Universidade do Minho
Escola de Engenharia

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Dissertação de Mestrado
Mestrado em Bioengenharia

Trabalho efetuado sobre a orientação da
Professora Doutora Cristina Pereira-Wilson
da
Professora Doutora Maria Alice Carvalho
e da
Professora Doutora Joana Azeredo

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DECLARAÇÃO

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Evaluation of the anticancer potential of new nitrogen heterocycles in human colon cancer

Abstract

Colorectal cancer (CRC) is one of the most common types of cancer, being its incidence higher in developed countries, where diets rich in fats and sedentary life styles are usual. The inexistence of a cure in addition with tumor resistance to the actually used drugs makes imperative the search for new and effective solutions. To worsen the actual treatment scenario is the fact that patients do not respond equally to the administered drugs (for ex. Fluorouracil-5FU) due to the tumors' different genetic profiles. This ineffective treatment in many patients leads to thousands of annual casualties.

Previously, two compounds belonging to a family of nitrogen heterocycles were identified as active in HCT116(p53-wt) cells of colon cancer. New compounds derived from these hits were synthesized for this project with the aim of potentiate their anticarcinogenic potential. To do so, different R and R_1 groups were tested and both seem to interfere in the activity of the compounds. From these new compounds, 2 were identified as extremely potent and promising (10 and 24) in the same cell line but also in the isogenic line HCT116(p53-null) and CO115, with IC_{50}s between 0.6μM and 1.55 μM, in comparison with the reference compound 5FU, whose IC_{50}s are between 15 μM and 252 μM. With this analysis it was also possible to discover that the high activity of the compounds is independent of the p53 status, what presents and advantage relatively to 5FU that it Is not active in the absence of p53. In addition it was discovered that compound 24 is active in cells with both KRas and BRaf activating mutations. Their effect was assessed in cellular processes like apoptosis and cell cycle, where it was verified a low induction of apoptosis and accentuated arrest of the cell cycle in the S and G_2 phases. An initial study about the effect of the compounds in various molecular markers related with the cell cycle and apoptosis was also performed, being the most visible alteration the expression of p53 and cdc25c, proteins involved in the cell cycle. In conclusion, with this project were identified 2 extremely potent compounds in different CRC genetic profiles, what confers a massive advantage relatively to the approved drug 5FU.

Avaliação do potencial anticarcinogénico de novos heterociclos de nitrogénio no cancro do cólon humano.

Resumo

O cancro colorectal (CRC) é um dos tipos de cancro mais comuns, sendo a sua incidência maior em países desenvolvidos, onde maus hábitos alimentares e sedentarismo são usuais. A inexistência de uma cura juntamente com a resistência dos tumores às drogas actuais torna imperativa a procura por novas e eficazes soluções. A piorar o cenário de tratamento actual está o facto de os pacientes não responderem de igual forma às drogas administradas (ex. fluorouracil-5FU) devido aos diferentes perfis genéticos dos tumores. Este tratamento ineficaz de muitos pacientes leva à vitimização de milhares de pessoas por ano.

Anteriormente, dois compostos pertencentes a uma família de heterociclos de nitrogénio foram identificados como activos em células HCT116(p53-wt) de cancro do cólon. Novos compostos derivados destes hits foram sintetizados com o objectivo de potenciar a sua acção anticancerígena. Para tal, diferentes grupos R e R_1 foram testados e ambos parecem interferir na actividade dos compostos. Destes novos compostos, 2 foram identificados como extremamente potentes e promissores (10 e 24) na mesma linha celular e também na linha isogénica HCT116(p53-null) e CO115, com IC_{50}s entre 0,6µM e 1,55 µM, em comparação com o composto de referência 5FU cujos IC_{50}s estão entre 15 µM e 252 µM. Com esta análise foi também possível descobrir que a alta actividade dos compostos é independente do status de p53, o que é uma vantagem em relação ao 5FU que não tem actividade na ausência de p53. Também se descobriu que o composto 24 é activo em células com Kras activamente mutado, assim como com BRaf. O efeito destes compostos foi avaliado em processos celulares como a apoptose e o ciclo celular, onde se verificou uma indução baixa de apoptose e uma acentuada alteração do ciclo celular com paragem do ciclo nas fases S e G_2. Um estudo inicial sobre o efeito dos compostos em diversos marcadores proteicos relacionados com o ciclo celular e apoptose foi também realizado, sendo que a alteração mais visível foi a expressão de p53 e Cdc25c, proteínas envolvidas no ciclo celular. Em conclusão, com este trabalho foram identificados 2 compostos extremamente potentes em diferentes perfis genéticos de CRC, o que confere uma enorme vantagem relativamente à droga aprovada 5FU.

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Abbreviations list

5FU	Fluorouracil
Akt/PKB	Protein kinase B
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor 1
APC	Adenomatous polyposis coli
APC/C	Anaphase-promoting complex
APS	Ammonium persulfate
ATM	Ataxia telangiectasia mutated kinase
ATR	Ataxia telangiectasia and Rad3-related kinase
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer protein
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2 protein
BH3-only	Bcl-2 homology domain 3 proapoptotic proteins
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA	Breast cancer susceptibility protein
BSA	Bovine serum albumin
BUB	Budding uninhibited by benzimidazoles mitotic checkpoint kinase
Cdc2	Cdk1, cell division cycle protein 2 homolog
CDC20	Cell-division cycle protein 20
Cdc25	Cell division cycle protein 25
CDK	Cyclin dependent kinase
Chk1/2	Checkpoint kinase 1/2
CIN	Chromosomal instability
CKI	Cyclin dependent kinase inhibitor
c-MYC	v-myc myelocytomatosis viral oncogene homolog
CRC	Colorectal cancer
DAPI	4,6-diamidino-2-phenylindole

DCC	Deleted in colorectal carcinoma
Diablo	Direct IAP-binding protein with low pI
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSBs	Double-stranded breaks
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal-regulated kinases
FADD	Fas-associated protein with death domain
FAP	Familial adenomatous polyposis coli
FasL	Fas ligand
FBS	Bovine fetal serum
FDA	Food and Drug Administration
FdUTP	Fluorodeoxyuridine triphosphate
FSC	Forward scatter
FUTP	Fluorodine triphosphate
G0 phase	Quiescent state of the cell cycle
G1 phase	Gap 1 phase of the cell cycle
G2 phase	Gap 2 phase of the cell cycle
GADD45	Growth Arrest and DNA Damage 45 protein
GF	Growth Factors
GSK3β	Glycogen synthase kinase 3 beta
HDAC	Histone deacetylases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNPCC	Hereditary non-polyposis colorectal cancer
IAP	Inhibitor of apoptosis
IC50	Half maximal inhibitory concentration
IGF	Insulin-like growth factor
JNK	c-Jun N-terminal kinase
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

M phase	Mitosis phase
MAD	Mothers against decapentaplegic
MAPK	Mitogen-activated protein kinases
MCM	Minichromosome maintenance protein complex
MDM2	Mouse double minute 2 homolog
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
MMR	Mismatch repair genes
MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
MSI	Microsatellite instability
mTOR	Mammalian target of rapamycin
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYT1	Myelin transcription factor 1
NOXA	“Damage”/Phorbol-12-myristate-13-acetate-induced protein 1
ORC	Origin recognition complex
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor 4B
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor 2A
$p19^{ARF}$	ARF tumor suppressor p19
$p21^{CIP1}$	Cyclin-dependent kinase inhibitor 1A
$p27^{KIP1}$	Cyclin-dependent kinase inhibitor 1B
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly-(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI	Propidium iodide
PI3k	Phosphatidylinositol 3-kinase
PIP2	Phosphoinositol 4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PLK1	Polo-like kinase 1
PMSF	Phenylmethylsulfonyl fluoride
pRb	Retinoblastoma tumor suppressor protein
PUMA	p53 upregulated modulator of apoptosis
RAF	Rapidly Accelerated Fibrosarcoma

RAS	Rat sarcoma
RB	Retinoblastoma
RNA	Ribonucleic acid
S phase	Synthesis phase of the cell cycle
SAR	Structure-activity relationship
SCF	Skp, Cullin, F-box containing complex
SDS	Sodium dodecyl sulfate
Smac	Second mitochondria-derived activator of caspases
SMAD3/4	Mothers against decapentaplegic homolog 3/4 (Drosophila)
SSC	Side scatter
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta protein
TP53	Tumor suppressor protein p53
TPBS	Tween Phosphate buffered saline
TRAIN	TNF-related apoptosis-inducing ligand
TS	Thymidylate synthase
VEGF	Vascular endothelial growth factor

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State of the Art

1. Cancer

Tissue homeostasis relies on the delicate balance between cell survival and death mechanisms, which ultimately determine life itself. If this balance is disturbed by some kind of unwanted modification in these mechanisms, homeostasis is lost. At the cellular level, loss of homeostasis can lead to abnormal cell growth and proliferation, a disease known as cancer. One of the main causes of death worldwide[5], cancer is the result of the accumulation of several mutations, which in turn generate damages and errors that the cell is unable to correct. Because of these damages, cancer cells can grow and multiply without any control.

Over the past decades, cancer research has brought to light a small group of biochemical, molecular and cellular traits acquired by the majority of human cancers. Tumorigenesis is a multistep process responsible for the transformation of normal cells into malignant ones and, in this process, some of these steps were identified in all tumors. It is widely accepted that tumor formation is the result of six main alterations on cell physiology, namely: self-sufficiency on growth signals, insensitivity to antigrowth signals, evasion of programmed cell death (apoptosis), infinite replicative potential, tissue invasion and metastases and angiogenesis[6] (Figure 1). Mitogenic growth signals bind to normal cells through specific transmembrane receptors, activating their proliferative state. Unlike cancer cells, in the absence of these exogenous signals normal cells are not able to grow[6]. Many of the mutated oncogenes of tumor cells mimic the effect of these growth signals, making cells independent to generate their own signals and less dependent on external stimulation. This way, the heterotypical signaling process, where one cell stimulates another, is stagnated and a autocrine stimulation system is established, breaking the homeostatic mechanism that sets the appropriate behavior of cells. One example of growth independency is the overexpression of growth factors (GF) receptors, found in many types of cancers, that makes cells hypersensitive to regular levels of GF that normally would not trigger proliferation[6]. Cells may also receive antiproliferative signals that help maintain organism homeostasis. These anti-growth signals can either be soluble growth inhibitors or immobilized inhibitors[6], which can stop proliferation by two ways. They can push cells out of proliferation into a static state (G0) or induce cells to leave their proliferative potential definitively, entering a post-mitotic state[6]. These effects of the anti-growth signals are related with the arrest of cell cycle, which is going to be described in more detail in subsequent sections. The majority of

antiproliferative signals are regulated by the retinoblastoma protein (pRb), that when hypophosphorylated blocks proliferation. The third hallmark of cancer is the ability of cancer cells to evade programmed cell death, known as apoptosis. Once this mechanism is triggered by some physiological signal, a precise cascade of steps takes place[6], normally in response to mitochondria. Found in all types of cancer, the most common strategy to escape apoptosis is the loss, by mutation, of the proapoptotic regulator *P53* gene. This results in inactivation of the p53 protein, a feature common to more than 50% of human cancers[6]. Another strategy is to enhance the activity of the PI3 kinase-Akt/PKB pathway that transmits antiapoptotic survival signals, decreasing this way apoptosis. These previous three hallmarks – sell-sufficiency of growth signals, insensitivity to antiproliferative signals and evasion of apoptosis – brings us to the next common cellular trait, which is unlimited replicative potential. Normal cells die after a certain number of divisions, unlike cancer cells that do not seem to have this control and grow indefinitely, gaining immortality. Immortality is also promoted by the upregulation of telomerase, the enzyme responsible for maintenance of telomeres[6], whose function is to regulate structural stability of the chromosomes. Because cancer cells grow at a fast pace, they have higher demands of oxygen and nutrients, which are matched with the formation of new vessels. Angiogenesis is the expansion of blood vessels to supply the cancer cells, what helps cancer cells grow and spread through the bloodstream[6].

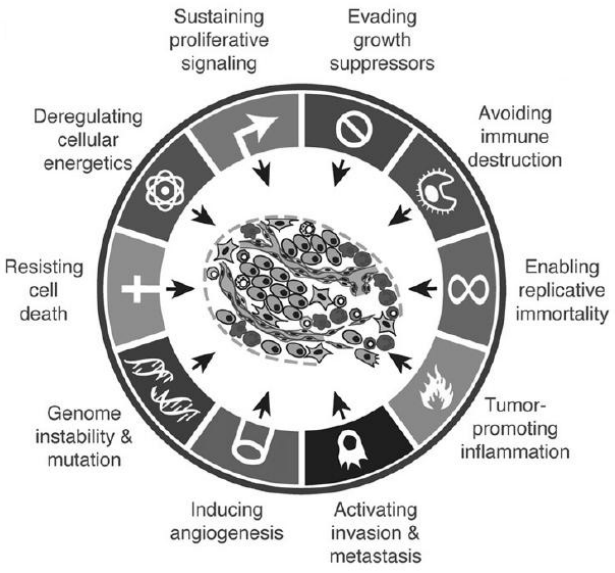


Figure 1 – Hallmarks of Cancer. Image extracted from: "Hallmarks of Cancer: next generation"[1].

Recently, a new vision of the process of tumor development has been established. In it, tumors were analyzed as a whole, taking into account their microenvironment, and not only the cancer cells individually[1]. Classic cancer hallmarks are supported by two enabling characteristics, genome instability and mutations and tumor-promoting inflammations, that confer an advantage in the development of cancer cells. With this new view, two emerging hallmarks were born, namely the reprogramming of energy metabolism and evasion of immune destruction[1] (Figure 1).

The consequence of the abnormal and unregulated proliferation of cancer cells is the formation of a mass, designated by tumor[7]. The distinction between a malignant and benign tumor resides on the fact that a benign tumor is confined. A tumor is classified as benign if cells tend to grow slowly and are confined to their original location. On the contrary, in malignant tumors, cancerous cells grow rapidly and gain the ability to invade the surrounding tissues, destroying and replacing them[7]. Only these tumors are considered cancers. Once malignant cells invade the lymphatic or/and circulatory systems, they can travel through the organism and invade other organs, where they can lodge, grow and multiply. The result of this process of invasion is the creation of new tumors – metastasis. Of all the cells present in the primary tumors only 0.01% are able to invade tissues and survive in the bloodstream, however metastases are a life threatening and recurrent diagnosis in cancer patients[8].

Cancers are classified according to the kind of tissue from which they originate or the location in the body where they first developed. Carcinomas make for 90% of all human cancers and arise from epithelial tissue[7]. Other less frequent classes of cancers are sarcomas, derived from connective tissues like bone, cartilage and fat, lymphomas, originated in the hematopoietic cells that abandon the bone marrow and mature on lymph nodes and leukemia known as blood cancer, that arises in the bone marrow, preventing it from producing platelet, white and red blood cells[7].

1.1. Eukaryotic Cell Cycle

The development of organisms and life itself depends on the ability of cells to create exact copies of themselves, in a process designated by cell cycle. All cells derive from pre-existing cells through a series of events that lead to cell division and duplication. This highly regulated process originates two genetically similar cells, assuming that everything goes according to plan. Taken into account that the human body is constituted by about 10^{14} cells,[7] all derived from just one, it is fundamental that cell cycle occurs without errors in a well regulated manner. Cellular division serves then to maintain homeostasis in tissues, replacing dying cells and creating new ones when needed. Any errors in division may lead to mutations in the genome or aneuploidy.

1.1.1. Phases of the cell cycle

Eukaryotic cell cycle is comprised of 4 phases, arranged into interphase and mitosis/cytokinesis. In interphase, cells prepare for division by growing, duplicating their DNA and accumulating nutrients and in mitosis, they split into 2 similar cells. Interphase encompasses three phases – G1, S, G2 phase – and mitosis is the last one[7] (Figure 2). These 4 phases vary in length, depending on the type of cell cycle and the signals that influence it[2]. In a human cell with a normal 24hours proliferation cycle, interphase occupies the majority of the cycle, circa 95%, leaving about 1 hour for mitosis and cytokinesis[7]. In interphase, duration values for G1, S and G2 phase are 11h, 8h and 4 hours, respectively.

Gap 1 phase - G1 phase - is the first of the cell cycle and functions as a resting state where the cell grows and prepares for chromosomal replication, which takes place in the next phase[2]. Millions of proteins and later on enzymes, required for DNA replication, are formed here. Located between mitosis and DNA replication, cells in this phase are metabolically active and grow but no DNA is replicated[7].

Synthesis of DNA is performed in the S phase, between G1 and G2 phases[2]. This phase starts when DNA replication begins and, at the end, DNA doubles in quantity. This process is completed in the minimum time possible, so that DNA base pairs are not exposed to external factors[9] (for example mutagens like radiation, DNA reactive chemicals, virus DNA, base analogs and intercalating agents[10]) too long.

In the gap 2 phase – G2 phase – cellular growth continues and cells get ready for mitosis[2]. Proteins required for preparation of mitosis are now synthesized[7].

The last phase, mitosis – M – consists in the nuclear and cell division[2]. Chromosomes and all cellular organelles are divided equally between the two daughter cells. Mitosis is itself constituted by several phases distinguished by the organization of the cellular constituents. They are prophase, metaphase, anaphase and telophase. In the end of mitosis cytokinesis happens[2]. This is not a part of mitosis but directly follows it, being responsible for the total separation of the cellular membrane and creation of the two new cells. Cells on mitosis are distinguishable by microscopy from cells on interphase[7].

When a new cell is originated, after passing through mitosis, it can either re-enter G_1 phase, giving rise to new cells, or stop growth for an undefined period of time, a condition

known as G_0 phase[2]. Cells stay in this quiescent state until new growth stimulatory signals emerge. An example are skin fibroblasts that will stay ”frozen” in G_0 phase until extracellular stimulation[7]. Without growth factors (eg. EGF, IGF) or nutrients, cells stop dividing and arrest in G_0[2].

After fertilization, embryonic cells present smaller cycles, which results in rapid rates of multiplication. These initial cycles do not show gap phases, only mitosis and S phase[7], what implicates that cells doo not grow and cell division times are rather diminished. As cells proliferate and the organism reaches adulthood, cycles become progressively longer. Adult cells end up by stopping their proliferation, some even permanently like nerve cells, resuming it only when necessary[7].

Even when no errors occur during eukaryotic cell cycle, ploidy varies among its phases[7]. In G1 phase cells have their characteristic DNA content, in other words, they are diploid (2n). When they transition to S phase, cells change from 2n to 4n and, in the G2 phase, then remain tetraploid[7]. This condition is only altered when cells divide through cytokinesis , returning to their natural diploid state. To ensure the correct completion of this cycle, an extensive mechanism of regulation is activated within cells.

1.1.2. Checkpoints

Like all mechanisms, cell cycle needs to be supervised and controlled to ensure a correct functioning. If this happens to fail, daughter cells will become unfaithful copies of their parents[7] and the principle of cellular division will be lost. In order to control the quality of the cycle, a series of rigorous steps, called checkpoints, exist along the cell cycle with the aim of verifying whether a previous event occurred correctly before starting a new one[2]. In the chance these checkpoints fail to act, genetic instability will be promoted and cells with damaged DNA or incorrectly partitioned chromosomes will be allowed to divide[2]. The result is propagation of cells potentially carcinogenic.

Cell cycle begins when the balance between growth-promoting and inhibitor factors is disturbed. The point at which stimulatory signals are favored and cells enter division is called restriction point[2]. Retinoblastoma protein (pRb) (result of the expression of the suppressor gene RB) controls this point by deciding whether the cell will pass to S phase or remain on G1. This protein has an inhibitory effect on the cycle progression when bound to

the transcription factor E2F[2]. After stimulation, during early G1, RB protein is phosphorylated and E2F factor is released, which will activate S phase genes, preparing the next phase[2]. Once cells pass this restriction point, cell cycle will continue even if growth-stimulatory factors cease[2].

Two major checkpoints regulate cell cycle – the G1/S and G2/M checkpoints. Right after cells commit to cell cycle, they are subjected to the control of the first checkpoint. In this, the cell ascertains mutations in DNA, arresting the cycle until DNA is repaired or the cell is eliminated by apoptosis[2]. p53 tumor suppressor is an important protein in this checkpoint. DNA damage activates reparation in this G1/S checkpoint. Here cells are arrested before entering the S phase and replication of DNA errors are avoided.

p53 is also important in the G2/M transition. G2/M checkpoint stops cells from entering mitosis if DNA's replication is not completed, preventing cell division[7]. Cells remain arrested in the G2 checkpoint until the complete genome is replicated. In addition to correct DNA replication, this checkpoint also examines the existence of damages in DNA[2].

The last checkpoint – spindle checkpoint – is situated inside mitosis. It function is to stop the progression of mitosis and prevent anaphase from happening in case something is wrong with the cell's organization prior to division[2]. Incorrect assembly of the spindle or disordered chromosomal orientation will arrest cells in mitosis, ensuring that chromosomes separate correctly between the daughter cells[7].

In the S phase, the integrity of the DNA and possible damages are also assessed. In this quality point, replication errors like insertion of incorrect bases or replication of wrong segments is evaluated[7]. Existence of errors, like stalled replication machinery or damages resulting from prolonged inability to synthesize DNA, activates the control process of this phase, what results in a arrest of mitosis that can be either dependent or independent of p53[11].

Cell arrest in G_1 and G_2 phases is mediated by two DNA damage related protein kinases – ATM and ATR[7]. These kinases recognize damaged or incorrectly replicated DNA and are activated in response to these damages. Their signaling leads to cycle arrest and activation of DNA repair systems. In cases where DNA repair is impossible, programmed cell death is activated[7]. Mutations on *ATM* gene, responsible for ataxia telangiectasia disease, are often found in cancer.

1.1.3. Cell Cycle Regulation

Cell Cycle is an intricate and delicate process that requires the activity of several molecules to control and coordinate the different phases. Some stimulate the progression of the cycle and others inhibit it, altering the biological functions of regulatory proteins through phosphorylation. These important regulatory proteins are divided into three groups: cyclins, cyclin dependent kinases (CDKs) and cyclin dependent kinases inhibitors (CKIs), that have the purpose of maintaining the cycle's delicate balance.

Figure 2 – Cell Cycle. The cell cycle is tightly regulated by CDK/cyclin complexes.

There are four types of cyclins, defined by the stage of the cell cycle in which they operate. All cyclins bind to cyclin dependent kinases (CDKs), other key class of cell cycle regulatory molecules, forming an active holoenzyme complex, as represented in Figure 2. CDKs are serine/threonine protein kinases that require binding to cyclins, the regulatory subunits[2]. Their activity is regulated by: association with cyclins; activating phosphorylation of threonine near position 160; inhibitory phosphorylation of threonine 14 and tyrosine 15, and association with CDK inhibitors (CKI's)[7]. Normally, levels of CDKs remain stable during the cell cycle, whereas levels of cyclins increase and decrease through the different phases, with the exclusion of cyclin D[2]. Variation of cyclins levels respond to the rate of synthesis and degradation by the ubiquin-protreasome pathway[2].

Starting at G_1, represented in Figure 3, cyclins of this phase – cyclins D (1, 2 and 3) - promote the passage through the restriction point[7] and, consequently, the initiation of the cycle. Hereafter, cyclins of the G_1/S transition – cyclins E (E1 and E2) - are activated in late G_1[7], leading the cell to commit with DNA replication and passage to S phase[2]. Once in S phase, the connection of cyclins A and CDKs is mandatory for the initiation of DNA replication. The last existing class of cyclins are in G_2/M phase, cyclins B, that promote mitosis[2].

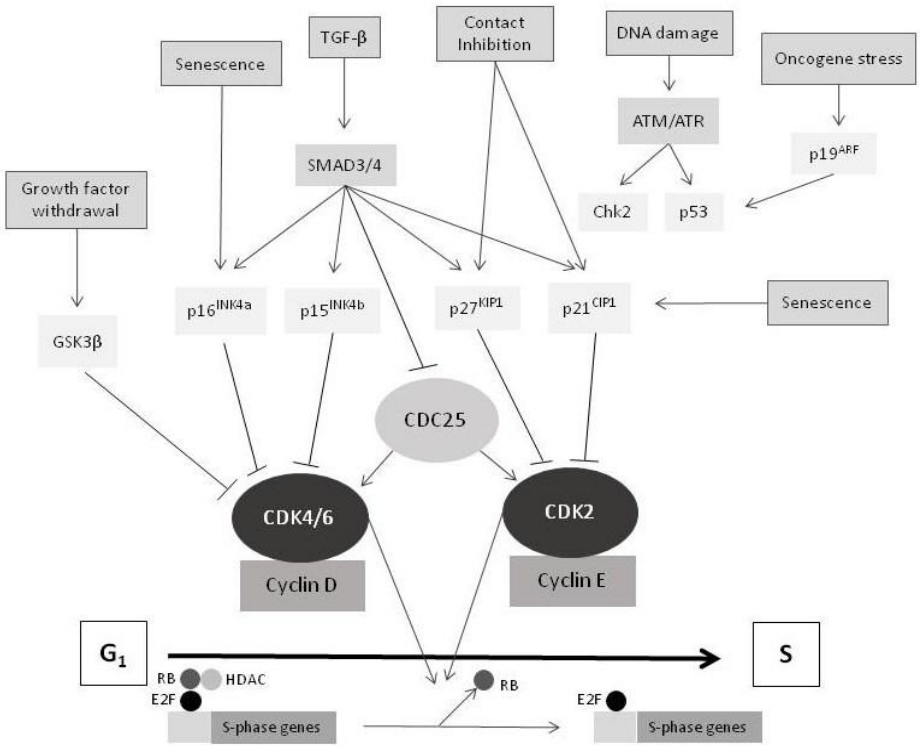


Figure 3 – G_1/S checkpoint. Regulation of the transition from the G_1 to S phase by several proteins. Adapted from "The Molecular Biology of Cancer"[2].

Cell cycle initiation in G_1 phase and passage to S phase are controlled by kinases CDK4/6-cyclin D and CDK2-cyclin E, but also by the complex RB-E2F[2] (Figure 3). As described previously, RB protein controls the restriction point, arresting the cycle when bound to E2F transcription factor (RB-HDAC-E2F). RB phosphorylation by CDK4/6-cyclin D and CDK2-cyclin E will remove RB from the complex and, as a consequence, E2F will be released[2], allowing the transcription of S phase genes, whose function is to encode proteins that enhance the transition between G_1-S and prepare DNA replication. Cells can arrest at G_1 due to the degradation of Cdc25A phosphatase. ATM pathway causes the phosphorylation of this enzyme by Chk2, what consequently leads the maintenance of the complex Cdk2/cyclin E in an inactive state[12]. G_1 phase checkpoint is controlled by several stimuli, namely, TGF-β; DNA damage; contact inhibition; replicative senescence; oncogenic stress; and growth factors removal[2]. The first five act via the induction of the families of CKIs INK4 and KIP/CIP. One of the key players of this checkpoint is p53 protein. It indirectly senses DNA damage and decides if the cell will arrest the cycle in G_1 until damage is repaired or trigger apoptosis in case repair is impossible.

Regulation of DNA synthesis in S phase is made with the assistance of complexes CDK2-cyclin E/A. To start DNA replication, three elements are required: the activation of replication origins, activation of DNA unwinding functions and of polymerases[2]. Replication origins are prereplication complexes located at chromosomes whereas DNA helicases are responsible for the unwinding of DNA through the opening of the DNA helix. Activation of these three elements is regulated by CDK activity levels[2]. When CDK activity is low, replication origins are formed and when CDK activity levels are high, DNA polymerases and helicases are recruited, leading to the beginning of DNA replication. High CDK activity prevents the formation of more prereplication complexes, remaining high until mitosis is complete, moment after which activity lowers again. In addition, the transition from low to high levels of CDK activity are crucial for correct DNA replication[2]. Damages caused during DNA replication by, for example, ionizing radiation activate the ATM/ATR pathway and their signals are integrated by Cdc25A, causing a rapid cell arrest[12].

During S phase, only one copy of DNA (4n) is made by each cycle. Like the rest of the process, this too has to be controlled so aneuploidy is prevented. With that purpose, molecular mechanisms of control exist to avoid the re-initiation of DNA replication until the cycle is completed. This mechanism involves mini-chromosome maintenance (MCM) helicase proteins in G_1 phase that bind to replication origins together with ORC proteins, forming the origin recognition complex - ORC[7]. This complex is then considered licensed for replication[13]. MCM proteins only bind to DNA in G_1 phase and, as soon as replication begins, MCM proteins leave the origin and initiation stops. These do not bind DNA in any other phases of the cycle as a consequence of regulatory protein kinases' activity[7]. Variations of CDKs activity in cell cycle dictate the distinction between the licensed state of replication origins in G_1-phase and the unlicensed state in the rest of the cycle. Licensing is only allowed when CDK activity decreases at the end of mitosis, relieving its restriction and allowing the beginning of a new cycle[13]. As cells transit from G_1 to S phase, activity of CDK increases what triggers initiation and prevents the formation of more complexes[13], as mentioned previously.

G_2/M checkpoint, whose key regulator is CDK1/cyclin B, prevents cells from entering mitosis in case the genome is damaged (Figure 4).

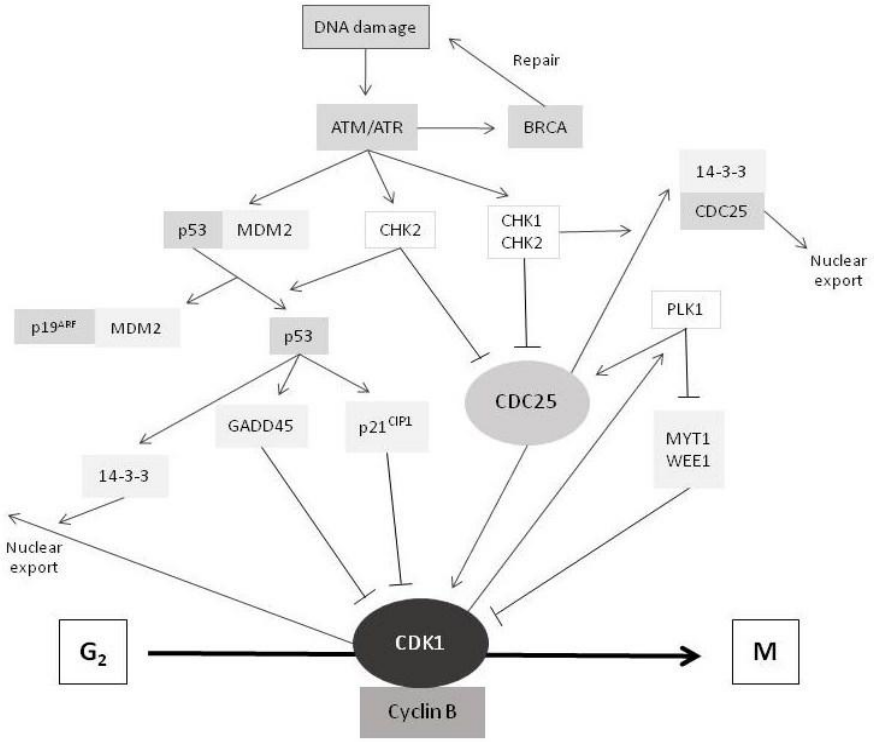


Figure 4 – G_2/M checkpoint. Regulation of the G_2/M transition by several proteins. Adapted from "The Molecular Biology of Cancer".

Because of the effect of Wee1 and MYT1 kinases on tyrosine 15 and threonine 14, CDK1 (also known as Cdc2[11]) remains inactivated during G2 phase and will only be activated near the end[7]. Due to the phosphorylating action of the polo-kinase PLK1, CDC25c is activated. This phosphatase activates CDK1 through the dephosphorylation of threonine-14 and tyrosine-15 residues[12]. The now active complex CDK1/cyclin B will create a positive feedback with the continuous phosphorylation of Cdc25c, which will amplify the signal and push cells towards mitosis[11]. DNA damage can arrest progression of this transition, independently of p53, through the activation of ATM/ATR kinases. This activation starts two parallel cascades, schematized in Figure 4, that will ultimately inhibit CDK1-cyclin B activity[2]. In the first cascade, which detects less profound damages, CHK activated kinases phosphorylate and, consequently, sequesters CDC25 in the cytoplasm where it cannot function[11]. This results in the inactivation of the CDK1-cyclin B complex. The other cascade, which detects major damages like double-stranded breaks (DSBs), counts with regulation by p53 protein. After DNA damage, p53 is phosphorylated, dissociating from the complex p53-MDM2[2]. This results in fully functional p53 that will bind to DNA. Genes activated by p53 will encode the proteins 14-3-3, GADD45 and $p21^{CIP1}$. These will bind to CDK1, promoting nuclear

export, complex dissociation and inhibition, respectively[2]. Relatively to Gadd45 protein, its dependency on p53 in order to cause G_2 arrest was identified, although it is not clear why[11]. The effect of 14-3-3 and p21 proteins on the G_2 arrest was also analyzed in HCT116 colon cell line, leading to the conclusion that 14-3-3 controls the duration of the arrest and p21 is involved in its stabilization[11].

Cell division in M phase depends on the attachment of kinetochores to the spindle fibers. These protein structures, located on chromatids, separate the sister chromatids to opposite poles of the cell, being this way directly related with the correct separation of chromosomes in cells. Therefore, in the spindle checkpoint an evaluation of the attachment of the kinetochores is performed[2]. If these structures are incorrectly attached to the spindle fibers, BUB and MAD proteins are sequestered. These proteins form an inhibitory complex that will inactivate the anaphase-promoting complex (APC/C)[2].

Several proteins, known as mitotic kinases, regulate the mitotic spindle. This group of serine/threonine proteins encompasses CDK1, PLK, NIMA-related kinases, WARTS/LATSI-related kinases and Aurora/IPL1-related kinases. The last group, Aurora kinases, is involved in the progression of cell cycle from G_2 phase to cytokinesis[2].

As described previously, p53 protein is a major key regulator of G_1/S and G_2/M checkpoints. This protein senses DNA damages and arrests the cycle until this damage is repaired or, in the case the damage is too extensive, induces apoptosis. G_2/M transition involves inhibition of cell division cycle protein 2 (Cdc2) - CDK1 - and, in addition to the already discussed mechanisms by which p53 is able to block cell cycle, other targets were identified. An example is the repression of *cyclin B1* gene by p53 that also contributes to the block of mitosis' entry[11]. Other mechanisms involve induction of the transcription of the *reprimo*, *B99* and *mcg10* genes by p53 that leads to G_2 arrest and repression of *topoisomerase II* gene that prevents entry into mitosis[11]. Overexpression of reprimo, a cytoplasmic protein, causes arrest of the G_2/M transition but its mechanism of action is unknown[11]. On the other hand, topoisomerase II is an important enzyme for DNA replication, since it binds to DNA creating DSBs and assists the repair process. This enzyme is downregulated by p53 and its inhibition stops cells from entering mitosis[11].

An experiment performed by Taylor and Stark (2001) with HCT116 colon cancer cells lead them to the conclusion that p53 is not a requisite for the initial G_2 arrest but is fundamental for a long-term stop[11]. This is due to the activation of p53-indepent pathways, ATM-ATR, discussed above. Other studies suggested that in addition to causing G_2 arrest on its own, p53 can also prolong the effect of DNA damage on it[11]. Nevertheless, p53 effects on G_2/M transition depends on the cell type under study[11].

Cyclin dependent kinases are periodically activated by cyclins, what drives the cell cycle forward. On the contrary, CDK are negatively regulated by CKIs[2]. Once CKIs act through inhibition of the cycle, it is not hard to understand that many of them are tumor suppressors in humans and loss of their function is associated with cancer. There are two families of CKIs – INK4 and CIP/KIP – whose function is to inhibit the activity of cyclin-CDK complexes. INK4s (p15, p16, p18, p19) act by binding to CDKs and CIP/KIP (p21, p27, p57) by binding to cyclins[2]. The INK4 family of proteins inhibits the activity of cyclin D-CDK4 of G_1 phase and prevents RB phosphorylation[2]. A protein of this family, ARF ($p14^{ARF}$ in man) also activates another important tumor suppressor, p53. ARF sequesters MDM2 (that targets p53 for degradation) and stabilizes p53 in response to oncogenic stress, what results in G_1/S and G_2/M arrest[2]. The other family of inhibitors are the CIP/KIP family. These inhibit CDK2-cyclin E and A and CDK1-cyclin B[7]. High levels of $p21^{CIP1}$, major transcriptional target of p53, arrest the cell cycle by inhibition of cyclin E-CDK2[2]. Two CKIs, $p16^{INK4a}$ and $p27^{KIP1}$, are also important for the control of the restriction point. They bind and inhibit the activity of cyclin D-CDK and cyclin E-CDK, arresting cells in G_1[2].

Control of the cell cycle is also dependent on the degradation of proteins. Two complexes of enzymes – SCF and APC/C – control the cycle by promoting degradation[2]. These enzymes are ubiquitin ligases that act on several regulators of the cycle, causing their destruction by the proteasome. SCF enzymes regulate the G_1/S transition, destroying cyclins of this phase and some CKIs that control S phase initiation[2]. These enzymes are activated by p53 and genotoxic stress, targeting regulators like cyclin E and c-MYC, gene commonly related with cancer that encodes transcription factors. APC/C enzymes regulate mitosis proteins like cyclin B and securin[2], being responsible for initiation of anaphase and exit of mitosis.

1.1.4. Cell Cycle and Cancer

The complex mechanisms of cellular regulation portrayed previously serve the purpose of avoiding the propagation of errors in populations of cells. They have the ability to arrest cell cycle and repair DNA, so that no mutations or aneuploidy spread through cells, although most of the times errors may occur. Mutations in two types of genes – proto-oncogenes and tumor-suppressor genes – are particularly relevant to cancer[2]. Proto-oncogenes encode proteins that operate on signaling pathways that stimulate cell division. In case of mutation, these genes have a gain of function and proliferation values are enhanced, aiding the tumorigenesis process[2]. Inactivating mutations on tumor suppressors, tumor-suppressor genes, also lead to enhance values of proliferation. These are responsible for the inhibition of the cell cycle, once they encode proteins involved in regulatory pathways (eg. RB and p53 proteins)[2].

The decision to divide is mainly dependent on the G1 phase. If cells pass the restriction point and transit to S phase, cell cycle will occur without any additional stimuli. Bearing this in mind, it is expected that, in cancer cells, the G1/S transition control is often disrrupted[2], making cells independent of mitogenic stimuli. The Rb pathway, whose protein phosphorylation is a key event of G1/S transition, is frequently altered in cancer. In fact, nearly 90% of human cancers show variations in the RB pathway[2]. Its non-functionality is due mostly to the inappropriate activation of CDK/cyclin or loss of CKIs[2].

Occasionally, mutations on CDKs occur in cancers. The result on some CDKs, for example CDK4 and 6, is to make them unresponsive towards CKIs[2]. Other effect, in some colonic tumors, is the overexpression of CDK1 and 2[2]. Inappropriate activation of CDK-cyclins, result of deregulation of CDC25, has been found in some cancers. CDC25 phosphatase family is responsible for CDK activation. Deregulation of cyclins are another target for tumorigenesis. For example, high levels of cyclin D1 expression, participant of early G1 phase, are often found in many types of cancer, for example colon cancer[2]. An important target for cancer pathogenesis is the dysregulation of cyclins that bind to S-phase CDK2[2], such as E-type and A-type cyclins. These regulate DNA replication and cellular proliferation, being high levels of expression associated with aggressive disease and poor

prognosis. Overexpression of cyclin B1, regulatory subunit of the G_2/M transition, is often found in colorectal cancer and is also associated with poor prognosis[14].

Adding to mutations in the CDK-cyclin mechanism, loss of CKIs is another important factor for cancer development, being Rb protein their major target[2] once the absence of CKIs will increase proliferation. An example of a relevant CKI is $p27^{KIP1}$, whose downregulation is associated with tumor aggressiveness and poor prognosis. In colorectal carcinomas, the diminished levels of $p27^{KIP1}$ are due mainly to increase of proteasome-dependent proteolysis rather than gene deletion[2]. Cells deficient in $p21^{CIP1}$ and p53, most common mutated gene in human cancers, are not able to arrest the cell cycle, in response to DNA damage[2]. $p21^{CIP1}$ is a downstream target of p53 that helps to maintain cell survival when DNA damage happens.

Since cell cycle checkpoints control the progression of the cycle, their loss is a sign of cancer and a common one in humans. In addition to p53, RB and several CKIs, checkpoints are also controlled by two DNA damage-response pathways – ATM and ATR-CHK1[2]. These signaling pathways function as checkpoint activators after DNA damage. Double-strand breaks (DSBs), consequence of the damage in DNA, are repaired by proteins activated by the ATM kinase[2]. Deficiency in these pathways results in DSBs accumulation and genomic instability.

Cells undergoing cell cycle are targets for cancer chemotherapy, once their DNA is more exposed and susceptible during DNA synthesis and cell division[9]. Because some tumor cells, eg. colon cells, present a slower growth rate than others (eg. leukemia), they become less susceptible to most anticancer agents. This is counteracted by shifting of G_0 cells to G_1, so they can re-enter the cycle. To do so, debulking of the tumors is performed with surgery or radiation[9]. As a consequence of this reduction of size, the remaining cells are pushed from G_0 to G_1 due to the increase of availability in nutrients and oxygen, among others. These now active cells are more susceptible to drug therapy[9].

1.2. Cell death mechanisms

As initially discussed, tissue homeostasis is fundamental for survival and the balance between life and death has to be carefully made through a variety of processes. In fact, about $5x10^{11}$ cells are eliminated every day in humans, but a continuous production in the bone marrow balances the cellular losses[7]. Regulation of growth and control of cell division was clarified previously. Now, some mechanism that control cellular death will be explored.

1.2.1. Programmed Cell Death – Apoptosis

Apoptosis, or programmed cell death, is a common endogenous process in which cells order their own destruction in a organized manner. This mechanism is activated whenever the organism needs to eliminate some cells or the cell itself senses something wrong[7], leading to some morphological transformations. The first event is the decrease of cell size and consequent aggregation of its components. This is followed by condensation of the nucleus and, in some cases, nuclear fragmentation. The final step is the formation of the apoptotic bodies[7], cell fragments surrounded by cytoplasmic membrane to be phagocytosed by leukocytes. Phosphatidylserine, normally located in the inner part of the membrane, is expressed in the surface during apoptosis and is recognized by receptors expressed by the phagocytes[7].

Caspases

Caspases are the executors of apoptosis, cleaving more than 100 different cellular targets like inhibitors of DNase and cytoskeletal proteins for example. There are at least 7 families of caspases that can be classified as initiators or effectors, depending on their function[7]. All caspases are initially inactivated and converted to an active form through proteolytic cleavage by other caspases, with the exception of the initiator – caspase 9[3]. This is activated by link with Apaf-1, forming the apoptosome that will then start a chain reaction, activating downstream effector proteins like caspase 3 and 7, similar in function. Formation of this complex requires the release of cytochrome c from mitochondria[15]. Given the importance of caspases to cellular death, their regulation is fundamental to survival. Caspases are regulated by a family of proteins, the inhibitors of apoptosis (IAP) family. IAP proteins interact directly with caspases, inhibiting their function. The Smac/Diablo and Omi/Htr2 proteins released from the mitochondria interfere with the action of IAPs[7].

Intrinsic pathway

The intrinsic or mitochondrial pathway, represented in Figure 5, is initiated in the interior of the cell by any stimuli that causes oxidative stress, mitochondrial disturbances and DNA damage[16]. This apoptotic process is regulated by the Bcl-2 family that acts in the mitochondria. Bcl-2 family members are divided into Antiapoptotic Proteins (bcl-2 itself), whose function is to inhibit apoptosis and Proapoptotic Proteins, that induce caspase activation and promote death[7]. The balance between these two types of proteins determines the fate of the cell. Proapoptotic Proteins are sub-divided into two groups that differ in function and extent of homology with Bcl-2. The proapoptotic multidomain proteins have three Bcl-2 homology (BH) domains (eg. Bax and Bak) and the BH3-only proapoptotic proteins (eg. PUMA and Noxa) only have one[7]. Bax and Bak members are inhibited by antiapoptotic members like Bcl-2. BH3-only members are regulated by signals that induce death or survival. Once activated, these proteins work against the antiapoptotic bcl-2 family and activate the Bax/Bak multidomain proteins, leading to caspase activation and cell death[3]. Bax and Bak bind to the mitochondria, releasing cytochrome c and other proteins like, for example, Smac/Diablo and Omi/Htr2, due to the subsequent membrane permeabilization. This promotes the apoptotic process by caspase activation[7].

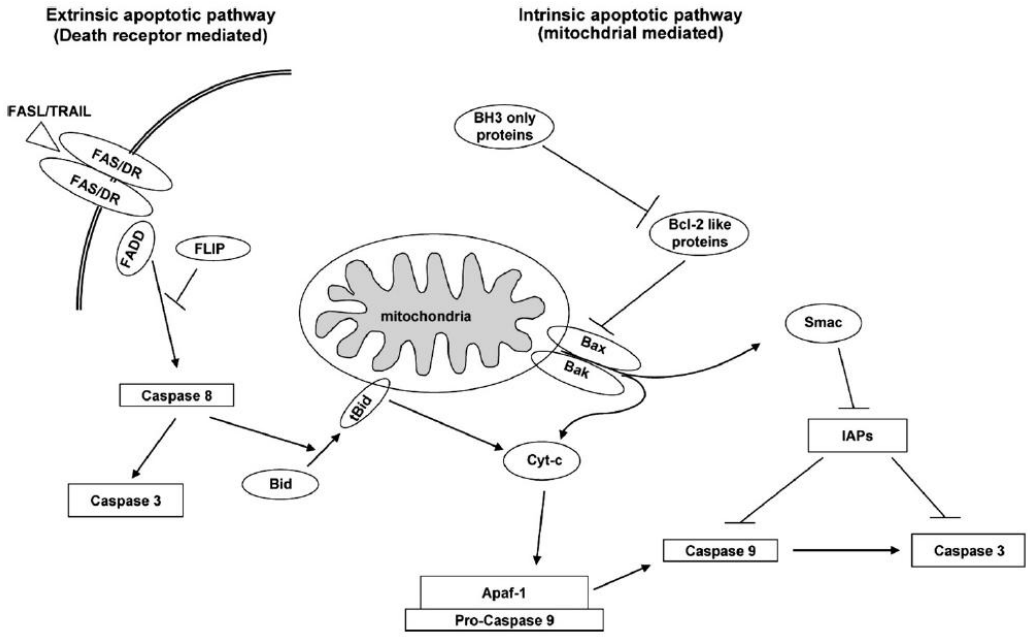


Figure 5 – Intrinsic and Extrinsic apoptotic pathways. Image extracted from "Apoptosis signaling proteins as prognostic biomarkers in colon cancer: A review"[3]

Extrinsic pathway

The apoptotic process can also be initiated from the exterior of the cell (extrinsic or death receptor pathway) by the activation of death receptors in the surface (Figure 5). Death receptors like, for example, FAS and death receptor 4 and 5, are activated by their specific ligands (i.e. FasL and TRAIL)[3]. This link results in the activation of caspase 8 by the accumulation of FADD molecule. Once activated, caspase 8 leads to the activation of the effector caspases. This pathway is not completely independent of mitochondria. In fact, caspase 8 also activates the Bid BH3 only protein, linking this way the two apoptotic pathways[3].

p53 function

The important role of apoptosis is the elimination of damaged cells. The ATM/ATR signaling pathway, main pathway of DNA damages, is mediated by p53. The choice between inhibition of the cell cycle and induction of apoptosis depends on the extension of the DNA damages. Normally, when apoptosis is a result of p53 mediation the intrinsic pathway is activated[17]. The increase of p53 in response to DNA damage leads to the synthesis of BH3-only proteins, what consequently causes an increase in the number of apoptotic cells[7]. P53 activates several genes important for the execution of the apoptosis' intrinsic pathway, for example NOXA and PUMA. The increase of apoptosis can also be due to the interaction of p53 with antiapoptotic proteins. The link between these bcl-2 like proteins and p53 destabilizes the mitochondrial membrane, causing a release of cytochrome c. In addition, p53 mediated apoptosis can occur via the extrinsic pathway, by the induction of genes encoding cell surface receptor like death receptor 5 and Fas [17].

1.2.2. Senescence and Necrosis

Senescence is a process of biological aging that can end up in cell death. With the passage of time, cells accumulate mutations and alterations that cause deterioration and even death. The cells' activity is controlled by the telomeres that protect the ends of the chromosomes and determine when the cell has to stop dividing. The length of the telomeres is shortened in each division of the cell and when they reach their limit, cells stop dividing. Some cells can also undergo senescence in response to stress. Cancer cells are not

susceptible to telomere-dependent senescence once they have the enzyme telomerase that regenerates telomeres and allow cells to reproduce without temporal control[18].

Another process worth mentioning is necrosis. This is a pathological and disorganized process of cell death, consequence of traumatic and acute cellular injury. It can be caused by toxic agents or biological agents that have a direct effect on the immune system like fungi, bacteria or viruses. After damage of the cell, its constituents are spread into the extracellular space, provoking an inflammatory response[19].

2. Colon Cancer

Main focus of this project and 3rd most common cancer in the world, colon cancer is the 4th leading cause of death due to cancer, according to the latest statistics[20]. Risk factors for colon cancer encompass increasing age, family history of colorectal cancer, colorectal polyps, specific genetic mutations and, most importantly, sedentary and unhealthy diets rich in animal fats[21]. Alteration of genetic markers causes predisposition to this disease and individuals with predisposition present a higher risk in the presence of favorable environment[21]. Higher incidence of this type of cancer is typically found in developed countries, where the intake of animal fat is coincidently larger[22]. The first stages of colon cancer are often asymptomatic but mortality rates have been decreasing globally in the last two decades[22]. This is the result of better screening processes and detection tools.

Before going any further, colon's function and morphology must be refereed. This organ is part of the digestive system, together with esophagus, stomach and small intestine. More specifically, colon constitutes the large intestine (with cecum and rectum) and its function is to extract water and salt from solid wastes prior to their elimination. Colon can be divided into 4 sections, namely the ascending, transverse, descending and sigmoid colon[23], represented in the figure 6A. Transversally and from the inside out, colon if formed by an epithelial layer denominated mucosa, made of epithelial cells which form invaginations towards the interior, the submucosa and a layer of muscle. In the external part of colon exists a moist membrane called serosa[23] (Figure 6B).

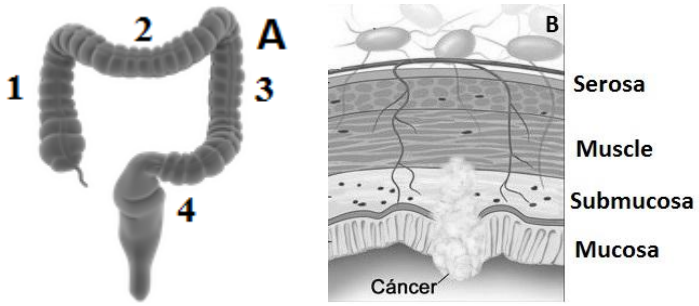


Figure 6 – Schematic representation of the colon morphology. In A it is represented the four sections of the colon: 1- ascending; 2- transverse; 3-descending; and 4- sigmoid colon. Image B represents a transversal incision on colon with stage II cancer, where it can be identified its different layers.

Colon cancer stages are classified taking into account the invasion of these layers. In stage 0, also referred as carcinoma *in situ*, no invasion is observed and cells on the mucosa wall can become cancerous and spread. In the 2nd stage, stage I, cancer is already formed in the mucosa and slips to the sub-mucosa. When the tumor has already passed the muscle till the serosa without transcending this layer, cancer is at stage II. At stage III, cancer reaches the lymph nodes and, finally, at stage IV, it spreads to other organs[24].

A new approach has been developed in the last years with the goal of reducing colon cancer mortality rates. The use of preventive agents is suggested to delay the carcinogenesis process and, logically, the safety of the compounds is one of the main concerns. For example, curcumin, a common spice in India, was proposed as a chemopreventive agent for colon cancer by Johnson and Mukhtar[25]. The promising results of its activity together with the safety and tolerability presented lead to the ongoing clinical trial phase II[25].

Regarding treatment, chemotherapy plays a central role fighting this disease but the diversity of patients' genetic profiles hampers efficacy. The discovery of drugs that show efficacy in all patients motivates the search of more efficient drugs.

2.1. Carcinogenesis process

The transformation of normal cells into carcinogenic ones is a multistep process that occurs through an extended period of time. This process is the result of the accumulation of several genetic mutations which change the mucosa, leading to the formation of carcinomas. In fact, about half of the human colon tumors show a mutation in the *Ras* oncogenes, which stimulates proliferation[6]. Many external and internal factors contribute to this

transformation like carcinogens, but the most critical factor is diets rich in animal fats, as discussed previously.

The carcinogenic process (Figure 7) can be divided into 3 parts. The first is the initiation, where the cells' genetic information is altered in consequence of mutations due to carcinogens[4]. In this stage of development, cells gain an intrinsic proliferative capacity that cannot be controlled, due to mutation on genes like APC[4]. Promotion of the initiated cells is the 2nd phase and leads to abnormal cell division and formation of cell masses, neoplasms, that are now detectable. Accumulation of mutations on genes like p53 and KRas happens due to the continuous exposure to genotoxic agents[4]. Tumor growth relies on the delicate balance between the number of cells in proliferation and the ones directed to death pathways[9]. Colon tumors have low growth rates, which is due to a small unbalance, being that the cellular production only exceeds cell losses by 10%[9]. As said previously, this growth rate has serious effects on the tumors susceptibility to chemotherapy. The last stage is the progression. In this, tumors turn malignant and gain the ability to invade other tissues and organs and create metastases[4].

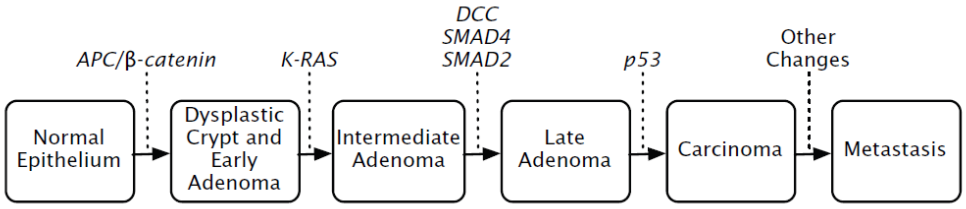


Figure 7 – Classical pathway for colon cancer progression[4]. In the scheme is represented some key genetic alterations responsible for the carcinogenesis process. Extracted from "Dynamics of cancer – Incidence, Inheritance and Evolution".

The majority of colon tumors follow this pathway of genomic instability. These have chromosomal instability (CIN) – CIN tumors - and present allele losses and aneuploidy[26]. Most solid tumors present CIN but its causative genes are not identified yet. The other pathway - microsatellite instability (MSI) - is due to defects in one of the mismatch repair system (MMR) genes. This less common pathway is only present in 15% of colorectal cancers and is characterized by several DNA mutations and diploidy. The most frequently inactivated genes seen in MSI are *MLH1* and *MSH2*. MSI leads to the HNPCC syndrome, which, consequently, follows different genetic steps of cancer progression[26].

The study of the genetic profiles of colon tumors lead to their categorization into the following groups: sporadic, inherited (about 10%) and familial (about 25%). Focusing on the syndromes with hereditary interference, the two most common forms are the familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC)[25]. The FAP syndrome is the result of germline defects on the *adenomatous polyposis coli* gene (APC) which is related with the initiation of tumors. APC is involved in the transmission of signals to the nucleus and patients with this mutation show predisposition to cancer and a high initiation rate of adenocarcinomas. About 30% of the FAP cases do not have hereditary origins and arise *de novo*[27]. Due to the large number of adenocarcinomas characteristic of this syndrome, it is probable that some of them progress and become malignant with the assistance of additional mutations in other genes like p53. The most common inherited cancer susceptibility is hereditary nonpolyposis colorectal cancer syndrome (HNPCC), the result of autosomal dominant mutations in a MMR gene, as described previously. These mutations result in an enhancement of the tumors' progression rate[27] and accounts for 15% of colon cancers[7].

2.2. Growth signaling pathways

Cell homeostasis and maintenance depends on the delicate balance between cell division versus senescence and survival versus death. These processes are induced by several regulatory signals that bind to receptors in the cell membrane. Regulatory growth signals can be either local – paracrine - produced by the own cell, or act at distance through the circulation - endocrine. In addition they can also be positive or negative[28]. Normally these GF are integrated in the G_1 phase of the cell cycle before the restriction point, deciding if the cell will either divide or arrest. Cancer cells do not depend on this regulation, creating their own growth signals[28]. These signals, commonly referred as GF, can be classified into three groups: mitogens, that promote cell cycle entry by induction of the G_1/S transition; growth factors, that promote protein synthesis, resulting in an increase of cell mass; and survival factors, that promote survival by elimination of apoptosis. Mitogens act on cyclin D-CDK complexes increasing their activity, and pushing the cell cycle forward[28]. Contrary, cytostatic factors inhibit replication. TGF-β, member of the Transforming growth factor (TGF)

family is an example. Regulation signals are integrated by different pathways, which serve as a communication road to the nucleus[28]. MAPK/ERK and PI3K/Akt signaling pathways (Figure 8) are frequently altered in colon carcinomas, due to Ras mutations for example, breaking the cell equilibrium and causing a concomitant increase of cell proliferation and decrease of apoptosis.

MAPK/ERK signaling pathway

The commonly known as MAP kinase (MAPK) pathway, schematized in Figure 8, is part of a larger signaling family, the MAPK signaling pathways family. In addition to this subfamily of kinases regulated by extracellular signals – MAPK/ERK – there are two others, the JNK and p38 pathways[29]. The Mitogen-activated protein kinases (MAPK) protein was originally denominated as ERK, once it regulates extracellular signals, being this pathway also known as RAS-RAF-MEK-ERK pathway.

This pathway, deregulated in about 30% of cancers, is one of the most important for cell increased proliferation and decreased apoptosis, creating a chain of communication between external signals (eg. growth factors) and the nucleus in a series of protein activations[29]. Phosphorylation of ERK protein (pERK) is a indicator of the activation of this pathway, which can be due to the upstream participants in the transmission of the signal, like KRAS and BRAF[30]. Mutations on these genes lead to uncontrolled proliferation. After activation of RAS by external signals, it binds to RAF and consequently activates MEK, which will then phosphorylate ERK[29]. This will provoke a response by the nucleus like, for example, cyclin D synthesis[7]. Since this pathway is frequently altered in colon cancer it provides excellent investigation targets when studying the effect of compounds in cancer cells.

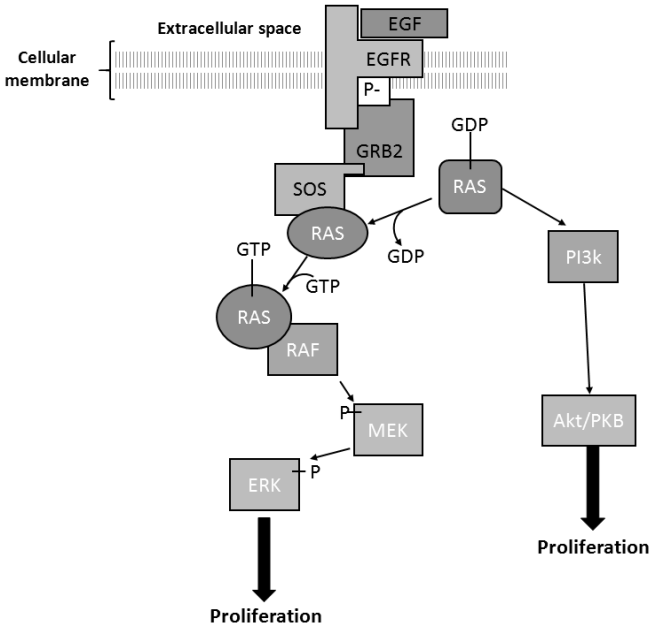


Figure 8 – MAPK/ERK and PI3K/Akt pathways.

PI3K/Akt signaling pathway

Another important and commonly altered pathway in colon cancer is the PI3K/Akt signaling pathway, schematized in Figure 8. This is one of the major intracellular pathways promoting cell survival. Deregulation of this pathway also leads to an increase of proliferation and decreased of apoptotic cells[30].

After activation of PI3K by Kras, for example, Akt is activated by phosphorylation of the membrane phospholipid PIP_2, which is then turned into PIP_3. Akt phosphorylation is a good indicator of the activation of this pathway. This process results in the activation of mTOR that promotes cellular growth. A key substrate of Akt is the Bad protein, member of the BH3-only proapoptoctic proteins of Bcl2 family[7].

3. Colon Cancer Treatment

Depending on the stage at which the tumor is detected and the patient itself, treatment of colon cancer can take an active form of action, by fighting and trying to find a cure for this disease or a palliative action. When this type of cancer is diagnosed early, surgery is often the treatment of choice. In addition to the surgical removal of the tumors, chemotherapy is normally used with the goal to eliminate remaining cancer cells that can spread through the organism. The discovery of an active drug effective on all the different genetic profiles found in colon carcinomas poses as a serious challenge.

3.1. Drugs approved for chemotherapy

Fluorouracil (5FU), represented in Figure 9B, is probably the most widely used drug for chemotherapy in several types of cancer. This FDA approved antimetabolite is used in advanced colon cancer that has recurred or not gotten better. 5FU has to be converted *in vivo* into its active form, fluorodeoxyuridine triphosphate (FdUTP) so it can bind to RNA and produce the desired effect. The incorporation of 5FU into RNA interferes with DNA synthesis by inhibition of thymidylate synthase (TS)[31], enzyme responsible for the generation of thymidine triphosphate. This is considered the main mechanism of action of this drug. Inhibition of TS is only possible during S phase of the cell cycle and only a few cells are found

in this phase, what would decrease 5FU's effect if it were not for other mechanisms of action[32]. A second mechanism of action of 5FU relies on fluorodine triphosphate (FUTP), another active metabolite. This replaces uracil and is able to incorporate RNA, inhibiting its processing and, consequently, cell growth. In order to overcome resistance to 5FU, biochemical modulation of its metabolism was examined with several purines and pyrimidines, but only some of them were successful[31]. In a previous study, the effect of 5FU was assessed in two isogenic cell lines of colon cancer, HCT116 (p53-wt) and HCT116 (p53-null) with a complete knockout of p53, which allowed to ascertain the importance of p53 for the activity of 5FU. It was concluded that HCT116 (p53-null) cells were resistant to the effect of 5FU, making p53 of extreme importance to the efficiency of this drug[33]. In addition, it became clear the dependence on p53 of 5FU's mechanism of apoptosis induction. In HCT116 (p53-wt) cells, where the compound is active, high levels of apoptosis were observed. In opposition to this, in HCT116 (p53-null) cells 5FU had no effect. This was corroborated by the cleavage of PARP-1 protein, which was only cleaved in HC116 (p53-wt)[33].

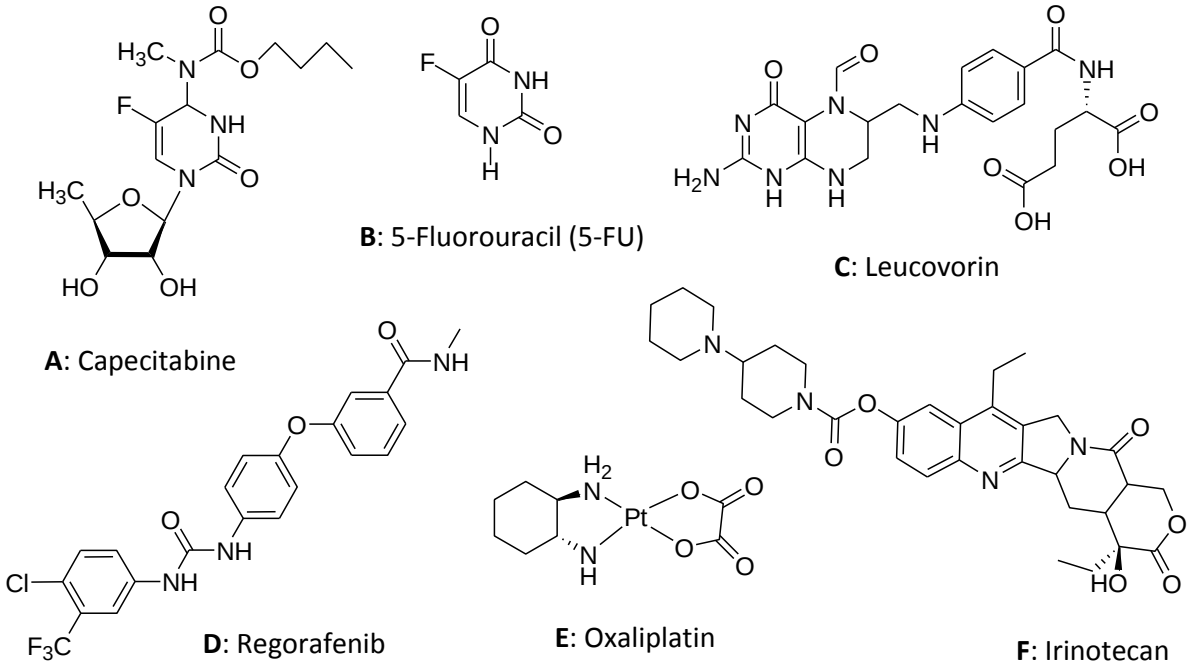


Figure 9 – Chemical structures of FDA approved drugs for CRC chemotherapy. Image obtained with ChemDraw software.

Capacitabine (Figure 9A) is another FDA approved compound for treatment of colon cancer in stage III patients who already had surgery. It is a prodrug, what means that serves as a precursor for the desired drug, being active by the normal metabolism of the organism. This fluoropyrimide carbamate is activated by thymidine phosphorylase of tumor cells, which

transform it into active 5FU. Consequently, the same mechanism of action as 5FU is to be expected for this small molecule, leading to inhibition of DNA and protein synthesis and cell division[34].

Patients with advanced or recurrent colon cancer at stage III can also be administered oxaliplatin (Figure 9E). This FDA approved drug is a organoplatinum complex that results in the inhibition of DNA replication and transcription with non-specific cytotoxicity. This is the consequence of the formation of inter and intra- strand cross links in DNA, which prevent DNA replication and transcription, what ultimately results in cell death[35]. The inexistence of a specific target results in low efficacy of treatment and strong side effects.

Another example is irinotecan (Figure 9F) used for patients that present metastases or which cancer has recurred. Irinotecan is also a prodrug, being converted into its biologically active metabolite SN-38. This antineoplastic small molecule inhibits the activity of topoisomerase I which leads to DNA breaks. The end result is the inhibition of DNA replication and induction of apoptosis[36].

Regorafenib (Figure 9D), developed by Bayer, is used for metastasized colon cancer in patients that already received other treatment without success. It targets VEGFR2 and PDGFR, preventing the formation of new vessels[37].

Leucovorin (Figure 9C) is used as a palliative treatment for chemotherapy. It is administered in addition with other antineoplastic drugs like 5FU. Leucovorin contradicts the toxic effects of these drugs, lowering their side effects. In addition allows their antineoplastic activity[38].

When CRC carcinomas gain the ability to spread and invade other organs, a different course of treatment has to be administered. So, in order to prevent the formation of metastasis, humanized monoclonal antibodies are used, namely Bevacizumab, Cetuximab and Panitumumab. The first antibody is directed against VEGFs (antiangiogenic) and the last two against EGF receptors(antineoplastic)[37]. A more recent drug approved by FDA against angiogenesis is Aflibercept. This recombinant protein is a VEGF inhibitor used as a second-line theraphy[37].

3.2. Drugs under development

Being cancer a complex disease that results from several mutations and cellular desregulations, the discovery of new and more effective compounds is a arduous task. Several processes can interfere with the cell's regulation and no compound is effective in all patients. So new drugs are synthesized everyday with different cellular targets in mind, a classic approach in drug development.

Several oncogenes commonly mutated in colon cancer like BRAF, KRAS, PI3K and SMAD, for example, are associated with lack of response to anti-EGFR therapies like Cetuximab and Panitumumab[37]. As a consequence, downstream components of these signaling pathways have become targets for drug development and small molecule inhibitors are already under clinical trials (Figure 10). Relatively to the MAPK/ERK pathway, two compounds, Selumetinib from Array BioPharma, and PD-0325901, from Pfizer, are under phase II and I, respectively, of clinical trial. These small molecules target the downstream proteins MEK. The PI3K/Akt pathway was also a target for drug development. BEZ235 and BKM120 molecules from Novartis and PX-866 from Oncothyreon are under phase I (BEZ235) and II of clinical trials and target the PI3K protein. Nevertheless, these drugs can become metastatic inducers depending on the proapoptotic tumor suppressor status of the tumor[37]. LOR-253, a compound from Lorus Therapeutics under clinical trial phase I, targets a different pathway, the WNT/β-catenin pathway. Disruption of this pathway causes a consistent growth inhibition and apoptosis on colon cancer. One way to interfere with this pathway is to alter the levels of Kruppel-like factor 4, which inhibits the expression of β-catenin. This is achieved by the inhibition of MTF1 that increases the levels of Kruppel-like factor 4[37].

Drug discovery for the prevention of angiogenesis is also an intended goal. Some new compounds, Tivantinib from ArQule, Brivanib from BMS and Vatalanib from Bayer/Novartis, are being tested (Figure 10). These target proteins related with the formation of new vessels, namely MET (Tivantinib), VEGFR2 (Brivanib) and VEGFR1-3 (Vatalanib). With the exception of Tivantinib, which is under phase II, the other two drugs are currently under phase III of clinical trials. A recently phase III trial was initiated with TAS-102 (Figure 10). This drug is a combination agent constituted with a pyrimidine analog and a thymidine phosphorylase inhibitor (TPI). TAS-102 inhibits the thymidine phosphorylase, a potent angiogenic factor.

Epigenetic mutations are also frequently associated with cancer. For that reason, the development of epigenetic drugs is a aim of drug discovery. The inhibitor of histone deacetylase (HDAC), Reminostat from 4SC, is currently under clinical trial phase II[37].

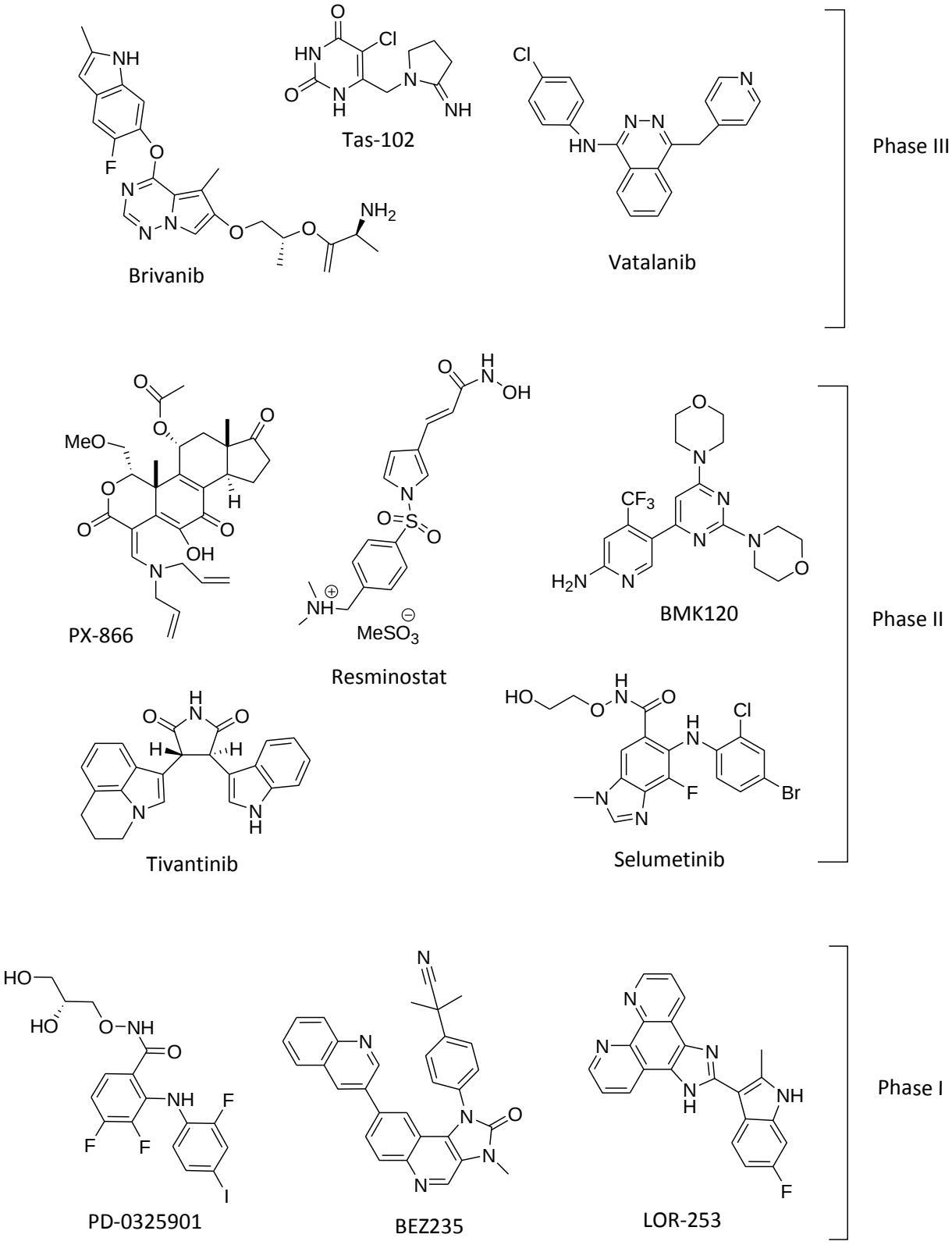


Figure 10 – Chemical structures of compounds under clinical trials for CRC chemotherapy. Image obtained with ChemDraw software.

Main Aims

Colon cancer is a major cause of cancer related death in developed countries. Despite the variety of drugs available, treatment efficacy is still reduced for some patients, particularly those presenting p53 deficiencies, which drives drug discovery research.

The main goal of this project is to identify a new compound with anticancer activity in colon cancer. Previously studied compounds, belonging to a family of nitrogen heterocycles, showed activity in HCT116 (p53-wt) colon cancer cells. It is now intended to enhance their anticancer potential by the study of the activity of a new set of compounds derived from them, using HCT116 (p53-wt) cells. IC_{50} values will be determined for the compounds that present higher activity. Concomitantly, a structure activity relationship (SAR) study will be made in order to unveil the effect of the substituents on the activity of the compounds.

Another important aim of this project is the assessment of the compounds' activity in the absence of functional p53 and KRas, normally associated with tumor resistance. To do so, compounds will be tested with HCT116 (p53-null) cells, with a complete knockout of p53, and CO115 cells, with an activating mutation on BRaf and KRas wildtype.

The mechanism of action of the most promising compounds is also a point of interest and it will be investigated in order to understand their interaction with the cell. This will be achieved by the assessment of the compounds effect on the induction of apoptosis and cell cycle progression.

Main Aims

Materials and Methods

In order to analyze the potential anti-carcinogenic effect of this family of compounds on colon cancer cells, several incubations of 48h were made with the selected compounds. After the incubations, different tests were performed depending on the desired parameters to analyze, described below.

1. Test compounds and reagents

All the compounds used in this project were kindly provided by the Chemistry Department of this university and synthesized by the group of Professor Maria Alice Carvalho. Stock solutions were made by dissolution of the compounds into dimetilsulfoxide (DMSO) and stored in aliquots at -20^{O}C. The concentrations of the stock solutions ranged from 300µM to 10 mM depending on the solubility of each compound. Compounds unable to dissolve at concentrations below 300µM were rejected from testing. Intermediate stock solutions were prepared taken into account the desired concentrations to be tested. Prior to incubation, stock solutions were diluted with culture medium so that the desired test concentration was reached, in such a way that the DMSO concentration did not surpass 0.5% (v/v), which otherwise would damage the cells.

Primary antibodies for the western blot assay were purchases from: anti-phospho-Akt (1:2000) and anti-phospho-ERK (1:2000) from Cell Signaling (Danvers, MA, USA);, anti-KRas (1:250), anti-PARP1 (1:500) and anti-phospho-JNK (1:500) from Santa Cruz Biotechnology, Inc.; and anti-β-actin from Sigma-Aldrich. Secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were purchase from Cell Signaling.

2. Solubility assay

The compounds' solubility was analyzed starting with the highest concentration intended to be tested. For each compound it was prepared a solution with 1mL of PBS (phosphate buffered saline) and 5µL of the highest stock concentration (5mM), which was kept at 37^{o}C for 24h. Posteriorly to this incubation, samples were observed to see if any crystals have been formed. If the solutions proved to be clear, they were considered suitable

for biological tests. On the other hand, if it was visible the presence of crystals in suspension, another incubation was performed, in the same conditions, at a lower concentration. This procedure was repeated at consequently lower concentrations until no crystals were observed. Compounds that were not soluble at the range of concentrations chosen to be tested were rejected from biological testing.

3. Cell lines and Culture

In this project, three human colon carcinoma derived cell lines with different genetic backgrounds (Table 1) were used as models to examine the effect of the compounds. The main cell line used in this project was HCT116, mutated in the codon 13 of the RAS proto-oncogene and wild type for p53 (p53-wt). In order to examine the role of p53, an isogenic HCT116 cell line was also used with a complete knockout for p53 (p53-null). These two cell lines were kindly provided by Dr. Vogelstein[39]. The other colon cancer cell line used, CO115, has an activating mutation on BRAF whereas Ras is wild type (Ras-wt) and was kindly provided by Dr. Raquel Seruca (IPATIMUP, University of Porto, Portugal).

Table 1 – Genetic profile of CRC cell lines used in this project.

	Altered gene expression due to mutations			
CRC Cell Lines	EGFR	KRas	BRaf	p53
HCT116 (p53-wt)	✓	✓	-	-
HCT116 (p53-null)	✓	✓	-	X
CO115	-	-	✓	-

Maintenance of the cells in culture was achieved in 25 cm^2 polystyrene flasks with RPMI-1640 medium (Sigma-Aldrich) supplemented with 6% of bovine fetal serum (FBS), 1% antibiotic-antimycotic solution, 10mM Hepes, 0.1 mM sodium pyruvate and 2 g/L sodium bicarbonate. Medium and antibiotic solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells were kept in a Sanyo COM-17 AIC incubator at 37^oC in a humidified atmosphere with 5% CO_2. Handling of the cells was done in a sterile environment using laminar flow chambers: Holten Lamin Air HBB 2448 or Telstar Bio II A.

3.1. SubCulture

Once cells reached a confluent state, a small amount of them (0.5×10^6 cells for HCT116 p53-wt and p53-null and 1×10^6 for CO115) were transferred into a new T25 culture flask, so they could grow. To do so, cells were washed with PBS, in order to eliminate possible remains of serum molecules, followed by the addition of a trypsin-EDTA solution, responsible for breaking the connections between the cells and the bottom of the flask. This reagent was acquired from Sigma-Aldrich, St. Louis, MO, USA. After a 5 minutes incubation at 37^{o}C, fresh culture medium was added to the flask in order to inactivate the trypsin. This way, it was obtained a cell suspension to be used in experiments and/or to grow into new flasks after cell count.

4. Cell count

Cell count is fundamental for plating and other procedures where it is required to know the specific concentration of cells in suspension. To count the number of cells, Neubauer Chambers were used with a solution of trypan blue 0.4% (p/v) diluted 1:1 with the cell suspension. Neubauer chambers are thicker crystal slides with 30x70mm of size and 4mm of thickness covered with a glass coverslip[40]. In this project it was used double chambers, which means that the chamber has two counting areas and, consequently, two counting grids. Each grid has 3x3mm in size, with 9 square subdivisions of 1mm. The distance between the bottom of the chamber and the cover is of 0.1mm[40]. It is this area that determines the volume of sample that can be analyzed ($1x10^{-4}$ mL/mm^3). After counting the number of cells in the four major squares, the mean of the values was calculated and multiplied by the dilution factor, in case trypan blue was used. The concentration was calculated by dividing this value by the volume of sample analyzed ($1x10^{-4}$ mL). Cell counting was done using an inverted optical microscope Olympus CK2.

5. Cell Plating

In order to correctly study the effect of the different compounds, it is necessary that the number of cells in each condition is equal. This is achieved by plating of a fixed concentration of cells. To do so, it is necessary to know the concentration of the initial cell suspension and, from this, a new suspension is made with the desired cell concentration (0.6×10^5 cells/mL for HCT116 p53-wt and p53-null and $1x10^5$ cell/mL for CO115). For the cellular viability assays, cells were plated into 24-multiwell culture plates (0.5 mL/well), for the nuclear condensation assays platting was done on 12-multiwell culture plates (1mL/well), and for flow cytometry and Western blot assays, cells were platted into 6-multiwell culture plates (2mL/well). Plates were kept at 37^{o}C and 5% CO_2 for 48h before incubation with the test compounds, to allow cell adhesion and growth. To eliminate any possible interference of DMSO, each individual plate had at least one control with DMSO at a concentration not higher than 0.5% (v/v). Incubation of cells with compounds was also done at 37^{o}C in a CO_2 incubator.

6. Microscopic observations

Images of the cells after incubation with the compounds were obtained through an inverted optical microscope Olympus IX71 with DP72 camera in phase contrast mode. For the nuclear condensation assays, the same microscope was used with the DAPI fluorescence filter, which enabled the identification of DNA and observation of the cells' apoptotic state caused by the compounds.

7. MTT assay

To analyze cell growth, cell viability was assessed through the colorimetric assay of MTT reduction, which is a fast, objective and reliable method, as previously described[41]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), an yellow tetrazolium salt, was purchased from Sigma-Aldrich. Aliquots of 5 mg/mL were made with PBS, in the

absence of light, and stored in aliquots at -20^{o}C. The MTT reduction assay is based on the fact that viable cells are able to reduce MTT into formazan crystals, unlike dead cells. This reaction is carried out by active reductase enzymes, namely mitochondrial enzymes succinate-dehydrogenase, non-functional in dead cells once they do not have functional mitochondria, which makes them unable to reduce MTT[42]. The formazan crystals have a characteristic purple color that, after dissolution with DMSO/Ethanol, can be measured by spectrophotometry. Taking this into account, a directly proportional relationship can be established between the number of viable cells and the color of the solutions, where wells with more viable cells result in solutions with a more prominent color.

After 48h of incubation with the test compounds, 50 µL of MTT (5mg/mL) was added to each well of 24 wells plates, which were then incubated for another 30 minutes at 37^{o}C, 5% CO_2. Posteriorly to this incubation with MTT, the culture medium was removed from the wells and the crystals were dissolved with DMSO/ethanol (500µL) for following analysis. These colorful solutions were pippeted onto 96 wells plates (200µL per well) and their absorbance was read in the spectrophotometer SpectraMax Plus^{384} Microplate Reader – Molecular Devices at 570 nm (with background subtraction at 690nm)[42]. As blank, DMSO/ethanol solutions were used. Results were expressed in percentage relative to control. In order to select the best compounds of the family under study, all of them were incubated at the same concentration (10 µM), with a few exceptions at lower concentrations, due to solubility issues. The results were expressed as mean from at least 3 independent experiments. Compounds that presented the lowest cellular viability were selected to continue in the study and, posteriorly, their IC_{50} was calculated using this same method. To do so, each compound was incubated at various concentrations and data from several experiments was arranged and expressed relatively to control. After data analysis using the GraphPad Prism 4.0 software it was possible to see the behavior of the compounds and to determine the IC_{50} value of each one. Compounds with the lowest values of IC_{50} were chosen, once again, to continue with the following assays.

8. Nuclear Condensation assay

To examine the possible apoptotic effects of the selected compounds on the cell lines under study, the nuclear condensation assay was performed. The objective of this assay is to identify apoptotic cells through Hoechst DNA staining. Apoptotic cells suffer a series of morphological changes that allow their identification like their decrease in size and aggregation of their components, condensation of the nucleus and the formation of the apoptotic bodies[7]. With this test is easy to find out the number of cells that suffer apoptosis due to the compounds' effects and their respective percentage in each sample.

Cells were incubated with the compounds for 48h in 12 wells plates, time after which both floating and attached cells were collect to falcons and washed with PBS. To do so, falcons were centrifuged two times in a Sigma 2-16K centrifuge at 500g for 10min and pellet was resuspended in 500µL of PBS. Then, cells were fixed with 4% paraformaldehyde (PFA) (2mL/sample) and kept in ice for 20min. Finally, they were washed once again with PBS, centrifuged as above and all the pellet was resuspended in 500µL of PBS. Samples were then ready to be attached to a polylysine coated slide using a cytospin. Once the apparatus of the Shandon Cytospin 4 (Thermo Scientific, Waltham, MA, USA) was assembled, samples were loaded onto the hoppers and centrifuged at 500rpm for 5min. The slides were washed with PBS two times for 5min. In the last step of this assay, slides were put in a dark chamber and incubated for 10min with 20µL of Hoechst stain. This stain was purchased from Sigma-Aldrich and kept at 4^{o}C in aliquots of 0.5 mg/mL. This BisBenzimide, used as a fluorescence stain for DNA, was diluted in PBS (1:100) prior to testing, so that the concentration in the samples was 5 µg/mL. After the incubation time, it was added 6µL of glycerol 50% (v/v) in order to fix the lamella to the slide. Finally, with the help of an inverted microscope with proper fluorescence filter, it was possible to identify apoptotic cells and calculate their percentage from the ratio between them and the total number of cells. For each condition, a minimum of 400 cells was counted. Apoptotic cells were identified by the presence of fragmented and/or shrunken nuclei (Figure 11). Data was organized taken into account all the conditions under study and their respective values of percentage of apoptosis.

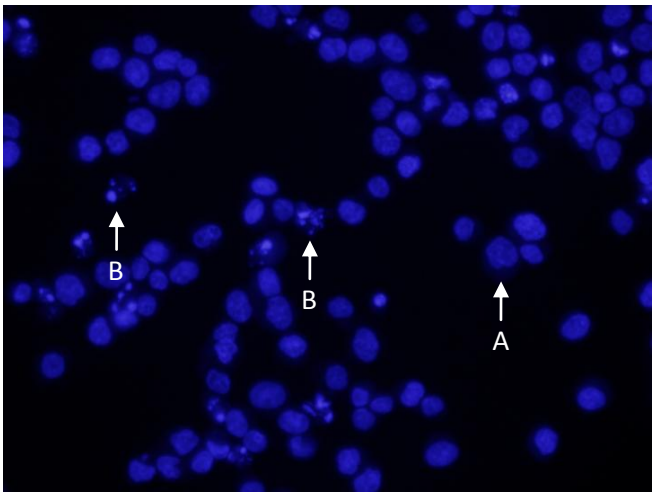


Figure 11 – Representative image of a nuclear condensation experiment. In the image can be identified normal cells (A) as well as apoptotic cells (B). Image obtained with an inverted optical microscope Olympus IX71 and DAPI fluorescence filter.

9. Cell Cycle Analysis by Flow Cytometry

The flow cytometer was used with the aim of study the effect of the test compound on the progression of the cell cycle. Several types of analysis can be performed with this technique, including detection of total DNA content for cell cycle analysis. With this test it was possible to analyze the cell cycle progression after treatment of the cells with the compounds, what gave us a more clear idea about their mechanisms of action[43]. Flow cytometry is a technology based on the ability of a cytometer to detect and count cells individually, with the aid of a laser, classifying them. Cells and other particles have to be suspended in a fluid as they pass the electronic detection mechanism. As they are conducted through this liquid stream, cells reach the interrogation point, also denominated analysis or observation point, where they are intersected by a laser. Today's lasers can be either gas (argon ions; helium-neon laser) or solid-state lasers (red or green diode or blue and violet lasers), being the most common the argon ion lasers[43]. In this point, the beam of light is scattered by the particle. Fluorescent chemicals present in the particles are also excited and emit light in a longer wavelength than the light source. Fluorochromes can also be used to identify apoptotic cells. The scattered and fluorescent lights are then detected by a set of detectors that gather the information about the particles and transmit them to a computer[43]. Typically, there is one detector in front, the forward scatter (FSC) and several perpendicular scatters, the side scatters (SSC) as well as fluorescent scatters. The FSC

analyses the volume of the cell and the side scatters give information about the interior of the cells, like the shape of the nucleus for example. The group of detectors is accompanied by a ADC system that converts the signals from light to electrical signals so they can be processed by a computer. Finally, signals are analyzed by a computer where they can be interpreted[43]. Data is plotted taking into account the number of cells and the fluorescence intensity, which is proportional to the DNA content[7].

To perform this assay, cells were incubated with the compounds for 48h. After this time, cells from each well were trypsinized and collected into falcons, in which they were submitted to a centrifugation at 500g for 3min in a Sigma 2-16K centrifuge. Pellet was resuspended in 500 µL of PBS and kept in ice for 15min. Posteriorly, another centrifugation was performed in the same conditions, in order to wash the cells with PBS. The resuspended pellet from this centrifugation was then pipetted into ice-cold 96 ethanol and maintained in ice for at least 15 minutes with the goal of fix and permeabilize cells. This step inhibits cellular activity and allows the dye to enter the cell while it remains intact[43]. Samples are then vortexed and centrifuged two more times at 700g for 3 minutes. After resuspension of the pellet, 50 µL of RNAse A is added to all samples, which are then incubated for 15 minutes at 37^{o}C, in order to destroy all RNA from the cells. This step certifies that the fluorescent dye only binds to DNA and results are more reliable. The last step is the addition of 60 µL of propidium iodide (PI) from Sigma-Aldrich per sample, a DNA-binding fluorochrome that intercalates the DNA double helix. After this procedure, samples were ready to be analyzed in the Coulter Epics XL Flow Cytometer (Beckman Coulter Inc., Miami, FL, USA), where at least 40000 single cells were counted per sample. Data was then fitted using the FlowJo Analysis Software (Tree Star Inc., Ashland, OR, USA), through the mathematical Watson Pragmatic model[44].

10. Western blot assay

The compounds' effect at a molecular level was studied resorting to the western blot method. In this technique, a sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was first used in order to separate the different cellular proteins. After incubation with the compounds, proteins are extracted from cells and protein concentration

quantified. Samples are then prepared in a way that the same quantity of proteins is loaded in all conditions, making possible the comparison between samples. Protein preparation involves the addition of a denaturant agent that linearizes proteins and grant them a negative charge proportional to their mass[45]. As said previously, the denaturant agent used was sodium dodecyl sulfate (SDS), an anionic detergent that denaturants non-disulfide-linked tertiary and secondary structures. In order to further denature proteins, heating of the samples in the presence of dithiothreitol (DTT) is a usual step. Heating of proteins promote their denaturation, as well as DTT, which is able to reduce disulfide links, not denature by SDS, and break quaternary structures[45]. Only after this treatment proteins can be separated accordingly to their mass and length. After loading the samples onto the electrophoresis gel, an electric field is applied, forcing ions to move from the negatively charged electrode - the cathode - to the positive one - the anode. This results in the migration of proteins (with negative charge) towards the anode[45]. Polyacrylamide gels consist, as the name implies, in acrylamide and bisacrylamide. Polymerization is possible due to bisacrylamide that can make cross-links between two polyacrylamide molecules. Ammonium persulfate (APS) and tetramethylethylenediamine (TEMED), a source of free radicals and a stabilizer respectively, are responsible for the beginning of the polymerization reaction, being for this reason the last components added[45]. Ethanol is poured over the resolving gel with the goal of removing possible bubbles and smooth the surface. After sample loading and start of the run, protein migration results from the action of chloride and glycinate ions present in the gel. Chloride ions run faster than the proteins linked with SDS and glycinate ions run in front of the running buffer, creating two barriers that restrain the proteins and force them to move through the staking gel. In the transition between the stacking and resolving gel the pore size decreases, leading to the separation of the proteins based in their molecular weight (MW) only[45]. Throughout this process, denatured proteins are separated accordingly to their mass, taken that small molecules will fit through the pores of the gel more easily than large ones. The second step of western blot is the transfer of the proteins from the electrophoresis gel into a membrane (blotting), where they become immobilized[45]. In order to detect the proteins of interest, specific antibodies for each one are used. Unoccupied binding sites of the proteins are occupied to prevent non-specific binding of the antibodies. After a first incubation with these primary antibodies, a second incubation is made with the secondary antibodies, which specifically bind the primary type

and possess a detectable group. This group is responsible for the visualization of the bands after incubation with the revelation solutions and detection[45]. The last step is then the detection of the protein bands. The choice of detection method depend on the imaging equipment available, being the chemiluminescence detection system the elected in this project. With these results, the effect of different drugs on different proteins' expression can be assessed.

This assay was performed after incubations of 24/48h with the compounds. Cells were then washed with PBS and lysed for 15min at 4^{o}C with ice cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris-HCl (pH 8), 2 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Roche, Mannheim, Germany), phosphatase inhibitors (20 mM NaF and 20 mM $Na_2V_3O_4$) and protease inhibitor cocktail (Roche). After the successful extraction of the proteins, their concentration by quantified through the Bio-Rad DC protein assay (Bio-Rad Laboratories Inc.). Bovine serum albumin (BSA) was used as the standard protein. From each sample, 20µg of protein was prepared and loaded onto the polyacrylamide gel. After electrophoresis, proteins were electroblotted to Hybond-P polyvinylidene difluoride membranes (GE Healthcare), which were then blocked in TPBS (PBS with 0,05% Tween-20) with 5% (w/v) of non-fat dry milk. When all non-specific binding sites were occupied, membranes were incubated with the primary antibodies overnight and then with the secondary antibodies for 1h. Immunoreactive bands were identified using the Immobilon solutions (Milipore, Billerica, MA, USA) and the chemiluminescence detection system, ChemiDoc XRS (Bio-Rad Laboratories, Inc.). Intensity of the bands was quantified with the aid of the Quantity One software (Bio-Rad Laboratories) and β-actin was used Gas a loading control.

11. Statistical analysis

All experiments were performed independently and at least three times, after which data was treated as means ± SEM. Results were evaluated statistically using the one-way ANOVA followed by a Dunnet's post-test. Statically differences were considered significant when p-values < 0.05 (* p≤0.05; ** p≤0.01;***p≤0.001). For the IC_{50} calculus of the various compounds, a nonlinear regression was used. With this analysis it was established a

relationship between the concentrations of the inhibitors (in logarithm) and their response in cellular viability. Outliers were excluded from the analysis in order to increase the accuracy of the analysis. All the statistical analyses were performed through to the GraphPad Prism 4.0 software (San Diego, CA, USA).

Results and Discussion

With the aim of assessing the anticarcinogenic potential of a variety of nitrogen heterocycles, several analyses were performed. The experimental work started with the compounds' screening, through their analysis of cellular viability by the MTT assay, using the HCT116 (p53-wt) cell line. The most active compounds, identified in the screening, were selected in order to study their mechanisms of action. To do so, the effect of the compounds on cellular proliferation/induction of death and on the progression of the cell cycle was analyzed. Expression of related molecular markers was assessed by western blot.

1. Compounds' biological activity

1.1. Screening in HCT116 (p53-wt) cells.

In order to ascertain the effect of the compounds on cellular viability by the MTT assay, these were incubated at 10µM for 48h with HCT116 (p53-wt) cells. This cell line was chosen to perform this assay, as it was the cell line used in the previous investigation[46]. Cellular viability was assessed through the performance of the MTT assay. All the tested compounds have the same central structure, the heterocyclic nucleus (Fig. 12a). Around the central structure different substituents R and R^1 were incorporated. In order to assess the importance of the R^1 group's position in the heterocyclic structure, the substituent R^1 was introduced in a different position of the heterocyclic nucleus (Fig. 12b).

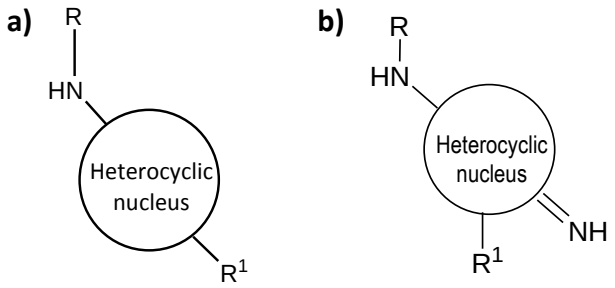
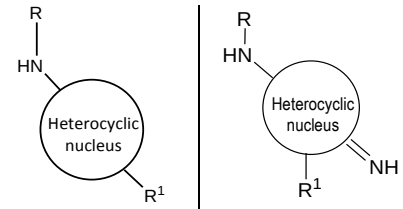


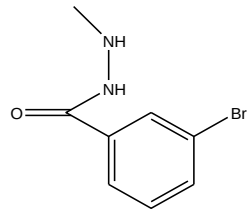
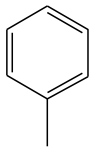
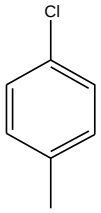
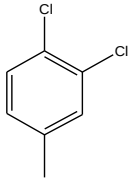
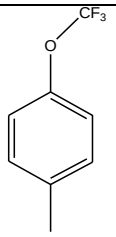
Figure 12 – Central structure of the compounds. All the compounds have the same central structure (**a**) with the exception of compound 36 (**b**).

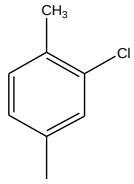
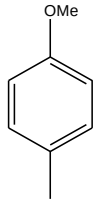
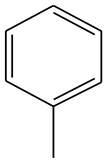
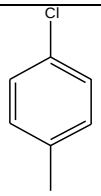
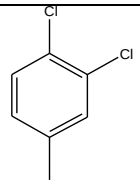
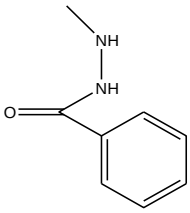
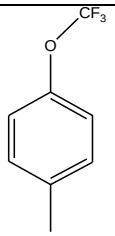
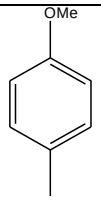
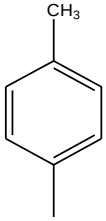
All the compounds were tested at the same concentration (10µM), so a comparison between their structure and activity could be established. Compounds 10; 22; 23 and 14; 17 showed low solubility and were impossible to test at 10µM, being tested at 5µM or 1,5µM. At the same concentration, variations on cell viability between the compounds reflect the influence of the substituents. Therefore, high values of cell viability correspond to a weak effect and vice versa. Compounds that present more activity (values of cellular viability lower than about 40%) had their IC_{50} determined. All the results are summarized in the Table 2.

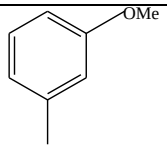
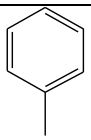
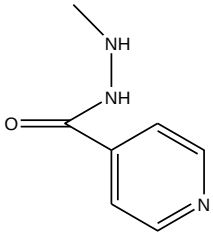
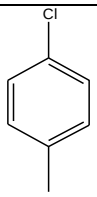
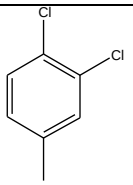
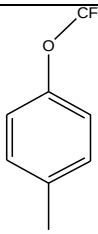
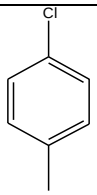
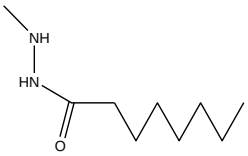
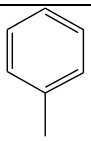
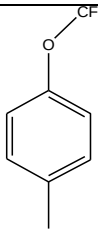
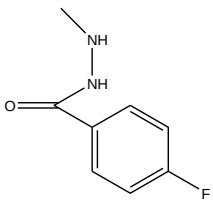
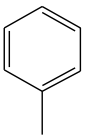


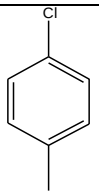
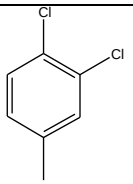
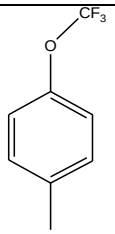
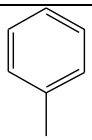
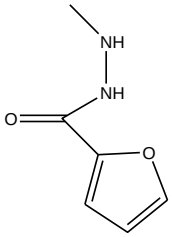
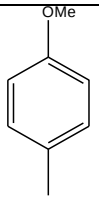
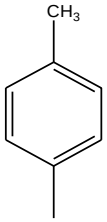
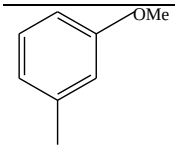
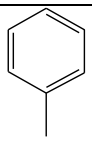
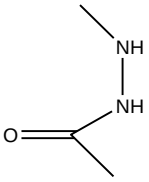
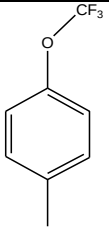
Compound 1-35 | Compound 36

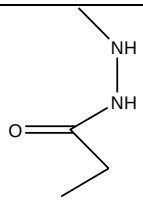
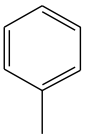
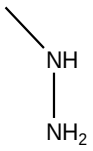
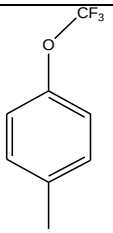
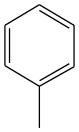
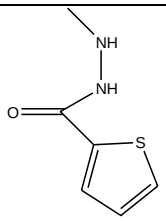
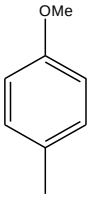
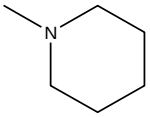
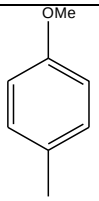
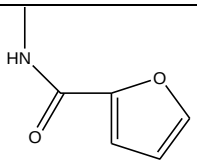
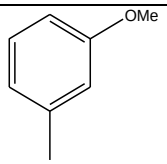
Table 2 – Cellular viability of HCT116 (p53-wt) cells in the presence of the test compounds at 10µM and IC_{50} of the more active compounds. Values result from the mean ± SEM of at least three independent experiments.

Compound	R^1	R	Cellular viability (% at 10 µM)	% Inhibition	IC_{50} (µM)
1			23 ± 4.3	77 ± 4.3	1.26
2			59 ± 4.7	41 ± 4.7	-
3			71 ± 3.5	29 ± 3.5	-
4			43 ± 3.7	57 ± 3.7	2.85

5		56 ± 2.8	44 ± 2.8	-
6		70 ± 0.2	30	-
7		23 ± 3.9	77 ± 3.9	2.99
8		31 ± 3.9	69 ± 3.9	4.03
9		30 ± 4.9	70 ± 4.9	3.22
10 *			40 ± 5.3	60 ± 5.3	0.89
11			90 ± 7.7	10 ± 7.7	-
12			39 ± 1.9	61 ± 1.9	-

13			39 ± 6.2	61 ± 6.2	-
14**			96 ± 3.5	4 ± 3.5	-
15***				-	
16***					
17**			78 ± 5.4	22 ± 5.4	-
18			76 ± 1.4	24 ± 1.4	-
19			64 ± 2.9	36 ± 2.9	-
20			78 ± 6.2	22 ± 6.2	-
21			38 ± 4	62 ± 4	2.72

22 *			93 ± 5.9	7 ± 5.9	-
23 *			62 ± 1.8	38 ± 1.8	-
24			19 ± 3.8	81 ± 3.8	0.72
25			23 ± 1.6	77 ± 1.6	4.58
26			17 ± 3.6	83 ± 3.6	2.82
27			18 ± 2.3	82 ± 2.3	1.23
28			18 ± 2	82 ± 2	2.29
29			95 ± 0.5	5 ± 0.5	-
30			35 ± 4.4	65 ± 4.4	5.61

31			65 ± 4.7	35 ± 4.7	-
32			44 ± 0.4	56 ± 0.4	-
33			35 ± 1.9	65 ± 1.9	7.94
34			38 ± 1.1	63 ± 1.1	-
35			93 ± 3.5	7 ± 3.5	-
36			31 ± 1.4	69 ± 1.4	4.57

In the table above are listed all the compounds tested and their effect on cellular viability at 10 μM. In a few cases, biological activity was measured at *5μM and **1.5μM due to low solubility. IC_{50} was calculated for the compounds that presented stronger inhibition (normally above 65%). *** These compounds were excluded due to their impossibility to dissolve, either in DMSO or water.

The results presented in Table 1 show that the biological activity of this new family of compounds depends on both substituents R and R^1 present in the heterocyclic nucleus. Compounds having R=Ph and different R^1 groups, **1**, **7, 14, 19, 21, 25, 29, 31** and **33**, show 23 ≤ % cellular viability ≤ 96. Compounds having R^1=NHNHCO(3´-BrC_6H_4) and different R groups, **1, 2, 3, 4, 5** and **6**, show 23 ≤ % cellular viability ≤ 71.

Compounds **24**, **25, 26, 27** and **28** having R^1=NHNHCOfuryl and different aryl groups as R registered the highest activity (17 ≤ % cellular viability ≤ 23). For this set of compounds,

the R group does not seem to influence the activity. IC_{50} was then determined for these potent compounds and compound **24** emerged as the most active with an IC_{50} = 0.72 µM. High activity (23 ≤ % cellular viability ≤ 40) was also registered for compounds **7, 8, 9** and **10** having the substituent R^1=NHNHCOPh and different R groups. From these, the most active compounds, **7** and **10**, were identified based on their IC_{50}. Compound **7** showed IC_{50} = 2.99 µM and compound **10** IC_{50} = 0.89 µM. However, compound **11,** that also has R^1=NHNHCOPh but R=4-$MeOC_6H_4$, presents a very low activity (90 % of cellular viability). This result shows that the group R has a high influence on the activity.

The activity registered for compounds **1, 2, 3, 4, 5** and **6** having R^1=NHNHCO(3´-BrC_6H_4) and for compounds **21, 22** and **23** having R^1=NHNHCO(4´-FC_6H_4) varies a lot with the substituent R. While compound **1** shows a cellular viability of only 23%, compound **22** shows a cellular viability of 93%.

When compounds have the substituent R^1=NHNHCOMe, NHNHCOEt or $NHNHCO(CH_2)_6CH_3$ they show low activity (compounds **18, 19, 20, 29** and **31** have 64 ≤ % cellular viability ≤ 95) with the exception of compound **30** (cellular viability = 35%), which substituent R is the bulky group 4-$CF_3OC_6H_4$. This set of results seems to suggest that a bulky alkyl group as substituent R^1 is not favourable for activity, however, the presence of a bulky group in the substituent R is favourable. The activity registered for compounds **32** and **33** (% cellular viability = 44 and 35 respectively) and for compound **35** (% cellular viability = 93) also support the previous assumption. The group R^1=$NHNH_2$ is a very small group and both the compounds show high activity, yet the activity is very low when R^1=piperidine, a very bulky group.

Compounds having R^1=NHNHCO(4´-pyridine) showed low solubility either in DMSO or in water. It was only possible to prepare solutions of the compounds **14** and **17** at 300µM, being these compounds screened at the maximum concentration possible of 1.5µM (200-fold lower than the stock). Compounds **15** and **16** were impossible to dissolve. Biological results showed that when R^1=NHNHCO(4´-pyridine) compounds are less active.

The activity displayed by compound **28** (% cellular viability = 18) and by compound **36** (% cellular viability = 31), whose structures differ only in the relative position of the R^1 group, suggest that the position of R^1 group in the heterocyclic nucleus also affects the activity of the compounds.

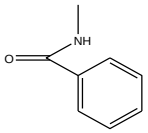
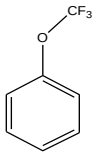
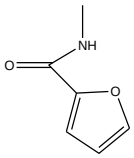
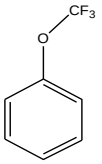
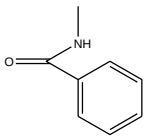
From the SAR analysis of this family of compounds two hits emerged, compounds **10** and **24** with IC_{50s} of 0.89μM and 0.72μM, respectively. Both the compounds have R=4-$CF_3OC_6H_4$ but different R^1 substituents (R^1=NHNHCOPh in compound **10** and NHNHCOfuryl in **24**).

1.2. Activity of selected compounds in HCT116 (p53-null) and CO115 cells.

The two most potent compounds, i.e. with lower IC_{50s}, tested on the HCT116 (p53-wt) cell line, compounds **10** and **24,** were selected to proceed in this study (0.89μM and 0.72μM respectively). Compound **7** was also selected in order to compare the activity of the 2 selected compounds with a compound that has simply phenyl groups as substituents R and R^1, being for that reason denominated "naked" compound. Fluorouracil (5FU) is one of the most frequently used drugs on chemotherapy for the treatment of colon cancer[31], reason why it was selected as a reference compound. The effect of p53 and KRas, frequently mutated genes in colon tumors[26], on the activity of the compounds was assessed with the assistance of two additional cell lines, HCT116 (p53-null) with a complete knockout of p53 and CO115 with an activating mutation on BRAF and KRas wildtype.

The results summarized in Table 3 show that the selected compounds are extremely active against all human colon cancer cells, in comparison with the reference drug 5FU. In the HCT116 (p53-wt) cell line, compounds **10** and **24** show a IC_{50} value (IC_{50}= 0.89 and 0.72μM) more than 15-fold smaller that the value of 5FU (IC_{50}= 15μM). The dependency of the compounds' effect on p53 was studied with HCT116 (p53-null) cells. The results clearly demonstrate that the effect of the compounds is independent of p53. This cell line, with a complete knockout of p53, is even more sensitive to the compounds than HCT116 (p53-wt). On the contrary, this cell line is resistant to 5FU, the reference compound. Since the mechanism of action of 5FU is dependent on p53[33], it is expected that its effect on a cell line without p53 would be smaller, with a much higher IC_{50} (IC_{50}=252.2μM). The "naked" compound presents a higher value of IC_{50} (IC_{50}=2.24μM) than compounds **10** and **24**, being still extremely potent.

Table 3 – IC_{50} of selected compounds and 5-FU in HCT116 (p53-wt), HCT116 (p53-null) and CO115 cell lines.

Compound	R_1	R	IC_{50} (μM)		
			HCT116 (p53-wt)	HCT116 (p53-null)	CO115
10*			0.89	0.59	-
24			0.72	0.55	1.55
7			2.99	2.24	3.6
5FU			15	252.2	29.5

*Compound 10 was tested at 5μM, maximum concentration able to test with it. IC_{50} of this compound was impossible to calculate in CO115 cell line since 50% of cellular viability couldn't be reached at this concentration.

With the CO115 cell line, the effect of the presence of normal RAS and a activating mutation on BRAF was analyzed. In this cell line, the IC_{50} of compound **10** was impossible to calculate, since 50% of cellular viability could not be reached at the maximum concentration. This result is particularly relevant since it allow us to imply that compound **10** may target mutant KRAS, only upstream component of BRAF in the MAPK/ERK pathway. This compound is extremely potent in both HCT116 cell lines, that harbour a mutation on KRAS. However, compound's **10** effect on CO115 cell line, that has a activating mutation in BRAF instead of KRAS, decreases greatly, being even impossible to calculate its IC_{50}. Recalling the MAPK/ERK pathway (Figure 8), HCT116 cells mutation is situated in the beginning of the pathway, so the targeting of any of its components results in a decrease of proliferation. On the other hand, CO115 activating mutation on BRAF is more downstream on the pathway and the targeting

of any component upstream will not provoke any effect. CO115 showed to be slightly more resistant to the compounds effect than the previous two cell line. The IC_{50} of compounds **24** and **7** increased to 1.55 and 3.6µM respectively, whereas 5FU shows an IC_{50} = 29.5µM, which is about 30 fold less active than the selected compounds.

In conclusion, the selected compounds **10**, **24** and **7** are extremely potent in the three cell lines: HT116 (p53-wt), HCT116 (p53-null) and CO115, with the exception of compound **10** in CO115. In addition, these compounds showed extremely high activity in HCT116 (p53-null) cells, unlike 5FU (Fig.13), demonstrating that they are also active in tumours with this genetic profile, which can be an advantage in patient treatment, since patients with mutant p53 tumours are more resistant to the available treatment.

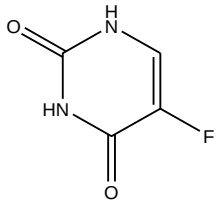
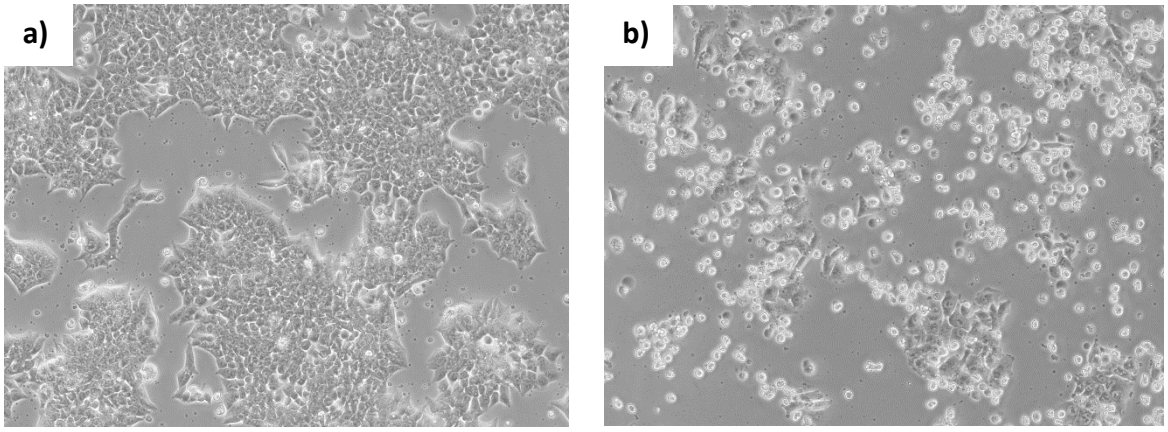


Figure 13 – Chemical structure of Fluorouracil (5-FU).

1.3. Cellular Morphology

The morphology of HCT116 (p53-wt) cells was analyzed after incubation with the selected compounds for 48 hours (Fig.14).



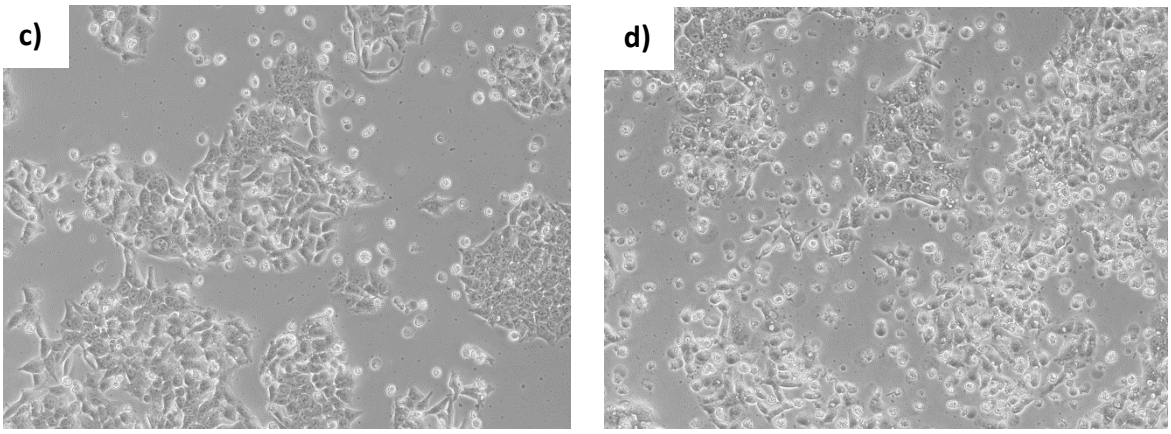


Figure 14 – Cellular Morphology of HCT116 (p53-wt) cell line. Images above were obtained with an Olympus IX71 inverted microscope and 10x1.6x objective lens in phase contrast mode after incubation with 5µM of compound **7** (b), 5µM of compound **10** (c) and 10µM of compound **24** (d). Image a) is the control.

Cells were incubated with 5µM of compound **7** (Fig.14b), 5µM of compound **10** (Fig.14c) and 10µM of compound **24** (Fig.14d). Relatively to control (Fig.14a) without the addition of any compound, it is visible an increase of dead cells (round and floating cells) with the selected compounds, particularly with the "naked" compound, i.e. compound **7**. Incubation with compound **24** exhibits a higher amount of dead cells what may be due to the fact that it was tested at a higher concentration. In fact, through the analysis of the incubation with 5µM of compound **24** (result not presented) fewer less dead cells were visible than with compound **7**, making compound **7** the better compound in death induction in this cell line.

A significant decrease in cell proliferation was produced by all compounds since the amount of cells attached to the bottom of the well decreases in comparison with control. Viable cells are attached to the bottom of the well and when they lose this attachment and, consequently, their shape, cells are no longer able to grow and proliferate.

2. Compounds' effect on apoptosis

With the goal of examining the effect of the best compounds on the induction of apoptosis, the nuclear condensation assay was performed. Compounds **10**, **24** and **7** were incubated with the three cell lines for 48h and at various concentrations, as well as 5FU.

Statistical analysis was impossible to execute on CO115 cell line once data is the result of only two independent experiments. Results are presented in Figure 15.

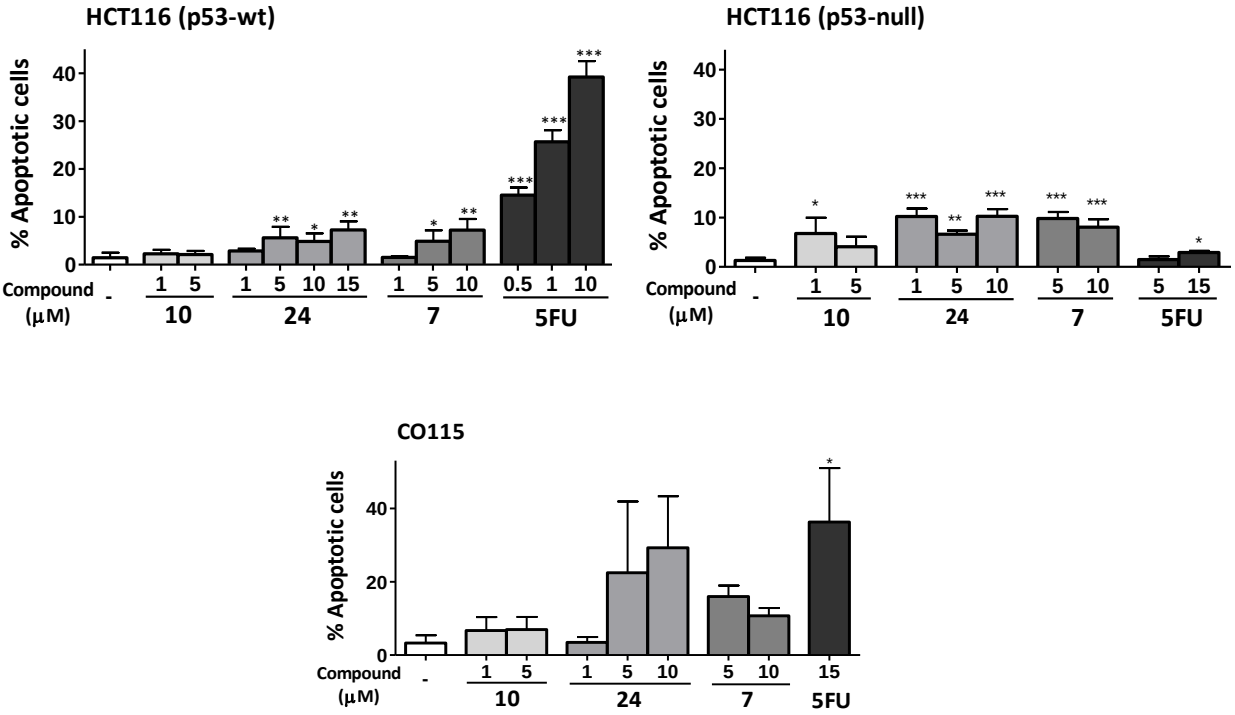


Figure 15 – Effect of the selected compounds and 5-FU on apoptosis, assessed by the Nuclear Condensation assay. Apoptosis was evaluated after treatment with the compounds for 48h in the three cell lines. Values are mean ± SEM of at least 3 independent experiments, with the exception of CO115 cell line which only has 2. * P ≤0.05, ** P ≤ 0.01 and *** P ≤ 0.001 in comparison with the control.

Observing the effect of the selected compounds on HCT116 (p53-wt) cell line, small increases in apoptosis are visible, in comparison with 5FU. The induction of apoptosis by compound **10** in this cell line is minimal and not significant, which indicates that the high activity of this compound is not due to the activation of this cell death mechanism. For compound **7**, a relationship between its concentration and the apoptosis' values can be established. At 1μM, neither compound **7** or **24** exhibit significant values of apoptosis. Increasing their concentration results in a higher induction of apoptosis, which values become significant. Examining the effect of 5FU, a directly proportional relationship between its concentration and the values of apoptosis is clearly visible. Even at concentrations much smaller than its IC_{50}, 5FU shows a very high increase of apoptosis. This corroborates the research made about this compound, which revealed that 5FU mechanism of action leads to the activation of cells' apoptotic pathways[33].

The effect of the compounds was then analyzed in the absence of p53 gene. To do so, compounds were incubated with HCT116 (p53-null) cell line that has a complete knockout of this gene. The most visible difference between effects with this cell line and the previous is for 5FU. An abrupt decrease on the effect of this compound was observed with HCT116 (p53-null) cells, making p53 of extreme importance for 5FU mechanism of action[47]. This is also in agreement with previous studies about this compound, where low apoptosis was associated with the absence of p53, relatively to HCT116 (p53-wt)[33;48;47] and patients' cells without p53 show resistance to 5FU[49]. Focusing on the selected compounds, no significant differences occur in comparison with the effect in the previous cell line. This leads to the conclusion that the effect of the compounds **10**, **24** and **7** does not depend on p53. In addition, no relationship between concentration and effect is established with these compounds. Furthermore, apoptosis is higher on the selected compounds than on 5FU, making them more active on this cell line.

The last cell line to be analyzed was CO115. This cell line was used in order to ascertain the effect of the compounds on apoptosis in the presence of mutated KRas gene versus BRaf. In this cell line, 5FU regains it activity due to the presence of p53, provoking the higher value of apoptosis from all the tested compounds. Relatively to the test compounds, there is a slight increase in apoptosis in comparison to the previous cell lines, making CO115 more susceptible to the apoptotic effect of the compounds. From these, compound **24** shows the higher increase in apoptosis' values, which are proportional to concentration.

Through the analysis of the data gathered until this point, it is perceptible that apoptosis is not the cause of the extremely high activity of compounds **10**, **24** and **7**, once its low levels of apoptosis can not justify their activity. Although compounds are much more active than 5FU, the apoptosis' induction rate of this approved drug is much higher, suggesting that these compounds act through different mechanisms of action. Another difference between 5FU and compounds **10**, **24** and **7** is the dependence on p53 gene. While the effect of 5FU is completely dependent on the presence of this gene, the apoptosis levels of the selected compounds almost did not vary in the absence of p53. The results obtained with 5FU are consistent with literature[50]. Finally, in CO115 cells with constitutively active RAF apoptosis increases slightly for all the compounds.

3. Effect of the compounds on cell cycle

The necessity to study the cell cycle came from the need to understand the compounds' mechanisms of action after discovering their poor induction of apoptosis. Once again, all the compounds, including 5FU, were incubated for 48h with the three cell lines and effects analyzed by flow cytometry. Results represent at least 3 independent experiments in HCT116 (p53-wt). On the other two cell lines results were obtained with less than three independent experiments. Below are exposed representative images of the progression of the cell cycle in the three cell lines and in the presence of all the compounds.

Control

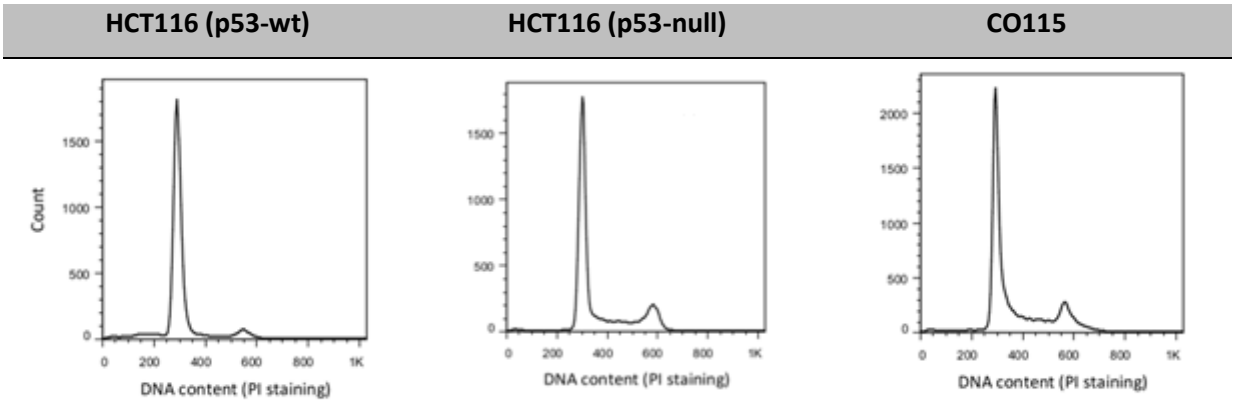


Figure 16 – Normal progression of cell cycle in the 3 cell lines. In the figure above is represented the distribution of single cells through the different phases of cell cycle, namely, G1, S and G2/M. Images are representatives from at least 3 experiments in HCT116 (p53-wt) and 2 experiments for the other two cell lines.

The three images above represent the normal distribution of cells through the different phases of the cell cycle. The three cell lines were analyzed in the same conditions. Data is shown as number of cells (count) vs fluorescence intensity, which is proportional to DNA content. The first and second peaks represent the G1 and G2+M phases of the cell cycle, where cells have a DNA content of 2n and $4n^7$, respectively. The majority of the detected cells are in G1 phase, being followed from afar by cells on G2/M phase. The sub-G1 phase is found before the G1 peak and, as expected in this state of equilibrium, the sub-G1 value is close to the baseline. Logically, the S phase is located between the G1 and G2

phases, being its value concordant to the expected, on the three cell lines. DNA synthesis on the S phase is a rapid process, resulting in the small number of cells found in this phase[7].

Compound 10

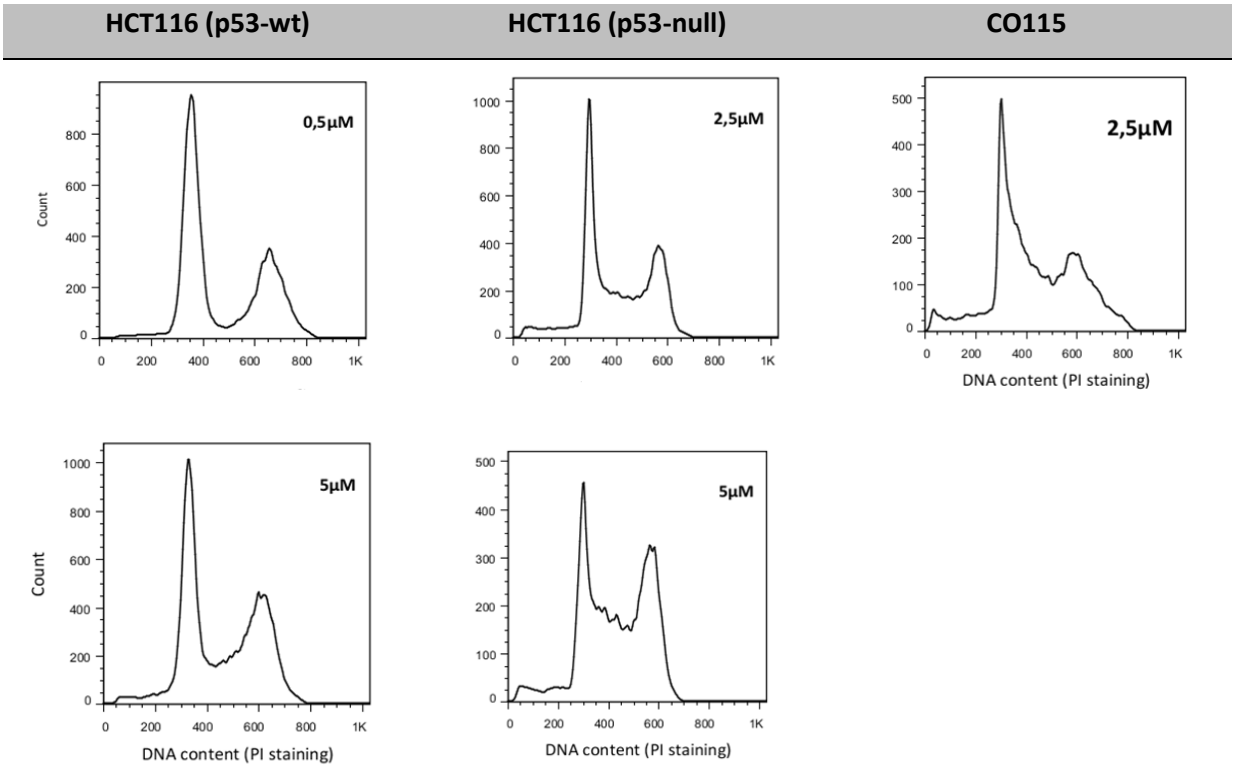


Figure 17 – Effects of compound 10 in cell cycle progression of the 3 cell lines. Assessment by flow cytometry was done after 48h of treatment. In the figure above is represented the distribution of single cells through the phases of cell cycle, namely, G1, S and G2/M. Images are representatives from at least 3 experiments in HCT116 (p53-wt) and less than 3 experiments for the other two cell lines.

The effect of the compound 10 on the progression of the cell cycle is represented above. Starting with the analysis of the main cell line of this project – HCT116 (p53-wt) – it is visible a very significant accumulation of cells on the G2/M phase, whose increase is proportional to the compound's concentration. Comparing the results of this cell line with the ones of HCT116 (p53-null), a even more prominent S and G2 arrest is visible. This is in agreement with the activity results, where compound **10** showed to be slightly more active in this cell line without p53. Finally, observing the results of CO115, it is clear that there is the highest S arrest and smallest G2 arrest in comparison with the results of the previous cell lines. This accumulation of cells in the S phase is near the G1/S transition in comparison with the other cell lines, where the S arrest is closer to the G2/M peak. The accumulation of cells in the sub-G1 phase is also slightly bigger in this cell line.

Compound 24

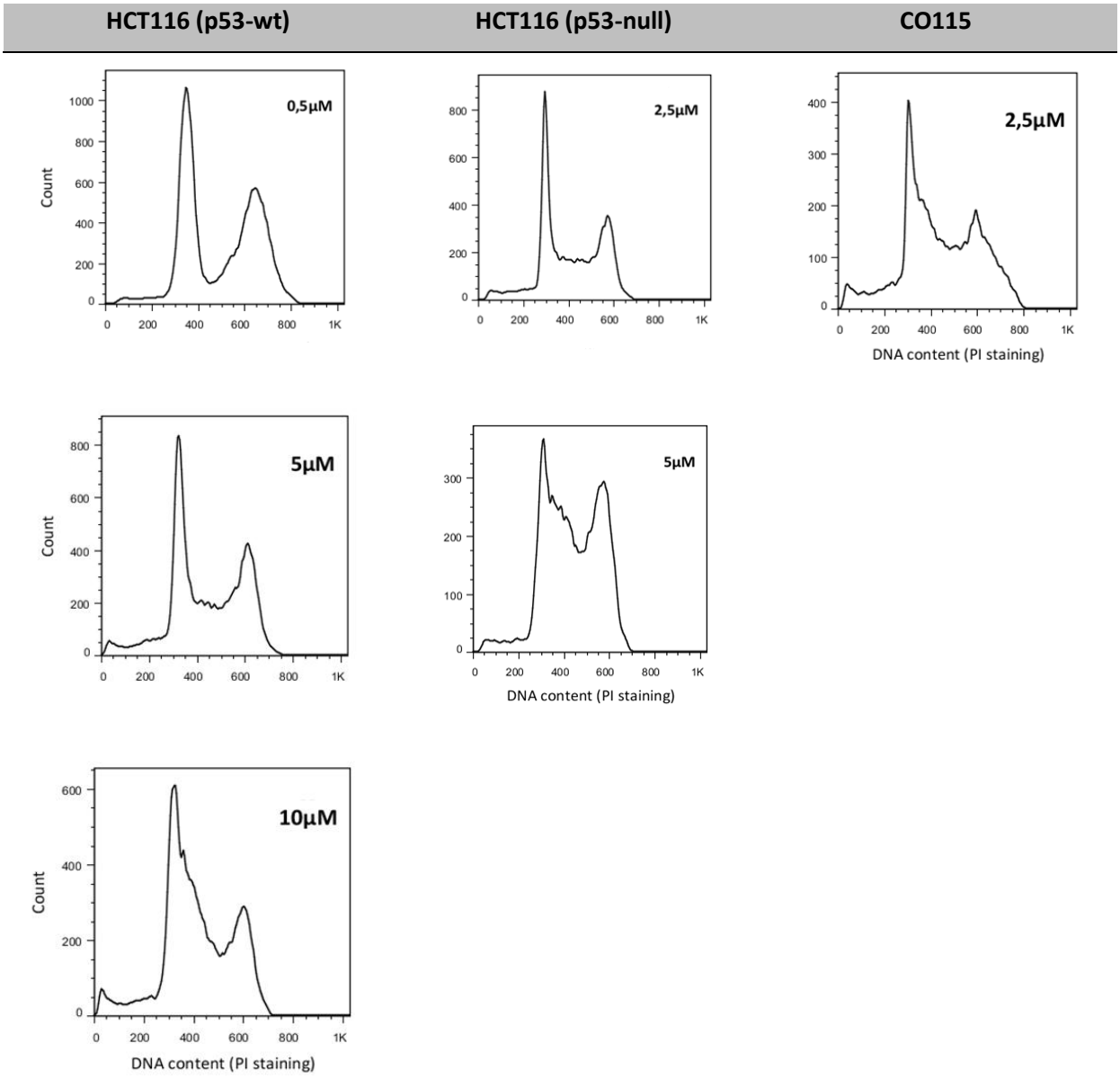


Figure 18 – Effects of compound 24 in cell cycle progression of the 3 cell lines. Assessment by flow cytometry was done after 48h of treatment. In the figure above is represented the distribution of single cells through the phases of cell cycle, namely, G1, S and G2/M. Images are representatives from at least 3 experiments in HCT116 (p53-wt) and less than 3 experiments for the other two cell lines.

The images above represent the effect of compound 24 over the progression of the cell cycle. Starting again with the HCT116 (p53-wt) cell line, an extremely high G2 arrest was observed even in the lowest concentration, 0.5µM. With the increase of concentration, the G2 peak remains about half of the G1 peak, but a substantial accumulation of cells on the S phase is evident. The sub-G1 phase increase is also directly proportional to the compound's

concentration. Analyzing now the HCT116 cell line with p53 knockout, a high S and G2/M arrest was observed. The increase of concentration provokes an abrupt augmentation on the levels of the S and G2/M phases. In comparison with the previous cell line at 5µM, these arrests are much higher, which is concordant with the IC_{50} results. Compound 24 showed more activity in HCT116 (p53-null) cells than on (p53-wt). Focusing now on CO115, more than the G2 arrest, a high S arrest is visible. In addition, there is also an accumulation of cells on the sub-G1 phase.

Compound 7

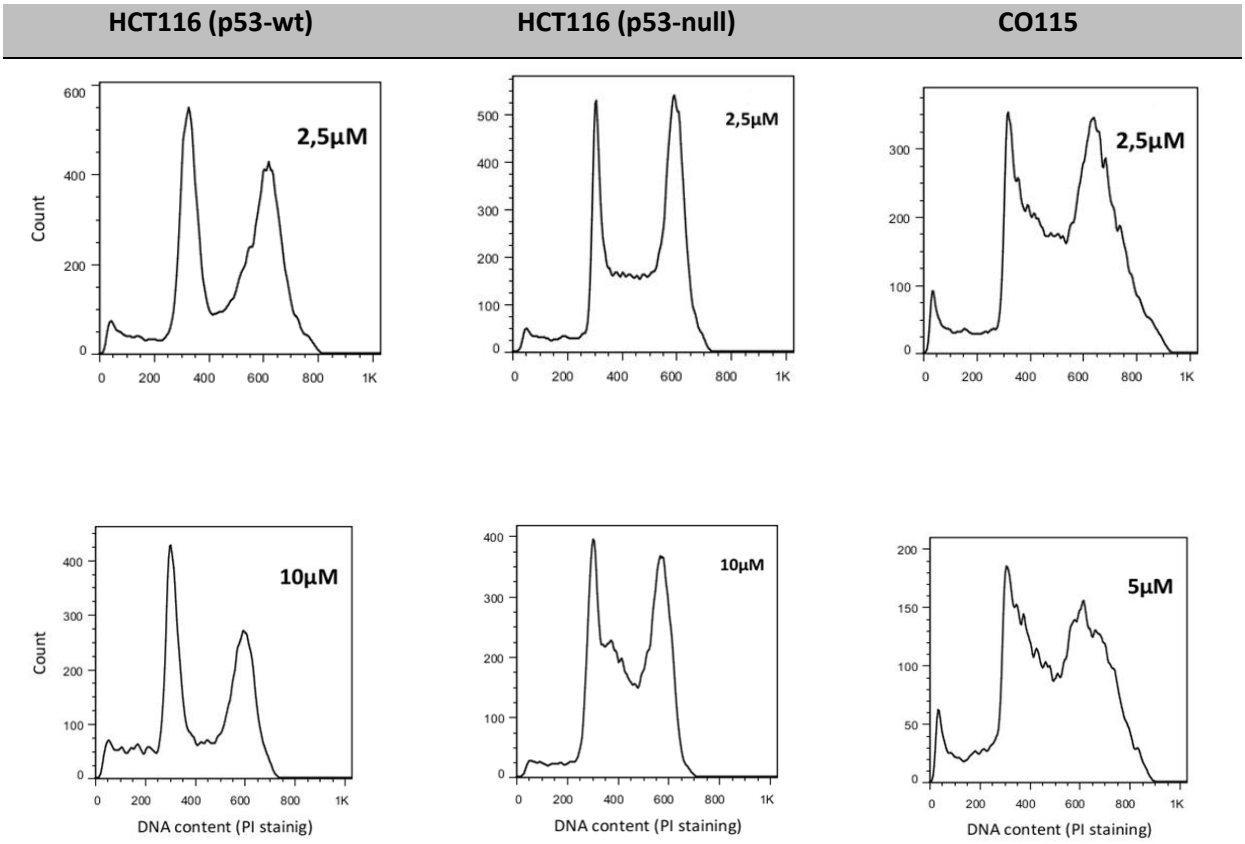


Figure 19 – Effects of compound 7 in cell cycle progression of the 3 cell lines. Assessment by flow cytometry was done after 48h of treatment. In the figure above is represented the distribution of single cells through the phases of cell cycle, namely, G1, S and G2/M. Images are representatives from at least 3 experiments in HCT116 (p53-wt) and less than 3 experiments for the other two cell lines.

Relatively to HCT116 (p53-wt) cells, a high accumulation of cells is observed in the G2/M phase with the presence of the compound **7**. Contrary to the previous compound, there is not a noticeable increase in the S phase but a slight increase of cells in the sub-G1 phase is visible. In both HCT116 (p53-null) and CO115 cell lines at 2.5µM, the G2 phase

suffered an extremely high arrest, being the G2+M peak at the same level of the G1. The increase of concentration in both cell lines, lead to a slight decrease of the G1 and G2+M peaks and an increase of S phase cells. In all the examples the accumulation of cells in the sub-G1 phase is bigger than with the previous compounds. This corroborates the previous morphological observations, where more cell death was detected with this compound.

5FU

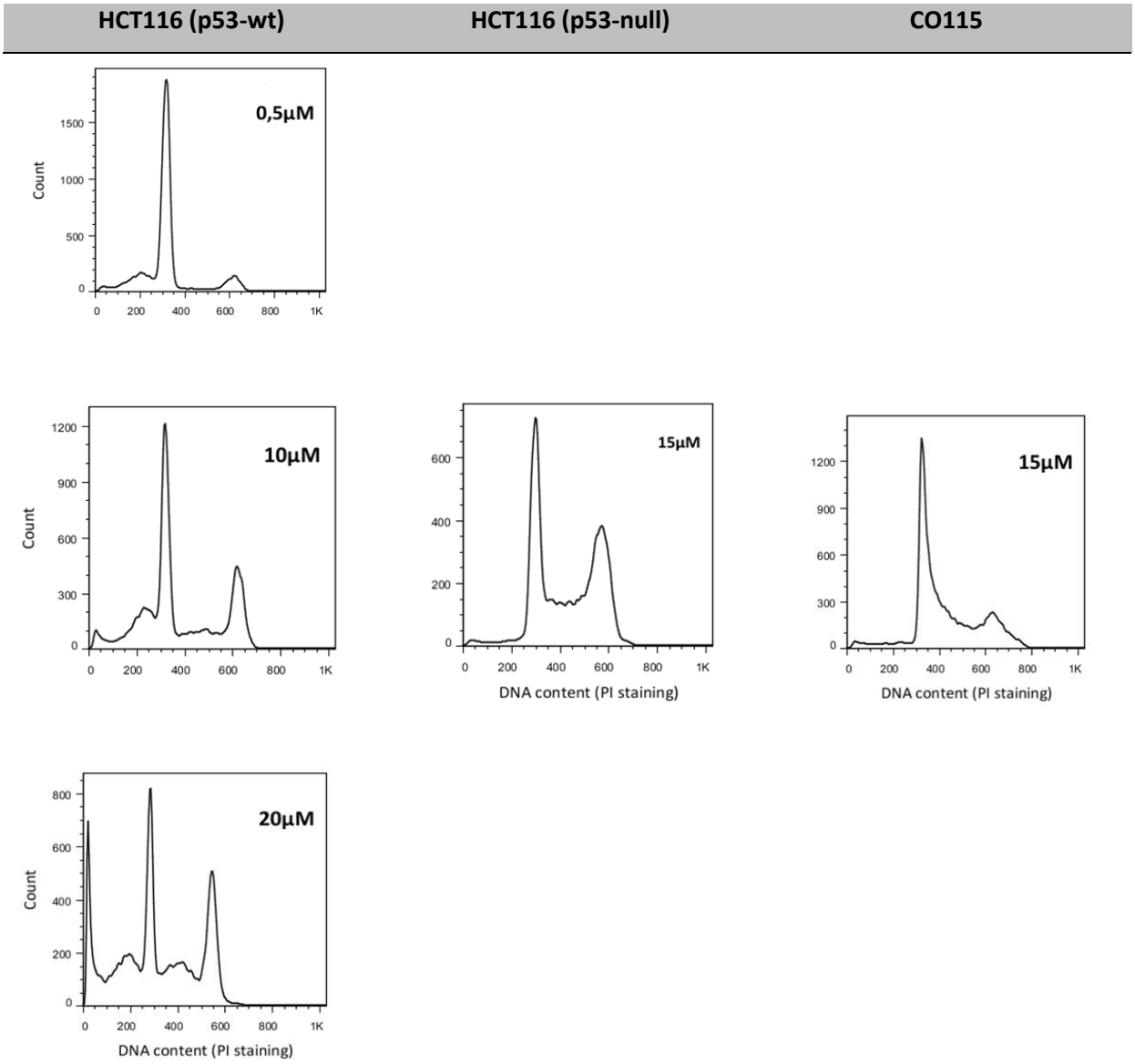


Figure 20 – Effects of 5-FU in cell cycle progression of the 3 cell lines. Assessment by flow cytometry was done after 48h of treatment. In the figure above is represented the distribution of single cells through the phases of cell cycle, namely, G1, S and G2/M. Images are representatives from at least 3 experiments in HCT116 (p53-wt) and less than 3 experiments for the other two cell lines.

Progression of cell cycle was also studied with 5FU in the three cell lines. Starting the analysis with HCT116 (p53-wt) cell line as usual, almost no disturbance was observed at 0.5µM, the lowest concentration, in comparison with control. Only the sub-G1 phase increased slightly. Taking into account that the IC_{50} of 5FU is 15µM in this cell line, this dosage is extremely low to provoke any significant effect. The increase of concentration is accompanied by a consequent increase in cells on the G2 and sub-G1 phases.

Cell cycle progression in HCT116 (p53-null) cells at 15µM was altered in comparison with HCT116 (p53-wt), in consequence of the p53 gene knockout. A high G2 arrest was also observed with these cells, accompanied by a higher accumulation of cells in the S phase but, contrary to the previous cell line, the cell cycle of HCT116 (p53-null) cells lacks the increase of sub-G1 phase observed with HCT116 (p53-wt), as reported in a previous work[33]. Once the sub-G1 fraction is an indication of DNA fragmentation[7], typical of apoptosis, nuclear condensation results are concordant with this observation. The nuclear condensation assay clearly revealed the dependence of 5FU's induction of apoptosis on p53 wt presence.

The incubation of CO115 cells with 5FU at 15µM was ineffective in the disturbance of the cell cycle. Both sub-G1 and G2 phases, typically arrested with this reference drug, did not present any significant accumulation of cells. Only S phase suffered a slight arrest in comparison with control and prior cell lines.

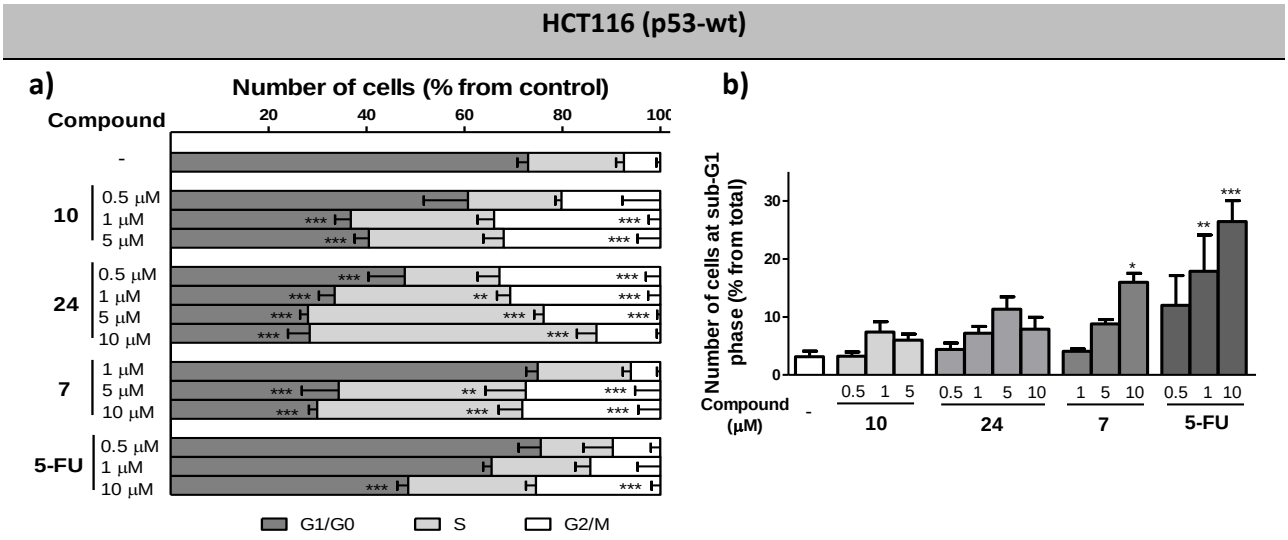


Figure 21 – Effect of the compounds in cell cycle (a) and in the percentage of cells at sub-G1 fraction (b) in HCT116 (p53-wt). Values are mean ± SEM of 3 independent experiments. * P ≤0.05, ** P ≤ 0.01 and *** P ≤ =0.001 in comparison with the control.

Data from at least three independent experiments of all the compounds tested in HCT116 (p53-wt) cells was treated statistically and presented in Figure 21a. Relatively to the cell cycle analysis on the left, all the compounds presented a lower G1 peak and higher S and G2/M phase arrest relatively to control, with the exception of compound **7** at 1µM. At its lowest concentration, 1µM, compound **7** does not provoke any significant variation in the progression of the cell cycle. The increase of compound **7** concentration causes the accumulation of cells in the S and G2/M phase and, consequently, the decline of cells in G1 phase. Compound **10** shows a significant G2 arrest on concentrations superior to 0.5µM. This concentration independent variation of the G2+M peak is accompanied by the decrease of the G1 peak. S phase remained constant at different concentrations with this compound. A more pronounced S phase arrest was seen with compound **24**. At its lowest concentration, 0.5 µM, compound **24** presents a very high G2 arrest, which diminishes with the increase of concentration. Concomitantly, cells rapidly accumulate in the S phase, resulting in the extremely high arrest observed at 10µM. Knowing this, it can be assumed that this compound interferes with the synthesis of DNA in the S phase, maybe by introducing errors in the DNA chain, recognized by the cell and that ultimately lead to the arrest of the cell cycle. In general, these three compounds cause the arrest of the S and G2/M phases in HCT116 (p53-wt) cells. The effect of the reference compound 5FU in the progression of the cell cycle was also analyzed. As represented above, 5FU also caused the arrest of the cell cycle in the G2/M phase. Only at 10µM this augmentation of cells in G2/M phase is significant. This result is concordant with a previous study where 5FU was related with G2/M arrest in HCT116 (p53-wt) cells[33].

The effect of the compounds on the sub-G1 phase of HCT116 (p53-wt) cells was also analyzed (Fig.21b). The sub-G1 fraction of the cell cycle, whose DNA content is lower than 2n, is representative of DNA fragmentation, typical of apoptosis. Through the observation of Fig.21b it is clear that the selected compounds do not cause any significant effect on the apoptotic levels of these cells, with the exception of compound **7** at 10µM. These results corroborate the nuclear condensation analysis, where low values of apoptosis were detected, with the same incubation parameters. From the three, only compound **7** reveals a concentration dependent effect, as observed with the nuclear condensation assay. As expected due to the previous analysis and reported works[33], 5FU caused a high

accumulation of cells in the sub-G1 phase, in concentrations much lower than its IC_{50}. This concentration dependent effect is also concordant with the nuclear condensation results.

Insufficient data did not allow the statistical analysis of cell cycle progression in the other cell lines.

Analyses made so far lead us to the conclusion that our test compounds and 5FU function through different mechanisms of action. While apoptosis cannot be taken as the main target of our selected compounds, 5FU clearly functions through its induction. On the other hand, it was proved that the selected compounds' mechanism of action interferes directly with the progression of the cell cycle, causing a higher arrest of the S and G2/M phases than 5FU.

4. Effects on molecular targets of proliferation and death

After the discovery of the compounds' main effect, the arrest of the cell cycle, levels of several molecular targets were analyzed by western blot with the goal of enlightening the compounds' mechanism of action in HCT116 (p53-wt) cells. This was achieved by the identification of the compounds effect on the expression of various proteins with some initial experiments of Western blot. In Figure 22 are represented the effects of compounds 10 (Fig.22a) and 24 (Fig. 22b), the most potent, in the expression of various proteins related with cell cycle and death.

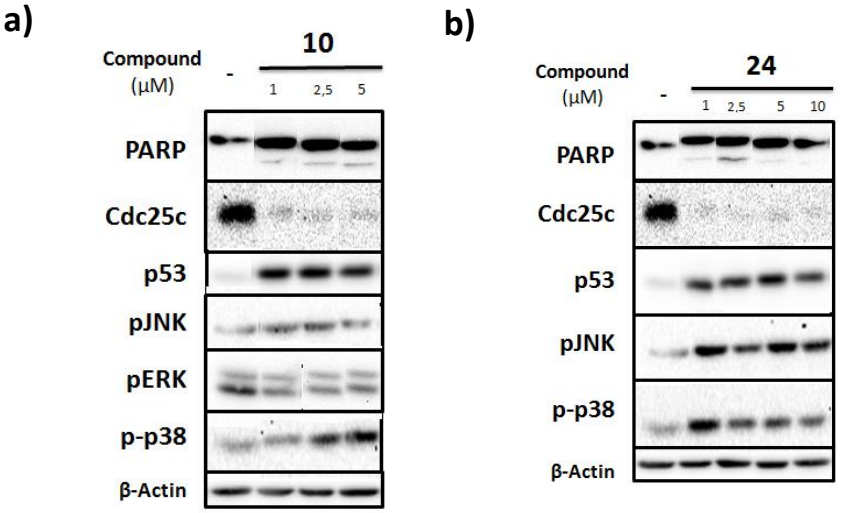


Figure 22 – Effect of compounds 10 (a) and 24 (b) on the expression of several markers involved in cell cycle and death in HCT116 (p53-wt) cells. Results were obtained by Western Blot. B-actin was used as a loading control. Images are representative of 3 independent experiments.

The phosphorylation of ERK, indicative of the activation of the MAPK/ERK pathway, was tested in order to assess the effect of the compounds in this pathway. The importance of MAPK/ERK comes from the fact that this pathway is activated in a high number of CRC cases, resulting in increased proliferation and inhibition of apoptosis[51]. As presented in Fig.22a, compound **10** decreases phosphorylation of this protein, what suggests a decrease in proliferation levels[30]. In addition to this signaling pathway involved in the control of proliferation and induction of apoptosis, two other stress-activated pathways with the same function of control were studied, namely the JNK and p38 pathways[52]. Both compounds **10** and **24** cause an increase in the levels of both pJNK and p-p38, particularly the last one. Compound **24** presents a higher expression of both proteins in comparison with compound **10**, what indicates a higher induction of apoptosis[30]. This inference is concordant to the previous nuclear condensation analysis, where compound **24** showed a higher induction of apoptosis than compound **10**. The expression of p-p38 in the presence of compound **10** also shows a concentration dependent effect. The expression of the apoptotic marker PARP-1 was also tested in order to assess the induction of apoptosis and its dependency on caspase activation. In the event of caspase mediated apoptosis, PARP-1 is cleaved by caspases and, consequently, inactivated which is associated with increased apoptosis [53]. Both compounds presented a slight increase of cleaved PARP-1 expression. The low levels of cleaved PARP shown, can be explained by the very small induction of apoptosis by the compounds, as

demonstrated with the previous results. Reminiscing of the cell cycle analysis, where a high G2/M arrest was identified, molecular markers related with the cell cycle progression were also tested, namely Cdc25c and p53. Cdc25c phosphatase activates the cdk1/cyclin B complex inducing, consequently, the transition from the G2 to the M phase[12] (Fig.4). In the presence of the 3 test compounds and 5FU (Figures 22 and 23), it is visible a high decrease in the expression of this phosphatase, what supports the cytometry results. Other result concordant to the G2/M arrest is the increased expression of the p53 protein in the presence of compounds **24** and **10**. p53 regulates the synthesis of many proteins involved in several stress activated responses like, for example, apoptosis and cell cycle arrest. Depending on cell type and stimulus, the p53 post-transcriptional network can be modified, leading to an increase of apoptosis levels and/or arrest of cell cycle[54]. Given the low values of apoptosis obtained with these compounds, it is expected that the high increase of p53 expression is related with the S and G2/M cell cycle arrest observed, possibly by induction of the synthesis of inhibitors of the cdk1/cyclin B complex, like p21, Gad45 and 14-3-3[2,54] (Fig.4).

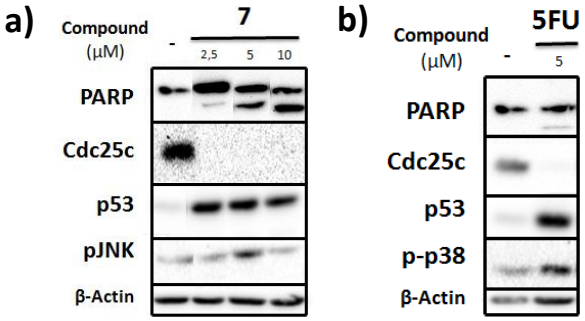


Figure 23 - Effect of compound 7 (a) and 5FU (b) on the expression of several markers involved in cell cycle and death in HCT116 (p53-wt) cells. Results were obtained by Western Blot. B-actin was used as a loading control. Images are representative of 3 independent experiments.

The effect of compound **7** (Fig.23a) and the reference compound 5FU (Fig.23b) in the expression of various proteins related with the cell cycle and death was also investigated. With compound **7** is visible a clear concentration dependent increase of the PARP cleavage, another apoptotic marker. The higher levels of cleaved PARP expression of compound **7** in comparison with **24** and **10** agree with to the higher induction of apoptosis by this compound. This induction of apoptosis by compound 7 does not seem to be JNK dependent. As with the preceding compounds, it is visible an abrupt decrease in Cdc25c expression and a

increase of p53, suggesting that this compound also acts on the cell cycle progression. 5FU presents a similar protein expression but, in light of the previous results, it needs to be interpreted in a different way. The reduction of cdc25c and increased p53 expression may be related with the cell cycle arrest in G2/M but, given the slightly lower arrest and much higher values of apoptosis that 5FU presents in comparison with the tested compounds, seems to indicate that p53 induction by 5FU leads to the induction of apoptosis. Previous studies have reported increased p53 expression after treatment with 5FU[55]. Concerning the expression of other apoptotic markers, a slight increase in the levels of p-p38 and cleaved PARP is visible. The small increase in the expression of these proteins can be a consequence of the small induction of apoptosis due to the low concentration at test (5μM) relatively to 5FU's IC_{50} in this cell line (15μM).

Other molecular markers, mostly related with the cell cycle progression, need to be analyzed, as well as a more extensive study of the expression of these proteins, in order to take more accurate conclusions.

5. Fluorescence

In the initial investigation, compound 2 has show fluorescence with a FITC filter[46], as demonstrated in Fig.24. This property was also investigated for the selected compounds but none of them showed fluorescence (data not presented).

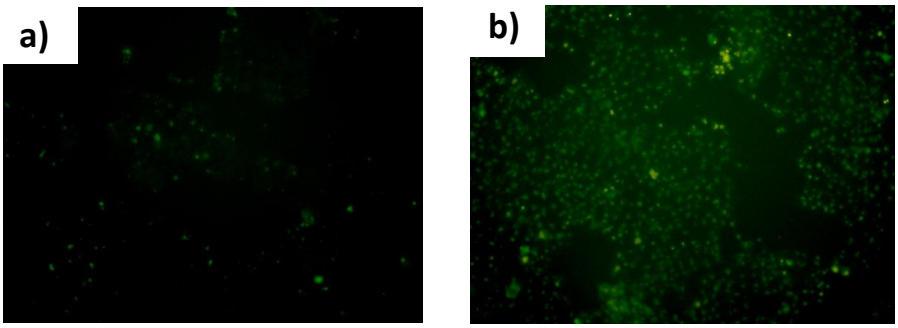


Figure 24 – Fluorescence assessed in HCT116 (p53-wt) cells in the absence (a) and presence of the previously studied compound 2 (b). Images above were obtained with an Olympus IX71 inverted microscope and 10x objective lens with FITC filter.

6. DNA damage and Senescence

The effect of the selected compounds on senescence and DNA damage was evaluated with a single preliminary experience in HCT116 (p53-wt) cells. Compounds were tested at different concentrations but results were inconclusive.

With this project a new and promising family of compounds was discovered. Starting with an initial structure, several substitutions were made in the R and R_1 groups. Both groups interfere with the activity of the compounds and some were identified as especially important for their activity, namely phenyl and furan for R_1 and phenyl and OCF_3 for R. From this group, 2 compounds (**10** and **24**) were identified as extremely potent and promising in comparison with the reference drug 5FU. These compounds were also tested in cell lines with a complete knockout of p53 (HCT116 (p53-null)) and with an activating mutation in the BRaf gene instead of KRas (CO115). Compounds 10 and 24 show independency of the p53 status, what confers an advantage relatively to 5FU in the treatment of CRC which is completely dependent on the presence of functional p53. Compound 24 was also active in cells with mutated BRaf but compound 10 lost all of its activity what indicates that this compound may target KRas.

The effect of the best compounds was tested in the induction of apoptosis in the 3 cell lines. The induction of apoptosis by these compounds was low in all cell lines and, once again, the independency of p53 was established. On the other hand, 5FU demonstrated again its dependency. In the presence of p53, 5FU caused a high induction of apoptosis but in its absence 5FU had no effect. Given the high activity of the compounds and their low induction of apoptosis, it can be assumed that their main mechanism of action does not induce apoptosis. These results were corroborated by the cell cycle analysis where the arrest of the sub-G1 phase in HCT116 (p53-wt) cells, indicative of apoptosis, was very low. Contrary, the arrest of the S and G2 phases was extremely high in this cell line with compounds 10 and 24. This effect may be due to the inhibition of cyclin B, regulator of the G2 transition, by the compounds. Although our compounds are not similar to any known inhibitor of the G2 transition, they may target the complex cyclin B/Cdk1 or any of its

regulatory proteins. Knowing that the high S and G2 arrest still happens in the absence of p53, the p53 independent ATM/ATR pathway is an interesting target of study. Compounds 10 and 24 may be interfering with components of this pathway like CHK1 and 2, for example, or even with the Ccd25 phosphatase. The expression of some molecular markers was studied with HCT116 (p53-wt) cells. The expression of cleaved PARP protein corroborated once again the low induction of apoptosis by our compounds. On the other hand, the expression of Cdc25c and p53 corroborated the cell cycle results, indicating a high G2 arrest.

Final Remarks and Future Work

1. Final Remarks

Throughout the course of this research, all aims set in the beginning were fulfilled with success. The biological activity of several nitrogen heterocycles belonging to the same family was evaluated by the MTT assay, in the HCT116 (p53-wt) cell line. With this study it was possible to conclude that both substituents R and R^1 present in the heterocyclic nucleus of the compounds interfere with their activity. More than this, results seem to suggest that a bulky alkyl group as substituent R^1 is not favourable for activity, however, the presence of a bulky group in the substituent R is. In this family of compounds two extremely active hits emerged, namely compounds **10** and **24**, with IC_{50s} of 0.89μM and 0.72μM, respectively. These compounds, in addition with the naked compound **7**, were selected to continued in the study.

The biological activity of compounds **10** and **24** was tested in different cell lines with p53 knockout (isogenic HCT116 (p53-null)) and mutant BRAF (CO115), in order to assess if their activity if dependent on p53 and KRAS/BRAF status. Compounds proved to be very promising by their extremely high activity in the studied cell lines, relatively to a reference drug (5FU). Particularly in the absence of functional p53, a mutation related with resistance to anticancer drugs such as 5FU, test compounds were extremely active, what provides an advantage in the treatment of patients. In CO115 cells, with an activating mutation in BRAF gene, the IC_{50} of compound **10** was impossible to calculate since its activity was very low. This lead to the assumption that this compound may targets KRAS gene.

The effect of the test compounds was then assessed in the induction of apoptosis and progression of the cell cycle. Relatively to the induction of apoptosis, the selected compounds presented a low effect, with no dependency in the p53 status. Contrary, 5FU presented a high induction of apoptosis in the presence of active p53 but almost no effect in its absence. The results obtained for the induction of apoptosis are concordant with the cell cycle analysis. The three compounds showed a low arrest of cells at sub-G_1 phase, representative of apoptosis, in comparison with 5FU in HCT116 (p53-wt) cells. Relatively to the progression of the cell cycle in HCT116 (p53-wt) cell line, compounds **10** and **24** cause a accentuated arrest of cells in the G_2/M and S phases, respectively. Given the very high S arrest provoked by compound 24 it is thought that this compound interferes with the DNA synthesis. 5FU also provokes a cell cycle arrest at G_2/M phase. These results clearly indicate

that the test compounds and the reference compound, 5FU, have very distinct effects. While the most evident effect of 5FU is on the induction of apoptosis, our compounds are more efficient in the arrest of the cell cycle at S and G_2/M phases. The initial analysis of molecular markers related with cell death and proliferation in HCT116 (p53-wt) cells sustains the previous conclusions. In addition, compounds **10** and **24** did not presented fluorescent with a FITC filter.

2. Future Perspectives

The work developed so far with this family of compounds paved the way for many lines of work in the future. Relatively to the work already performed, it would be beneficial to conclude the statistical analysis of the flow cytometry analysis in the HCT116 (p53-null) and CO115 cell lines. It would be also interesting to study possible targets, by the test of new molecular markers related with cell cycle arrest at S and G2 phase, like for example Chk1, topoisomerase and Cdc25A[56], as well as a statistical analysis of the markers already tested in the three cell lines. In the same way, the preliminary studies of senescence and DNA damage could be developed with the β-galactosidase and comet assay, respectively.

The study of the mechanism of action of the two hit compounds, **10** and **24**, can also be deepened. In order to do so, the possibility of compound **10** to target KRAS protein can be ascertain, for example, by the expression of KRAS and downstream proteins in HCT116 and CO115 cell lines or in cell lines with a KRas knockout. Relatively to compound **24**, that causes a strong S phase arrest, it would be relevant to compare this arrest with a reference compound that also causes S phase arrest like Roscovitine or even 5FU, for example.

Knowing that the absence of a functional MMR system is related with drug resistance[57] and that compounds **10** and **24** were very active in MMR-deficient cell lines, it will be important to assess the effect of the compounds in a cell line with functional MMR, like Caco-2 for example. This cell line is able to detect certain DNA damages due to the presence of functional MMR system, that translate them into DNA breaks, visible through the COMET assay. HCT116 cells do not detect these damages and, in consequence, no comets appear. In this project, the independency of the compounds' effect in the presence of p53 was established with cell lines with and without p53. In the future it would also be interesting to study the effect of compounds **10** and **24** in cells with mutated p53 like, for example, HCT15 cells.

Although the focus of this project Is the treatment of colon cancer it would also be interesting to assess the effect of our compounds in other types of cancer like breast cancer. An important characteristic to have into account is the compounds' specificity to cancer cells. This can be determined by the analysis of the compounds' effects on "normal" cells, namely ASF-2 fibroblasts. Synergy studies with the selected compounds and reference drugs,

like 5FU and cisplatin for example, can be made in the hope of maximizing their anticancer activity.

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