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Study of the combined effects of CDK1 inhibitors and senolytic drugs for the clearance of aneuploid-senescent cells

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Summary

I. Abstract

Despite the progresses in discovering new therapeutic drugs and treatments, cancer is still one of the main causes of death. The biggest part of available treatments, moreover, is not always effective against tumour spread and it also has negative effects on the healthy tissues of the individual. For this reason, it is extremely relevant to find new strategies to avoid side effects during the anti-cancer therapies.

Aneuploidy, an aberrant number of chromosomes in the cell, is a typical condition of cancer cells caused mainly by segregation errors and chromosomal instability (CIN). CIN is a process by which higher rate of chromosome segregation defects occurs by different mechanisms (chromosome mis-alignments, spindle alterations, mitotic defects cytokinesis failure…) resulting in aneuploidy that, by inducing proteotoxic stress, energy stress and DNA damages, affects proliferation of normal cells. On the other hand, CIN and aneuploidy allow cancer cells to escape pathways leading to cell death (apoptosis), cell cycle arrest and cellular senescence. The reactivation of pathways leading to apoptosis or cellular senescence is a powerful strategy to halt proliferation of cancer cells. In particular, cellular senescence, an irreversible cell cycle arrest, presents itself as an effective mean to stop proliferation of cancer cells that could be then killed specifically, for example by senolytic drugs. Generally, cellular senescence originates from a G1 arrest of the cell cycle caused by DNA damage or other cellular stresses/alterations (such as defects in chromosome segregations).

The cyclin-dependent kinase CDK1 is the most overexpressed kinase in malignant tumours compared to other CDKs. Its function is important for the correct cell cycle progression and its action is highly regulated, in order to preserve the right progression of mitosis. Once CDK1 is degraded, cell can successfully ultimate mitosis, proceeding from anaphase to telophase. Evidence showed how CDK1 and not its partner cyclin B1 is the main responsible of the right progression of mitosis[1].

Aneuploidy has been suggested also as a trigger for cellular senescence and aging. Thus, I hypothesized that inducing a cell cycle arrest in the G2/M phase by inhibiting CDK1 could trigger mitotic errors leading to a subsequent G1 arrest and likely senescence of cancer cells. To this aim I have inhibited CDK1 by RNAi and the selective inhibitor RO-3306, an ATP-competitor which interferes with CDK1 activation, in cultured cancer cells and not transformed aneuploid human cells. Moreover, as a positive control of senescence, the flavonoid Curcumin was used due to its known senescence inducer activity in cancer cells. After senescence induction, cells have been treated with the senolytic drugs Fisetin and Quercetin in order to specifically eliminate senescent cells. Globally, my work shows that CDK1 depletion, by RNAi and pharmacological inhibition, leads to G2/M arrest followed by $p21^{waf1/cip1}$ rise and triggers cellular senescence in cancer cells as shown by SA-βGal positive cells. Accordingly, even though with different mechanisms, Curcumin has induced cell cycle arrest in the G2/M phase, as well as senescence, in tumor cells. Interestingly, the percentage of senescent cells reduces following treatment with the drugs Quercetin and Fisetin, confirming the senolytic action of these compounds. Thus, combining CDK1 inhibition with senolytic drugs can be a powerful strategy for the clearance of aneuploid – senescent cancer cells.

II. Introduction

1. Chapter I: Aneuploidy, cancer and the cell cycle

1.1. Aneuploidy and cancer

Eukaryotic cells display a number of chromosomes, named ploidy, which is species-specific, well-defined, stable during development and transmitted to the progeny without alterations. The most part of eukaryotic organisms is diploid, with two copies of each chromosome. Aneuploidy is a highly common condition among malignant tumours and it is characterized by the aberrant number of chromosomes in the cells. These cells divert from the normal ploidy, losing or gaining chromosomes, or part of them, during the division process[2]. Clearly, an imbalance in the chromosomes copy number influences the genetic background. For example, in aneuploid cells genes located on gained chromosomes will be overexpressed, stressing their replication machinery. In fact, not only the products of the overexpressed genes, but also transcription factors and proteins involved in transcription and translation are more represented. In the end, cells will have an altered number of proteins, suffering of proteotoxic stress. Moreover, aneuploidy is strictly correlated with chromosome instability (CIN), the increased rate of mitotic errors that lead to gains and losses of parts or of entire chromosomes. These two conditions influence themselves in a bi-univocal manner: aneuploidy causes CIN, and CIN feeds aneuploidy in a positive-feedback loop[2] (**Fig.1**).

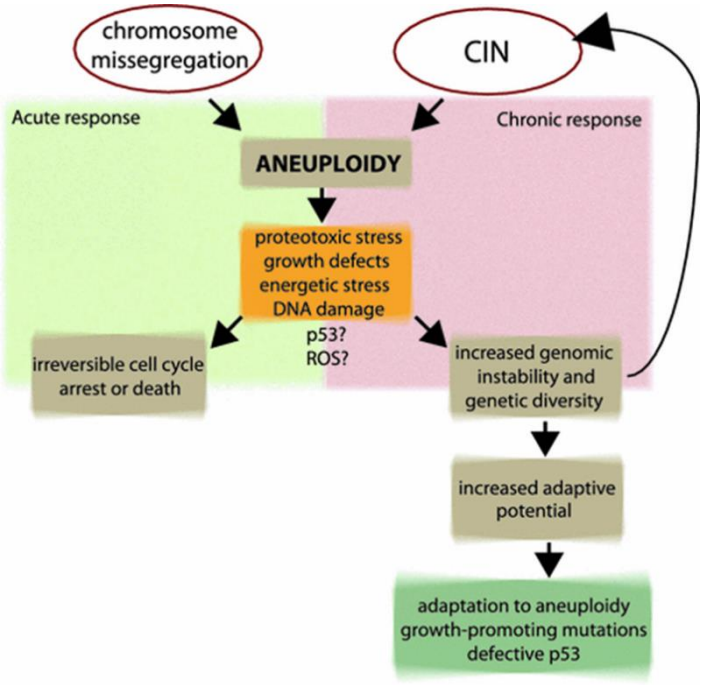


Fig. 1 – *Schematic representation of aneuploidy establishment and mantainance*[2]

On the other hand, alterations in the gene copy numbers can also be an advantage for cells, allowing them to survive in different environmental conditions. In fact, genetic heterogeneity increases the adaptive potential of the cells, which can adapt to and deal with new, changed, or

adverse environments. Tumours are the most explicative example of this feature, since cancer cells are able to survive to different conditions and to counteract stimuli that normally are deleterious for healthy cells. This increased survival rate is strongly correlated to the altered genetic balance that aneuploidy confers to cancer cells.
Transcriptomic and proteomic analysis confirmed that aneuploidy strongly reflects in gene and protein expressions in cancer cells, even if in a fluctuating and sometimes weak manner[2]. Also, even if CIN gives a very big contribution to the alterations observed in cancer cells, aneuploidy seems to be the most influent factor affecting DNA replication, protein folding and cell cycle regulation, with these being the most altered pathways in human cancers.

1.2. Altered pathways in cancer

Cell proliferation is the basis of growth and tissue regeneration. Living cells grow, replicate their genetic information and then divide, generating two daughter cells with identical DNA content. These processes need to be highly regulated in order to avoid the accumulation of errors, especially those related to DNA replication, and to prevent the propagation of wrong information to the progeny. In fact, cells can be subjected to many deleterious stimuli to which they need to respond in order to maintain their homeostasis. However, in some cases, these stimuli are too aggressive resulting in damage of cell components, like DNA, that must be repaired. Otherwise, if the damage is too large and cannot be fixed, the injured cell must be blocked or killed. Tumour-suppressors, as p53 and pRb, are in charge of controlling that those severe alterations are corrected or not transmitted to the progeny. To this aim, these two proteins are able to induce cell cycle arrest (likely causing cellular senescence) or apoptosis, embroiling a huge number of mediators that act on the cell cycle (i.e. $p21^{waf1/cip1}$ and the CDKs) and on the programmed cell death (apoptosis).
Given their chromosomal instability and the high rate of aneuploidy, cancer cells appear to accumulate alterations in many genes, included the ones required to counteract tumour progression. Tumours show a great imbalance between oncogenes that increase the proliferation rate and tumour-suppressor genes that hamper it. In particular, the formers are overexpressed while the latter are downregulated. Normally, the opposite action of these genes is kept in balance in order to guarantee the correct execution of cell proliferation and division. However, in cancer cells tumour-suppressor genes as the ones coding for p53 or pRb, which are involved in the principal pathways of protection of the genome from mutations and damages[3], are frequently repressed or are less represented compared to normal cells. Their action is fundamental to stop proliferation in cells with DNA alterations that can be deleterious if transmitted to the daughter cells. Moreover, the absence of signals that arrest the proliferation leads to uncontrolled cell division, enhanced by the overexpression of oncogenes. All these factors are the main cause of (and contribute to) the uncontrolled spread and to the elevated invasiveness of cancer cells.
Clearly, tumour cells have developed the skill to bypass all those pathways that can hinder their survival. Apoptosis and cellular senescence are the main strategies used by cells to protect the surrounding and the daughter cells from the transmission of mutations, DNA damage or chromosome number alterations. Both of these pathways are mediated by p53, which in turns activates a signal cascade leading to one of these processes. While apoptosis is a kind of death, which differs from necrosis due to its programmed, structured nature, cellular senescence is the

irreversible arrest of the cell cycle. A senescent cell is still viable and metabolically active, but it stops proliferating. It is clear that aneuploidy, inducing stochastic loss or gain of chromosomes in cancer cells, gives them the ability to escape apoptosis or cellular senescence, improving the proliferation and survival rates of malignant cells.

1.3. Cell cycle, checkpoints and cyclin-dependent kinases (CDKs)

1.3.1. *The cell cycle*

As mentioned above, cellular division is one of the main processes a cell must go through successfully in order to multiply. Cell division is the final step of a bigger sequence of events which is the cell cycle, constituted by the M phase (mitosis), the S phase, and two gap phases, G1 and G2, preceding the S phase and the M phase, respectively.
A cell requires the completion of some steps before completing mitosis. First of all, the DNA must be duplicated before division, in order to guarantee the maintenance of the right ploidy in the daughter cells. Nevertheless, since both DNA duplication and mitosis are highly complex and regulated processes, cells must be ready to accomplish them: for this reason, during the G1 and G2 phases cells check that both the internal and the external environments are favourable for the successive phases (adequate amount of nutrients, proteins and organelles) and, as well, they use this time to grow. The G1 phase is especially involved in these roles: in fact, it can be very long, lasting up to 10 hours depending on the circumstances.
During the S phase (where "S" is for "synthesis"), the DNA is replicated for the successive division. This phase lasts 10-12 hours, representing almost the half of the entire cell cycle. Globally, the S phase and the G1 and G2 phases are usually grouped together to a bigger phase called *interphase*. The interphase lasts on average 23 hours in the majority of human cells (even if it can also reach 36 hours, especially in *in vitro* cultured cells), while the mitosis only lasts 1 hour. Finally, in the G2 phase cells starts preparing for mitosis: chromatin begins to condensate and the microtubules begin to organize to form the future mitotic spindle. During mitosis, the nuclear envelope breaks down, chromosomes align on the mitotic spindle and, once perfectly distributed, they segregate equally towards the two poles of the mitotic spindle in order to constitute the genetic pool of the daughter cells that will be formed in the end of mitosis. The mitosis is organized in five different phases (prophase, prometaphase, metaphase, anaphase and telophase) which terminate in the cytokinesis, the division of one cell in two daughter cells harbouring exactly the same genetic pool.

1.3.2. *Cell cycle checkpoints*

The passage from a phase of the cell cycle to another is highly dependent on both intracellular and extracellular signals. For example, if cells in G1 phase live in a hostile environment, they can enter a resting state called G_0 phase, during which they wait for more favourable conditions to proceed in the cell cycle. On the other hand, if all the conditions are satisfied, cells can proceed in the S phase: this progression is controlled by a checkpoint

named "*restriction point*", after which cells start duplicating the DNA even if external nutrients are removed.

Cell cycle checkpoints, as the name suggests, are in charge of controlling the correct prosecution of the cell cycle. Their existence has been hypothesized, at first, as main protagonists of the control of the cell size. Two cell size checkpoints are, in fact, active in correspondence of the G1 and G2 phases. In particular, a small cell will slow down its progression in the cell cycle compared to a cell of larger dimensions. Bigger cells, on the other hand, will be considerably faster in entering the successive phase of the cell cycle. Globally, three checkpoints regulate the cell cycle: two of them, the G1 (restriction point) and the G2 checkpoints, which are in charge of assuring the absence of DNA damages; the third, the SAC (M-phase) checkpoint, is linked to the correct alignment and biorientation of chromosomes on the mitotic spindle.

Cell size checkpoints are only one type of checkpoints: many other alterations can affect the correct prosecution of the cell cycle, especially the ones regarding the DNA or the steps of the mitosis. For this reason, stages as DNA replication, entry into mitosis and the disposition of chromosomes on the mitotic spindle are finely controlled by specific mediators. Cells harbouring damaged DNA cannot enter the S phase unless the damage is fixed, otherwise cells arrest in G1.

If during DNA replication something goes wrong, such as, for example, the presence of a break, the replisome is blocked until the errors are corrected (for example, by recruiting specific mutagenic bypass polymerases or by using the other, not mutated strand as template)[4] and only after damage correction the cell can enter the G2 phase. In any case, correct DNA replication is verified by the G2 checkpoint, together with the cell size: both these parameters must be satisfied for the progression in the M-phase.

Once the cell enters the M-phase, other conditions must be satisfied to allow the optimal completion of mitosis. Fully and correctly replicated DNA must be equally distributed among the daughter cells. For this reason, chromosomes must be correctly aligned on the mitotic spindle during mitosis and this only occurs if the kinetochore of each chromosome makes contact with the microtubules emanating from each pole of the mitotic spindle. Chromosomes contact the microtubules through the kinetochore, a part of the centromere with an inner layer, making contact with the centromeric chromatin, and an outer layer, (composed by the KMN network: Knl1, Mis12 and Ndc80 complexes) making contact with the microtubules (**Fig.2**, top). In order to proceed to the last steps of the mitosis, all the chromosomes must be bound to the microtubules, in order to guarantee the correct segregation of the sister chromatids. The Spindle Assembly Checkpoint (SAC) is the mitotic checkpoint, activating, in turn, the effector mitotic checkpoint complex (MCC).

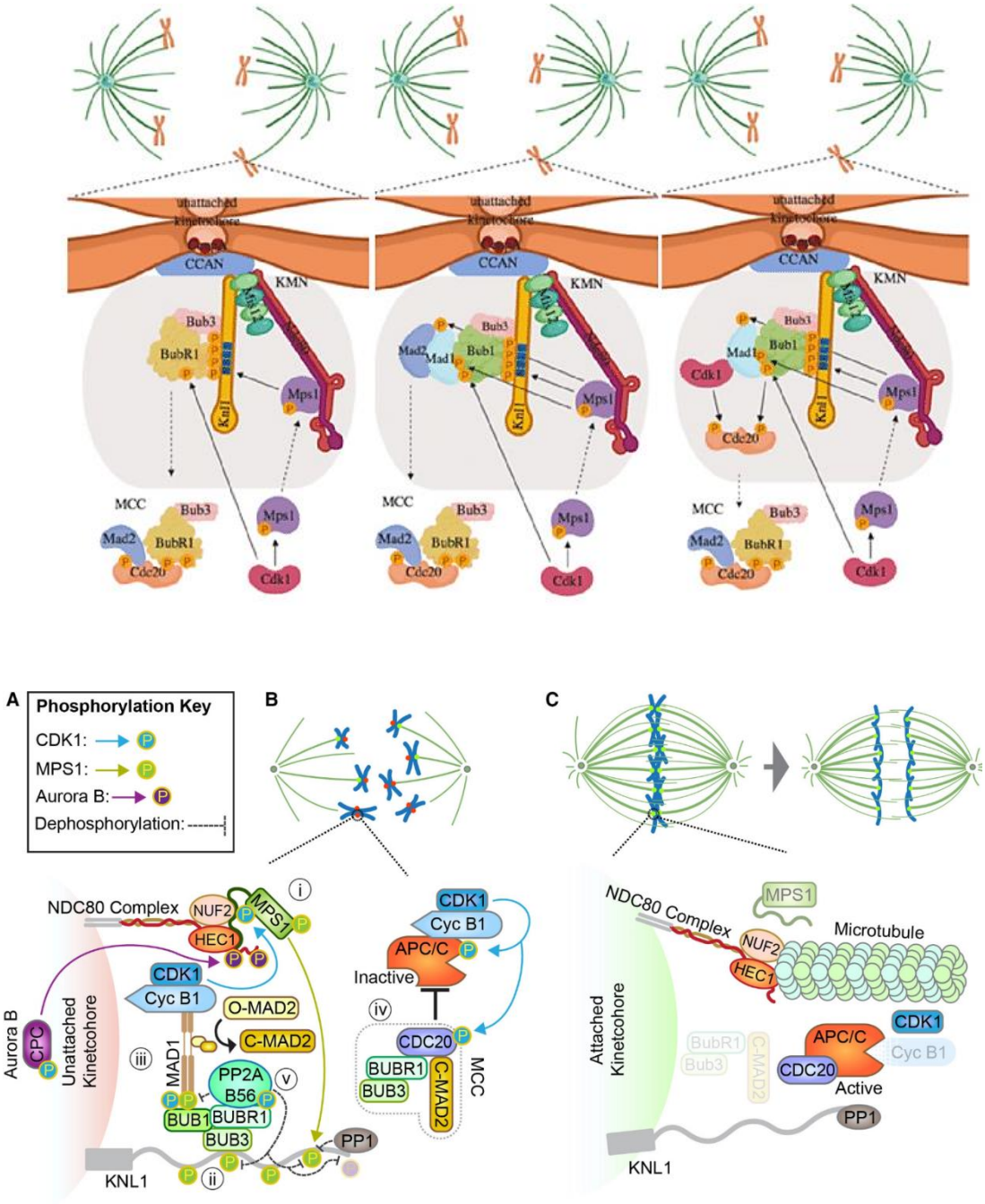


Fig. 2 – *SAC composition and functioning[5, 6]. Interactions between CDK1 and the SAC (top). SAC components and their role with unattached and attached kinetochores (bottom).*

The SAC is composed by several proteins, such as Mps-1, Mad1 and Mad2, Bub1, Bub3 and BubR1, and checks for the right chromosome bi-orientation on the mitotic spindle. During the prometaphase, the SAC proteins, once phosphorylated, are recruited to the kinetochores: Mps-1 interacts with the KMN network, especially with the Ndc80 and the Knl1 complexes, phosphorylating them. These modifications allow the recruitment of BubR1-Bub3 and Bub1-Bub3 complexes, the last of which phosphorylates Mad1 which, in turn, phosphorylates Mad2. At this point, all the SAC proteins are recruited at the

kinetochores, verifying that all the chromosomes contact the microtubules correctly (**Fig. 2**, bottom). Mps-1 is displaced out from the kinetochore once it takes contact with the microtubule. The SAC recruitment allows the catalytic action of the proteins that constitute it, determining the rate of production of the MCC. If all the conditions are satisfied, the MCC will in the end detach from the kinetochore freeing the APC/C^{Cdc20}(Anaphase Promoting Complex) which, once active, can degrade the cyclin B1 and securin, letting the separase protein free to-separate the sister chromatids. The cell can thus proceed through anaphase, telophase and cytokinesis, going towards the exit from the mitosis.[7] The kinetochore – microtubule attachment is also mediated from a motor protein, CENP-E, which is fundamental for the migration of the chromosomes on the mitotic spindle. Alterations on the expression or functionality of CENP-E produce severe consequences on chromosomes segregation causing aneuploidy.

1.3.3. Cyclin-dependent kinases (CDKs)

Cell cycle progression is regulated by specific proteins, the cyclin-dependent kinases (CDKs). These serine/threonine protein kinases, available in large excess in the cytosol, are however inactive until they bind their specific partners: the cyclins. Each CDK has its specific cyclin. Cyclins not only activate the kinases, but also regulate, with their presence, the timing of their activation during the cell cycle. Cyclins, in fact, are synthesized in a highly regulated manner when needed, and they are degraded through proteasome when the cell needs to proceed to the successive phase of the cell cycle.

According to sequence homology of the kinase domain, CDKs are members of the CMGC group of kinases, of the DYRK family (dual-specificity tyrosine – regulated kinases) and CDK-like kinases. Their dependency on the cyclins as partner, regulatory subunits is what distinguish CDKs from the kinases of the other families.

Despite their common function in the regulation of both gene transcription and cell division, CDKs differentiated a lot during evolution, assuming more specialized roles during time. CDKs can be divided in two main categories: CDKs that regulate the cell cycle, which can bind multiple cyclins, and transcriptional CDKs, whose role is to phosphorylate transcription factors, other kinases or the carboxy terminal domain (CTD) of the RNA polymerase II, and which are independent from the cyclin oscillation during the cell cycle. CDK7, for example, is a CDK activating kinase (CAK) and is able to phosphorylate the CTD of RNApol II, the T-loop of other CDKs, nuclear receptors and to coordinate the progression of the mitosis when associated with the DNA-dependent helicase Xpd (a subunit of the TFIIH)[8].

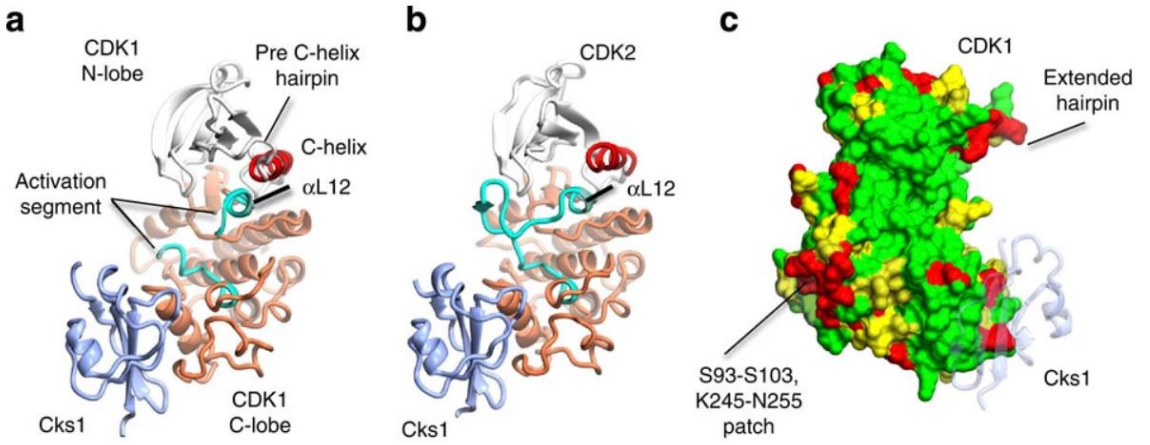


Fig. 3 – *CDK1 structure*[9].

Despite their individual features all the CDKs display common characteristics. For example, they are proline-directed serine/threonine kinases: this means that they recognize S/T-P-X-K/R sequence, because of a hydrophobic pocket near the catalytic site which can accommodate the proline. However, for many CDKs the K/R residues are not necessary, and some are even not proline directed. The size of the CDKs is variable as well: it can range from 250 to 1500 amino acid residues, depending on the length of the N-term or the C-term sides. CDKs have the typical two-lobed structure of the kinases, with the catalytic site between the N-term, constituted of beta-sheets, and the C-term, full of α-helices. The amino terminal lobe contains an inhibitory G-loop rich in glycines and a C-helix. The C-lobe, instead, contains the activation segment called T-loop, which harbours the phosphorylation sensitive segment and which is positioned in the active site in the unbound form of the CDK. After the binding with the cyclin, the T-loop moves, freeing the catalytic cleft. Taking as a model the CDK2, the binding with the cyclin occurs between the C-helix of the CDK and another specific helix on the cyclin. This association boosts a rotation on the axis of the C-helix, promoting interactions which allow the binding of the ATP, and the shift of the T-loop, as mentioned, making the threonine accessible for the phosphorylation by CAK. Moreover, once phosphorylated, the threonine stabilizes the activated form of the kinase. However, this mechanism of association and activation differs for some CDKs, with each CDK having its particular conformation, activation and interaction modes. It is not sufficient for CDKs to be activated in order to perform their function: in fact, their inhibition from the G-loop should be removed. The G-loop residues, once phosphorylated, inhibit the CDK, and so the action of the Cdc25 phosphatase is in its turn required for the cell cycle progression.

Human cell cycle CDKs can be grouped in 3 main subfamilies: CDK1 subfamily (including CDK1, CDK2 and CDK3), CDK4 subfamily (constituted by CDK4 and CDK6) and CDK5 subfamily (encompassing CDK5, and CDKs from 14 to 18). Cyclin-dependent kinases and cyclins can be divided in accordance with the specific phase of the cell cycle during which they act. For this reason, G1-, G2- and M-CDKs – cyclins complexes can be distinguished[10]: CDK4/6 – Cyclin D and CDK2/Cyclin E for G1 progression towards the S phase, responding to mitogenic stimuli; CDK2/Cyclin A for the entry in the S phase; CDK1/Cyclin A and CDK1/Cyclin B for G2/M progression.

The synthesis of every cyclin is tightly regulated and oscillates ("cycles") during the cell cycle. This assures that CDKs activation only occurs when necessary, avoiding mistakes in the activation of substrates when they are not needed.

1.4. CDKs as anti-cancer targets

One of the distinctive traits of cancer is its unlimited and uncontrolled proliferation. This peculiar feature derives from particular mutations affecting genes involved in cellular division or in the cellular responses and whose main role is to counteract cell growth in presence of severe alterations. For example, pathways leading to apoptosis or cellular senescence are inactivated in many malignancies, and many important components of the cell cycle do not work properly. For this reason, reactivating or targeting these pathways and components has frequently been used as a strategy to contrast the spread of tumour cells.

In particular, CDKs are one of the principal targets in cancer therapy. Overexpression of certain CDKs, such as CDK2, is linked to uncontrolled proliferation and malignancy development. Acting towards cell cycle kinases can impact on proliferation in healthy cells, triggering the response of the cell cycle control mechanisms. However, cell cycle checkpoints are altered in several malignancies, conferring tumours the ability to proliferate despite the presence of anomalies.

Given these assumptions, CDKs removal was studied during time. Deletion of CDK2[11], CDK4 and CDK6[12], for example, showed no effects on cell proliferation and on cell viability, despite their fundamental role in the cell cycle. The possible explanation of this phenomenon can be found in the redundancy of the CDKs: since cells can overcome the lack or the impaired activity of a CDK counting on other CDKs having structural and functional homologies with the lacking one. On the other hand, it should be considered that even if not arrested and alive, cells or organisms lacking CDKs still show some alteration, such as a reduced lifespan or sterility: these variations, though, do not produce severe consequences. However, this can be true for a partial subset of CDKs: removal or inhibition of CDK1, instead, leads to proliferation arrest and cell death, showing that CDK1 not only is not dispensable for cell cycle progression, but it is also fundamental for cell viability.

1.5. CDK1: structure, functions, targets

CDK1 is the progenitor of the CDK family, being the first that has been discovered in *Xenopus laevis* eggs as a maturation factor. It has been successively identified as the human homologue of the yeast Cdc28/Cdc2, which is the only kinase essential for cell viability. CDK1 regulatory partners are cyclin A and cyclin B. In particular, it is the only kinase able to bind cyclin B1, essential for the entry into the mitotic phase of the cell cycle. Cyclin A starts accumulating during the late G1-phase, allowing the entering into the S-phase when it binds CDK2. While it accumulates, it is also bound by CDK1 during the S-phase, activating it, and its level in the cell rise until many complexes of CDK1/CycA are detectable in the late-S/G2 phase. This complex is fundamental to trigger the entry into the M-phase, together with the most mitotic specific complex CDK1/CycB1. CDK1/CycA persists until the degradation of the cyclin which is ubiquitinated and then degraded by the proteasome during the early pro-metaphase. Cyclin B1, instead, is degraded during the anaphase by the APC/C (ubiquitination) and the

proteasome (degradation), triggering the inactivation of CDK1 and the consequent exit from mitosis. While the CDK1/CycA complex activity is strictly dependent on cyclin A levels in a directly proportional manner, the CDK1/CycB activity not only depends on the cyclin levels, but also on the action of the two phosphatases Wee1 and Cdc25, which act on the phosphorylation degree of the CDK1's active site. Clearly, due to its essential role, CDK1 activity is highly and finely regulated, especially if compared with the other cell cycle CDKs.

1.5.1. CDK1 structure

Because of their fundamental role in the cell cycle, the CDKs domains are generally highly conserved. During evolution, each CDK developed specific feature to better accomplish its proper task, but all of the cell cycle kinases derive, as mentioned, from CDK1: for this reason, the first CDK shares common features with the others. When observed in the monomeric, inactive form, it assumes the typical bi-lobal structure (**Fig. 3**), with the C-helix out of the catalytic cleft and the activation segment (T-loop) not phosphorylated, so that it cannot bind the substrate peptide. CDK1 shares a high degree of homology in particular with the sequence and the structure of CDK2, with only few differences as those in the C-termini and in the activation segment. Specifically, for this last sequence, CDK1 possess 40-46 residues in the active site cleft, beyond the ATP-binding site and before the start of the C-helix, that form a protruding hairpin which is absent in CDK2. The inhibitory G-loop is mobile and flexible, as well as the C-termini of the protein: this last feature differentiates CDK1 structure from the CDK2 one.

Another important aspect in analysing CDK1 structure is the specificity of its bonds with Cyclin B1 and Cyclin A, compared with other cyclins. First of all, the CDK1 region involved in cyclin recognition seems to be the C-terminal sequence, which binds a complementary side on cyclin B1. Secondarily, the specificity of this interaction has to be attributed to three aromatic residues (Y170, Y177 and Y258) which are highly conserved in Cyclin A and B1, but absent in other cyclins (such as Cyclin E). These aromatic residues interact with the CDK1 surface, while smaller amino acids in these positions present in other cyclins prevent this binding.

Thus, despite their common origin which gave rise to homologies and similarities in both structure and functions, CDKs maintain proper, specific features and this is particularly true for CDK1, progenitor of the other CDKs and main protagonist of cell cycle regulation.

1.5.2. Role of CDK1 in the cell cycle: functions and targets

CDK1 is involved in various processes that take place in the cells. For example, its role in epigenetic modifications on ESCs chromatin has been already revealed, showing that CDK1 is fundamental in maintaining the undifferentiated state of stem cells by inhibiting the methyltransferase Dot1l, responsible of H3K79 methylation[13]. Moreover, CDK1 appears to be essential for the regulation of cellular adhesion, acting on the regulators of actin, intermediate filament, tubulin and RhoGTPases[14]. However, as mentioned above, the main role of CDK1 is to allow the enter and the correct prosecution of mitosis during the cell cycle. Like other CDKs, CDK1 fulfils this role by phosphorylating specific proteins downstream, allowing them to execute their tasks or, on the other hand, inhibiting their

action.

First, it has been shown that CDK1 presence is required until anaphase to allow the normal progression of mitosis. For many years, Cyclin B1 degradation by the APC/C^{Cdc20} complex was thought to be the trigger for mitotic exit. However, it has been shown that after CDK1 degradation, which is also operated through the APC/C^{Cdc20}, cells complete telophase and cytokinesis, exiting mitosis[15].

During mitosis, CDK1 is involved in different tasks as cytoskeleton remodelling for the mitotic spindle formation, allowing SAC's components to take contact with centromeres, interacting with the APC/C^{Cdc20} complex and reinforcing its activation by the creation of positive feedback loops. During the S and G2 phases, CDK1 is kept inactive by the two kinases Myt1 and Wee1, which phosphorylate the CDK1 Thr14 and Tyr15 residues: these modifications allow the accumulation of CDK1/CyclinB1 complexes during S and G2 phases maintaining them inactive at the same time. In this way, once the cell is ready to enter mitosis, there will be enough CDK1/CycB1 complexes to proceed with the cell cycle that can be activated simply by the removal of the phosphates. This removal is operated by the CDC25C phosphatase, which is, in turn, phosphorylated by CDK1 (now active) to reinforce its action. Successively, CDK1 starts phosphorylating its target proteins, such as the APC/C^{Cdc20} complex, the Gwl (Greatwall) kinase, the PP1A phosphatase (which, together with PP2A, antagonize CDK1 activity), Mps-1 (member of the SAC) and the CPC (chromosomal passenger complex), which is in turn in charge of phosphorylating the Aurora B kinase for its proper localization in proximity of the kinetochores. While phosphorylation of Gwl (which, once active, induce a phosphorylation cascade leading to the inhibition of the PP2A phosphatase through inhibition of its protein partner B55, blocked by the now phosphorylated complex Ensa/Arpp19) and PP1A are directed to a positive feedback loop which guarantees that CDK1 activation persists during mitosis, the phosphorylation of Mps-1 is involved in a more complex mechanism. As mentioned above, Mps-1 is a member of the SAC checkpoint, which forms the MCC involved in the overcome of metaphase and in the inhibition of Cdc20 until all the conditions are satisfied. By phosphorylating Mps-1, CDK1 starts the phosphorylation cascade, which is necessary for the SAC assembly and functioning, inhibiting Cdc20 until all the chromosomes are bound to microtubules. Moreover, Cdc20 phosphorylation appears to increase the affinity of this protein for the MCC, reinforcing its inactivation, and to reduce the affinity for the APC/C. Clearly, CDK1 is responsible for the correct accomplishment of all the processes of the mitosis, avoiding the precocious activation of the APC/C and the consequent exit from the mitosis until all the conditions are satisfied.

1.6. CDK1 inhibition

Due to its pivotal role in cell cycle regulation, CDK1 has been often targeted in the context of anticancer therapies. Mice embryos knocked-out for CDK1 could not proceed development, and $CDK1^{NULL}$ blastocyst showed altered nuclei, bigger in size, and a reduced division capability[16]. Moreover, CDK1 total inhibition frequently leads to apoptosis or cell cycle arrest, indicating that cells absolutely depend on CDK1 for their survival.

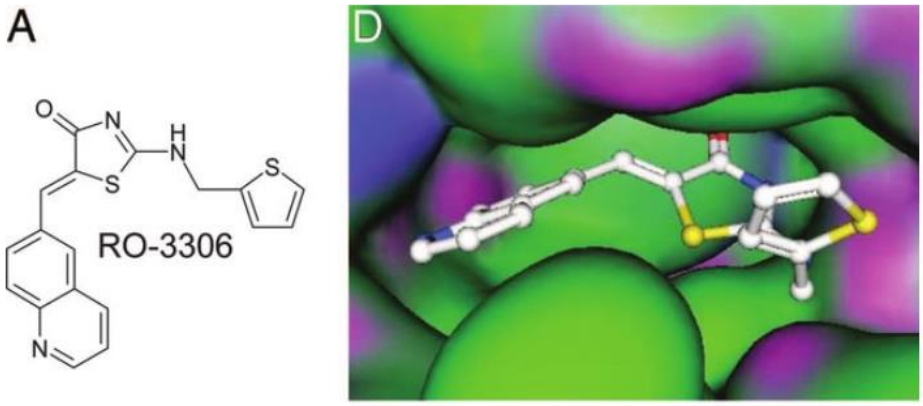


Fig. 4 – *RO-3306 inhibitor and its interaction with the cell cycle kinase CDK*[17].

CDK1 structure has been unknown for long time, due to various difficulties in isolating it with crystallography assays. Moreover, being the progenitor of the whole cell cycle CDK family, it shares many common features with the other kinases, giving rise to challenging efforts in researching inhibitory molecules. For these reasons, there are not specific and selective CDK1 inhibitors currently available, but the molecules in use also have off-target effects, mainly directed towards other kinases.

The inhibitors currently available are mainly ATP competitors, with high affinity for the ATP binding site of the kinases. The most used CDK1 inhibitor is RO-3306 ((5Z)-5-Quinolin-6-ylmethylene-2-[(thiophen-2-ylmethyl)-amino]-thiazol-4-one, 5-(6-Quinolinylmethylene)-2-[(2-thienylmethyl)amino]-4(5H)-thiazolone)[18], being to date the only molecule known to be specifically selective for CDK1, with the highest affinity for it (Ki = 35 nM) compared to the other cell cycle kinases (**Fig. 4**). Treatments with RO3306 in cancer cells generated impaired proliferation, mitotic spindle disorganization, defects in chromosome alignment, a reduced migration rate and apoptosis, with a range of doses going from low (1 μM) to high (10 μM) concentrations[19,20].

Other CDK1 inhibitors, such as resveratrol, dinaciclib or flavopiridol, despite their high affinity for CDK1, are, as a matter of fact, pan-CDK inhibitors. This means that they preferentially bind CDK1, but that they can also bind the other kinases when dispensed at higher doses. For this reason, it is necessary to find empirically the concentration of these inhibitors that can act selectively towards the desired target in order to avoid side effects or not-specific results[21].

1.7. Cell cycle arrest and mitotic slippage

As mentioned above, cell cycle checkpoints are in charge of stopping the cell proliferation in absence of satisfying environmental conditions or in presence of DNA damage and chromosome misalignments. Many triggers can lead to cell cycle arrest: DNA damage (by radiations or other stimuli), for example, induces cell cycle arrest in the G1 phase in order to prevent the propagation of the damage in the S phase and, subsequently, to the daughter cells. This transition is controlled by the G1/S checkpoint, responsible for detecting alterations in both DNA content or quality and cell size.

The cell cycle can be stopped in the other two cell cycle checkpoints as well. In particular, the SAC checkpoint satisfaction is fundamental to allow the mitosis prosecution in response to the

correct bipolar alignment of chromosomes at the mitotic spindle. Several attempts have been performed during years to understand the consequences of perturbed mitosis, that prevents the SAC inactivation and induces the cell cycle arrest in mitosis. Cells that arrest before SAC satisfaction frequently undergo cell death in order to tackle the abnormal cell division, through both mitotic catastrophe (that follows the activation of the apoptotic machinery before Cyclin B1 degradation) or the premature exit from mitosis, entry into the G1 phase and the subsequent apoptosis. However, the scenario is slightly different for cancer cells.

Cancer cells frequently harbour genetic alterations that confer them an increased adaptative potential compared to their healthy counterpart. This altered genomic background allow them to develop new strategies to escape the cell cycle arrest, for example by SAC defects. Clearly, these modifications exponentially increase the genetic alterations, fuelling the abnormal genotype and leading to a condition which is called genomic instability (GIN). GIN is, in turn, involved in most of all the acquired new features of tumour cells, such as resistance to chemotherapeutic drugs or the survival in very strict and hostile conditions. This instability of the genetic heritage also produces a second, alternative road, in addition to the apoptosis, in response to mitotic arrest: the *mitotic slippage* (**Fig. 5**).

As the name suggests, cells that undergo mitotic slippage simply skip the mitosis when arrested in this phase of the cell cycle by escaping the SAC arrest and slowly degrading Cyclin B1[21]. This premature exit brings to an incorrect chromosome segregation and to an incomplete cytokinesis, producing tetraploid or multinucleated cells. The fate of these cells can be apoptosis or cellular senescence.

How the cell makes the choice between mitotic catastrophe or mitotic slippage is still a debated question. It has been hypothesized[22] that cells fluctuate between these two possible outcomes creating a balance between apoptosis or escaping mitosis. This model, defined "competing networks-threshold model", is based on the evidence of the fundamental role of the Bcl-2 family proteins, mainly known for their role in the regulation of apoptosis, such as Mcl-1, whose competition with Cyclin B1 for the degradation tips the scale in favour of mitotic cell death. Other studies have shown the involvement of Myc, BIM, ERK proteins, the centromeric protein CEP55, Plk1, the SAC proteins and, recently, of the $p31^{comet}$ in the settlement of mitotic slippage[23]. Clearly, all these mediators are dependent from CDK1 activation, which in any case is the principal contributor to the establishment and accomplishment of the M phase.

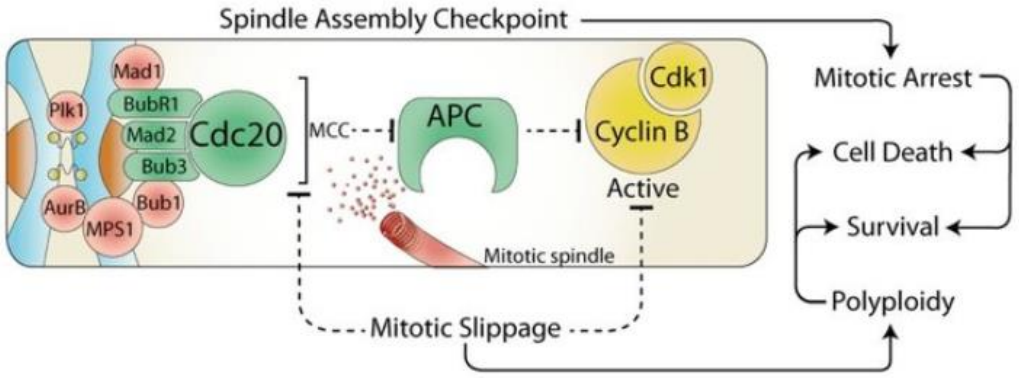


Fig. 5 – *During mitosis, the SAC controls that anaphase is not started until all chromosomes are attached to the mitotic spindle. Unsatisfied SAC produces mitotic arrest, which often leads to cell death. However, the release of Cdc20 from the mitotic checkpoint complex (MCC) or by direct inhibition of Cdk1 can bypass SAC-induced mitotic arrest. This mitotic slippage can result in polyploidy and constitutes a potential mechanism for escaping mitotic cell death and increase survival*[24].

2. Chapter II: Senescence and senolytic drugs

2.1. Cellular Senescence

Cellular senescence is defined as the irreversible cell cycle arrest in a still viable, metabolically active cell. It was first defined by Leonard Hayflick in 1960, who observed that cells stop proliferating after a certain number of divisions (**Fig. 6**). Differently from aging, which is a wider process involving a whole organism, cellular senescence is a more circumscribed phenomenon interesting the single cell. A senescent cell has well defined features that make it easily recognizable from both a macroscopic and a molecular point of view (**Fig. 7**). In detail, senescent cells appear to be enlarged, flattened and vacuolated: their dimension increases dramatically, and vacuoles derived from stress start to accumulate in the cytoplasm. Moreover, senescent cells show senescent associated heterocromatic foci (SAHF), regions of heterocromatin in which pRb localizes, an overexpression of the β-Gal enzyme, often used as senescence marker, and lipofuscin clumps: these latter are aggregates deriving from catabolism of carbohydrates, lipids and proteins which accumulate in the lysosome. While normal cells divide themselves and dilute the amount of lipofuscin between the daughter cells, senescent cells, which do not divide, accumulate it, showing dark dots visible under transmitted light microscopy. Another distinctive trait of a senescent cell is the senescent associated secretory phenotype (SASP). Senescent cells are able to secret cytokines and interleukines in order to produce an inflammatory response with both autocrine and paracrine action. While the autocrine action is thought to reinforce the inflammatory response in a positive feedback loop, the paracrine action serves to propagate the senescent phenotype to the surrounding cells, in order to create a barrier to isolate the damaged cell in a more effective manner. IL-6 and IL-1α are the most representative mediators of the SASP, and this is why they are used as senescence markers.

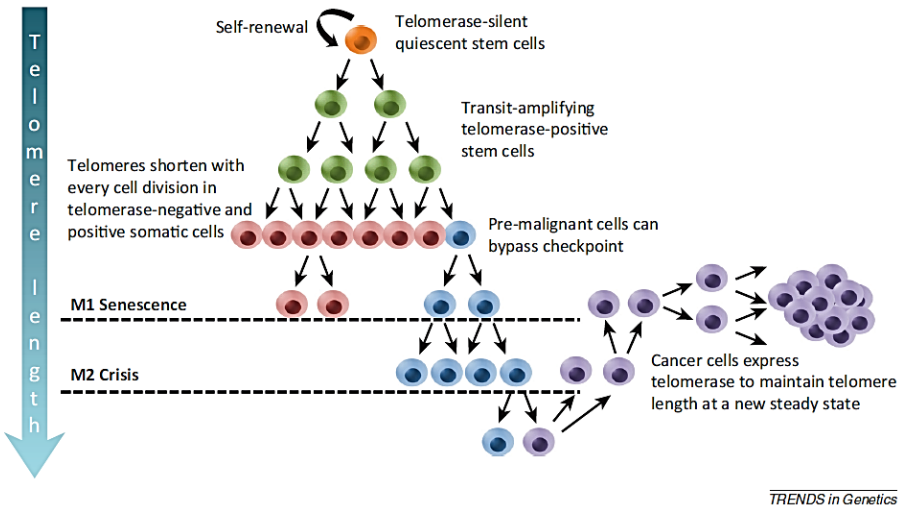


Fig. 6 – *Telomere erosion and cellular senescence establishment*[25].

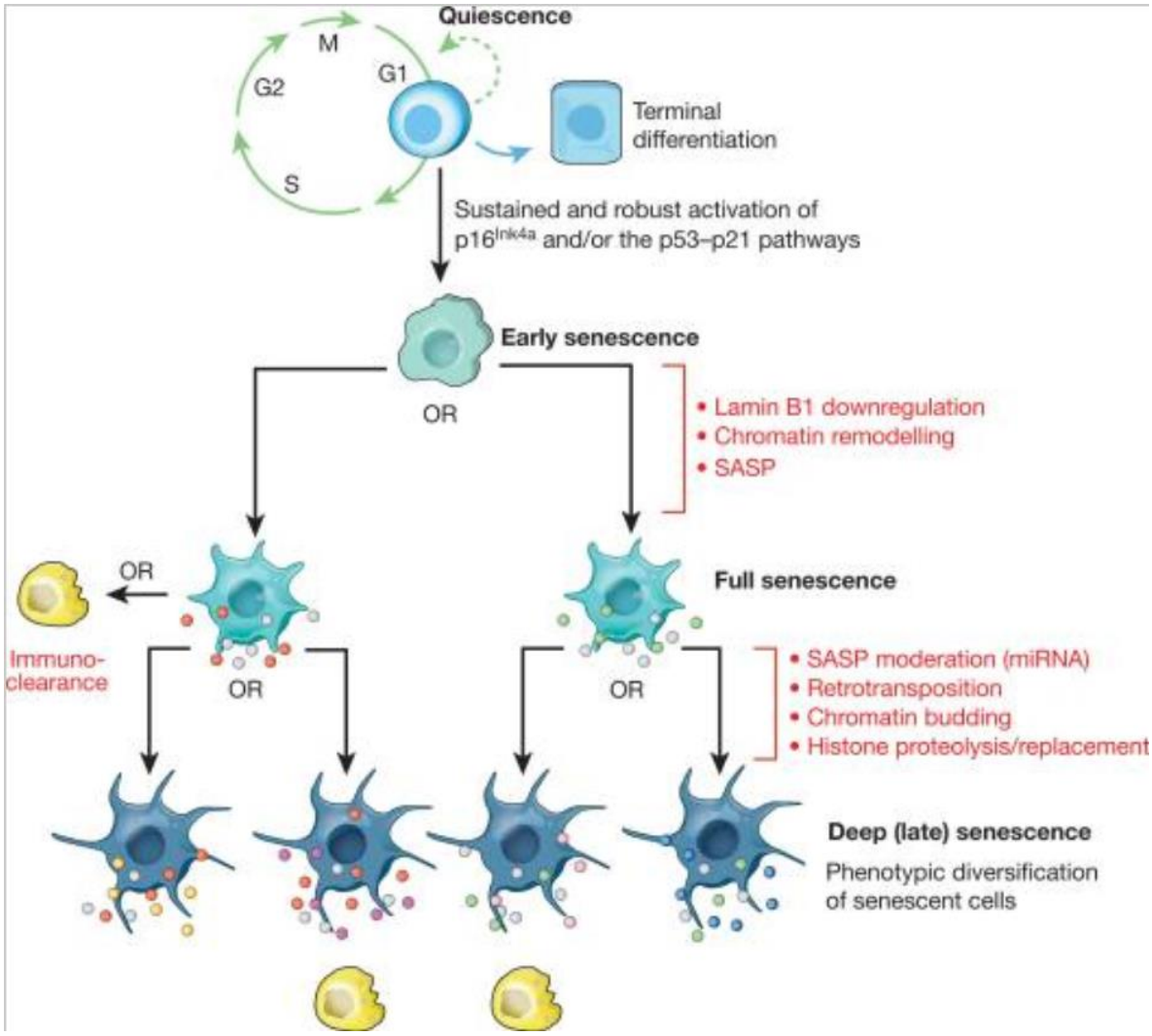


Fig. 7 – *Cellular senescence induction and establishment during time*[26].

Four main types of senescence are known, classified according to the inducing trigger: replicative senescence (RS), induced by telomeric erosion during time, oxidative stress induced senescence (OSIS), derived from mitochondrial stress following ROS accumulation, oncogene induced senescence (OIS) and ionizing radiation induced senescence (IRS). Even if each kind of senescence has its specific pathway, some mediators, as p53, pRb and $p21^{waf1/cip1}$, as well as the component of the DNA damage response, are in common and act synergistically to stop proliferation of damaged cells.

Senescence is induced by p53 in response to various stresses as, for example, DNA damage, radiations or oxidative stress. At first, cells try to repair those damages. However, if the healthy condition cannot be rescued, p53 is activated and the CDKs inhibitors $p21^{waf1/cip1}$ and p16 are recruited. $p21^{waf1/cip1}$, a protein belonging to the KIP/CIP family that plays important roles in the cell cycle and in cytoskeleton modification during mitosis, is an inhibitor of cyclin D/CDK4 or CDK6, cyclin E/CDK2, cyclin A/CDK1 (Cdc2) or CDK2 and cyclin B/CDK1. This inhibition leads to the cell cycle arrest in the G1 phase which, if protracted during time, will then cause an irreversible arrest mediated by pRb. pRb that will not be phosphorylated by the CDKs, is inactive

and will not bind the E2F transcription factor, responsible of the progression from the G1 to the S phase.
However, it has been discovered that a G2-derived cell senescence exists too. This type of senescence, even if distinct from the G1-derived one, is still induced by $p21^{waf1/cip1}$ but the exact mechanisms still remain to be understood[27]. Studies has shown that the involvement of APC/C(Cdh1) - mediated cyclinB1 degradation in cell cycle exit from G2 phase[28]. However, this process seems to be mostly dependent on CDK1 degradation. CDK1, which is the major responsible of the cell cycle progression from G2 phase to M phase, communicates with the Aurora B kinase[29] another important mediator of mitosis. Together, these two kinases decide the right moment for the cell to end mitosis by ending the anaphase. It has been shown that CDK1 inhibition leads to premature mitotic exit, triggering the accumulation of mitotic errors. This mitosis skipping is called "mitotic slippage", and it appears to be one of the potential roads that drive the cell to senescence.

2.2. Aneuploidy and cell senescence

Cancer and cellular senescence share an ambiguous, double-faced relationship based on a bi-univocal correspondence. On one hand, cellular senescence presents itself as an antipodal cell status compared to the uncontrolled proliferation that typically characterize cancer cells. On the other hand, however, recent evidence has shown that these two phenomena are more connected than it seems at first sight. In fact, it has been demonstrated that aneuploidy, intended as chromosomal instability (CIN), has a strong causal relationship with senescence, which, in this case, also shows pro-tumorigenic effects (**Fig. 8**). Induction of aneuploidy with drugs like Reversine (an MPS-1 inhibitor) or Nocodazole clearly leads cells into senescence in a p53-dependent manner. This appears to be true not only for cancer cells like HCT116, but also for non-tumoral cell line like RPE-1 hTERT (retinal pigment epithelium), meaning that aneuploidy, more than the malignant background of the cells, is the only trigger for senescence[30]. Moreover, the SASP produced by these senescent cells acts in a pro-tumorigenic manner, increasing the migration rate and the proliferation of the malignant cells.
Other studies have corroborated the strong link between aneuploidy and senescence. It has been revealed that during aging the number of mitotic defects increases[31], producing alterations in chromosomes segregation, anaphase delay and the rise of aneusomy levels. This mitotic decline derives from the transcriptional repression of the mitotic genes in those cells, pre-senescent or senescent, expressing the SASP. The deriving aneuploidy, in turn, continues promoting the senescent response through accumulation of chromosomal alterations. Though many factors are involved in senescence establishment, the FoxM1 transcription factor (one of the main protagonists of the G2/M transition) appears to have a fundamental role in this process, since its repression leads to cell cycle arrest and senescence[32].
Globally, the role of cell senescence is to stop the propagation of cells harbouring damages or alterations. Together with apoptosis, cell senescence represents one of the most valid mechanisms of defence used by organisms against the development of malignancies. So, even if cancer cells have the ability to escape those defensive pathways, aneuploidy is one of the triggers to establish a senescence response, linking, in this way, the cancer and the irreversible cell cycle arrest.

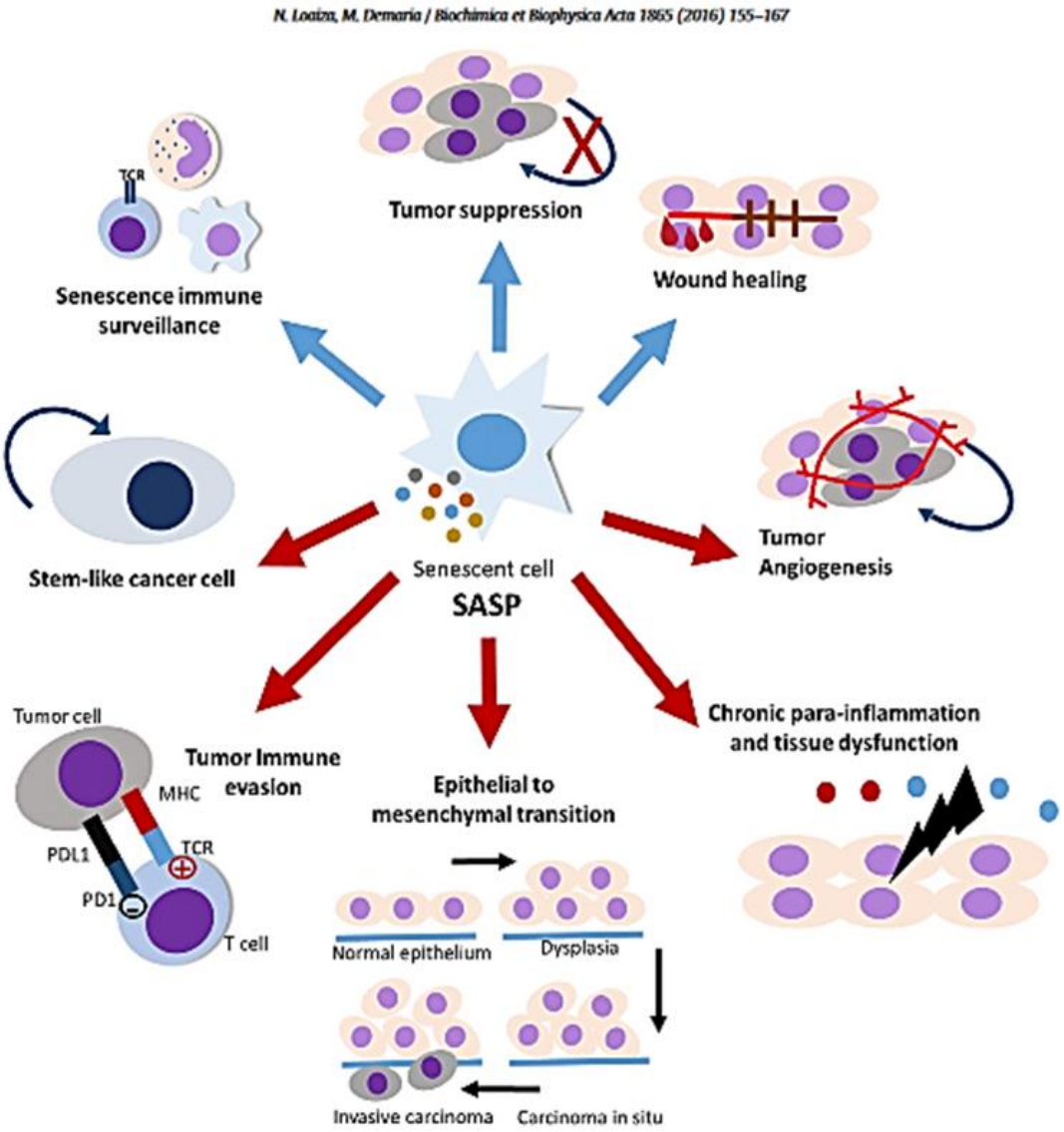


Fig. 8 – *Effects of senescence-associated secretory phenotype (SASP) on tumorigenesis and tumor suppression*[33].

2.3. Cancer and senescence: friends or foes?

While cell senescence, by inducing the cell cycle arrest, represents a strong weapon against the spread of malignancies, it is not always an ally in this battle. The cell cycle arrest *per se* clearly is an advantage, however, as explained above, the description of cellular senescence as the mere block of the cell cycle is a bit simplistic, given that it is a more complex phenomenon. What obstacles the process of tumour arrest in this case is the presence of the SASP. The chemokines and interleukines secreted by the pre-senescent and senescent cells generate and fuel an inflammatory environment which can act as a double-edged sword. Moreover, the SASP is not only constituted by soluble, pro-inflammatory factors but, at the same time, it is composed of proteases and insoluble protein and components of the extracellular matrix. The synergic action of these mediators creates an extracellular environment in which the matrix is

more elastic and less dense than normal, promoting the movements of the cell and, in the case of cancer cells, metastasis. Furthermore, the SASP has been linked with angiogenesis, increased cell proliferation and motility, cell differentiation and tumour immunology, presenting itself as a pro-tumorigenic phenomenon[34]. This concept is corroborated by the fact that p53, which protects the genome from DNA damages and acts as tumour-suppressor, also inhibits the SASP production, confirming the tumorigenic potential of the secretory phenotype of senescent cells. At the same time, the SASP also contributes to limiting tumour propagation by reinforcing the cell cycle arrest through its paracrine action and by recruiting immune cells in sites of injury[35]. In particular, the immune system appears to play both a supportive and/or a suppressive role towards cancer cells, depending on the stage of tumour development, the tissue involved, the senescence-inducing stimulus and, consequently, on the type of SASP factors secreted[36]. Globally, senescence and SASP appear as dynamic processes whose deriving effect is dependent not only on the kind of cells involved, but also on the external environment and on the stimuli that fire the senescent response. However, the roles that these phenomena play in their relationships with tumours represent, if deepened, a possible strategy to counteract cancer cells proliferation and migration.

2.4. Senescent cells elimination (senolysis) and senolytic drugs

It is not surprising, in the light of the discovered negative effects of senescence, that senescent cells can impair the normal growth and the wellbeing of the surrounding cells. While, on one hand, cancer cells proliferation is hampered, on the other hand healthy tissues are damaged by the presence of the so called dysfunctional senescent cells (SNCs)[37]. For example, studies in obese mice showed that the elimination of senescent cells significantly reduced the obesity-related inflammation and, in parallel, alleviated the dysfunctions observed both in metabolism and in the adipose tissue. This clearance of the dysfunctional senescent cells was performed by the activation of suicide genes triggered by $p16^{Ink4a}$ promoter or by the use of a specifical class of molecules: the senolytic drugs[38]. Another example is the IPF (idiopathic pulmonary fibrosis) which is partially maintained by senescent cells, whose elimination considerably ameliorate pulmonary function[39]. Last, aging is correlated with side effects and increased fatigue following chemotherapy[40], while the elimination of senescent cells delays tumour recurrency and metastasis ameliorating, at the same time, the patient response to chemotherapeutic drugs. Thus, the clearness of senescent cells (senolysis) is a mandatory aim to reach in the scenario of tissues damages. Senolysis can be triggered by 1) senomorphic drugs, which abolish p53 inhibition through MDM-2 inhibition and allow the consequent p53-mediated SASP inhibition[41], 2) HSP-90 inhibitors, which reduce consequently the expression of the AKT protein (overexpressed in senescent cells) that produces apoptosis in senescent cells[42] and 3) senolytic drugs. The senolytic drugs are a class of molecules that can target and clear selectively senescent cells. The mechanisms through which these molecules accomplish their task is not completely clear, and, given the poor availability of senescent cells in an organism (with them representing only a small percentage of the total cells), studies about these drugs have been challenging. While comparing senescent cells with dividing non-senescent cells and with serum-starved non senescent cells gave rise to artifacts, focusing on the SCAP (senescent-associated anti-apoptotic

pathways) led to more interesting results[43]. Senescent cells, in fact, protect themselves from SASP-induced apoptosis through the expression of anti-apoptotic mediators (such as Bcl-2 or Bcl-xL). The elimination of those factors, and the successive bioinformatics comparison of molecules targeting them specifically, gave birth to a set of 46 potential senolytic compounds, like Dasanitib and Quercetin[44]. The senolytic specific action seem to depend on the amount of SCAP proteins targeted, in a direct proportional manner. On the contrary, senolytic compounds that only target one or few components of the SCAP (such as Navitoclax or other Bcl-2 inhibitors) present several off-target effects, inducing apoptosis even in not-senescent cells and playing a more "panolytic" action. Moreover, their effect is strongly tissue-related, acting on some senescent cell types and not on other types. The cytotoxic effect observed also depends on the SCAP protein targeted: for example, Bcl-xL inhibitors appear to be less toxic than the Bcl-2 ones[45]. For these reasons, the combination of more senolytic compounds is more specific for senescent cells, reducing the off-target effects on non-senescent, dividing cells.

2.4.1. Features of the most known senolytic drugs

The class of senolytic drugs is highly heterogeneous. These small molecules can be both of natural origin or synthetic, with different structures, targets and effect. The administrable dose varies according to the molecule, as well as the bioavailability and the biodistribution. Last, their action is often variegated, since some molecules that carry out the senescent cells' clearance can also act, depending on the dosage and on the cell type, as senescence or apoptosis inducers.

It has been shown that most of the senolytic molecules currently used share an action on the Nrf2-Keap1 pathway, a main regulator of the ROS-induced stress response. This pathway plays a protective role on cells, has an anti-apoptotic activity and its inactivation is linked to cancer development or premature senescence[46]. Tocotrienols, curcumin, quercetin, fisetin, piperlongumine and phloretin are some of the small molecules acting on Nrf-2-Keap1 pathway that also showed senolytic activity and they all share a natural origin.

Tocotrienols

Tocotrienols are derivatives of Vitamin E, they have four homologs (alpha, beta, gamma and delta) and display several positive effects for human health, showing a protective action on the cardiovascular and nervous system, on the skin and against malignancies[47]. Even if they are still not confirmed as proper senolytics, tocotrienols considerably ameliorate the lipid profile and reduce inflammation, probably acting on senescent cells or on the SASP[48].

Curcumin

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a flavonoid, main component of turmeric (Curcuma longa), that has been widely used in the past for its anti-inflammatory and antioxidant properties. Curcumin has a highly pleiotropic activity on cells, due to its ability of acting on several proteins and pathways.

The first effect of curcumin is observed on the cell cycle, where at the micromolar concentrations it induces the accumulation of cells in the G2/M phase. For example, it

reduces CDK1/Cyclin B1 expression by inhibiting p38 MAPK, Smad 2/3 and ERK signaling in human cardiac fibroblasts[49] Moreover, it induces mitotic catastrophe and consequent cell death[50]. It is also able to induce apoptosis at high micromolar doses, and, paradoxically, it can also trigger senescence in and ATM and ROS independent manner in vascular smooth muscle cells[51], but it triggers G2/M arrest and apoptosis in an ATM-dependent manner in the head and neck squamous carcinoma[52].
In particular, senescence induction was also observed after 10 and 15 μM treatment of curcumin for 24 h in both human colon cancer cells HCT116 and in breast cancer MCF-7 cells[53]. This occurs following cell cycle arrest in the G2/M phase, when the mitotic cells escape the block and enter in the successive phase of the cell cycle, showing increased SA-βGal activity and increased γH2AX foci also. The senescence induction is almost certainly dependent on p53, $p21^{waf1/cip1}$ and p16.
The potential senolytic activity of curcumin is currently under study. However, given its high protective action in ageing and its ability to increase organisms' lifespan, it is a promising molecule towards the elimination of senescent cells[54].

Fisetin

Fisetin (2-(3,4-dihydroxyphenyl)-3,7-dihydroxy-4H-1-benzopyran-4-one; 3,3',4',7-tetrahydroxyflavone, or 5-deoxyquercetin) is a flavonoid discovered in the venetian sumach (Rhus cotinus) and found in the green parts, hardwood and barks of many plants. Compared with other flavonoids, it displays high senotherapeutic effects. In fact, treatment with fisetin strongly decreased markers of senescence in mice, as well as markers of inflammation and oxidative stress[55]. Moreover, it has been shown that it clear senescent, but not proliferating, HUVEC cells, demonstrating selectivity towards the senescent phenotype. However, fisetin's senolytic activity has not been observed in senescent IMR90 fibroblasts, corroborating the variegated effects of senolytic small molecules.

Quercetin

Another important member of the class of flavonoids is Quercetin (3, 3', 4', 5, 7-pentahydroxyflvanone). Contained in several plants, fruits and vegetables, it displays anti-inflammatory activity (by acting on COX and LOX) and promotes immunity[56]. As already said for Curcumin, Quercetin is also able to induce G2/M arrest in human promonocytic U937 cells. Moreover, these cells also underwent caspase-mediated apoptosis, meaning that Quercetin is also able to regulate programmed cell death[57]. The same result was observed in A549 lung cancer cells exposed to quercetin-metabolite-enriched plasma, but not in the ones exposed to control plasma, by the reduced expression of CDK1 and Cyclin B1, meaning that also the deriving metabolites of Quercetin displays a role in the cell cycle arrest[57,58].
The most known action of Quercetin as a senolytic drug is in combination with Dasanitib: this "senolytic cocktail" showed a strong effect specifically oriented on senescent cells clearance[59].

III. Aim of the project

The main purpose of my work during my PhD is to couple two different phenomena: aneuploidy, on the one hand, and senescence, on the other hand. This coupling, in turn, would allow the selective clearance of aneuploid, senescent cancer cells by senolytic drugs. This project arises from the necessity of finding a new strategy for the elimination of cancer cells, reducing or possibly eliminating the impact on the healthy cells of the organism. In fact, as mentioned above, cancer cells can easily become resistant to chemotherapeutic drugs, thanks to their ability to escape the defensive mechanisms (apoptosis) that the cells normally use to protect themselves and to tolerate even hostile environments due to their altered genetic background. In particular, aneuploidy plays a key role in the genomic alteration observed in cancer cells. In the light of these assumptions, the re-activation of the protective pathways in aneuploid cancer cells tolerating adverse stimuli configurates itself as a powerful strategy for the elimination of malignancies. Last, coupling of these aneuploid cells with senescence would make cancer cells recognizable to the class of senolytic drugs: in that way, it could be possible to selectively target aneuploid senescent cells, preserving, at the same time, the euploid proliferating healthy cells.

1. **First objective: CDK1 inhibition and cell cycle arrest**

   The first aim to pursue is the block of the cell cycle in the G2/M phase in order to induce senescence in aneuploid cancer cells. Given their altered genetic background, cancer cells should be able to evade the cell cycle arrest exiting from mitosis (mitotic slippage), re-entering in the G1-like phase. At this point, the mitotic aberrations derived from this process should trigger senescence in response to alterations in the DNA content (polyploidy).
   In order to induce the G2/M block, the cell cycle kinase CDK1 was targeted. Several examples already exist in literature for other cell cycle kinases as CDK4/6 whose some inhibitors are currently on clinic trial or already used in chemotherapy. However, a lower number of attempts has been performed towards CDK1 inhibition and this make this protein even more interesting to be targeted.
   To reach this goal, CDK1 was targeted both with RNAi, to modulate its expression and with drugs to reduce its activity. The cell cycle arrest was verified by cytofluorimetry.

2. **Second objective: senescence**

   To ascertain if the observed cellular senescence was derived from aneuploidy, three different cellular systems were used: euploid, non-tumoral cells (RPE-1 hTERT), aneuploid cancer cells (HCT116, with microsatellite instability (MIN); MDA-MB 231 and MCF-7, triploid, showing chromosomal instability (CIN)) and a third, hybrid system between the first two constituted by RPE-1 hTERT cells in which aneuploidy has been induced and that can, thus, be used as a control system for establishing the causal relationship between aneuploidy and senescence.

3. **Third objective: senolysis of aneuploid, senescent cells**

   Once observed the senescent phenotype, cancer cells were treated with senolytic drugs to induce

their selective clearance. In particular, the flavonoids Fisetin and Quercetin were selected, both of natural origin, due to their known senolytic action.

IV. Results

1. Creation of an aneuploid, non-cancerous cell line

To evaluate at what extent cellular senescence is dependent on aneuploidy and not to additional alterations found in aneuploid tumor cells, I created clones of the immortalized RPE-1 hTERT cells (retinal pigmented epithelium), each one with a different aneuploidy level, which are considered 90% diploid. Various strategies exist to alter the genetic background of the cells, but most of them are usually cytotoxic. To obtain a not transformed aneuploid cell line I have used two strategies: CENP-E inhibition and the accumulation of segregation errors deriving from high passage cultured cells.

1.1. CENP-E inhibition: GSK923295

Perturbations of the centromere-microtubule interactions normally cause the cell cycle arrest in the M phase due to the SAC checkpoint that when activated allows cell cycle progression only in the presence of correct microtubule-centromere (kinetochore) attachment. Alterations in the centromeric sequence or in the centromeric proteins (kinetochore) have been correlated with aneuploidy by generating mitotic defects. Thus, inhibition of the centromeric protein CENP-E turned out to be a promising strategy to induce aneuploidy in normal cells. GSK923295 is a specific inhibitor of CENP-E, affecting its capability of dissociating from microtubules. The molecule acts by inhibiting the release of the inorganic phosphate and thus stabilizing the motor domain of CENP-E: this action impairs the formation of the metaphase plate, induces mitotic arrest and, after long exposition to the molecule, mitotic death.
To evaluate the effects of CENP-E inhibition on RPE-1 hTERT, I have used two different concentrations of GSK923295 high, (50 nM) and mild (5 nM) for 4 h and 72 h, respectively. The selected GSK923295 concentrations were based on literature, according to the observed effects. Previously, it has been shown[58] that GSK923295 actually blocks cells in prometaphase after 4 h of treatment at the dose of 50 nM. The 5 nM dose, instead, has been shown to reduce the activity of CENP-E to 50% after 72 h of treatment.

1.1.1. GSK923295 50 nM

Initially, I examined the effects of GSK923295 in HCT116 asynchronous cells to confirm the block in prometaphase. After treatment, cells were released for a total time of 60 minutes during which they were fixed every 15 minutes to evaluate if the mitotic arrest was reversible and the mitotic duration shortened. In fact, perturbing mitosis during certain mitotic sub-phases could trigger an accelerated mitotic exit[59]. Data showed that GSK923295 actually arrests HCT116 cells in mitosis. The mitotic index increases from the 7% of the control to the 20% after 4 hours of treatment (**Fig. 9B**). To further investigate in which phase of mitosis GSK923295 arrests cells, the single mitotic figures were analysed and the most part of them were actually prometaphases, confirming the data previously obtained (**Fig. 9C**). Interestingly, data collected after the release from GSK923295 demonstrate that the effect of CENP-E inhibition is reversible and that the mitotic index strongly decreases after GSK923295 removal. After 30 minutes, cells resume mitosis reaching a mitotic index of >7% which increases to >10% after 45 minutes of release, lasting until 1 hour. The heterogeneity of mitotic figures rises proportionally with the time of release, reflecting the loss of the synchronization obtained with GSK923295. Whether these cells exit mitosis, or simply relapse in the mitotic phase successive to the one in which they were stuck during treatment, it is still to be investigated.

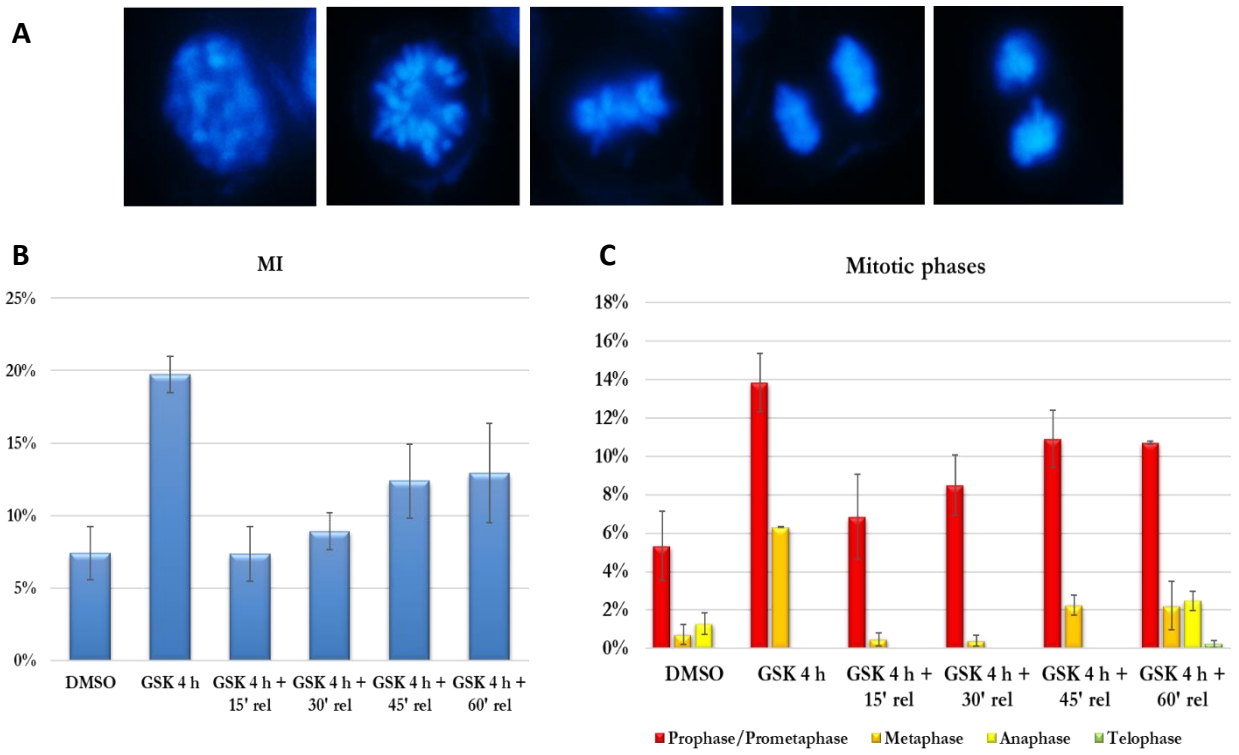


Fig. 9 - A. *DAPI-stained nuclei for mitotic phases analysis after treatment with GSK923295 50 nM 4 h and after the indicated minutes of release in HCT116 cells.*
B. *Mitotic index.* **C.** *Percentage of different mitotic phases at each time of release. Statistic analysis was made on the mean values of two replicates.*

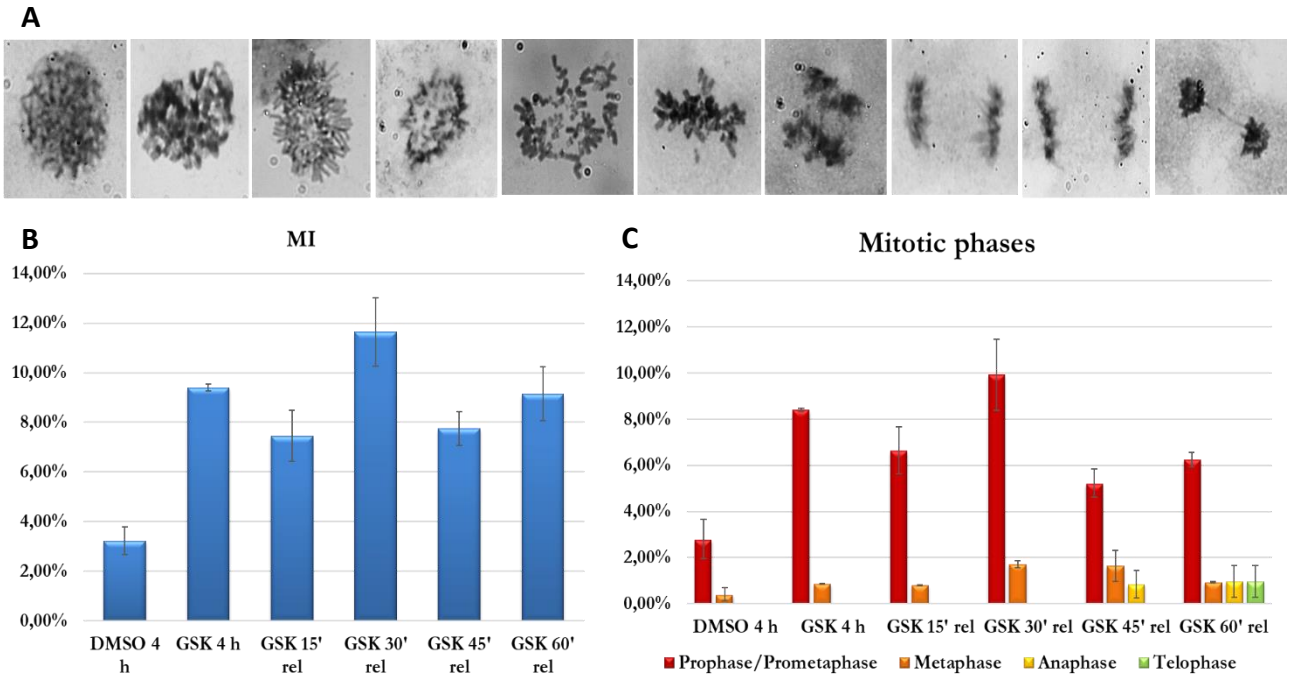


Fig. 10 - A. *Cytogenetics for mitotic phases analysis after treatment with GSK923295 50 nM 4 h and after n minutes of release in RPE-1 hTERT cells.*
B. *Mitotic index.* **C.** *Percentage of mitotic phases for every time of release. The standard deviation was calculated on the mean of two replicates.*

A slightly different trend was observed, instead, in RPE-1 hTERT treated in the same way as HCT116 cells (**Fig. 10**). In fact, even if after 4 h of treatment with GSK923295 the mitotic index rises from the 3% to almost the 10%, it stays unchanged during all the times of release, except for the 30 minutes of release, where the mitotic index overcomes the 10% (**Fig. 10B**). This might be related to the fact that RPE-1 hTERT, being an epithelial/fibroblastic cell line, shows highly increased duration of the cell cycle compared to the tumour cell lines. Interestingly, the mitotic phases (**Fig. 10C**) reproduce the ones observed in HCT116, with cells slowly restarting the mitosis from the phase in which they were blocked and proceeding through anaphase and telophase from the 45 minutes of release on. Once proven the CENP-E's activity inhibition, I decided to analyse the variation in the ploidy of RPE-1 hTERT after treatment with GSK923295 through metaphase spread assay. Since CENP-E inhibition induces mitotic arrest, I used Reversine, an inhibitor of the Mps-1 SAC protein, to allow cells the overcome the cell cycle arrest induced by the SAC checkpoint.

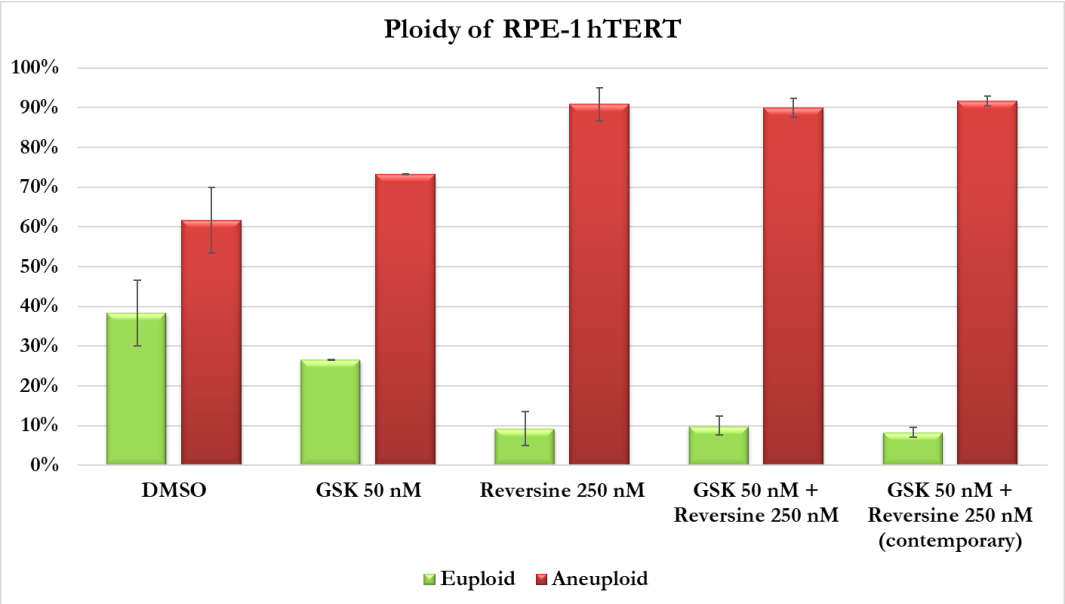


Fig. 11 - *Metaphase spread quantification of RPE-1 hTERT cells after treatment with GSK923295 and Reversine, 50 nM and 250 nM respectively. Statistics analysis was performed on two replicates, analyzing 30 metaphases/condition.*

Interestingly, RPE-1 hTERT cells appeared to already have a 50% aneuploidy level in the control, with a high percentage of ipodiploid cells. However, this aneuploidy rate increases in response to all the treatments performed (**Fig. 11**). While GSK923295 alone produces a 20% increase of aneuploid cells in respect to control, Reversine alone increased the aneuploidy rate until the 90%. Cells treated with GSK923295 50 nM for 4 hours followed by the treatment of 250 nM Reversine for 24 hours, also reach the 90% of aneuploidy A higher percentage of aneuploid cells was obtained when cells were treated with GSK923295 and Reversine at the same time, removing the media after 4 hours and re-administering Reversine until 24 hours. Thus, it appears that Reversine and GSK923295 have a synergistic effect on aneuploidy induction, especially when given one after another and not contemporary.

1.1.2. GSK923295 5 nM (acute and chronic treatment)

The second experimental approach that I decided to use involves the 5 nM dose of GSK923295 that has been shown to inhibit the activity of CENP-E by 50%. Given the reduced dose, the effects of the molecule have been revealed only after 72 h. In this case, GSK923295 was administered according to two different approaches: an acute treatment, where the molecule was given only once, and a sub-chronic treatment, in which the drug was given every 72 h. In both cases, the end point was settled for 2 weeks (**Fig. 12**).

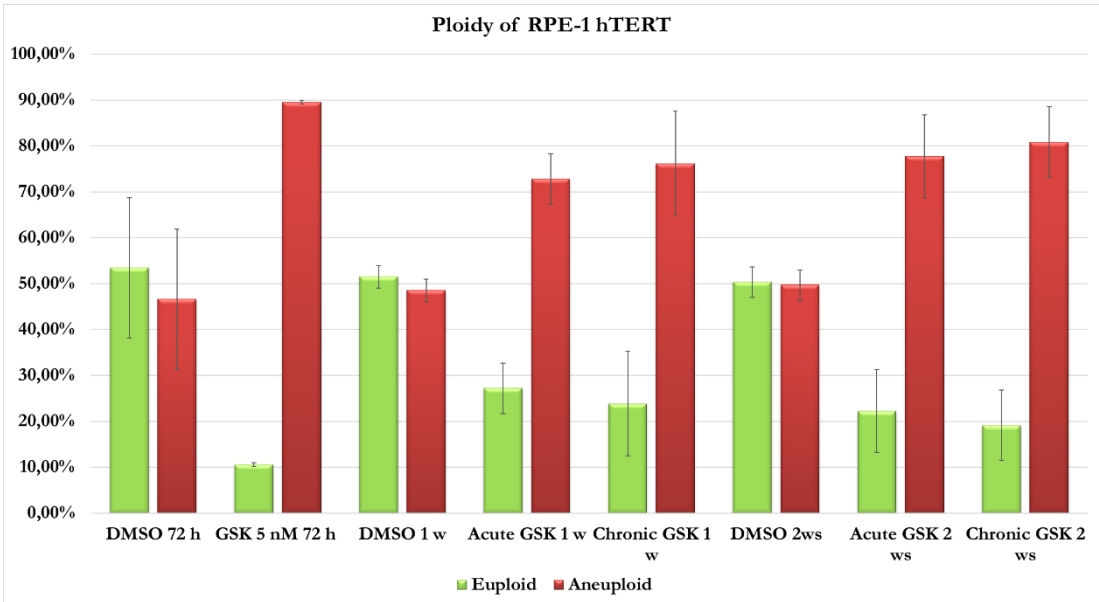


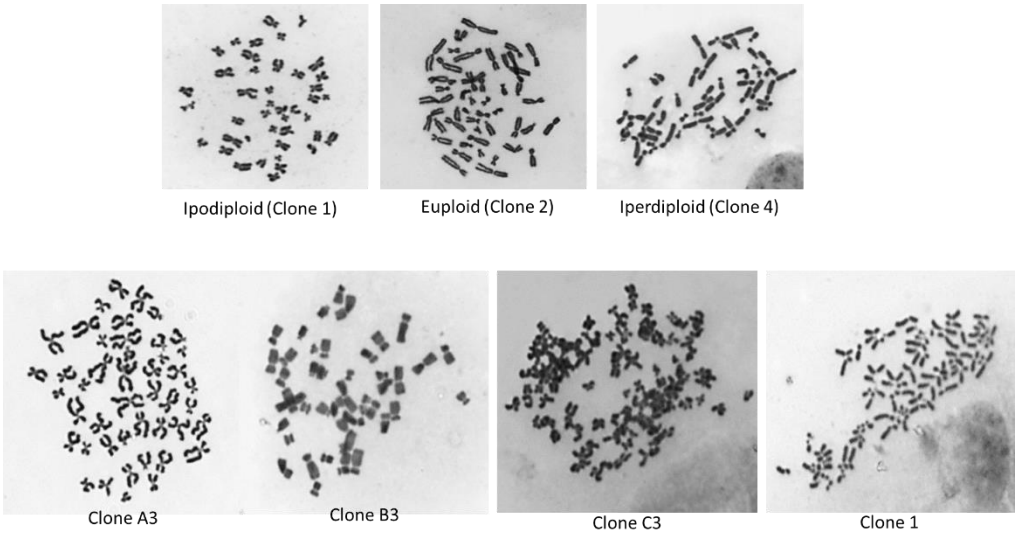
Fig. 12 - *Metaphase spread quantification of RPE-1 hTERT cells after treatment with GSK923295 in an acute (administered once) and a chronic (given every 72 h) manner. The percentage of aneuploidy increases during time following both treatments. Statistical analysis was performed on data deriving from two replicates, counting 20 metaphases/condition.*

Interestingly, even in this case a high number of aneuploid cells was observed in the control after 72 h. This aneuploidy level appears to remain almost stable after 1 week and 2 weeks in the control, while it grows consistently in response to GSK treatment, starting from 72 h and decreasing until 2 weeks. In particular, though both the acute and the sub-chronic treatments induce an increase in the percentage of aneuploid cells, the chronic treatment appears to be slightly more effective than the acute in increasing the aneuploidy level. Collectively, my results show that the treatment with a low dose of GSK923295 can be efficacious in inducing aneuploidy, and that this treatment clearly produces important changes in the number of chromosomes.

1.1.3. Drug free approach for aneuploidy establishment and maintainance

Despite the successful results obtained with GSK923295, with both the high and the low doses, and in all the various experimental approaches, the presence of aneuploidy in the control cells, and its increase during time, were a little odd. RPE-1 hTERT cell line is reported to have only a 10% level of aneuploidy. However, the observed percentage was higher in my hands (almost the 30%), especially if cells were left in culture for several days. Taking advantage from this observation, I cultured these cells for 3 weeks, to enrich the

aneuploid population, and then I seeded them in a 100 mm Petri dish at a very low density (300 cells) and in a MW24 with a concentration of 1 cell/well in order to generate aneuploid clones. After two weeks, clones were formed and then amplified. In the end, I could isolate 11 different clones, which were analysed through metaphase spread to establish their ploidy.



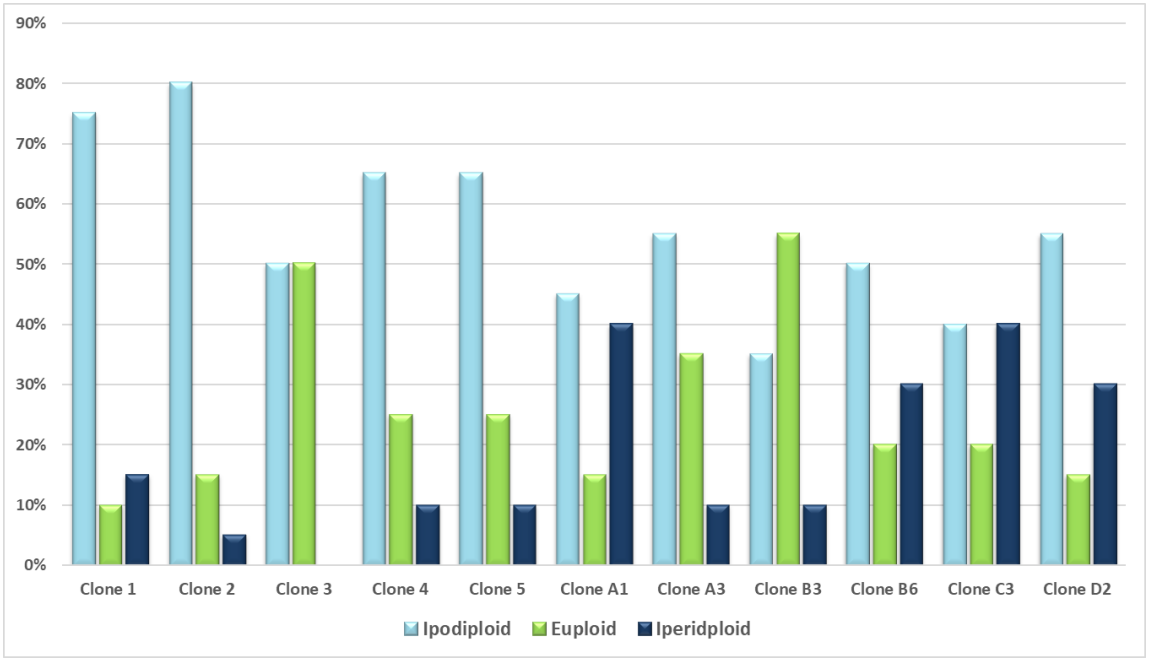


Fig. 13 – *RPE-1 hTERT clones' characterization by ploidy. 10 different aneuploid clones were isolated, each one with a specific aneuploidy rate. 20 metaphases/clone were counted.*

The clones derived from the 100 mm Petri dish were named from 1 to 5, while those derived from the 24-well plate were named A1, A3, B3, B6, C3 and D2. Several chromosomal aberrations were observed in almost all the clones (endoreduplications, broken chromosomal arms; **Fig. 13**, top). Moreover, except for the clones 3 and B3 that mantained an euploid rate of almost the 60%, all the other clones showed high rates of aneuploidy

(**Fig. 13**, bottom), with the clones A1 and C3 being the ones with a large number of iperdiploid cells (40%). Consequently, these two clones were used in the successive experiments as an aneuploid, non-tumour cell line, in order to observe the effects of aneuploidy on the cellular responses to treatments.

2. The G2/M arrest and senescence induction

In order to induce senescence in the cell lines that I used for my project, I decided, at first, to target CDK1, being this kinase often overexpressed in cancer and being the main regulator of the G2/M transition. The basic idea is to force, through CDK1 inhibition, the arrest of the cell cycle in the G2/M phase. At this point, cells can engage into two different destinies: either they die during mitosis (*Dim*, death in mitosis), either they skip mitosis (mitotic slippage). In this last case, cells become polyploid and usually go through apoptosis or senescence[59]. Some cancer cells harbouring mutations, instead, can continue cycling, adapting to the new genetic framework. My idea was to induce senescence through polyploidy establishment after mitotic slippage (**Fig. 14**).

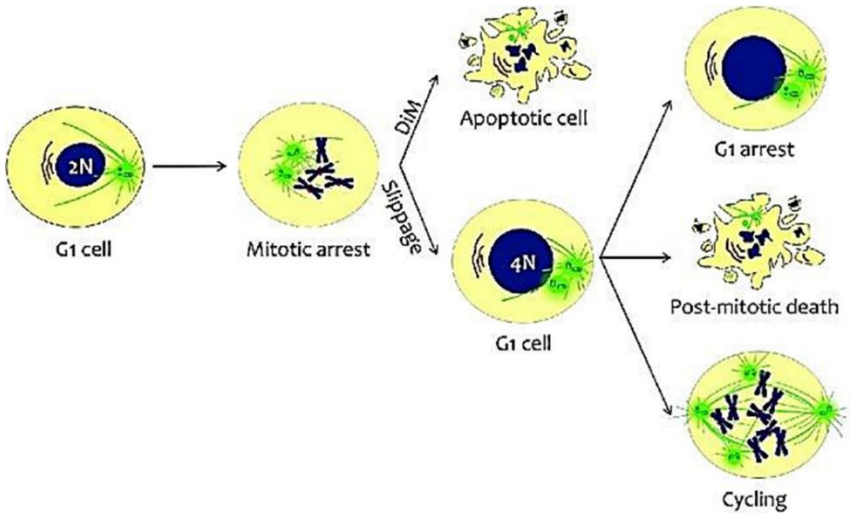


Fig. 14 – *Schematic representation of cell fates following prolonged mitotic arrest*[60].

However, given the uncertainty of the outcome of this approach, I decided to pursue an alternative path for inducing senescence. It has been shown that many natural compounds, particularly the ones belonging to the flavonoids family, can trigger a cell cycle arrest in the G2/M phase and senescence. Though these molecules present an elevated degree of pleiotropy, acting on several different targets inside the cells, they represent a powerful alternative for senescence induction. In particular, I decided to use Curcumin and Quercetin, both having a double-faced action as senescence inducers and senolytics. Thus, I could count on a sort of "plan B" for senescence induction, as well as a certain senescence inducing drug for the subsequent treatment with senolytics, in the case that CDK1 depletion was not successful for the purposes of my project.

2.1. CDK1 depletion

Given the difficulties encountered to isolate CDK1 structure and due to the strong similarities shared by the various CDKs, it has been and it still is a hard task to find a selective, specific CDK1 inhibitor. In fact, kinase inhibitors usually are ATP competitors that actually hamper the

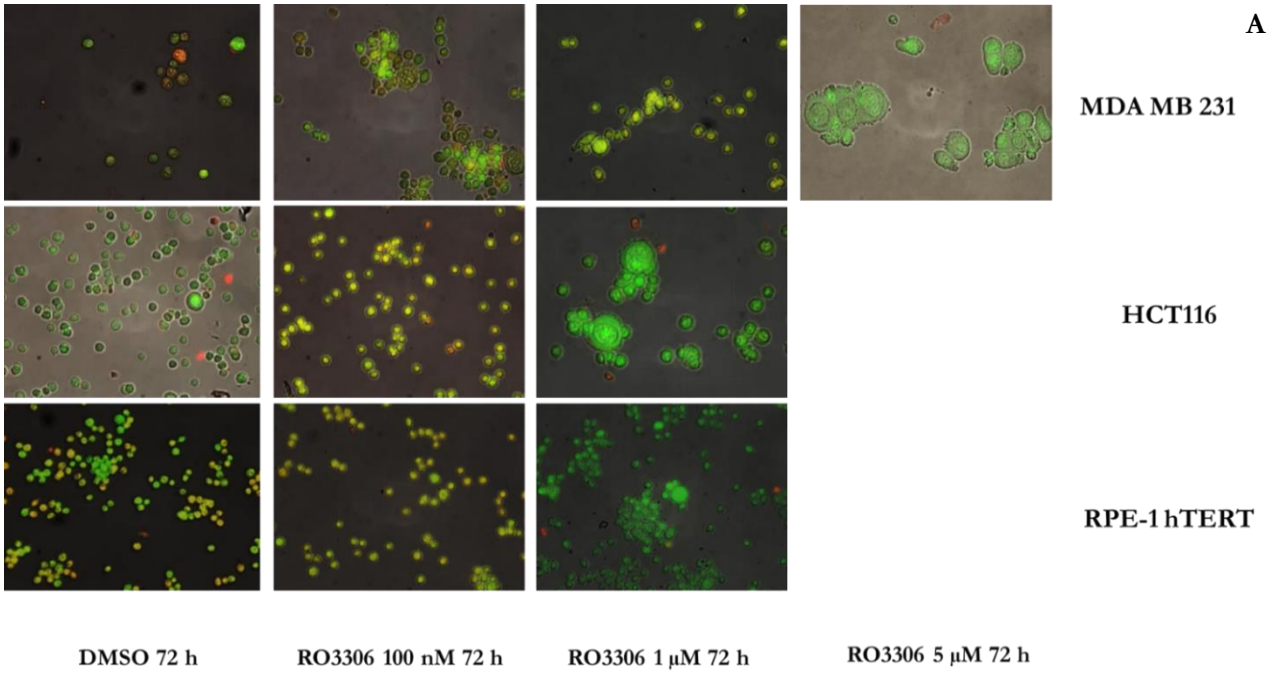
protein catalytic activity and for this reason these molecules often show high affinity for different kinases at the same time. Nowadays, the most used and selective known inhibitor of CDK1 is RO3306, with a Ki of 20 nM (>15 folds selective against several kinases). Even if at higher concentrations this molecule can also target the other cell cycle kinases, it has a specific action on CDK1 activity. For this reason, RO3306 has been selected for CDK1 inhibition to induce the arrest of the cell cycle. In parallel, considering that few data were available about CDK1 activity reduction and since I did not know if this pharmacological approach could effectively lead to positive results, I decided to perform, at the same time, RNA interference against CDK1. In fact, it has been shown that reducing CDK1 expression[61] triggers the onset of polyploidy and, at the same time, it could be another potential strategy to remove CDK1 from the cells acting upstream respect to the mature protein.

2.1.1. RO-3306: preliminary assays

The effect of a molecule on its target is dependent on various aspects, especially when administered to a cultured cell line. The culture media, the nature of the solvent, the cell line used are all parameters that must be kept in count when performing a treatment. In particular, no preexisting doses were recommended in regard to RO3306. By contrast, several experiments were performed and can be found in literature, where a high heterogeneity in cellular responses was observed after the treatment with RO3306. For these reasons, preliminary assays were necessary to establish the most accurate dose for the used cell line.

2.1.1.1. Cytotoxicity and cell cycle arrest

First of all, I needed to find a concentration that could allow the G2/M arrest and, at the same time, that was not cytotoxic. At first, two high doses were tested, 5 and 7 μM, on three different cell lines: HCT116, MDA-MB 231 and RPE-1 hTERT. These first cytofluorimetric assays revealed an effective block of the cell cycle in the G2/M phase, in the tumor cell lines, with the arrested population rising proportionally with the dose. However, RPE-1 hTERT started to arrest in G2/M only with the dose of 7 μM and higher. As a collateral effect, the G2/M arrest of these cell lines was accompanied by a strong cytotoxic effect. A huge mortality rate was observed and further confirmed with acridine orange/ethidium bromide assays, in both HCT116 and MDA-MB 231 cells, while RPE-1 hTERT seemed to better tolerate the action of RO3306, showing no cell death (**Fig. 15A**). As an ulterior confirmation of these results, the 5 μM dose was tested for 24 h in another cell line, the MCF-7, showing, even in this case, cell cycle arrest in G2/M phase and toxicity. Due to the observed toxicity, I decided to test lower doses of the molecule, decreasing it to a panel of different concentrations going from 100 nM to 1 μM. The toxicity was assessed through acridine orange/ethidium bromide assay for different times in MDA-MB 231, HCT116 and RPE-1 hTERT. In general, these low doses did not produce apoptosis, which starts to be visible from the concentration of 1 μM in HCT116 and of 5 μM in MDA-MB 231 after 72 h treatment. Unfortunately, neither the 100 nM nor the 1 μM dose of RO3306 triggered a G2/M arrest of the cell cycle in none of the cell lines analysed. Successive experiments analysing the 2 and 3 μM doses showed no cell cycle arrest as well. For all these reasons, the final selected dose was the 5 μM one, inducing the arrest of the cell cycle in G2/M phase for the half of the viable population (**Fig. 15B**)and a moderate toxicity compared to the higher concentration of the drug.



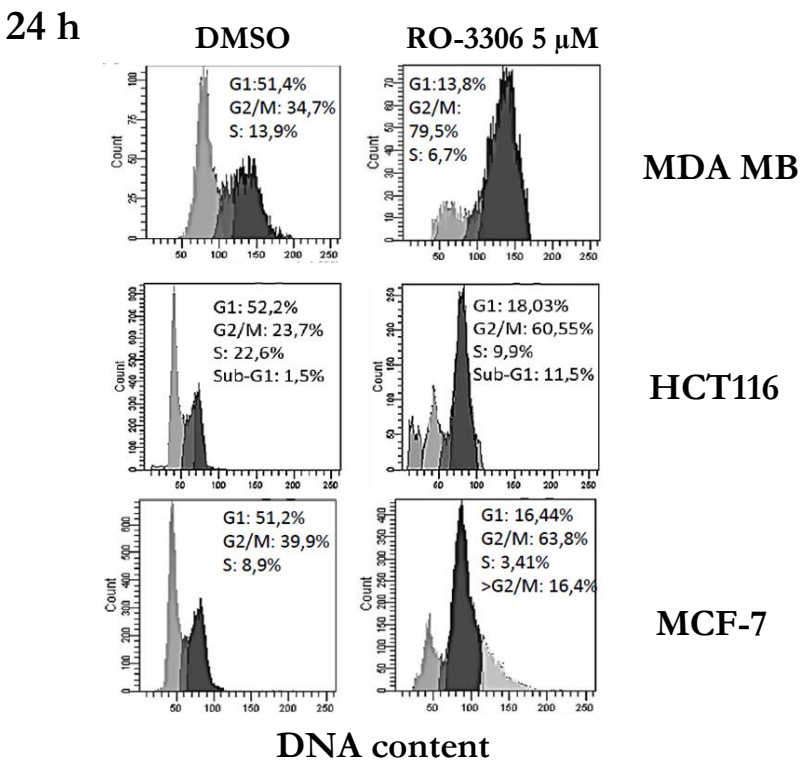
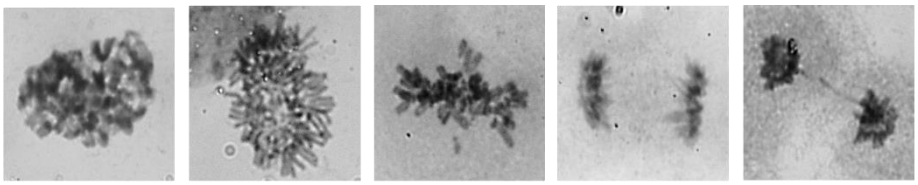


Fig. 15 – A. *AO/EB assay of cancer cell lines MDA-MB 231 and HCT116 and not tumor cell line RPE-1 hTERT treated with RO-3306. Low dose 100 nM reveal no toxicity in cancer cells. Apoptosis, with visible blebbings and fragmented chromatin, starts to be observed in HCT116 at the 1 µM concentration and in MDA-MB 231 at the 5 µM concentration. RPE-1 hTERT do not reveal cytotoxicity in response to the treatments.*

B. *Cytofluorimetric analysis of MDA-MB 231, HCT116 and MCF-7 cell lines following 5 µM treatment with RO-3306. G2/M population, in dark grey, is identified by 4 n DNA content and increases after the treatment with CDK1 inhibitor. G1 and S phase are stained light grey and grey, respectively.*

2.1.1.2. Cell cycle arrest: G2 or M phase?

Once selected the concentration to be used, my next aim to reach was the senescence following mitotic slippage and iperdiploidy. The cell cycle arrest after treatment with RO3306 was effectively confirmed, however, cytofluorimetric analysis cannot discriminate between the G2 or the M phase. Given that CDK1 is necessary to both the initiation and the prosecution of mitosis, it is not easy to understand at which boundary the cells arrest to when the kinase is inhibited and cells need to be halted in mitosis rather than in the G2 phase, for the mitotic slippage to occurr. Moreover, this separation it is even less defined in asynchronous cell populations I used for my experiments. For all these reasons, the mitotic index after treatment with RO3306 was evaluated through cytogenetic assay in HCT116 cells. The drug was administered at the dose of 5 μM for 24 h, and cells were lysed and fixed after releases of 15 minutes each. Interestingly, the mitotic index appeared to be lower than in the control at 24 h of treatment, while it started rising from the first 15 minutes of release to the following endpoints, reaching a peak of the 13% after 60 minutes of release (**Fig. 16**). This first result shows that after the pharmacological inhibition of CDK1, HCT116 cells reduce their mitotic population even respect to the control, meaning that the treatment forces their arrest in the G2 phase. When released, cells restart cycling, entering mitosis and enriching their mitotic sub-population. This finding is corroborated by the successive analysis of the mitotic phases, where the relative abundance of each phase (included in the previously obtained mitotic index percentage) was evaluated. As can be seen in **Fig. 16**, the mitotic population is initially composed mainly by prophases, prometaphases and metaphases, becoming always more differentiated proportionally with the length of the time of release from the treatment. At the final endpoint of 60 minutes of release, anaphases and telophases are observed, even if prophases and prometaphases still remain the most numerous figures.



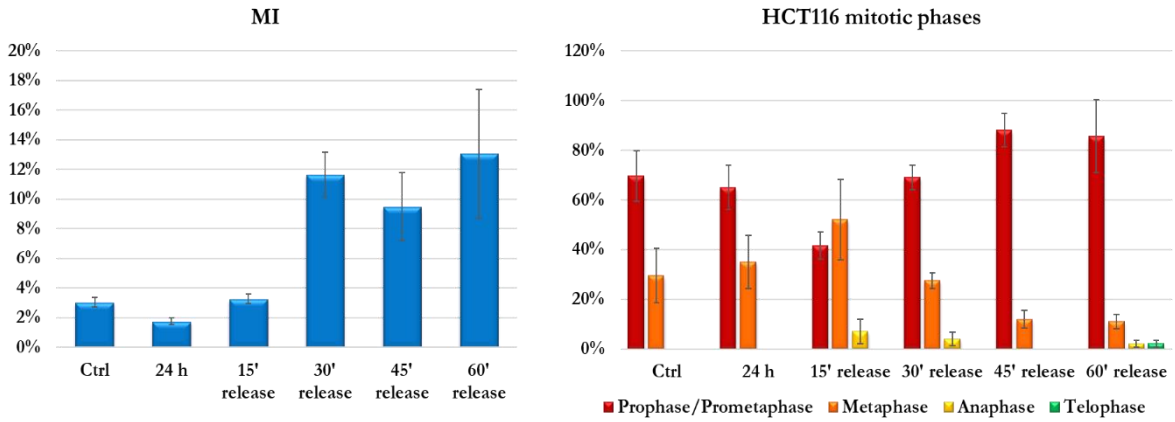


Fig. 16 – *Cytogenetic analysis of HCT116 treated for 24 h with RO-3306 5 μM and then released for 1 hour. Mitotic index and the percentage of each mitotic sub-phase were calculated every 15 minutes during the hour of release. The mitotic index reduces at 24 h of treatment with CDK1 inhibitor, revealing cell cycle arrest in G2. The mitotic figures become always more variegated, from the precocious to the more advanced, as release time increases.*

As a further confirmation of the obtained result, immunofluorescence assays were performed labelling Cyclin B1 protein, a marker of M phase. In fact, it is expressed throughout the mitotic phase (until metaphase), and it starts accumulating from the late G2 phase. The immunofluorescence assays were performed on both HCT116 and RPE-1 hTERT cells (the latter used as a negative control).

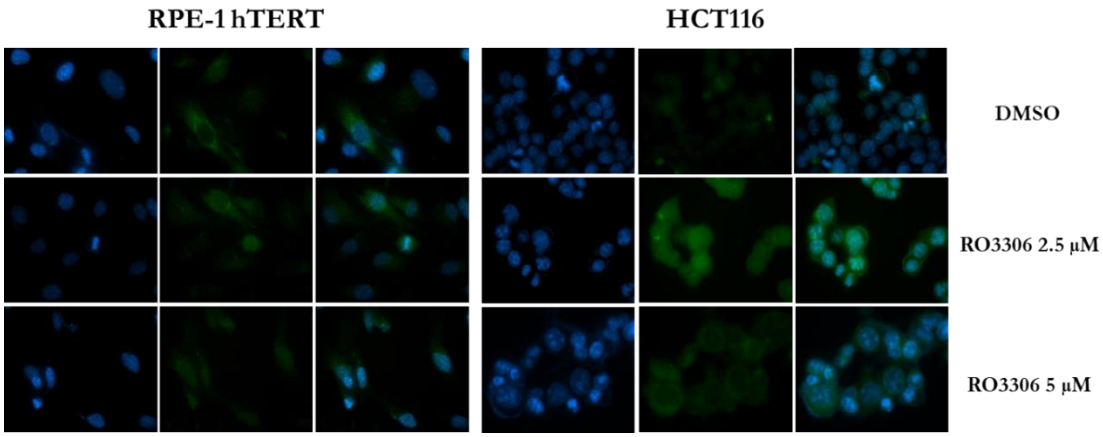


Fig. 17 – *Immunofluorescence assay for Cyclin B1 on RPE-1 hTERT and HCT116 following treatment with two different concentrations of RO-3306 (2.5 and 5 μM) for 24 h. Fluorescence intensity reduces in a dose-dependent manner, meaning that cells are blocked in the G2 phase.*

As shown in figures, while no difference in fluorescence intensity can be observed in RPE-1 hTERT cells after treatment with RO3306, in HCT116 the signal strongly increases when cells are treated with 2.5 μM of the molecule, decreasing at the concentration of 5 μM (**Fig. 17**). The low dose of RO-3306 do not probably affect the mitotic entry, as confirmed from the high fluorescence intensity of Cyclin B1, while the 5 μM concentration, instead, prevent cells to enter mitosis, by arresting them in the G2 phase. This result is also confirmed from the uncondensed state of the chromatin in the cells treated with the high dose of RO-3306, which is a clear marker of the G2 phase. Collectively, these results show that the pharmacological inhibition of CDK1 by RO3306 5 μM induces cell cycle arrest in the G2 phase in aneuploid cancer cells.

2.1.1.3. Cellular senescence

The cell cycle arrest can be induced by various triggers. For example, DNA damages that cannot be recovered are recognized as a obnoxious stimuli from the cell, which stops its proliferation to avoid the propagation of an altered offspring. After cell cycle arrest, the cells can go towards different fates: apoptosis or senescence are the most common pathways that are activated. Since the treatments with RO-3306 effectively arrested cells in the G2 phase, I decided to verify if, despite the observed apoptotic response, also the senescence induction could be noticed. To this aim, several approaches were used, focusing both on the high (5 μM) and the low (100, 250, 500 nM and 1 μM) doses of RO-3306. In particular, the effects of the high dose were analysed after 24 h and after 72 h of release, and after 72 h of treatment; the low doses were tested at longer endpoints, for different cell lines. As previously observed, the 5 μM dose of RO-3306 mainly triggered apoptosis in cancer cells. RPE-1, instead, revealed no cytotoxicity in response to the treatment, acting as a negative control. The treatment

was performed for 24, 48 and 72 h in HCT116 (**Fig. 18**). Despite the high rate of mortality, few cells stained positive to the SA-βGal assay after 72 h of continued treatment. However, to define these cells as senescent other assays are needed, since their morphology does not reflect the typical senescent phenotype.

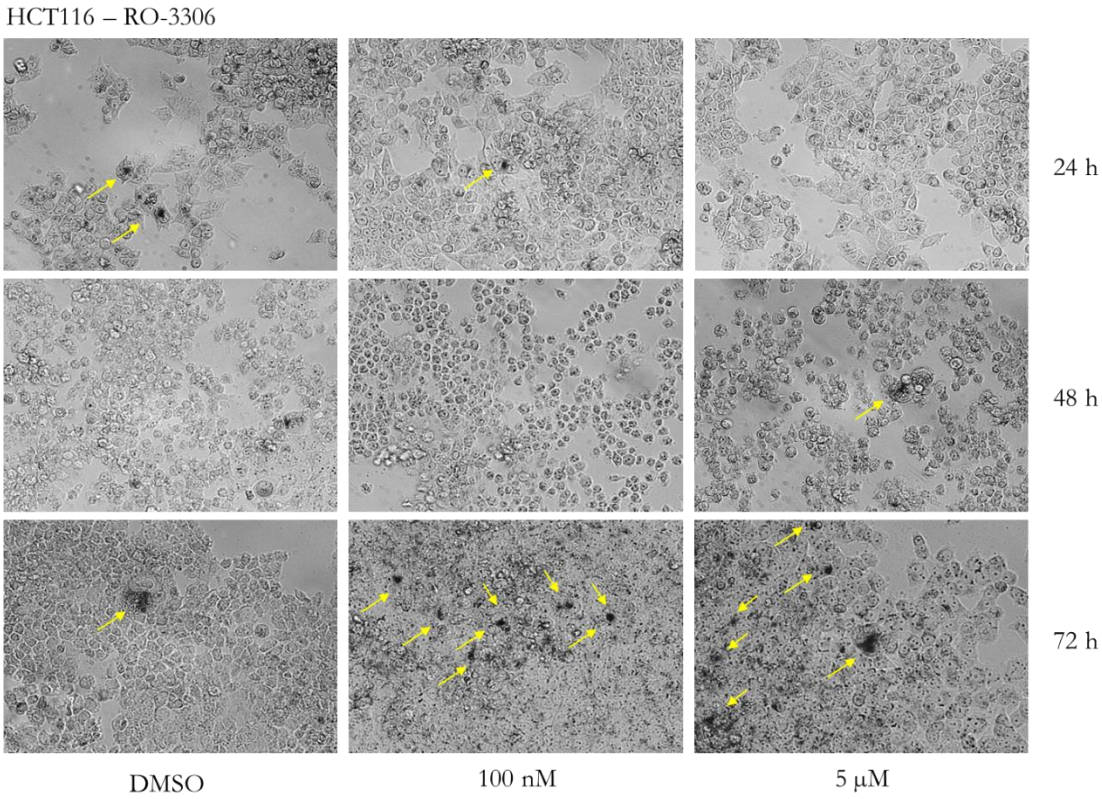


Fig. 18 – *SA-βGal assay for HCT116 cells following low (100 nM) and high (5 μM) doses of RO-3306 for three different endpoints (24, 48 and 72 h). The percentage of cells staining blue increases with time, though the cells morphology does not properly reflect the senescent phenotype.*

For this reason, a different approach was settled for the high dose of RO-3306. Senescence establishment is not immediate: instead, it requires several days, according to the trigger, and each stage of senescence can be differently characterized. This concept can explain why the majority of the SA-βGal positive cells can be observed at 72 h. However, another successful strategy is to perform an acute treatment and then release cells for long times: in this case, cells can respond to the induced stress in absence of the obnoxious stimulus, which can result in increased toxicity. Thus, HCT116 and MCF-7 were treated with 5 μM RO-3306 for 24 h and then released for 72 h. RPE-1 hTERT and the aneuploid RPE-1 clone were used as controls.

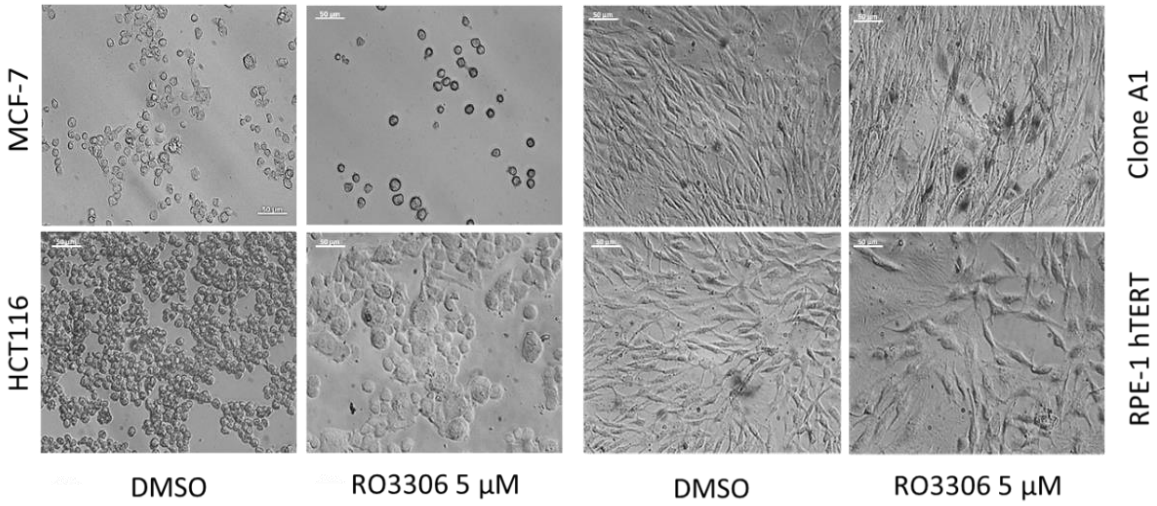
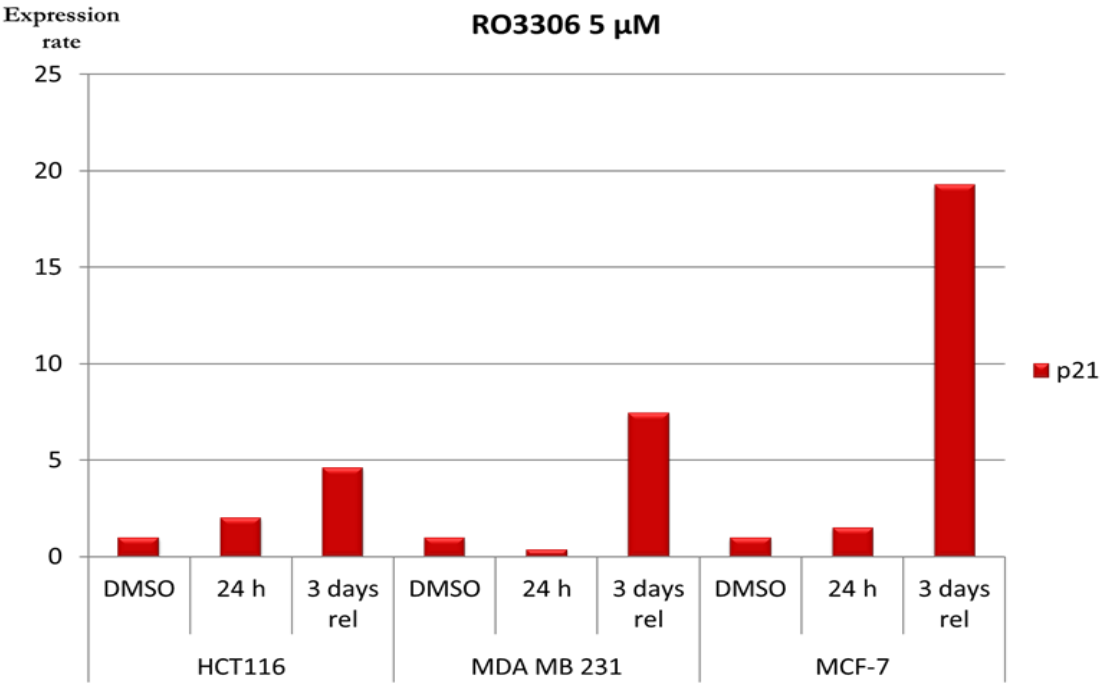


Fig. 19 – *SA-βGal assay on cancer cell lines HCT116 and MCF-7 and on non-tumor cell lines RPE-1 hTERT and aneuploid Clone A1 following 24 h treatment with RO-3306 5 µM. Interestingly, each cell line reacts differently, confirming the heterogeneous action of the molecule. HCT116 are larger and flatter compared to the control but do not stain blue; MCF-7 cells show an apoptotic phenotype; Clone A1 cells stain blue, showing senescence, while RPE-1 hTERT become larger but not blue, suggesting that the action of the CDK1 inhibitor is more specific for aneuploid cells.*

As can be observed in **Fig. 19**, the cellular responses to this treatment are heterogeneous. MCF-7 cells appear to be small and stressed, suggesting an apoptotic phenotype; HCT116 cells show considerably altered shape and dimensions, becoming wide and flat, without staining positive to the SA-βGal assay; WT RPE-1 hTERT cells show an increased size and no positivity to the staining, while A1 clone cells maintain more or less their dimension (though a slight increase is still detectable) while staining blue. As these differences can be attributed to variables that are strictly related to the SA-βGal assay (whose reactivity changes across the cell lines) and to the heterogeneity of RO-3306 action on differfent cell lines, clearly this experimental approach induces an increase in the cell size and, when visible, an accumulation of the β-Galactosidase enzyme, specifically in aneuploid cells. Interestingly, the RT-qPCR analysis (**Fig. 20A**) for $p21^{waf1/cip1}$ expression following RO-3306, under the same conditions as before, reveals a decreased expression after 24 h and an increased expression after 72 h of release in all the three tumor cell lines analysed (HCT116, MDA MB 231 and MCF-7). However, if, on one hand, $p21^{waf1/cip1}$ increased levels can be indicative of a pre-senescent state, some of these cell lines experience apoptosis and cell death, meaning that this experimental approach is probably not the most effective for senescence induction.

A



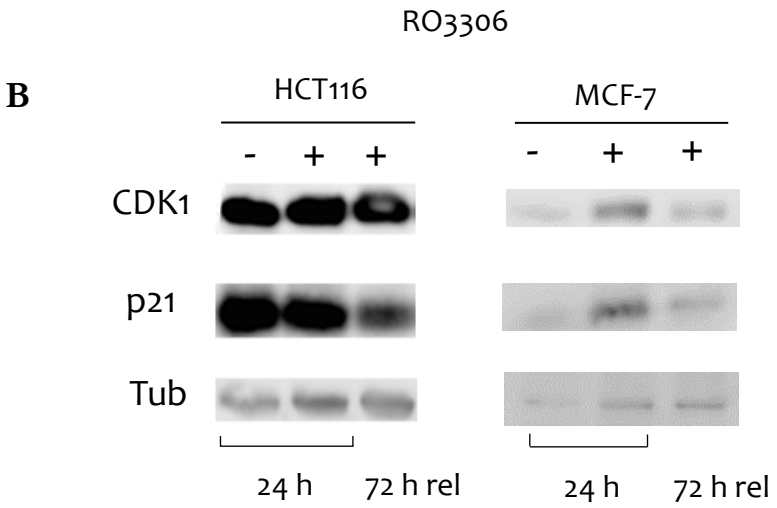


Fig. 20 – A. *RT-qPCR in three different cancer cell lines (HCT116, MDA MB 231 and MCF-7) after RO-3306 5 µM treatment for 24 h and 72 h from release for evaluation of* $p21^{waf1/cip1}$ *expression.*
B. *Western blot analysis for* $p21^{waf1/cip1}$ *protein expression in HCT116 and MCF-7 cells.*

Moreover, the protein levels of $p21^{waf1/cip1}$ evaluated by western blot (**Fig. 20 B**) in HCT116 and MCF-7 do not increase after 72 h of release from the treatment. Instead, the protein expression appears to be reduced compared to the control and the 24 h of treatment (where no increase is expected to be observed).
To solve this ambiguity, also observed in the SA-βGal assay in which cells appear to be bigger and flatter, but not blue, two other RT-qPCR were performed (**Fig. 21**).

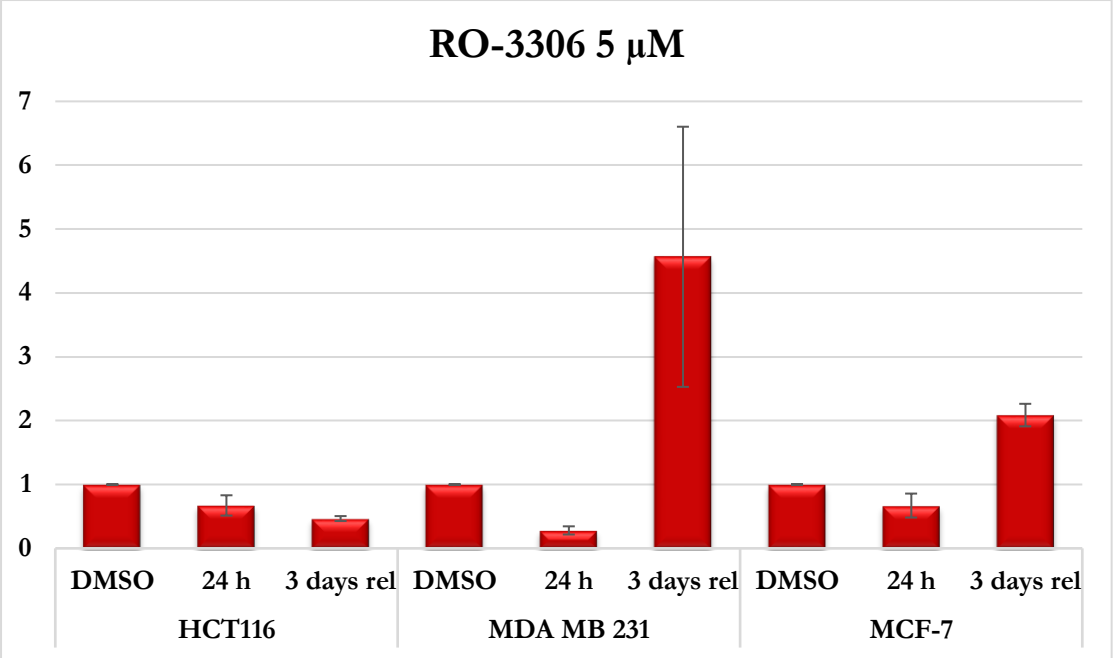


Fig. 21 – *RT-qPCR in three different cancer cell lines (HCT116, MDA MB 231 and MCF-7) after RO-3306 5 μM treatment for 24 h and 72 h from release for evaluation of* $p21^{waf1/cip1}$ *expression. Statisical analysis was performed on two replicates.*

This time, the analysis revealed a reduction of $p21^{waf1/cip1}$ transcript in HCT116 cells, confirmed the increase already observed in MCF-7 cells, and also showed an increase, but huge variability, in MDA MB 231. While the result observed in MDA MB 231 can be probably explained through the high heterogeneity of their genetic background, the reduction seen in HCT116, as previously observed in the western blot analysis for $p21^{waf1/cip1}$, might significate that senescence induction, whenever observed, it is not probably dependent on $p21^{waf1/cip1}$.
Low doses of RO-3306 for longer times of exposure trigger, instead, an increase of the SA-βGal positive cells compared to the control. In particular, 100 nM and 1 μM of RO-3306 were tested on MDA-MB 231, HCT116 and RPE-1 hTERT cells for 72 h and for 1 week on MDA-MB 231 cells. As can be observed in **Fig. 22**, the effects of the drug changes according to the cell type. While MDA-MB 231 show an increase in the number of senescent cells following the treatment with 100 nM RO-3306, HCT116 rate of cells positive to the assay increases after the dose 1 μM. RPE-1 hTERT, on the contrary, show no staining after 72 h of treatment, but this could depend on their reduced speed of cycling compared to the tumor cells. In fact, >90% of RPE-1 can be found, normally, in the G1 phase of the cell cycle, and inhibiting

CDK1 in this phase could produce no effect since it starts being expressed in late G2.

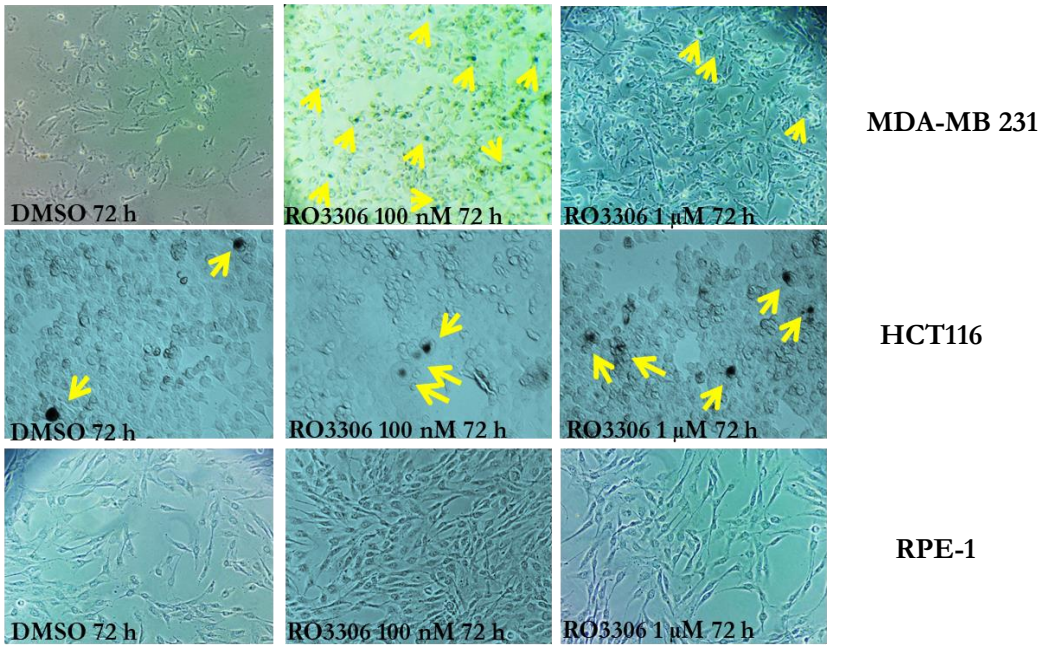


Fig. 22 – *SA-βGal for HCT116, MDA-MB 231 and RPE-1 hTERT following long exposure (72 h) with low doses (100 nM and 1 μM) of RO-3306. A slow increase in the percentage of senescent cells is detectable in HCT116 cells with the micromolar concentration, and in MDA-MB 231 with the nanomolar dose. RPE-1 hTERT, as expected, do not stain blue.*

Collectively, the results obtained following treatment with RO-3306 revealed a heterogeneous response about senescence induction. In general, at least 72 h are required to observe positivity to the SA-βGal assay and longer times of exposure to the drug seem to be more favourable to senescence induction. A lower dose (100 nM or 1 μM) seems to be more effective in inducing senescence in cancer cells, while normal cells are more affected by a higher dose (>5 μM), probably for their reduced proliferating speed. However, because of the great deviation observed in the results, MDA MB 231 were removed from all the following analyses.

2.1.1.4. *Combining RO-3306 with SAC-targeting drugs*

Since the treatment with RO-3306 alone triggered a reduced, heterogeneous senescent response, I decided to try another experimental approach. The mechanism by which cells should enter senescence after CDK1 inhibition is based on the subsequent cell cycle arrest in G2 phase. However, there is no warranty that the prolonged arrest alone leads to senescence, since cells can also decide to go through programmed cell death (which has been effectively observed after RO-3306). Another way to obtain senescence is the already cited G1 arrest following mitotic slippage. The main idea was to combine CDK1 inhibition mediated by RO-3306 with the inhibition of the spindle assembly checkpoint (SAC), which is primarily involved in mitotic arrest after the detection of misaligned chromosomes. Many of the SAC proteins are phosphorylated by CDK1, such as Mps-1: this double inhibition of one of the key SAC proteins should allow the

forced escaping of mitosis, with cells dragging back errors deriving from the lack of CDK1 action. Given these assumptions, HCT116 cells were treated with both RO-3306 and Reversine (Mps-1 inhibitor), contemporary or one after the other, for 24 h and then released for 72 h.

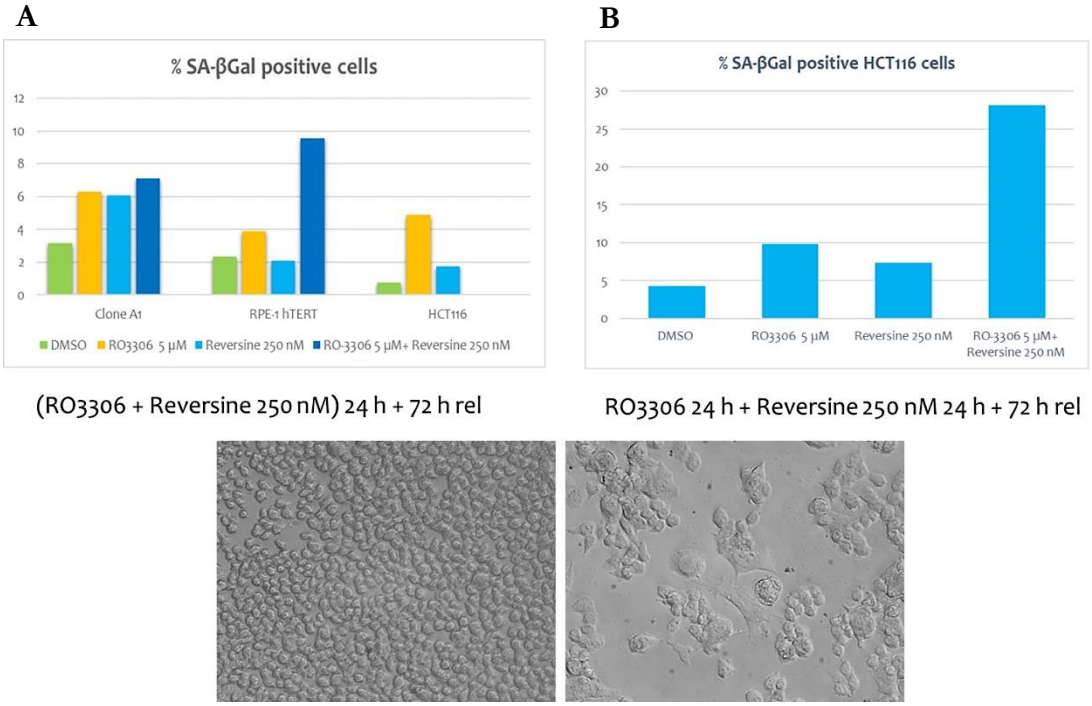


Fig. 23 – A. *Quantification of SA-βGal assay in different cell lines (HCT116, Clone A1 and RPE-1 hTERT) following combined, contemporary treatment of RO-3306 and Reversine. While RO-3306 alone induces an increase in the percentage of senescent cells in all the aneuploid cell lines, in the case of the double treatment HCT116 do not stain blue. However, as can be observed in the bottom panel of the image, cells are huge and flat compared to the control, mimicking the senescent phenotype.*

B. *Quantification of SA-βGal assay in HCT116 following double treatment with RO-3306 and Reversine administered one after 24 h from the other. Here, cells stain blue reaching the 28% of positivity to the assay. Thus, different experimental approach produces different responses even in the same cell line.*

As can be seen in **Fig. 23**, the two different approaches produced two different responses. When the two molecules are given contemporary, RPE-1 hTERT and Clone A1 show an increase in the percentage of senescent cells, whereas HCT116 do not stain blue. However, HCT116 appear huge and flat compared to the control, meaning that, though not blue, these cells can still be senescent. Interestingly, the RO-3306 treatment alone produce an increase in the percentage of senescent cells which is specific for the aneuploid cell lines. On the other hand, in HCT116 cells to which the two molecules were given one by one, the percentage of SA-βGal positive cells increases after the treatments. Interestingly, the amount of senescent cells reaches almost the 30% following RO-3306 and Reversine treatment, while it only slightly increases when the two molecules are administered alone. Globally, this indicates that RO3306 and Reversine act synergically, inducing senescence.

2.1.2. CDK1 RNAi: preliminary assays

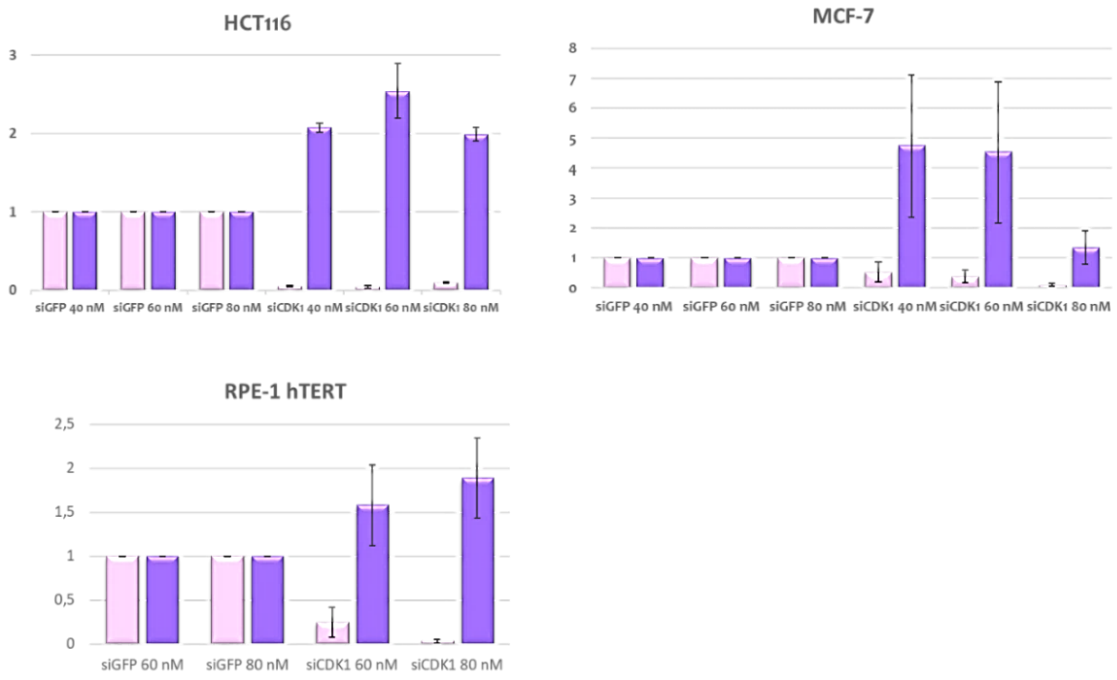
Also in this case, preliminary assays were necessary to define the amount of siRNA and the exposure time to it to obtain CDK1 posttranscriptional silencing in different cell lines. RNAi was performed on HCT116 and MCF-7 cells, using RPE-1 hTERT cell line as a control.

2.1.2.1. *Transcript and protein expression*

First of all, the right siRNA concentration was needed to be established. I transfected three different doses in the nM range (40, 60 and 80 nM) for 48 and 72 h in HCT116 cells. Clearly, in order to evaluate the percentage of CDK1 mRNA reduction, the first assay to be performed was RT-qPCR, followed by WB analysis to evaluate the related protein concentration. siGFP was used as a control, since the cells used do not express the green fluorescent protein. As shown in **Fig. 24**, CDK1 levels (pink) reduce considerably after 48 h of siRNA transfection already with the 40 nM dose. CDK1 mRNA levels keep constant even increasing the amount of siRNA. In this assay, $p21^{waf1/cip1}$ levels were also investigated, revealing a strong increase in parallel with CDK1 reduction. In this case, the only observable exception is for the 80 nM concentration, in which the $p21^{waf1/cip1}$ levels are always higher than the control, but considerably lower than the samples treated with the lower siRNA doses.

This information is particularly relevant in the context of senescence induction, meaning that CDK1 depletion might trigger senescence already after 48 h. WB analysis confirms what already observed for the mRNA expression, with CDK1 protein levels decreasing already after 48 h of siRNA transfection, while $p21^{waf1/cip1}$ protein levels increase. Interestingly, the posttranscriptional silencing of CDK1 has a major impact on $p21^{waf/cip1}$ for the doses of 40 and 60 nM, while protein levels are less affected at the 80 nM concentration. This might be explained with the fact that a higher amount of siRNA is often susceptible of off-target effects, while lower amounts normally target the correct mRNA in a more specific manner[62].

A



B

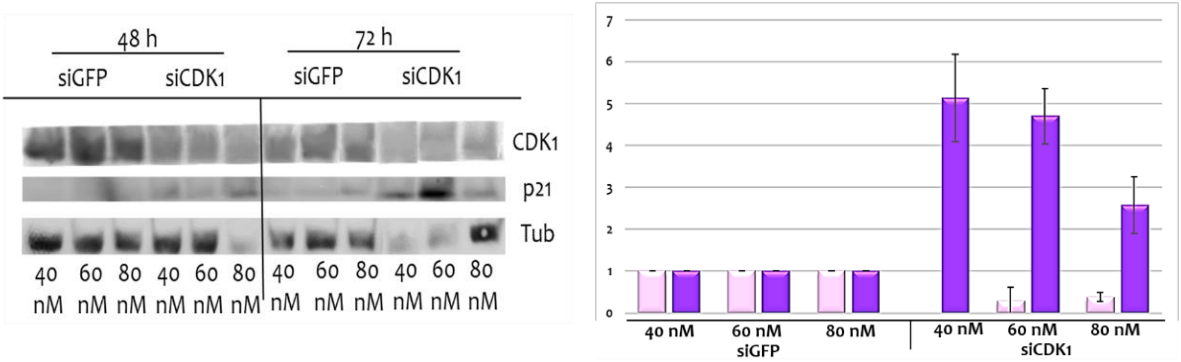


Fig. 24 – A. *RT-qPCR following 48 h of CDK1 RNAi for the evaluation of CDK1 and p21$^{waf1/cip1}$ expression. RNAi was successful in all the cell lines tested reducing almost totally CDK1 mRNA. In parallel, p21$^{waf1/cip1}$ expression increases, showing promising basics for senescence establishment*.

B. *Western blot analysis for CDK1 and p21$^{waf1/cip1}$ following CDK1 silencing. CDK1 appear to be reduced, as already seen for the mRNA, while p21$^{waf1/cip1}$ levels increase.*

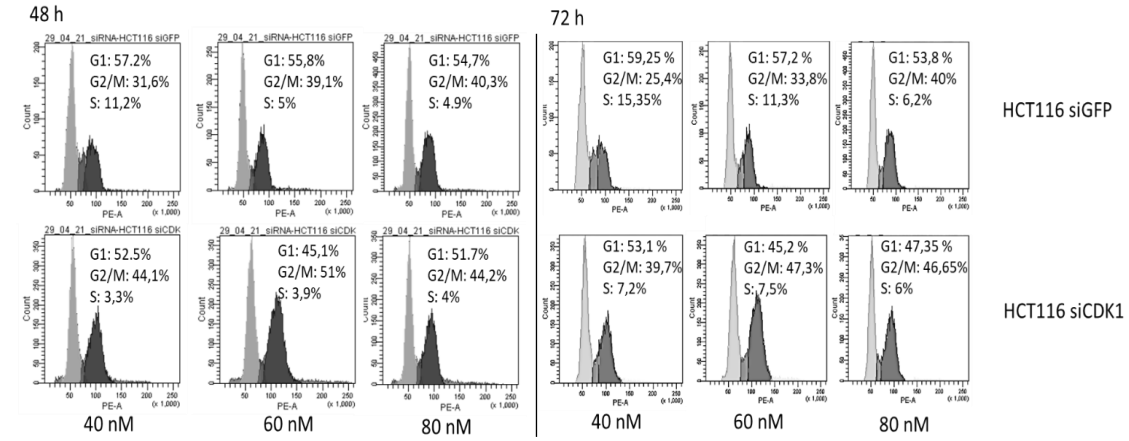


Fig. 25 – *Cytofluorimetric analysis of HCT116 following 48 h and 72 h of CDK1 silencing. An increase of the G2/M population can be observed for all the doses and times. Light grey: G1 phase; Grey: S-phase; Dark grey: G2/M phase.*

2.1.2.2. *Effects of CDK1 RNAi on the cell cycle*

Considering that the previous results showed an effective posttranscriptional silencing of CDK1 and, in parallel, an increase of the senescence marker p21$^{waf1/cip1}$, I asked whether the cells treated with siCDK1 could undergo cellular senescence. For this reason, as a first information, I decided to check the cell cycle profile of the transfected cells to see if an arrest was detectable.

HCT116 cells were transfected for 48 and 72 h and then collected for cytofluorimetric analysis. As shown in **Fig. 25**, a G2/M-rise was detected after 48 h of exposure to the siRNA, which reaches its maximus at the 60 nM dose. Interestingly, the 80 nM dose induced an accumulation of cells in the G2/M phase which is identical to the 40 nM dose. Also in this case, the reduced effect could be attributed to an off-target effect of the higher dose of siRNA. Considerably, this discrepancy disappears after 72 h of treatment, with the G2/M subpopulation being lower at the 40 nM dose, and higher at the 60 and 80 nM doses, which show an identical percentage of cells. In this case, given the longer exposure time to the treatment, the lower dose of siRNA is exhausted, and cells restart cycling; the higher doses, instead, can bypass the off-target effect since a longer time can allow the binding of the target RNA despite the non-specific interactions. Thus, posttranscriptional silencing of CDK1 induces an arrest of the cell cycle in almost the 50% of cell population after 48 and 72 h, at different concentrations. These results, together with the data obtained with RT-qPCR and WB, paved the way for investigating senescence induction after CDK1 depletion.

2.1.2.3. *Cellular senescence*

The promising results obtained after CDK1 depletion encouraged me to perform SA-βGal assay on HCT116 cells. The first experiments, however, did not produce significant results: despite the observed G2/M accumulation of cells following siRNA transfection, the same treatment for 48 or 72 h did not give rise to senescence. Nevertheless, these time points could be too restricted to induce a senescent response. For this reason, I decided to release cells from transfection and observe them after 72 h of release. Even in this case, I could not observe cell senescence; however, the presence of increased $p21^{waf1/cip1}$ senescence marker, together with the cell cycle arrest, was too promising to not try other experimental strategies.
It should be considered that CDK1, being necessary for the cells, is highly expressed. Silencing for 72 h could not be sufficient to trigger an irreversible cell cycle arrest since, at the time of silencing, cells already express CDK1 protein and can continue the cell cycle even if the CDK1 mRNA is partly degraded. Moreover, previous studies revealed that a massive removal of CDK1 is deleterious for cells, which undergo apoptosis. As so, a continuous RNAi for longer times at lower concentrations of siRNA should assure a constant depletion of the majority of CDK1 mRNA, while, at the same time, the protein already present is degraded (for the normal turnover) and very few proteins are only synthesized. After several attempts at different timepoints, administering siCDK1 every 72 h, at the concentration of 40 nM, I finally could observe cellular senescence in HCT116 cells after 10 days, followed by 3 days of release. As can be observed in **Fig. 26**, the 28%, percentage of cells staining blue compared to only the 2% of the control cells. Collectively, these results show that continuous, mild silencing of CDK1 for long times effectively induces cellular senescence in HCT116 cells.

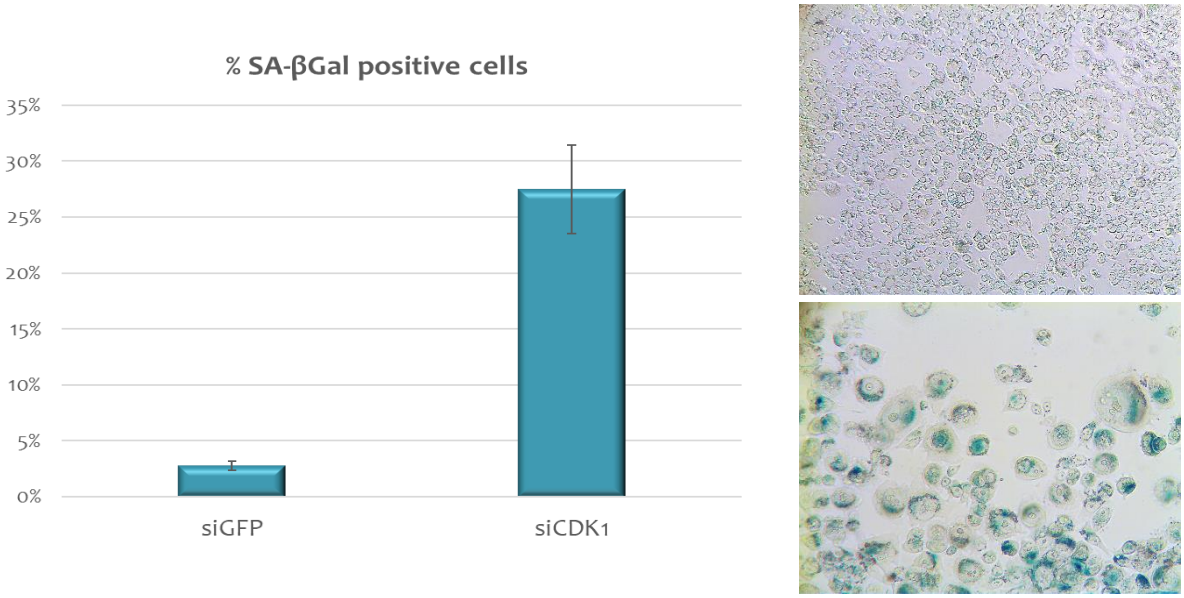


Fig. 26 – *Following three transfections with siCDK1 40 nM, HCT116 stain blue to the SA-βGal assay reaching the 28%. As can be observed in the right panel, cells are blue, bigger, and flatter compared to the control (above). Statistic analysis was performed on three replicates.*

2.1.3. Combined effect of pharmacological and post-transcriptional inhibition of CDK1

To address the problem of the presence of CDK1 protein during the first hours of RNA interference, I decided to combine the siRNA against CDK1 and RO-3306, in order to reduce the time of the treatment as well. At the same time, to increase the amount of cell population to be subjected to the treatment, I decided to synchronize HCT116 cells with the CENP-E inhibitor GSK923295. After an *overnight* treatment with 50 nM GSK followed by shake-off, cells were transfected with siCDK1 40 nM for 48 h and then treated with RO-3306 5 μM for 24 h. Mitotic shake-off would allow to select only the cells in mitosis, which express the highest level of CDK1. Once re-seeded, cells were left in culture for 4 hours before treatments, in order to allow them to attach. At the end of the treatments, almost the 35% of the cells was found dead, as expected. However, once analysed through cytofluorimetry, the desired outcome was observed, with the majority of the HCT116 cells blocked in the G2/M phase of the cell cycle after the treatments.

As can be seen in **Fig. 27 B**, 72 h after cells re-seeding following mitotic shake-off, half of the cell population is stopped in G1. In absence of other treatments, this is expected, since after 72 h cells have lost the synchronization induced. However, 52% of the cells is instead blocked in G2/M phase after RNAi treatment alone (while the 40 nM dose of siCDK1 induces, without synchronization, the accumulation in G2/M of only the 44% of the cells) immediately performed after the 4 h following mitotic shake-off.

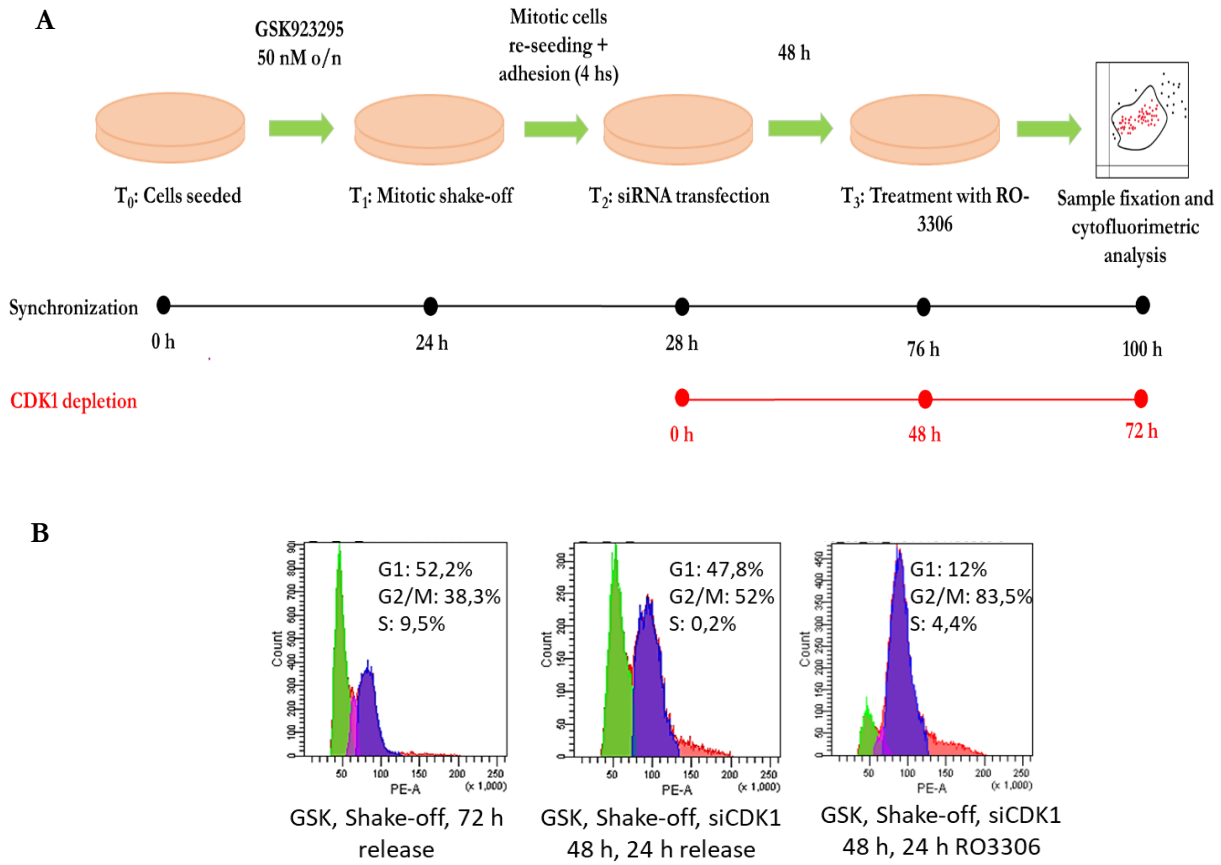


Fig. 27 – A. *Schematic workflow of the HCT116 cells synchronization followed by CDK1 depletion.* **B.** *Cytofluorimetric profiles of HCT116 cells following 5 h of GSK923295 treatment, mitotic shake-off and re-seeding (4 hours). Cells released for 72 h show normal cell cycle profile. Cells treated with siCDK1 for 48 h and then released for the remaining 24 h, display an increase of the G2/M (blue) population; cells treated with both siCDK1 and RO-3306 (24 h) show the 83,5% of cells blocked in the G2/M phase. G1 phase: green; S-phase: violet; cells with >4n DNA content: red.*

Furthermore, following the double treatment with RNAi and RO-3306, more than 80% of cells arrest in the G2/M phase, meaning that the combination of RNAi and pharmacological depletion of CDK1, following synchronization, produces a synergic effect for the G2/M arrest. Despite the remarkable rate of mortality, a high number of cells was available to be analysed, thus the treatment is not as toxic as it could seem at the beginning. Further analysis, following those different times of release, may probably show senescence or at least polyploidy after this double approach to CDK1 inhibition.

2.2. Flavonoids as senescence inducers

2.2.1. Preliminary assays

The first aspect to be investigated was the arrest of the cell cycle following the treatment with Curcumin and Quercetin (**Fig. 28**). In particular, basing on the literature, the concentrations of 10 μM for Curcumin and from 5 to 15 μM for Quercetin were selected[35,40].

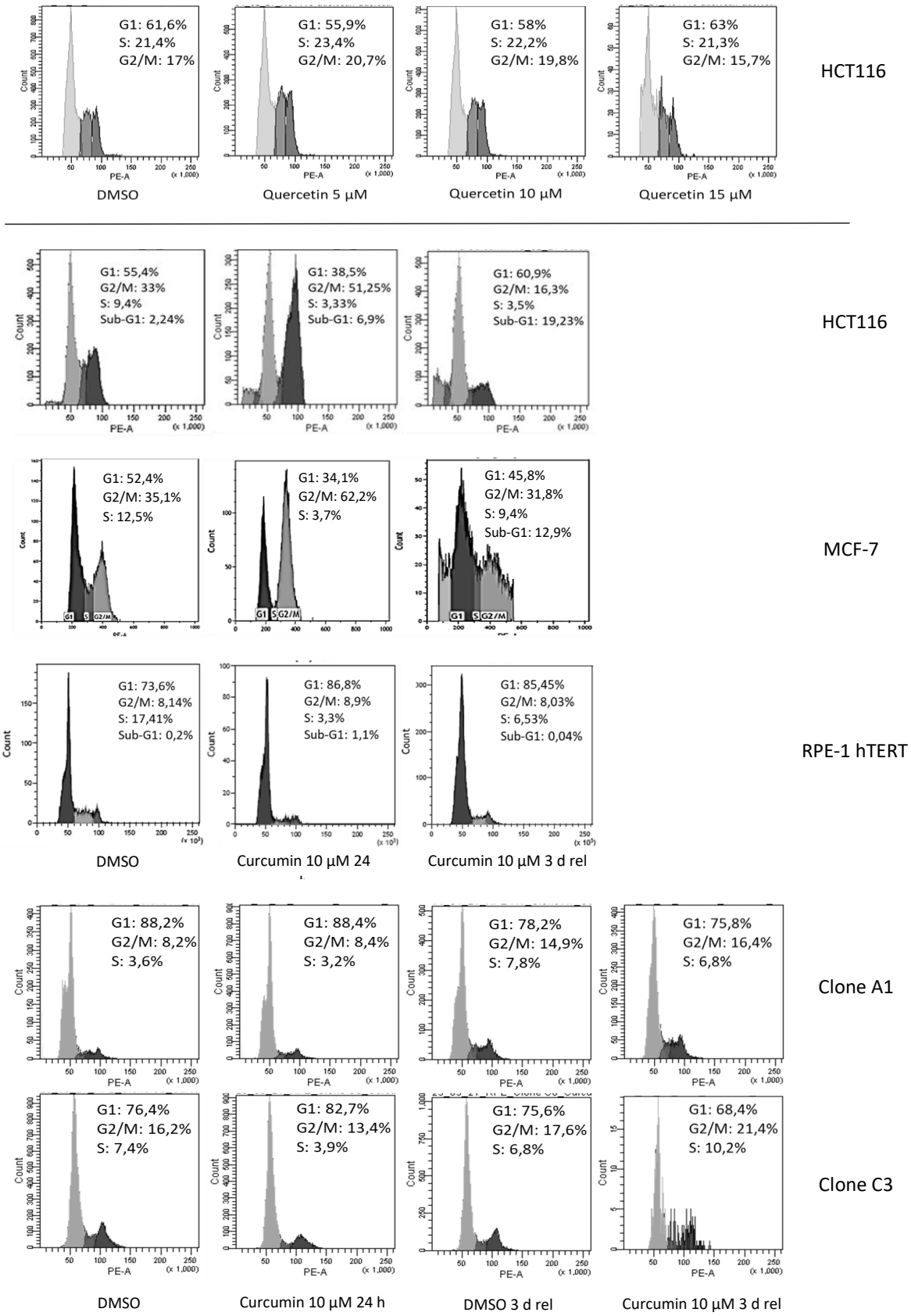


Fig. 28 – *Cytofluorimetric analysis of various cancer and non-tumor cell lines in response to Curcumin and Quercetin treatment. Quercetin (above) does not influence the cell cycle in HCT116 cells. Curcumin, instead, can induce cell cycle arrest in G2/M in both HCT116 and MCF-7, but not in RPE-1 hTERT and Clones, meaning that this effect can not be related to the cells' ploidy (bottom).*

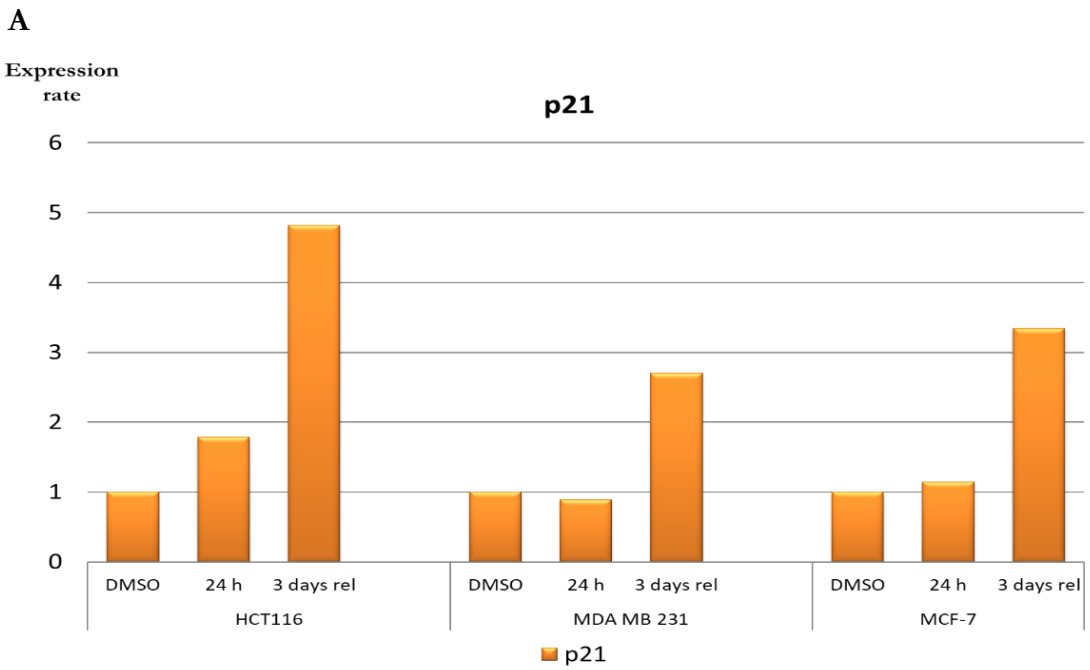
The two molecules were administered for 24 h before fixing and analysing them. Unfortunately, Quercetin did not give the expected result: HCT116 only slightly accumulate in G2/M following the 5 μM dose (3,7% more than the control); however, the G2/M subpopulation seems to decrease at higher doses of the molecule, returning to the control conditions at the highest concentration tested. Thus, Quercetin as senescence inducer was discarded.

On the other hand, treatment with Curcumin 10 μM for 24 h triggered a strong cell cycle arrest (>50%) in G2/M in both HCT116 and MCF-7 cells. Driven by these encouraging results, I tested the molecule on RPE-1 hTERT as well as on the RPE-1 aneuploid clones. Cytofluorimetric analysis of cell cycle profile after Curcumin treatment showed a roughly homogeneous response. All the three lines of RPE-1 do not arrest in G2/M, but they accumulate more in G1. This can be due to the fact that RPE-1 have a slower proliferation rate compared to cancer cells, so the treatment with Curcumin, if acting at the G2/M transition, might not be effective in those cells; however, given the broad spectrum of Curcumin targets, another explanation can be attributed to the malignancy of the cells, with cancer cells reacting to the drug in a stronger manner than the non-tumor ones.

In conclusion, Curcumin gave better results than Quercetin for what concerns the cell cycle arrest. For this reason, I decided to proceed with further analysis in the attempt of inducing senescence in cancer cells.

2.2.2. Evaluation of Curcumin action on senescence induction

To assess senescence establishment after Curcumin treatment, I evaluated the presence of the senescence markers $p21^{waf1/cip1}$ and β-Galactosidase enzyme. Senescence marker expression was tested in three cancer cell lines (HCT116, MDA MB 231 and MCF-7), treated with Curcumin 10 μM for 24 h followed by 72 h of release.



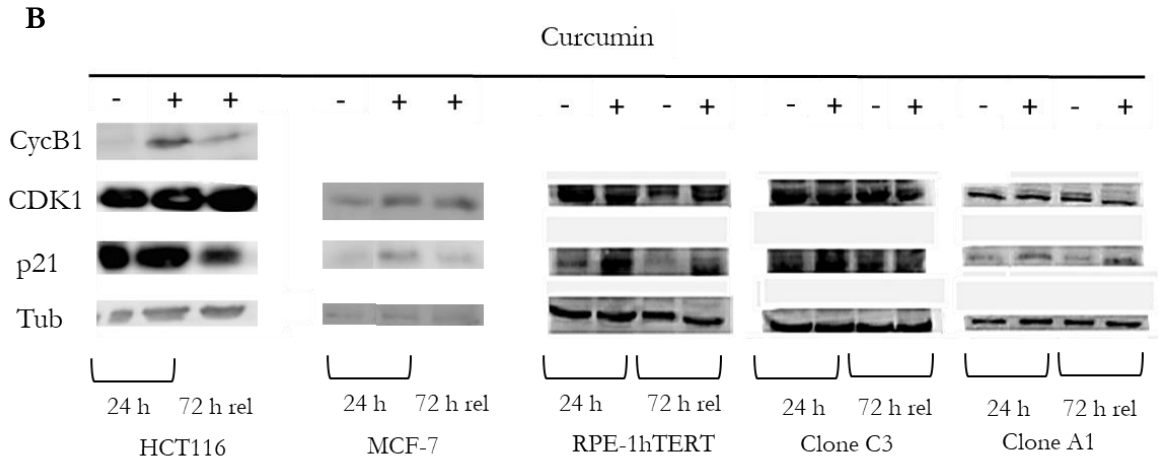


Fig. 29 – *RT-qPCR for p21$^{waf1/cip}$ expression (top) and western blot (bottom) for evaluation of protein expression following Curcumin 10 μM treatment. In all the three cancer cell lines p21 transcript increases at 72 h from release; on the other hand, p21 protein levels seem to decrease at the same endpoint in HCT116 and MCF-7 cells and have a fluctuating trend in RPE-1 hTERT and Clones, confirming the independence from cell ploidy.*

The RT-qPCR showed a consistent increase of p21 transcript in all three cancer cell lines treated with Curcumin (**Fig. 29A**). The highest outcome is observed for HCT116 cells, with a rise of almost 5 folds of the transcript after 72 h of release from the treatment. However, a small rise is detected after 24 h of treatment as well. Conversely, p21$^{waf1/cip1}$ protein appears to be reduced after 72 h of release from the treatment in HCT116 cells (**Fig. 29B**). As already observed for RO-3306, this might be caused by the fact that senescent cells express a high catabolic activity leading to protein degradation. Cyclin B1 protein level, as expected, rises at 24 h of treatment, indicating the cell cycle block in the G2 phase. RPE-1 hTERT cells, instead, display a heterogeneous pattern, with p21 resulting more expressed after 72 h of release in the WT cells and in the Clone A1, and showing no considerable differences in the Clone C3. These changes are probably related to the different genetic background of the cells. The SA-βGal assay was performed on the same cell lines revealing, even in this case, a positive, even if slight, staining after Curcumin treatment. In particular, the positivity percentage of senescent cells is of the 11% in HCT116, compared to the 2% of the untreated cells (**Fig. 30**). Moreover, cells appear to be bigger and flat, with many stress granules and vacuolizations typical of senescence and also show dark granules which probably are lipofuscin aggregates. Thus, even if probably by the activation of a different pathway, the treatment with curcumin led to senescence in cancer cells.

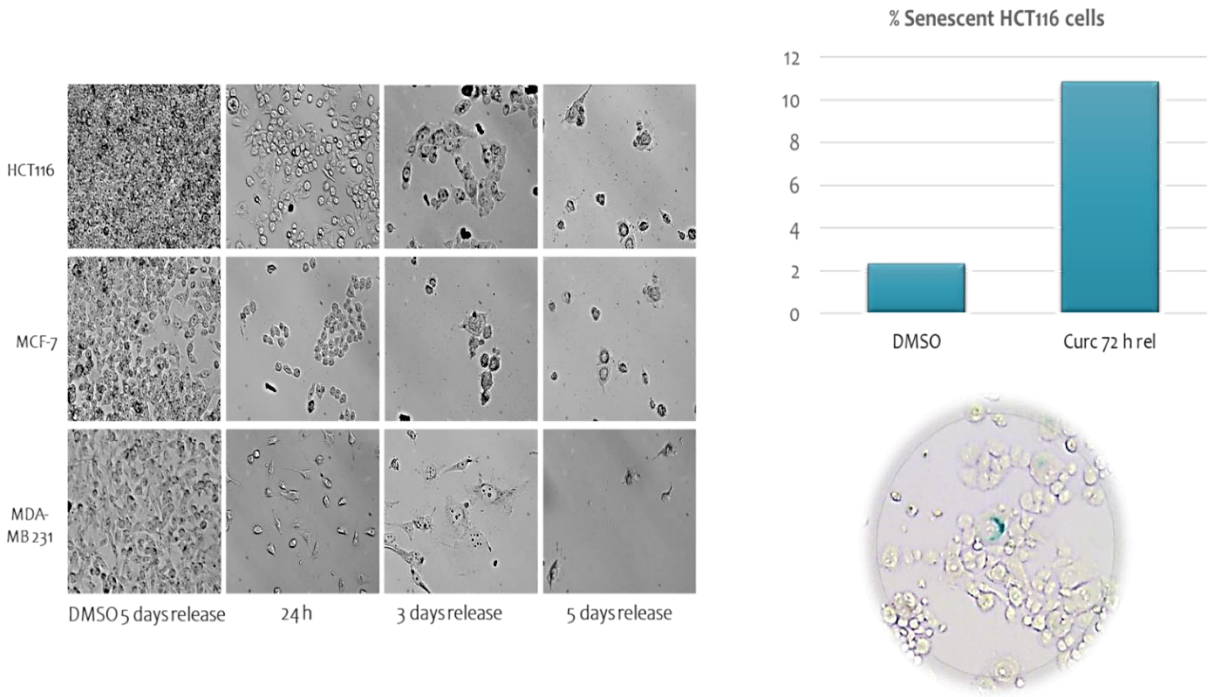


Fig. 30 – *SA-βGal assay on HCT116, MDA MB 231 and MCF-7 following Curcumin treatment (left) showing bigger, flatter cells 3 and 5 days from release. On the right, quantification of HCT116 senescent cells compared to the control condition.*

3. Senolytic drugs

Given that all the three treatments performed (CDK1 depletion through post-transcriptional and pharmacological inhibition, and curcumin treatment) successfully induced senescence in the studied cancer cells, the clearance of senescent cells through senolytic drugs was the next aim to reach. In particular, Fisetin and Quercetin were selected as senolytic drugs, two natural polyphenols which have been shown to have senolytic activity both alone and in combination with other drugs.

3.1. Preliminary assays

In order to exclude a broad and aspecific effect of the drugs due to cytotoxicity, the right concentration was needed to be found. Thus, the Trypan-Blue assay was performed in order to establish the mortality rate, if detectable, following the treatment with senolytics. HCT116, RPE-1 hTERT and Clone A1 were treated with Quercetin and Fisetin with different concentrations, starting from 5 until 20 μM for 72 h. Globally, a dose-dependent toxicity was observed, as expected, with the major effects detected for the 15 and 20 μM doses especially in HCT116 cells (**Fig. 31**). RPE-1 hTERT and Clone A1 react less and differently to the molecules, coherently with what was observed previously with other treatments.

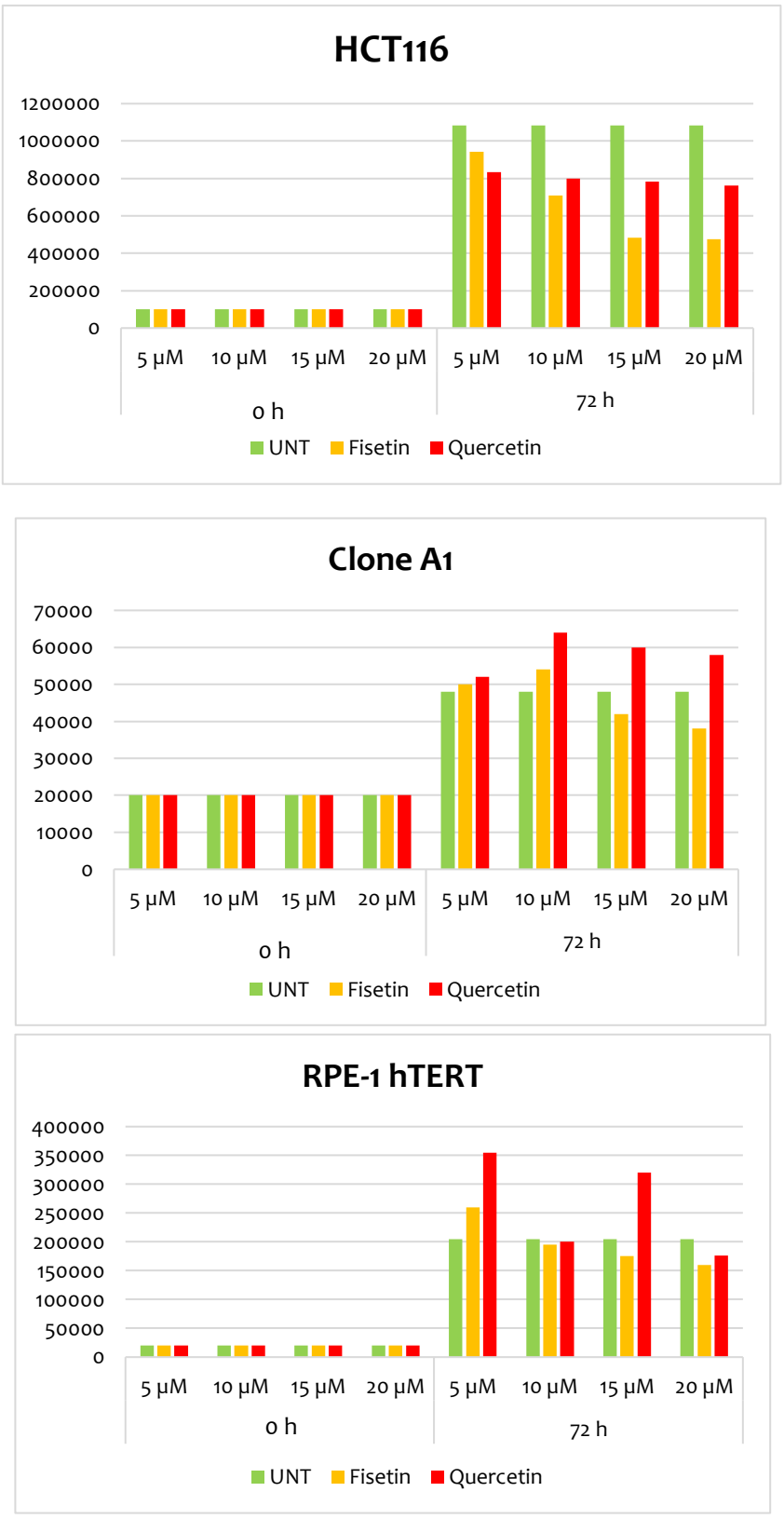


Fig. 31 – *Trypan Blue cell count for the establishment of cytotoxicity in response to senolytic treatment in HCT116, Clone A1 and RPE-1 hTERT. No mortality rate was observed, however cells proliferated less as the senolytics concentration increased. The cell number is indicated in the Y axis.*

As can be observed in **Fig. 31**, the two molecules act with two different trends: while Fisetin effect is related to the concentration used, Quercetin action appears to be more heterogeneous, in particular in RPE-1 hTERT cells. Moreover, the interesting aspect of this assay was that cells stained for Trypan Blue were extremely rare; at the same time, the cell number reduced considerably according to the increasing dose. Thus, the molecules are not toxic at the tested concentrations but they probably halt cell proliferation. In order to avoid synergistic effects of the senescence inducer (Curcumin or siCDK1) and of the senolytics the lower doses of 5 and 10 μM were selected and tested, as a control, on HCT116 cells transfected with the control siGFP for three times to evaluate the expression of the β-Galactosidase enzyme.

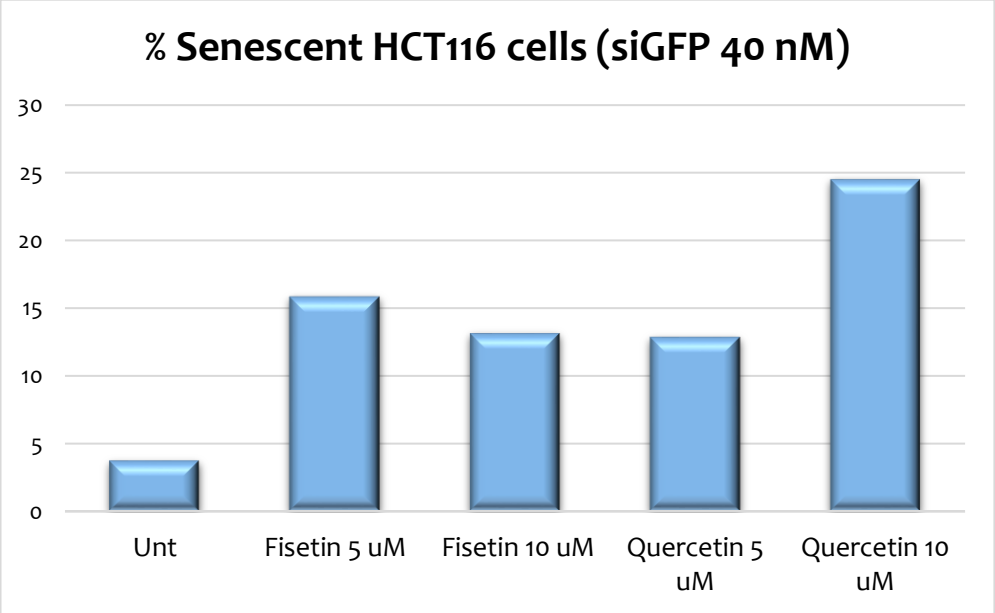


Fig. 32 – *siGFP-transfected HCT116 cells following treatment with Fisetin and Quercetin 5 and 10 μM for 72 h. In not-senescent cells, senolytic drugs act as senescence inducers.*

Surprisingly, cells treated with senolytics following siGFP transfections show a higher percentage of senescent cells compared to the untreated ones (**Fig. 32**). Even if unexpected, this result correlates with what already observed for cell proliferation, which appeared slowed or halted according to the count of the number of the cells. Thus, to understand if the observed effect was an artefact, the same experiment was performed in the presence of the senescence inducers siCDK1, administered with the same modalities of the siGFP, and Curcumin 10 μM.

3.2. Selective clearance of senescent cells

Both Fisetin and Quercetin action were analysed in HCT116 cells in which senescence was induced with RNA interference against CDK1, with the same modalities shown above, or with curcumin, as a "positive" control for senescence. In fact, the curcumin-induced senescence does not seem to be related to the aneuploid set-up of the cells, contrary to the senescence observed after CDK1 depletion which correlates with the altered chromosome number of the cells. HCT116 cells were seeded treated with curcumin 10 μM for 24 h and released for 72 h; for RNAi, cells were transfected thrice (every 72 h) and then released for 72 h. At this point, the culture media was replaced and senolytics were administered for 72 h. After the treatment with both molecules, the percentage of remaining cells was very low, with a

dose-dependent trend. In particular, very few senescent cells were still present in comparison to the normal cells, whose quantity increased proportionally with the senolytic concentration. As shown in **Fig. 33**, the SA-βGal positivity was reduced compared to the cells only treated with the senescence inducer. Moreover, the remaining senescent cells showed many blebbings and vesicles which suggested an apoptotic phenotype, indicating that the senolytics probably triggered apoptosis specifically in senescent cells, as expected.

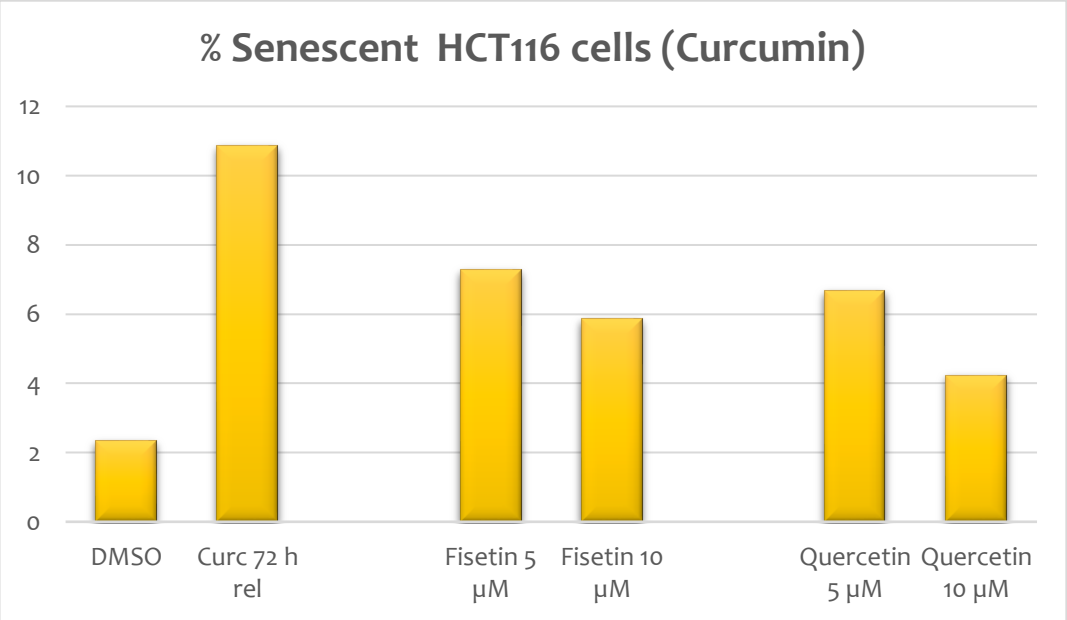


Fig. 33 – *Percentage of HCT116 senescent cells following treatment with Fisetin and Quercetin senolytic drugs. Senescence was induced with Curcumin as previously explained. The number of senescent cells decrease for both drugs in a dose-dependent manner.*

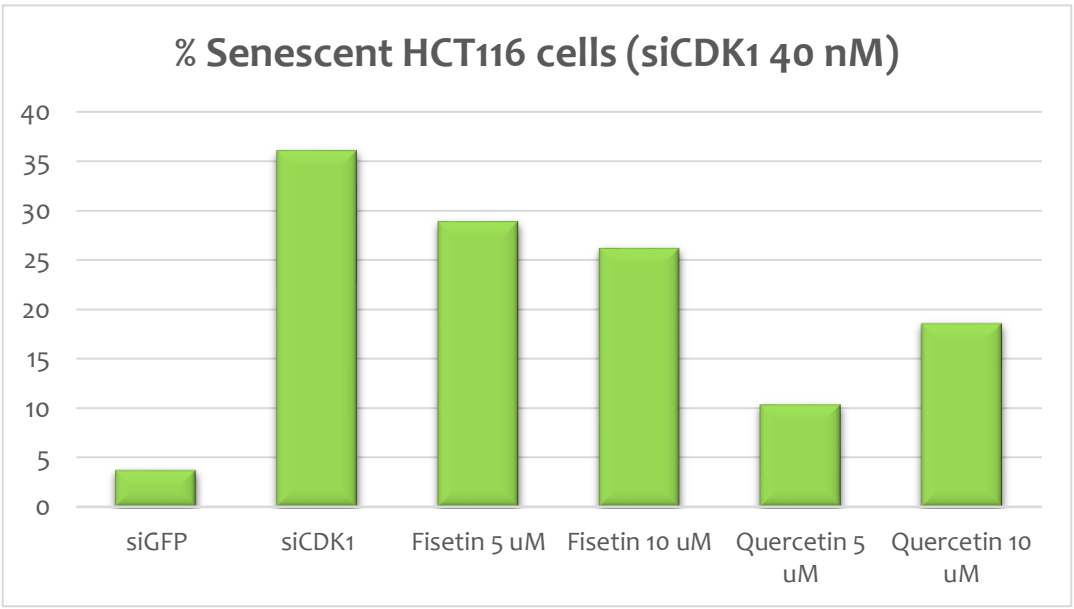


Fig. 34 – *HCT116 in which senescence was induced by CDK1 RNAi as previously explained were treated with Fisetin and Quercetin as mentioned above. Even in this case, the number of senescent cells decrease after the treatment with senolytics, even if a small rise is observed following treatment with the highest dose of Quercetin. This is probably due to the fact that the drug also act on the not-senescent cells which are still present at the moment of the treatment and in which the senolytics induce senescence.*

Interestingly, the trend observed in the percentage of RNAi - senescent cells analysis following senolytic treatment appears to be identical to the one seen in the siGFP treated cells (**Fig. 34**). Undoubtedly, the amount of senescent cells is reduced after senolytic administration; however, there still is a population of senescent cells which slightly increases with the highest concentration of Quercetin (though remaining lower than the siCDK1 treated cells alone). Probably, this remanence is attributable to the double action of flavonoids, senescence inducers on one hand, and senolytic drugs on the other. A great part of the senescent cells disappears and shows apoptotic markers (blebbings) after senolytic treatment, but the not-senescent part probably reacts as in the control condition, entering senescence after flavonoids treatment (**Fig. 35**). Unfortunately, since only one replicate for condition could have been performed until now, these data can be only defined as preliminary. However, these results represent promising preconditions to confirm the senolytic action of Quercetin and Fisetin.

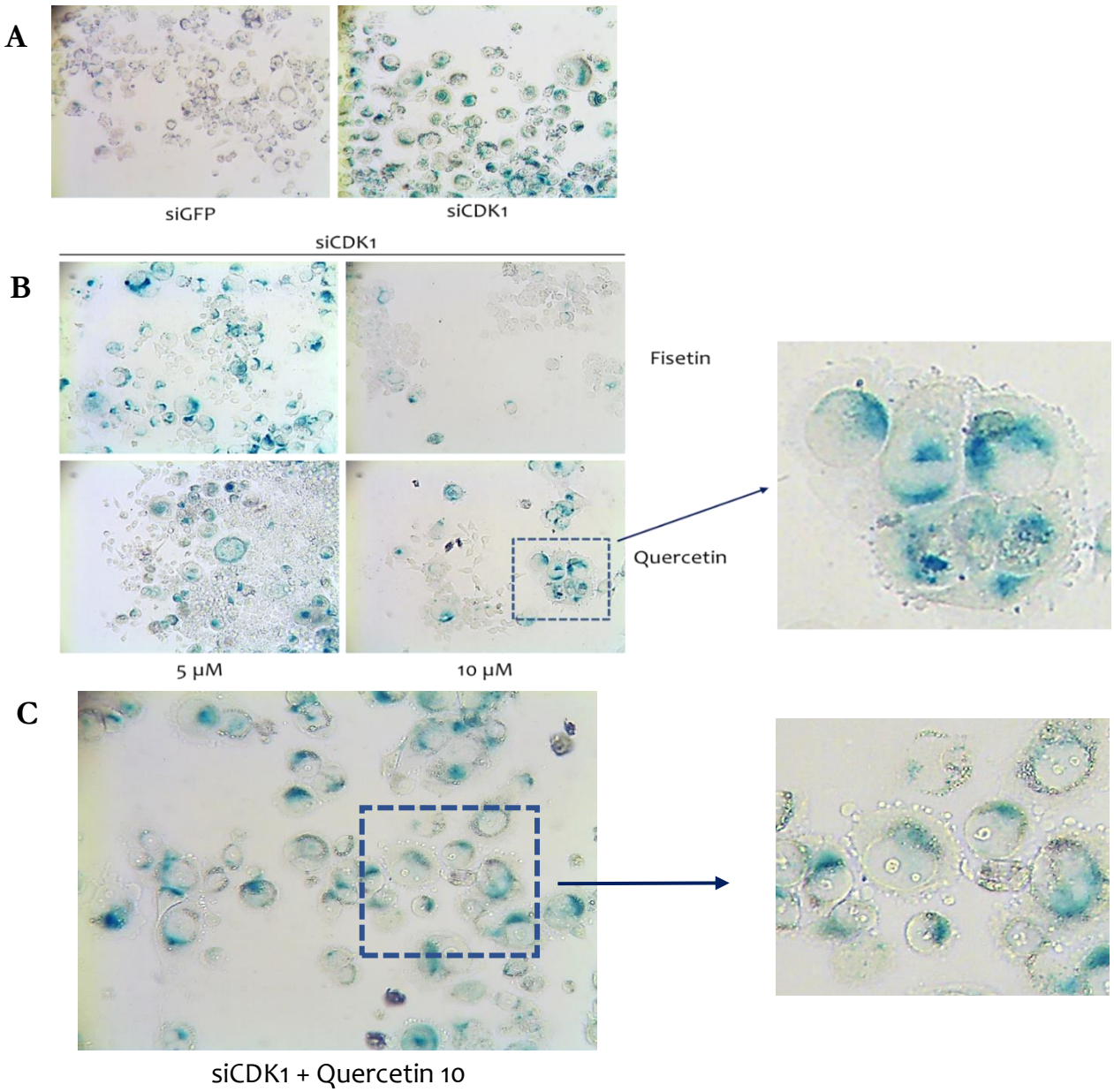


Fig. 34 – *SA-βGal assay in HCT116 cells in which senescence was induced with CDK1 RNAi and senolytics were given following senescence induction.* **A.** *Comparison between control cells (siGFP) and siCDK1 – transfected cells showing the successful senescence induction.* **B, C.** *Dose-dependent effect of senolytic drugs. Blebbings, which show apoptosis, can be observed in the magnification on the right.*

V. Discussion and conclusions

It is largely known that the clearance of cancer cells from an organism is extremely challenging. Most of the current therapies are highly invasive and frequently harm not only the tumor cells, but also the healthy cells of the patients. The principal difficulty in contrasting cancer cells is their genetical heterogeneity to which participate the altered number of chromosomes that they possess. This condition, named aneuploidy, is the main responsible for the majority of the altered features of tumor cells (uncontrolled proliferation, survival in adverse conditions, improved migration capacity). In particular, tumors can easily escape all those pathways, as apoptosis or cellular senescence, that normally stops the propagation of damaged and dangerous cells. Thus, the re-activation of these pathways can be a potential ally in fighting uncontrolled proliferation and spread of malignant cells. The cell cycle master regulator CDK1, the cyclin-dependent kinase progenitor of all the other cell-cycle related kinases, has been frequently found to be overexpressed in tumors. CDK1 controls the passage from the G2 to the M-phase of the cell cycle allowing the phosphorylation of proteins involved in cell division. CDK1 depletion, as already observed for other cell cycle CDKs, can lead to cell cycle arrest which, if prolonged, may trigger senescence especially in those cells that harbor genetic/chromosoma aberrations. The work presented in this thesis was aimed to induce cellular senescence in aneuploid cancer cells through CDK1 depletion, in order to make them recognizable to a specific class of drugs, the senolytics that have been proved to induce apoptosis selectively in senescent cells. Thus, induction of cellular senescence and treatment with senolytics can lead to the specific elimination of aneuploid cancer cells.
Here I show that CDK1 depletion effectively induces senescence in cancer cells. Pharmacological inhibition with the ATP-competitor, CDK1/2 inhibitor, RO-3306 impacts severely on cell viability in a dose-dependent manner. However, an intermediate dose of 5 μM results in cell cycle arrest in the G2/M phase in three different cancer cell lines (HCT116, MDA-MB 231 and MCF-7) with low toxicity. Cytogenetic analysis revealed that most of these cells arrest in the G2-phase, as can be deducted from the decline of the mitotic index. In addition, following 72 hours of release from the treatment, in order to allow cellular senescence establishment, cells show an increase of the $p21^{waf1/cip1}$ senescence marker. This result corroborates the expected senescence induction, which is furthermore highlighted by the presence of giant, flat and vacuolized cells. These morphological features, together with the presence of dark pigmented granules (lipofuscin) and with the increase of the β-Galactosidase expression, demonstrate that cells in which CDK1 was inhibited can enter senescence following the G2 arrest. Moreover, the observed apoptosis suggests that CDK1 depletion can be a good, effective strategy in cancer cell elimination even without senescence induction. In fact, RO-3306 appears not to be as toxic as in tumor cells in the normal cell lines RPE-1 hTERT and IMR90. Thus, its action is selective for tumor cells and, to increase its effect and reduce its toxicity, new delivery systems are currently on trial, such as hallosyte nanotubes whose have already shown to be able to carry RO-3306 inside the cells. These organic nanotubes allow the molecules delivery without the intermediary of organic and potential cytotoxic solvents such as DMSO. Further experiments will be performed to consolidate their delivery capability of RO-3306.
RNA interference targeting CDK1 proved to be the best approach in inducing cellular senescence. One transfection alone only induces a slight accumulation of cells in the G2/M phase. The possible explanation of this result is that some CDK1 still remain, fulfilling CDK1 function, despite RNAi targeting has worked out. Cell cultures are asynchronous and CDK1 starts to be expressed only from the late S-phase, meaning that CDK1 post-transcriptional silencing can produce a detectable effect only if started in the G1 phase and continuing for at least 24 hours (the normal duration time of the cell cycle). After synchronization in M-phase, mitotic shake-off and cell re-seeding in order to make them re-enter in G1 phase, the percentage of G2/M arrested siCDK1 cells increased in respect to the asynchronous ones. The most consistent cell cycle arrest was observed with a combination of RNAi and RO-3306

inhibition, suggesting the presence of high levels of CDK1 in these tumor cells and that a combined treatment is required for CDK1 complete depletion. For what concerns RNAi alone, after three transfection rounds, 72 hours interval, with a low dose of siCDK1, HCT116 cells started to appear bigger, flatter, and positive to the SA-βGal assay. Thus, three transfections are demanded, to observe senescence, due to the high expression of the protein. Last, cellular senescence establishment is a slow process: 48 hours are probably not sufficient, since at least 10 days are needed for a cell to become fully senescent. The same approach does not halt RPE-1 hTERT, suggesting that CDK1 silencing leads to cell cycle arrest and cellular senescence specifically in cancer cells. As a positive control of cellular senescence, the flavonoid Curcumin was selected due to its proven senescence-inducer activity. This molecule has a widespread target network, being able to act on various proteins such as the NF-*k*B factor[63], involved in the inflammatory response, and the p300 HAT[64], one of the main responsible of chromatin acetylation. Moreover, an inhibitory activity of this molecule on CDK1 transcription was also revealed. According to the studies of Mosieniak et al., the 10 μM concentration of Curcumin has the ability to block the cell cycle at the G2/M phase and, after a release of at least 3 days, to induce senescence. This result was confirmed in HCT116, MCF-7 and MDA-MB 231 cells, where, in particular, at least half of the cells were arrested in G2/M and 11% of HCT116 cells were senescent in comparison to the control. Immunofluorescence and successive cytogenetic analyses for Cyclin B1 and CDK1 show that Curcumin treated cells are more likely to arrest in mitosis than in G2, so that the trigger of cellular senescence in this case is probably related to a forced, premature exit from mitosis leading to mitotic errors and possibly to DNA damages. Interestingly, only a slight increase in the percentage of senescent cells was observed in the aneuploid RPE-1-clone A1, meaning that senescence induction by Curcumin, unlike RO-3306, do not depend on aneuploidy, but it is probably related on the activation of other pathways. Moreover, results following Curcumin treatment in RPE-1 hTERT and the derivative aneuploid clones appeared to be heterogeneous for $p21^{waf1/cip1}$ expression; since RPE-1 hTERT already show an aneuploidy level of at least 10%, and given that RPE-1-clones were generated by exploiting their ability to lose euploidy during culturing, the variegated results observed might depend on the different chromosomes that are lost or gained in every cells, though some chromosomes appear to be more prone to be lost or gained[65].

After inducing senescence in tumor derived cells, I tested the senolytic drugs Quercetin and Fisetin to elicit the selective clearance of senescent cells. The percentage of senescent cells reduces after the treatment in a dose dependent manner for both molecules and for cells in which senescence has been induced with two different approaches (CDK1 RNAi and Curcumin). However, a small rise in the percentage of senescent cells has also been observed with the highest dose of Quercetin, as well as in the control, not-senescent cells (siGFP- and DMSO-treated cells) which show a slight increase of senescent cells after treatment with senolytics in a dose-dependent manner. This probably happens because flavonoids carry on a double-edged action as both senescence inducers and senolytics mediated by (or caused by) the activation of the Nrf-2 pathway. In the siCDK1 and Curcumin treated cells, the more the senolytic concentrations increase, the more senescent cells disappear, leaving space to the not senescent ones which react to Fisetin and Quercetin as the control conditions do. This probably occur only with the highest concentration of Quercetin because, since its major dose, it is more available and also acts on the not-senescent cells. Coherently, the slight rise of senescent cells with the highest dose of Quercetin observed in the cells treated with both siCDK1 or Curcumin can be a train of the effect observed in normal cells which are still present in the treated conditions. Senolytic drugs, furthermore, are often used in combination with other drugs (for example: Quercetin + Dasanitib) in order to increase their action which changes according to the dose and the cell type. Thus, further assays based on the combination of the two molecules may probably result in an increase of the senolytic action.

Globally, my PhD work shed some light on the effects of CDK1 inhibition on the cell cycle of cancer cells. Even if more investigations would be needed, I can conclude that CDK1 depletion has a strong effect in halting proliferation and, consequently, in spreading of the malignant cells, confirming the original hypothesis and what already observed for the other CDKs inhibition. For example, CDK4/6

inhibition is already on clinical trial through drugs as Palbociclib[66] which efficiently induces apoptosis in cancer cells. Despite the scarce availability of CDK1-specific inhibitors, the results obtained with RO-3306 and RNAi are promising for continuing targeting this kinase. Last, combining this approach with senolytic drugs can surely be advantageous for preserving the healthy cells of the organisms while targeting the malignant ones. Considering that many other senolytics are available, both from natural (flavonoids and phenols) and synthethic origin (Navitoclax), the possibilities are huge and new combinations can be found that result in a more effective action.

VI. Materials and methods

Cells and cell culture

RPE-1 hTERT, MCF-7, MDA-MB 231 and HCT116 with MIN phenotype (near-diploid) cells (kindly provided by Dr. B. Vogelstein, John Hopkins University, Baltimore, MD) were cultured in D-MEM with 10% FBS (Corning), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 and at 37° C. All the used molecules (GSK923295, Reversine, Curcumin, RO-3306, Fisetin, Quercetin) were solved in DMSO. Cells were usually divided twice/week, according to the confluency degree reached.

Cells transfection

Cells were transfected at a confluency of 70% according to the Lipofectamine 3000 protocol instructions. siRNAs against GFP (5'GGC UAC GUC CAG GAG CGC ACC-3') and CDK1 (5'ACU UCG UCA UCC AAA UAU AdTdT3'; based on the work of Xiao et al., 2019) were solved in siMax universal buffer and then diluted in RNAse-DNAse free sterile H_2O. For transfection in cancer cells, siRNAs were first incubated in OptiMEM (ThermoFisher Scientific, Monza, Italy) and then added to the Lipofectamine 3000 – OptiMEM mix. The mix was incubated for 8 minutes and then added to culture medium. The highest suggested volume of Lipofectamine 3000 (7,5 μL/well in a MW6 plate) was used. For transfection in RPE-1 hTERT, K2 transfection reagent (Biointex Laboratories GmbH) was used according to producers' instructions. The intermediate volume of K2 transfection reagent was used (6,4 μL/well of a MW6 plate). For both transfection protocols, the culture medium was never replaced, and data were collected after at least 48 h of incubation.

Cells viability

Cells were detached by incubation with Trypsin 0,05%-EDTA 0,5 M at 37° C for 5 minutes or until they were round, then collected with PBS 1X and diluted 1:2 with Trypan Blue in a final volume of 400 μL. Then the cells-Trypan Blue mix was loaded in a Burker chamber and cells were counted.

Cell cycle phase analysis PI staining and cytofluorimetric analysis

Cell cycle stage was analysed by flow cytometer using a Propidium Iodide (PI) staining assay based on the binding of PI, a DNA dye that binds the DNA in a stoichiometric way. The DNA amount allows to resolve cell cycle phases in a given cell population into G0/G1, S, and G2/M phases. Aliquots of 1,000,000 cells were obtained by detaching them with Trypsin-EDTA and by collecting them with cold PBS 1X. Successively, cells were harvested by centrifugation, washed with PBS and incubated in the dark in a PBS solution containing 20 μg/mL propidium iodide and 40 μg/mL RNase, for 30 min, at room temperature. Then, samples were immediately subjected to fluorescence-activated cell sorting 64 analysis by FACS Diva flow cytometer using FACS Canto software (BD Biosciences). At least 20,000 cells were analyzed for each sample.

AO/EB staining for apoptosis evaluation

Cells were cultured in MW12 plates in triplicate. The day of the analysis, cells supernatants were collected and cells were detached with Trypsin-EDTA, collected with 1X PBS and centrifuged at 1000 rpm for 5

minutes. Cells pellet was manually re-suspended in cold PBS and newly centrifuged to eliminate all the culture medium. Then, each cell pellet was re-suspended in 25 µL of AO/EB mix (1X in PBS), put on microscope slides and covered with cover glass for fluorescence microscopy observation.

SA-βGal

Senescence was evaluated by SA-βGal assay. Cells were cultured in triplicate in a MW12 plate and, after medium removal, fixed the day of the analysis with pre-heated 4% PFA (paraformaldehyde) for 5 minutes at room temperature. Then cells were washed twice with 1X PBS for 5 minutes and incubated for 24 h at 37° C with a staining solution containing:

- X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside; Promega Italia S.r.l. Società Unipersonale, Milano, Italy) 0,1%;
- 5 mM of potassium ferrocyanide and 5 mM of potassium ferricyanide
- 150 mM Sodium chloride
- 2 mM Magnesium chloride

in 40 mM citric acid/sodium phosphate solution, pH 6.0.
The next day, the staining solution was removed, cells were washed twice with distilled water and then observed in transmitted light with a 20x objective. The percentage of senescent cells was evaluated on 400 cells for HCT116 and on 100 cells for RPE-1 hTERT. Standard deviation was calculated on the mean of three replicates.

Immunofluorescence

$7x10^5$ cells were seeded the day before treatment on glass coverslips put on the bottom of wells of a MW12 plate. The day of the analysis cells were fixed after two washes with 1X PBS with ice-cold methanol for 5 minutes at room temperature or until evaporation. After two washes with PBS, cells were permeabilized with 0,01% Triton-X solved in mQ water for 10 minutes at room temperature and then washed again and blocked with 0,1% BSA (bovin serum albumin) for 30 minutes at room temperature. Antibody labelling was made by solving antibodies in 0,1% BSA according to the producers' suggested dilutions. The primary antibodies were incubated over night at 4° C and the secondary antibodies were left for 1 h at room temperature. Each labelling was followed by 1X PBS washes. DAPI (1 mg/mL) was added prior to observation with Zeiss Axioskop microscope.

RT-qPCR

RNA extraction was performed through the ReliaPrep™ RNA Cell Miniprep System (Promega S.r.l., Milan, Italy) starting from at least $1x10^6$ cells. RNA was quantified with Nanodrop™ and loaded on 1% agarose gel to verify bands integrity. 1 µg of RNA was reverse-transcribed in cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems with the following conditions:

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

RT-qPCR was performed through Applied Biosystems 7300 Real-Time PCR System Software. Each sample was analyzed in triplicate (50 ng of cDNA/replicate), with a final volume of 25 μL for each replicate. The reaction mix is prepared as follows (the indicated volumes and concentrations are referred to one replicate):

- 12,5 μL1X Master Mix SyBR Green (Applied Biosystems)
- 2 μM Forward and Reverse primer mix
- RNA-se/DNA-se free H_2O

Gene expression was quantified by comparing the ΔΔCt of each gene to the one of the endogenous gene GAPDH (Forward primer: 5'-CTCATGACCACAGTCCATGCC-3'; Reverse primer: 5'CAATCCACAGTCTTCTGGGT3'). CDK1 was targeted with the Forward primer 5' GATTCTATCCCTCCTGGTC 3' and the Reverse primer 5' AATATGGTGCCTATACTCC 3', based on the work of Xiao et al., 2009. $p21^{waf1/cip1}$ sequence was targeted with the Forward primer 5' CTGGAGACTCTCAGGGTCGA-3' and the Reverse primer 5' CGGATTAGGGCTTCCTCTTG-3'.

Metaphase spread

Ploidy of the cells was analyzed through metaphase spread assay. Cells were seeded to be at the 70% of confluency at the day of the analysis on microscope slides in order to avoid artifacts due to drip. The day of the analysis, cells were treated with Colcemid 0.2 μg/mL for 2 h (HCT116) and 3 h and 40 minutes (RPE-1 hTERT), in order to maximize the number of mitotic cells. After rinse with PBS, cells were incubated with KCl solution 75 mM for 8 minutes to induce swelling and osmotic lysis. Nuclei were then fixed with ice-cold Carnoy fixative (3:1 metanol : glacial acetic acid) and stained with 5% Giemsa staining solution for 8 minutes. Microscope slides were then rinsed with distilled H_2O prior to observation with 20X objective (for the search of metaphases and the calculation of mitotic index) and with the 63X and 100X obectives (for chromosome count).
For each sample, 30 metaphases were analysed.

Western blot

Protein expression was evaluated through western blot. Proteins were extracted from $1x10^6$ cells, trypsinized and centrifuged, in Laemmli buffer 4X (48 mM Tris-HCl pH 6.8, 20% glycerol, 1,6% SDS, 11,52 mM β-mercaptoethanol and 0.08% bromophenol blue). Samples were denatured at 95° C for 10 minutes. 30% Acrylamide/Bis solution (Biorad) was used to obtain 10% SDS-Page solved in Tris-HCl buffer (pH 8.8 for the resolving gel; pH 6.8 for the stacking gel), with SDS. Electrophoresis was performed in Mini-PROTEAN System (Biorad), with Tris-Glycin pH 8.3 running buffer (0.025 M Trizma base, 0.192 M Glycin and 0.1% SDS) with constant voltage (50 V until samples exit wells; 80 V for the rest of the run). The gel was incubated in the towing buffer for 10 minutes before proceeding with protein transfer in PVDF membrane prior activated with methanol. Transfer buffer, stored at 4°C before the use, contained 25 mM Tris-HCl pH 8.8, 150 mM Glycin and 20% methanol. Transfer was performed for 90 minutes with constant voltage (V=100) with ice. After Ponceau revelation, membrane was washed with PBS-Tween 0.1% and incubated for 1 h in 5% Milk solved in PBS-Tween. Finally, incubation with primary antibodies (α-CDK1, Santa Cruz Biotechnology, 1:1000; α-$p21^{waf1/cip1}$, Santa Cruz Biotechnology, 1:200; α-Cyclin B1, Santa Cruz Biotechnology, 1:200; α-β-Tubulin, 1:10000) was performed sequentially and overnight at 4° C, each one followed by incubation with α-Mouse-HRP secondary antibody for 1 h at room temperature. Protein bands were revealed with Chemidoc XRS^+

(Biorad) following SuperSignal West Femto Maximum Sensitivity Substrate (Thermofisher Scientific) incubation. Bands were quantified with ImageJ software.

VII. Bibliography

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