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Escola de Ciências

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In vitro analysis of novel chemical compounds for the treatment of lung cancer

Tese de Mestrado
Mestrado Bioquímica Aplicada - Ramo Biomedicina

Trabalho efetuado sob a orientação do
Professora Doutora Maria Alice Carvalho
Doutora Marta Oliveira

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***In vitro* analysis of novel chemical compounds for the treatment of lung cancer**

Abstract

Lung cancer is one of the most diagnosed types of cancer and the leading cause of cancer-related deaths worldwide. The 5-year overall survival rate associated with this malignancy continues to be very low (17%) and the number of new cases and deaths expected to increase by 72.5% and 76.3%, respectively, in the next years.

Despite the identification of various molecular alterations associated to the development of a lung malignant phenotype, several possible treatment options and development of novel therapeutic strategies and combinations, improvement of lung cancer survival has not changed substantially. These facts highlight the urgent need for a continuous search for newer and better lung cancer therapies.

In this context, this work aimed at evaluating the anti-cancer potential of a series of novel family-related chemical compounds for lung cancer treatment. Compounds containing the same base scaffold decorated with different R and R1 substituent groups in specific positions were tested and their activity assessed and compared in two distinct lung cancer cell lines (A549 and H292). An initial screening revealed two extremely active compounds, C6 and C3, in both cancer cell lines, with IC_{50} values bellow 2 μM. Of note, it was also found that the position and the type of substituent group affected compound's efficiency. Further studies were conducted to better understand the mechanism of action of the two most promising drugs, namely their impact on cell death and cell cycle progression using flow cytometry. Results showed that both C3 and C6 are able to increase the percentage of apoptotic A549 and H292 cells but are not strong inducers of apoptosis. On the other hand, C3 and C6 also affected cell cycle progression, mainly by increasing cell arrest in S and G_2/M phases. To complement this analysis, evaluation of expression/activation of different molecular markers related with cell death (ex: caspase-3) and cell cycle progression (ex: CDC25c phosphatase) following cell treatment with C3 and C6 was performed by Western blot, with most results supporting flow cytometry data. Overall, the findings obtained herein suggest that A549 cells were more sensitive to compound C6, whereas H292 cells were more affected by compound C3. Although further experiments are required to validate this data, these results suggest a great potential for this family of compounds, and in particular C3 and C6 as novel anti-cancer therapeutic drugs for the treatment of lung cancer.

Análise *in vitro* de novos compostos químicos para o tratamento do cancro de pulmão

Resumo

O cancro do pulmão é um dos tipos de cancro mais diagnosticados, sendo considerado a principal causa de morte ligada a cancro em todo o mundo. A taxa de sobrevivência ao fim de cinco anos associada a esta malignidade continua a ser muito baixa (17%) e é esperado que nos próximos anos o número de novos casos e mortes aumente em 72.5% e 76.3%, respectivamente.

Apesar da identificação de várias alterações em marcadores moleculares associados com o desenvolvimento do fenótipo maligno do cancro do pulmão, de várias opções de tratamento e desenvolvimento de novas estratégias e combinações terapêuticas, o aumento da sobrevivência não tem sido substancial. Estes factos destacam a necessidade urgente da pesquisa contínua de novas e melhores terapias contra o cancro do pulmão.

Neste contexto, este trabalho teve como objetivo avaliar o potencial de uma nova família de compostos químicos para o tratamento do cancro do pulmão. Compostos contendo o mesmo núcleo central com diferentes grupos substituintes R e R1 em posições especificas foram testados e a sua atividade avaliada e comparada em duas linhas diferentes de cancro do pulmão (A549 e H292). Uma avaliação inicial permitiu a identificação de dois compostos extremamente ativos nas duas linhas celulares de cancro, compostos C6 e C3, com um valor de IC_{50} inferior a 2 μM. Adicionalmente, foi também descoberto que o tipo e posição do grupo substituinte afeta a eficiência dos compostos. Outros estudos foram também realizados com o objetivo de melhor perceber o mecanismo de ação dos dois compostos mais promissores, nomeadamente o seu impacto na morte celular e progressão do ciclo celular com citómetria de fluxo. Os resultados mostraram que os dois compostos têm a capacidade de aumentar a percentagem de células A549 e H292 apoptóticas, porém não são fortes indutores de apoptose. Por outro lado, foi também verificado que os dois compostos afetaram a progressão do ciclo celular, desencadeando, principalmente, um aumento do número de células em fase S e G_2/M. Para complementar esta análise, a expressão/activação de diferentes marcadores moleculares relacionados com a morte celular (ex: caspase 3) e progressão do ciclo celular (ex: Fosfatase CDC25c) após tratamento com C3 e C6 foi também avaliada por Western Blot, com a maioria dos resultados a suportar os dados obtidos por citómetria de fluxo. No geral, os resultados

obtidos sugerem que as células A549 são mais sensíveis ao composto C6, enquanto que as células H292 são mais afetadas pelo composto C3. Embora mais estudos sejam necessários para validar estes dados, estes resultados sugerem que esta família de compostos tem um grande potencial, e em particular os compostos C3 e C6 como novos fármacos anti-tumorais para o tratamento de cancro do pulmão.

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Abbreviations

7-AAD	7-Amino-Actinomycin
ALK	Anaplastic Large Cell Lymphoma Kinase Gene
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-related Protein
BSA	Bovine Serum Albumin
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CTLA-4	Cytotoxic T-lymphocyte-Associated Antigen 4 pathway
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EGFR	Epidermal Growth Factor Receptor
EML4	Echinoderm Microtubule-Associated Protein-like 4
ERK	Extracellular Regulated Kinase
FACS	Fluorescent-activated cell sorter
FBS	Fetal Bovine Serum
G_1 Phase	Gap-1 phase
G_2 Phase	Gap-2 phase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HER-2/neu	Human Epidermal growth factor Receptor 2
IC_{50}	Half Maximal Inhibitory Concentration
KRAS	v-Ki-ras2 Kirsten Rat Sarcoma Viral Oncogene Homolog
M Phase	Mitosis
MAPK	Mitogen-Activated Protein Kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSCLC	Non-Small Cell Lung Carcinoma
p53	Tumor Protein p53

PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline + Tween
PD-1	Programmed Death-1 pathway
p-ERK	Phosphorylation of ERK protein
phH3	Phosphohistone H3
PI	Propidium Iodide
PS	Phosphatidylserine
PTEN	Phosphatase and Tensin Homolog
RAS	Rat sarcoma
RB	Retinoblastoma Tumor Suppressor
SCLC	Small-Cell Lung carcinoma
SQCLC	Squamous Cell Lung Cancer
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
S Phase	Synthesis Phase
STS	Staurosporine
TBS	Tris-buffered Saline
TBS-T	Tris-buffered Saline + Tween
TK	Tyrosine Kinase
TKIs	Tyrosine Kinase Inhibitors
VEGF	Vascular Endothelial Growth Factor

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1. State of Art

1.1. Lung Cancer

Lung cancer is one of the most common cancers and the leading cause of cancer related deaths worldwide.[1] According to the International Agency for Research on Cancer, 2.093.876 new cases were diagnosed worldwide in 2018 and 1.761.007 people died from the disease (Fig. 1).[2] Until 2040, is expected an increase of 72.5% and 76.3% on the number of incident cases and deaths, respectively.[3] Strikingly, the 5-year overall survival i.e., the percentage of people who live at least 5 years after being diagnosed with lung cancer is less than 17%, and most patients die within one year of diagnosis.[4]

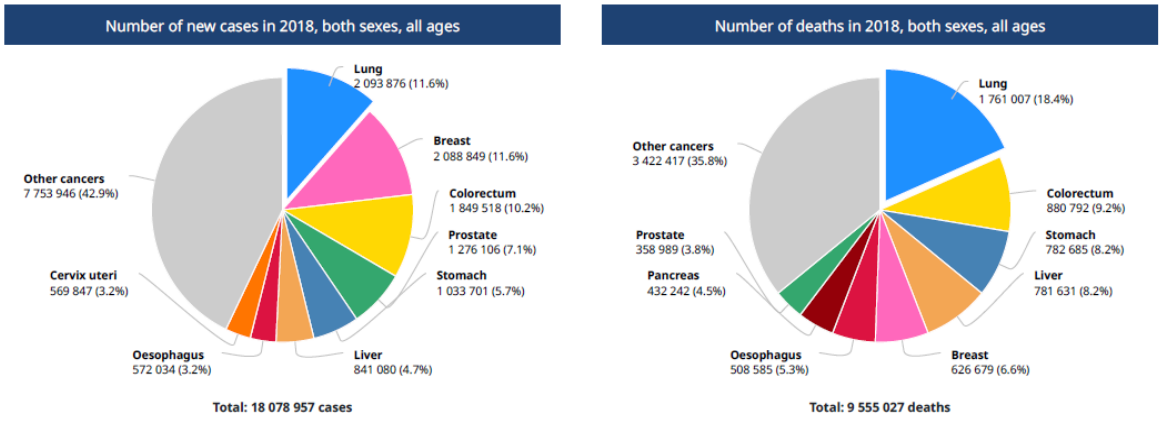


Figure 1. **Estimated number of new cases and deaths of different types of cancer in 2018**.[2]

In Europe, lung cancer is the fourth most common and the one with the highest mortality[5], similarly to Portugal, where 51,22% of the cases are diagnosed at an advanced stage[6].

Several risk factors have been associated to the development of lung cancer. Smoking is considered the main cause, being associated with 90% of the cases worldwide.[7] Others include second hand or passive smoking, pollution[8] and age, since at the time of diagnosis 50% of the patients are over 70 years of age and, within this group, 15% are older than 80 years.[9]

1.1.1. Lung Tumorigenesis

In humans, the process by which a normal cell becomes a malignant cell is considered to be a multistep process, which results from the acquisition of one or more genetic changes that alter normal cell homeostasis.[10,11] The development of preneoplastic lesions in the lung and progression to a malignant tumor (Fig. 2) is still unclear and continues to be investigated to further understand the biology of the disease, to unravel potential biomarkers as well as to develop novel and more accurate strategies for early cancer detection.[12]

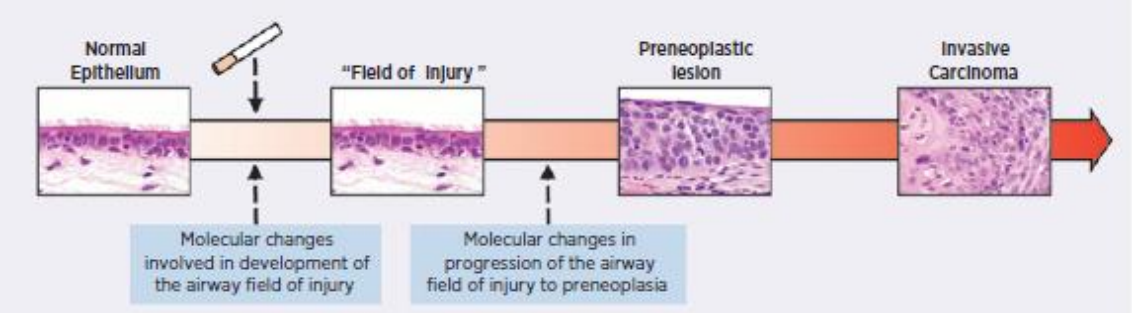


Figure 2. **Progression of the molecular airway field of injury to preneoplasia and lung malignancy**.[15]

1.1.2. Histological Classification

Lung Cancer is a heterogeneous disease, both histologically and molecularly. It can arise in different sites of the bronchial tree, and thus lead to different symptoms and signs.

From a histological point of view, lung cancer can be divided in two main types: Small Cell Lung carcinoma (SCLC) and Non-Small Cell carcinoma (NSCLC), which account for 15% and 85% of all lung cancers, respectively (Fig. 3). Although less abundant, SCLC is the most aggressive form, with 95% of diagnosed patients ultimately dying.[13] As for NSCLC, it can be further divided into three major histological subtypes (Fig. 3):

- Squamous Cell Lung Cancer (SQCLC), this subtype is strongly associated with smokers. The number of affected patients has been declining in the last decades.[12]

- Lung adenocarcinoma, which is the most common histological subtype of lung cancer[12]. Compared to other types of lung cancer, it tends to grow slower.[4]
- Large Cell Lung Carcinoma that accounts for 5-10%[4] of lung cancers. It is usually diagnosed by exclusion when the tumor does not have any morphologic characteristic of adenocarcinoma, Squamous cell carcinoma or Small cell carcinoma.[12]

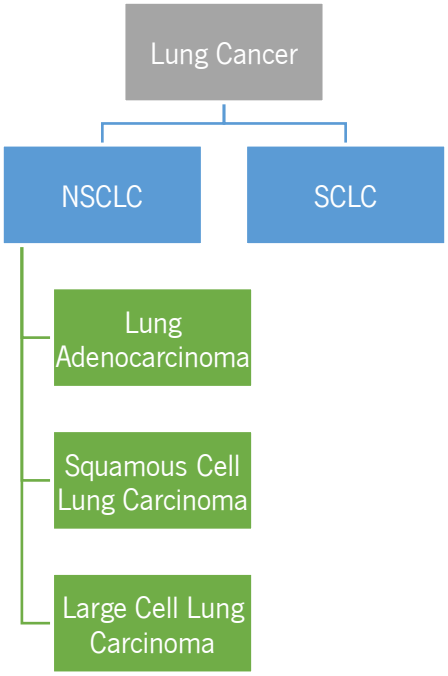


Figure 3. **Histological classification of lung cancer.**

1.1.3. Staging

Additionally, to the histological classification of the tumor, it is also very important to assess its extent, i.e, determine its location and how much it has grown and/or spread to distinct tissues. Cancer staging is important for therapeutic reasoning and assess prognosis, being currently the only proven prognostic marker that helps in the diagnostic of the disease aggressiveness.[7] Depending on

the type of lung cancer distinct staging systems are used. NSCLC has various stages (0-IV) (Fig. 4), and the lower the stage, the less the cancer has spread. At early stages (0 and II) (Fig. 4), the primary tumor is localized and restricted to the lung and nearby lymph nodes. In contrast, in more advanced stages (III and IV), cancer has spread to other areas of the lung, chest or even more distant organs.[7] Remarkably, the vast majority (70%) of lung cancer patients are diagnosed at an advanced stage of the disease (stage III or IV)[4,8] (Fig. 4).

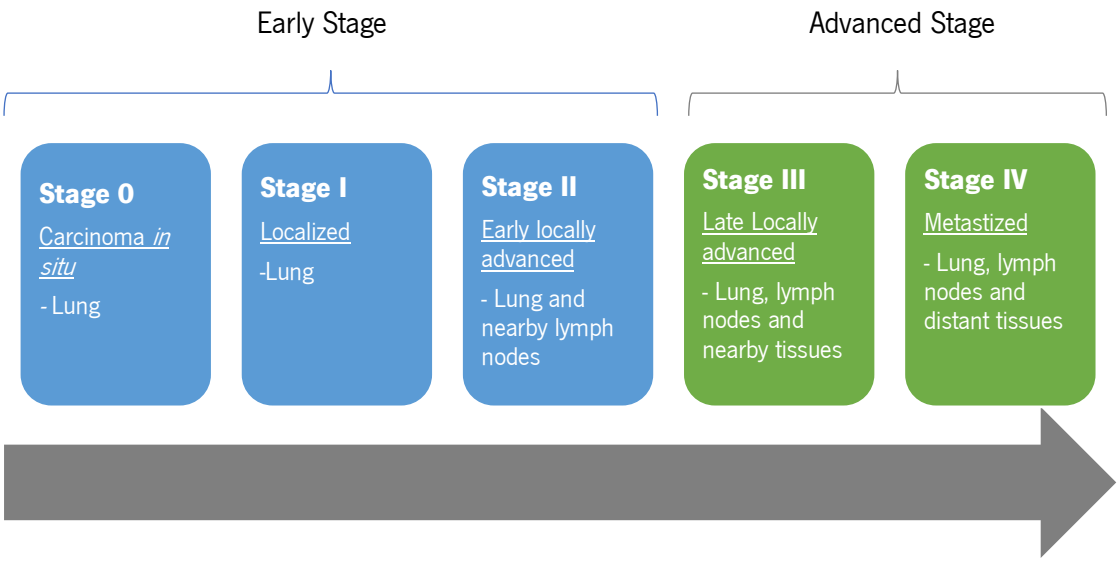


Figure 4**. Schematic representation of the various stages of lung cancer**. Early stages are represented in blue and advanced stages represented in green.

Regarding SCLC, two main stages can be considered: limited stage when the tumor is found only in one lung and possibly nearby lymph nodes and extensive stage when the tumor has spread to other areas of the lungs, chest or body.[13]

1.1.4. Molecular biology of lung cancer

Molecular biomarkers have the potential to identify and predict patients that will more likely benefit from a specific therapy or respond to it[12], so understanding these events at different levels and their functional significance is expected to impact lung cancer prevention, diagnosis and treatment[14]. A large body of evidence suggests that a sequence and/or accumulation of complex and heterogeneous

molecular abnormalities is the basis for the development of a lung malignant phenotype.[15] Those alterations can result in important functional cellular abilities as described below.

1.1.4.1. Oncogene-driven growth stimulation

An oncogene is generally a mutated form of a normal gene (proto-oncogene), that when activated can disrupt normal cell growth and differentiation leading to uncontrolled proliferation. In lung cancer, several activation mutations in several proto-oncogenes, such as the epidermal growth factor receptor (EGFR), Human Epidermal growth factor Receptor 2 (HER-2/neu), and Rat sarcoma (RAS) have been identified. Interaction of EGFR with its ligand, triggers the initiation of downstream signaling pathways involved in cell proliferation, survival and angiogenesis, such as PI3K/AKT and Mitogen-Activated Protein Kinase (MAPK) pathways (Fig. 5).[14,16,17] Dysregulation of EGFR by activating mutations or overexpression is thus critical and highly associated with lung cancer development, invasion and metastasis.[14,16,17] In advanced NSCLC, particularly, EGFR overexpression is associated with poor survival and resistance to some chemotherapy agents, including cisplatin.[17] Likewise, dysregulation of the MAPK pathways ERK 1/2, JNK, p38 and ERK 5, that transmit signals of several stimuli from the cell membrane to the nucleus promoting inflammatory and/or proliferative responses[18] (Fig. 6), is shown to be linked to lung and other types of cancers.

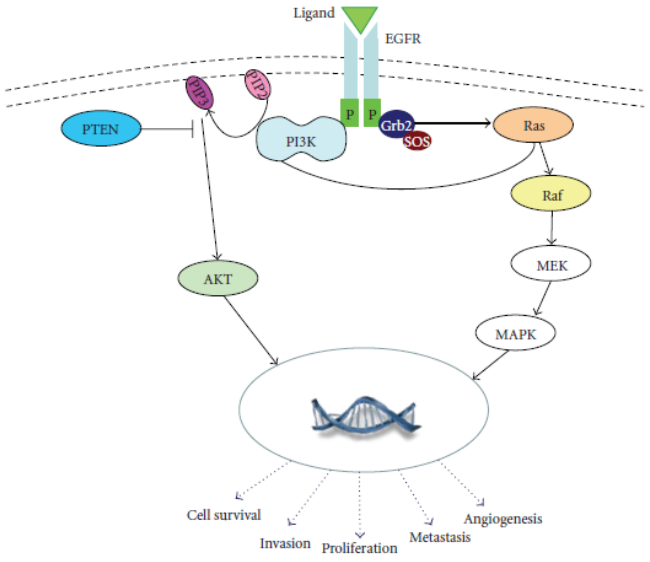


Figure 5. **EGFR signaling pathway.** [96]

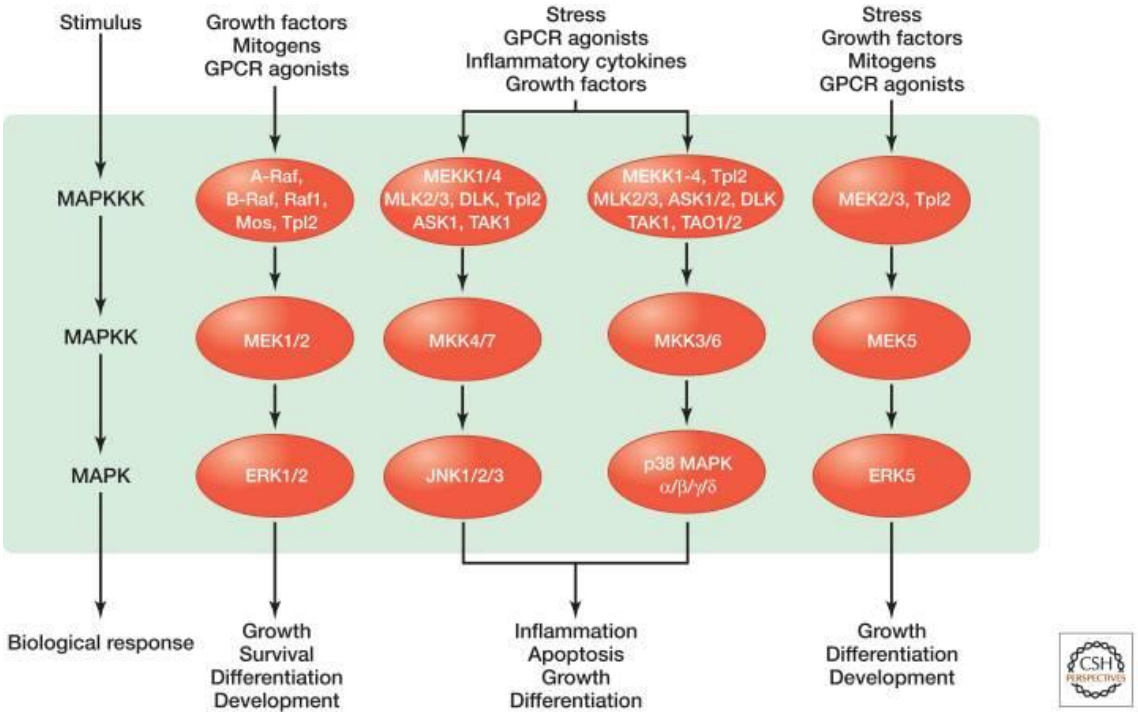


Figure 6. **MAPK pathways.**[97]

HER2 or ErbB-2 is a proto-oncogene that, similarly to EGFR, belongs to receptor tyrosine kinase family. However, unlike other members of that family HER2 does not have a specific ligand but can form a heterodimer with other members of its family to become activated. Overexpression of HER2 occurs in 30% of NSCLC and is associated with poor prognosis, multiple drug resistance and high prevalence of metastases.[14,17,19,20]

The RAS oncogene family, which includes v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), NRAS and HRAS, plays a crucial role in cellular proliferation.[21] Upon activation, intracellular signals are transmitted by several downstream pathways, including the MAPK pathways (Fig. 6), and will culminate with the activation of transcription factors.[14] RAS mutations are seldom or absent in SCLC, but can be found in 15-20% of NSCLC. Up to 50% of the lung adenocarcinomas carry RAS mutations, most of them (25-40%) affecting the KRAS gene, while HRAS and NRAS mutations are uncommon.[14] Even though EGFR can be responsible for RAS activation, normally EGFR and KRAS mutations are mutually exclusive but exceptions can happen.[14] Usually KRAS mutant tumors are

resistant to EGFR tyrosine kinase inhibitors (TKIs), as KRAS mutations leads to constitutive activation of signaling pathways downstream of EGFR, thus becoming an important target for the development of new therapies.[14]

A molecular rearrangement linked to increase of cell proliferation and consequent inhibition of apoptosis is anaplastic large cell lymphoma kinase (ALK) gene, a transmembrane receptor tyrosine kinase.[16] ALK fuses with echinoderm microtubule-associated protein-like 4 (EML4) genes, creating a chimeric protein with constitutive kinase activity[19], which activates the MAPK signaling pathway (Fig. 6) and consequently promotes malignant growth and cell proliferation.[14,19] Rearrangements on ALK are mostly associated with lung adenocarcinomas, being present in 3 to 7% of NSCLCs.[19] Although ALK mutations are typically mutually exclusive with EGFR and KRAS, cases of coexistence with EGFR have been reported.[14,19]

1.1.4.2. Insensitivity to anti-proliferative signals by inactivation of tumor suppressor genes

Tumor suppressor genes have a critical role in controlling the cell antiproliferative mechanisms and are involved in cellular response to DNA damage and consequent repair processes.[17] The loss or inactivation of tumor suppressor genes is frequent during lung cancer tumorigenesis.[17] The tumor protein p53 (p53) gene, found to be inactivated in 75% of SCLCs and about 50% of NSCLCs is considered the "guardian of the genome".[17,22] It protects cells from DNA damage, stress or excess of proliferative signals, by interfering with the cell cycle progression (Fig. 7) and/or inducing apoptosis, thus preventing a normal cell from becoming malignant (Fig. 8).[17,22,23] Under physiological conditions and in the presence of a DNA damage, cell cycle checkpoints halt the cell cycle in G_1 or G_2, (Fig. 7), giving the cell time to activate the repairing pathways and let them operate before the cell progresses to the next phase or start the process of programed cell death.[22] Upon DNA damage, the main regulators Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3-related Protein (ATR) kinases are activated, resulting in the phosphorylation and activation of p53 (Fig. 8).[23] Additionally, ATM and ATR kinase activation triggers CHK1 and CHK2 activation and CDC25c phosphatase phosphorylation, ultimately blocking cells from entering into mitosis (Fig. 8).[24]

However, when mutated, p53 tumor suppressing activity is abolished and G_1/S or G_2/M checkpoints impaired (Figs. 7 and 8). As for triggering apoptosis, p53 has several pro-apoptotic

transcriptional targets, like BAX which induces the release of cytochrome *c* to the cytosol and consequently can activate the caspase cascade.[22] This will be discussed in more detail in section 1.1.4.3. In lung carcinomas, p53 mutations have been associated with poorer prognosis and cellular resistance to treatment. Also, it is claimed that coding mutations on p53 arise in the early stages of development of lung cancer and are required to maintain the malignant phenotype during tumor progression and metastasis, since they are preserved during those processes. However, this kind of abnormality is mostly diagnosed in SCLC, whereas in NSCLC, it is most common in SQCLC and less common in adenocarcinomas.[14,25]

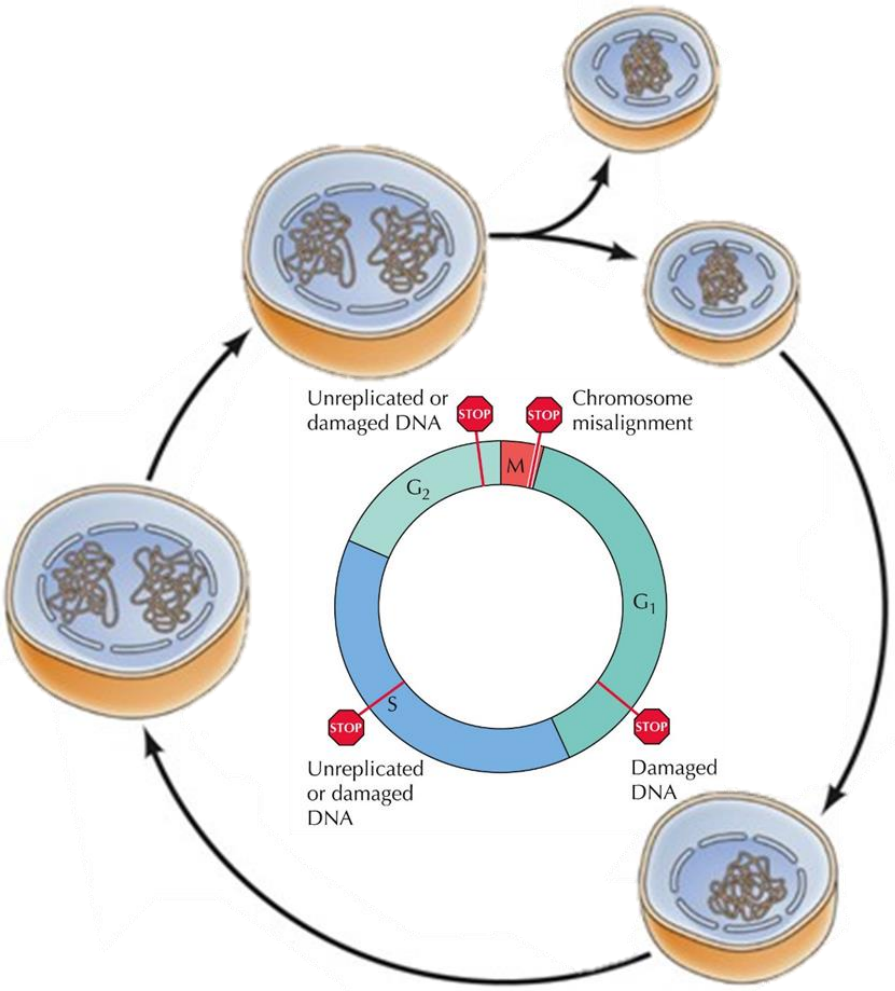


Figure 7. **Cell Cycle and Checkpoints.** The eukaryotic cell cycle is divided in four phases M, G_1, S and G_2. In Gap 1 phase (G_1 phase) cell growth occurs, being followed by synthesis phase (S phase), in which the DNA is replicated. The last phase of interphase is Gap 2 phase (G_2 phase), where cell growth continues and proteins essential for Mitosis (M phase) are synthetized. The cell cycle ends with Mitosis where, the separation of chromosomes and cell division or cytokinesis occurs.[72]The DNA damage checkpoints in G_1, S, and G_2 phases can lead to cell cycle arrest and/or apoptosis in response to damage or stress.[72]

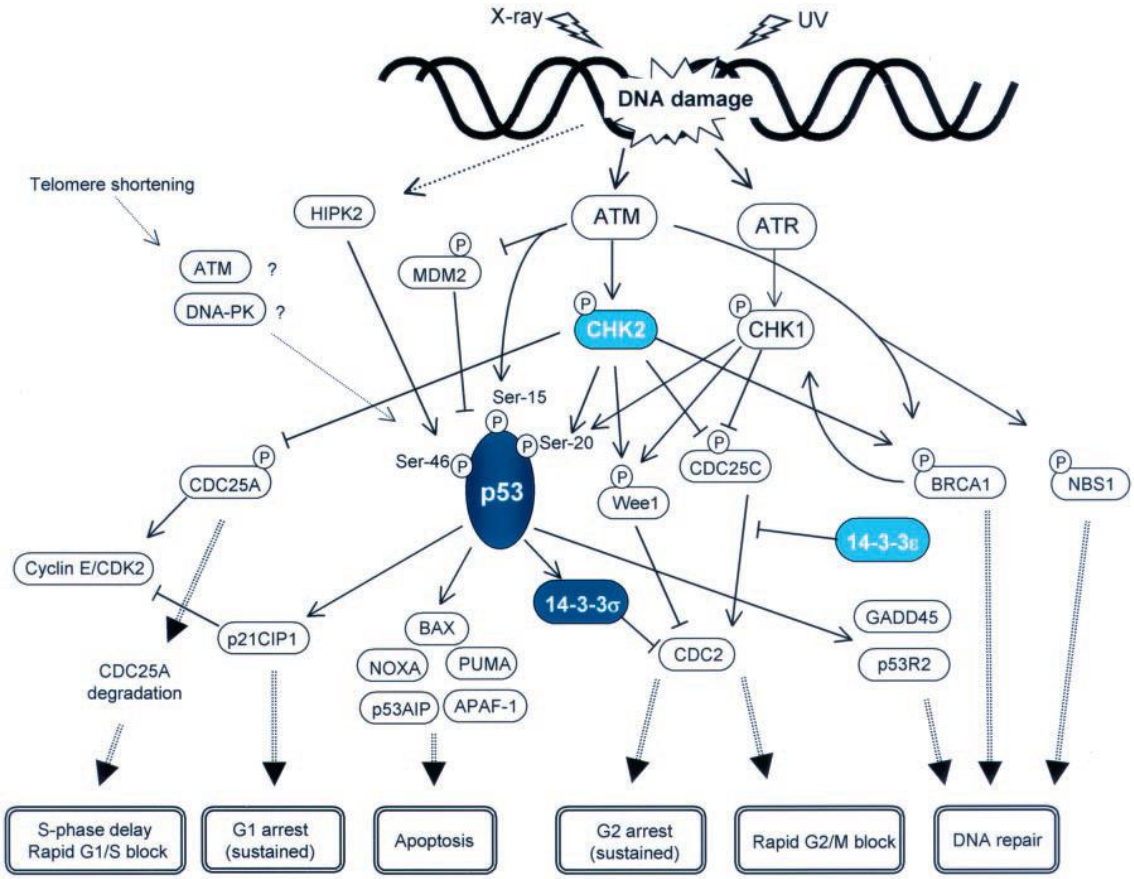


Figure 8. **DNA damage response signaling pathway.** [23]

Another major pathway involved in the pathogenesis of lung cancer is the RB pathway, which negatively regulates cell cycle progression and cooperates with p53 to control it.[17] The interaction between retinoblastoma tumor suppressor (RB) and transcription factor E2F is important for the regulation of G_0/G_1 cell cycle progression.[17] When RB protein is hyphosphorylated, it binds to E2F transcription factor, blocking S-phase entry and cell growth.[17] However, alterations in this pathway, allow release of E2F transcription factors, stimulating cell proliferation and conferring cell insensitivity to antigrowth factors. Remarkably, functional inactivation of RB is found in almost all (90%) SCLC and rather rare in NSCLC (15-30%).[17]

Phosphatase and Tensin Homolog (PTEN), is an additional tumor suppressor gene found to be altered lung cancer, but at a lower extent. It encodes a protein phosphatase that inhibits the PI3K/AKT/mTor signaling pathway, linked to cell growth, survival and apoptosis (Fig. 5).[14,19] Inactivation

of PTEN function leads to an unrestricted activation of AKT independently of ligand biding[14], thus allowing deregulated cell growth. PTEN mutations are relatively rare (5%) in NSCLC, and are associated with ever smokers, as opposed to reduced protein expression which has been reported in 75% of NSCLC.[14]

1.1.4.3. Evading apoptosis

Apoptosis or programmed cell death is an organized and controlled process that is essential for tissue remodeling during embryo development and maintenance of homeostasis balance on adults.[17] Defects in apoptosis are, in turn, also closely linked to tumorigenesis and drug resistance.[17] The mechanism of apoptosis is complex and may take place essentially via two distinct pathways: the extrinsic (death receptor), which is activated by the engagement of death receptors on the cell membrane, and the intrinsic (mitochondrial) pathway, that involves the release of cytochrome *c* by mitochondria (Fig. 9). Despite the differences, both pathways activate a group of enzymes belonging to the cysteine protease family named caspases and converge to a common pathway initiated by caspase 3. Downstream caspases then trigger cleavage of various proteins, induce DNA fragmentation and have an effect on the cytoskeleton, provoking characteristic morphological alterations of apoptotic cells.[17,22]

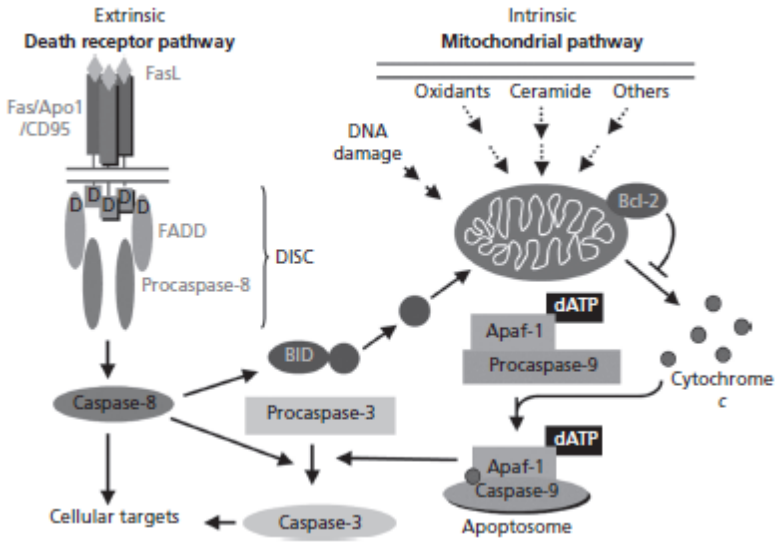


Figure 9.**Major apoptotic pathways of mammalian cells.** [98,99]

The p53 tumor suppressor gene and the Bcl-2 gene family are key regulators of apoptosis. While p53 promotes cell death, BCL-2 can have pro-apoptotic and anti-apoptotic activity. The balance between the pro- and anti-apoptotic members of the Bcl-2 family determines if the cell survives or enters in the apoptosis process.[17,22] In lung cancer, overexpression of BCL-2 is found in between 75 to 95% of SCLC, being also associated with NSCLC, more specifically to squamous cell carcinomas and adenocarcinomas.[17]

1.1.4.4. Cellular immortality

At the end of each chromosome there is a heterochromatin structure named telomere. Telomeres have the function to serve as protective caps, maintain chromosome integrity, reversely repress the transcription of neighbor genes and prevent the end-to-end fusion or degradation of chromosomes.[17] During DNA replication, DNA polymerases are unable to replicate the 5'-end of linear DNA, so telomeres become shorter after each somatic cell division.[17] This shortening is known as end-replication problem and does not lead to loss of essential genes.[17] The shortening leaves the cell with a potential to undergo 50 to 70 divisions, and after that cell growth arrests and enters in senescence.[17]

Elongation and maintenance of the telomeres of eukaryotic chromosomes is assured by telomerase, a specific ribonucleoprotein enzyme complex, allowing to extend the number of divisions that a cell can undergo.[17] In adult organisms, telomerase activity is absent in the majority of normal cells.[17] However, in tumor cells, the increase of telomerase activity is extremely frequent, allowing unlimited proliferation and consequently immortality.[17] In lung cancer, activation of telomerase in frequently observed (100% in SCLC and 80% in NSCLC).[26]

1.1.4.5. Tumor angiogenesis

Angiogenesis, or the formation of new blood vessels, is a vital process that ensures the supply of oxygen and nutrients.[10] The process involves several angiogenic mediators, such as vascular endothelial growth factor (VEGF), angiopoietins and platelet-derived growth factor.[27,28] In normal tissue, the process is highly regulated by the counterbalance of positive and negative signals that can promote or block the angiogenic process.[10] In tumor cells, the new blood vessel growth is required not only to sustain growth but also for metastasis[17,29]. However, the new blood

vessels are abnormal, poorly organized and have altered permeability, leading to an irregular tumor growth, decrease of drug delivery[29], and consequently poor survival[17]. High levels of VEFG have been detected in about 50% of lung cancers.[27,28]

1.1.5. Current Therapies

The main aim of cancer treatment is to remove or destroy the cancerous cells without affecting normal cells.[30] The different treatment options for lung cancer are summarized in Table 1 and depend mainly on three factors: the specific stage of the disease, the molecular features of the tumor and the general status of the patient[31] . A more detailed description of each treatment option is provided below.

Table 1. **Different types of lung cancer, stages and treatment**.[7,31]

Cancer	Tumor Stage	Treatment
NSCLC	0	Surgery
	I	Surgery (resectable) or radiotherapy (unresectable)
	II	Radiotherapy (unresectable) or surgery (resectable) followed by chemotherapy, targeted therapy or combined therapy
	IIIA	Surgery combined with chemotherapy, chemoradiation, combined therapy or targeted therapy
	IIIB	Chemotherapy, immunotherapy, targeted therapy or combined therapy
	IV	Chemotherapy, immunotherapy, targeted therapy or combined therapy
	Recurrent	Chemotherapy, immunotherapy or palliative radiation therapy
SCLC	Limited	Chemotherapy, radiotherapy, targeted therapy or combined therapy
	Extensive	Chemotherapy, Targeted therapy, immunotherapy, combined therapy or prophylactic cranial irradiation
	Recurrent	Chemotherapy or palliative therapy
Metastasis	-	Systemic surgery, radiosensitization or radiotherapy

1.1.5.1. Early Stage therapies

1.1.5.1.1. Surgery

For patients diagnosed with lung cancer in stages 0, I, II and IIIA (Table 1), surgical resection is the best and most effective choice, since it improves long-term survival.[7,8,31] However, for this option to be viable it is important that the tumor can be completely resectable and the patient is capable to endure the surgical intervention.[8] The standard procedure of surgery in lung cancer is lobectomy, where a lobe of the organ is removed.[8] This has proven to increase long-term survival by 30% and decrease local recurrence rate by 75% in patients that have been treated with this kind of surgery in comparison to the ones with less aggressive procedures.[8,32] Nevertheless, new minimal-access surgical procedures, such as video-assisted lobectomy, are being offered to an increasing number of patients with the same results as a normal lobectomy.[8]

1.1.5.1.2. Radiotherapy

In radiotherapy, death of cancer cells is caused by high-energy beams that damage DNA, allowing the control or even destruction of tumors localized at specific sites.[4] Although surgery is the first treatment choice for patients with early stage lung cancer (Table 1), some patients cannot undergo that procedure due to a number of factors, such as old age, presence of other types of illness and also patient refusal.[33] So, for those patients radiotherapy becomes their primary treatment.[33]

Despite its effectiveness, radiotherapy has some disadvantages since the thorax is still a challenging anatomical site for radiation delivery[34] and because radiation also damages the healthy cells in its path. Another downside of this treatment is that it leads to lower survival rates when compared to surgery.[33]

1.1.5.1.3. Adjuvant Therapies

Even though surgery has a high percentage of success, some patients may benefit from adjuvant therapy, a post-surgery treatment which can contribute to reduce the chances of cancer relapse.[4]

One example is chemotherapy, which consists in the use of drugs to destroy cancer cells and will be further discussed in section 1.1.5.2.1. When used as adjuvant therapy in NSCLC patients, it has shown good results in several trials[33,35], namely an improvement of 5.4% in the 5-year survival as compared to patients submitted only to surgery.[35] Chemotherapy can be used alone or combined with radiation therapy, and both may also be used before surgery to shrink cancers and make them easier to remove.

Other examples of adjuvant therapies include radiotherapy and more recently target therapy and immunotherapy, that will be described in sections 1.1.5.2.2 and 1.1.5.2.3, respectively, as these are given to patients with advanced disease.[4]

1.1.5.2. Advanced stage therapies

Approximately 40% of lung cancer patients are diagnosed in stage IV, when the tumor has already spread to other organs.[4] In NSCLC, near 70%, are diagnosed in late stages of development, usually with locally advanced or even metastasized tumors.[4,8] As such, these widespread tumors are very hard to cure, being the main goal to control disease progression. Treatment selection will thus depend on the type of tumor, on how far it has disseminated, on the number of existing tumors and on the patient's overall health. Below are summarized the different alternatives that can be included in a treatment plan for patients with advanced lung cancer.

1.1.5.2.1. Chemotherapy

Chemotherapy consists in the use of drugs or other chemicals to kill cancer cells, being its effects systemic.[30] It consists in a non-targeted approach, which can be used in different situations: before or after surgery, to reduce the size of the tumor or kill possible remaining tumor cells, respectively, or even as the main treatment and palliative care[36], for more advanced cancers (Table 1). Chemotherapeutic agents can be given intravenously or orally, but in the case of lung cancer the majority is given intravenously. Several different chemotherapeutic drugs for lung cancer are currently available, which have different mechanisms of action, as discussed below.[30]

Platinum-based therapies, such as carboplatin and cisplatin, applied as single agent or in combination are considered the first-line therapy in lung cancer.[9] These kind of anticancer drugs induce

cell cycle arrest and apoptosis by forming intra- and inter-strand adducts with DNA.[30] However, platinum compounds attack both healthy and malignant rapidly dividing cells indiscriminately, leading to the development of severe side effects and mechanisms of drug resistance.[9]

Another problem of platinum-based therapies is that patients who respond to this treatment and even show a temporary stabilization of the disease, will eventually face a cancer recurrence.[37] In almost 30% of NSCLC patients, the first-line therapy fails.[38] However, many of those patients are still eligible to receive further treatment, so second- and third-line chemotherapeutic compounds are considered, being usually used as palliative as they increase patients' quality of life and prolong their survival.[37]

Taxanes, other category of chemotherapeutic agents, act by perturbing tubulin polymerization, the "building block" of microtubules, thus blocking cell division.[30] Drugs belonging to this category can affect the cell cycle at different phases.[30] Docetaxel, the first agent approved as second-line therapy agent, targets centrosome organization and affects the cell cycle in S, G_2 and M phases, while paclitaxel causes cell damage by disturbing the mitotic spindle in G_2 and M phases of cell cycle (Fig. 7).[30,38]

Other types of chemotherapeutic agents considered as second- and/or third-line therapies are antimetabolites, which block the synthesis of nucleic acids by acting as decoys that can be misincorporated into nucleic acids or limit deoxyribonucleoside triphosphates availability, e.g. gemcitabine, and topoisomerases inhibitors, which inhibit the enzymes responsible for relaxing DNA supercoiling during replication and transcription, e.g. Irinotecan.[39]

The use of different chemotherapeutic agents, either alone or in combination, is associated with undesirable side effects, since chemotherapy has poor selectivity for cancer cells.[31,40] The side effects are specific to each type of chemotherapeutic agent, but there are some common ones such as hair loss, mouth sores and fatigue.[40]

1.1.5.2.2. Targeted Therapy

In cancer cells many of the networks regulating cellular activities (e.g. proliferation, survival and death) are drastically modified and new approaches capable of specifically interfering with these

dysregulated pathways (e.g a cancer's specific gene or protein), led to the beginning of the "targeted therapy" era.[41]

Targeted therapies block the growth and spreading of the tumor[42] while reducing normal cell damage. This is based on the fact that for the targeted treatment to have an efficacy-to-toxicity profile, the molecular target must be overexpressed, unique or mutated in tumor cells, so it kills tumor cells but does not significantly affect normal cells.[43] One of the main ways to achieve that purpose is using monoclonal antibodies, which act by blocking a receptor in the cell surface, consequently blocking its function, and indirectly recruiting immune cells to complement their cytotoxic effect[44]. To further enhance their function, these monoclonal antibodies can be coupled with toxins or cytotoxic radionucleotides, i.e. nucleotides labeled with a radionuclide.[44] The other option is the use of synthetic small molecules, that besides being able to act on the same pathways and targets as monoclonal antibodies, have also the capability to enter the cell and inhibit some downstream enzymatic functions.[44]

In lung cancer, only a limited group of all identified molecular targets have been considered relevant to the development of targeted therapeutics.[45] An obvious one is EGFR, an important component of PI3K/AKT and MAPK pathways (Fig. 5) that has an integral role in tumor growth and spread.[46] Currently several anti-EGFR drugs (Table 2) have already been approved. Other molecular targets that have also been considered, are ALK, with some drugs already approved and others under development (Table 2), and more recently HER2, with drugs still under development (Table 2). Angiogenesis, more specifically VEGF, has also been considered as a target for the development of targeted therapy with some monoclonal antibodies anti-VEGF already approved. All these options will be discussed in more detail below.

Table 2. **Developmental phases of available drugs for the treatment of lung cancer.**[1,29]

Target	Drug	Status
EGFR	Gefitinib	Approved
	Erlotinib	
	Afatinib	
	Osimertinib	
	Necitumumab	
ALK	Crizotinib	Approved
	Alectinib	
	Ceritinib	
	Lorlatinib	Phase 2
	Brigatinib	
HER2	Trastuzumab	Phase 2
	Afatinib	
	Dacomitinib	
VEFG	Bevacizumab	Approved
	Ramucirumab	

Despite the variety of targeted therapies available, this kind of approach is mostly used to treat advanced lung cancer patients with specific mutations[1], other way the treatment will not be effective. So, for patients who have mutations that do not fit in an approved targeted therapy, the standard first-line therapy continues to be platinum-based chemotherapy.[1]

Anti-angiogenesis therapy

Angiogenesis is a fundamental process in the development of primary tumors and secondary metastatic lesions, being VEGF the major regulator and promotor of angiogenesis in normal and malignant tissues.[33,47] Two main approaches have been adopted to target tumor angiogenesis: monoclonal antibodies that block VEGF receptor binding and small molecule TKIs that inhibit downstream VEGF receptor mediated signaling.[29]

Bevacizumab (Table 2), a humanized monoclonal antibody against VEGF was the first anti-angiogenic agent approved to treat NSCLC.[29,33] Bevacizumab binds to VEGF, inhibiting its binding to downstream receptors.[29] The efficacy of this drug has been supported by several studies, in which the

use of bevacizumab in combination with other therapies versus the other therapies alone showed an increase on progression free survival and overall survival.[48,49]

EGFR Inhibitors

One of the most studied therapeutic targets is EGFR, present in 50 to 90% of NSCLC.[43] As above mentioned, the overexpression of EGFR has been associated to a more aggressive phenotype and worse prognosis of the disease.[43] Observations that tumor cells with mutations on kinase domain of EFGR are sensitive to EGFR -TKIs, led to a significant advance on NSCLC treatment.[43] Erlotinib and Gefitinib (Table 2) are two first-generation inhibitors of the tyrosine kinase domain of the intracellular part of EGFR[33], having the capability to stop dysregulated cell growth. The efficacy of these therapeutics has been maintained by the results of several phase III trials, in which the progression free survival and overall survival of first-line treatments of NSCLC were compared with Erlotinib or Gefitinib. Results showed a significant increase in terms of progression free survival of 2.9 and 5.4 months with Gefitinib[50,51] and 8.5 months with Erlotinib[52] in patients with EGFR-mutated tumours.[50–52]

Despite the demonstrated efficacy of this kind of therapy, patients treated with first-generation EGFR-TKIs eventually develop resistance and will possibly experience a relapse, which means that if EGFR-TKIs do not destroy the tumor, will make it stronger.[53] The main resistance-associated mutation includes a T790M mutation in the EGFR tyrosine kinase (TK) domain, reported in 50 to 60% of EGFR TKI resistant tumors.[20] The development of a second and third generation of EGFR-TKIs, with different mechanisms of action, was needed to solve the acquired resistance, however both solutions also had problems with acquired resistance.[16]

ALK Inhibitors

ALK is also one of the molecular targets that can be used for the treatment of NSCLC, being detected in 3 to 7% of the patients.[19,54] Crizotinib (Table 2) is a ALK-TKI, that induces cell cycle arrest in G_1/S phase and apoptosis.[47] A phase III trial of Crizotinib versus platinum-based doublet chemotherapy, showed an increase of response rate of 45% to 74% and an increase of progression free survival of 7 months to 10.9 months. The results of this study contributed to the approval of the use of Crizotinib in first-line ALK-positive advanced NSCLC.[54] Despite the positive results, Crizotinib has shown some side effects, such as nausea and visual disturbances.[54]

Other inhibitors

HER2 mutation has shown some potential as a therapeutic target, since it was observed that nearly 50% of the patients with advanced NSCLC showed a reduction on the size of the tumor using Afatinib and Trastuzumab.(Table 2).[19,55]

KRAS, BRAF and FGR1 are other potential molecular altered genes that have been considered for the development of targeted inhibitors for the treatment of lung cancer, existing already several compounds with those characteristics in different stages of clinical development.[12] Still, despite all the advances made and results of some clinical trials using combination therapies, until now there is no targeted therapy approved to treat patients with detected KRAS mutation.[19]

1.1.5.2.3. Immunotherapy

Immunotherapy, which in fact comprises a broad class of therapies, uses the body own natural defense system to cause an immune mediated cancer cell death.[4,56] The purpose is to improve the immune system allowing it to target cancer cells slowing or even stopping their growth.[4] To achieve that goal several approaches have been used, such as immunomodulators, therapeutic vaccines, autologous cellular therapies and monoclonal antibodies directed against checkpoint inhibitor signals on activated T-cells and/or cancer cells.[56]

Yet, until recently, it was thought that lung cancer was non-immunogenic, because immunotherapies had low to none effect on treating this disease.[56] Many studies, however, revealed that lung cancer cells are able to evade the immune system and that the tumor microenvironment induces immunosuppression, by secretion of immunosuppressive cytokines and expression of co-inhibitory molecules.[56,57]

In healthy cells, the immune system uses immune checkpoint pathways to maintain and regulate the immune response to a pathogen, protecting tissues from damage, but in tumor cells this mechanisms can be deregulated by immune-modulating mechanisms.[4] So, to respond to these alterations, new strategies targeting immune-modulating mechanisms of tumor cells are being developed.[4]

One of the approaches is the use of immunomodulatory agents, such as immune checkpoint inhibitors and cytokines.[57] Monoclonal antibodies targeting cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and the programmed death-1 (PD-1) pathway have shown some positive results in advanced NSCLC.[56]

Monoclonal antibodies against CTLA-4, block the inhibitory signal provided by CTLA-4, by preventing the interaction between CTLA-4 and its ligands. This causes an increase in the activation and proliferation of tumor specific T-cells, thus allowing an effective immune response against the tumor.[56] Ipilimumab is an anti-CTLA-4 monoclonal antibody, that has improved the progression free survival and overall survival when administrated after chemotherapy in advanced NSCLC patients.[57]

1.1.5.2.4. Combined Therapy

Combined therapy has the capacity to fill some gaps of chemotherapy alone or single agent targeted therapy.[58] The gathered knowledge from clinical experience and animal models has showed that when different pharmaceuticals are administrated in combination, it is easier to reach a synergetic effect, even with lower doses or concentrations of each drug. This improves safety, reduces side effects and increases of patient compliance.[31,58] In addition, improves therapeutic efficacy, decreases development of drug resistance mechanisms and/or less adverse effects usually associated with mono-chemotherapy.[31] In NSCLC, three different combinations have been used: two different chemotherapeutic drugs, one cytotoxic drug and one pathway inhibitor, and two different pathway inhibitors.[31]

The use of two distinct chemotherapeutic drugs combined, more specifically platinum doublets, is more active, with nearly a double response rate than single-agent treatments in advanced NSCLC, although with more severe toxicity.[59] Although other combinations of cytotoxic drugs have also shown some promising results when compared to single agents, platinum doublets continue to be used as first-line therapies.[59]

The addition of targeted therapies to cytotoxic drugs, came from the basis of synergism between cytotoxic drugs with different pharmacologic properties, and also because of the superior efficacy of doublet chemotherapy over single-agent treatment.[59] In that context, in a randomized phase 2 clinical trial, Bevacizumab, an anti-angiogenic agent, was added to the standard carboplatin-paclitaxel

regimen in previously untreated patients with advanced NSCLC. The results showed an increase in longer median time to progression (4.2 to 7.4 months) and an increase in survival (14.9% to 17.7%).[48]

Following the success of combining Bevacizumab with chemotherapeutic regimens, other approaches have been used. One of them is the combination of immunotherapy agents with chemotherapy. In a phase 2 clinical trial, pembrolizumab, a anti PD-1 antibody with positive results as single agent, was combined with carboplatin-pemetrexed chemotherapy regimen in advanced stage NSCLC patients.[60] The results showed an increase on response of 29% to 55% and an increase on progression free survival of 8 to 13 months.[60]

The results of combining targeted therapy and immunotherapy agents to double chemotherapy regimens provided a significant and clinically relevant improvement in antitumor activity compared with chemotherapy alone.[48,60]

1.1.6. Novel Compounds under development

Despite intensive research and enormous progresses, the treatment of lung cancer is still challenging, particularly in metastatic disease.[61] As such, novel compounds continue to be developed, with some of them showing promising results.

Pyrimethamine, a folic acid antagonist, with capability to indirectly block DNA synthesis[61] and proven antitumor activity in melanoma[62] and prostate cancer[63], was used by Lin *et al.* to access its antitumor effect on lung cancer *in vitro* using A549 cells, and *in vivo*, using BALB/c Nude mice.[61] *In vitro* results showed an increase of apoptosis and cell cycle arrest.[61] *In vivo,* pyrimethamine, induced a decrease of tumor volume, demonstrating the compound-mediated suppression of tumor growth.[61]

Another approach considered for the development of novel compounds with anti-tumor activity is the use of natural plant products.[64] Natural products are considered an important source of chemotherapeutical drugs, since around 59% of commercially available cancer drugs are direct or indirectly originated from natural sources.[64]

From that point of view, cucurbitacins, i.e. derivatives of *Cucurbitaceae* family, and their byproducts have a proven cytotoxic effect in some types of cancer such as breast and prostate cancer.[64]

A new semisynthetic derivative of cucurbitacin B, the 2-deoxy-2-amine-cucurbitacin E, was used by Silva *et al.* to evaluate their potential in lung cancer.[64] In that study, the cytotoxic potential of this compound was demonstrated both *in vitro* and *in vivo*, using A549 cells and a transgenic mouse lung cancer model, respectively.[64] The results showed in both conditions anti-proliferative and apoptotic activity, suggesting that the compound can be considered a promising lead compound for the development of an anti-lung cancer drug.[64]

One of the problems of EGFR mutated lung tumors is the eventual development of resistance to the first-generation EGFR inhibitors due to mutations, despite the initial positive response to the therapy.[65] As previously mentioned, in 50 to 60% of the cases the resistance is due to the T790M mutation.[20] EGF816 is a novel, irreversible mutant-selective third generation EGFR TKI with the capacity to specifically target EGFR-activating mutations, while sparing wild-type EGFR.[65] A study performed by *Jia et al.*, using several *in vitro* and *in vivo* models, showed that EGF816, in fact, inhibited the most common EGFR mutations, with minimal inhibition of wild-type EGFR.[65] The results translated into strong tumor regressions *in vivo* and provided basis for phase I/II clinical trials in lung cancer patients with EGFR mutations.[65]

1.2. Aims

Despite the existence of several therapeutic options, the overall cure and 5-year survival rates of lung cancer patients remain low[36]. This highlights the urgent need for the development of new anticancer agents in order to improve clinical outcomes in the deadliest cancer worldwide.

In this context, the main objective of this work was to perform a pre-clinical evaluation of the anti-tumorigenic potential of a set of novel chemical compounds for the treatment of lung cancer. To accomplish this goal, different lung cancer cell models were used, and the following specific aims pursued:

1. Perform an initial drug screening;
2. Determine the IC_{50} value of the most active compounds;
3. Conduct a structure/activity relationship (SARS) study;
4. Investigate the molecular mechanisms underlying the effect of the two most promising compounds, namely impact on cell cycle progression and cell death.

2. Material and Methods

2.1. Cell lines and culture conditions

The ability to maintain cells in culture is very important since it allows to perform functional studies and evaluate cell behavior is the presence of external factors, such as an artificial compound or a new drug. Thus, it will be imperative for this work.

In this work, two well characterized human tumor cell lines from lung cancer, with different characteristics were used.

The human lung carcinoma cell line A549 (already existent in the group) and human mucoepidermoid carcinoma cell line H292 (DSMZ) were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen). DMEM medium was supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Invitrogen) and 1% penicillin/streptomycin (HyClone). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

2.2. Cell treatment

Compounds stock solutions were prepared in sterile Dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Prior to all treatments, cells were allowed to adhere for 24 hours and then exposed to test compounds diluted in culture medium for 48 hours. All experiments were performed in parallel with untreated control (only cells and the corresponding culture medium), DMSO drug vehicle control (0.1% (v/v)), death control (cells treated with a high concentration of DMSO that leads to cell death, 23% (v/v)) and apoptosis control (100 nM of Staurosporine (STS)).

2.3. Microscopic Observations

Images of the cells after 48 hours of treatment with test compounds were obtained with inverted microscope Nikon Eclipse TS100 and Nikon Digital Sight DS-Fi1 camera. The images were obtained at different magnifications (5x, 10x and 20x)

2.4. Cell viability assay

Cell viability assays allow the evaluation of the effect of the test compounds on cell responses, namely cell proliferation or toxicity that can lead to cell death. To access cell viability, we performed the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, a coulometric assay. This

assay is based in the capacity of the mitochondrial enzymes to cleave the yellow tetrazolium salt, MTT, into a purple colored formazan.[66] So the amount of formazan produced is directly proportional to the number of living cells, once the dead cells lose the capacity to metabolize MTT.[66] Normally, formazan is accumulated inside the cells as a precipitate that needs to be dissolved with DMSO or other solubilization methods, prior to absorbance readings at 570 nm.[67]

In order to select the best compounds with anti-tumorigenic activity from a large group, an initial screening was performed by testing the same concentration (10 μM) of the different test compounds in lung cancer cell models. Then, the compounds that induced lower cell viability were selected and their concentration that inhibited cell growth by 50 (IC_{50} value) was determined by a dose-response curve using GraphPad Prism 7.0 software (San Diego, CA, USA). The test compounds with lower IC_{50} value were selected to further studies.

To perform the MTT assay, cells were seeded in 96-well plate at a density of 7.500 cells/well in A549 and H292 cell lines. After treatment with test compounds for additional 48 hours cell viability was assessed. The culture medium was removed and replaced with fresh culture medium containing 0.5 mg/ml of MTT. After 2 hours of incubation at 37°C and 5% of CO_2 (protected from light), the media was carefully removed, and the intracellular formazan crystals were dissolved in a solution of DMSO and 2-Propanol in a ratio of 1:1 and transferred to a new well. Absorbance was measured at 570 nm using microplate reader SYNERGY H1 (Biotek). Each condition was tested in triplicate in a single experiment which was repeated independently at least in three times.

2.5. Flow cytometry

Recently, through the screening of novel compounds it became essential and important to study their effects on living cells.[68] Flow cytometry has the capacity to detect and efficiently sort cells based on fluorescent signals that were combined with cellular marks of proteins or DNA. This technique can be used to isolate cells and to access apoptosis or cell viability.[66]

The main advantage of this technique is the fact that it can analyze and distinguish multiple cell populations within a heterogeneous sample. In this technique, after the sample being sucked inside of the flow cytometer it is mixed with Phosphate buffered saline (PBS) and lead through a narrow channel where the cells are lined in a single row that allows each single cell to be analyzed individually

in the interrogation point. Here the cells pass through a laser beam, that scatters in multiple directions. The flow cytometer can detect the scattered light in two directions: forward scattered light by a detector located in front of the beam laser and cell and side scattered light by a detector located parallel to the laser beam and cell. The forward scattered light signal is proportional to the cell size and the side scattered light signal is proportional to the cell shape and internal complexity. So, by the analysis of both signals is possible to understand the size, shape and internal complexity of the cell, allowing to divide a heterogeneous cell population into different groups. Additionally, the flow cytometer has the capacity to detect light emitted by excited fluorescent molecules, e.g. monoclonal antibodies fluorescently labeled or fluorescent dyes.

In this work, this method was used to analyze the effect of the compounds on the cell cycle distribution and cell death.

2.5.1. Cell Death Evaluation

Dead cells can be distinguished from viable cells in flow cytometry by the alteration of the light scatter properties, since they show an decrease in forward scatter signal and an increase in the side scatter signal. Though, these differences are not sufficient to distinguish death cells from viable cells, so the use of fluorescent indicators of cell membrane integrity has been important to achieve that goal.[69]

During the process of cell death one of the early changes on the cell occurs on the membrane by the translocation of the phosphatidylserine (PS), a negatively charged phospholipid, from the inner side of the plasma to the external layer. Annexin V is a protein that binds to phospholipids and has a high affinity to PS. However, the PS translocation is not an exclusive characteristic of apoptosis occurring also during the necrosis process. One of the biggest differences between apoptosis and necrosis is that during apoptosis the cell membrane integrity is maintained, this way the use of another fluorescent dye is necessary. 7-Amino-Actinomycin (7-AAD) is a fluorescent dye with high affinity with DNA, more specifically a G-C base specific intercalator. This dye can only reach the DNA if the cell has lost membrane integrity.[70,71]

The combination of Annexin V and 7-AAD allows to distinguish between early apoptosis, late apoptosis and necrosis.

A549 and H292 cells were plated in 6-well plates (75.000 cells/well) for 24 hours. After 48 hours of incubation with test compounds at the IC_{50} concentration, the culture medium of each condition containing potential death cells was collected and centrifuged at 2500 rpm for 10 minutes. Meanwhile, the adherent cells were trypsinized, centrifuged at 1200 rpm 5 minutes, and washed twice with PBS. After final centrifugation, the supernatant was discarded, and the death cell pellet was resuspended in 100µl of 1x Annexin V Binding buffer (eBioscience™), and then transferred into the corresponding tube of adherent cells pellet. Subsequently, 100 uL of cell suspension were mixed with 5µl of 7-AAD (eBioscience™) and 5µl of Annexin V-PE (eBioscience™). Following the staining procedure, sample acquisition and data analysis of at least 80.000 events per sample were performed using LSR II Flow Cytometer System (BD Bioscience) and BD FACSDIVA™ software. The results were then treated with FlowJo (Tree Star).

2.5.2. Cell Cycle distribution analysis

Cells at different phases of the cell cycle can be distinguished by its DNA content, since that at different stages of the cell cycle the quantity of DNA varies. During G_1 phase, cells are diploid, that is contains two copies of each chromosome (referred as 2n). In S phase, since DNA replication occurs the quantity of DNA increases from 2n to 4n, this way in this phase the DNA content can fluctuate between 2n and 4n. Lastly, during G_2 and M phases the DNA content stays at 4n, decreasing to 2n after cytokenesys.[72] This way the different cell cycle phases can be distinguished by Fluorescent-activated Cell Sorter (FACS), by the use of a fluorescent dye that binds to DNA. In this work, the fluorescent dye used was Propidium Iodide (PI), a fluorescent DNA intercalating dye.

A549 and H292 cells were plated in 6-well plates (75.000 cells/well) for 24 hours. After treatment with the test compounds at the IC_{50} concentration for additional 48 hours, adherent cells were trypsinized, centrifuged at 1200 rpm during 5 min at 4°C, and washed twice with PBS at 4°C. Subsequently, the supernatant was discarded, and the cell pellets were resuspended in a solution of 80% ice cold ethanol drop by drop, while vortexing gently. Samples were stored overnight at 4°C until data acquisition. For cell cycle analysis, cells were centrifuged again at 2000 rpm for 5 min at 4°C, washed twice with PBS, and the cell pellets were resuspended on 100 µl of PBS containing 50 µg/mL of PI and 100 µg/mL of RNase (Invitrogen) and further incubated for 30 minutes at room temperature.

Sample acquisition and data analysis were performed using: LSR II Flow Cytometer System (BD Bioscience) and BD FACSDIVA™ software (BD Bioscience), with the acquisition of at least 80.000 events/sample. The results were then treated with FlowJo (Tree Star).

2.6. Western Blot

The Western Blot is a semi-quantitative technique that allows separation identification of specific proteins from a complex mixture. Proteins are separated is based on the molecular weight through gel electrophoresis.[73] In this work, this method will be used to observe the signaling pathways that may be affected in treated versus non-treated tumor cells. This will allow the comparison of the levels of activation or silencing of the signaling pathway on both conditions To address this, the levels of protein phosphorylation can be determined, since this is a fundamental mechanism in cell signaling[74]. Also, it is possible to evaluate the protein expression levels of a given target.

A549 and H292 cells were plated in 6-well plates (100.000 cells/well) for 24 hours. After 48 hours of incubation with test compounds, cells were washed with PBS and lysed in cold lysis buffer, containing RIPA Buffer (50 mM Tris-HCl pH=8, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate (SDS), 150 nM NaCl and 5 mM Ethylenediamine tetra-acetic acid (EDTA)) and protease and phosphatases inhibitors (1x Complete, 10 mM NaF, 1 mM Na_3VO_4 and 1 mM $Na_4P_2O_7$) for 5 minutes on ice. After scraping cell monolayers, the cell suspensions were centrifuged at 14000 rpm for 10 minutes at 4°C. Quantification of total protein content of each cell lysate was performed using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

Equal amount of total cell lyates (20 µg) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), after the addition of elution buffer (Laemmli 2x (Bio-Rad) and β-mercaptoethanol 5% (v/v)) to each sample and performing a denaturing step (98°C, 5 minutes). After electrophoresis, the separated proteins were transferred for 1 hour 20 min at 400 mA to nitrocellulose membranes.

In order to evaluate the expression levels of p-ERK, Caspase3, CDC25c, p-P38 and p53, membranes were blocked in a solution of 5% (w/v) non-fat milk in PBS with 0.1% Tween 20 (PBS-T) or 5% (w/v) BSA in TBS with 0.1% Tween 20 (TBS-T) for one hour at room temperature, under agitation. Then, the membranes were incubated overnight at 4°C under agitation with: anti-p-ERK (1:500, Rabbit,

Cell signaling), anti-Caspase 3 (1:500, Mouse, Santa Cruz Biotechnology Inc.), anti-CDC25c (1:250, Mouse, Santa Cruz Biotechnology Inc.), anti-p-P38 (1:500, Mouse, Santa Cruz Biotechnology Inc.) and anti-P53 (1:500, Mouse, Santa Cruz Biotechnology Inc.). The membranes were then washed with PBS-T or TBS-T three times for ten minutes, under agitation at room temperature and incubated with anti-Rabbit (1:3.000, Cell Signaling) and anti-Mouse (1:2.000, Cell Signaling) antibodies during one hour at room temperature, under agitation. After washing, the membranes were developed using the ECL Prime Western Blotting Detection System (GE Healthcare).

For loading control analysis, membranes were probed with monoclonal anti-GAPDH (1:5.000, Mouse, Santa Cruz Biotechnology Inc) with a secondary antibody anti-Mouse (1:2.000, Cell Signaling).

Intensity of the bands was quantified with the aid of Quantity One software (Bio-Rad Laboratories, Inc).

2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.0 software (San Diego, CA, USA).

All experiments were performed independently and at least two times. Data of all experiments was analyzed as means ± SEM. Results were evaluated using a non-parametric one-way ANOVA followed by a Dunnet's post-test. Statically differences were considered significant when p-values <0.05 ($^{*}p<0.05$ and $^{**}p<0.01$). The IC_{50} value was determined using a by a dose-response curve, where a relationship between the concentration of the inhibitors (in logarithm) and their response in cellular viability was established.

3. Results and Discussion

3.1. Biological evaluation: first-screening

The potential of this family of chemical compounds as anti-cancer agents for the treatment of lung cancer was firstly evaluated in two distinct NSCLC cell lines, A549 and H292, using the MTT assay. This quantitative colorimetric method is used to evaluate cell viability upon treatment with a given substance. Thus, the most active compounds will induce greater cell viability loss. Importantly, all compounds tested herein have the same central core and two substituent groups (R and R1) which can vary (Fig. 10). As such, at the same drug concentration, a structure/activity relationship can be established and differences in cell viability reveal the effect of divergent substituents.

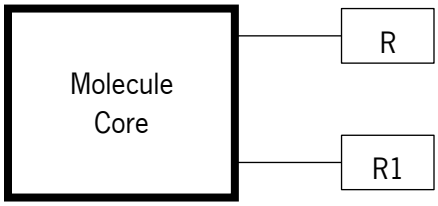


Figure 10. **Central structure of compounds with two substituent groups: R and R1**.

In this work, 11 distinct family-related compounds were evaluated, and cell viability assessed 48h post-treatment at a concentration of 10 μM. As indicated in Table 1, some compounds induced similar cell viability in both cell lines: compounds C1, C6, C7, C8 e C11.

Compounds C5, C6 and C7 have the same R substituent group. In compounds C6 and C7, the R1 substituent group is different, Ph and Furyl, respectively, however that does not lead to differences in cell viability between the two cell lines and even among compounds. In C5 the presence of 3-BrC_6H_4 in substituent group R1 increases cell viability in both cell lines, being the increase more perceptible in A549 cells. Other examples are compounds C9 and C10, in which the presence of different groups R leads to an increase of cell viability on H292 cells.

Other compounds that induced different cell viability on the different cell lines are compounds C2, C3, C4. These compounds induce low cell viability in H292 cells, between 7.10% and 17.34%, indicating that the presence of Ph in substituent group R can have great importance in the compound's activity on this cell line. However, in A549 cells the same compounds induced a higher cell viability, between 12.93% and 38.49%. This shows that different groups present in R1 influence cell viability on this cell line.

Table 3. **Cellular viability of A549 and H292 cells after 48h of treatment with the indicated compounds at 10μM**. Each value is the mean ± SD of at least three independent experiments

			Cell Viability (%) (10 μM)	
Compound	**R1**	**R**	**A549**	**H292**
C1	3-BrC_6H_4	Ph	29.70±7.92	30.87±11.44
C2	Ph	Ph	12.93±2.91	7.10±1.65
C3	4-FC_6H_4	Ph	22.80±0.46	8.13±5.45
C4	Furyl	Ph	38.49±3.89	17.34±6.07
C5	3-BrC_6H_4	4-$CF_3OC_6H_4$	54.40±7.14	42.83±12.52
C6	Ph	4-$CF_3OC_6H_4$	28.20±9.62	28.23±9.12
C7	Furyl	4-$CF_3OC_6H_4$	24.50±9.59	26.17±3.94
C8	Ph	3,4-$Cl_2C_6H_3$	50.67±6.14	52.53±4.67
C9	Furyl	4-$MeOC_6H_4$	38.87±7.26	20.79±7.39
C10	Furyl	4-MeC_6H_4	37.54±17.02	30.77±2.10
C11	Furyl	3-$MeOC_6H_4$	41.95±17.65	40.12±4.88

3.2. Determination of IC_{50}

After the initial screening, compounds inducing a cell viability lower than 40%, were further investigated and their IC_{50} determined. This included a total of 8 compounds, i.e. excluding compounds C5, C8 and C11.

In order to determine the IC_{50} values, the previously selected compounds were incubated for 48h at a defined range of concentrations that were then transformed in a dose-response curve, which allowed the determination of the IC_{50} value (Table 4).

Table 4. **IC_{50} value of the selected compounds in A549 and H292 cell lines.** Values result from at least three independent experiments.

			IC_{50} Value (μM)	
Compound	**R1**	**R**	**A549**	**H292**
C1	3-BrC_6H_4	Ph	3.281	1.994
C2	Ph	Ph	3.294	2.508
C3	4-FC_6H_4	Ph	1.999	1.722
C4	Furyl	Ph	6.860	3.896
C6	Ph	4-$CF_3OC_6H_4$	1.723	0.867
C7	Furyl	4-$CF_3OC_6H_4$	5.473	1.069
C9	Furyl	4-$MeOC_6H_4$	4.595	3.717
C10	Furyl	4-MeC_6H_4	4.538	2.200
Cisplatin	-	-	5.000[75,76]	4.000[77]

Comparing the results of the IC_{50} values summarized in Table 4, it is possible to observe that the IC_{50} value of all compounds was lower in H292 cell line than in A549 cell line. This means that the compounds are more active against H292 cell line than against A549 cell line. In order to discuss the structure activity relationship (SAR), and a more comprehensive analysis of the results, compounds were divided in four categories or classes of activity (Table 5):

i) IC_{50}<2 μM: Extremely active compounds (+++)

ii) 2<IC_{50}<4 μM: Very active compounds (++)

iii) IC_{50} >4: Moderately active compounds (+):

iv) IC_{50} not determined as cell viability was higher than 40%: Not active compounds (-).

Table 5. **Classification of activity of the compounds.**

			Class of activity	
Compound	**R1**	**R**	**A549**	**H292**
C3	4-FC_6H_4	Ph	+++	+++
C6	Ph	4-$CF_3OC_6H_4$	+++	+++
C1	3-BrC_6H_4	Ph	++	+++
C7	Furyl	4-$CF_3OC_6H_4$	+	+++
C2	Ph	Ph	++	++
C4	Furyl	Ph	+	++
C10	Furyl	4-MeC_6H_4	+	++
C9	Furyl	4-$MeOC_6H_4$	+	++
C5	3-BrC_6H_4	4-$CF_3OC_6H_4$	-	-
C8	Ph	3,4-$Cl_2C_6H_3$	-	-
C11	Furyl	3-$MeOC_6H_4$	-	-
Cisplatin	-	-	+[75,76]	+[77]

Considering this classification, compound C3 and C6 are extremely active against the two cell lines. Compounds C1 and C7 are also extremely active in H292 cell line. Compound C2 is very active against both cell lines, compound C1 is very active against A549 cell line and compounds C4, C9 and C10 are very active against H292 cell line. Compounds C4, C7, C9 and C10 are considered moderately active in A549 cell line. The influence of each substituent on compound's activity is discussed below.

Compounds C5, C6 and C7, have the same R substituent group and different R1 substituent groups. Compound C5 is not active in A549 and H292 cell lines, C6 is extremely active in the two cell lines and C7 is moderately active in A549 cell line. These results allow us to conclude that R1 substituent group has high influence in the activity of these family-related compounds. The same conclusion may be achieved from the results obtained with compounds C1, C2, C3 and C4, which have the same R substituent group and different R1 substituents.

When R1 substituent group is a Ph and R group is variable, which happens in compounds C2, C6 and C8 (Tables 3, 4 and 5), compounds are very active, extremely active and not active against A549 and H292 cell lines, respectively, so it is possible to say that the R group has also impact in the level of compound´s activity. Additionally, comparing the level of activity of compounds C2 and C6,

since compound C6 has higher activity than C2 this means that the group 4-$CF_3OC_6H_4$ as R is very important for the compound´s activity.

Compounds C4, C7, C9 and C10, have a furyl group as R1 and Ph, 4-$CF_3OC_6H_4$, 4-$MeOC_6H_4$, 4-MeC_6H_4 as R, respectively. These compounds show moderate activity in A549 cells, maintaining a very high or extremely high activity in H292 cells. This result seems to indicate that the furyl group is important for high activity in the H292 cell line however it seems that it is not favorable for activity against the A549 cell line.

Finally, comparing the most active compounds in both cell lines, C3 and C6, both compounds have different substituent groups as R and R1, however both have a Ph group as substituent, which seems to indicate that the Ph group, in either position, has an important role in the compound´s activity.

Since, compounds C3 and C6 have the highest level of activity in both A549 and H292 cancer cells, they were selected for further studies.

3.3. Analysis of compounds effect on cell morphology

The morphology of A549 and H292 cells was analyzed after 48h of incubation with compounds C6 and C3 at IC_{50} value. For control purposes, cells were also left untreated i.e, incubated with culture medium only or treated with 0.1% DMSO (as a drug vehicle control), as negative controls. As a positive control or death control, cells were incubated with 100nM Staurosprine (STS), a known inducer of apoptosis in an extensive range of cell lines.[78]

In control conditions, the morphology of A549 cells, was found to be normal, with cells growing adherently as a monolayer with close cell-to-cell contacts and displaying typical epithelial characteristics (Figs. 11A and 11B). In addition, in both negative control conditions, a high cell density was observed, together with a few unattached cells. However, when incubated with the compound C6 at the IC_{50} concentration, cells changed their morphology from cuboid to a more a long spindle-shape (Fig. 11C). Cell density appears to be also affected by the presence of the compound as evidenced by the reduced number of cells present (Fig. 11C). On the other hand, for compound C3, A549 cell morphology remained unaltered and cell confluence similar to that observed in the negative conditions (Fig. 11D). These results indicate that A549 cells are more sensitive to compound C6 exposure rather than

compound C3 treatment. As expected, cells incubated with STS exhibited severe changes in their shape and morphology, with cells retracting and becoming round-shaped. This is in agreement with previous reports.[79]

Regarding the H292 cell line, in control conditions, cells showed a classical epithelial morphology and high cell density, despite the presence of some unattached cells (Figs. 12A and 12B). Upon incubation with compounds C6 (Fig. 12C) and C3 (Fig. 12D) at the respective IC_{50} concentration, both cell morphology and density remained unaltered, in contrast to what was observed with compound C6-treated A549 cells (Fig. 11C). However, in the presence of STS, H292 cell morphology changed dramatically (Fig. 12E).

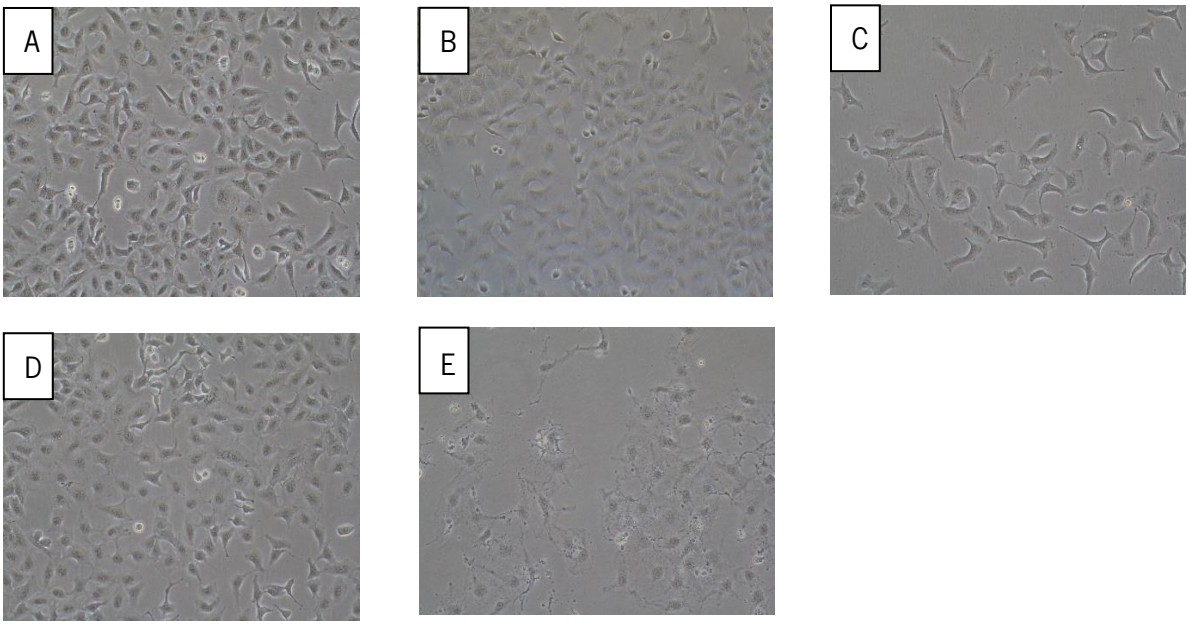


Figure 11. **Cellular Morphology of A549 cells**. Images above were obtained with an inverted microscope Nikon Eclipse TS100 and Nikon Digital Sight DS-Fi1 camera and 10x Magnification. (**A**) Untreated Control, (**B**) Vehicle Control, (**C**) C6 at IC_{50} concentration, (**D**) C3 at IC_{50} concentration and (**E**) Death Control.

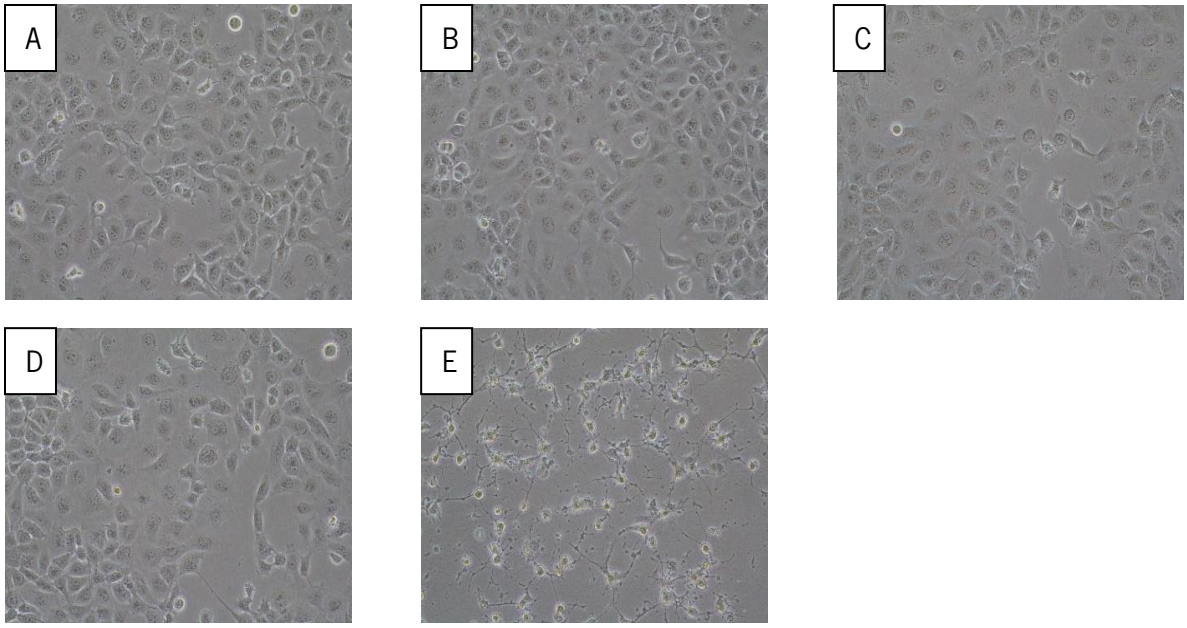


Figure 12. **Cellular Morphology of H292 cells.** Images above were obtained with an inverted microscope Nikon Eclipse TS100 and Nikon Digital Sight DS-Fi1 camera and 10x Magnification. (**A**) Viability Control, (**B**) Vehicle Control, (**C**) C6 at IC_{50} concentration, (**D**) C3 at IC_{50} concentration and (**E**) Death Control.

3.4. Impact on Cell Death

To access the mechanisms underlying compound's mediated loss of cell viability, their impact on cell death was evaluated by flow cytometry. Two fluorescent indicators of membrane integrity[69] were combined: Annexin V which detects cells undergoing apoptosis and the live/dead dye 7-Amino-Actinomycin (7-AAD) which binds to DNA if the cell membrane has been disrupted.[70,71] So, Annexin V in conjunction with 7-AAD allows to distinguish early apoptotic cells from dead cells.

As depicted in figure 11A, A549 cells treated for 48h with compounds C6 and C3 were differentiated among viable (Annexin V and 7-AAD negative cells, located in the lower left quadrant-Q4), early apoptotic (Annexin V positive and 7-AAD negative cells, located in lower right quadrant-Q3), and late apoptotic (Annexin V and 7-AAD double positive cells, located in the upper right quadrant-Q2). Cells located in the upper left quadrant-Q1 and thus staining positive for 7-AAD but negative for Annexin V are also considered dead cells, but undergoing necrosis, a distinct death process. Of note, cells were incubated with compounds both at IC_{50} and $2xIC_{50}$ values to investigate a possible dose-response effect. Incubation of cells with 0.1% DMSO was also performed and used as a control since during the

optimization process of this assay no differences were observed in the cellular distribution between untreated cells and cells exposed to 0.1% DMSO. As positive or death control, cells were incubated with 100nM of STS, a well-accepted inducer of apoptosis.

Results shown in figure 13A are representative of one experiment, whereas bar charts presented in Figure 13B display the mean of three independent experiments. As expected, in control conditions the vast majority (78.6%) of A549 cells are viable. Treatment with compounds C6 and C3 at IC_{50} equitoxic concentrations increased the percentage of early apoptosis from 14.3% to 19.2% and 19.4% respectively and late apoptosis from 6.1% to 23.1% and 17.1% respectively (Fig. 13B), indicating that both compounds can induce apoptosis of A549 lung cancer cells, although no statistical significance could be detected. In addition, a dose-response, albeit moderate, was observed when a $2xIC_{50}$ concentration was used, particularly for late apoptosis (Fig. 13C). Still, results suggest that both compounds C6 and C3 are not strong inducers of apoptosis (1.8 and 1.4-fold increase for early apoptosis and 3.8 and 2.8-fold increase for late apoptosis, respectively), in comparison with STS (1.8 and 9.4-fold increase for early apoptosis and late apoptosis, respectively) (Fig. 13B). A residual percentage of necrotic cells was detected in the presence of both compounds, which was statistically significant ($p<0.05$) for compound C6 at its $2xIC_{50}$ value when compared with control conditions (3.6% vs 1%, respectively) (Fig. 13C).

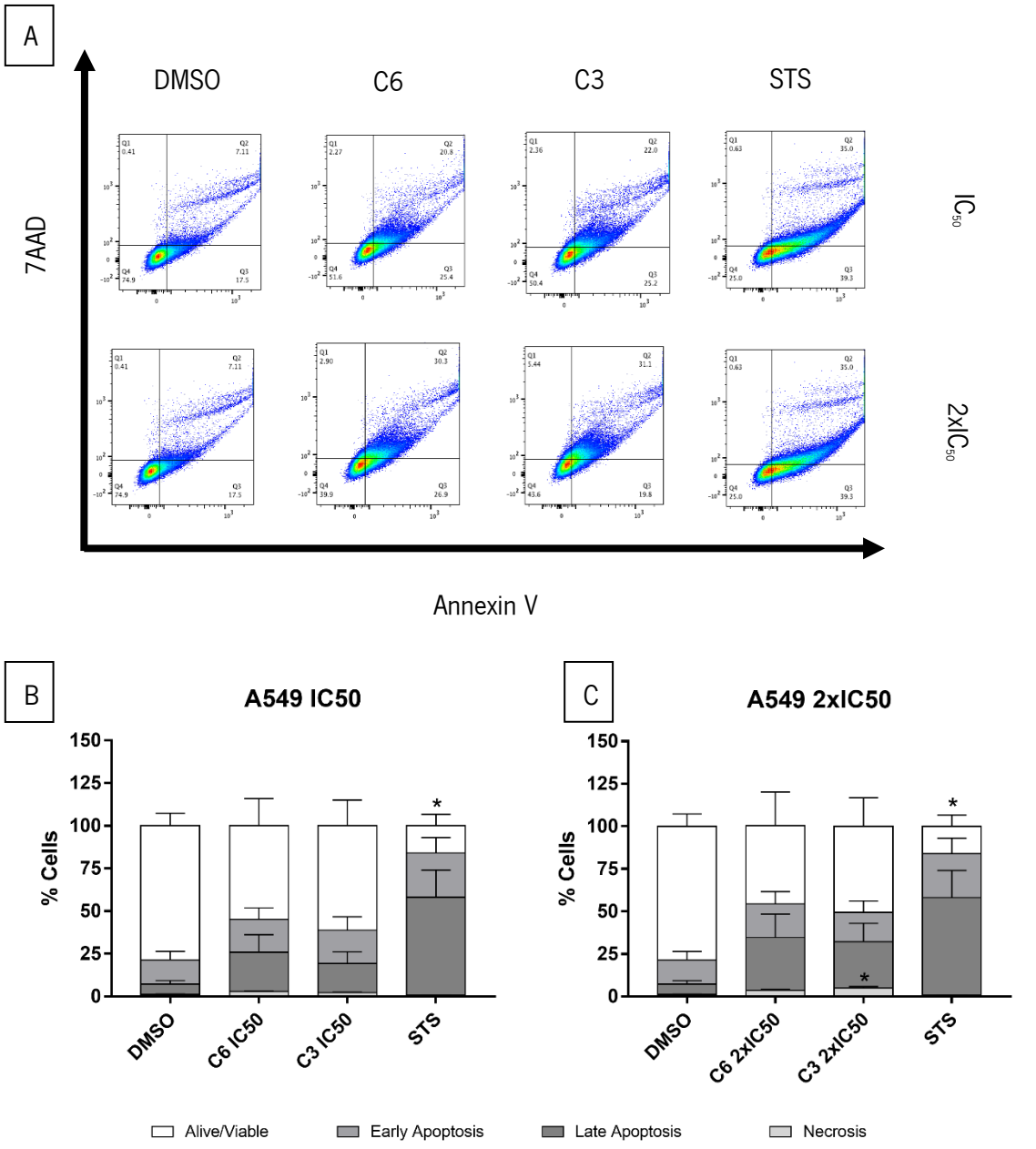


Figure 13. **Compounds C6 and C3 show a tendency to induce apoptosis of A549 cells**. Evaluation of apoptosis using Annexin V and 7AAD assay, following cell incubation with compounds C6 and C3 at equitoxic concentrations (IC_{50} and $2xIC_{50}$) or DMSO (Vehicle Control) for 48h. Representative flow cytometry profiles (**A**) and respective quantification (**B**) and (**C**). Data represent mean ± SEM of at least three independent experiments. *p<0.05 and **p<0.01 analyzed by one-way analysis of variance (ANOVA) followed by a Dunnet's post-test.

Analyzing the data gathered with H292 cells (Fig. 14), it is possible to observe that in comparison to control conditions, the percentage of early apoptotic cells, with compounds C6 and C3, increased from 10.4% to 17.6% and 21.1%, respectively and late apoptotic cells from 8.2% to 15.5%

and 22.3%, respectively (Fig. 14B), although no statistically significance could be detected. This suggests that, alike A549-treated cells, both compounds show a tendency for inducing the apoptosis of H292 lung cancer cells. Remarkably, a dose-response effect was again detected but only for late apoptosis, as the percentage of cells on early apoptosis does not change when doubling the concentration of either compounds (C6: IC_{50} 17.6%, 2xIC_{50} 18.5 %; C3: IC_{50} 21.1%, 2xIC_{50} 19.1 %). Furthermore, the effect on late apoptosis is more pronounced with compound C3 treatment than with compound C6 (2.6 and 4.8-fold, respectively), with compound C3 showing a significant ($p<0.05$) increase at 2xIC_{50} (Fig 14C). In fact, the percentage of the percentage of total dead cells induced by C3 at 2xIC_{50} is close to that induced by STS (62.5% vs 70.8%), although differences can be seen when analyzing the different stages of apoptosis (19.1% and 39.5% for C3 vs 10.6% and 56.9% for STS, in early and late apoptosis, respectively) (Fig. 14B). Again, the percentage of necrotic cells in the presence of compounds C6 and C3 is negligible (Fig 14B and 14C).

Comparing the results obtained with both cell lines, it is possible to conclude that, once again, A549 cells are more sensitive to compound C6 whereas compound C3 has a higher effect in H292 cells. Although C6 triggers slightly different levels of early apoptotic cells in both cell lines (1.3-fold increase for A459 vs 1.7-fold for H292) at IC_{50} values, its effect on late apoptosis doubles in A549 compared to H292 (1.9-fold vs 3.8-fold). On the other hand, no considerable differences are observed for late apoptosis induction upon treatment with C3 at IC_{50} values in both cell lines (2.8-fold increase for A549 vs 2.7-fold for H292), however a stronger response is found for early apoptosis in H292 (1.4-fold increase for A549 vs 2-fold for H292). Yet, further experiments are necessary to statistically validate these data and thus confirm these assumptions.

Induction of apoptosis is the basis for most chemotherapeutic drugs. Cisplatin, a platinum agent used as a first-line therapy in lung cancer, has an anti-tumor effect that results from DNA damage induced apoptosis.[80] In a study where A549 cancer cells were incubated with cisplatin at its IC_{50} value, results showed an increase on early and late apoptotic cells, being the increase stronger on early apoptosis.[76] However, high toxicity and drug resistance frequently limit the response to platinum-based therapy. Thus, novel anticancer agents have been screen and shown to improve the proapoptotic effect of cisplatin or overcome cisplatin resistance, like tetrandrine, a bis-bezylisoquinoline[81] and sanguinarine, a phenanthridine analog[82]. More importantly, it was also shown that combining two

different compounds triggering apoptosis via different mechanisms resulted in improved efficacy, which was further enhanced through the addition of a third apoptosis-inducer agent, TRAIL.[82] Taking this and the results described above into consideration, it can hypothesized that drug combination experiments involving C6 and C3, as well as a cisplatin or other chemotherapeutic drug may result in improved efficacy, thus warranting further analysis.

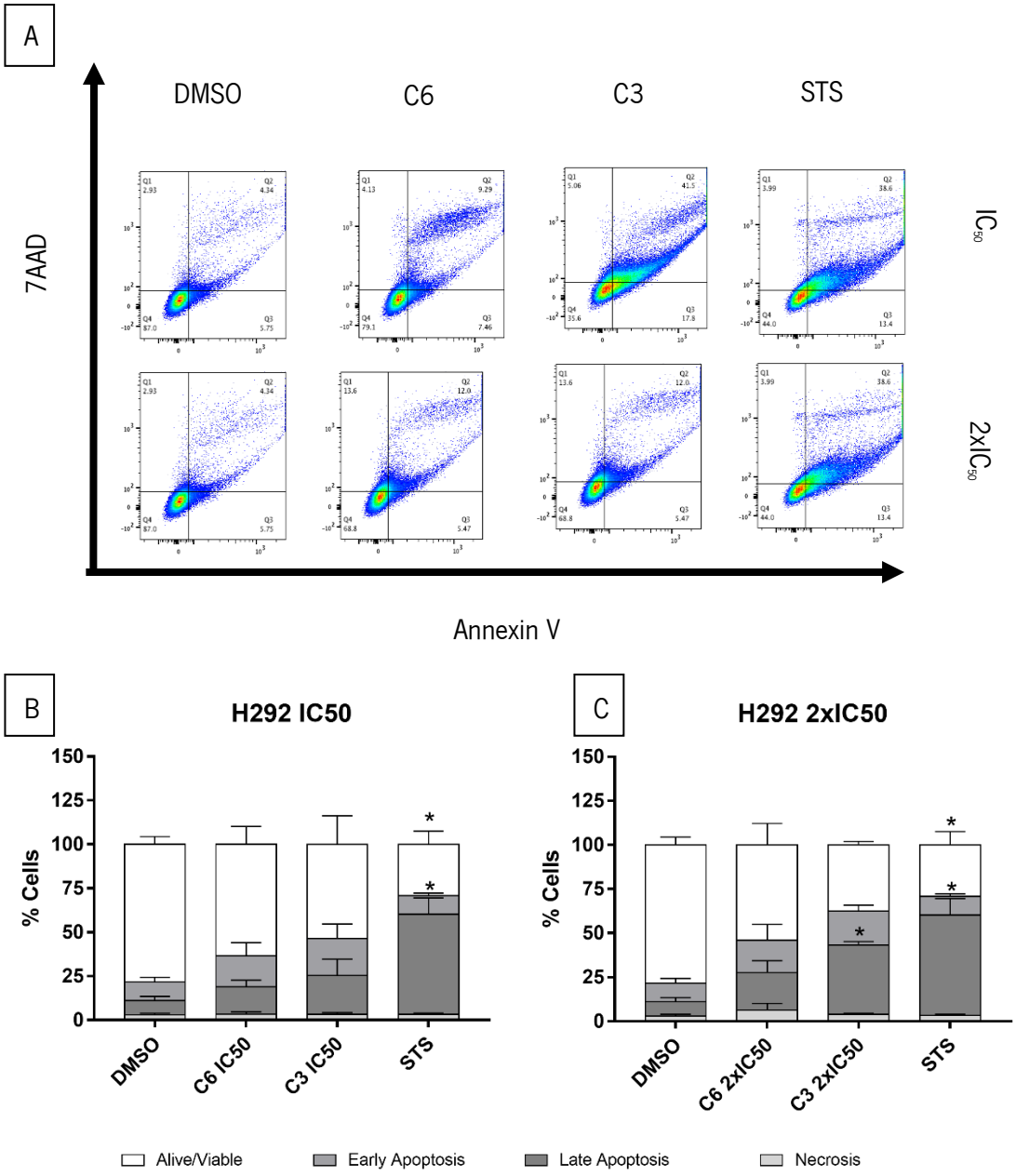


Figure 14. **Compounds C6 and C3 show a tendency to induce apoptosis of H292 cells**. Evaluation of apoptosis using Annexin V and 7AAD assay, following cell incubation with compounds C6 and C3 at equitoxic concentrations (IC_{50} and $2xIC_{50}$) or DMSO (Vehicle Control) for 48h. Representative flow cytometry profiles (**A**) and respective quantification (**B**) and (**C**). Data represent mean ± SEM of at least three independent experiments. *p<0.05 and **p<0.01 analyzed by one-way analysis of variance (ANOVA) followed by a Dunnet's post-test.

3.5. Impact on cell cycle distribution

In order to investigate the influence of compounds C6 and C3 on cell cycle progression, A549 and H292 cells were treated for 48h with both drugs at IC_{50} values, and cell cycle distribution measured by flow cytometry. Different phases of cell cycle, G_1, S, and G_2/M can be evaluated by its DNA content using propidium iodide (PI), a fluorescent DNA-intercalating dye. For control proposes, cells were incubated with 0.1% of DMSO. During the optimization process of this assay no differences were observed in the cellular distribution of untreated versus 0.1% DMSO-treated cells, thus the latter were selected as negative control.

Figure 15 summarizes the results obtained for A549 cells, and includes representative histograms of one experiment, as well as bar charts with the mean of the percentage of cells in each phase of the cell cycle from four independent experiments. The left images of panel A illustrate the normal distribution of cells in the different phases of the cell cycle. The higher and first peak, highlighted in pink corresponds to G_1 phase, where cells are diploid (2N). In the S phase, highlighted in blue, DNA replication occurs (4N), whereas during G_2 and M phases, highlighted in green, cells continue to grow and undergo cell division (2N), respectively.

After 48h of exposure, compound C6 induced a significant ($p<0.05$) S-phase arrest, as detected by the presence of 24% of A549 cells, whereas only 12.9% of control cells progressed to this phase. Concomitantly, a significant ($p<0.05$) decrease of cells in G_0/G_1 phase upon compound C6 treatment was observed. In addition, the percentage of C6-treated A549 cells at G_2/M also increased 5.2% in comparison to control conditions. An equivalent effect was observed in the presence of C3 (21.7% in phase S and 26.7% in G_2/M versus 12.9% and 19.7% of cells incubated with DMSO, respectively), although no statistical significance could be detected.

The results of FACS analysis of H292 cells showed, in control conditions, 56.6% of the cells at G_0/G_1 phase, 15.5% at S phase and 25% at G_2/M phase. Incubation with compound C6 at IC_{50} revealed an increase of 4.6% and 3.7% of cells in S phase and G_2/M phase, respectively, comparing to the control. Regarding the results of compound C3 at IC_{50}, it was also observed an increase of 7.25% of cells in S phase and 3.78% in G_2/M phase in comparison with control conditions. Additionally, with both compounds was possible to observe a small decrease in the percentage of cells present on G_0/G_1

phase (7.1% and 10.2% with compound C6 and C3, respectively). In contrast to A549 cells, the differences observed with H292 cells were not statistically significant, indicating that more experiments are needed to confirm these results.

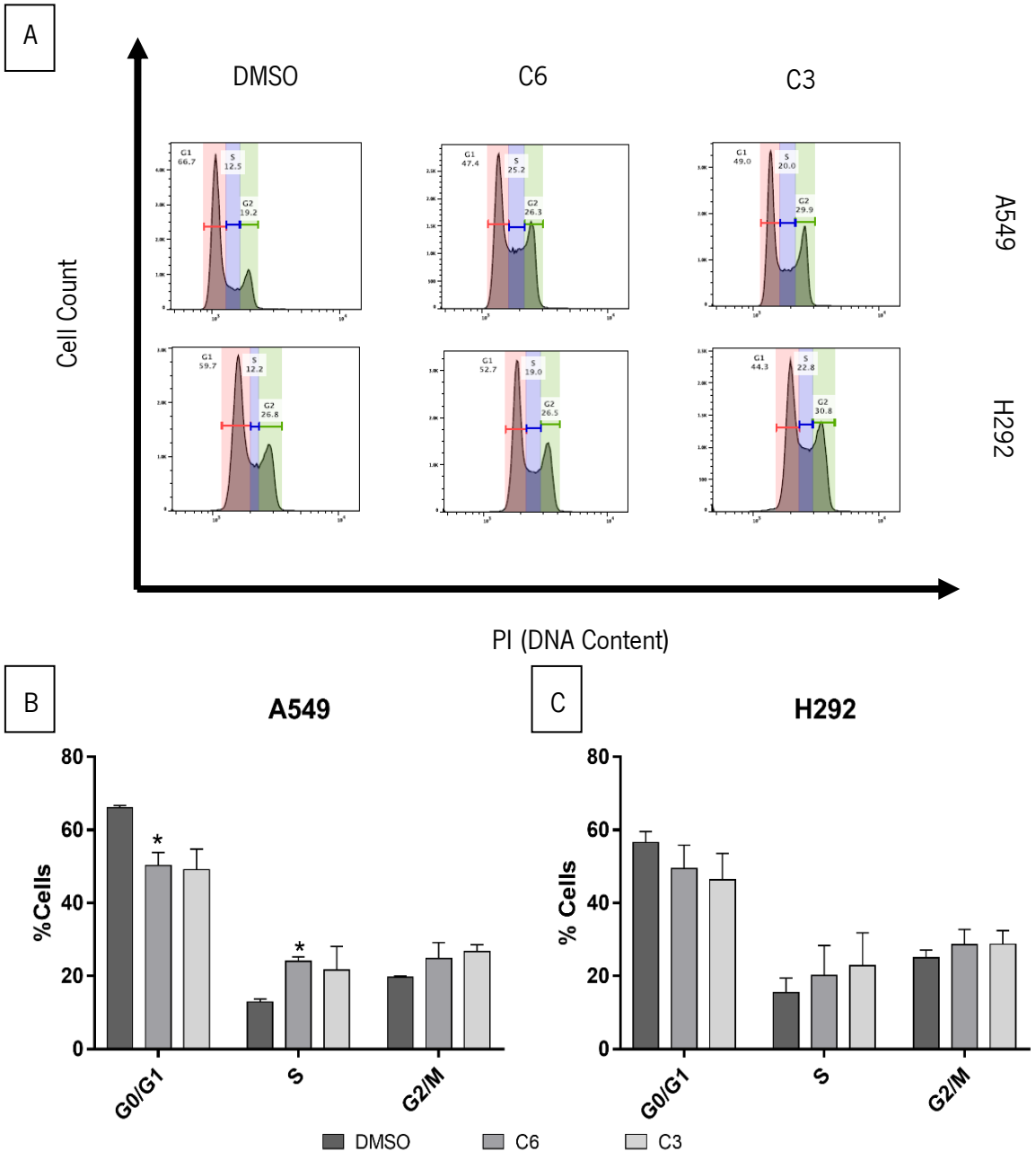


Figure 15. **Effects of compounds C6 and C3 on cell cycle progression**. A549 and H292 cells were treated with compounds C6 and C3 at equitoxic (IC_{50}) concentration or DMSO (Vehicle control) for 48 h. Cellular DNA was stained with PI, and flow cytometry analysis was performed to determine cell cycle distribution. Histograms show one representative example from three independent experiments of both cell lines (**A**) respective quantification of A549 (**B**) and H292 (**C**). Data and represent mean ± SEM of at least four independent experiments. *p<0.05 and **p<0.01 analyzed by one-way analysis of variance (ANOVA) followed by a Dunnet's post-test.

Altogether, the results suggest that compound C6 induces cell cycle arrest of A549 cells at the S phase, which is a consequence of DNA damage accumulation. A similar trend is observed for compound C3 in this cell line and for both compounds in H292 cancer cells. As such, these results suggest that both compounds interfere with DNA synthesis, maybe by introducing errors in the DNA chain, which are recognized by the cell control mechanisms, leading to an arrest of the cell cycle. The increase of cell cycle arrest in G_2/M phase, usually promoted by p53 activation in response to DNA damage, can also be linked to the increase of apoptotic cells observed in the previous assay (Figs. 13 and 14). Therefore, it can be assumed that treatment of A549 and H292 cells with these compounds affects both cell division and apoptosis, with a more pronounced effect of compound C6 in A549 cell cycle arrest at IC_{50} concentration. Some chemotherapeutic agents have also been described to interfere with cell cycle progression, namely triggering an S-phase cell arrest. One of them is Gemcitabine, as reported by Tolis *et al.*, using two NSCLC cell lines.[83] Another drug that also has an effect in cell cycle arrest is Topotecan, an inhibitor of Topoisomerase I.[84] A study by Giovannetti *et al.*, where A549 cells were exposed to Topotecan, showed an accumulation of cells in S-phase[84]. Altogether results indicate that the anti-proliferative effect of C6 and C3 is likely caused by induced cancer cell apoptosis and S/G_2/M arrest. The underlying molecular mechanisms were explored and are described below.

3.6. Impact on the expression of cell proliferation and cell death markers

To enlighten the mechanism of action of the compounds, the expression or phosphorylation levels of various molecular targets involved in cell cycle, cell proliferation and/or apoptosis were evaluated by Western Blot. A549 and H292 cells were lysed after 48h of incubation with compounds C6 and C3 at IC_{50} value. For control purposes, cells were also treated with 0.1% DMSO (as a drug vehicle control). Figure 16 summarizes the results obtained.

Considering the previous cell cycle analysis, expression of the CDC25c phosphatase, which promotes cell cycle progression and is usually expressed during G_2 to M transition[85], was evaluated. In figure 16B, it is possible to observe that in A549 and H292 cells, both compounds show a tendency to a similar decrease in expression of CDC25c, although no statistically significant differences could

be found. CDC25c downregulation leads to cell cycle arrest at the G_2 to M transition, thus supporting the flow cytometry results for cell cycle arrest at G_2/M phase obtained post treatment with both compounds in both cell lines (Fig. 15B).

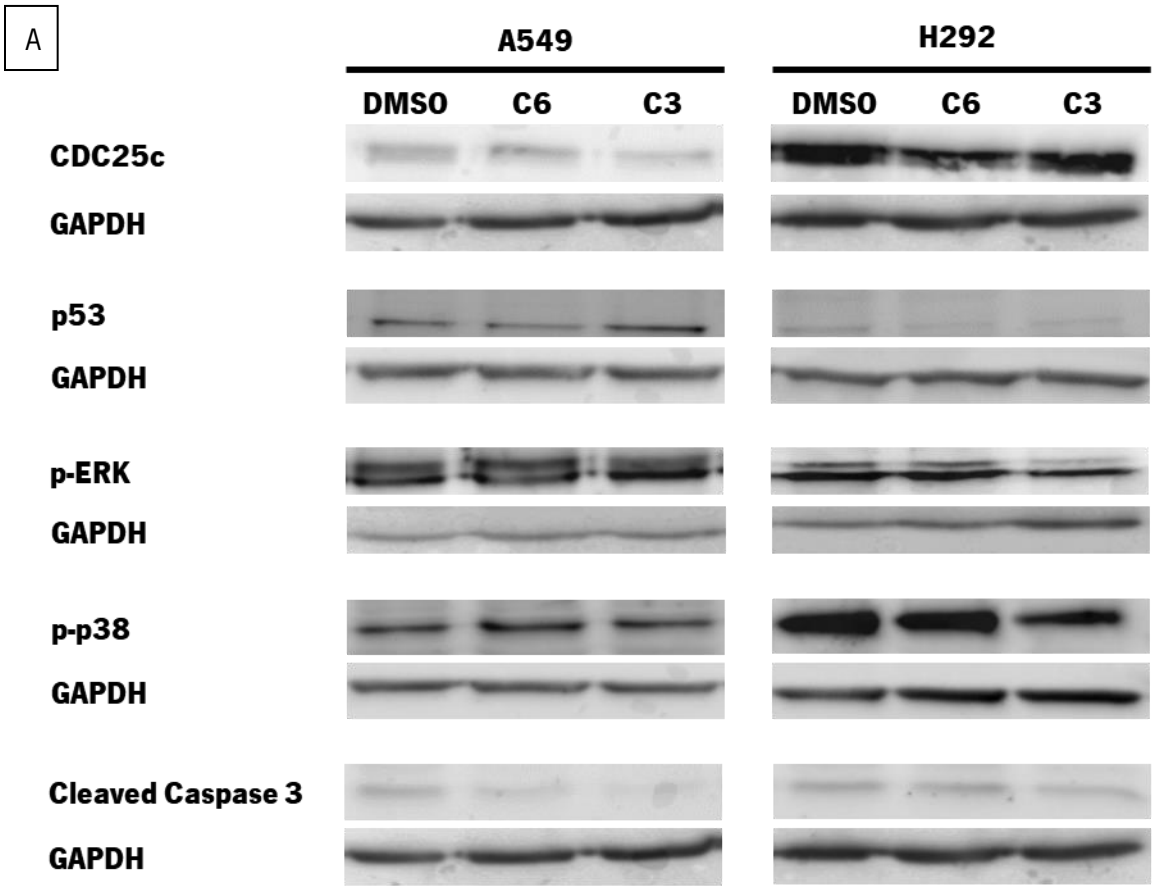
In parallel, a possible increase of expression levels of p53, which vary in response to stress factors, e.g. DNA damage, leading to cell cycle arrest and in more extreme conditions apoptosis[86] was also analyzed. Despite the fact that, in this case, results were obtained for only one biological replicate due to technical constrains, no great differences were observed for p53 expression upon compound C6 treatment in any of the cell lines tested (Fig. 16B), whereas compound C3 tends to slightly lower p53 levels, both in A549 and H292 cells. This way, data indicates that compound C6-induced G_2/M phase arrest of A549 cells appears not to be related with differences in p53 expression, but rather linked with decrease of expression levels of CDC25c.

MAPK signaling pathways are involved in a wide variety of cellular processes, namely cell proliferation, survival and apoptosis. MAPK/p38 cascade is activated in response to stress stimuli[87], is linked to p53 cell arrest in G_2/M checkpoint[88], and also closely associated with cell apoptosis. As such, the phosphorylation levels of p38, indicative of activation were evaluated. As shown in figure 16B the phosphorylation levels of this protein seem to increase in A549 cells, in the presence of compound C6. In H292 cells, instead, compound C3 shows a tendency to decrease p-p38 levels, despite the lack of statistical significance. As previously mentioned, cell cycle analysis showed a tendency for an increase of G_2/M cell arrest in both cell lines when exposed to the compounds, being it stronger in A549 cells. Given that p-p38 is also related to cell apoptosis[89], increased p-p38 phosphorylation levels may be also associated with the increase of apoptosis observed in A549 cells. Furthermore, the fact that p38 MAPK has been associated with apoptosis activation by cisplatin and other chemotherapeutic agents[89] may show the potential of compound C6 as an antitumor agent with A549 cancer cells. Although compounds C6 and C3 induced apoptosis in H292 cells, the results do not support the involvement of MAPK/p38 pathway.

Additionally, activation of another MAPK signaling pathway, the MAPK/ERK which is also linked to regulation of cell proliferation and apoptosis[87] was assessed. Compound C6 (Fig. 16B) appears to decrease the phosphorylation of this protein in A549 cells and, also appears to a have a slight effect

in H292 cells. In turn, compound C3 only seems to decrease p-ERK levels in H292 cells. The apparent decrease of p-ERK on A549 and H292 cells induced by compounds C6 and C3, respectively, suggests a decrease of proliferation levels and/or apoptosis induction. This is in agreement with the flow cytometry data showing higher levels of apoptosis in A549 cells upon treatment with compound C6, whereas a stronger effect was observed for compound C3 in H292 cells.

To further elucidate compounds effect on cell apoptosis, the levels of the intracellular apoptosis-related protein caspase-3 major executioner caspase[90], were accessed. During apoptosis pathway, procaspase 3 is cleaved to form the active caspase 3 enzyme.[90] Hence, in figure 16 the levels of cleaved caspase-3 following cell treatment are shown. Results are indicative of a downregulation of cleaved caspase-3 in A549 in the presence of both compounds. More subtle differences were observed in H292 cells in comparison to control conditions. Although an accurate interpretation of the results is hampered by the lack of statistically significant differences, and more experiments are necessary to validate this data, the apparent decrease of cleaved caspase-3 in A549 cells and the similar levels in H292 cells does not supports the results of cell death analysis, which revealed higher apoptotic rates for both cell lines in the presence of both compounds. The expression of PARP-1, another apoptotic maker, was also assessed in this work but results were inconclusive, requiring further optimization, and for that reason were not included herein.



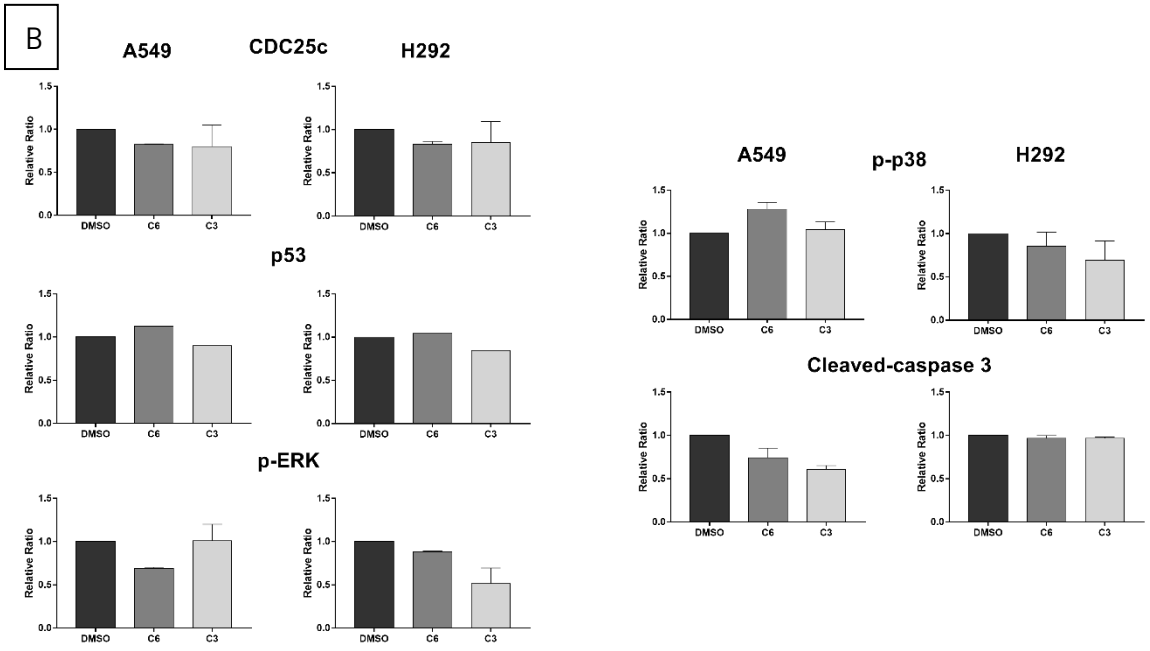


Figure 16. **Effect of compounds C6 and C3 on the expression of several markers involved in cell cycle and death.** Total proteins were isolated from A549 and H292 cells following incubation with compounds C6 and C3 at IC_{50} concentration or DMSO (Vehicle control) for 48h. (**A**) Images are representative of at least two independent experiments, (**B**) Blots were normalized to GAPDH. Data and represent mean ± SEM of at least two independent experiments.

4. Final Remarks and Future Work

4.1. Final Remarks

Throughout the course of this research, all aims set in the beginning were fulfilled with success. The biological activity of a series of novel family-related chemical compounds was evaluated by MTT assay, in A549 and H292 cancer cell lines. It was also possible to identify, in this group of compounds, two extremely active compounds, namely compounds C3 and C6, with an IC_{50} value bellow 2 μM, which were selected for further studies. The results also showed that the type and position of both substituent groups, R and R1, influenced the compounds activity. One of them was identified as especially important for their activity, specifically Ph, which is present in the two most potent compounds C6 and C3 either in position R or R1.

The compounds effect in cell morphology of A549 and H292 revealed that in general the compounds do not appear to affect cell morphology, except in A549 where compound C6 led to a decrease on cell density and changes on cell morphology, as well.

The impact of the compounds C3 and C6 on cell death was also accessed. The results showed the compounds can induce cell apoptosis, although they cannot be considered as strong inducers of apoptosis when compared with Staurosporine. Remarkably, the increase of compounds concentration lead to a dose response effect, this effect being mainly observed when H292 cells were treated with C3 at $2xIC_{50}$ value which led to a percentage of apoptotic cells very similar with the one obtained with Staurosporine. Additionally, the results shown that the compounds have a low effect in inducing necrosis.

The impact of the compounds on cell cycle progression was also evaluated, showing an increase on S and G_2/M phases on both cell lines and once again compounds C6 and C3 had a stronger effect in A549 and H292 cells, respectively.

The results of an initial analysis of molecular markers related with cell cycle progression, cell proliferation and cell death of A549 and H292 cells generally supports the previous conclusions, except for the decrease of cleaved-caspase 3.

4.2. Future Perspectives

The results obtained in this work were very interesting and promising. It was possible to identify two very potent compounds that should be further investigated, not only to decipher in more detail their mechanism of action, but also to evaluate their efficiency in other lung cancer cells lines as well as in other tumor models. Yet, to first confirm the findings presented herein a higher number of experiments are required, in order to obtain statistically significant differences. Moreover, it would be interesting to access the dose-response effect and flexible drug exposure times in all the assays performed.

In order to complement the data, it would be relevant to analyze cell proliferation levels in the presence of C3 and C6, for example by studying the expression levels of phopho-histone 3 (phH3), either by western blot or immunohistochemistry. This protein is phosphorylated during chromatin condensation in mitosis[91], allowing to access the effect of the compound on cellular mitotic activity. Considering the results obtained on the impact of the compounds on cell death and cell cycle arrest, it would be interesting to access other molecular markers such as telomerase, anti-apoptotic factors Bcl-2 and Survivin[61,64], Cyclin-Dependent Kinases 1 and 2, linked to G_2 to M progression[92], Cyclin B1, a cell cycle regulator[64], and STAT3, a transcription factor linked to regulation of some cell cycle regulators and anti-apoptotic proteins[64]. Optimization of cleaved-PARP1 expression analysis, which is a product of PARP1 cleavage by caspase 3 and 7 in early apoptosis[93] or other apoptosis-related molecules, would also more clearly reveal compound's effect on cell death. Additionally, it could be accessed if the compounds induce apoptosis by the extrinsic or intrinsic apoptotic pathway. For that the expression levels of caspase 8, associated to the extrinsic pathway, and caspase 9, linked to the intrinsic pathway, could be evaluated. Taking into account the results obtained in the impact of the compounds on cell cycle arrest, the Comet Assay (single-cell gel electrophoresis), in which the DNA strand breaks are measured, could be used to access the compounds effect on DNA damage since it is a simple and reliable assay.[94]

Besides the efficacy of the compounds, one of the main issues related to new drugs is their cytotoxicity towards normal cells. As such, viability of human immortalized non-malignant lung cells and primary cells should be verified in the presence of both compounds. In addition, comparative

studies with a biosimilar reference drug for lung cancer treatment (such as Erlotinib or Gefitinib), would be crucial to further evaluate the potential of these compounds.

Finally, it has been demonstrated that combining different drugs in cancer treatment can have advantages in comparison with the single-therapy approach, such as the possibility to target key pathways in an addictive or synergetic manner and, also, a reduced probability of development of drug resistance.[95] So, the combination of these compounds with each other or even with already approved drugs could lead to interesting results. Overall, this work contributed to the identification of new chemical compounds with strong anti-cancer properties, paving the way for further advances in anti-cancer drug discovery and development.

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