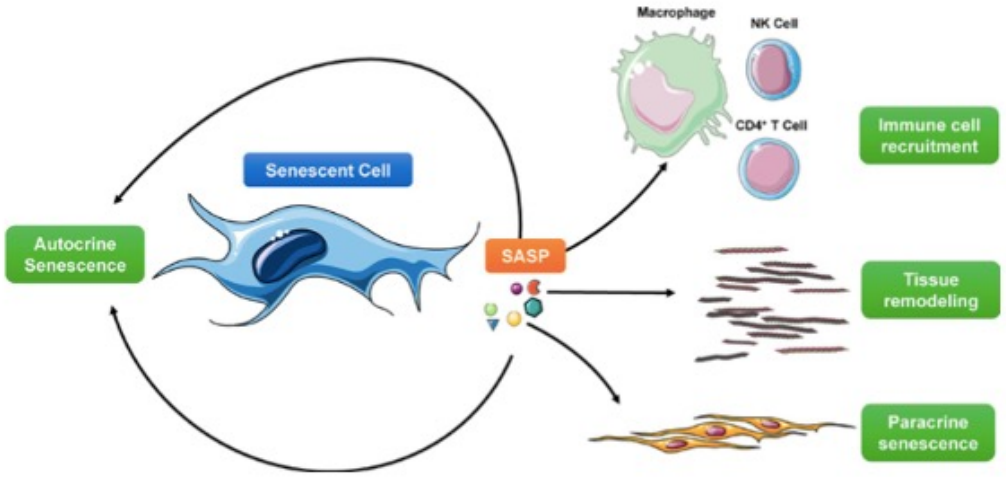


Figure 5. ***SASP is a key mediator of extrinsic cellular functions, acting in both autocrine and paracrine signaling.*** In the autocrine signaling it stops cell growth. In the paracrine action, it recruits immune cells, such as macrophages, neutrophils, and natural killer cells (NKs), to induce phagocytosis and eliminate senescent cells. MMP secretion and growth factors such as VEGF contribute to the remodeling of surrounding tissue, inducing angiogenesis, and reducing fibrosis. Molecules such as TGF-β can transmit the phenotype of senescence to surrounding cells [21].

1.4 Senescence in aging

Ageing is considered a critical risk factor for several human pathologies, including neurodegenerative, cardiovascular and musculoskeletal diseases, metabolic disorders, dementia, osteoporosis, osteoarthritis, idiopathic pulmonary fibrosis, glaucoma and cancer [119] [120] [121] [122] [123]. The ageing process is determined by impairment and deterioration of physiological functions in tissues and organs [124]. Nevertheless, the causes of ageing remain unknown, and our understanding of its mechanisms is also limited. In recent decades, research has identified some molecular aspects related to ageing [124]. Recent studies in animals have shown that while animals do not have a life expectancy as long as humans, aged organisms are characterized by similar features, specifically telomere shortening, mitochondrial dysfunction, genomic instability, epigenetic changes, stem cell exhaustion, immune system reduction, and senescent cell accumulation [125] [126]. These ageing features are generally conserved from yeast to humans and have been classified into three categories: i) primary, i.e., the main causes associated with ageing damage; ii) antagonistic, i.e., the responses to the damage; and iii) integrative, i.e., the consequences in response to the damage [18]. In this context, senescence belongs to the antagonistic class and has been identified by the scientific community as a key factor involved in the complex process of ageing [18]. The relationship between senescence and ageing has been found in two mechanisms: first, the accumulation of senescent cells can affect the functionality of tissues and at the same time, ageing can be caused by this accumulation; on the other side senescence limits the regenerative potential of adult stem cells causing the exhaustion of the regenerative potential of stem cells finally leading to ageing [27]. While the beneficial role of replicative senescence aims to prevent the proliferation of malignant cells, but also to promote tissue repair and regeneration during embryonic development [127], the increased accumulation of senescent cells stimulates innate immune surveillance [112] [111].
During ageing, (***Fig. 6***) the physiological elimination of senescent cells does not function properly anymore. In fact, senescence in aged organisms promotes the decline of the immune system, which is referred to as "immune senescence" [18] and

leads to the formation of chronic proinflammatory niches that result in various pathological manifestations [128].

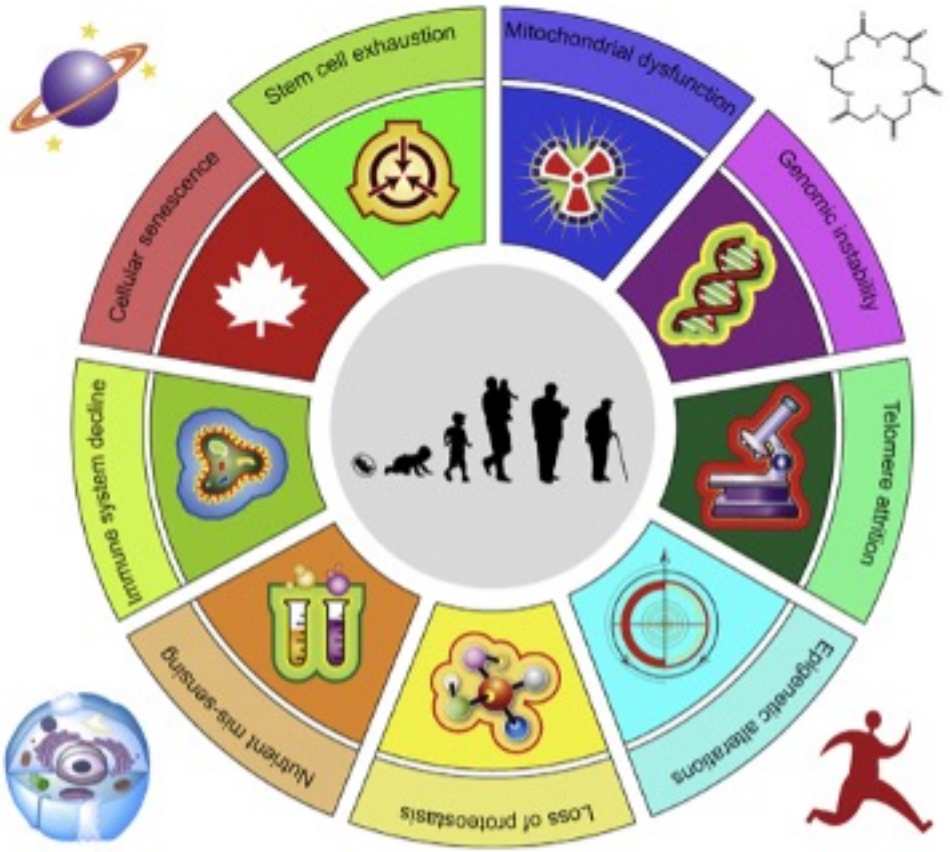


Figure 6. ***Aging is a complex process that contributes to an irreversible decline of the physiological processes of the organism that support its survival and fertility***. It is considered an important risk factor for cancer, cardiovascular and neurodegenerative diseases. Aging process is the progressive loss of physiological integrity and decreased function of tissues and organs. Scientific research has recently focused on the mechanisms of senescence (cellular aging, biological aging) as one of the key factors in a complex aging process [142].

2. DNA damage response

Throughout human life, each cell is subjected by several DNA injuries every day [129]. The injuries can come from exogenous or endogenous sources. UV, ionizing radiation, and a plethora of genotoxic chemicals are among the exogenous stimuli, with UV radiation causing helix-distorting lesions, while genotoxicants inducing interstrand cross-links (ICLs) and DSBs [130]. Endogenous damage, which includes alkylation or hydrolysis of DNA chemical bonds and ROS [131] [132] are responsible for the formation of single-strand breaks (SSBs) [133]. In response to DNA damage, cells activate a complex mechanism known as the DDR. To prevent the spread of aberrant genetic information, the cell can stop the cell cycle to allow the repair enzyme to resolve the lesions, and when they are successfully repaired, the cells restart the cell cycle and resume their physiological functions. ATM and ATR are the major protein kinases recruited and activated by DSBs and ssDNA, respectively [134] targeting CHK1/CHK2 involved in cyclin-dependent kinase (CDK) inhibition.

Inhibition of CDKs causes the cell cycle to arrest at the checkpoint in G1-S, S, and G2-M phases. During this time, DNA repair proteins are transcriptionally or post-transcriptionally induced, recruit repair factors to the damaged site, and modulate their functions through acetylation, phosphorylation, ubiquitination, or SUMOylation [134]. In the presence of irreparable damage, DDR-signalling transits to a chronic state that requires triggering of cellular senescence or cell death by apoptosis [135] [136] [137].The different nature of DNA lesions requires a lesion-specific repair system. In DSBs repair, two mechanisms are activated, namely non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ repair occurs when two DNA fragments with short or no sequence homology are joined and repaired [138]. There is the classical NHEJ pathway that involves protein complexes such as DNA-PKcs, Ku70/Ku80 heterodimers, XRCC-1, DNA ligase 4, instead the alternative pathway is known as alt-NHEJ in which PARP1 and DNA ligase 3 act [139]. It has been observed that NHEJ is more error-prone in senescent cells, promoting genomic instability and cancer [140] [141].

HR, in order to repair the damage, uses the information in the DNA sequence present in the sister chromatid. Moreover, proteins involved in this repair pathway, such as RAD51, RAD52, MRE11 and others are drastically reduced during cell ageing [142] [143]. Three main mechanisms are involved in the response to SSBs: mismatch repair (MMR), nucleotide excision repair (NER) and BER. MMR is involved in the correction of short deletion/insertion loops caused by errors during the replication process [144] and consists of four complexes: MutL (α,β), MutS (α,β), where MutS α/β is involved in the recruitment of MutL α/β, thus forming a tetrameric complex that works as a repair machinery to correct the mismatched lesions [144]. NER removes helix-distorting DNA lesions [145]. It is composed of two sub-pathways: transcription-coupled NER (TC-NER), which involves the interaction between RNA pol II and CSA, CSB and XAB2, and global genome NER (GG-NER), in which the complex of UV-damaged DNA-binding protein (UV-DDB) and xeroderma pigmentosum protein (XPC) scans the entire genome to repair the lesions [145]. Another DNA repair system, activated in response to DNA single strand breaks, is the well-known BER, that will be discussed in more detail in the following paragraph.

2.1 Base excision repair pathway

Exposure to ROS, genotoxic compounds, irradiation, alkylation, and deamination are deleterious stimuli that may cause DNA non distorting lesions [146] [147] and can be corrected by the BER pathway, first described in the paper by Lindhal T. [148]. BER takes place in the nucleus and mitochondria (using different isoforms of mammalian proteins) and proceeds in sequential steps: damage detection with excision of the base, strand incision, end processing, repair synthesis with gap filling, and ligation [149] (***Fig.7***). BER is initiated by a specific DNA glycosylase that recognizes the damaged base and promotes repair [150]. There are two types of glycosylases: Monofunctional and bifunctional. Monofunctional DNA glycosylases (e.g., UNG2, SMUG1, MPG) act by hydrolyzing the N-glycosidic bond, leaving the apurinic/apyrimidinic (AP) site for processing by the enzyme APE1. Bifunctional DNA

glycosylases (e.g., OGG1, NEIL1/2) have mainly AP-lyase activity and are more specific for oxidized bases [151] (***Table 1***).

Table 1. Mammalian DNA glycosylases [164].

Enzyme	Subcellular localization	Mono-/ bifunct.	Substrates and (minor substrates)	Mouse knockout	Human disease[a]
UNG2	Nuclei	M	U, 5-FU in ss and dsDNA, U:A and U:G context (alloxan, 5-hydroxyuracil, isodialuric acid)	Partial defect in CSR, skewed SHM, B-cell lymphomas	Complete defect in CSR, HIGM syndrome, infections, lymphoid hyperplasia
UNG1	Mitochondria	M	Like UNG2	Unknown	Unknown
SMUG1	Nucleus	M	5-hmU, U:G > U:A > ssU, 5-FU, εC in ss and dsDNA	Viable and fertile, SMUG1/UNG/MSH triple k.o. reduced longevity	Unknown
TDG	Nucleus	M	U:G > T:G (5-hmU in dsDNA, 5-FU)	Embryonic lethal, epigenetic role in development	Unknown
MBD4 (MED1)	Nucleus	M	U:G and T:G, 5-hmU in CpG context (εC, 5-FU in dsDNA)	Viable and fertile, C to T transitions, intestinal neoplasia	Mutated in carcinomas with microsatellite instability
MPG (AAG)	Nucleus	M	3meA, 7meG, 3meG, Hx, εA	Viable and fertile, triple knockouts in MPG/ AlkBH2/AlkBH3 hypersensitive to inflammatory bowel disease	Unknown
OGG1	Nucleus	M/B	8-oxoG:C, FapyG:C	Viable and fertile, OGG1/ MUTYH double knockouts cancer prone	OGG1 activity associated with CAG repeat expansion in Huntington's disease
MUTYH	Nucleus	M	A opposite 8-oxoG/C/G	OGG1/MUTYH double knockouts cancer prone	MUTYH variants associated with colon polyposis
NTHL1	Nucleus	B	Tg, FapyG, 5-hC, 5-hU in dsDNA	Viable and fertile, NTHL1/NEIL1 double knockouts cancer prone	Unknown
NEIL1	Nucleus	B	Tg, FapyG, FapyA, 8-oxoG, 5-hU, DHU, Sp and Gh in ss and dsDNA	Viable and normal at birth, obese after 7 months, NTHL1/NEIL1 double knockouts cancer prone	Unknown
NEIL2	Nucleus	B	Similar to NEIL1	Unknown	Unknown
NEIL3	Nucleus	M/B	FapyG, FapyA, Sp and Gh in ssDNA	Viable and fertile, memory and learning deficit	Unknown

[a]Association of germline alterations in DNA glycosylases NTHL1, NEIL1, NEIL2, MPG, TDG, UNG, and SMUG1 with colorectal cancer has been reported, but significance uncertain [Broderick et al. 2006]. bifunct., bifunctional; k.o., knockout.

The process can proceed via a short or a long pathway (***Fig. 7***). The nature of the pathway chosen is poorly understood. One hypothesis is that the switching depends on the ATP concentration near the AP-site or on the nature of the lesion, and on whether the glycosylases are mono- or bifunctional. In short patch repair, the strand incision step involves X-ray repair cross-complemeting 1 (XRCC-1), a scaffold protein that brings the endonuclease Apurinic/Apyrimidinic endodeoxyribonuclease 1 (APE1) to the lesion site [152], which carry out the incision of the 5' strand at the abasic site, generating 3'OH and 5'deoxyribose phosphate ends. DNA polymerase β, which performs the repair synthesis step, cleaves the deoxyribose phosphate moiety and generates the 5'-phosphate end, allowing the DNA ligase III complex to seal the nick and restore the integrity of the DNA structure [153] [154]. Long patch takes place when a gap of 2-10 nucleotides is created and filled [155] [156] [157]. Specifically, DNA glycosylase generates an unsaturated hydroxyaldehyde at the 3'-end and a phosphate at the 5'-end through its β-lyase activity [177]. The 3' end is efficiently processed by APE1 to form the 3' OH end. The enzymes recruited are DNA polymerase beta (POL-β) or DNA polymerase delta (POL-δ), PCNA, Flap structure-specific endonuclease 1 (FEN1) and DNA ligase 1 (LIG1). When cells are in a low-energy state, the enzyme poly (ADP -ribose) polymerase1 (PARP1) performs poly (ADP)-ribosylation of proteins and stimulates the long-patch [158], whereas the short-patch BER is generated by a mechanism involving increased ATP concentration [159]. HMGB1 is a chromatin protein involved in the regulation of DNA chromatin structure and gene expression and also interacts with APE1 and FEN1 [160]. The importance of BER resides in its ability not only to repair DNA damage, but also to respond to DNA-damaging chemotherapies. In cancer therapy BER proteins are considered good therapeutic target [161].

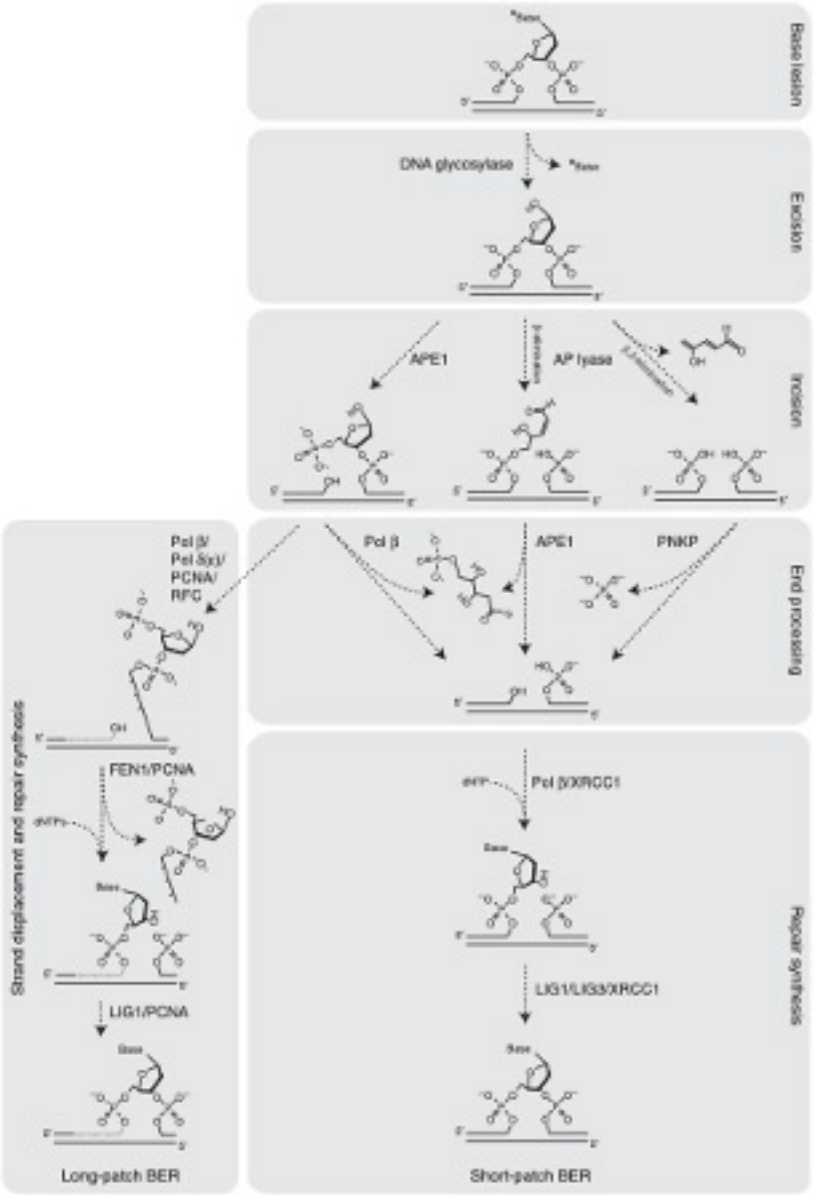


Figure 7. ***Representation of BER pathway***. The short patch and the long patch repair are shown. The repair process consists of several steps: base excision, incision, end processing, and repair synthesis, including gap filling. and ligation [164].

2.2 Human Apurinic/Apyrimidinic endodeoxyribonuclease 1/Redox factor 1 (APE1/Ref1)

Human apurinic/apyrimidinic endodeoxyribonuclease 1/redox factor 1 (APE1/Ref1) was first purified by the laboratories of Linn and Grossman using HeLa cervical cancer cells as a protein that can act at AP sites of DNA substrates [162]. APE1 is encoded by APEX1 gene (approximately 3 Kb) on chromosome 14.q11.2, consisting of five exons [163]. The open reading frame encodes a 318 amino acid protein that is homologous to the exonuclease of *Escherichia coli III* but differs in the N-terminal region [162]. APE1 is a monomeric protein in which the first 33-35N terminal unstructured sequence comprises the intranuclear localization signal and is essential for interaction with nucleic acids and several interacting proteins. Also located within the N-terminal region, the lysine residues 27, 31, 32, 35, some of which are acetylated in cancer cells, are involved in the binding of various DNA and RNA substrates and can simultaneously act on RNA by processing oxidized abasic RNA [164]. Under certain circumstances, the first 33 amino acid residues can be proteolytically removed, resulting in an endogenous truncated form [165] [166]. The redox domain is localized from amino acids 35 to 127 and the c-terminal domain takes place from residue 161 to residue 318 [167] (***Fig. 8***).

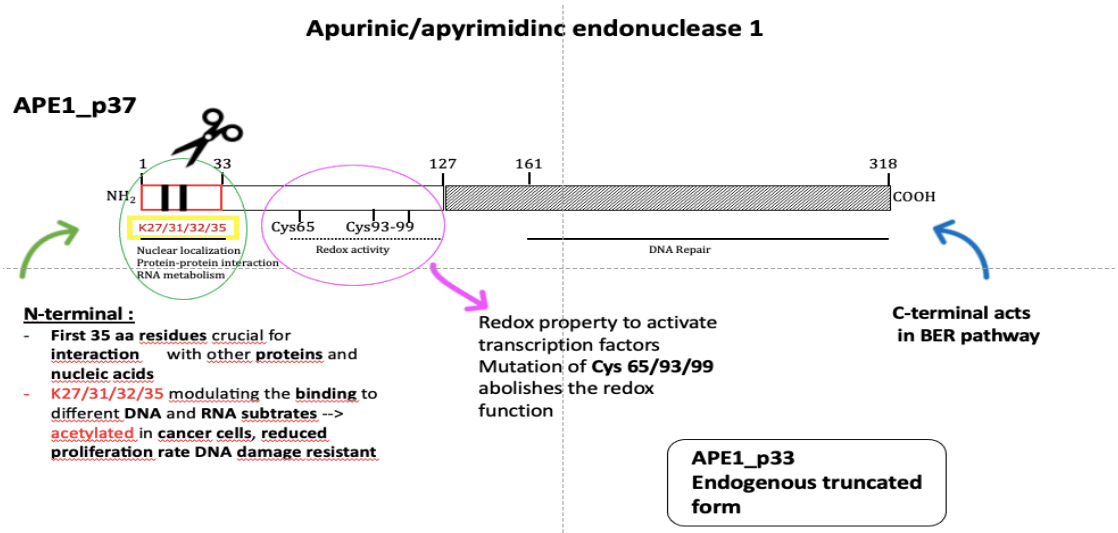


Figure 8. ***Ape1 protein structure***. The first 35 aa residues of the N-terminal sequence are involved in the interaction with other proteins, while important lysins are 27,31,32,35 for DNA/RNA binding activity. The region containing the amino acids 35 and 127 is the redox domain; this activity is associated to Cysteine residues 65,93,99. C-terminal domain has the DNA repair property acting in BER pathway. When APE1 protein is cleaved, is called p33. Adapted from [191].

2.2.1 APE1 and the redox activity trough N-terminal domain

The region that plays a major role in redox regulation is located between 34-127 residues [168]. Structural analysis of this region revealed that it is organized in a β-strand with a spherical core and the Cys 65 residue, which is mainly involved in redox activity, is located in a hydrophobic pocket, [169] making direct interaction with other proteins impossible [170] [171]. This requires a conformational change to expose Cys 65 as a binding site for various transcription factors, as shown by Georgiadis and co-workers [171]. As a redox factor, APE1 is able to make intermolecular disulfide bonds and form a complex with thioredoxin (TRX) through disulfide bonds [172]. The cysteine (Cys) residues: Cys65, Cys93 and Cys99, can change their oxidative state by forming disulfide bonds and switch to the thiol state to exert redox activity [172]. APE1 induces the DNA binding activity of several transcription factors such as: NF-κB, p53, Myb, AP -1 by maintaining the reduced state of Cys residues via the Cys 65 [173] [174] [175]. APE1 has also been described as a redox chaperone, where the reduced state of the transcription factor targeting Cys is due to GSH and thioredoxin [169]. The redox chaperone property of APE1 is thought to be a protective mechanism by which cells can be preserved from genotoxic damage caused by elevated levels of ROS.

2.2.2 APE1 and the repair DNA damage with C-terminal domain

APE1 is mainly involved in base excision repair through its C-terminal domain, which exerts endonuclease activity and processes apurinic/apyrimidinic sites (AP sites), as described in the previous paragraph (**2.2**). In the BER reaction, APE1 hydrolyzes the 5'-phosphodiester backbone at the AP site, resulting in a DNA single-strand break with a 3'-hydroxyl and deoxy-ribose 5'-phosphate end [176]. Moreover, proper rearrangement of 3'-hydroxyl by APE1 can be achieved in an alternative manner: it exerts a 3'-5' exonuclease activity and converts it to a 3'-phospho-a,b-unsaturated aldehyde [177].

Another important role of APE1 is the coordination of the BER steps where it can interact with other repair pathway proteins such as OGG-1, POL-β, XRCC-1, FEN1, ensuring the maintenance of genomic stability [178].

2.2.3 APE1 is considered a pleiotropic protein

In addition to its canonical role, APE1 is involved in transcriptional regulation, either as a cofactor in a transactivator complex or as a redox signaling factor by stimulating the DNA-binding activity of other transcription factors, e.g., Fos, Jun, Pax, and NF-κB [179] [180]. Regarding the transactivating property, APE1 can bind the negative calcium-responsive elements (nCaRE) nCARE-A and nCARE-B, and can exert, for example, negative regulation of parathyroid hormone (PTH) or positive regulation of SIRT1 gene in oxidative stress condition [165] [181]. Another important point observed by Bhakat, A. and coworkers, is that increased concentration of extracellular calcium induces acetylation of APE1 at lysine residues 6 and 7 by p300, which enhances the ability of APE1 to bind nCARE sequences [182]. In recent years, a proteomic analysis performed by our research group has shown that a large part of the APE1 interactome consists of proteins involved in ribosome biogenesis and RNA processing nucleophosmin (e.g., NPM1) [183] [166]. APE1 has been shown to play an important role in RNA quality control and is able to regulate ribosome biogenesis within nucleoli through the interaction with NPM1 through its N-terminal domain [184] [166]. APE1 has been found to cleave damaged oxidized/abasic RNA. Specifically, Lee *et al*. have shown that APE1 is involved in cleavage of the coding region of c-myc mRNA affecting the transcript turnover. For this reason it was defined as a "cleasing" factor [185] [184] [180] (***Fig. 9***).

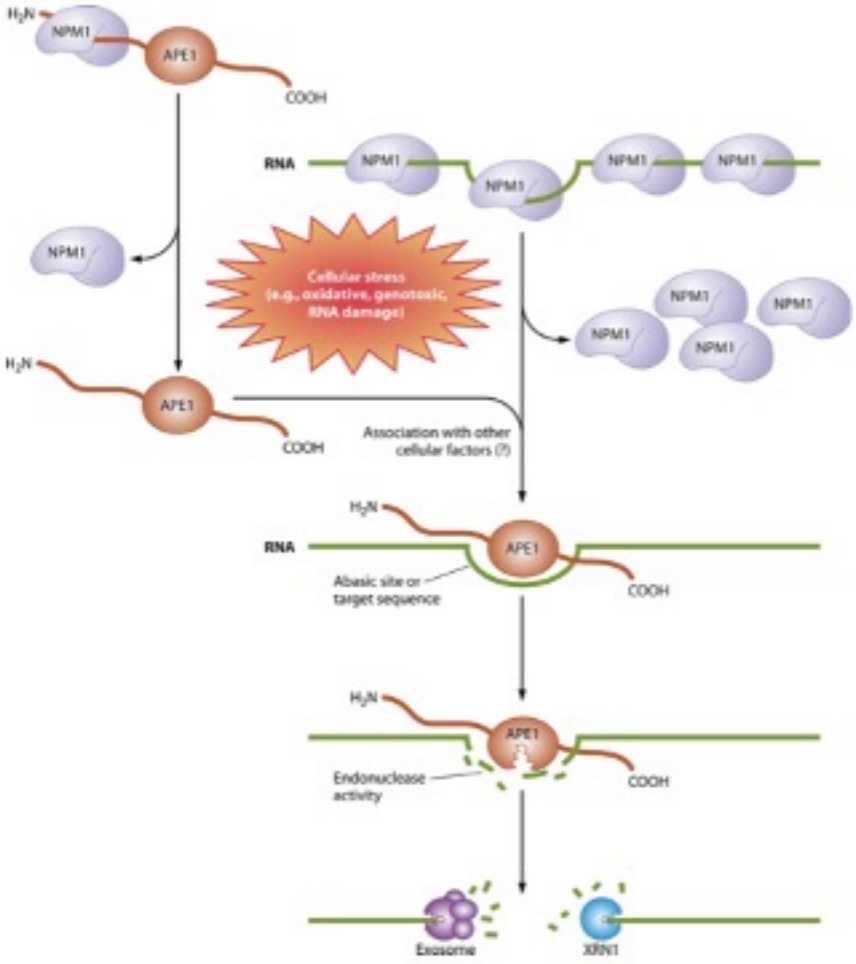


Figure 9. ***Model of APE1 role in RNA metabolism***. Nuclear protein 1 (NPM1) binds the N-terminal domain of APE1 and competes with RNA for binding. After a genotoxic insult, the affinity between NPM1 and APE1 is reduced, allowing APE1 to act in processing RNA [191].

In a recent paper, the involvement of APE in RNA metabolism, particularly in the processing and stability of pri-miRNA, was demonstrated by its association with the Drosha processing complex during genotoxic stress [186]. These studies have shown that APE1 plays a role in regulating miRNA processing. Nanostring analyses performed in HeLa cell lines with APE1 knocked down, revealed an association between decreased levels of mature miRNAs. Subsequently, experiments performed with genotoxic stress (H_2O_2) showed upregulation of some miRNAs, such as miR-221 and miR-222, whose target is the tumour suppressor PTEN, indicating a possible role of APE1 in regulating modified oxidized miRNAs. These results indicate that APE1 plays a role in regulating PTEN expression through the processing of miRNA-221 and 222, not only as a transcriptional regulator, but also at the post-transcriptional level, during genotoxic stress [186].

2.3 BER and cell senescence

There are several interesting studies that address the relationship between ageing and defects in BER. However, the involvement of BER enzymes in cellular senescence has not been well defined, yet. In the present section, we will discuss data from several authors to fix our knowledge of BER enzymes in the context of senescence. The starting point is the observation that oxidative stress has been defined as one of the major causes of senescence, affecting the integrity of DNA structure in nuclear and mitochondrial genes [187]. In this context, the major signaling pathway involved is BER [187]. Since in mammals APE1 (described in more detail in the following paragraph 2.2) accounts for the majority of AP-endonuclease activity, it has been suggested that exposure to oxidative stress leads to rapid accumulation of APE1 in the nucleus [188]. When the subcellular distribution of some BER proteins was analyzed in different tissues of aged mice, different APE1 activities were observed in mitochondria and in the nuclear fraction when comparing, for example, brain and liver tissues, whereas in the cytoplasmic compartment of presenescent cells, OGG1, NTH1, and APE1 activity were decreased of about 50% [189]. The relocalization of BER enzymes was explained as a consequence of the increase in ROS produced by mitochondria during ageing and senescence [187]. Atamna *et al*. found that the number of AP sites was higher under basal conditions in senescent human lung fibroblasts (IMR90 cells) and in human leukocytes compared to young cells [190]. The increased levels of AP sites in old cells could be explained by the large amount of endogenous oxidative damage, leading to increased DNA damage with age. It can be also explained by the lower BER enzymes repair ability in an age-dependent manner [191] [192]. Indeed, when young and senescent cells were pulse-treated with H_2O_2, it was observed that in young cells the AP sites were repaired normally by AP endonuclease and POL-β, whereas senescent cells showed a reduction in repair of AP sites and also loss the 39% of cell viability, reflecting a severe state of oxidative damage [190]. In a paper published in 2009, Heo *et al*. found that reduced APE1 levels were strictly correlated with the development of the senescence phenotype. This experiment was performed with mesenchymal stem cells (MCS) from older donors. These cells exhibited the same characteristics of senescent cells, with particularly

elevated levels of ROS, which lead to apoptotic cell death or senescence through activation of p53 [193]. To protect cells from oxidative damage, APE1 inhibits NAD(P)H oxidase regulated by rec1, thereby reducing the intracellular concentration of ROS [194]. In addition, APE1 prevents the accumulation of oxygen intermediates and plays a role as an anti-senescent agent in an antioxidant system [195]. In a study conducted by Li and co-workers, the altered expression of BER proteins in primary human fibroblasts during senescence was investigated. While APE1 and endonuclease three homolog 1 (NTH1) were downregulated in fibroblasts at late passages, XRCC-1 was increased, and the levels of other proteins such as POL-β, 8-oxoguanine DNA glycosilase 1 (OGG1) were not altered [196]. The observation that deficiency of APE1 can induce DDR-induced senescence suggests that APE1 plays a fundamental role in telomere protection among BER proteins, as it can bind telomeric repeats to facilitate DNA repair [197]. Studies on the enzymatic activity of APE1, whose transcriptional function appears to be subject to age-related changes, revealed that the acetylated form of APE1 decreased in the livers of aged mice compared to young mice, while total levels remained unchanged. Indeed, APE1 was unable to bind nCaRE-B, indicating an age-dependent decrease in APE1 transcriptional function [187].

Intano *et al.* observed decreased levels of APE1 in germ cell extracts from old mice, the activity of which was restored by the addition of purified recombinant APE1 [198] [199]. Reduced POL-β was found in the brains of old mice and rats [200] [201] [202]. It is suggested that the gradual accumulation of DNA damage and the impairment of gene functions involved in the DNA damage response may contribute to the onset of ageing and cancer [135].

3 Role of senescence in lung cancer and in chemoresistance

The World Health Organization ranks lung cancer as the second leading cause of death, with lack of diagnostic tools being the main obstacle to determining treatment. Lung cancer can be divided into the following categories: non-small cell lung cancer (NSCLC), which is the most common (85% of lung cancer cases), and small cell lung cancer (about 15%). Several strategies can be used to counteract the progression of NSCLC: Chemotherapy, radiotherapy, immunotherapy, surgical resection [203] [204]. Several studies have shown that treatment of lung cancer with platinum-containing agents (such as cisplatin or carboplatin) improves overall survival (OS) and slows cancer progression [205]. Since chemotherapy is the most commonly used therapeutic approach, it can promote tumor regression by inducing cell death through apoptosis and cellular senescence, leading to growth arrest (***Fig. 10***). Platinum compounds are able to covalently bind to DNA, resulting in cell cycle arrest, apoptosis, and DNA damage response [206]. While treatment with platinum compounds is the best strategy to combat lung cancer, it can unfortunately lead to relapse in less than twenty-four months in patients undergoing a second cycle of chemotherapy [207]. Studies conducted on lung cancer relapses after treatments with CDDP have shown that therapy-induced senescence is one of the molecular mechanisms underlying such tumors [208]. Indeed, CDDP can induce senescence in several tumor types, as has been observed in melanoma and ovarian cancer [209] [210] and such senescent cells have a more aggressive phenotype [211]. Failure to respond to therapy in many cases is attributed to intrinsic or acquired treatment resistance.

Mechanisms of resistance may include: activation of oncogenic pathways, alternative splicing, dysregulation of a drug's target, ineffective drug delivery, and mutation in such genes, e.g. EGFR (T790M, which occurs in 60% of affected lung cancer patients) [212].

Figure 10. ***How cancer cells respond to chemotherapy***. Most chemotherapeutic compounds induce DNA damage with activation of the DDR. In the case of severe damage, cells are driven into apoptosis. In case of mild damage, cells decide to activate senescence. Secretion of SASP factors may contribute to tumor regression or progression depending on the strength of the stimuli [214].

The role of senescent cells in the response to chemotherapy is more complicated and depends on the nature of the stimulus [93]. Senescent cells generally act by producing SASP factors, but some of them can also secrete small extracellular vesicles called exosomes (which contain proteins and small RNA) and are potentially harmful to cancer progression [213]. Considering that senescent cells have a pleiotropic function, they could be a reservoir for cancer resistance. If the cells are not properly removed (e.g., by apoptosis), the remaining cells continue to secrete proinflammatory cytokines with beneficial or harmful effects [214] . They in fact, can support chemoresistance or can be reprogrammed to re-enter the cell cycle [215] [216] [217], acquiring the characteristics of senescent stem cells, which determine the renewed growth and the development of the tumor [218] [219].

Cancer treatments also have side effects, in particular, the therapy may reduce the effectiveness of the intervention in the short term. At the same time, excessive toxicity of the treatment promotes cancer recurrence and the appearance of a secondary tumor [238]. After chemotherapy, a chronic pro-inflammatory state occurs, causing an overexpression of several cytokines such as IL-6, IL-8, chemokines CCL2 and

CXCL12 and many others [220]. Patients show cardiovascular morbidity, fatigue and decline in physical function [221] [222] [223] (***Fig.11***). The link between senescent cells/SASP and cancer therapy needs more insights, especially related to the involvement of senescent cells in modulating short- and long-term side effects in various cancers.

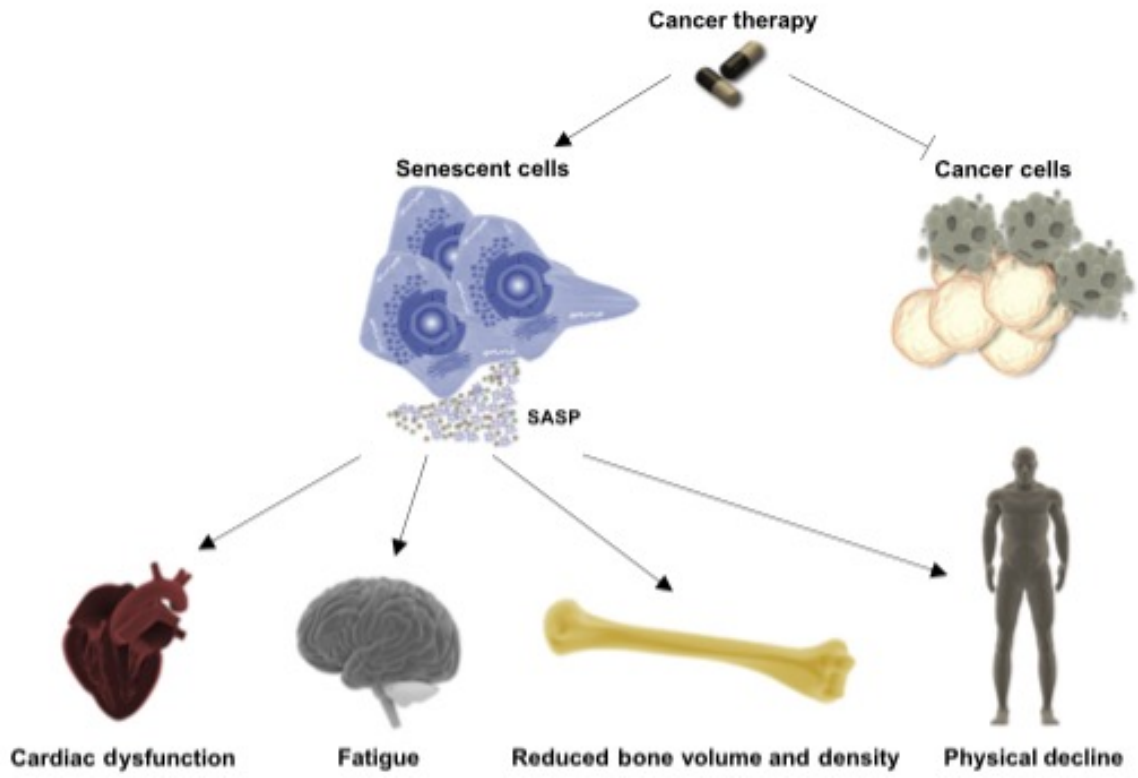


Figure 11. ***Cancer therapy is an effective method against cancer progression, but it can also induce cellular senescence***. In this context, SASP factors are responsible for external side effects, such as cardiac dysfunction, bone loss, impairment of physiological functions and consequently physical decline. Adapted from [242]

3.1 MicroRNAs as potential cancer biomarkers and role in cell senescence

Recent advances in molecular medicine have identified miRNAs as valid biomarkers implicated in several human oncological and non-oncological diseases [224]. Lee *et al.* demonstrated in 1993 that a short RNA sequence of about 22 nucleotides called *lin-4*, identified in *C. elegans*, is able to bind the 3' untranslated region (UTR) of *lin-14* mRNA and regulate its translation through RNA-RNA interaction, [225]. miRNAs are small single-stranded non-coding RNAs (approximately 18-24 nucleotides in length). miRNA genes may be organized in clusters, localized in the intronic regions or transcribed [226]. The function of miRNAs is to regulate gene expression at the post-transcriptional level, leading to the degradation or the arrest of translation of the target mRNAs. miRNA genes are transcribed into long primary transcripts of 100-200 nucleotides in length (pri-miRNAs) by RNA polymerase II and III in the nucleus. pri-miRNAs have 5' capping and are polyadenylated at the 3' end, with a characteristic structure of stem (33 base pairs) and loop [227]. Pri-miRNAs are processed to generate mature miRNAs. In fact, pri-miRNAs are cleaved into pre-miRNAs (70 nt, with hairpin structure) by Drosha, an RNase III-type protein [228]. Drosha, in combination with the cofactor DGCR8 (DiGeorge syndrome critical region gene 8), forms the microprocessor complex (650 kDa) [229] [230]. Specifically, DGCR8 binds the pri-miRNAs and facilitates the cleavage of the substrates mediated by Drosha [231] [232]. In this step, the pri-miRNAs are processed into pre-miRNAs, which are considered precursors of mature miRNAs. After their production, the pre-miRNAs are exported from the nucleus to the cytoplasm by Exportin 5 and its cofactor Ran-GTP [232], where the maturation is completed. The RNAse specific for double-stranded RNA (ds-RNA) III, called Dicer, acts on pre-miRNAs to release miRNA duplexes of 22 nt [233], which form a complex in conjunction with Argonaute (Ago) proteins to generate the RNA-induced silencing complex (RISC). In this complex, one strand of the RNA duplex remains bound to Ago as mature miRNA (termed the guide strand), while the other strand (termed the passenger strand) is degraded [232]. The mature miRNA drives RISC to recognize and target mRNAs, especially miRNAs present at the 5' end the seed sequence, from position 2 to 8, which matches the 3' UTR of the

mRNAs. RISC determines the degradation of mRNA when miRNA and mRNA target are perfectly matched, otherwise it promotes the repression of mRNA translation. This process leads to an overall decrease in protein levels by mRNA targets [234] (***Fig. 12***). miRNAs regulate cancer processes, in particular they are involved in tumorigenesis, from early stages to metastasis and chemosensitivity [226] [235]. Researches have investigated the differences in the levels of circulating miRNA in healthy and diseased patients, and the observed changes provided evidence for different pathologies. Indeed, these differences lead to the consideration that miRNAs have diagnostic and prognostic value (Biomarker Working Group).

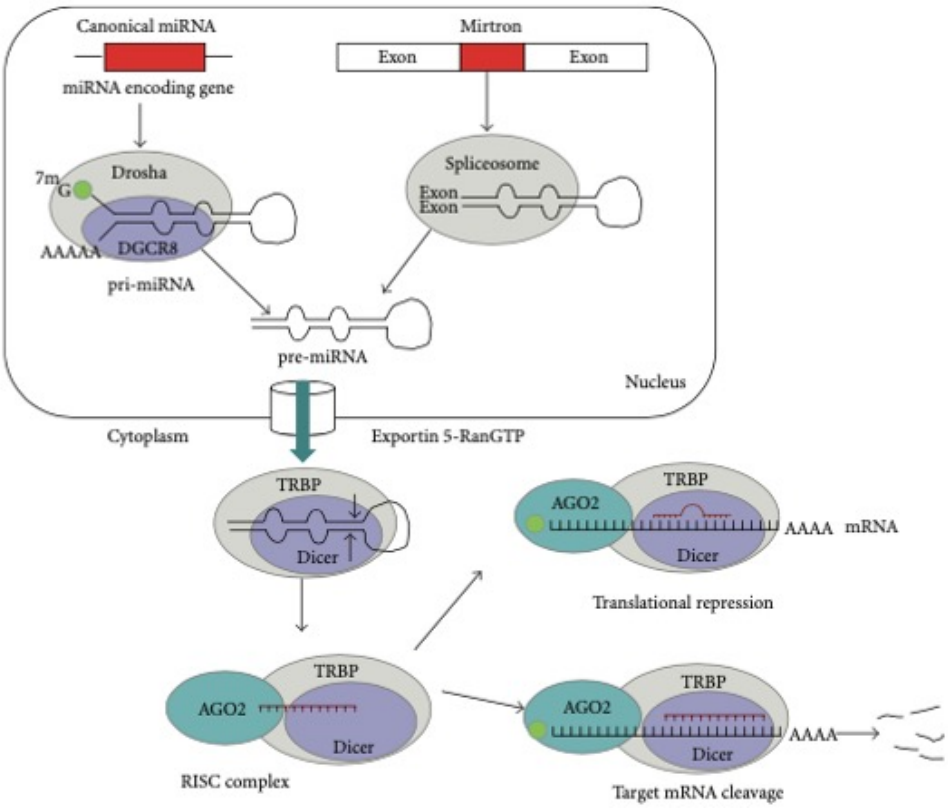


Figure 12. ***miRNA biogenesis begins with the endoribonuclease Drosha, which processes pri-miRNAs into pre-miRNAs.*** Exportin-5 transports pre-miRNAs from the nucleus to the cytoplasm. Pre-miRNAs are converted into mature miRNAs by Dicer and incorporated into the RISC complex. Once in the RISC complex, miRNAs can inhibit translation or promote degradation of the mRNA target [234].

3.1.1 MicroRNAs in senescence

Since senescence is considered a new approach for cancer therapy, the analysis of miRNAs in senescence has attracted great interest [4]. Targeting miRNAs may provide a protective mechanism against the deleterious effects of SASPs, because miRNAs activated during senescence, in response to stress signals, are critical for modulating the tumor suppressor pathway. It is known that the tumor suppressor protein p53 and the retinoblastoma (Rb)-associated protein (RB1) are able to induce cellular senescence when cells are hit by different stressors [236]. p53 regulates the cell cycle at G1/S and G2/M checkpoints and activates the transcription of several genes involved in cell cycle surveillance, such as $p21^{CIP1}$. Moreover, miRNAs regulating p53 are crucial for the control of senescence suppression. Indeed, p53 is bound and repressed by miR-504, miR-125, miR-25 and miR-30d [237] [238]. The expression of miR-34a can be stimulated by p53, in an autoregulatory loop, this miRNA targets and represses SIRT1, inducing senescence in endothelial progenitor cells [239]. $p21^{CIP1}$ takes part of the DDR proteins together with p53, p16 and γ-H2AX, and it has also been identified as an important marker of senescence [240] [241]. Suppression of $p21^{CIP1}$ expression by miRNAs affects cell senescence and consequently cell proliferation. In a study conducted by Borgdoff *et al.,* miRNAs screening showed that some of them, including miR-130b, miR-302a-d, miR-512-3p and miR-515-3p, are able to inhibit the expression of $p21^{CIP1}$ and thus prevent RAS (G12V)-induced senescence in human mammary epithelial cells [242]. On the contrary, there are other miRNAs that enhance the expression of $p21^{CIP1}$, such as miR-159, whose level is increased in senescent cells and contributes to cell cycle arrest and growth arrest [243]. miRNAs may influence senescence through interaction with the Rb/p16 pathway. Indeed, decreased expression of miR-24 correlates with increased p16 levels, as observed in senescence associated with osteoarthritis [244]. p16 expression can also be suppressed by other miRNAs: miR-300, miR-514, miR-663 and miR-141 [245], [246].

In human lung cancer cell experiments, miR-449a was reported to regulate Rb by targeting E2F3, a key regulator of G1/S cell cycle transition [247] [248].

The proinflammatory environment created by the secretion of interleukins, chemokines, growth factors and proteases promote tumor progression in surrounding tissues [249] [114]. The main inflammatory mediators IL-6 and IL-8 are negatively regulated by miR-146a/b, reducing the activity of IRAK1 and NF-κB to decrease the status of inflammatory cells [250] [251] (***Fig.13***). The properties attributed to the functions of miRNAs may be useful to develop new therapies in the context of their role in carcinogenesis, chemosensitivity and cellular senescence.

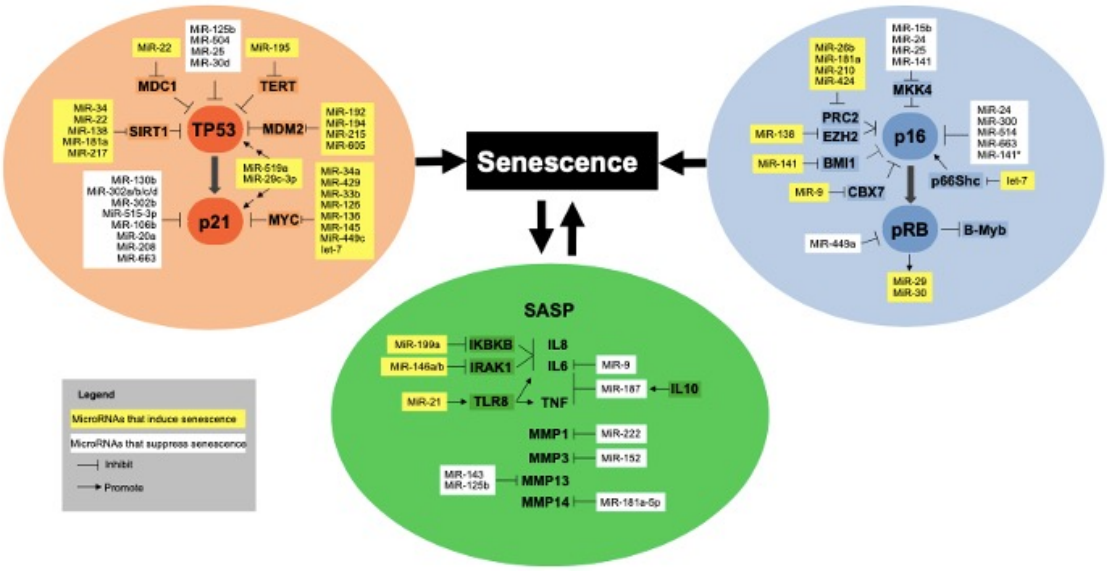


Figure 13. ***Three major pathways involved in cellular senescence***. These are regulated by different miRNA: p53/p21, p16/RB and SASP. miRNAs can promote or inhibit senescence by modulating their gene targets [271].

3.2 APE1 and miRNAs in lung cancer: where are we?

Several studies have shown that APE1 is involved in regulating the expression of several genes related to chemoresistance [176]. The overexpression of APE1 observed in several human tumors, including NSCLC, has strengthened the hypothesis that APE1 may have prognostic and/or predictive significance in cancer [252]. Wang and coworkers conducted an interesting study in NSCLC patients in which they analyzed APE1 expression levels in relation to tumor response to CDDP-based adjuvant therapy. The results show that a decrease in APE1 levels improves the sensitivity of NSCLC cells to CDDP. Therefore, determining APE1 expression in NSCLC cells prior to chemotherapy may help predict the effect of adjuvant chemotherapy [253]. Indeed, APE1 is considered a predictive marker of sensitivity to chemotherapy [254]. Recently, a miRnome analysis was performed by our research group, in a context of APE1 depletion in the A549 cell line a cellular model for NSCLC. The APE1 depletion was carried out with the use of specific siRNA against APE1. Thanks to this analysis it was possible to obtain a list of about 61 miRNAs that are differentially expressed (DE-miRNAs) respect to the A549 control cells, silenced with a control scramble siRNA. Some of the obtained DE-miRNAs have prognostic value in NSCLC and were found also dysregulated in HeLa cells upon APE1-depletion (Manuscript under preparation). Specifically, thirteen DE-miRNAs were selected as candidate miRNAs, based on their prognostic value and chemoresistance after cisplatin treatment (***Table 2***).

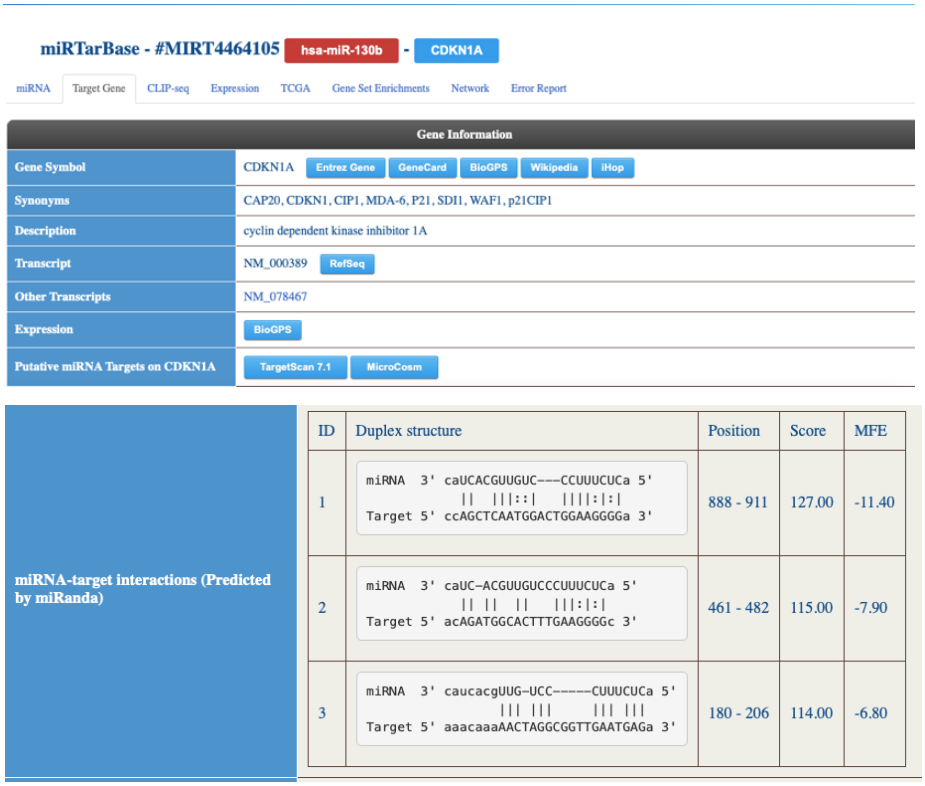
Table 2: Analysis miRNAs profiled with the Nanostring and RNA-seq technology carried out in A549 cell line

	Nanostring			*RNA-seq*	
miRNA	**Dysregulation**	**LogFC**	**q-value**	**LogFC**	**q-value**
miR-4488	Up	2.00	5.37E-03	2.33	NA
miR-1246	Up	1.41	8.47E-05	-0.07	NA
miR-24-3p	Down	-1.01	1.14E-07	NA	NA
miR-183-5p	Down	-1.12	2.25E-07	-1.20	6.94E-23
miR-660-5p	Down	-1.12	7.33E-04	-0.38	5.80E-01
miR-130b-3p	Down	-1.21	4.71E-02	-1.08	1.06E-02
miR-543	Down	-1.24	1.38E-04	-1.43	1.18E-02
miR-200c-3p	Down	-1.55	2.94E-03	-0.27	NA
miR-376c-3p	Down	-1.58	3.69E-08	-0.60	2.57E-01
miR-218-5p	Down	-1.64	7.55E-12	NA	NA
miR-146a-5p	Down	-2.00	1.82E-11	NA	NA
miR-92b-3p	Down	-24.08	4.72E-03	NA	NA
miR-33a-5p	Down	-24.92	1.53E-02	0.22	8.06E-01

In the present work, we elaborated the study of miR-130b and miR-146a in the context of CDDP-induced senescence.

3.2.1 Main functions of miR-130b

Mir-130b (***Fig. 14***) belongs to a family of miRNA that is deregulated in various cancers and functions as an onco-mir or onco-suppressive mir, promoting cell migration and invasion, as observed in bladder cancer, glioma, and esophageal cancer [255] [256]. In the context of DNA damage-induced senescence, overexpression of miR-130b leads to decreased cell viability, triggers apoptosis, and arrests the cell cycle in an irreversible manner. As we know, during cellular senescence there is an accumulation of DNA damage [257], the overcoming of which by miR-130b in turn stimulates the expression of genes involved in DDR and DNA repair [258]. Another function associated with high levels of miR-130b in colorectal cancer is related to epithelial-mesenchymal transition (EMT) and angiogenesis [259]. miR-130b is also involved in the induction of chemoresistance in breast and lung cancer through the activation of phosphoinositide 3-kinase (PI3K)-AKT signalling and Wnt/β-catenin [260]. Data from the literature have shown that miR-130b correlates with poor overall survival in patients with NSCLC [261], while in prostate cancer (PCa) it has a tumour suppressive function affecting cell viability, cell death and invasion. Since CDKN1A and CDKN1B are two validated target genes of miR-130b [262] [263], it stands to reason that miR-130b may play a potential role in modulating senescence cellular process [264].



miRTarBase - #MIRT4464105 hsa-miR-130b - CDKN1A

miRNA | Target Gene | CLIP-seq | Expression | TCGA | Gene Set Enrichments | Network | Error Report

Gene Information

Gene Symbol	CDKN1A Entrez Gene GeneCard BioGPS Wikipedia iHop
Synonyms	CAP20, CDKN1, CIP1, MDA-6, P21, SDI1, WAF1, p21CIP1
Description	cyclin dependent kinase inhibitor 1A
Transcript	NM_000389 RefSeq
Other Transcripts	NM_078467
Expression	BioGPS
Putative miRNA Targets on CDKN1A	TargetScan 7.1 MicroCosm

miRNA-target interactions (Predicted by miRanda)

ID	Duplex structure	Position	Score	MFE
1	miRNA 3' caUCACGUUGUC---CCUUUCUCa 5' Target 5' ccAGCTCAATGGACTGGAAGGGGa 3'	888 - 911	127.00	-11.40
2	miRNA 3' caUC-ACGUUGUCCCUUUCUCa 5' Target 5' acAGATGGCACTTTGAAGGGGc 3'	461 - 482	115.00	-7.90
3	miRNA 3' caucacgUUG-UCC-----CUUUCUCa 5' Target 5' aaacaaaAACTAGGCGGTTGAATGAGa 3'	180 - 206	114.00	-6.80

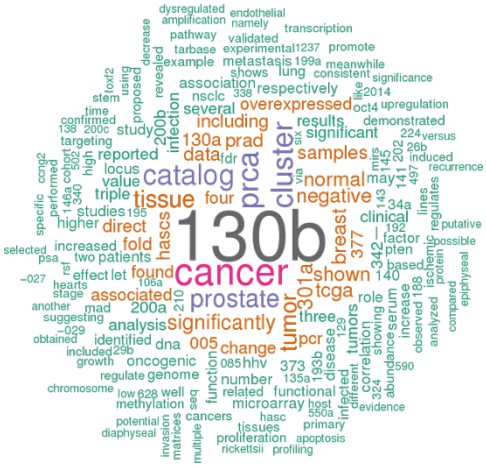


Figure 14. ***miRTarBase for has-miR-130b-3p and target gene CDKN1A***. miRNA-target interaction prediction by miRanda tool assessing CDKN1A as target gene of miR-130b-3p.

3.2.2 Main functions of miR-146a

miR-146a was identified by David Baltimore and his collaborators on chromosome 5 [265] [266]. Its main function is to promote cell death and limit cell proliferation and migration in NSCLC [267]. In several NSCLC cell lines, miR-146a expression is very low, especially in advanced stages of lung cancer with lower progression-free survival [268]. In a cellular model in which senescence was induced, miR146a was observed to increase starting from undetectable levels in proliferating and quiescent cells [269] [270]. The main role of mir-146a/b is to negatively regulate the inflammatory process. Specifically, in the immune system, high levels of miR-146a regulate IRAK1 and TRAF6 via a negative feedback loop [271]. Indeed, these proteins are part of the signaling pathway IL-1- Toll like receptor in which NF-κB is activated [23] [266]. In the context of senescence, miR-146a/b has been identified as a negative regulator of inflammation, i.e., it prevents the excessive release of SASP factors such as IL-6 and IL-8 [250]. Overexpression of miR-146a in human fibroblasts (HCA2) has been associated with a dramatic reduction in the levels of IRAK1 and IL-6 and IL-8 in both proliferating and senescent cells [250]. This reduction is mainly observed at the transcriptional level, as a consequence of NF-κB inactivation due to the action of miR-146a on its target gene IRAK1 [23].

Investigating the role of miRNAs in a model of induced cellular senescence and understanding how APE1 might contribute to this cellular process by modulating miRNAs targets (***Table 3***) could be an interesting area of research, as it has high potential as diagnostic biomarkers that may be useful as therapeutic agents in the treatment of various pathologies related to ageing.

Table 3. miRNAs involved in senescence in A549 cell line

Upregulated	Downregulated	Pathway	Targets	Cancer Type	References
hsa-miR-210		p16/RB	EED, EZH2, suz12	Venous metastasis of hepatocellular carcinoma (HCC)	Ji et al., (2018) Borgdorff et al., (2010) Bhaumik et al., (2009) Wang Y et al., (2011) Gysler et al., (2016)
	hsa-miR-130b	p53/p21	p21	Prostate cancer (PCa), Xeroderma Pigmentosum (XP), NSCLC	Ramalho-Carvalho et al., (2017) Val Cervo, et al., (2011) Hirono et al., (2019) Han et al., (2020) Ye et al., (2017)
	hsa-miR-146a/b	SASP	IRAK1, TLR8	Anaplastic thyroid cancer cells, NSCLC	Philipot et al. (2014) Giglio, et al (2013) Marasa et al. (2009) Liu et al., (2020) Huang et al, (2017)
	hsa-miR-183	SASP	ITGB1	Colorectal cancer (CRC)	Davis et al. (2017) Wang et al. (2019) Li et al. (2010
	hsa-miR-200c	p53/p21	ZEB1, BMI1	Gastric Cancer (GC)	Magenta et al. (2011) Yu et al., (2021)
	hsa-miR-24	p16/RB	MKK4	Hepatocellular Carcinoma (HCC)	Bu et al. (2016), Chen et al., (2016)

4 Targeting senescent cells as a promising therapeutic approach

Recently, selective elimination of senescent cells has attracted considerable interest and represents a promising therapeutic approach in the field of age-related diseases. In the article published by Baker *et al.* in 2011, a transgenic mouse model INK-ATTAC was used for inducible elimination of p16INK4A in positive senescent cells following drug treatments, which delayed the onset of age-related phenotypes [272]. The strategies developed to target senescent cells can be applied at different levels, i.e.: i) inhibition of pro-survival signaling pathways with senolytics in single or in combination, ii) activation of the immune system, iii) use of fluorescent probes *in vivo* and *in vitro*, and the use of nanoparticles containing chemotherapeutic substances for the administration of medicinal products used both for diagnostic purposes and for the development of therapeutic agents.

The most widely used pro-survival pathway is represented by the BCL-2 family, whose inhibitor Navitoclax (ABT -263) induces apoptosis of cells undergoing senescence and also promotes reduction of the expression of SASPs, including CDKN2A, TNFα and Ccl5 [273]. Senolytic activity was achieved with a combination of two agents: Desatinib, an inhibitor of tyrosine kinases such as SRC, c- KIT and Quercetin which acts on kinases and receptors, selectively targeting senescent cells [274] [275] (***Fig. 15***).

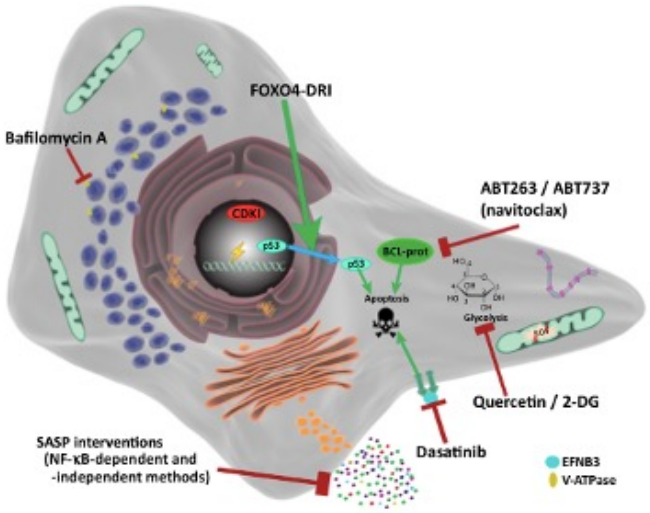


Figure 15. ***Representation of the senolytic compounds and their action in senescent cells***. Senolytics target senescent cells to induce cell death. One mechanism of action provides the targeting the Bcl-2 antiapoptotic family of proteins (e.g., ABT263 and ABT737). The second strategy exclude p53 from the nucleus by using Quercetin or Dasatinib to induce apoptosis [70].

combination of these two drugs used in an aged mouse model improved lifespan and physical dysfunction [276]. Quercetin (QC) (3,3',4',5,7-pentahydroxyflavone) is a polyphenolic flavonoid found in plants and foods that possesses anticancer and antiproliferative properties [277]. Quercetin treatment of A549 cell line was shown to affect cell growth by inhibiting cell survival pathways such as PI3K/Akt/NF-κB [278]. Quercetin has been shown to arrest the cell cycle in G0/G1 phase in leukemia, in S phase in colorectal carcinoma and in G2/M phase in adenocarcinoma of the breast and esophagus [279] [280]. In a paper published by Klimaszewska-Wisniewska *et al.* it was reported that quercetin induces apoptotic cell death in A549 cells at three different concentrations (10, 30 and 60 μM) during 24 hours of treatment. The occurrence of apoptosis was examined by image cytometry, following annexin V/propidium iodide double staining and by real-time PCR measurements, revealing downregulation of BCL2 and upregulation of BAX mRNA expression levels [281].

Regarding the role of the immune system, the elimination of senescent cells mediated by CD4(+) T cells and monocytes/macrophages is driven by a process called senescence surveillance [282]. The immunogenicity of senescent cells makes these cells more interesting for developing clinical strategies involving their manipulation [127]. Since senescent cells are characterized by increased levels of lysosomal β-Galactosidase, in recent years several fluorescent probes have been developed to monitor β-galactosidase activity both *in vivo* and *in vitro* (***Fig.16***).

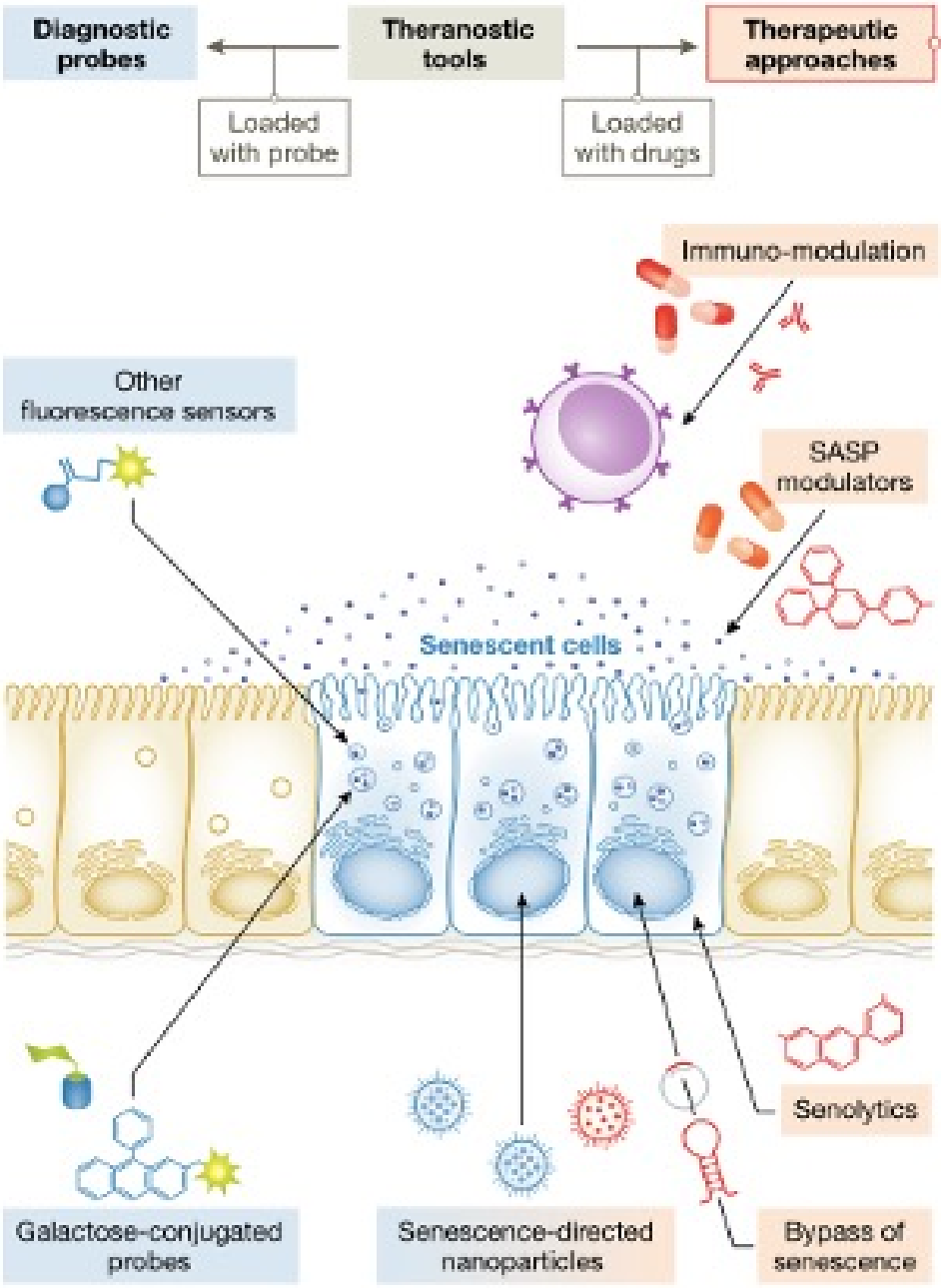


Figure 16. ***Cellular senescence is associated with numerous human diseases and thus offers potential targets for therapeutic and diagnostic interventions, including galactose-conjugated and fluorescent probes for the detection of senescent cells.*** This provides an important opportunity for longitudinal monitoring of senescence in clinical trials. The development of pharmacologically active compounds called senolytics inhibits survival pathways in senescent cells and lead to programmed cell death. The use of senolytics as therapeutic strategy is enhanced by the immunomodulators, that promote the natural elimination of senescent cells. Nanoparticles (NPs), which can encapsulate cytotoxic agents, tracers and/or small molecules, can be used both therapeutically (drug delivery) and diagnostically. Adapted from [141].

In particular, for *in vivo* experiments, it has been developed the AGHa fluorescent probe, an OFF-ON two-photon probe, that can be hydrolyzed by β-Galactosidase activity, which shifts a fluorescent signal in tumors of mice treated with palbociclib compared to untreated tumors used as controls [283]. Gal-Pro is another valid fluorescent probe, generally used for *in vitro* testing.

It consists of hemocyanin conjugated to a D-Galactose residue, emits in the near infrared, and shows low background fluorescence and high photostability [284].

Recent advances in the field of nanomedicine have led to the development of a novel drug delivery system based on functionalized nanoparticles (NP) of mesoporous silica, containing a specific chargo ready for delivery [285]. The nanoparticles can be coated with galacto-oligosaccharides (Gos) that encapsulate rhodamine and penetrate by endocytosis into senescent cells where they deliver their contents. This system was improved by using 6-mer galacto-oligosaccharide (Gal) on the surface of the NPs and tested in a model of chemotherapy-induced senescence. To enable drug delivery, GalNPs have been loaded with chemotherapeutic agents such as doxorubicin, palbociclib or navitoclax, which are used as senolytic agents and are able to kill senescent cells, offering valid therapeutic potential [286]. The most interesting type of nanoparticles are the periodic mesoporous organisilicas (PMOs), which were first described by Inagaki and colleagues [287]. PMOs are composed of an organic-inorganic hybrid scaffold, and some properties make PMO materials unique, such as porous channels with adjustable size, the ability to functionalize the inner and outer surfaces of the wells, and low citotoxicity, which allows these NPs to be biocompatible [288]. From a biomedical perspective, NPs are mainly used for drug and protein delivery. Chengzhoung Yu and colleagues were able to perform protein delivery in cancer cells using phenylene-bridged PMO NPs whose pores were coated with a RNase A [289]. Another application of NPs is in combination with high-intensity focused ultrasound (HIFU). This system generates energy and consequently increased temperature in damaged tissue to cause destruction [289].

AIMS

Aims of the study

Cells that are hit by a stress signal may activate a protective mechanism in which the cells stop the cell cycle to allow the DNA repair system to repair the damage. However, if the repair is not efficient the cells go into apoptosis to prevent the damage from spreading. Otherwise, the second option is to irreversibly arrest the cell cycle by acting on the $p21^{CIP1/}$p53 or $p16^{INK4a}$/Rb axis and entering the state of cellular senescence. Consequently, the generation of a proinflammatory state by the secretion of SASP factors determines the propagation of the senescence phenotype, generally in response to DNA-damaging chemotherapy. Cellular senescence plays a role not only in tumor suppression, but also in tumor progression and chemoresistance. BER enzyme, are involved in chemoresistance and DNA damage response mechanism, and the intermediates of BER pathway, especially the AP site and SSB, are toxic in the cell.

Based on the observation that there are relatively few studies in the literature on the contribution of the BER pathway of DNA lesions to extrinsic senescence, we developed a model of cell senescence induced by two different DNA-damaging agents: CDDP and DOXO, in the A549 cell line. Then, we analyzed the expression of BER enzymes during cell senescence induced by CDDP and DOXO focusing on the role of APE1 through specific miRNAs involved in cell senescence phenotype.

In the end, we tested the potential role of APE1 specific inhibitors as novel senolytic compounds. Additional preliminary studies on the development of modified nanoparticles to analyse cell senescence are also reported.

RESULTS

In order to induce extrinsic cell senescence in A549 cells we used two genotoxicants currently used in chemotherapy: i.e., CDDP and DOXO. These compounds are used as chemotherapeutic agents [290] and lead to various cellular final outcomes such as apoptosis, autophagy or at least senescence [291] [292].

1 Induction of a senescence phenotype in A549 cells by CDDP-treatment

In the present work, we first induced senescence phenotype in A549 lung cancer cell line by using CDDP as DNA damaging compound. CDDP covalently binds DNA, producing adducts in the form of inter-strand and intra-strand DNA crosslinks and DNA-protein crosslinks [293].

First, we determined the concentration, time and dose of treatment that can induce the activation of the senescence marker $p21^{CIP1}$ in A549 cells. Based on a previous analysis of cell viability (***Fig. 17A***), we set the maximum dose at 6.25 μM and the minimum dose at one tenth of the highest. The concentration of 6.25 μM was chosen given the sublethal effects of these treatments, which cause loss of 50% of cell viability, as in our recent work [294]. Hence, we treated cells with three different concentrations of CDDP (0.625 - 3.0 - 6.25 μM) only for twenty-four hours and the cell morphology upon release was monitored for several days. Upon five days from drug release, with the dose of 6.25 μM, using bright field Leica DMi1 microscopy, we observed changes in the cellular phenotype, in cells treated with CDDP, that resulted enlarged and flattened, compared to proliferating cells treated with *N*,*N*-dimetilformammide (DMF), as a control (***Fig. 17 B-C***). The accumulation of $p21^{CIP1}$, was measured by Western blot analysis (***Fig. 17 D-E***) and it was detectable after five and nine days of treatment with 6.25 μM CDDP.

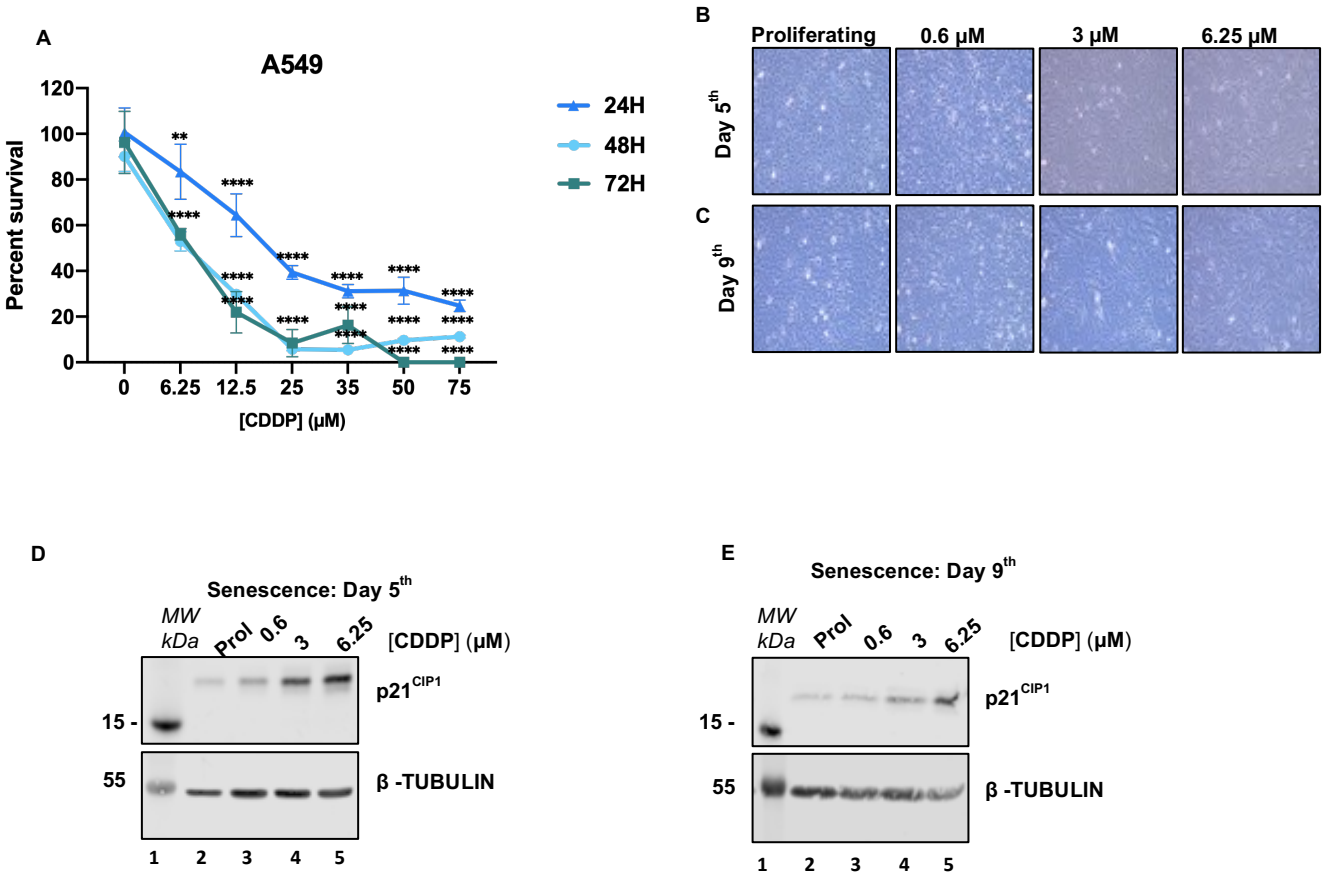


Figure 17. ***Phenotypic changes in A549 cell line induced senescence with CDDP.*** **(A)** Analysis of cell viability in A549 cell line after CDDP treatment. MTS assay was performed using the indicated concentrations of CDDP at 24, 48, 72 hours. The viability of untreated cells at the indicated time points was evaluated as a control. Asterisks represents the means ± SD of three independent experiments. Asterisks indicate statistical significance of the data. **P < 0.001, **** P < 0.0001, Student's t-test. Images of Proliferating (Prol) and senescent cells were acquired in bright field at 5 **(B)** and 9 **(C)** days after drug release using Leica DMi1 microscope. Cells treated with CDDP 6.25 µM became enlarged, flat and irregular. Cell cycle arrest was assessed using $p21^{CIP1}$ as a marker of senescence, its level increases during the senescence period, as shown in **(D)** and **(E)**.

Based on these preliminary results, we decided to use this cellular senescence model obtained by using CDDP treatment for twenty-four hours at a final concentration of 6.25 µM. Upon treatment with DMF and CDDP, the cells were washed extensively, and complete medium was added to release them at following time points: twenty-four hours, five and eight days after drug release. Cells treated with DMF were used as proliferating control. Then, the senescent phenotype was assessed at each time

points, by using the β-Galactosidase assay [8]. ***Fig. 18A*** shows representative images of β-Galactosidase-positive cells (in shades of blue) compared with corresponding proliferating cells, as controls. Quantification of the percentage of β-Galactosidase-positive cells for each condition is shown in the graph on the right. The data obtained clearly show that after five days of release from CDDP-treatment, the morphological changes were significant and about 80-85% of the cells were positive for SA-β-Gal staining compared to the proliferating cells. These results suggest that the treatment with CDDP at the chosen dose promotes the development of a senescent phenotype in A549 cells, as expected.

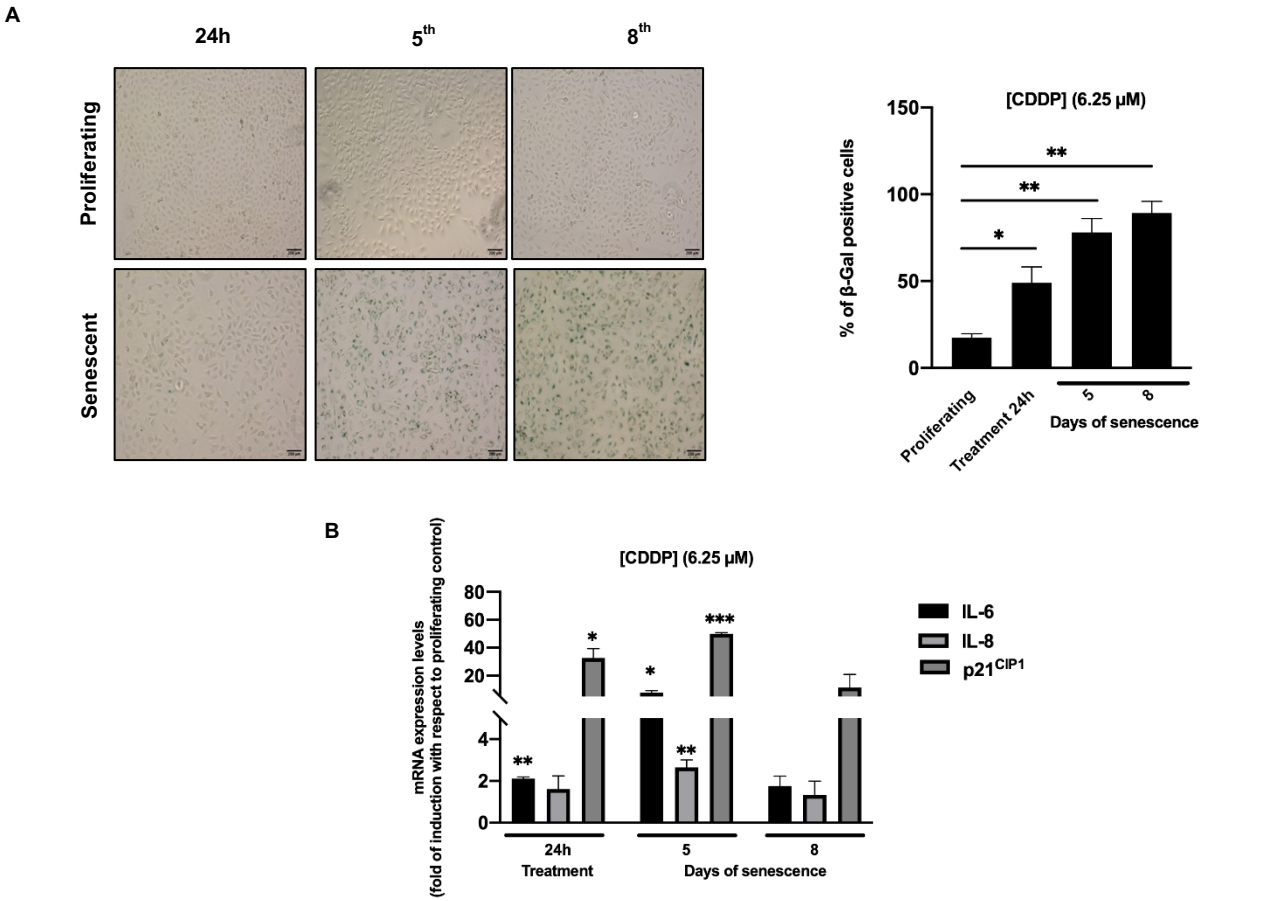


Figure 18. ***CDDP treatments induce the senescent phenotype in A549 cells.*** **(A)** Cells were photographed with a microscope at 20× magnification. Proliferating and senescent cells were plated at a density of 0.2 x 10^6 cells per 6-well and treated once using DMF in the case of proliferating cells and CDDP in the case of senescent cells, with a dose of 6.25 μM for 24 hours. Then were washed extensively, replated in a drug-free medium, and stained to detect SA- β-gal activity. The percentage of cells positive for SA-β-gal was determined by counting seven random fields under bright field, Leica DMi1 microscope. At least 200 cells were counted for each independent determination. The analysis was performed twenty-four hours (24h treatment), five and eight days after drug release. The average of diameter for proliferant cells was around 50-70 μm, for senescent cells around 80-120 μm. Representative scale bars of 200 μm. The histogram on the right shows the percentage of β-gal-positive cells. Asterisks indicated the means ± SD of three independent experiments. *P < 0.05, **P < 0.001, Student's t-test. **(B)** $p21^{Cip1}$ IL -6 and IL -8 mRNA expression levels analyzed by real-time reverse transcription-PCR. Histogram shows an upregulation of senescent markers after 5 days of release. Expression of $p21^{Cip1}$ also mediates G2/M cell cycle arrest. Data are expressed as fold of induction respect to proliferating control and normalized using GAPDH as internal control. Asterisks indicated the means ± SD of three independent experiments. *P < 0.05, **P < 0.001, ***P < 0.0002, Student's t-test

Several senescence markers are known to be expressed during the senescence process, such as the cell cycle inhibitors $p15^{INK4B}$, $p16^{INK4A}$, $p21^{CIP1}$, p53 and Rb. In addition to these markers, cells also express a variety of SASP proteins, including IL-6 and IL-8 [295].

In order to complete the characterization of the senescent phenotype in our CDDP-induced senescence model, we analyzed the mRNA expression levels of, IL-6, IL-8 and $p21^{CIP1}$. As shown in ***Fig.18B***, the mRNA expression levels of IL-6, d IL-8 and $p21^{CIP1}$ were significantly increased compared with proliferating control cells, with maximal expression at day five of release. These data demonstrate that A549 senescent tumor cells develop a proinflammatory secretion profile upon genotoxic treatment associated with the senescent phenotype.

1.1 Expression of BER enzymes during extrinsic cell senescence induced by CDDP-treatment in A549 cells

The effect of extrinsic cell senescence on the expression of BER enzymes is still completely unknown. For this purpose, we investigated the regulation of the expression levels of BER enzymes during the CDDP-induced senescence process. In particular, we focused on the following enzymes: APE1, XRCC-1, POL-β, POL-δ, whose expressions were tested at both mRNA and protein levels under the experimental conditions described above. In CDDP-induced senescent cells, we found that APE1 mRNA expression was downregulated at days five and eight of senescence compared to the twenty-four hours of treatment, as shown in the histogram of ***Fig.19***. Regarding XRCC-1, we observed a slight increase of expression just upon CDDP-treatment for 24-hours, while a decrease at five and eight days of senescence, compared to the twenty-four hours of treatment. POL-δ were downregulated during CDDP-induced senescence, in particular at eight days of senescence. A small increase in POL-β expression upon 24-hours of CDDP-treatment was observed, and also for this enzyme the mRNA expression shown a downregulation at five and eight days of senescence, with respect to twenty-four hours of treatment (***Fig.19***) Because mRNA expression of XRCC-1, POL-β, and POL-δ did not reach statistical significance at five and eight days, these results may indicate a trend toward decline during senescence.

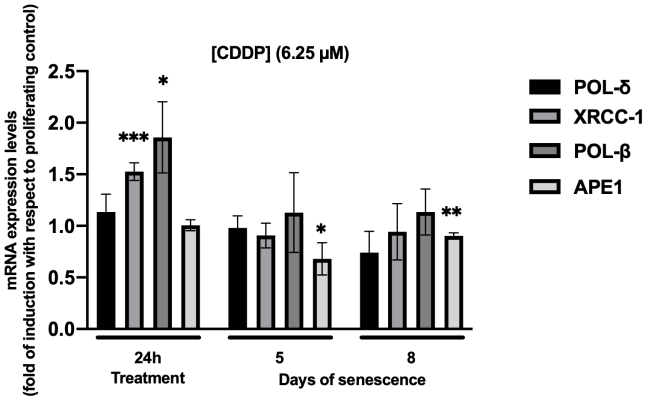


Figure 19. ***Effect of senescence on the mRNA expression of BER enzymes.*** The mRNA expression levels of endogenous BER enzymes were analyzed by real-time reverse transcription-PCR in CDDP-induced senescent cells twenty-four hours (24h treatment), five and eight days after drug release. The histogram shows a general downregulation of APE1, XRCC-1, POL-ß and POL-δ at days five and eight of senescence comparing to twenty-four hours of treatment. Data are expressed as fold of induction with respect to proliferating control. Asterisks indicated the means ± SD of three independent experiments and represent a significant difference from proliferating cells. *P < 0.05, **P < 0.001, ***P < 0.0002, Student's t-test

We also tested the expression of the BER enzymes at the protein level. As it can be observed in ***Fig. 20A, C***, under senescence conditions, we observed important changes in APE1 protein expression, which was downregulated on day five and eight of senescence. As with POL-δ, we observed downregulation in senescent cells in the late phase of senescence (corresponding to day five and eight), compared with proliferating control cells. XRCC-1 and POL-β showed a variable pattern among proliferating and senescent cells. In particular, for XRCC-1 the expression levels slightly increase upon five days of release and then decrease. For POL-β, the trend was very similar to XRCC-1, with a slight increase upon five days downregulation remaining stable in the expression during senescence. The upregulation of $p21^{CIP1}$, only in senescent cells, further indicated that the cell cycle was arrested in G1/S phase, as expected (***Fig. 20A-B***). All these data showed differential expression of BER enzymes analyzed under proliferating and senescent conditions, showing an overall trend of downregulation, which was more prominent for replicative POL-δ and APE1, in particular, being strongly downregulated in senescent cells both at the mRNA and, in particular, at the protein levels (***Fig. 20C***).

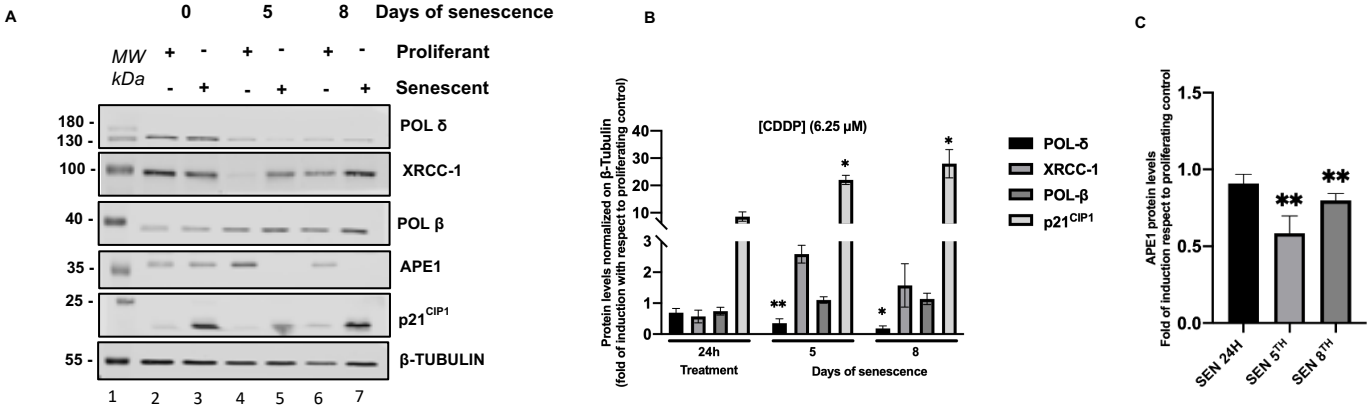


Figure 20. ***Effect of senescence on the protein expression of BER enzymes.*** (**A**) The image shows a representative Western blot of protein expression of BER enzymes at the indicated time points during drug treatment. Extracts from uninduced cells were used as controls. Anti-β-tubulin antibody was used as loading control and protein expression of $p21^{Cip1}$ was evaluated. (**B-C**) Histograms show densitometric analyses of BER enzymes expressed as fold of induction compared to proliferating cells as control. Asterisks represent a significant difference from proliferating cells and the means ± SD from three independent experiments are shown. *P < 0.05, **P < 0.001, ***P < 0.0002, Student's t-test.

The significant down-regulation of APE1, which was partly paralleled by a similar down-regulation of its mRNA levels under senescence conditions, prompted us to investigate a possible effect on its protein stability associated with proteasomal degradation [296]. To promote proteasomal inhibition, MG132-treatment at the dose of 40 µM [297] was performed for four hours, in senescent cells at five days after drug release. Then, protein expression analysis, through western blotting, demonstrated an accumulation of APE1 protein compared to their untreated counterpart upon MG132-treatment (***Fig. 21A***). This result suggests that the proteasome is responsible for the decrease of APE1 protein level in senescent cells. Proteasome have been shown to promotes the degradation of the IKK kinase complex (IKKα and IKKβ) and thus of IkB proteins [298].

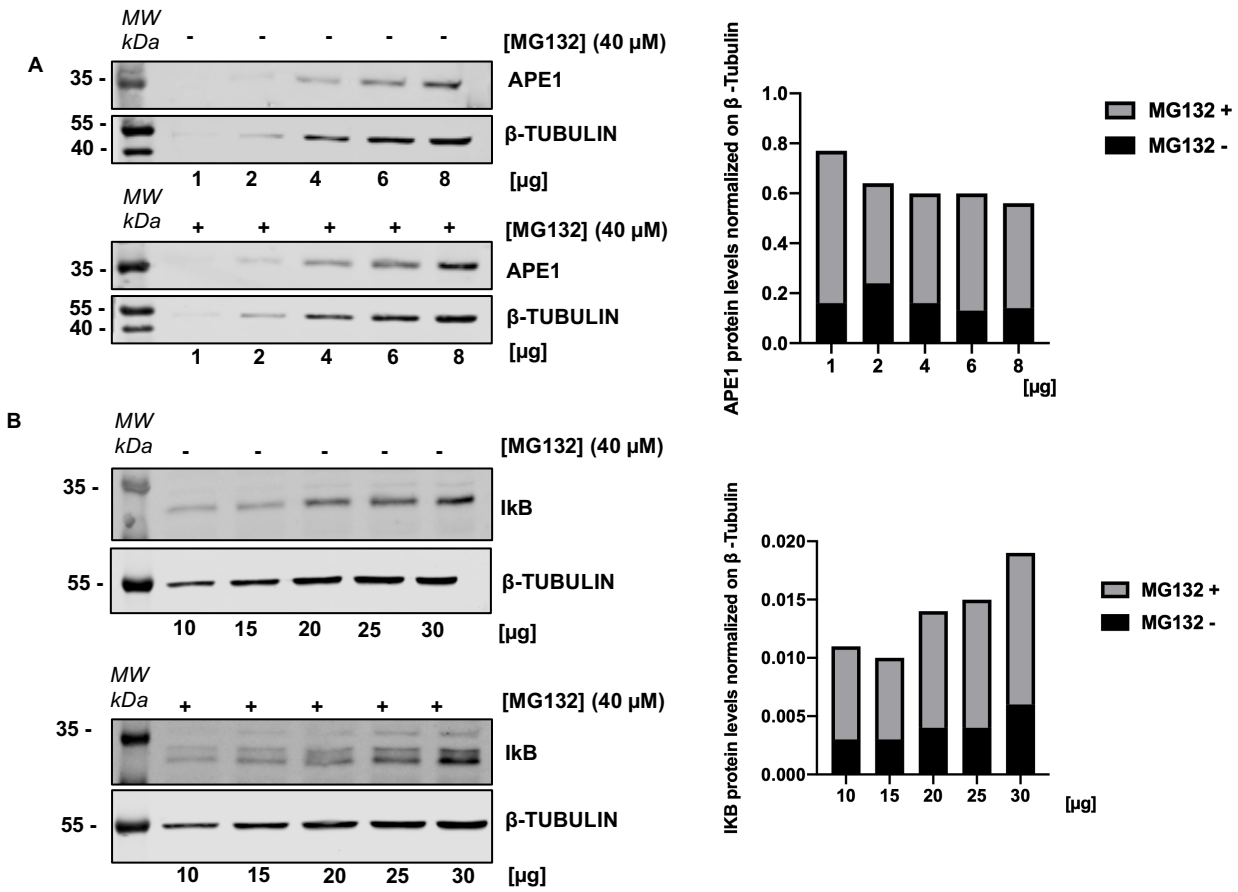
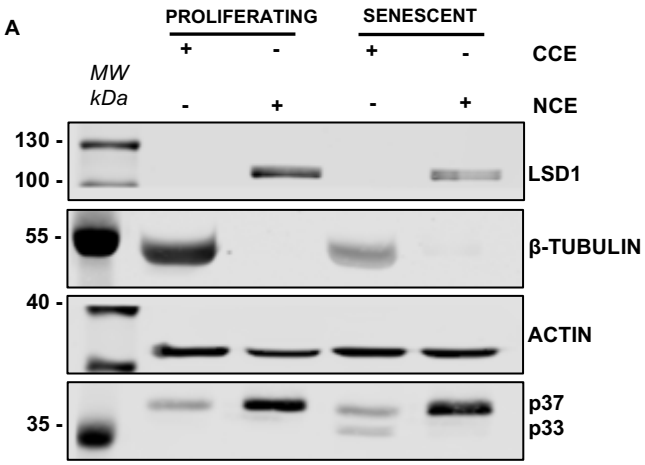


Figure 21. ***Analyses of quantitative protein levels of APE1 in senescent cells.*** **(A)** Analysis of APE1 protein content was performed using different concentrations of whole cell extract (expressed in µg) of A549 senescent cells treated with 40 µM of MG132 for four hours compared with their respective untreated control. Anti-β-tubulin antibody was used as a loading control. The histogram on the right shows the accumulation of APE1 as a result of proteasome inhibition in MG132 treated cells. **(B)** IkB detection in senescent cell was used as a control for MG132 treatment. Analysis of IkB protein content was performed using different concentrations of whole cell extract (expressed in µg) of A549 senescent cells treated with 40 µM of MG132 for four hours compared with their respective untreated control. Anti-β-tubulin antibody was used as a loading control.

In this context, we used the IkB antibody in the same experimental setup as in ***Fig. 21A*** to ensure the effect of MG132, shown in ***Fig. 21B***.

In order to analyze the distribution of APE1 during senescence in nuclear and cytoplasmic extracts of proliferating and senescent cells, we performed cellular sub-fractionation. As shown by Western blot analysis, only the full length 37 kDa APE1 protein form (p37) was detected in nuclear extracts, both in proliferating and senescent cells. Conversely, a reduced amount of APE1 was found in cytoplasmic fractions, especially in senescent cells, where it was observed also an accumulation of the truncated p33 form (***Fig. 22A***), in accordance with its degradation through involvement of the proteasomal machinery [296].
We then examined the cell distribution of APE1 through immunofluorescence (IF) experiments using Live-cell imaging microscope Leica AF6000LX. We measured the mean fluorescence intensity in the nucleus, normalized to DAPI fluorescence intensity, in both proliferating and senescent cells. As shown in the ***Figure 22B***, APE1 was less abundant in the nuclear fraction of senescent cells compared with proliferating cells.



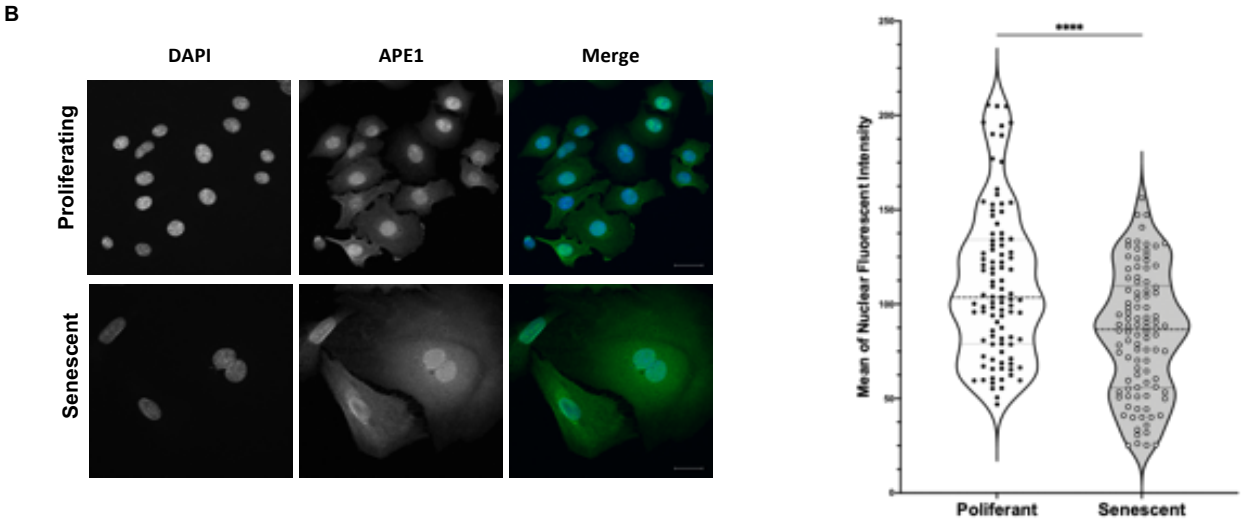


Figure 22. ***Analyses of subcellular localization of APE1 in proliferant and senescent cells*****. (A)** Subfractionation of APE1 performed in proliferant and senescent cells. LSD1 and β-tubulin antibodies were used to assess respectively nuclear (NCE) and cytosolic fractions (CCE), and anti-actin antibody was used as a loading control. **(B)** Confocal immunofluorescence images of proliferating and senescent cells show a change in cell morphology and a general redistribution of APE1 subcellular localization upon CDDP-induced senescence in A549 cell line; a specific polyclonal α-APE1 antibody and a secondary α-rabbit antibody conjugated to Alexa Fluor 488 were used to stain the cells; images were acquired with a 63x objective; representative scale bars of 25 μm. The graph on the right shows quantification of the mean value of nuclear fluorescence intensity between proliferating and senescent cells. Means ± SD of three independent experiments are indicated. ****P < 0.0001, Student's t-test.

2 Induction of senescence phenotype with DOXO

Induction of the senescence phenotype in A549 cancer cells was also performed with DOXO, which is known to have the ability to induce DNA cross-links by inserting itself into DNA and blocking its replication and transcription [299]. Similar to the previous analysis (***Fig. 17A***), we first examined cell viability (***Fig. 23A***), taking into account the DOXO dose that causes a 50% loss of cell viability. Using the same approach as for CDDP-treatment, we first tested the doses of 0.5 μM and 0.6 μM DOXO for seventy-two hours of treatment and then analyzed the phenotype of the cells after the release of the drug up to fourteen days. In ***Fig. 23B-C***, we show representative images of proliferating cells treated with dimethyl sulfoxide (DMSO) alone and senescent cells treated with 0.5 μM and 0.6 μM DOXO, three and seven days after drug release, photographed in brightfield using the Leica DMi1 microscope. The morphological cellular changes obtained, similar to those observed in the CDDP senescence-induced model (***Fig. 17B-C***), were apparent as early as three days after drug release, while $p21^{CIP1}$ protein levels increased at three and seven days, as shown in ***Fig. 23 D-E***.

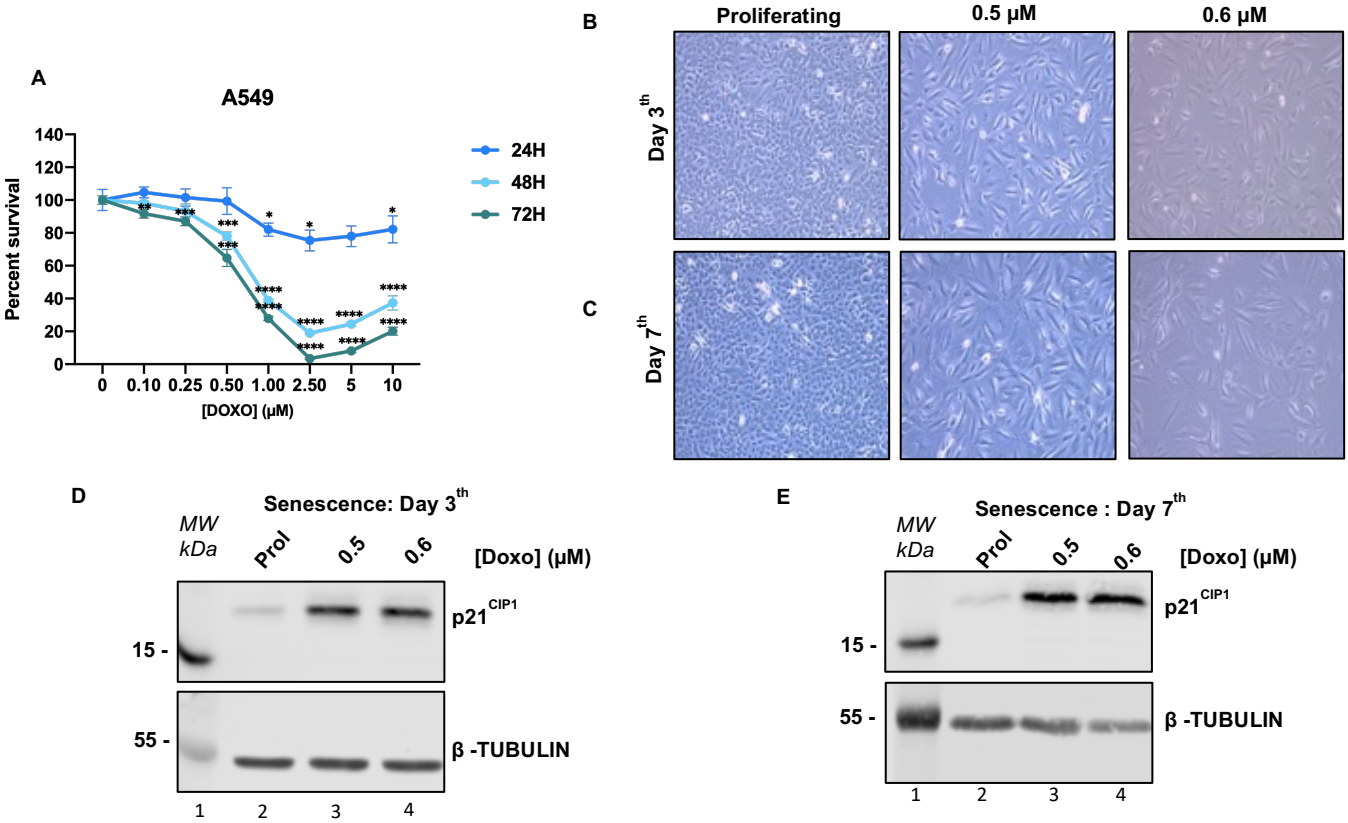


Figure 23. ***Phenotypic changes in A549 cell line induced senescence with DOXO.*** **(A)** Analysis of cell viability in A549 cell line after DOXO treatment. MTS assay was performed using the indicated concentrations of DOXO at 24, 48, 72 hours. The viability of untreated cells at the indicated time points was evaluated as a control. Asterisks represent a significant difference from untreated as control. *P < 0.05, **P < 0.001, ***P < 0.0002, ****P < 0.0001 Student's t-test. **(B)** and **(C)** show the phenotypic changes of A549 cell line treated with 0.5 and 0.6 μM of DOXO. The field was photographed under bright field, Leica DMi1 microscope 3 and 7 days after drug release, respectively. In **(D)** and **(E)**, $p21^{CIP1}$ protein levels were analyzed, which increase during the senescence period.

The results of these preliminary experiments, in which we followed the development of the senescence phenotype for fourteen days with two different doses of DOXO, were useful to find the conditions for obtaining a model of DOXO-induced cancer cell senescence. Thus, in order to obtain the phenotype of induced cell senescence, we chose the following condition: 0.5 μM for seventy-two hours followed by release up to nine days. We discarded the dose of 0.6 μM due to its toxic effects on the cells. The treated cells were analyzed by β-Galactosidase assays. Histogram (***Fig. 24)*** shows quantification of the percentage of β-Galactosidase. We found that six days after release, approximately 85% of the cells were β-Galactosidase positive compared to the proliferating untreated cells, as a control. These data suggest that we were able to develop a senescent phenotype with another genotoxic drug, namely DOXO (***Fig. 24***).

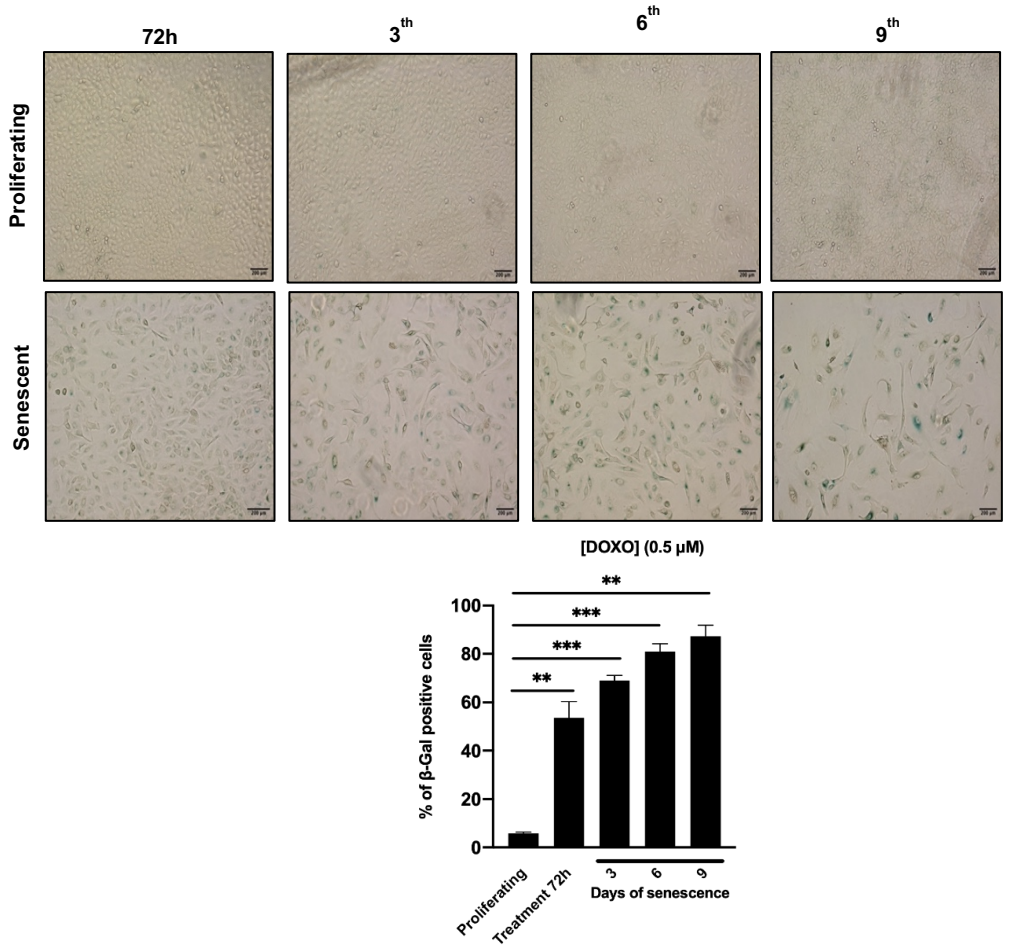


Figure 24. ***DOXO treatments induce senescence phenotype.*** A549 cells were photographed with Leica DMi1 microscope at 20× magnification. Proliferating and senescent cells were plated at the density of 0.6 x 10^6 cells per 6-well and treated with DOXO 0.5 μM for seventy-two hours, washed extensively, replicated in drug-free medium, and stained to detect SA-β-gal activity to analyze the senescent phenotype. The analysis was performed seventy-two hours (72h treatment), three, six and nine days after drug release. The experiment was carried out up to nine days of senescence, since at ten days the number of senescent cells was considerably reduced owing to the high cell mortality. The average of diameter for proliferant cells was around 50-70 μm, for senescent cells around 80-120 μm. Representative scale bars of 200 μm. At least 200 cells were counted for each independent determination. The graph below shows the percentage of β-Gal-positive cells. Asterisks indicated the means ± SD of three independent experiments. **P < 0.001, ***P < 0.0002 Student's t-test.

Similar to the previous characterization with CDDP, we characterized the DOXO-induced cell senescence model by analyzing the mRNA expression levels of IL-6, IL-8 and $p21^{CIP1}$ at days three, six and nine of senescence. Consistent with the complete senescence phenotype, the mRNA expression levels of IL-6, IL-8, and $p21^{CIP1}$ were always upregulated compared to the corresponding proliferating cells, with maximal expression after six to nine days for IL-6 and IL-8 (***Fig. 25A***). The expression of BER enzymes was also analyzed in DOXO-induced senescence. We observed that the transcript levels of endogenous APE1 were down-regulated after six and nine days of senescence compared to the proliferating control. Comparing the expression levels of POL-δ between senescent and proliferating cells, they were lower after seventy-two hours and three days of senescence, whereas they remained unchanged after six and nine days. We observed a different trend for POL-β, which is much more highly expressed in senescent cells than in proliferating cells. POL-β mRNA level begins with low expression, as observed at seventy-two hours of treatment, and then maintains comparable expression levels from day three to day nine of senescence. Finally, for XRCC-1, transcript levels are slightly lower in senescent cells compared to proliferative cells from the start of treatment at seventy-two hours to the sixth day of senescence. At day 9, XRCC-1 expression levels are similar between senescent and proliferating cells (***Fig. 25B***). We then examined the protein levels of BER enzymes. The data in ***Fig. 25 C,E*** show that APE1 protein expression was downregulated in DOXO-induced senescent A549 cells compared with the corresponding proliferating cells. A different trend was seen in POL-δ protein expression, whose protein levels were detectable in proliferating cells seventy-two hours after treatment with DOXO and three days after DMSO release, whereas they were downregulated in the corresponding senescent cells. Protein levels of POL-δ remained downregulated in both proliferating and senescent cells six and nine days upon drug release. Analysis of XRCC-1 showed a variable pattern, in which seventy-two hours after treatment there was a slight increase in senescent cells, while protein levels in both proliferating and senescent cells were similar three and six days after drug release, while protein levels nine days after treatment were similar to seventy-two hours of treatment. In the case of POL-β, protein concentrations seventy-two hours after treatment, three and nine days after drug release were similar in both proliferating and senescent cells,

while a comparable reduction was observed six days after drug release in both proliferating and senescent cells (***Fig.25 C,D***). Taken together, these results suggest that there was variable expression of BER enzymes that needs further investigation. In this regard, APE1 showed a distinct pattern of downregulation only in senescent cells compared to proliferating cells, as previously observed in the CDDP senescent induced model (***Fig. 20 A,C***), and compared to the others BER enzymes analyzed previously.

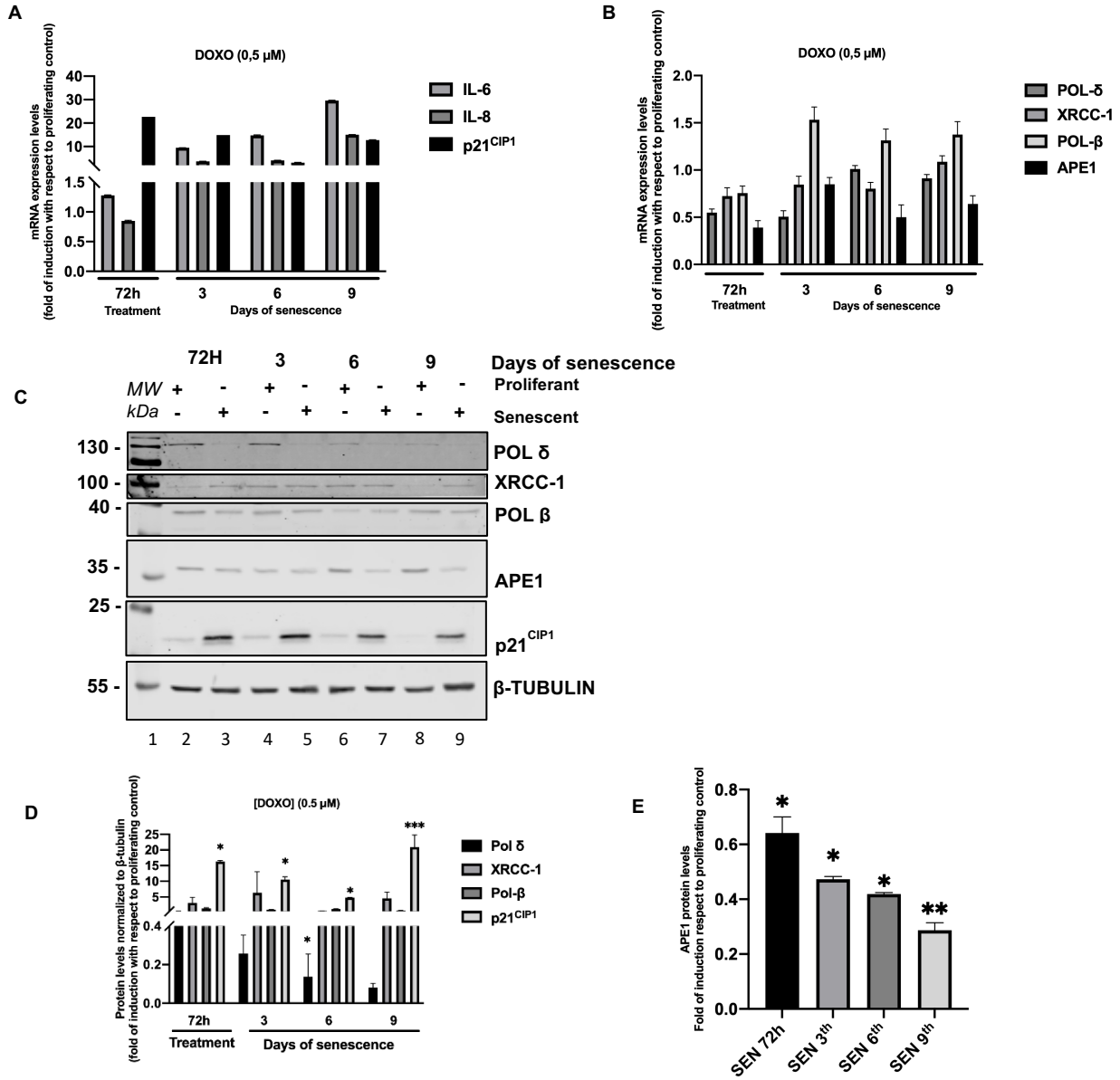


Figure 25. ***Effect of senescence on the expression of BERs.*** Real-time reverse transcription-PCR was performed in DOXO-induced senescent cells at the indicated time points. **(A)** IL-6, IL-8 and $p21^{CIP1}$ were significantly induced during the late senescence period. The mRNA expression levels were normalized using GAPDH as an internal control. **(B)** BER Enzyme mRNA expression levels were tested by real-time reverse transcription-PCR. APE1 shows an overall downregulation for each day analyzed compared to proliferating cells used as control. **(C)** Endogenous protein expression of BERs were detected by Western blotting at the indicated time points during drug treatment and development of the senescent phenotype. Extracts from uninduced cells were used as controls. Filters were probed with anti-β-tubulin antibody as a loading control and anti-p21 Waf1/ Cip1 antibody as a marker for G1/S cell cycle arrest. **(D,E)** Plots show densitometric analysis of BERs and APE1 protein levels, respectively, as fold of induction with respect to proliferating cells. Asterisks represent significant difference compared to proliferant cells used as control *P < 0.05, **P < 0.001, ***P < 0.0002 Student's t-test.

3 Effect of APE1 silencing on SASP factors and on p21^{CIP} gene expression during extrinsic senescence

IL-6 and IL-8 expression can be regulated by APE1 [300]. To better understand the role of APE1 during extrinsic cell senescence, we downregulated APE1 by transiently transfecting specific siRNAs in our CDDP-induced senescence model before the induction of cellular senescence. With this aim, A549 cells were transfected with siSCR and siAPE1 for seventy-two hours. To induce senescence, cells were treated with CDDP for twenty-four hours at a final concentration of 6.25 μM forty-eight hours after the beginning of APE1 silencing, under the same conditions as in the previous experiments (see ***Fig. 26*** for experimental details).

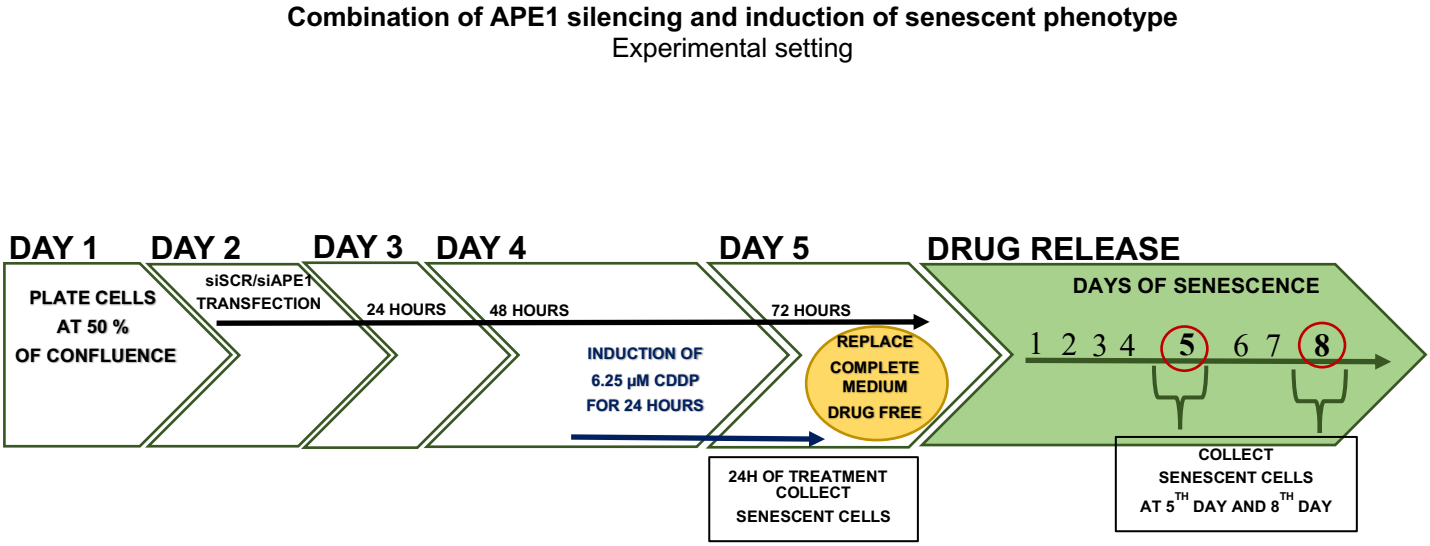
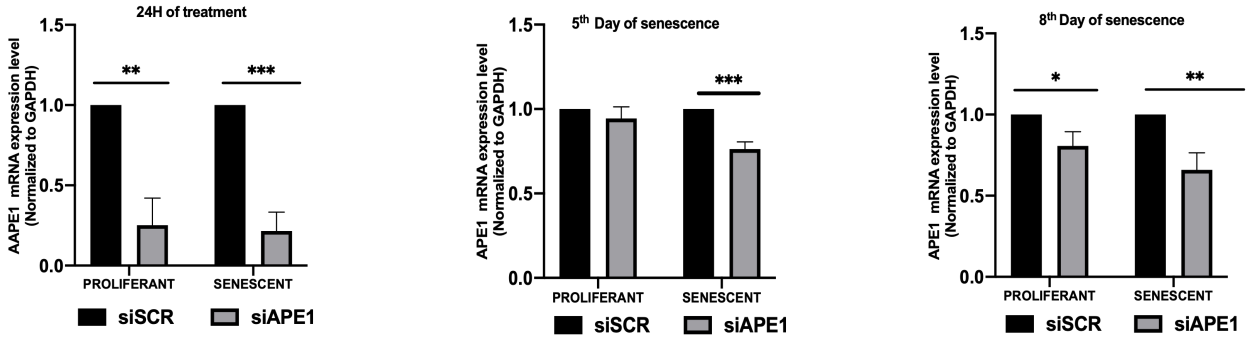


Figure 26. ***Experimental procedures of A549 induced senescent cells APE1 downregulated***. A549 cells were plated at 50% confluence. After twenty-four hours of seeding, cells were transfected with siSCR and siAPE1 for seventy-two hours. Forty-eight hours after the beginning of silencing, the media were removed and replaced with media containing DMF in proliferant or CDDP in senescent cells. Twenty-four hours later, the media were removed, the cells were washed extensively twice with wash buffer, and the free media were replaced. Proliferant and senescent cells were collected twenty-four hours after treatment, five and eight days after drug release.

The ***Figure 27A*** shows the silencing effect of APE1 itself, that is visible after twenty-four hours of CDDP treatment (corresponding to seventy-two hours of silencing) in both proliferating and senescent cells, while after five and eight days it is lost in proliferating cells compared to siSCR, while the downregulation of APE1 observed in senescent cells, is probably due to the effect of senescence. At the protein levels the downregulation of APE1 in proliferating and senescent cells confirms the silencing effect of APE1 after seventy-two hours of silencing (twenty-four hours of CDDP treatment), while after five days of senescence, in proliferating cells transfected with siSCR and siAPE1, the protein levels were almost unchanged, and senescent cells transfected with siAPE1 showed only a small decrease compared to siSRC senescent cells (***Fig. 27B***). When analyzing $p21^{CIP}$ expression, we observed that the activation of $p21^{CIP1}$ was promoted only upon CDDP-treatment for twenty-four hours (***Fig. 27C***). In fact, in proliferating cells, downregulation of APE1 is accompanied by an increase in $p21^{CIP1}$, at both the transcriptional (***Fig. 27C***) and protein levels (***Fig. 27B***, compare lanes two and four), which is visible after twenty-four hours of treatment. Western blot analysis showed that induction of $p21^{CIP}$ was apparent upon siAPE1 in both proliferating and CDDP-treated cells upon twenty-four hours of silencing, while $p21^{CIP1}$ levels are very similar in cells transfected with siSCR and siAPE1 (***Fig. 27B*** lanes three and five, seven and nine). These results indicate that the activation of $p21^{CIP1}$ by CDDP is independent of APE1 at five and eight days of senescence, possibly due to the limited silencing efficacy at later times of transfection.

Data obtained upon transfection suggest that APE1 promotes the regulation of $p21^{CIP1}$ in proliferating cells but not under cell senescence being, the dependence of $p21^{CIP1}$ expression by APE1, lost after five and eight days of senescence.

A



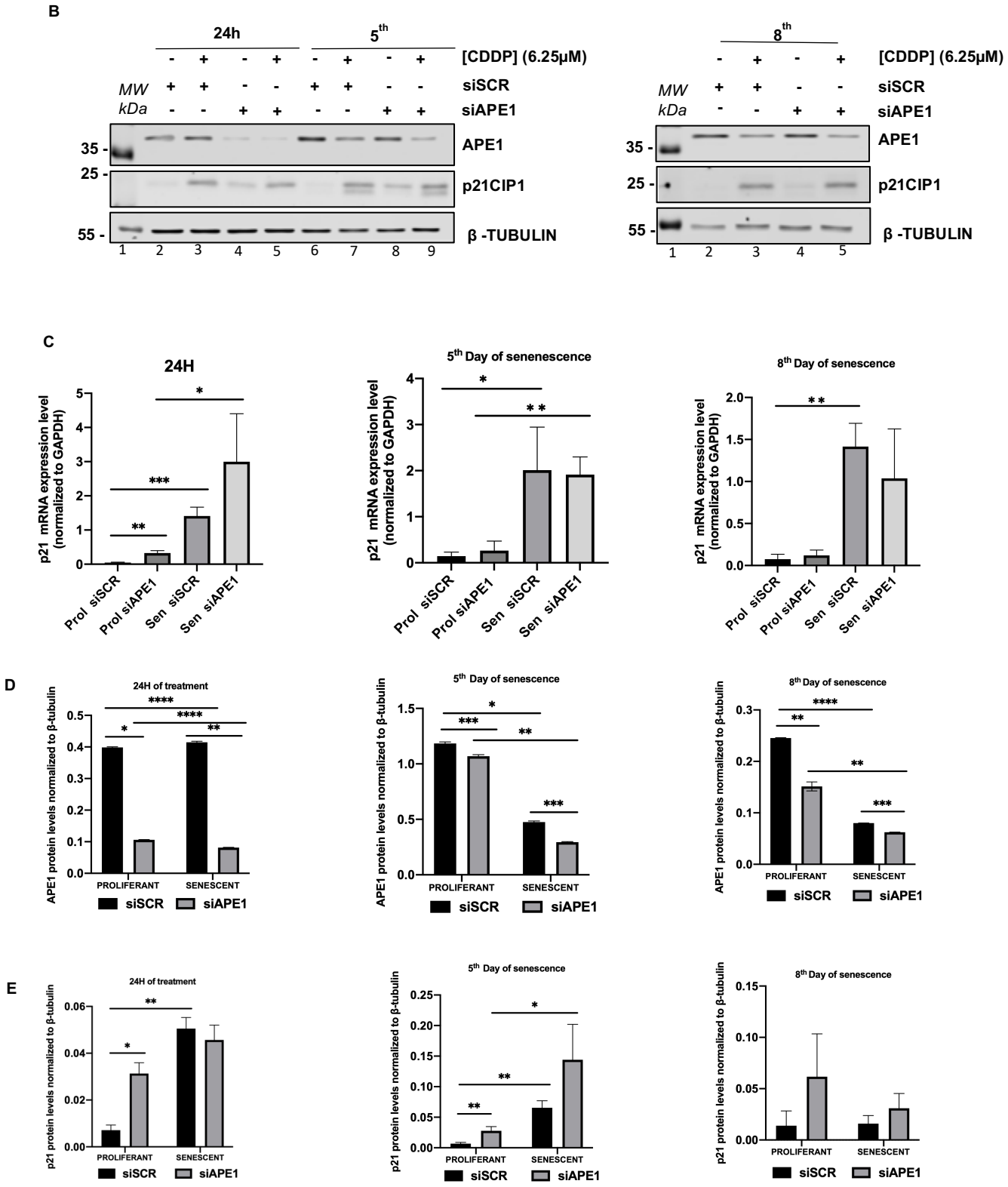


Figure 27. ***APE1 mRNA and protein level evaluated in A549 cell line silenced for APE1 expression.*** **(A)** APE1 mRNA levels assessed by qRT-PCR analysis of proliferating and senescent cells silenced for APE1 expression. Total RNA was extracted from the A549 cell line expressing APE1 (WT) or whose APE1 was silenced (siRNA), and reverse transcribed. Detected APE1 mRNA levels were normalized to GAPDH levels. **(B)** Representative Western blotting analyses of extracts of proliferating and senescent cells. Filters were probed with anti-APE1, anti-p21 Waf1/ Cip1 antibody and anti-β-tubulin antibody as a loading control. **(C)** Histogram shows detected levels of mRNA $p21^{CIP1}$ normalized to GAPDH levels, at indicated time points. Data represent means ±SD of three independent experiments. Statistical analysis was achieved by applying Student's t-test. *P < 0.05, **P < 0.001, ***P < 0.0002. **(D-E)** Histogram D indicates densitometric analyses of APE1 showed in **(B),** histogram **E** shows densitometric analyses of $p21^{CIP1}$ showed in **(B).** The means ± SD from three independent experiments are shown. Asterisks represent significant difference compared to siSCR cells used as control *P < 0.05, **P < 0.001, ***P < 0.0002, ****P < 0.0001 Student's t-test.

The expression of IL-6 and IL-8 SASP factors was then analyzed at the mRNA levels. In ***Fig. 28A***, IL-6 expression induced by twenty-four hours of CDDP-treatment was dependent on APE1 expression, as its induction was significantly reduced upon APE1 silencing. On the contrary, during later times of senescence, the inhibitory effect of APE1 depletion exerted no effects on IL-6 induction. Similar data were obtained in the case of IL-8 expression (***Fig. 28B***). These results showed that the expression of SASPs IL-6 and IL-8 genes is clearly APE1-dependent twenty-four hours upon CDDP-treatment, but the effect is lost at later times, as we can observe at day five and eight of senescence, where SASP factors are upregulated, while APE1 expression is reduced (***Fig.28A-B***).

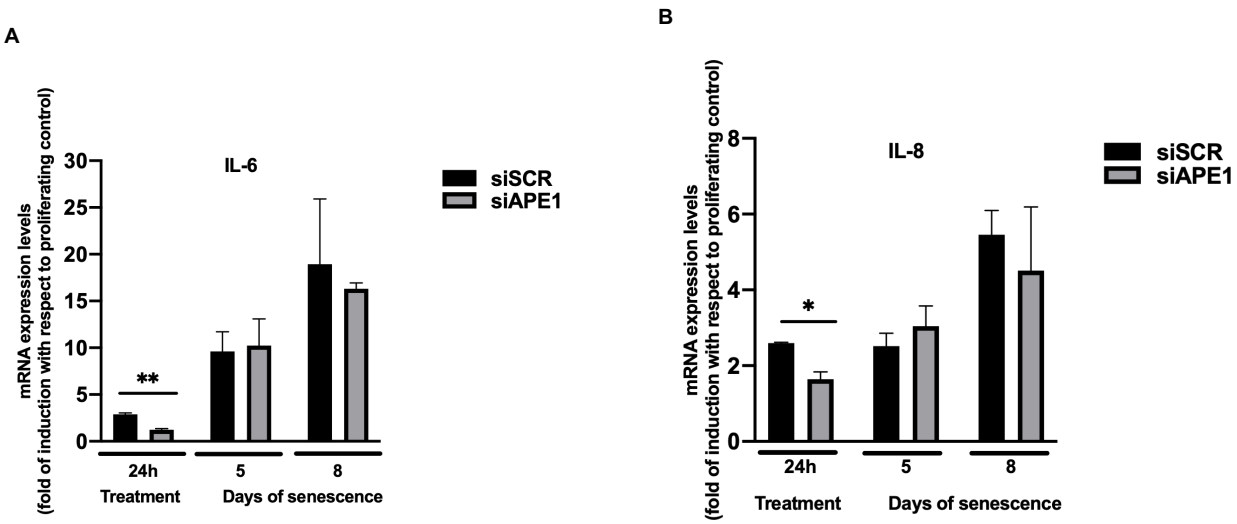


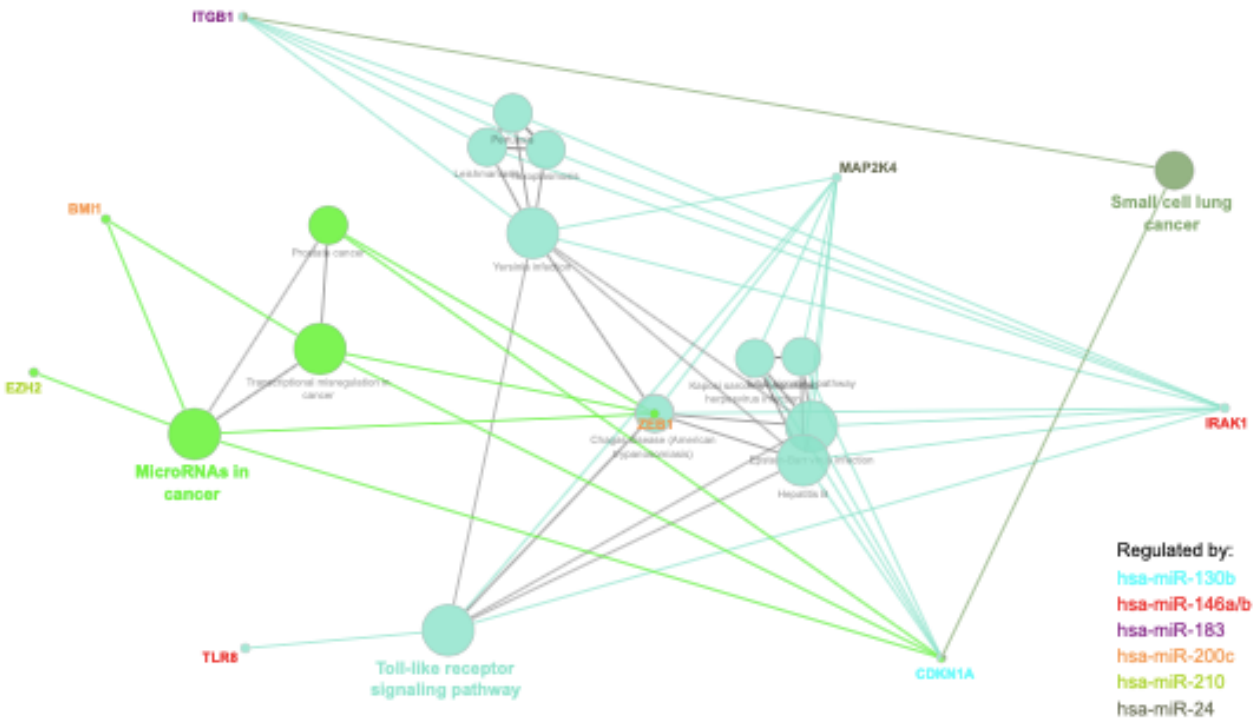
Figure 28. ***APE1 expression in relation to SASPs in CDDP induced cellular senescence.*** **(A-B)** SASPs mRNA levels assessed by qRT-PCR analysis of proliferating and senescent cells silenced for APE1 expression. Total RNA was extracted from the A549 cell line expressing APE1 (WT) or whose APE1 was silenced (siRNA) and reverse transcribed. Histograms show detected levels of IL-6, IL-8 normalized to GAPDH levels. Asterisks represent significant difference compared to siSCR cells used as control *P < 0.05, **P < 0.001, Student's t-test.

4 APE1 could regulate the expression of senescent markers through miR-130b

In a recent work, we have shown the involvement of APE1 in RNA metabolism, in particular pri-miRNA processing and stability via association with the Drosha processing complex, during genotoxic stress conditions [186]. Data not shown obtained from unpublished work in the Lab and obtained through miRNA differential expression analysis in A549 cells upon APE1 knock down, revealed that some miRNAs regulated by APE1 are involved in the expression of cellular senescence biomarkers.

We assembled a literature-based [301] [251] list of human miRNAs regulating the SASP, p53/p21^{CIP} and p16/RB senescence-associated pathways, globally identifying fifty-seven miRNAs (Antoniali *et al.,* in preparation). We then assessed the regulatory effect exerted by APE1 on these miRNAs, through the silencing of the APE1 gene in the A549 cell line (Antoniali *et al.,* in preparation), finding five miRNAs that were down-regulated (miR-130b, miR-146a, miR-183, miR-200c, miR-24) and one that was up-regulated (miR-210) upon siAPE1 (***Fig. 29A-B***). Afterwards, we recovered the validated targets associated with these six differentially expressed miRNAs (DEmiRNAs), representing the main effectors of the senescent phenotype: CDKN1A (hsa-miR-130b); IRAK1 and TLR8 (hsa-miR-146a/b); ITGB1 (hsa-miR-183); ZEB1, BMI1 (hsa-miR-200c); EED, EZH2, SUZ12 (hsa-miR-210); MKK4 (MAP2K4) (hsa-miR-24). Finally, we used the Cytoscape plugin ClueGO [302] [303] annotate these ten target genes, querying the KEGG database (Min number of genes 1, Min % Genes 1%, Two-sided hypergeometric test (Benjamini-Hochberg correction) and representing the miRNA-target-pathway relationships as a functional network (***Fig.29A***).

A



B

Upregulated	Downregulated	Pathway	Targets	Cancer Type	References
hsa-miR-210		p16/RB	EED, EZH2, suz12	Venous metastasis of hepatocellular carcinoma (HCC)	Ji et al., (2018) Borgdorff et al., (2010) Bhaumik et al., (2009) Wang Y et al., (2011) Gysler et al., (2016)
	hsa-miR-130b	p53/p21	p21	Prostate cancer (PCa), Xeroderma Pigmentosum (XP), NSCLC	Ramalho-Carvalho et al., (2017) Val Cervo, et al., (2011) Hirono et al., (2019) Han et al., (2020) Ye et al., (2017)
	hsa-miR-146a/b	SASP	IRAK1, TLR8	Anaplastic thyroid cancer cells, NSCLC	Philipot et al. (2014) Giglio, et al (2013) Marasa et al. (2009) Liu et al., (2020) Huang et al, (2017)
	hsa-miR-183	SASP	ITGB1	Colorectal cancer (CRC)	Davis et al. (2017) Wang et al. (2019) Li et al. (2010
	hsa-miR-200c	p53/p21	ZEB1, BMI1	Gastric Cancer (GC)	Magenta et al. (2011) Yu et al., (2021)
	hsa-miR-24	p16/RB	MKK4	Hepatocellular Carcinoma (HCC)	Bu et al. (2016), Chen et al., (2016)

Figure 29. ***Network of the significantly enriched KEGG functional terms and of the associated miRNA-regulated targets.*** **(A)**The validated targets (n=10) of the six senescence-associated miRNAs differentially expressed in A549 cells after APE1 silencing were annotated using the Cytoscape plugin ClueGO querying the KEGG database. For each enriched functional cluster (adjusted p-value ≤ 0.05), the most significant term is highlighted in bold, with each additional enriched term belonging to the same cluster indicated by a node in the same color. Each annotated gene is linked to its associated functional terms and color-coded according to its regulating miRNA(s). **(B)** validated targets associated with six differentially expressed miRNAs.

In the data shown above, APE1 decreases during A549 cell senescence induced by CDDP treatment (***Fig. 20A,C***). The expression levels of six miRNAs in A549 cell line obtained from bioinformatical analysis (***Fig. 29B)***, are reported, as shown in the histogram in the ***Fig. 30A***. Subsequently, the miRNAs were validated in A549 cell line, by qRT-PCR, following APE1 downregulation (***Fig. 30B***). We observed that miR-130b, miR-146a and miR-183 were strongly down-regulated, while only miR-200c showed an upregulation (***Fig. 30B***). The endogenous expression levels of the six miRNAs were analyzed also in senescent cells and shown in the histogram in ***Fig. 30C***. In the senescence model, the endogenous levels of miRNAs were compared with those in proliferating cells after twenty-four hours of CDDP-treatment and five days of senescence (***Fig. 30C***). According to the bioinformatic analysis, miR-130b, miR-146a and miR-200c show very low expression levels following APE1 down-regulation, during cellular senescence.

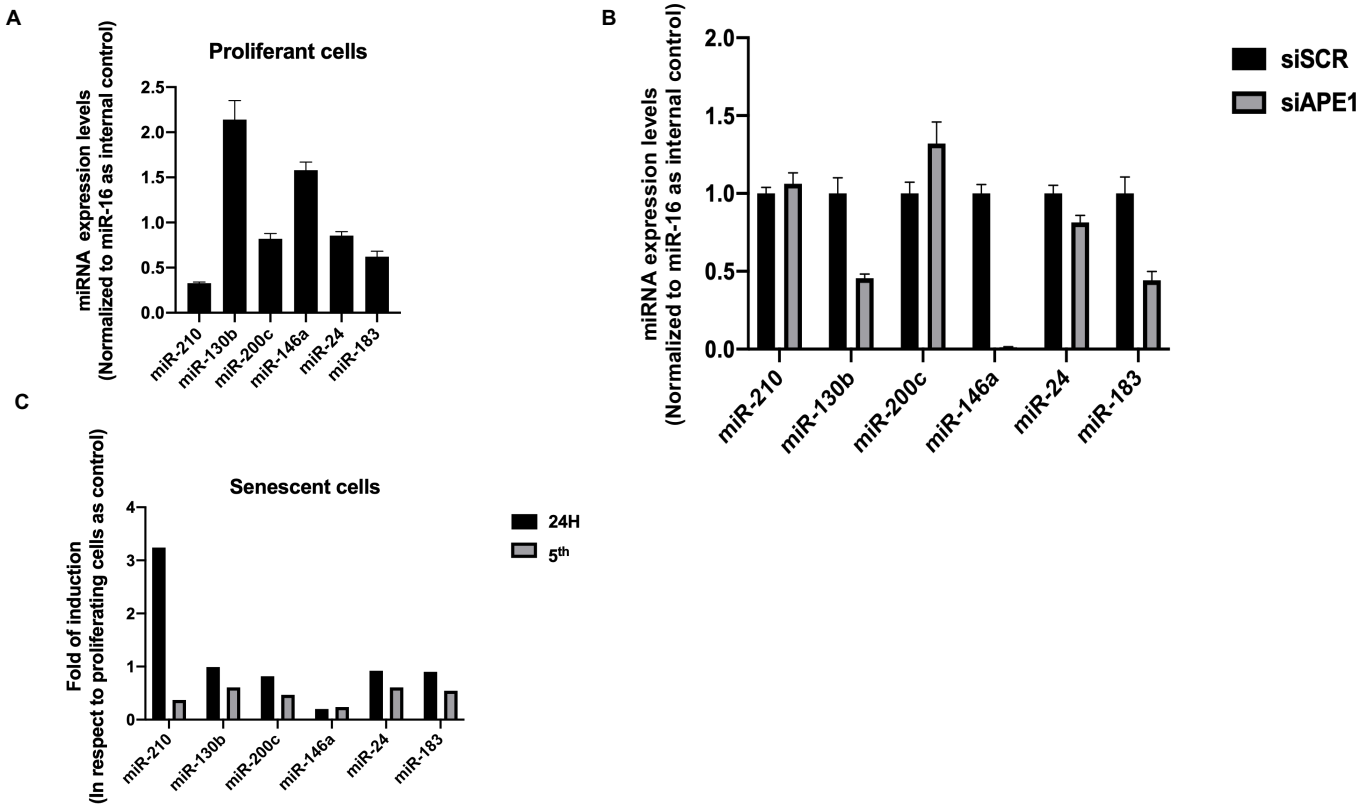


Figure 30. ***Six miRNAs differentially expressed were validated in A549.*** **(A)** Basal miRNAs expression levels analyzed by qRT-PCR in proliferating cells. Expression of miRNAs was normalized to the level of miR-16a as internal control. **(B)** The expression of miRNAs was measured by qRT-PCR in proliferating cells. Total RNA was extracted from the A549 cell line transiently silenced for APE1 and reverse transcribed. Data obtained from bioinformatics analysis confirmed the downregulation of miR-130b, miR-146a and miR-183, except miR-200c, after silencing of APE1. **(C)** The expression of miRNAs was measured by qRT-PCR in senescent cells at twenty-four hours of treatment and five days after drug release. Total RNA was extracted from the A549 cell line in which APE1 had been transiently knocked down and reverse transcribed. Expression of miRNAs was normalized to the level of miR-16a as internal control.

Among all miRNAs, we were interested to investigate miR-130b, which was down-regulated during senescence compared with the proliferating control, in agreement with the previous bioinformatics analysis, and in view of its target genes. A query of the KEGG database revealed that miR-130b is involved in the p53/p21^{CIP} pathway activation, having p21^{CIP1} as a target gene. In order to assess the involvement of APE1 in the regulation of p21^{CIP1} through miR-130b, we analyzed p21^{CIP1} at mRNA and protein levels following the overexpression of miR130b in a context of APE1 downregulation. In the present experimental setting, we first transfected A549 cells with siSCR and siAPE1 for seventy-two hours, and at forty-eight hours of silencing we overexpressed miR-130b using specific mi-RNA mimic for twenty-four hours. p21^{CIP1} was analyzed at transcriptional level as reported in the histogram in the ***Fig. 31 A***, which shows that the effect of APE1 on p21^{CIP1} occurs, at least in part, through miR-130b in proliferating cells, since overexpressing miR130b-mimic caused p21^{CIP1} downregulation. p21^{CIP1} expression was evaluated also using western blot analysis (***Fig. 31B***), where p21^{CIP1} protein levels increased in the lane four, six and eight corresponding to cells in which APE1 was silenced. In the lane seven and eight, cells were transfected with mimic-miR130b, and the increased level of p21^{CIP1} due to APE1 depletion resulted decreased, supporting the hypothesis in which APE1 plays a key role in the p21^{CIP1} regulation in the absence of genotoxic treatment. In the ***Fig. 31C*** we analyzed the expression of miR-130b following the overexpression with corresponding mimic, confirming that the effect on p21^{CIP1} is due to the overexpression of miR-130b, as shown in the histogram.

Based on the observation that miR-130b is downregulated upon APE1 silencing, we checked the effects on p21^{CIP1} at the transcriptional and protein levels in the context of APE1 silencing and overexpression of miR-130b in senescent cells at twenty-four hours of CDDP treatment. As the graph shows (***Fig. 31D***), the transcript levels of p21^{CIP1} remain unchanged upon both downregulation of APE1 and overexpression of miR-130b. Even at the protein level (***Fig. 31E***), the differences in p21^{CIP1} expression between senescent cells siSCR and siAPE parallel the transcript levels. These results indicate that the upregulation of p21^{CIP1} in senescent cells is not dependent on APE1–mediated miR-130b regulation.

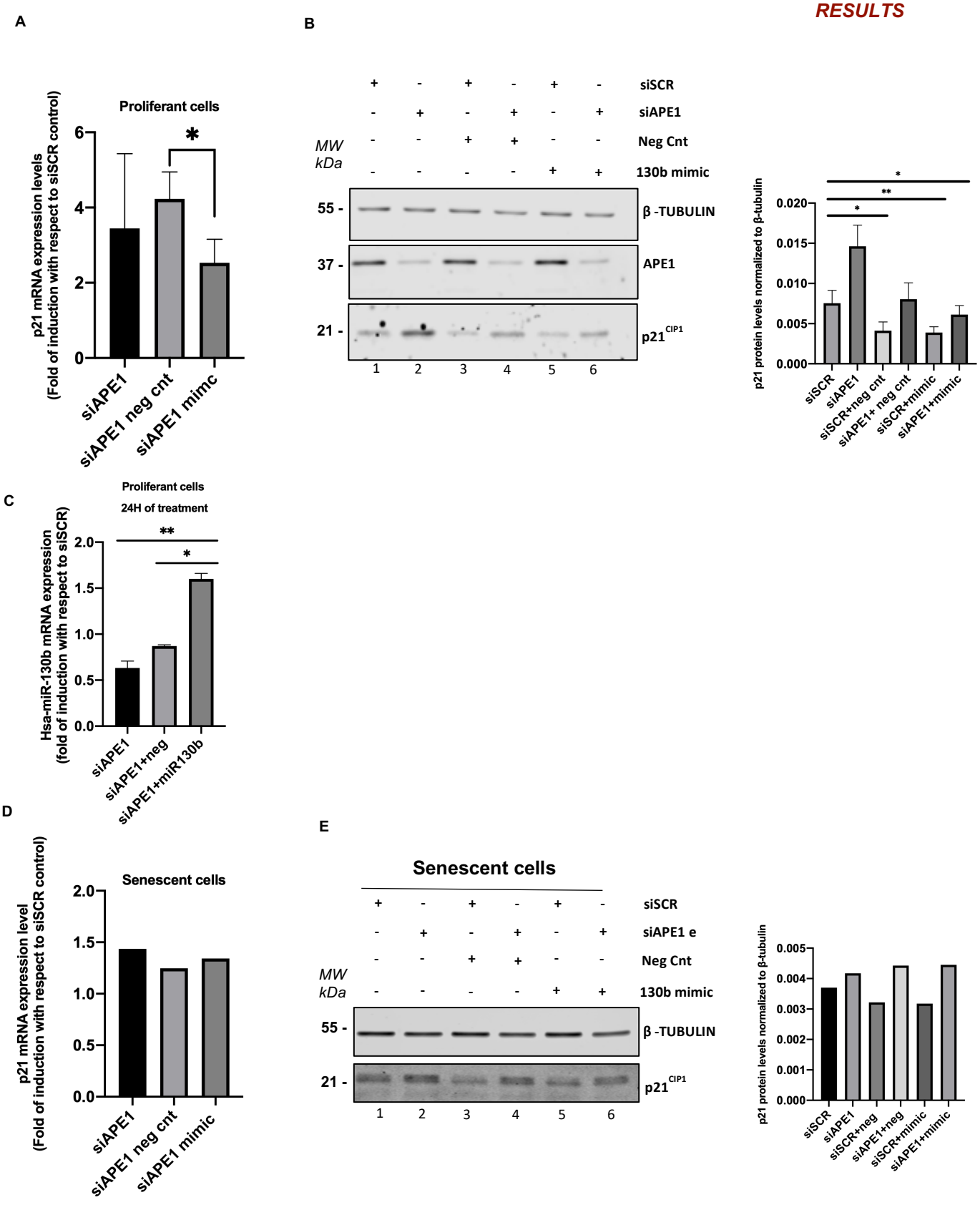


Figure 31. ***p21^{CIP1} is modulated by APE1 via miR-130b.*** **(A, D)** The p21^{CIP1} mRNA level was assessed by qRT-PCR analysis of proliferating **(A)** and senescent cells **(D)** silenced for APE1 expression, and overexpressing miR-130b. Total RNA was extracted from the A549 cell line expressing APE1 scr or whose APE1 was silenced (siRNA) and reverse transcribed. Histogram shows detected levels of p21^{CIP1} normalized to GAPDH levels. Asterisks represent a significant difference from control (SCR). *P < 0.05, Student's t-test. **(B, E)** Representative Western blotting analyses of extracts of proliferating and senescent cells obtained 24 hours after treatment with CDDP. p21^{Cip1} and APE1 protein expression was evaluated, anti-β-tubulin antibody was used as loading control and. Histogram shows densitometric analyses of p21$^{CIP1.}$ Data represent means ±SD of three independent experiments. *P < 0.05, **P < 0.001. Student's t-test. **(C)** Evaluation of miR-130b mRNA levels assessed by qRT-PCR of proliferating cells overexpressing miR-130b. Asterisks represent a significant difference from control (SCR). *P < 0.05, **P < 0.001. Student's t-test.

5 APE1 inhibitors exert senolytic activity

Cancer cell senescence is a mechanism responsible for the occurrence of cancer resistance phenomena [304]. Therefore, we tested whether CDDP-induced senescent cells are more resistant to treatment with higher doses of CDDP. To this end, we performed a cell viability assay by treating proliferating and senescent cells with CDDP for forty-eight hours. As shown in the graph (***Fig. 33A***), the results indicate that senescent cells are more resistant to additional CDDP-treatment than proliferating cells. In the experiments performed so far, we have observed a reduction in APE1 levels during cell senescence at both the transcriptional and protein levels. We examined whether the residual fraction of APE1 could be a good candidate to be targeted for sensitization of senescent cells to chemotherapy treatment. We wondered whether inhibition of APE1 functions would allow senescent cells to be more sensitive to treatment with CDDP. We used two different inhibitors of APE1: compound #3 (which blocks endonuclease activity) and E3330 (which impairs redox function) [305][306] (***Fig. 32***) to assess cell viability when these inhibitors were used in combination with CDDP.

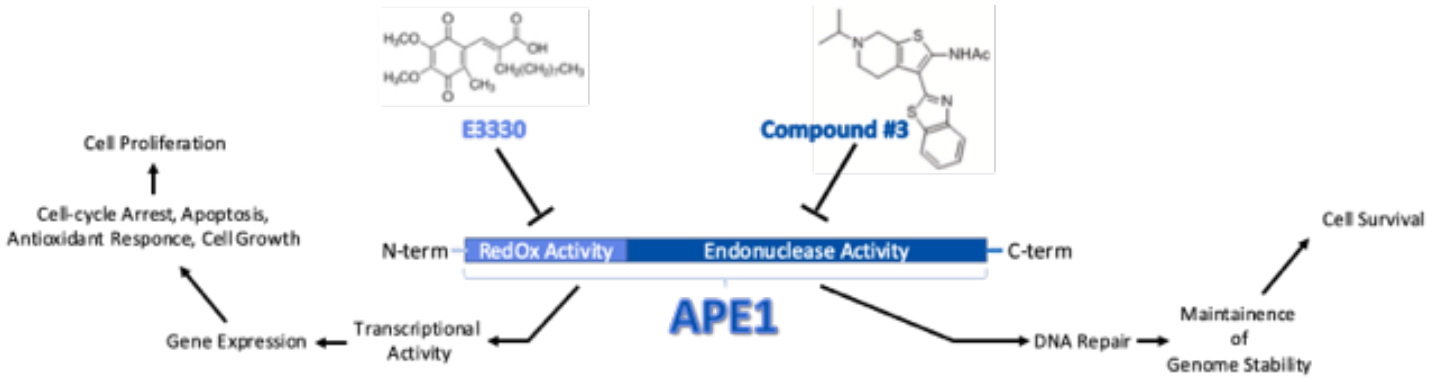


Figure 32. ***APE1 enzymatic activity domains: biological functions and respective small molecule inhibitors.*** The APE1 RedOx activity domain is localized in the N-terminal region of the protein and is mainly involved in regulating gene expression by acting on various transcription factors, e.g., NF-Kβ. APE1 RedOx activity is known to be inhibited by a small molecule compound called E3330. APE1 endonuclease activity is mainly achieved by the core part of the protein, this domain is mainly involved in the recognition of apurinic/apyrimidinic sites of DNA repair. Compound #3 is a small molecule with high specificity for inhibiting the AP-endonuclease activity of APE1.

The graph in ***Fig. 33B*** shows that senescent cells treated with #3 were less sensitive than senescent cells to CDDP treatment. The same result was obtained when E3330 was used in combination with CDDP (***Fig. 33C***). These data showed that in a model of chemoresistance related to senescence, reduction or blocking of APE1 functions leads to sensitization to chemotherapy, similar to the use of senolytic compounds such as the well-known Quercetin, that was used here as a senolytic compound as a control (***Fig. 33D***).

Overall, these data clearly show that the use of two different APE1 inhibitors leads to the same effect, proving that APE1 can be considered a good senolytic target. In particular, inhibitors #3 and E3330 can be promising senolytics. Further studies are needed to support these evidences.

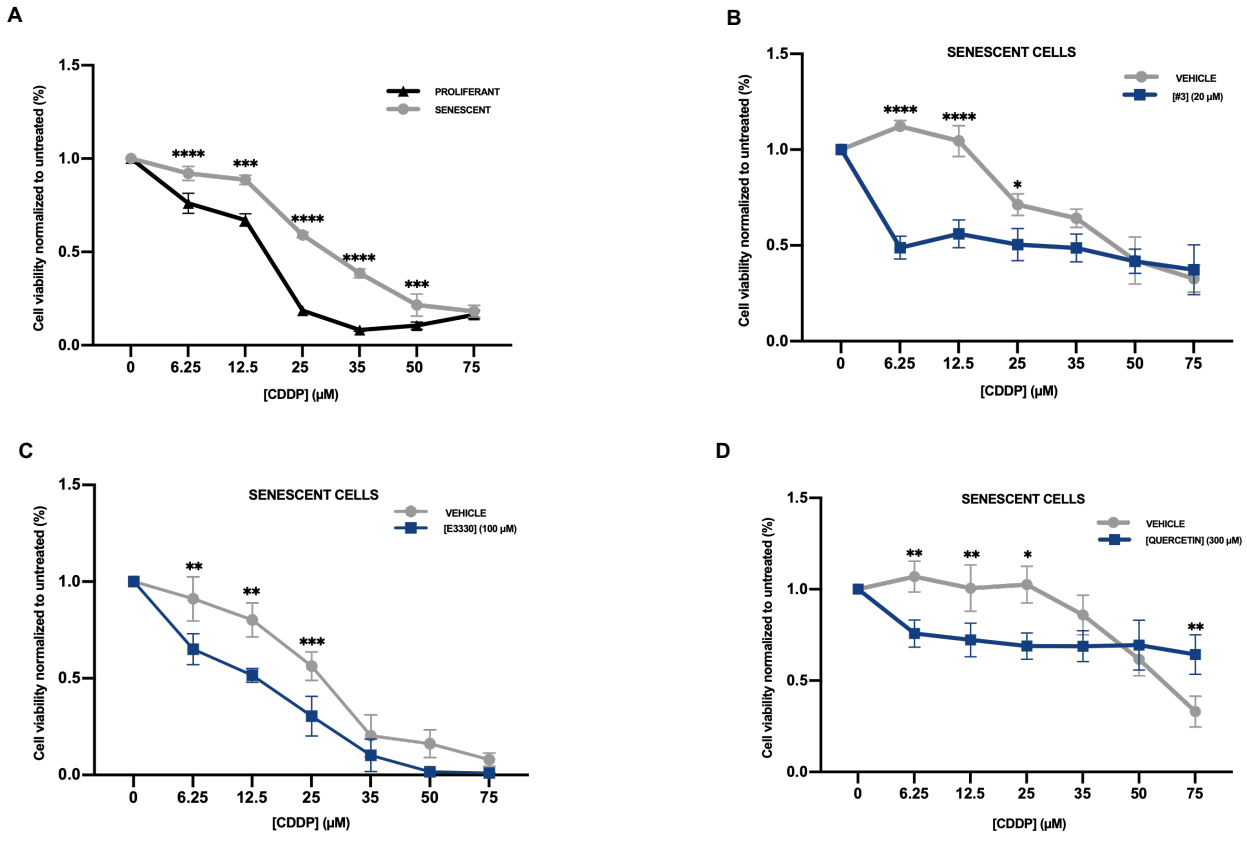


Figure 33. ***Inhibition of APE1 functions affect cellular viability of proliferating and senescent cells.*** Senescence of A549 cells was induced with 6.25 μM CDDP for twenty-four hours. Three days after drug release, proliferating and senescent cells were propagated at a density of 4 x 10^3 cells per 96-well and one day later treated with elevated concentrations of CDDP [6.25 μM-75μM] for forty-eight hours. The viability of the cells was determined using the MTT assay. Results presented in the Histogram **(A)** shows cell viability of proliferating and senescent cells normalized for the untreated condition. Data are expressed as percentages and are given as mean ± SD of three independent replicates. **(B)** Senescent cells treated with 20μM #3 + [6.25 μM-75μM] CDDP for forty-eight hours, **(C)** 100μM E3330 + [6.25 μM-75μM] CDDP for forty-eight hours were less viable than senescent cells treated with CDDP alone [6.25 μM-75μM] for forty-eight hours. **(D)** The natural senolytic Quercetin 300μM in combination with CDDP [6.25 μM-75μM] for forty-eight hours exerts the same effect. Data are presented as mean ± SD of three independent replicates. Asterisks represent a significant difference from untreated cells as control. *P < 0.05, **P < 0.001, ***P < 0.002, ****P < 0.0001 Student's t-test.

FUTURE STUDIES

Preliminary data 1

APE1 could regulate the expression of senescent markers through miR-146a

In addition to the study already performed on the regulation of $p21^{CIP1}$ by APE1 via miR-130b (***Fig.31***), we decided to investigate the role of miR-146a in CDDP-induced cellular senescence. Regarding miR-146a study, literature data show the involvement of miR-146a in numerous molecular processes, including senescence [250]. Among SASP factors, we decided to investigate the modulation of IL-6 by miR-146a and APE1.

From the bioinformatic analysis, miR-146 is comprised among the six miRNAs that were downregulated after silencing APE1 (***Fig. 29***). These data were then confirmed by qRT-PCR analyses in both the A549 cell line in which APE1 was silenced and the CDDP-induced senescence model (***Fig. 30***). We demonstrated that IL-6 is downregulated in proliferating cells in which APE1 was silenced, compared to cells transfected by siSCR (***Fig. 34A***). To assess the involvement of APE1 in the regulation of IL-6 through miR-146a, we analyzed IL-6 mRNA levels following the overexpression of miR146a in a context of APE1 downregulation. In the present experimental setting, we first transfected A549 cells with siSCR and siAPE1 for seventy-two hours, and at forty-eight hours of silencing we overexpress miR-146a using specific mimic for twenty-four hours (***Fig 34B***). These data suggest that the expression of IL-6 is not dependent on APE1 in proliferating cells. Conversely, repeating the same experimental setting performed in the ***Fig. 31A-B***, in senescent cells (at twenty-four hours of CDDP treatment), IL-6 mRNA expression was strongly reduced (***Fig. 34C***). Data in the histogram revealed that APE1 and miR-146a, have an important role in the CDDP induced senescent cells, determining a downregulation of IL-6.

In the model proposed by Bhaumik *et al.* [250], miR-146a indirectly acts on IL-6.

Based on this experimental evidence, we do not have a plausible explanation on how APE1 may act in the regulation on IL-6 through miR-146a.

We are currently investigating the molecular mechanism by which APE1 regulates IL-6 via in our CDDP-induced senescence model (***Fig. 34D***).

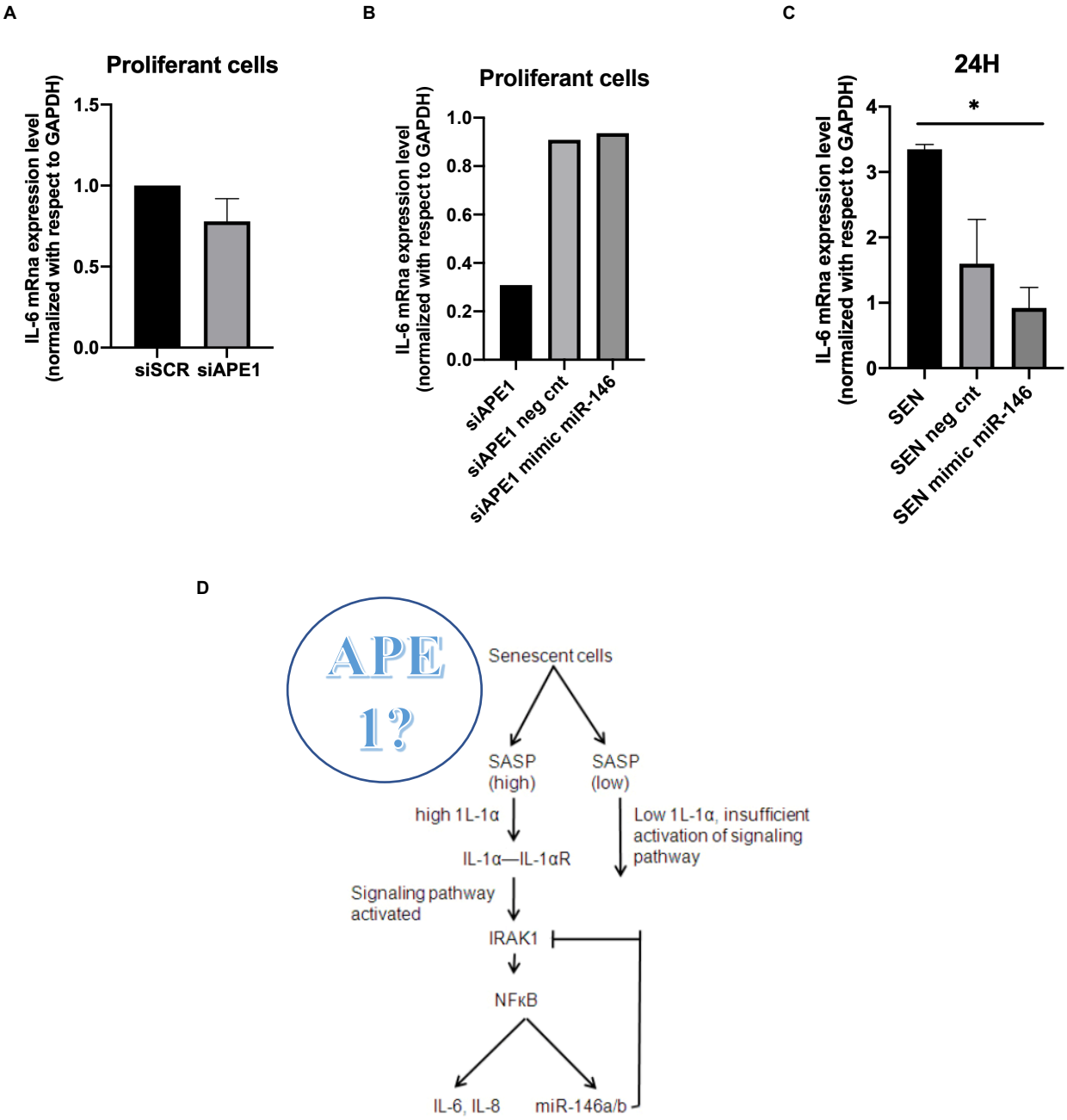


Figure 34. ***IL-6 is regulated by APE1 via miR-146a*****. (A)** IL-6 mRNA level was assessed by qRT-PCR analysis of proliferating cells silenced for APE1 expression. Total RNA was extracted from the A549 cell line expressing APE1 scr or whose APE1 was silenced (siRNA) and reverse transcribed. Histogram shows detected levels of IL-6 normalized to GAPDH levels. **(B)** Histogram shows IL-6 mRNA level assessed by qRT-PCR analysis of proliferating cells silenced for APE1 expression, and overexpressing miR-146a. mRNA level of IL-6 was normalized to GAPDH. **(C)** IL-6 mRNA level was evaluated by qRT-PCR analysis of senescent cells and overexpressing miR-146a. mRNA level of IL-6 was normalized to GAPDH. Data represent means ±SD of two independent experiments. Statistical analysis was achieved by applying 0ne-way ANOVA test. *P < 0.05. **(D)** In this model was proposed the role of miR-146a in cell senescence: in high SASP condition, IL-1α was activated, and interacting with IL-1α R promotes the activation of IRAK1, with consequently activation of NF-kB and production of IL-6, IL-8 and miR-146a. miR-146a may acts downregulating IRAK1 decreasing the IL-6 and IL-8 expression. Adapted from [268].

Preliminary data 2

Evaluating senescent cells using NPs

One of the most commonly used biomarkers to detect cellular senescence is the β-Gal assay. Indeed, senescent cells overexpress lysosomal endogenous beta-galactosidase, which catalyzes hydrolysis in the presence of the X-GAL substrate, leading to the formation of an indole compound. The dimerization of indole, determines the formation of the characteristic blue precipitate that accumulates in the perinuclear region of senescent cells, allowing to distinguish them from those that are not senescent [80].

However, this test can sometimes have limitations, such as the presence of false-negative cells. We can have negative cells in the β-GAL test, due to the substrate-uptake inability. We have an underestimated percentage of senescent cells. With this method, in addition to the β-gal assay, we can determine whether all the cells that uptake the substrate, are actually senescent or not, which is not possible with the classical assay. It allows us to measure the uptake and senescence of these specific cells.

In the present part of the thesis, we worked on the development of a new method for the detection of cells in senescence. Galactose and X-GAL were separately bound to PMO nanoparticles (NPs) previously functionalized with IR -780 as a dye. Galactose was used as a negative control. The synthesis of NPs (***Fig. 35***) was performed in collaboration with Dott. De Marco R., Department of Food, Environmental and Animal Sciences (DI4A), University of Udine. For the generation of NPs, the following protocol has been followed:

Experimental section

Materials and methods

Standard chemicals were purchased from Sigma Aldrich and used as they are. The nanoparticles were purified by centrifugation and analyzed through Dynamic Light Scattering (DLS) (***Fig. 36***) and Zeta Potential (***Table 4***) measurements were

carried out with a Zetasizer Nano ZS (Malvern), the laser He-Ne at 633 nm, Max 4 mW, in polystyrene cuvettes (with optical path length 1 cm).

The infrared spectrum AT-IR was performed by Bruker. H NMR spectra (***Fig. 38***) were recorded using a Bruker apparatus at 400 MHz in 5 mm tubes in D_2O, water suppression was performed by the solvent presaturation procedure implemented in Bruker (PRESAT) [307].

Synthesis of PMO-OH

CTAB (486 mg) was dissolved in H2O/ethanol (88 mL and 33 mL), and 32 wt% ammonia (1.2 mL) solution. The reaction mixture was stirred at room temperature for 1 h before the addition of BTME (1.18 mL).

The above reaction mixture was continuously stirred for an additional time of 48 h at room temperature. The CTAB mesoporous template was removed by stirring with ethanol acid solution (50 mL) and HCl (1.5 g) at 50°C for 6 h. The resulting solid was recovered by centrifugation (6000 rpm, 20 min.), washed with ethanol for 3 times and dried at 60°C under vaccum [307].

Loading with IR-780

The dye IR-780 was dissolved in DMSO to give a final concentration to 2 mg/mL and it was added at 100 mg of the nanoparticles in water. The mixture was sonicated for 10 minutes and stirred for 12 hours at dark. The solid was recovered by centrifugation (6000 rpm, 20 min.), washed with water 3 for times and dried at room temperature [308][309].

Synthesis of PMO-NH_2

To prepare PMO-NH_2 the mesoporous nanoparticles were treated with APTES in presence of TEA in toluene and stirrer overnight. The nanoparticles were recovered by centrifugation and washed with ethanol as above [310].

Loading with Galactose and X-GAL

Galactose or X-GAL (40 mg) were added a mixture of $^{IR\text{-}780}$PMO-NH_2 (20 mg) in water/DMSO (ratio 1:1) and sonicated for 10 minutes. The suspension was followed placed on a stirrer for 48 hours. The solid was recovered by centrifugation and washed with water for 3 times [310].

To quantify the amount of X-GAL functionalized on the PMO was used UV-visible (***Fig. 35***). The galactose amount was quantified by NMR analysis because the UV-visible cannot detect it.

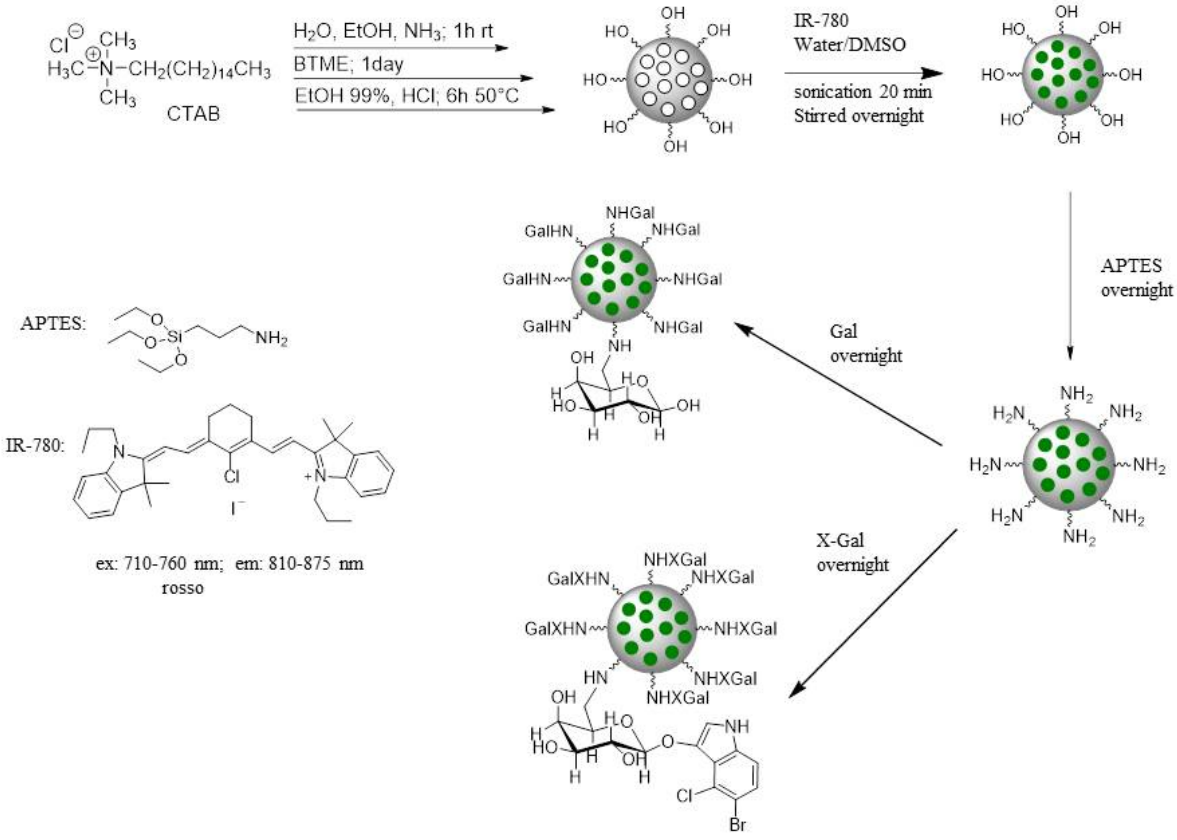
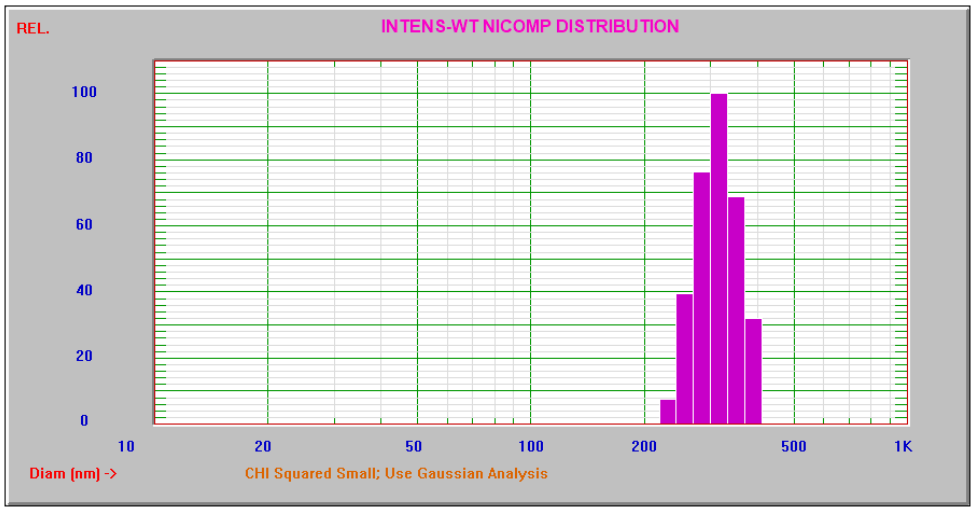


Figure 35. ***Schematic procedure synthesis of*** $^{IR\text{-}780}$***PMO-GAL /XGAL***

NPs	ZP, mV
PMO-OH	-6.0
PMO-NH_2	7.72

Table 4. Zeta potential values of the nanoparticles PMO-OH and PMO-NH_2



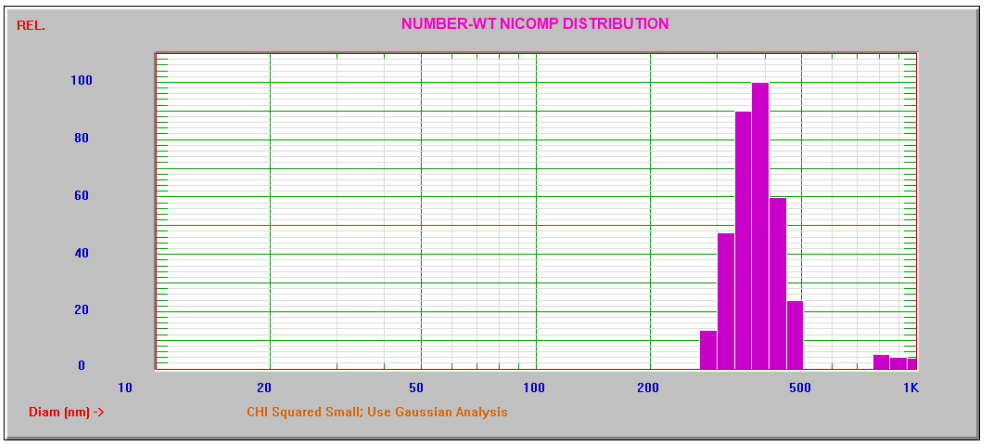


Figure 36. ***Dynamic Light Scattering (DLS) of PMO-OH (top) and $^{IR-780}$PMO-XGal/Gal (bottom).*** PMO-OH showed size between 200-350 nm. $^{IR-780}$PMO-XGal/Gal showed size ca. 300-450 nm.

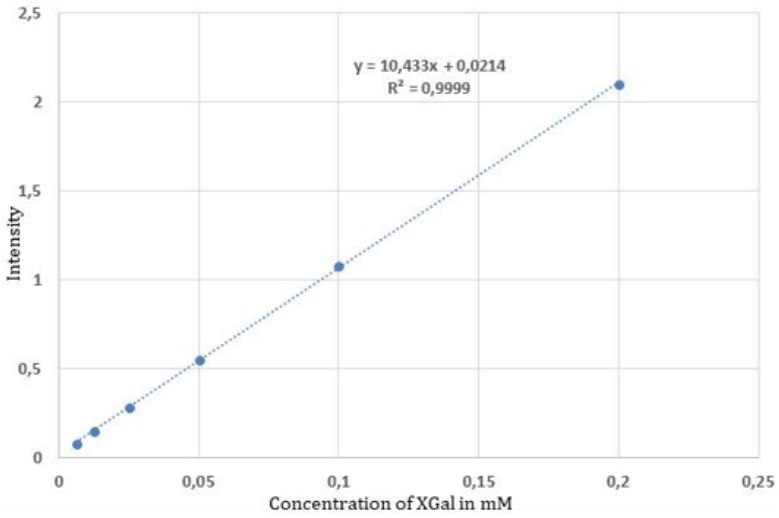


Figure 37. The calibration curve of X-GAL which was obtain by mixing different concentration of X-GAL in water.

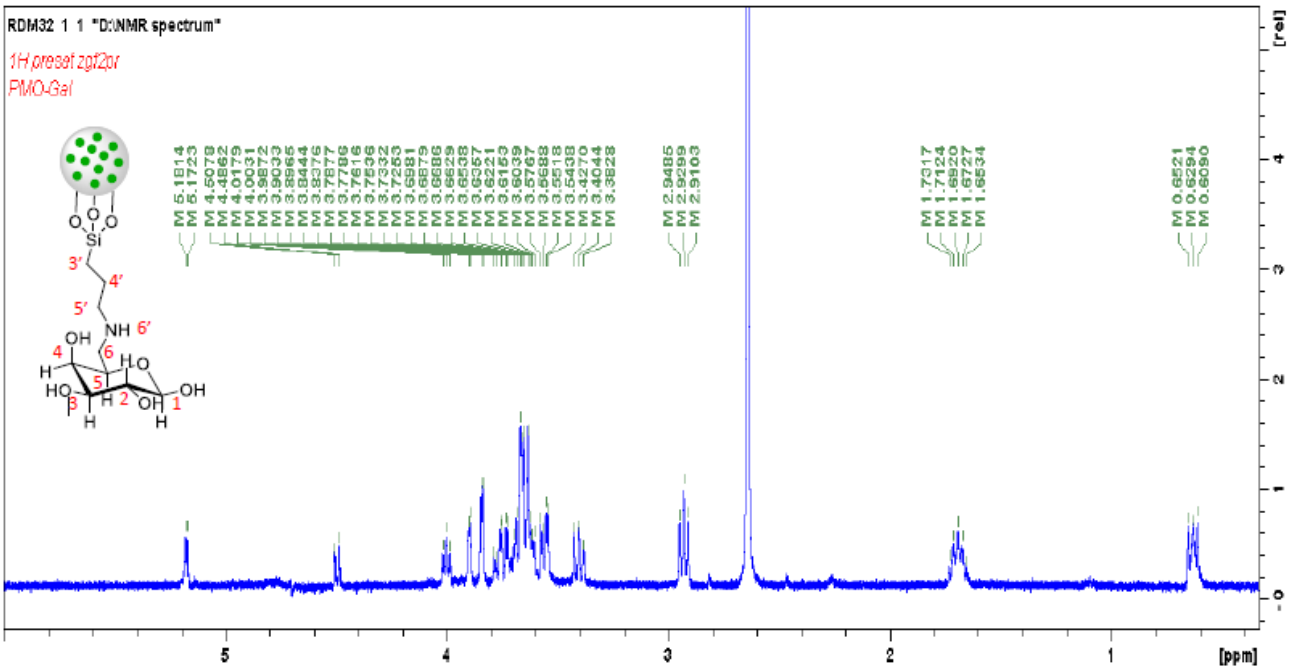


Figure 38. ***NMR analysis of*** $^{IR\text{-}780}$***PMO-Gal.*** The amount of galactose is 0.075 mg for 1 mg of PMO, PMO-Galactose, H NMR (400 MHz, D_2O) δ: 0.63 (t, J = 8.6 Hz, 2H, $AptesH_3$); 1.73-1.65 (m, 2H, $AptesH_4$); 2.93 (t, J = 7.7 Hz, 3H, $AptesH_5$+NH); 3.41 (t, J = 8.8 Hz, 1H, $GalactoseH\beta_2$); 3.56-3.54 (m, 4H, $GalactoseH\alpha_{2,5}+H\beta_{3,5}$); 3.74-3.63 (m, 3H, $GalactoseH\alpha_{3,6}+H\beta_{6'}$); 3.90-3.78 (m, 2H, $GalactosH\alpha_{4,6}$); 4.00 (t, J = 6.1 Hz, 2H, $GalactoseH\beta_{4,6}$); 4.50 (d, J = 8.6 Hz, 1H, $GalactoseH\beta_1$); 5.18 (d, J = 3.6 Hz, 1H $GalactoseH\alpha_1$).

Test on NPs using recombinant β-Galactosidase *in vitro*

To validate the functionality of the X-GAL compound, that is able to generates blue precipitate, 2 mg of PMO-OH, PMO-X-GAL and PMO-GAL NPs were suspended in 5 ml of water at pH 4.5, and β-Galactosidase from Aspergillus oryzae (Sigma, #G5160) was added at 1,000 ppm (5 mg). The same procedure was performed without adding the enzyme to the NPs suspension, as a "blank" control. Suspensions were stirred and placed on rocker in agitation for 1 hour at room temperature. Different aliquots were taken and analyzed. The sample with the addition of PMO-X-GAL was the only that develops a blue precipitation (***Fig. 39A***). These results suggest, the X-GAL conjugated with NP is converted to indole, and then in indigo, in the presence of the recombinant enzyme β-Galactosidase.

Test on A549 cell line using NPs *in vivo*

To test the up-take of PMO-OH, PMO-X-GAL and PMO-GAL NPs *in vivo*, NPs were prepared in 100% DMSO at concentration of 10mg/ml. NPs were subsequentially sonicated with the following programme: number of cycles 5, time 60 sec., pause 60 sec., at low intensity, using Sonicator Bioruptor Plus.

We have used dose and time-dependent cytotoxicity assays to determine the concentration of NPs that does not cause cytotoxicity in cells (data not shown).

Finally, we identified the dose of 50 μg/ml as the optimal concentration to use in cell culture.

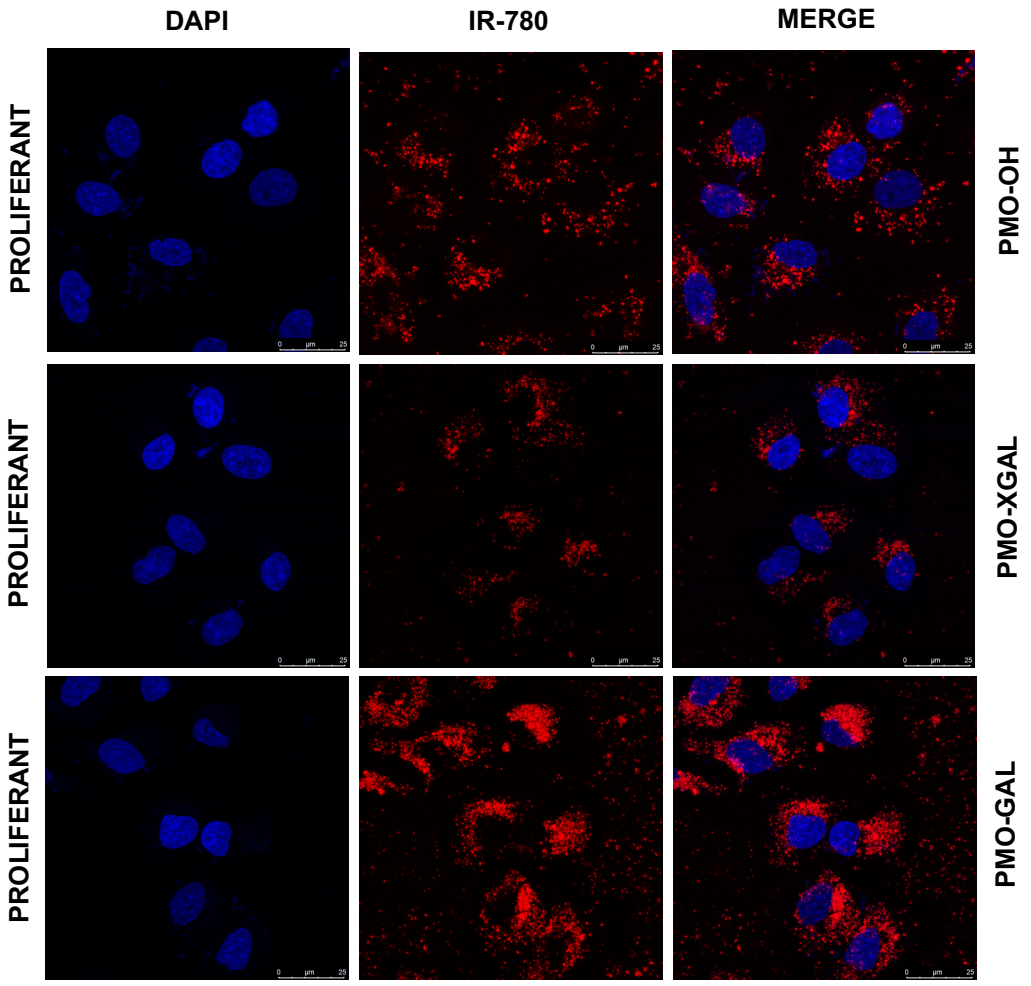
For this purpose, proliferant and CDDP-induced senescent cells, at day four of senescence were seeded at confluency of 0.3 $*10^5$ in glass coverslips, and the following day, cells were treated using the NPs at a final concentration of 50 μg/ml for twenty-four hours in complete medium. Nuclei were counterstained with DAPI mounting medium.

We then checked whether the NPs had integrated properly into the cells. For this purpose, we took multi-color images through sequential scanning of confocal microscope, visualized through a Leica TCS SP laser-scanning confocal microscope (Leica Microsystems), with a 63X oil objective. The images (***Fig. 39 B, C***) show in blue

the cell nuclei stained with mounting medium DAPI and in red the NPs labeled with IRdye-780.

A

B



C

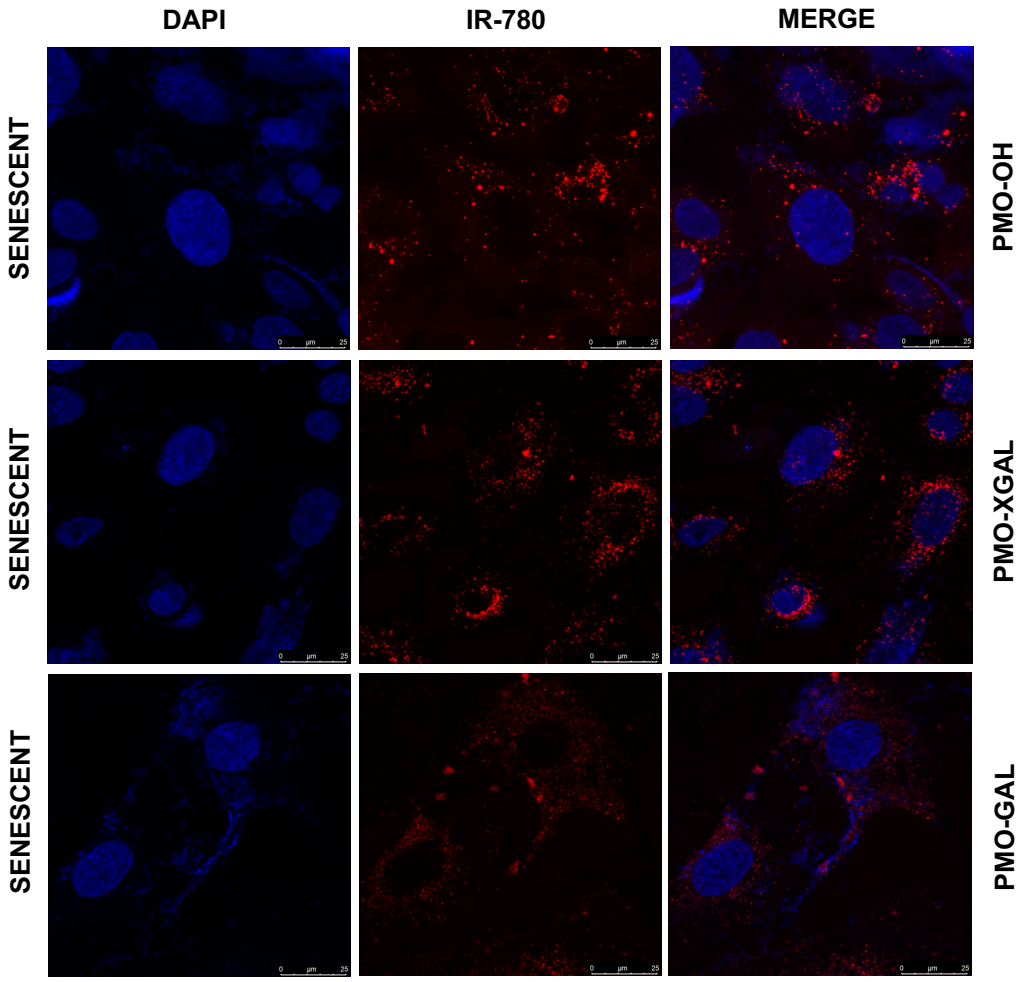


Figure 39. Test on NPs *in vitro* and *in vivo*. (A) The functionality of NPs was assessed by incubating 2 mg PMO-OH, PMO-X- GAL and PMO-GAL NPs with 5 mg recombinant β-Galactosidase enzyme. The blank shown in the figure refers to PMO-X-GAL without β-Galactosidase enzyme. A blank control was also performed for PMO-OH and PMO-GAL NPs (data not shown). 100 µl of each suspension was then applied to slides and the developed staining was observed as shown in the figure on the right. *Confocal immunofluorescence images of proliferating and senescent*. Proliferating **(B)** and CDDP-induced senescent cells **(C)** were treated with NPs at final concentration of 50 µg/ml. Cells show a general redistribution of NPs visible in red, in both proliferating and senescent. Images were acquired with a 63x objective; representative scale bars of 25 µm.

The experiments performed so far, have been useful in finding conditions for non-toxic reactions so that NPs can be used for *in vivo* testing.

In addition to test the uptake of NPs *in vivo*, we also sought to assess the ability of NPs to form the indigo precipitate in senescent cells. For this test, we used the same experimental conditions as for the "uptake of NPs" experiment (***Fig. 39 B,C***). Proliferating and senescent cells were propagated until four days after drug release and treated for twenty-four hours with PMO-OH, PMO-X- GAL and PMO-GAL NPs at a final concentration of 50 µg/ml in complete medium. The day after, the cells were photographed under bright field microscope. As shown in ***Fig. 40***, proliferating and senescent cells treated with PMO-OH, as expected, showed no blue precipitate, whereas with respect to proliferating and senescent cells treated with PMO-GAL and PMO-X- GAL, we could not understand whether the reaction occurred, we need to investigate it further. However, the next step will be to improve the development of an assay protocol to test NPs as an alternative to the classical β-galactosidase assay commonly used for *in vitro* experiments.

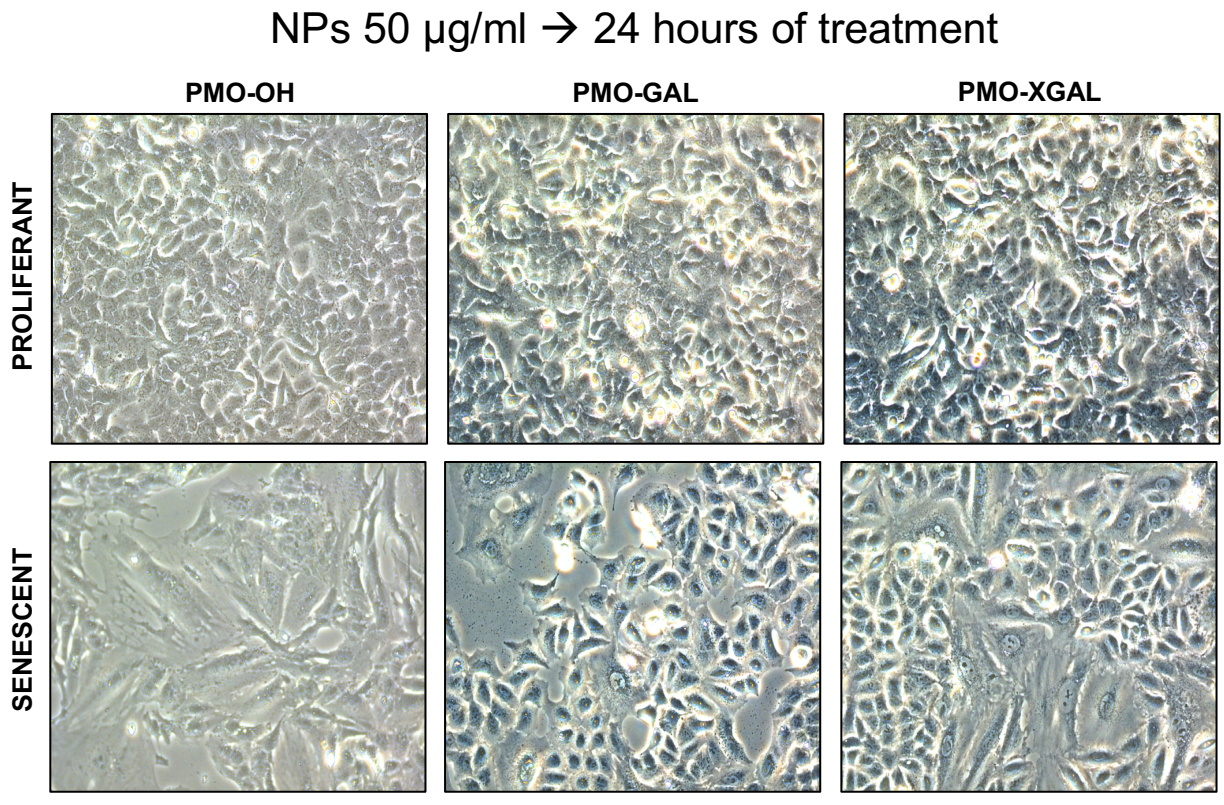


Figure 40. ***Test with NPs in vivo.*** Proliferating and CDDP-induced senescent cells were treated with PMO-OH, PMO-X- GAL and PMO-GAL NPs at final concentration of 50 µg/ml, for twenty-four hours. Images of Proliferating and senescent cells were acquired in bright field using Leica DMi1 microscope.

DISCUSSION

In recent years, several studies focused on understanding the mechanism at the basis of cellular senescence. It has become apparent that both replicative senescence, triggered by telomere shortening, and premature senescence, initiated by various stressors (including oxidative and oncogenic), share a common factor, which is DNA damage [311]. Indeed, in parallel with ageing, there is an accumulation of DNA damage, which could be related to an increase in the production of ROS and a decrease in the repair capacity of DNA [312].

While DNA damage contributes to cellular senescence, DNA repair factors are involved in preventing cell senescence and premature aging by ensuring genomic stability [313]. Indeed, genetic studies in humans and mouse models have shown that mutations in some components of DNA repair pathways are associated with cell senescence, such as that observed in segmental progeroid syndromes (PSs) [314]. It is known that DSBs are repaired by HR and NHEJ (as described in the introduction) [315]. The most common mutations identified in HR involve the Breast Cancer gene 1 (BRCA1) [316]. Although the role of BRCA1 in HR has not been precisely elucidated yet, it is suggested that the dysfunction of this gene may be responsible for the phenotype of accelerated senescence. Instead, deficient factors involved in NHEJ, such as Ku80, Ku70, XRCC-4 and the DNA ligase IV, are responsible for accelerated senescence, as shown in experiments on mouse embryonic fibroblasts (MEFs) [317]. In a recent study of NER (one of the two systems used to repair SSB along with BER), inactivating ERCC1 in a mouse model showed that the mice aged significantly faster. [318]. Among the causes of senescence is ROS production, whose oxidative damage is repaired by BER. However, embryonic elimination of genes essential for BER leads to lethality [319], making it difficult to understand their role in cell senescence. The uncontrolled increase in intracellular levels of ROS can lead to DNA damage and shorten telomeres [320]. It was found that in senescence human bone marrow derived mesenchymal stem cells (hBMSCs), APE1 provides the balance of intracellular ROS. However, prolonged exposure of hBMSCs to oxidative stress has been shown to determine the onset of senescence phenotype, in which expression levels of APE1

are greatly reduced [195]. Other studies have examined the correlation of APE1 with the development of senescence in different cell types [196]. For example, Heo *et al.* observed reduced levels of APE1 in human mesenchymal senescent stem cells, whereas overexpression of APE1 indicated regression of the senescent phenotype [195]. Moreover, the degradation of APE1 was also detected in embryonic stem cells during senescence [321]. Compared to other BER proteins, protein levels of APE1 are significantly reduced, which has been associated with senescence of hTERT-negative primary human fibroblasts in culture over a long period of time [196]. Furthermore, the correlation between the increase in $p16^{INK4a}$ and $p21^{CIP1}$ levels and the decrease in APE1 levels following knockdown or knockout suggests that telomere-induced senescence and/or DDR-signalling pathways are triggered precisely by the lack of APE1 [196]. Therefore, it is emerging that APE1 is a central factor in controlling cell senescence processes. However, APE1 appears to play a role in intrinsic cellular senescence, but its role in an extrinsic cell senescence model has not yet been investigated.

In the present work, we investigated the role of APE1 in the context of extrinsic senescence induced by genotoxic DNA damage. For this purpose, two models of premature senescence were generated, using the A549 cell line. CDDP and DOXO were chosen as genotoxic compounds. In the senescence model induced by CDDP, an increasing number of cells positive for the detection of the enzyme β-Galactosidase was observed in the late phase of senescence (five and eight days after drug release) compared to controls (proliferating cells). In the senescence model induced by DOXO, β-galactosidase enzyme increases as early as the third day after drug release and remains stable until the ninth day. Transcript levels of IL-6 and IL-8, two major SASP factors active in senescent cells compared with control cells, and transcript and protein levels of $p21^{CIP1}$, which is a senescence marker, confirm the establishment of senescence phenotype. These results have allowed us to establish two models of senescence on which subsequently performed further studies. Similar to our data, Sun and coworkers demonstrated a model of induced-cellular senescence by CDDP using the A375 melanoma cell line, in which they observed activation of the SASP response

including IL-1α and IL-1β, followed by increased expression or activity of IL-8 and β-GAL, in addition to upregulation of $p21^{CIP1}$ [322].

Hu and Zhang found that acute Doxorubicin treatment induced senescence of HeLa cells. When the cells grew in drug-free medium for another six days, the transcription and secreted proteins of IL-6 and IL-8 were significantly upregulated [323].

Concerning the analysis of BER enzymes, previous studies ***(Table 5)*** have shown that in parallel with cellular aging, the expression of POL-δ decreases and is thus also related to senescence [324]. A decrease in POL-δ levels leads to a decrease in cell proliferation, DNA synthesis and a delay in repair at the level of DNA strand breaks, which is affected by oxidative stress [325]. It has been shown that during senescence, the link between factor E2F1 and the POL-δ promoter, decreases and methylation of CpG Island 3 increases, leading to a decrease in POL-δ [326]. Although the expression of POL-δ is known to be down-regulated during replicative senescence [326], the mechanisms of POL-δ decline are not yet clear. Ahmed and coworkers provided direct evidence that loss of POL-β is sufficient to induce cellular senescence [327]. They found that homozygous loss of POL-β in mice resulted in an 11.7-fold increase in SA-β-gal activity and a 2-fold increase in p16 expression [327]. In a paper published by Li's research group, they measured XRCC-1 protein levels in the early, mid, and late steps, during replicative senescence using western blot analysis and found a significant increase [196]. The researchers also examined the effect of knockdown of XRCC-1 on senescence in the three normal fibroblasts and found that targeting XRCC-1, increased senescence [196].

In order to more broadly assess the contribution of BER enzymes in our model of CDDP and DOXO inducing senescence, we first characterized the expression of the following BER enzymes: POL-δ, XRCC-1, POL-β, and APE1, both at the transcriptional and protein levels. ***(Table 6)*** Regarding POL-δ in the two models has similar protein expression and follows the same trend of downregulation in the senescent cells compared to the proliferating counterpart. XRCC-1, in both transcription and protein expression levels shows a variable trend between CDDP and DOXO-induced senescence model.

As for POL-β, in the CDDP model, the induction is higher in senescent cells than in proliferating cells twenty-four hours after treatment, while at protein level the induction is observed five and eight days after drug release. Otherwise, in the DOXO model, POL-β mRNA levels are induced from day three to nine. This induction is paralleled what is observed at the protein level.

Unlike all other BER enzymes analyzed in the present work of Thesis, APE1 shows very similar expression in both models. We have shown that upon induction with CDDP and DOXO, the levels of mRNA and protein of APE1 are down-regulated in the senescent cells at an advanced stage compared to the control cells. From the study of the correlation between the enzymes of BER and extrinsic senescence, the effect of senescence on the expression of APE1 is much more evident, confirming what has already been reported in the literature in models of intrinsic senescence [195] [196]. Therefore, as for POL-δ, XRRC-1 and POL-β, whose trends are rather variable. Comparing the data from the literature with our results, the transcriptional and protein expression of the enzymes of BER might be related not only to the type of cell line but also to whether premature or replicative senescence is induced. It is necessary to perform additional detailed studies to understand the effects of senescence on these enzymes. It is known that overexpression of APE1 observed in various tumors correlates with the ability of APE1 to confer resistance to chemotherapeutic treatments. [328] [329]. It was found that when p53 is activated in response to genotoxic treatment, APE1 gene expression is negatively regulated by p53 in human colon carcinoma cells p53(+/+) but not in p53 null mutants [330]. Downregulation of APE1 was also achieved when p53WT is able to disrupt the binding of Sp1 to the APE1 promoter region where histone deacetylase (HDAC) was recruited [330]. However, to better understand when p53 regulates APE1 expression after a genotoxic insult, further studies will be conducted.

In our experiments, in the case of APE1, we hypothesized that its protein downregulation during senescence was due to the stability of the protein.

We could examined this regulation, only in the model of senescence induced by CDDP. The accumulation of the protein after proteasomal inhibition by MG132-

treatment suggests that this downregulation could be mainly due to protein degradation by the proteasome.

Table 5. BERs expression extrapolated from literature data

ENZYME	REGULATION	CELL LINE	REGULATION	REFERENCE
POL-δ	Decreased	Human lymphocytes	POL-δ subunit1 down-regulation was age-dependent	Wang *et al.*, 2012
POL-δ	Decreased	Human embryonic lung diploid fibroblast (2BS) Human fetal lung fibroblast (WI-38)	CpG island 3 methylation, downregulated POL-δ in replicative senescence	Gao S. *et al.*, 2019
XRCC-1	Increased	Primary Human fibroblast (BJ)	XRCC-1 significantly increased during replicative senescence	Li *et al.*, 2018
POL-β	Decreased	Rat brain/neurons	Decline of POL-β was observed in post mitotic tissues than the proliferating tissues	Vyjayanti *et al.*, 2012
POL-β	Unchanged	Primary Human fibroblast (BJ)	POL-β remained unchenged during the exended culturing	Li *et al.*, 2018
APE1	Decreased	Primary Human fibroblast (BJ)	APE1 was down-regulated during replicative senescence	Li *et al.*, 2018
APE1	Decreased	Humane bone marrow derived (hBMSCs)	Replicative senecence and stress induced senescence with hydrogen peroxide	Heo *et al.*, 2009

Table 6. BERs expression in A549 CDDP/Doxo-induced senescence during senescence development.

ENZYME	A549 CDDP-induced senescence 24H → 8TH DAY mRNA Senescent cells Protein Senescent cells	A549 DOXO-induced senescence 72H → 9TH DAY mRNA Senescent cells Protein Senescent cells
POL-δ	Decreased Decreased	Increased Decreased
XRCC-1	Decreased Decreased	Increased Increased
POL-β	Decreased Increased	Increased Increased
APE1	Decreased Decreased	Decreased Decreased

The data described above are consistent with those previously published by our research group, in which an inhibition of the N-terminal cleavage of APE1 was observed following inhibition of the proteasome by MG132-treatment in OCI/AML3 cells [296]. We do not know whether there is also a contribution from Post-Translational Modification (PTM) on APE1. For example, it is reasonable to assume that APE1 could be ubiquitinated during extrinsic senescence and then degraded by the proteasome. Further studies are therefore required to elucidate this mechanism.

We also aimed to analyze the cellular distribution of APE1 during CDDP-induced senescence. It is known that APE1 is mainly localized in the nucleus, [331] [332]. Although a small fraction is also present in both cytoplasm of proliferating and senescent cells, we were able to also detect the truncated form (p33) of APE1 only in senescent cells in addition to the full-length form (p37), as determined by sub-fractionation of nuclei and cytoplasm. We then analyzed, through immunofluorescence, the distribution of APE1 within the cells and observed a general distribution of APE1 at the cytoplasmic level in the senescent cells, which was much more evident than in the proliferating cells. Moreover, the quantification analysis of fluorescence intensity performed on nuclei of proliferating and senescent cells confirmed the reduction of APE1 amount associated with extrinsic senescence. It has been previously demonstrated that APE1 is able to promote the binding of p53 to DNA and the transactivation the $p21^{CIP1}$ gene (CDKN1A), a cyclin-dependent kinase inhibitor 1A [168] [168]. It is known that p53 is a transcriptional activator involved in DNA damage response and causes the following: cell cycle arrest in phase G1, senescence, apoptosis [333] [334]. We investigated the APE1 effect on $p21^{CIP1}$ and later on IL-6/8 in our CDDP-induced senescence model by using specific APE1 siRNAs. Analysis of silencing on APE1 itself showed that the effect of APE1 depletion is clearly visible in cells as early as twenty-four hours after CDDP-treatment, both at the transcriptional and protein levels. However, in the later stages of senescence, the effect of silencing is lost, even though the down-regulation of APE1 in senescent cells is still visible. This reduction is probably related to the effects of senescence on APE1 and not on the silencing effect ‘per se’. CDDP treatment induces the expression of $p21^{CIP1}$, as already observed in the two models of senescence. By analysing the expression level of p21, we found that in proliferating cells, the decrease in APE1

regulation is accompanied by an increase in $p21^{CIP1}$, both in mRNA and protein levels after twenty-four hours of treatment (corresponding to seventy-two hours of APE1 silencing, as described in the Results section). In senescent cells, activation of $p21^{CIP1}$ expression is observed after twenty-four hours of treatment with CDDP, as expected. In the analysis performed up to the fifth day after drug release, we observed the activation of p21CIP1 in proliferating cells in which APE1 is downregulated. This activation could be the consequence of the loss of the effect of silencing APE1.

In senescent cells, the activation of $p21^{CIP1}$ in cells transfected with siSCR and siAPE1 are very similar, indicating that the activation of $p21^{CIP1}$ by CDDP is independent of APE1.

As a multiple functional protein, APE1 can increase the DNA-binding activity of several transcription factors, including NF-κB, AP -1, and p53, through its redox function [335]. IL-6/8 are known to be regulated by NF-κB and AP-1 [336] [337], and APE1 has been shown to be involved in the secretion of IL-6 and IL-8 and the activation of NF-κB in BMSC [338] [339].

Since our previous results showed that IL-6 and IL-8 were significantly increased in the senescence model induced by CDDP and DOXO, we decided to investigate in detail the possible role of APE1 in the regulation of IL-6/8 in the senescence model induced by CDDP. To confirm the relationship between APE1 levels and IL-6/8 expression, we downregulated APE1 with a specific siRNA. The results of our study showed that both IL-6 and IL-8 expression were closely related to the expression level of APE1 in the CDDP model. Indeed, knockdown of APE1 during twenty-four hours treatment was more effective on the expression of IL-6/8 in proliferating and senescent cells. Since the depletion of APE1 by RNAi was transient, the effect of silencing APE1 after five and eight days from drug release, was not as evident as in the beginning. Altogether these results demonstrated that the expression of SASPs IL-6 and IL-8 genes is dependent on APE1 in the early phase of senescence, that we indicated as twenty-four hours of treatment, while, in advanced senescence stages (at day five and eight of senescence) the effect is lost, suggesting that the regulation of SASP factors is not dependent on the APE1 expression.

Recent studies from our research group have shown that APE1 can affect post-transcriptional gene expression by regulating miRNAs, thus influencing the chemoresistance phenomenon [186]. Indeed, APE1 was found to interact with the microprocessor DROSHA under baseline conditions, promoting the post-transcriptional maturation of miR-221/222 and affecting the gene expression of the PTEN gene and the signaling pathway of AKT [186]. Considering the role of APE1 also in RNA biology, and specifically on miRNAs regulation, we therefore investigated this modulation in our CDDP-induced senescence model.

Senescence and miRNAs may interact in several types of molecular mechanisms, especially those related to chemoresistance and aging [251]. Indeed, some miRNAs, such as miR-130b, have been identified as cell cycle regulators associated with senescence. Since $p21^{CIP1}$ is a well-known validated target genes of miR-130b [262] [263], as reported in the introduction section, we examined the relationship between miR-130b and $p21^{CIP1}$, under conditions of transient silencing of APE1.

We found that downregulation of miR-130b in A549 cells, upon APE1 depletion, was significantly correlated with upregulation of $p21^{CIP1}$, which is capable of promoting cell cycle arrest in response to various stimuli [340]. Indeed, overexpression of miR-130b leads to a decrease in the expression of $p21^{CIP1}$, which was confirmed at both the transcriptional and protein levels. However, when A549 cells treated with CDDP, where the $p21^{CIP1}$ level has been already activated, the effect of regulation by APE1 through miR-130b was not very strong, indicating that $p21^{CIP1}$ expression is not only dependent on APE1 through miR130b during cell senescence. These results are suggestive for a possible role of APE1 through miR-130b, in controlling cell cycle progression by regulating $p21^{CIP1}$ [341] [342] **(*Fig. 41*).**

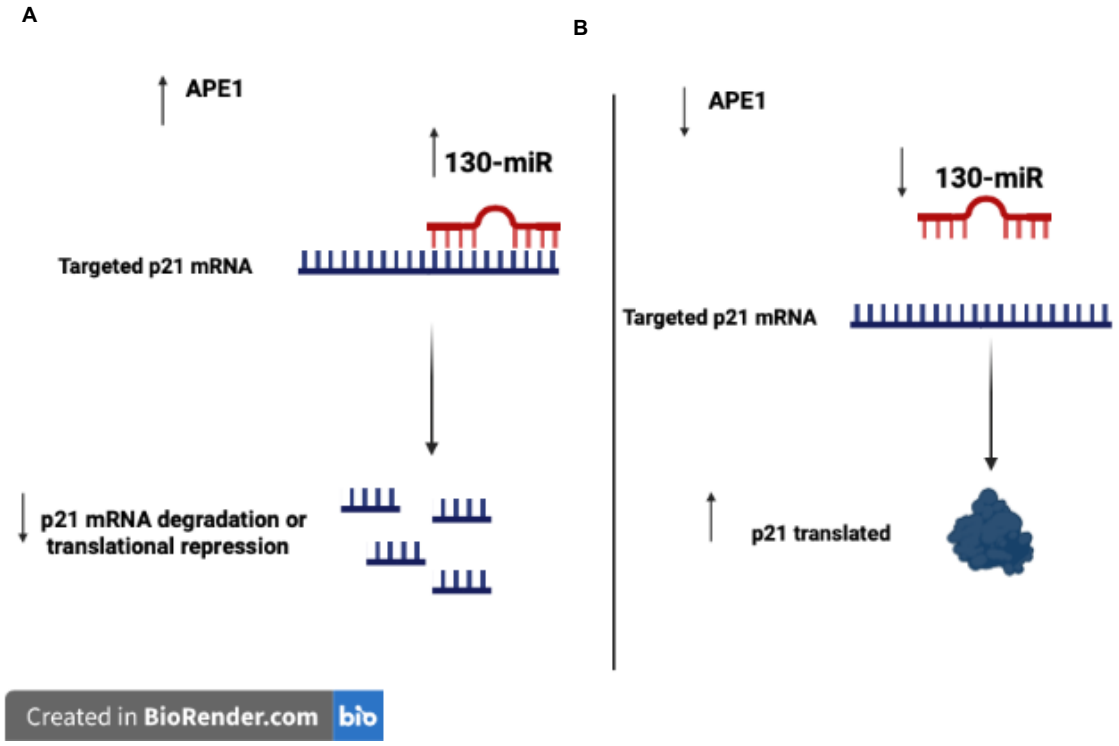


Figure 41. ***Schematic representation of the modulation of p21^{CIP1} by APE1 via miR-130b.*** **(A)** APE1 determines the downregulation of p21^{CIP1} by miR-130b, which recognizes p21^{CIP1} mRNA and promotes degradation or repression under basal conditions. (*See Results section for more details*) **(B)** Down-regulation of APE1 determines down-regulation of miR-130b, and under these conditions, p21^{CIP1} is correctly transcribed and translated. (*For more details, see the Results section*) Schematic representation was generated using BioRender software**.**

Cellular senescence is known to be a phenomenon related to cancer resistance [304]. However, the impact of senescence on cancer therapy is not fully understood [343]. Currently, the most widely used strategy is the use of agents that selectively kill senescent cells and are applied to tumors and many aging-related diseases, these agents are called senolytics [211].

In this context, since we observed that APE1 was downregulated in the senescence CDDP-induced model, our work was aimed to assess the involvement of the APE1

residual fraction in sensitization of senescent cells after inhibition of APE1 functions and after chemotherapy exposure.

APE1 is activated in a variety of cancers, such as lung, prostate, ovarian, colon, pancreatic, and leukemia cancers, resulting in increased aggressiveness [344]. APE1, as a redox signaling protein, is able to activate the transcriptional activity of STAT3, HIF-1α, NF-kB, and other transcription factors to promote cancer cell growth, migration, and survival, while also promoting inflammation and angiogenesis in the tumor microenvironment [344]. The downstream transcription factors of APE1 are involved in the promotion and progression of many cancers, and inhibition of APE1 redox signaling reduces growth and progression in various tumors. Although increased expression of APE1 in various tumors was correlated with their resistance to radiation and chemotherapy, inhibition of APE1 endonuclease/redox activity restored the sensitivity of cancer cell lines to radiation and anticancer drugs [345].

Analyzing our model of CDDP-induced senescence, we found that senescent cells were more resistant to CDDP-treatment compared to proliferating cells. Our data showed that we achieved sensitization to chemotherapy when we used two different APE1 inhibitors such as compound #3 (which blocks endonuclease activity generating an accumulation of abasic sites on DNA) and E3330 (which impairs redox function on NF-κB and other Transcription Factors). When we performed treatment with the two APE1 inhibitors on senescent cells, the observed effect was similar to the effect of treatment with quercetin, a known senolytic agent [366], when used alone or in combination with CDDP.

Therefore, the results obtained suggest a possible use of APE1 inhibitors as potential senolytic drugs. APE1 can be considered a good candidate as target for senolytic compounds. Further studies are needed to corroborate these findings.

CONCLUSIONS

In a model of premature senescence induced with two different genotoxic compounds, i.e., CDDP and DOXO, APE1 mRNA and protein levels decrease during CDDP/DOXO-induced senescence in A549 cells. We demonstrated that gene expression of IL-6 is dependent on APE1 24 hours after CDDP-induced senescence, while this effect is lost at later stages.

We investigated on miR-130b, a miRNA regulated by APE1, that is downregulated in senescence in concert with APE1 downregulation. In a model of CDDP-induced cancer cell senescence, down-regulation of miR-130b is associated with deregulation of APE1. The observed upregulation of $p21^{CIP1}$ protein level under APE1 silencing confirms the hypothesis that APE1 may contribute to the onset of senescence with the induction of SASP factors as observed in the early phase of senescence and modulating $p21^{CIP1}$ (validated target gene of miR-130b) by regulating miR-130b expression.

We also demonstrated that, CDDP-induced senescent cells are resistant to treatment with higher doses of CDDP. The APE1 inhibitors Compound #3 and E3330 sensitize senescent cells to CDDP, indicating that APE1 can be considered a good senolytic target (***Fig. 42***).

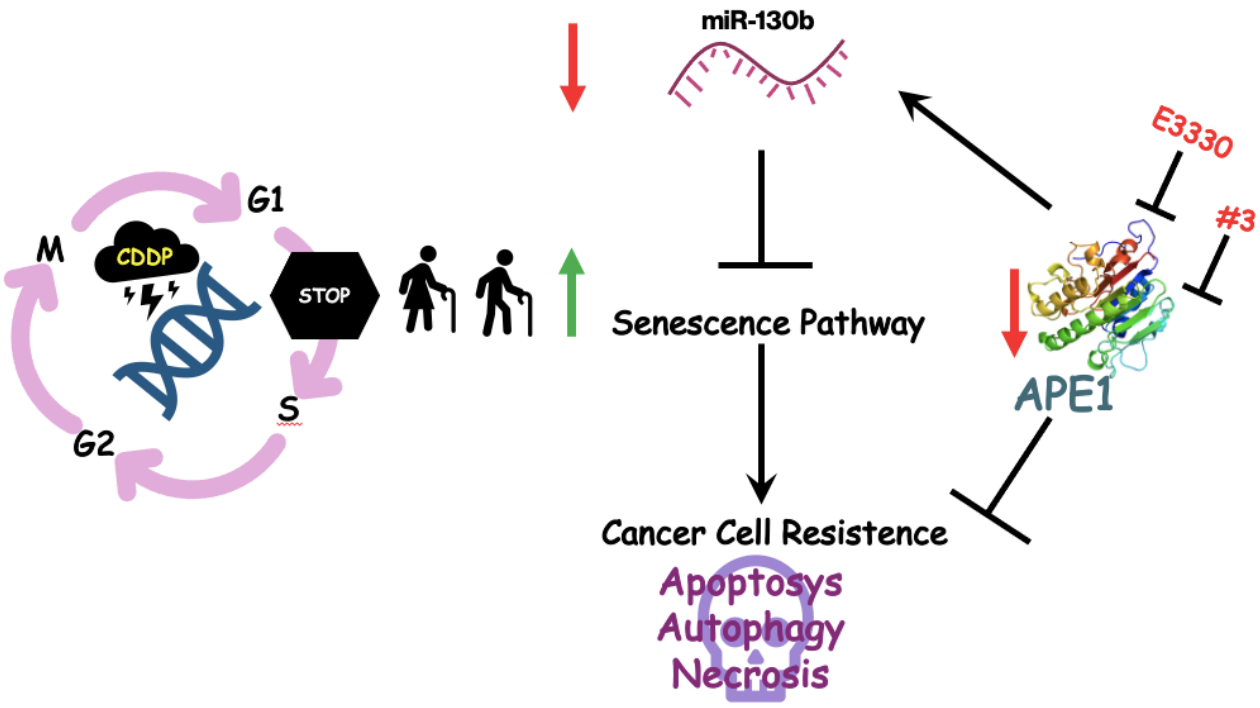


Figure 42. ***Proposed senescence CDDP-induced model.*** In the present proposed model of CDDP-induced senescence, the cell cycle is down-regulated in G1 phase, promoting cellular senescence and chemoresistance. APE1 plays a role in the early stages of senescence through the induction of SASP factors and the dysregulation of miRNAs. Inhibition of APE1 activity by COMPOUND #3, which blocks endonuclease activity, and E3330, which blocks redox function, exterts a senolytic effect and renders senescent cells more sensitive to CDDP treatment.

FUTURE PERSPECTIVES

The results shown in this work have provided evidence for the first time that APE1 is downregulated during extrinsic senescence. A similar result has previously only been obtained in the context of intrinsic senescence, as shown in the work of Heo *et al.* 2009 and Li *et al.* 2018. In addition to demonstrating that APE1 down-regulation occurs through the proteasome [296], it will be interesting to define a molecular mechanism that explains this degradation in the models of senescence proposed in this work. By silencing APE1, we simulated the same conditions of down-regulation of APE1 in senescence, and we checked the effect of silencing APE1 on the expression of SASP factors (IL-6/8) and $p21^{CIP1}$ in the CDDP-induced senescence model. However, we do not know whether silencing of APE1 can also affect the expression of SASP and $p21^{CIP1}$ factors in the DOXO-induced senescence model. Therefore, an investigation also in the DOXO-induced senescence model might be useful both to confirm the data obtained so far and to provide new elements for further speculation on the role of APE1.

Recently, our research group has shown that APE1 plays a role in RNA metabolism, pri-miRNA processing and stability under genotoxic stress conditions, through association with the Drosha processing complex [186]. Regulation of miRNAs by APE1 was also investigated in our model of CDDP-induced senescence, in particular, we first analyzed modulation of $p21^{CIP1}$, a target gene of miR-130b [262] [263], by APE1 via miR-130b and then also investigated APE1-miR-146a axis regulation in relation to SASPs. In preliminary section 1, experiments performed in proliferating cells show that the expression of IL-6 is not dependent on APE1 under conditions of APE1 silencing and miR-146a overexpression. On the contrary, when the same experimental setup is repeated in the CDDP-induced senescence model, the mRNA levels of IL-6 are downregulated, suggesting that this downregulation may be dependent on APE1. However, because IL-6 is not a direct target of miR-146a [262], we do not know the molecular mechanism behind this regulation. Therefore, our future studies will aim to better investigate this molecular mechanism by which APE1 is able to modulate IL-6 through miR-146a in the context of extrinsic senescence.

APE1 is a very interesting protein that is being studied for its multiple functions [179][180]. However, there are limited informations on the functions of APE1 in the context of intrinsic and extrinsic senescence. One of the future goals is to answer the question whether it is possible to observe effects on the modulation of $p21^{CIP1}$ by APE1 via miR-130b in both early and advanced stages of senescence when the functions of APE1 are specifically inhibited. That is, when compound #3 (which blocks endonuclease activity and produces an accumulation of abasic sites on DNA) and compound E3330 (which alters redox function on NF-kB and other transcription factors) are used.

Finally, we are currently working on a new method to detect senescent cells (*see Preliminary Data 2*) based on the development of nanoparticles-PMO (NPs) previously functionalized with IR-Dye-780 and then conjugated with X-GAL located on the outer surface of the particle. The NPs can be used to determine whether all cells that take up the substrate are indeed senescent. Tests using NPs on senescent and non-senescent cells are currently under investigation in our laboratory.

To increase internalization and selectivity of the previous system, we will bind the NPs with the RGDFK cycle (peptide cycle, has the side chains of arginine, glycine, and aspartic acid and the phenyl group on lysine) simultaneously with Galactose and X-GAL (***Fig. 43***). The cycle selectively binds the overexpressed integrins on the extracellular matrix of cancer cells. Synthesis is currently ongoing. With this system, we can apply these NPs to cancer cells to stop the cells overexpressing integrins, which are the ligand of the cyclic peptide.

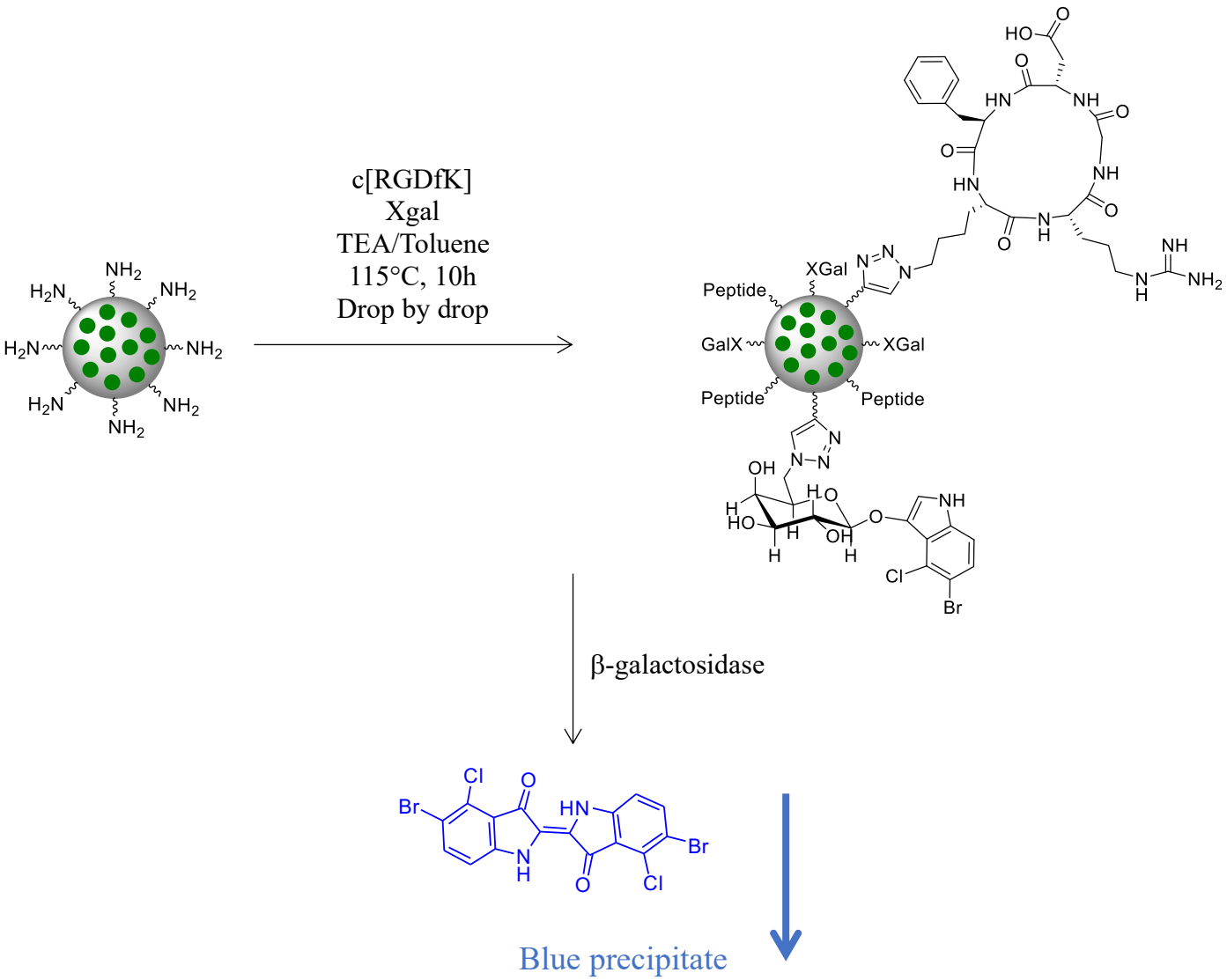


Figure 43. ***Internalization system using NPs conjugated with cyclic peptide RGDFK.*** The picture shows the synthesis of the NP, on which the cyclic peptide RGDFK (consisting of side chains of arginine, glycine, and aspartic acid and the phenyl group on lysine) is conjugated on the outer surface. In addition to RGDFK, the NP is combined with X-GAL. Uptake of NPs by the senescent cell, in which high levels of the enzyme β-Galactosidase are present, results in the formation of a blue precipitate due to dimerization of the compound, which contains an indole.

Currently, nanomaterials are used in nanomedicine as diagnostic and therapeutic tools in the treatment of chronic human diseases through the delivery of site-specific and targeted drugs [346].

EXPERIMENTAL METHODS

Cell line

A549 (adenocarcinomic human alveolar basal epithelial cells) cells were grown in RPM1 (Euroclone); were supplemented with 10% v/v fetal bovine serum, 100 U/ml penicillin, 10 µg/ml streptomycin sulphate and cultured in a humidified incubator containing a 5% CO_2 atmosphere, at 37 °C.

Cell viability

A549 cell viability. A549 cells were seeded in triplicate at the density of 4*10^3 cells in 96-well plates. After 24 hours, cells were washed with PBS and treated for 24, 48 and 72 hours with the following genotoxic agents: cis- Diammineplatinum(II) dichloride (CDDP), (Sigma Aldrich, Milan Italy), dissolved in dimethylformamide (DMF), Doxorubicin hydrochloride (Sigma Aldrich, Milan Italy), dissolved in UltraPureTM DNase/RNase-Free Distilled Water (InvitrogenTM, Thermo Fisher Scientific, Waltham, Massachusetts, United States) at the indicated concentrations (see results section) .

Cell viability on proliferant and senescent cells. Cell viability was performed five days drug relase. Proliferant and senescent cells were propagated at density of 4*10^3 per 96-well, three days after drug relase, and the day after treated with the indicated concentration of CDDP (see results section), or with CDDP in combo with APE1 redox inhibitor E3330 (Fishel, et al., 2007, Kelley et al., 2011), or APE1 endonuclease inhibitor #3 (Rai, G. et al. 2012), or Quercetin (Sigma-4951) at the indicated concentrations (see results section) for 48 hours. Cell viability was assessed through the MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay – Promega) as per manufacturer's instructions. The values of absorbance were indicative of cellular metabolic activity and were measured at 490nm, with EnSpire 2300 Multilabel reader, (Perkin Elmer, Waltham, Massachusetts

United States). Three biological replicates were performed for each treatment. Each recorded absorbance value was standardized with the absorbance value of the wells that contained medium only.

Induction of extrinsic senescence and senescence associated-beta-galactosidase (SA-β-gal) activity. A549 cells were seeded 0.2*10^6 cells in 6-well plates. Senescence was induced by treating cells with the DNA-damaging agent DOXO (0.5 μM) for 72 hours, and with CDDP (6.5 μM for 24 hours). After 72 hours and 24 hours for DOXO and CDDP treatment respectively, cells were extensively wash twice with PBS, and complete medium was added. The development of a fully senescent phenotype was obtained from 3 to 9 days (DOXO), from 5 to 8 days (CDDP), after drug relase.

Senescence associated-beta-galactosidase (SA-β-gal) activity. Staining for SA-β-gal was performed using Senescence β-Galactosidase Staining Kit #9860 (Cell Signaling Technology) according to the manufacturer's instructions. Cells were photographed with Leica DMi1 microscope at 20× magnification. The percentage of cells positive for SA - β-gal was determined by counting seven random fields under a bright field microscope and analyzed with ImageJ software. At least 200 cells were counted for each independent determination.

Preparation of cell extracts and protein quantification.

Whole cell extracts (WCE). Cells were washed with PBS and collected by trypsinization, centrifuged at 1200 rpm for 3 minutes at room temperature. Cellular pellets were resuspended with lysis buffer (RIPA - 50mM Tris HCl pH 7.5, 150mM NaCl, 1mM EDTA pH 8.0, 1% Triton X-100), supplemented with 1× protease inhibitor cocktail (Sigma), 1 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, at 4 °C, for 30 min. The lysates were centrifugated at 12,000 × g for 30 minutes, at 4 °C. In the end the supernatant was collected.

Nuclear cell extracts (NCE). Cells were washed twice with PBS, collected in PBS complemented with DTT 0.1mM and PMSF 0.5mM and centrifuged at 1000rpm for 10 minutes at 4°C. Cellular pellets were resuspended with T1 solution (HEPES pH 7.9, 10mM, KCl 10mM, MgCl2 0.1mM, EDTA pH 8.0, 0.1mM) and centrifuged at 2400 rpm for 10 minutes at 4°C. Cytosolic proteins were collected in the supernatant fractions, and kept on ice. Nuclear pellet was lysated in T1 solution, centrifuged at 2400 rpm for 10 minutes at 4°C in order to obtain nuclei. The supernatant was discarded and pellet consisting in the nuclear fraction was resuspended with T2 solution (HEPES pH 7.9, 20mM, NaCl 420mM, MgCl2 1.5mM, EDTA pH 8.0, 0.1mM, Glycerol 5%), for 30 minutes. The samples were centrifuged at 12000rpm for 20 minutes at 4°C and the supernatant comprises nuclear proteins was collected and kept on ice. Both cytosolic and nuclear extracts were subsequentially quantified for protein analysis.

Western blot analysis

Antibodies and western blotting analysis. The Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) was used in order to determine the protein concentration, according to the manufacturer's instructions. For western blotting analyses, whole- cell lysates, cytosolic and nuclear lysates were prepared, and 10 µg of proteins were resolved on 10%/12% gel SureCastTM Acrylamide Solution (40%) (Invitrogen, California, United States) and transferred in nitrocellulose membrane, (Amersham, United Kingdom). Western blot analyses were executed using the primary antibodies listed in the following table:

Antibody	Code number - Company	Dilution
APE1	13B8E5C2-Novus	1:1000
β-tubulin	T0198-Sigma-Aldrich	1:2000
DNA Polymerase β	Ab26343-Abcam	1:1000
DNA Polymerase δ	Ab10362-Abcam	1:1000
$p21^{Cip1}$	#2947-Cell Signaling	1:1000
XRCC-1	MA5-12071-Invitrogen	1:1000
LSD1	Ab129195-Abcam	1:5000
Actin	A2066-Sigma-Aldrich	1:2000

The incubation with primary antibodies were followed by washing membranes three times with PBS-0.1% Tween-20, (Sigma Aldrich, Milan, Italy), incubated for 1 hour at room temperature with the appropriate IRDye800/IRDye600 labelled secondary antibodies (diluted to 1:5000). Data normalization was performed by using monoclonal anti-tubulin and anti-Actin as indicated. Detection and quantification was performed with the Odyssey CLx Infrared imaging system, the membranes were scanned in one channel using an Odyssey IR imager; the relative signal of protein bands were quantified using Odyssey software (Image Studio 5.0).

Immuno-fluorescence

For immuno-fluorescence analyses A549 proliferant and senescent cells grown on glass coverslips were fixed with 4% paraformaldehyde (PFA) for 20 min; PFA was removed by rinsing the cover glass three times with Wash Buffer A (WBA – 150mM NaCl, 10mM Tris HCl, 0.05% Tween 20). To permeabilize cells, Triton X-100 0.25% in PBS was used for 5 min at room temperature on a rocking platform. Cells were incubated with 10% fetal bovine serum (FBS) in WBA for 30 minutes at room temperature on a rocking platform to avoid the a-specific binding oof the primary antibody. The incubation with the primary antibody (Anti APE1 – Vovus, NB 100-101) occurred over night on a humified chamber at 4°C. AlexaFluor®488-conjugated secondary antibodies (Jackson ImmunoResearch) were used for detection. Nuclei were counterstained with DAPI mounting medium (Invitrogen). Cells were visualized through a Leica TCS SP laser-scanning confocal microscope (Leica Microsystems)

equipped with a 488-nm argon laser, and a 63X oil objective (HCX PL APO 63X Leica). Multi-color images were captured through sequential scanning.

siRNA transfection

A549 cells cells were seeded at $0.2*10^6$ in six-well. The day after, cells were transfected with 100 pmol of custom hAPE1 siRNA per well (see hAPE1 siRNA sequence below). As a control was used - targeting siRNA pool (siSCR) transfection. Both siRNAs were supplied by GE Healthcare Dharmacon, (Colorado, Unites States). The transfection was performed with DharmaFECT transfection reagent (Thermo Fisher Scientific, Massachusetts, United States), according to the manufacturer's instructions. After 6 hours of transfections, fresh medium was added to the complexes for reducing cytotoxicity. The day after transfection cells were washed twice with PBS, fresh media was added and after 48 hours from transfection cells were collected.

Custom hAPE1 siRNA sequence:

Sense: 5' UACUCCAGUCGUACCAGACCUUU 3'

Antisense: 5' AGGUCUGGUACGACUGAGUAUU 3'

siSCR used is a pool of selected siRNAs, for this reason no sequences were provided by GE Healthcare Dharmacon.

Gene expression analysis - Quantitative real-time reverse transcriptase-PCR.

For the analysis of gene expression profiles, cells were collected and total RNAs were extracted with NucleoSpin ® RNA, (Macherey Nagel, Germany), according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using SensiFAST TM cDNA Synthesis Kit, (Bioline, Ohio, United States) according to the manufacturer's instructions, setting the following program:

- 25 °C for 10 min (primer annealing)
- 42 °C for 15 min (reverse transcription)
- 48 °C for 15 min (additional step for highly-structured RNA)
- 85 °C for 5 min (inactivation)
- 4 °C hold

cDNA were tested for IL-6, IL-8, $p21^{Cip1}$, APE1, DNA polymerase δ, DNA polymerase β gene expression. The housekeeping gene GAPDH was used for normalization. SensiFAST TM SYBR® No-ROX Kit, (Bioline, Ohio, United States), was used in qRT-PCR, according to the manufacturer's instruction. DNA was amplified in 96-well plates and 10 μM of the specific sense and antisense primers in a final volume of 15 μl for each well. Each sample analysis was performed in triplicate. The negative control was prepared with a sample without template. Reactions were run on CFX96 Real-Time System, (Bio-Rad, California, United States), applying the following cycling parameters:

- 95° C for 10 seconds (denaturation)
- 95° C for 30 seconds
- 60° C for 30 seconds repeated for 40 times (annealing/extension)
- 65° C for 31 seconds (melt curve 65 to 95 increment)
- 65° C for 10 seconds

The analysis was executed performing three biological replicas. Data were resolved applying ΔΔct method.

Primer list

Primer	Sequence
APE1	Forward 5´ to 3´- CCTGGACTCTCTCATCAATACTGG Reverse 5´ to 3´- AGTCAAATTCAGCCACAATCACC
GAPDH	Forward 5´ to 3´- TCTCTGCTCCTCCTGTTC Reverse 5´ to 3´- - GCCCAATACGACCAAATCC
DNA Polymerase β	Forward 5´ to 3´- AGTACACCATCCGTCCCTTG Reverse 5´ to 3´- AAAGATGTCTTTTTCACTACTCACTG
DNA Polymerase δ	Forward 5´ to 3´- GCTCCGCTCCTACACGCTCAA Reverse 5´ to 3´- GTCTGGTCGTTCCCATTCTGC
$p21^{Cip1}$	Forward 5´ to 3´- AAGTCCAGAGCCATTTCC Reverse 5´ to 3´- AATATAGGTCAAGTCTAAGTCG
XRCC-1	Forward 5´ to 3´- CTGGGACCGGGTCAAAAT Reverse 5´ to 3´- CAAGCCAAAGGGGGAGTC
IL-6	Forward 5´ to 3´- CAAAGATGTAGCCGCCC Reverse 5´ to 3´- GTTCAGGTTGTTTTCTGCC
IL-8	Forward 5´ to 3´- CTGGCCGTGGTCCTCTTG Reverse 5´ to 3´- CCTTGGCAAAACTGCACCTT

miRNA MIMIC Transfection

A549 cells cells were seeded at $0.2*10^6$ in six-well. The day after, cells were transfected with 30 pmol of miRNA mimic (hsa-miR-130b-3p, hsa-miR-146a-5p) per well (see hsa-miR-130b-3p, hsa-miR-146a-5p sequence below). Non- targeting negative control transfection was in parallel performed as a control. Both siRNAs were supplied by Ambion-Life Technologies. The transfection was executed with Lipofectamine RNAimax Reagent (Ambion-Life Technologies), according to the manufacturer's instructions. After 6 hours of transfections, fresh medium was added to the complexes for reducing cytotoxicity. The day after transfection cells were washed twice with PBS and collected.

Mature miRNA Sequence:

hsa-miR-130b-3p: CAGUGCAAUGAUGAAAGGGCAU

hsa-miR-146a-5p: UGAGAACUGAAUUCCAUGGGUU

Quantitative real-time reverse transcriptase-PCR

For the measurement of microRNA-expression from cell line, total RNA, including small RNAs, were extracted with miRNAs extraction and performed using miRNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Ten nanogram of total RNA per reaction, was reverse transcribed using TaqMan Advanced miRNA Assays (Applied Biosystems, Carlsbad, CA), according to the manufacturer's instructions. DNA was amplified in 96-well plates and 10 ng of total RNA were coincubated with 1 µl of miRNA-specific probe-TaqMan Advanced miRNA assay 20x -(See below the miRNAs probe), 10 µl of TaqMan Fast Advanced Mastermix 2x, in a final volume of 20 µl for each well. Each sample analysis was performed in triplicate., a sample in which was not added template was used as negative control.

Reactions were run on CFX96 Real-Time System, (Bio-Rad, California, United States), applying the following cycling parameters:

- 95° C for 5 min (Enzyme activation) – 1 cycle
- 95° C for 3 seconds (Denature) repeated for 40 times
- 60° C for 30 seconds repeated for 40 times (annealing/extension)

The analysis was executed performing three biological replicas, utilizing the expression of has-miR-16-5p as the housekeeping gene. Data were resolved applying ΔΔct method.

TaqMan probe list

TaqMan Advanced miRNA assay (20x)	Code
hsa- miR-16-5p	477860_mir
hsa-miR130b-3p	477840_mir
hsa-miR-146a-5p	478399_mir
hsa-miR-183-5p	477937_mir
hsa-miR-200c-3p	478351_mir
hsa-miR-24-3p	477992_mir
hsa-miR-210-3p	477970_mir

Statistical Analyses

Statistical analyses were achieved performing T-test using GraphPad Prism7 data analysis program. $P<0.05$ was considered statistically significant.

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ABBREVIATIONS

AP sites Apurinic/Apyrimidinic sites

APE1/Ref1 Apurinic/Apyrimidinic endodeoxyribonuclease 1/ redox factor 1

ATM Ataxia-Telangiectasia mutated

ATR ATM and Rad3-related

BER Base Excision Repair

BrdU 5-Bromo-2'-deoxyuridine

C/EBPβ CCAAT/enhancer-binding protein beta

CDDP cis-Diamineplatinum(II) dichloride

CDK Cyclin-Dependent Kinase

CDKi Cyclin-Dependent Kinase inhibitor

Cys cysteine

DAMP DNA Damage Associated Molecules

DDR DNA damage response

DDSP DNA Damage Associated Secretory Program

DE-miRNAs differentially expressed miRNA

DGCR8 DiGeorge syndrome critical region gene 8

DMF N, N-dimethylformamide

DMSO dimethyl sulfoxide

DOXO doxorubicin

DSBs Double-Strand Breaks

ECM Extracellular Matrix

EMT Epithelial to mesenchymal transition

FEN1 Flap specific endonuclease 1

GG-NER Global Genome NER

GM-CSF Granulocyte-Macrophage Colony-Stimulating Factor

Gos galacto-oligosaccharides

H_2O_2 hydrogen peroxide

HMGB1 High Mobility Group B 1

HMGB2 High Mobility Group B 2

HR homologous recombination

hTERT human Telomerase Reverse Transcriptase

ICLs Interstrand Cross-Links

IL-6 interleukin-6

IL-8 interleukin-6

LIG1 DNA ligase 1

MCSs Mesenchymal Stem Cells

miRNAs microRNA

MMR Mismatch Repair

MMS methyl methanesulfonate

mTOR mechanistic Target of Rapamycin

nCaRE Negative calcium Responsive Elements

NCE Nuclear Cell Extracts

NER Nucleotide Excision Repair

NF-κB Nuclear Factor κB

NHEJ Non-Homologous End Joining

NPM1 Nucleophosmin 1

NPs Nanoparticles

NSCLC Non-small cell lung cancer

NTH1 Endonuclease Three Homolog 1

$O_2^{\cdot -}$ superoxide radicals

OGG1 8-oxoguanine DNA glycosilase 1

OIS Oncogene-Induced Senescence

OS Overall Survival

PARP1 Poly ADP Ribose Polymerase 1

PCa prostate cancer

PCNA Proliferating cell nuclear antigen

PI3K phosphoinositide 3-kinase

PMOs Periodic Mesoporous Organisilicas

POL-ß DNA polymerase beta

POL-δ DNA polymerase delta

RB1 retinoblastoma (Rb)-associated protein

RISC RNA-induced silencing complex

ROS Reactive Oxygen Species

RS Replicative Senescence

SA-β-gal Senescence-Associated β-galactosidase

SAHF senescence-associated heterochromatin foci

SASP Senescence-Associated Secretory Phenotype

SIPS Stress-Induced Premature Senescence

SMS Senescence-Messaging Secretome

SOD Superoxide Dismutase

SSBs Single-Strand Breaks

TC-NER Transcription-Coupled NER

TGF-ß Transforming Growth Factor-ß

UV Ultraviolet light

UV-DDB UV-Damaged DNA-Binding protein

WCE Whole cell extracts

x-GAL 5-bromo-4-chloro-3-indoyl ß-D-galactopyranoside

XRCC1 X-ray repair cross-complemeting 1

PUBBLICATION

Paper published during Ph.D course