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Evaluating the chemosensitizing effect of Pentoxifylline in pairs of sensitive and multidrug-resistant non-small cell lung cancer cell lines

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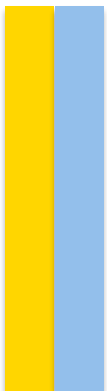

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Dissertação de Candidatura ao grau de Mestre em Oncologia submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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POSTER

- <u>Beatriz Matos</u>, Sara Peixoto da Silva, M. Helena Vasconcelos, Cristina P. R. Xavier Estudo *in vitro* do efeito de novas terapias combinadas no cancro do pulmão de não-pequenas células. ***5º Edição ENJIO (Encontro Nacional de Jovens Investigadores em Oncologia)***, Porto, Portugal, 22 Setembro 2023

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Abstract

Lung cancer is the leading cause of cancer-related death and the second most diagnosed cancer worldwide. Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases, being considered a heterogeneous disease. Despite recent and ongoing improvements in the treatment of NSCLC, this type of cancer still has a 5-year relative survival rate of 16%. The remaining therapeutic approach for NSCLC patients not eligible for single-agent immune or target therapies is platinum-based chemotherapy. However, multidrug resistance (MDR) is frequent in NSCLC, mainly due to its fibrotic stroma. Thus, our hypothesis is that combining an anti-fibrotic drug with currently conventional chemotherapy would improve treatment of this highly resistant and fibrotic cancer.

Interestingly, it has been reported that some non-anticancer drugs have the potential to be repurposed for anticancer therapy, allowing the development of new therapeutic options with shorter timelines and less expensive development costs. Pentoxifylline is an anti-fibrotic drug approved for the treatment of vascular diseases and that has been gaining interest in the oncology field, either by its anticancer potential or by its chemosensitizing effect in different cancer types. Moreover, pentoxifylline is known to inhibit Chitinase 3-like-1 (CHI3L1), which is a glycoprotein that activates several cancer-related pathways. Preliminary results by our research group, from analysis of “The Cancer Genome Atlas” (TCGA), have demonstrated that high levels of CHI3L1 are associated with poor prognosis and reduced survival of NSCLC patients under different drug treatments. In addition, preliminary results showed that pentoxifylline sensitized the NCI-H460 NSCLC sensitive cell line, and its MDR counterpart cell line (RH460), to paclitaxel and carboplatin cytotoxic drugs.

Therefore, the main aim of this dissertation was to assess the sensitizing effect of pentoxifylline to different chemotherapeutic regimens, in two pairs of sensitive and MDR counterpart NSCLC cell lines, and to disclose the mechanism of action of pentoxifylline.

To accomplish these goals, two pairs of sensitive and MDR counterpart NSCLC cell lines (NCI-H460 / RH460 and A549 / A549-CDR2) were used, to assess the sensitizing effect of pentoxifylline to paclitaxel, vinorelbine, carboplatin and to vinorelbine plus carboplatin treatments. This was possible by assessing the drugs effect on cell growth, using the Sulforhodamine B (SRB) assay. Then, the cytotoxic effect of these combined therapies was evaluated in a non-tumorigenic cell line, MCF-10A. Regarding the mechanism of action of pentoxifylline, its effect on the expression levels of CHI3L1 and its downstream activated proteins (e.g., β-Catenin, p-Akt and p-ERK), as well as on the expression levels of P-glycoprotein (P-gp) was assessed by Western blot. Finally, the effect

of pentoxifylline on the cell cycle profile, cell death and activity of P-gp, in the pairs of sensitive and MDR counterpart NSCLC cell lines, were evaluated by flow cytometry.

Our results clearly demonstrated the sensitizing effect of pentoxifylline to different chemotherapeutic regimens (paclitaxel, vinorelbine, carboplatin and to vinorelbine plus carboplatin) on both pairs of NCI-H460 / RH460 and A549 / A549-CDR2 NSCLC cell lines. All these drug combinations caused a significant reduction in the % of cell growth, when compared to the treatment with each of the drugs individually.

Indeed, the Fold Change of the percentage of cell growth inhibition resulting from the combined therapies versus conventional chemotherapies, in all the NSCLC cell lines under study, demonstrated that all the combined therapies studied (consisting of pentoxifylline plus conventional chemotherapy) led to a higher inhibition of cell growth, when compared to the conventional chemotherapies on their own. Importantly, none of the combined therapies caused a statistically significant increase in toxicity in the non-tumorigenic cell line tested, when compared to the conventional therapy regimens used in the clinical practice. Importantly, our results demonstrated that pentoxifylline decreased (but not statistically significantly) the expression of P-gp and CHI3L1, in both sensitive and MDR counterpart cell lines, and decreased the expression of β-catenin in both MDR cell lines. Importantly, pentoxifylline at 1 mM significantly decreased the levels of CHI3L1 expression in the RH460 cell line. Interestingly, pentoxifylline induced cell cycle arrest through an increase in the percentage of cells in G0/G1 phase, in both pairs of sensitive and MDR cells. In addition, pentoxifylline increased the levels of cell death in NCI-H460 cells. Finally, our results revealed that pentoxifylline did not inhibit P-gp activity.

Future work will validate the chemosensitizing effect of pentoxifylline in sensitive and MDR NSCLC spheroids, under the influence of cells from the tumour microenvironment, such as macrophages and fibroblasts. Lastly, future *in vivo* studies will allow to confirm the possibility of repurposing pentoxifylline to be used in combination with some chemotherapeutic drugs, to improve treatment of NSCLC.

Keywords: Chitinase 3-like-1, Combined Therapies, Drug Repurposing, Multidrug Resistance, Non-Small Cell Lung Cancer, Pentoxifylline

Resumo

O cancro do pulmão é a principal causa de morte relacionada com o cancro e é o segundo cancro mais diagnosticado em todo o mundo. O cancro do pulmão de não-pequenas células (CPNPC) representa cerca de 85% de todos os casos de cancro do pulmão e é considerado uma doença heterogénea. Apesar das melhorias recentes e contínuas dos tratamentos disponíveis para o CPNPC, este tipo de cancro ainda apresenta uma taxa de sobrevida relativa a 5 anos de 16%. A quimioterapia à base de platinos é a única abordagem terapêutica disponível para doentes com CPNPC que não são elegíveis para terapias alvo ou para imunoterapias. No entanto, a multirresistência (MR) à quimioterapia é frequente no CPNPC, principalmente devido à presença de tumores com um estroma fibrótico. Posto isto, a nossa hipótese é que a combinação de um fármaco anti-fibrótico com os regimes quimioterapêuticos convencionais atualmente utilizados na clínica, melhoraria o tratamento deste tipo de cancro altamente fibrótico e resistente.

Alguns fármacos aprovados para outras doenças que não o cancro, têm vindo a ser reportados como potenciais candidatos a serem reposicionados para o tratamento de cancro, permitindo o desenvolvimento mais rápido e menos dispendioso de novas abordagens terapêuticas. A pentoxifilina é um fármaco anti-fibrótico, aprovado para o tratamento de doenças vasculares, que tem vindo a ganhar interesse na área oncológica, tanto pelo seu potencial anticancerígeno, como pelo seu efeito quimiossensibilizador em diferentes tipos de cancro. Para além disto, a pentoxifilina está descrita como sendo um inibidor da quitinase 3-like-1 (CHI3L1). Esta glicoproteína está relacionada com a ativação de várias vias de sinalização relacionadas com o cancro, logo, o estudo da pentoxifilina apresenta uma vantagem adicional. Resultados preliminares obtidos pelo nosso grupo de investigação, com base na análise da base de dados “The Cancer Genome Atlas”, demonstraram que elevados níveis de CHI3L1 estão associados com o mau prognóstico e a reduzida sobrevivência dos doentes com CPNPC, sob o tratamento de diferentes regimes de fármacos. Adicionalmente, resultados preliminares demonstraram que a pentoxifilina sensibilizou a linha celular sensível de CPNPC, NCI-H460, e a sua contraparte MR (RH460), aos fármacos citotóxicos paclitaxel e carboplatina.

O principal objetivo desta dissertação foi estudar o efeito sensibilizador da pentoxifilina a diferentes regimes quimioterapêuticos, em dois pares de linhas celulares de CPNPC sensíveis, e as suas contrapartes MR, assim como estudar o mecanismo de ação da pentoxifilina.

De modo a alcançar os objetivos propostos, foram utilizados dois pares de linhas celulares sensíveis e as suas contrapartes MR de CPNPC (NCI-H460 / RH460 e A549 /

A549-CDR2) para estudar o efeito sensibilizador da pentoxifilina ao tratamento com paclitaxel, vinorelbina, carboplatina e vinorelbina com carboplatina. Isto foi possível avaliando o efeito dos fármacos no crescimento celular, através do ensaio de Sulforodamina B (SRB). O efeito citotóxico destas combinações terapêuticas foi posteriormente analisado numa linha celular não tumorigénica, MCF-10A. Quanto ao mecanismo de ação da pentoxifilina, o seu efeito nos níveis de expressão da CHI3L1, e das proteínas ativadas pela CHI3L1 (ex. β-Catenina, p-Akt e p-ERK), e da glicoproteína-P (P-gp), foi analisado por Western Blot. Por último, o efeito da pentoxifilina no perfil do ciclo celular, na morte celular e na inibição da atividade da P-gp foi analisado por citometria de fluxo.

Os nossos resultados demonstraram que a pentoxifilina apresenta um efeito sensibilizador aos diferentes regimes quimioterapêuticos estudados (paclitaxel, vinorelbina, carboplatina e vinorelbina com carboplatina). Este efeito foi verificado em ambos os pares de linhas sensíveis e MR de CPNPC, NCI-H460 / RH460 e A549 / A549-CDR2. As diferentes terapias combinadas causaram uma redução estatisticamente significativa na percentagem do crescimento celular comparativamente com o tratamento de cada um dos fármacos individualmente.

De facto, o Fold Change da percentagem de inibição do crescimento celular resultante das terapias combinadas versus o das quimioterapias convencionais, calculado para todas as linhas celulares de CPNPC em estudo, demonstrou que todas as terapias combinadas em estudo (que consistem na combinação da pentoxifilina com a quimioterapia convencional) levaram a uma maior inibição do crescimento celular, quando comparado com o efeito dos regimes quimioterapêuticos convencionais isolados. É importante notar que nenhuma das terapias combinadas causou um aumento estatisticamente significativo da toxicidade na linha não tumorigénica em comparação com os regimes quimioterapêuticos utilizados na prática clínica. Os nossos resultados também demonstraram que a pentoxifilina levou a uma diminuição (mas não estatisticamente significativa) da expressão da P-gp e da CHI3L1, em ambos os pares de linhas sensíveis e as suas contrapartes MR, e a uma diminuição da expressão da β-catenina nas duas linhas MR. De salientar que a pentoxifilina a 1 mM reduziu de forma estatisticamente significativa os níveis de expressão da CHI3L1 na linha celular RH460. A pentoxifilina levou também ao bloqueio do ciclo celular, resultando num aumento da percentagem de células na fase G0/G1, em ambos os pares de linhas sensíveis e MR. A pentoxifilina também aumentou os níveis de morte celular na linha NCI-H460. Por último, os nossos resultados revelaram que a pentoxifilina não inibiu a atividade da P-gp.

Num trabalho futuro pretendemos validar o efeito quimiossensibilizador da pentoxifilina em esferoides com origem em células sensíveis, ou MR, de CPNPC, sob a influência de células do microambiente tumoral, como macrófagos e fibroblastos. Por fim, futuros estudos *in vivo* permitirão confirmar a possibilidade do reposicionamento da pentoxifilina, de modo a ser utilizada em combinação com alguns regimes quimioterapêuticos, para melhorar o tratamento de CPNPC.

Palavras-chave: Cancro do pulmão de não-pequenas células, Quitinase 3-like-1, Multirresistência a Fármacos, Pentoxifilina, Reposicionamento de Fármacos, Terapias Combinadas

List of abbreviations

A

Akt	Protein Kinase B
ALK	Anaplastic Lymphoma Kinase
ATCC	American Type Culture Collection

B

BRAF	Raf Murine Sarcoma Viral Oncogene Homolog B1
BSA	Bovine Serum Albumin

C

CBP	Carboplatin
CEA	Carcinoembryonic Antigen
cfDNA	Cell Free DNA
CHI3L1	Chitinase 3-like-1
COX	Cyclooxygenase
CTCs	Circulating Tumour Cells
ctDNA	Cell Tumour DNA
CTLA-4	Cytotoxic T-lymphocyte-associated Antigen

D

DMEM	Dulbecco’s Modified Eagle Medium

E

EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular Signal-Regulated Kinase
EVs	Extracellular Vesicles

F

FAK	Focal Adhesion Kinase

FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FN1	Fibronectin 1

G

GLOBOCAN	Global Cancer Statistics
GI_{50}	50% Cell Growth Inhibition Concentration

H

HER2	Human Epidermal Growth Factor Receptor 2
HPS	High Performance Status

I

IL	Interleukins

K

KRAS	Kristen Rat Sarcoma Viral Oncogene Homologue

L

LPS	Low Performance Status

M

MDR	Multidrug Resistance
MET	Mesenchymal Epithelial Transition Factor
MMP	Matrix Metalloproteinase

N

NSAIDs	Non-steroidal Anti-inflammatory Drugs
NSCLC	Non-small Cell Lung Cancer
NSE	Neuron Specific Enolase

P

PAC	Paclitaxel
PBS	Phosphate Buffered Saline

PD-1	Programmed Death-1
PD-L1	Programmed Death-1 Ligand
P-gp	P-Glycoprotein
PTX	Pentoxifylline

R

RB1	Retinoblastoma 1
RET	Ret Proto-oncogene
rh123	Rhodamine-123
ROS1	ROS Proto-oncogene 1
RPMI	Roswell Park Memorial Institute

S

SCC-Ag	Squamous Cell Carcinoma Antigen
SCLC	Small Cell Lung Cancer
SRB	Sulforhodamine B

T

TAM	Tumour Associated Macrophages
TCA	Trichloroacetic Acid
TCGA	The Cancer Genome Atlas
TGF	Transforming Growth Factor
TKIs	Tyrosine Kinase Inhibitors
TME	Tumour Microenvironment
TNF-α	Tumour Necrosis Factor Alfa
TP53	Cellular Tumour Antigen p53
TTF-1	Thyroid Transcription Factor 1

V

VR	Vinorelbine

W

WB	Western Blotting

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Introduction

1. Lung Cancer

1.1 Epidemiology and Etiology

Lung cancer is the leading cause of cancer-related death and is the second most incident cancer worldwide. According to Global Cancer Statistics (GLOBOCAN), in 2020 lung cancer had the second highest number of new cases diagnosed (11.4%), after breast cancer (11.7%), and was responsible for almost 1.8 million deaths (18%) (**Figure 1**) [1]. One of the main factors contributing to this high mortality and for a 5-year survival rate of 4%, is the late diagnosis of the disease [2]. In the last decade, changes in the epidemiology and prevention of lung cancer have been noticeable, mainly as a result of changes in smoking habits, innovative lung cancer genetic discoveries and new therapies against lung cancer. Nevertheless, the rate of mortality of lung cancer is still estimated to increase up to 62% by 2040, partly related to a global increase in cigarette consumption [1, 3, 4].

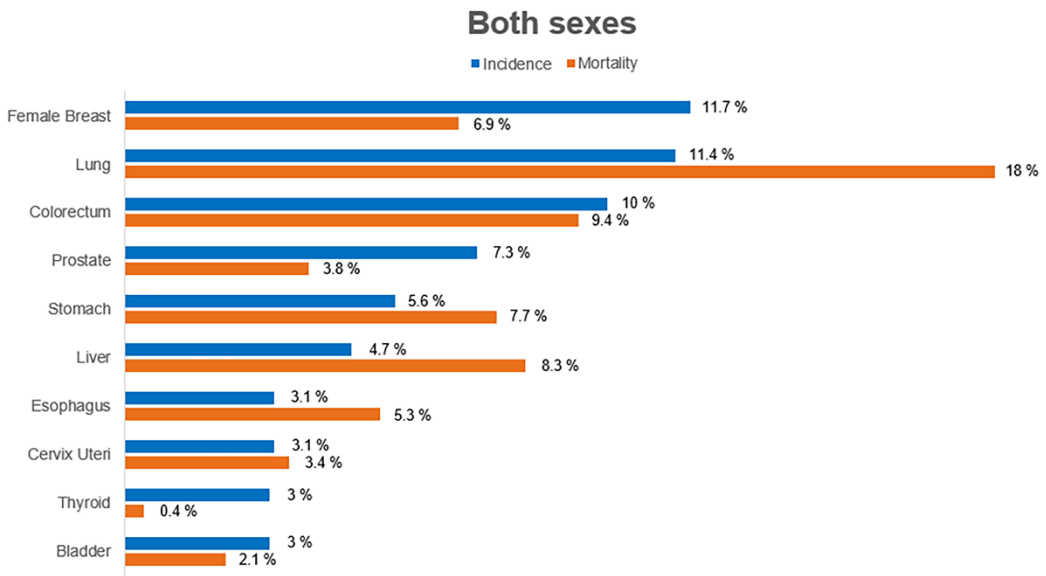

Figure 1 - Estimated 2020 worldwide cancer incidence and mortality rates for both sexes. The 10 most common cancers are shown in descending order. Adapted from GLOBOCAN. [1]

In fact, historical smoking patterns play a relevant role in lung cancer incidence and demographic distribution across the world. Moreover, lung cancer incidence has been associated with gender, age, race/ethnicity and socioeconomic levels [4]. The prevalence of smoking has been rising in the so-called "developing" countries and, as a result, lung cancer incidence is anticipated to rise in these countries. In the case of "developed" countries, the enforcement of laws and public education about the risks of smoking have led to a decline in tobacco use, and as such, it is expected that there will be a decrease in the number of lung cancer cases in these areas [5]. However, the rates in industrialized regions, namely in Europe and Northern America, still tend to be higher when compared to the rates seen in underdeveloped areas, like Africa or South America [1, 6]. In respect to gender, both lung cancer incidence and mortality rate are remarkedly higher in men when compared to women [1, 4]. However, in recent years, there has been a significant increase in the number of new diagnosed cases in women, primarily as a result of new smoking behaviours. Therefore, if this trend persists, by 2045 it is expected that more women might die from lung cancer than men [7]. Additionally, lung cancer is typically diagnosed at older ages, like many other oncological diseases. The median age of lung cancer diagnosis is around 70 years, while death occurs on average at age 72 [4]. Until the ages of 80 to 85 lung cancer mortality rises with age, but after that heart disease overtakes cancer as the leading cause of death in both genders [5].

As previously stated, smoking is believed to be the primary environmental factor contributing to the development of lung cancer [1]. Even so, up to 25% of lung cancer cases are not associated with smoking. Environmental risk factors, such as air pollution, exposure to uranium, radium or radon, as well as to asbestos may contribute to this variation in cancer risk [4]. Furthermore, genetic predisposition also increases the susceptibility of having lung cancer. Differences in sensitivity to mutagens may also have an impact on individual vulnerability to carcinogens [1, 8]. Importantly, genetic susceptibility has been associated with lung cancer cases in younger age groups [9].

1.2 Classification

Lung cancer is considered a heterogeneous set of diseases, composed by two main groups, the small-cell lung cancer (SCLC) and the non-small cell lung cancer (NSCLC), depending on their histological type, prognosis and treatment implications [2] (**Figure 2**).

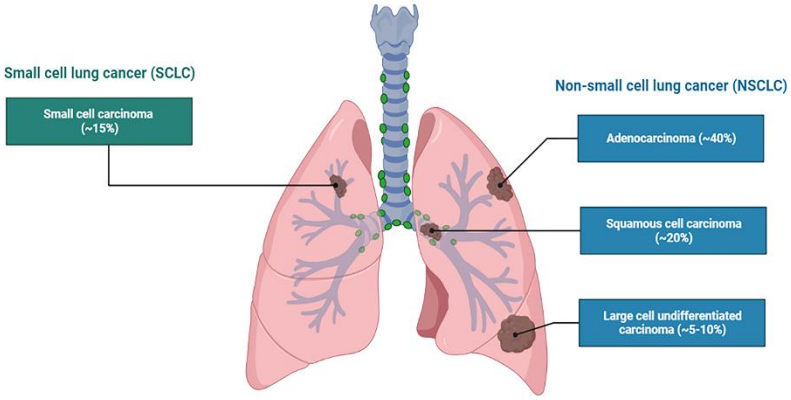

Figure 2 - The two main histological types of lung cancer, their most common anatomical sites with their respective prevalence. Created with Biorender (app.biorender.com)

1.2.1 Small Cell Lung Cancer (SCLC)

Small cell lung cancer (SCLC) accounts for almost 15% of all lung cancer cases, being a high-grade, poorly differentiated, and the most aggressive form of neuroendocrine lung cancer [2]. SCLC develops from neuroendocrine cells of the basal bronchial epithelium and is classified into limited stage, if it occurs within a single radiation point, and extensive stage, if the SCLC has already metastasized. SCLC-type carcinomas are often composed of small, fusiform, or spherical cells with minimal cytoplasm, granular chromatin and necrosis [10]. SCLC has the potential to metastasize to the brain, liver, and bones, and is characterized by having a faster tumour growth rate and widespread metastasis. Thus, most of the patients are diagnosed at advanced stages [10]. This high aggressiveness may be due to the hight mutational rate of SCLC tumours, with cellular p53 (TP53) and retinoblastoma 1 suppressor genes being found biallelically inactivated in almost all tumours [11].

Regarding risk factors, SCLC has the strongest epidemiological relation to tobacco among the different subtypes of lung cancer, whereas inherited genetic factors are thought to have a minimal impact on SCLC development [12]. Concerning the recommended treatment for SCLC patients, chemotherapy combined with radiation is the standard therapy [13].

1.2.2 Non-small Cell lung Cancer (NSCLC)

Non-small cell lung cancer (NSCLC) corresponds to the most prevalent type of lung cancer, representing approximately 85% of all lung cancer patients [2]. Adenocarcinoma, squamous cell carcinoma, and large-cell lung carcinoma are the three major histological phenotypes of lung cancer. Other types, such as salivary gland-type tumours, sarcomatoid carcinomas, and others, comprise a very small proportion of NSCLC patients [2, 14].

The adenocarcinoma corresponds to the majority of NSCLC cases (≈ 40 %), having an epithelial origin, namely from alveolar cells located in the smaller airway epithelium. This histological type of lung cancer has glandular histological features and is typically located in the periphery of the lung, arising in more distal airways [2, 15]. Moreover, it is characterized by expressing immunohistochemical markers that are consistent with an origin in the distal lung, such as the thyroid transcription factor 1 (TTF-1), Napsin A, as well as Keratin-7 [14, 16]. According to World Health Organization, adenocarcinoma can be divided into three patterns based on its invasiveness: adenocarcinoma *in situ* (pre-invasive lesion), minimally invasive adenocarcinoma, and invasive adenocarcinoma, and all may be occurring in the same patient [2, 10, 16]. Unlike the other subtypes of lung cancer, adenocarcinoma does not have smoking as its main risk factor. In fact, this type of NSCLC frequently arises in non-smoking women. The presence of activating mutations that affect driver genes, for example the oncogenes kirsten rat sarcoma viral oncogene homologue (KRAS), B-raf proto-oncogene serine/threonine kinase (BRAF) and the epidermal growth factor receptor EGFR, are key factors in the development of this type of tumours [10].

The squamous cell carcinoma accounts for 20% of NSCLC cases and is defined as a poorly differentiated tumour with epithelial origin [2, 16]. This type of cancer is most usually found in a central position, developing in a major or lobar bronchus. Squamous cell carcinoma is characterized by keratinization, considered its hallmark, and/or intercellular bridges, but it can also express immunohistochemical markers of squamous cell differentiation, such as p46 and p60 [2, 14, 15]. This type of cancer is strongly linked to smoking and chronic inflammation [14].

The large-cell carcinoma is the NSCLC subtype that has the lowest representation (5 – 10%) [15]. Since these tumours lack histological or immunohistochemical evidence of glandular, squamous cell, or small-cell differentiation, they are considered poorly differentiated [16]. Although these tumours are characterized by the presence of large cells with abundant cytoplasm and large nucleoli, its diagnostic is very complex as it needs an extensive sampling of a surgical resected material, after eliminating squamous cell carcinoma, adenocarcinoma, or small cell lung cancer as a hypothesis. Concerning immunohistochemical analysis, large-cell carcinomas may be positive for cytokeratin and

negative for TTF-1 and p40. As a result of improvements in immunophenotyping methods, the incidence of this type of cancer has been decreasing, since it has made it feasible to more accurately classify squamous cell carcinomas and poorly differentiated adenocarcinomas [2, 14, 15].

1.2.2.1 Diagnosis of patients with NSCLC

As already mentioned, one of the major reasons to the high mortality rate of NSCLC is due to late diagnosis. Thus, the implementation of screening programs, which aim to identify cancer cases at an early stage with low rate of false positives, and being economical for the healthcare system, would be of value [17].

Low-dose computer tomography screening is advised for people who meet the selection requirements outlined by the National Lung Screening Trial, namely that they are older than 55 years and are considered in high risk (smoking history of 30 pack-year) [15, 17, 18]. However, the implementation of NSCLC screening protocols is still low given a variety of difficulties, such as high costs, high rates of false-positive results, increased patient distress as a result of the need for extended follow-up, and the possibility of overdiagnosis, since this individuals are at higher risk for other potentially life-threatening comorbidities [15]. Regarding clinical findings, the most common symptoms of NSCLC patients are cough (50 to 75% of the patients), haemoptysis, chest pain, dyspnoea, and weight loss. Some other symptoms may be associated with paraneoplastic syndromes, such as superior vena cava syndrome [15, 17]. Since these symptoms are common to other respiratory illnesses, this malignant disease is frequently diagnosed at a more advanced stage.

The diagnosis of NSCLC comprises both biopsy analysis, for histological confirmation, and the establishment of TNM stage, which will influence cancer treatment options [15]. The TNM staging system was stablished by the American Joint Committee on Cancer, and considers the size of the primary tumour (T), the lymph node involvement (N), and the presence of distant metastasis (M), to classify the disease into four stages (I-IV), being the stage IV the most aggressive one [10, 19]. Concerning diagnosis techniques, less or more invasive methodologies are performed depending on the stage of the tumour. Less invasive techniques include imaging diagnosis, such as computerized tomography that allows to define some characteristics of the tumour (e.g., size, shape, position and relationship to nearby structures), as wells as positron emission tomography, which provides details about the lung tumour's metabolic profile and any lesions potentially associated. Moreover, magnetic resonance imaging, transoesophageal ultrasonography, or endobronchial ultrasound are other options. Some of the more invasive procedures include

cytological, histological and molecular analysis through surgical open or needle biopsy for a full diagnostic profile [2, 10, 17].

The application of biomarkers in the diagnosis of NSCLC is an approach that is still being improved [10]. For instance, liquid biopsy, which is a non-invasive method already used in the clinical practice, allows to analyse circulating tumour cells (CTCs) and circulating free DNA (cfDNA), thus identifying genetic mutations and establishing molecular patterns of an individual's disease [10, 20]. Importantly, for NSCLC diagnosis, CYFRA 21-1, carcinoembryonic antigen (CEA), neuron specific enolase (NSE), and squamous cell carcinoma antigen (SCC-Ag), are the most studied circulating antigen biomarkers. Even though there are promising results using these approaches, improvements in sensitivity and specificity are still needed [10].

1.2.2.2 Treatment options for patients with NSCLC

Since NSCLC is a heterogeneous set of diseases, patient's clinical condition (performance status), histological cell type as well as the molecular profile of this type of tumour, should be considered, when deciding on the best course of treatment. Furthermore, NSCLC treatment is stage specific, being classified into resectable, locally advanced, and advanced stages (**Table 1**) [15, 17].

Table 1 - Summary of the current therapeutic approaches available for Non-Small Cell Lung Cancer (NSCLC)

NSCLC		Treatment Options		References
Early Stages	**Stage I**	Surgery (HPS) Radiofrequency ablation (LPS) (+) Adjuvant Platinum Based-Chemotherapy / Adjuvant Radiotherapy		[17, 21, 22]
	Stage II / IIIA	(Neo-adjuvant Chemotherapy / Radiotherapy / Chemoradiotherapy) + Surgery (HPS) Radiofrequency ablation (LPS) (+) Adjuvant Platinum Based-Chemotherapy / Adjuvant Radiotherapy		[15, 17, 21-23]
Locally Advanced Stage	**Stage IIIB**	Chemoradiotherapy (HPS) Radiotherapy / Chemotherapy (LPS)		[21, 22]
Advanced Stage	**Stage IV**	Molecular Target Therapy	Immunotherapy / Immunotherapy plus Chemotherapy	[21, 22, 24-27]

HPS – High Performance Status, LPS – Low Performance Status

Patients with early stages of the disease (Stage I, II and IIIA) have surgery as the primary treatment option if tumour is classified as resectable and operable. Lobectomy, meaning the surgical removal of just one lobe, is taken as the best approach [17, 22]. Although there are other strategies, such as wedge resection that consists in removing a slice of tissue triangle-shaped, or segmentectomy that uses veins, arteries and airways to guide the tissue removal, lobectomy is still seen as the optimal procedure. This is because the rate of local recurrence is higher in patients undergoing sublobar surgeries, since during the procedure cancer cells may spread and undetectable micrometastases may be present [15, 17, 28]. In case of recurrence, or of positive surgical margins, patients can also be submitted to adjuvant chemotherapy or radiotherapy [21]. Patients with resectable stage II / IIIA NSCLC are also submitted to adjuvant radiotherapy or adjuvant platinum-based chemotherapy, which includes therapeutic duplets consisting of cisplatin or carboplatin with paclitaxel, gemcitabine or vinorelbine. Some patients with IIIA stage NSCLC can also be treated with chemotherapy, often combined with radiotherapy (chemoradiation), before the surgery, in order to downstage the tumour, enabling potential total excision and allowing an early treatment of micro-metastases [15, 17, 21, 23]. Patients who refuse surgery, or have unresectable tumours, or cannot be submitted to surgery for medical reasons, have radiofrequency ablation as a treatment option, if the tumours are small, or to reduce the margins of the tumour. Radiotherapy can also be applied, namely stereotactic ablative radiotherapy, having similar efficacy to surgery [15, 17, 21, 22].

The standard treatment for patients with locally advanced NSCLC disease, meaning that the cancer has progressed to neck or lung-near lymph nodes and may also have invaded significant chest structures [21], is chemoradiation, which consists of sequential or concurrent combination of chemotherapy and radiotherapy. However, patients who cannot receive combined therapy owning to their performance status, are only submitted to radiation therapy or, less often, chemotherapy alone [21, 22]. Patients with advanced stages of NSCLC with stage IV (that accounts for 40% of the newly diagnosed NSCLC patients) have treatment options relied on performance status, comorbidity, histology and molecular features of the cancer [22]. The standard treatment option for this stage is chemotherapy, or chemotherapy combined with targeted therapy or immunotherapy, when palliative care is not an option yet. Laser therapy, photodynamic therapy or radiation therapy are also treatment options that can be applied to mitigate the symptoms [21, 22].

Importantly, the establishment of the molecular profile of the individual´s tumour has been increasingly valued with several targetable alterations being associated with lung cancer carcinogenesis [14, 27]. For instance, the epidermal growth factor receptor (EGFR) mutations occur in 10% to 50% of NSCLC adenocarcinoma cases, usually more commonly

in female, Asian and non-smokers [29]. Tyrosine kinase inhibitors (TKIs) and monoclonal antibodies are the two main approaches used to target EGFR. Indeed, EGFR-TKIs are associated with a high tumour response rate when compared to the standard treatment of chemotherapy [17]. Although with promising results, most of the patients develop EGFR-TKIs resistance in a short time. In these cases, third generation Osimertinib is used, allowing to target both EGFR-sensitizing mutations, as well as the T790M mutation (which causes the EGFR-TKI resistance in almost 60% of the cases) [17, 24]. Unfortunately, subsequently mutations might occur, such as the EGFR C797S mutation, which is still an off-target mechanism of resistance [27]. Moreover, not all patients with EGFR mutations are eligible for treatment with TKIs due to rapid progression of the disease [27]. In these cases, platinum-based chemotherapy is the standard treatment [17].

There are other genes commonly mutated in NSCLC. For instance, KRAS is mutated in around ≈25% of patients with NSCLC adenocarcinoma. Although this mutation is generally mutually exclusive of EGFR, as well as other driver mutations, it can co-occur especially as a resistance mechanism in TKI-treated patients [27, 30]. Other clinically relevant molecular alterations in NSCLC, although less frequent, include genetic alterations in the anaplastic lymphoma receptor tyrosine kinase (ALK), chromosomal rearrangements in the c-ROS proto-oncogene 1 (ROS1) and alterations in the mesenchymal epithelial transition factor proto-oncogene (MET). TKIs can be used to target these alterations, but the multiple mechanisms of resistance end up leading to ineffective treatments [15, 24, 27]. Moreover, genetic alterations in human epidermal growth factor receptor 2 (HER2) and rearrangements in the Ret proto-oncogene tyrosine-protein kinase receptor (RET) can also be present in NSCLC patients, in an almost exclusive way, particularly in adenocarcinoma patients. Patients with mutations in HER2 are sensitive to chemotherapy and to some target therapies, such as Trastuzumab [27, 31], whereas patients having RET-rearranged NSCLC are responsive to a variety of inhibitors [27]. Finally, mutations in BRAF can also be present, accounting for 1-3.5% of NSCLC patients. In this case, patients are treated with dabrafenib or vemurafenib, if the BRAF V600E specific mutation is present [27, 32].

Despite the variety of studies conducted each year to improve treatment options for patients with NSCLC, there are still several off-target alterations, such as TP53 mutations that occur at a high rate in many of the NSCLC subtypes. In such cases, patients undergo other therapeutic approaches, such as chemotherapy or immunotherapy.

Importantly, most recently, immunotherapy has arisen as one of the most promising therapeutic strategies. This type of therapy primarily targets immunological checkpoints, which are essential pathways for keeping the balance between immune system and tumour, preventing tumour growth [15, 33]. Regarding the immune checkpoint inhibitors available

for NSCLC patients, they are designed to target: 1) cytotoxic T-lymphocyte-associated antigen (CTLA-4), which is an inhibitory T cell co-receptor (e.g. Ipilimumab and Tremelimumab); 2) programmed death-1 (PD-1) that is responsible for controlling the activity of T cells in peripheral tissues (e.g. Nivolumab and Pembrolizumab); 3) PD-1 ligands, such as B7-H1/PD-L1 and B7-DC/PD-L2 (e.g. Atezolizumab) [22, 25, 26]. As already mentioned, patients with advanced stages of NSCLC without targetable alterations have immunotherapy alone or in combination with chemotherapy, as the standard treatment option. However, immunotherapy can also be recommended for early stages of NSCLC, if the first lines of treatment fail and patients are eligible. [21].

1.3 Multidrug Resistance (MDR) and Lung cancer

Despite the wide range of therapeutic approaches and the growing availability of targeted and personalized therapies, lung cancer relative survival rate at 5 years is still very low. Furthermore, because of the disease's potential for metastasis and eventual emergence of drug resistance, lung cancer continues to be associated with a poor prognosis [34, 35].

Drug resistance is responsible for almost 90% of cancer related deaths, which means that pharmacological treatments are quite ineffective [36, 37]. Drug resistance can be classified as innate, when a patient already presents resistance before starting a specific treatment, or acquired, when resistance appears gradually within the start of the drug treatment causing a reduction in its effectiveness [37, 38].

Importantly, a major challenge is when patients display multidrug resistance (MDR), i.e. when cancer cells become cross-resistant to a variety of different drugs, each presenting unique structures and mechanisms of action [39], leading to a drawback in the successful treatment of cancer. The major factors leading to MDR comprise host and tumour factors, and also tumour-host interaction factors. Regarding host factors, some examples include genetic alterations, which particularly affect genes that encode for drug-metabolizing enzymes, drug uptake / efflux pumps, drug targets and mechanisms of DNA repair, [39], as well as drug-drug interactions [39, 40]. Among the various tumour factors that may play an important role in MDR are 1) evasion from cell death, 2) increased ability to repair DNA damage and subsequently avoid apoptosis, [39] 3) intratumor heterogeneity, which may lead to the relapse of tumours due to proliferation of already-existing low-frequency subclones [39, 41], and 4) alterations in the intracellular concentration of a drug, through

barriers created by impaired plasma membrane transporters [39]. Importantly, extracellular vesicles (EVs) have also been related to MDR, since EVs can reduce the intracellular concentration of chemotherapeutic drugs [39, 42]. Moreover, EVs are known to be important mediators of intercellular or horizontal communication between cells, transferring resistant phenotypes to sensitive cancer cells, through their cargo, as they carry multiple MDR-associated proteins, miRNAs or other cargo associated with MDR [39, 43-45].

1.3.1 Drug Resistance in NSCLC

Despite promising results in NSCLC patients treated with targeted therapies, responses to these treatments are often incomplete and temporary, as most patients eventually develop resistance [34]. In fact, during targeted therapy an "on-target" resistance is frequently observed, which means that secondary mutations occur to the primary target of the drug, or, less commonly, there is amplification or loss of the targeted oncogene, which limit the drug's effectiveness [34]. One of the most common recognized secondary mutations is the EGFR T790M, which confers resistance to first- and second-generation of TKIs, with tertiary mutations in response to third-generation TKIs already being found [34]. "Off-target" resistance can also occur through the activation of alternative signalling pathways and downstream modifications. [34] For instance, the activation of RAS-MAPK pathway, which can occur at multiple sites of the pathway, is responsible for tumour cell survival during treatment with ALK TKIs [34, 46]. Moreover, an activation of NF-κB, a transcription factor that regulates cell proliferation, apoptosis and inflammation, may also occur during EGFR TKI therapy, being responsible for early tumour cell survival [34, 47].

Recently, the development of resistance to immune checkpoint inhibitors has been also demonstrated, with the appearance of secondary genetic alterations and/or modifications of the tumour microenvironment [48]. For instance, the three main pathways of immunological escape from immune checkpoint inhibitors are: 1) the survival of tumour clones lacking or unable to present (neo)antigens, 2) T cell exhaustion, and 3) genetic modifications that result in an immunosuppressive microenvironment. Additionally, a potentially new relevant mechanism of resistance is the positive selection of NSCLC cells exhibiting mesenchymal characteristics during immunotherapy. The activation of WNT and NOTCH signalling, as well as genes that activate cytokines, including interleukin (IL) 6 and 8, and transforming growth factor (TGF), may also play an important role in the epithelial to mesenchymal transition [34, 49].

1.3.2 Multidrug Resistance (MDR) in NSCLC

Multidrug resistance (MDR) is also frequent in NSCLC, as its highly fibrotic stroma creates a favourable and supportive environment for cancer cells [39, 50]. The dense fibrotic stroma, which is composed by considerable amounts of extracellular matrix and abundant cancer-associated fibroblasts, creates a physical barrier that significantly prevents the delivery of drugs to tumour cells, resulting in suboptimal treatment effectiveness [50, 51]. Moreover, other major MDR alterations in NSCLC include genetic and epigenetic alterations. For instance, platinum-based chemotherapies are designed to induce DNA damage, however mutations in the components of DNA damage response pathways are frequent, which leads to evasion from cell death [39, 52]. Efflux pumps, such as P-glycoprotein (P-gp), are also overexpressed in NSCLC resistant-cells, resulting in a reduction in the concentration of drug that accumulates inside the cells, and thus reducing drug efficacy [39].

To conclude, in order to better understand the multi-factorial biological basis of resistance, new therapeutic approaches are needed.

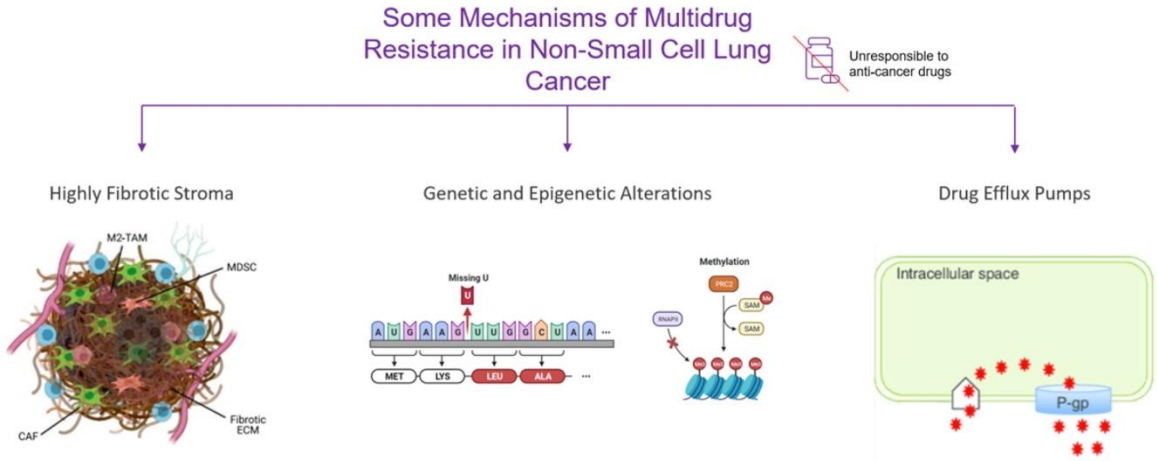

Figure 3 - Some mechanisms of Multidrug Resistance in Non-Small Cell Lung Cancer. Created with Biorender (app.biorender.com)

2. Drug repurposing: a breakthrough in drug discovery

De novo drug development is a complex, time-consuming, and expensive process that requires the evaluation of the drug's pharmacokinetic and pharmacodynamic characteristics, as well as of its efficacy and toxicity. Unfortunately, this process is associated with a high level of uncertainty of success [53, 54]. Drug repurposing has emerged as a strategy to overcome the disadvantages associated with the traditional drug development process. Drug repurposing, also known as “drug repositioning”, “drug reprofiling”, or “drug re-tasking”, aims to identify new clinical applications for already Food and Drug Administration (FDA) approved and/or investigational drugs, based on the fact that similar biological pathways may play a role in different diseases, and the polypharmacological effects of a drug may help treat other diseases [53, 55-57].

Drug repurposing consist of three main steps: 1) identification of the drug candidate, 2) assessment of the drug outcome in pre-clinical models; and 3) estimation of its effectiveness in clinical trials. Computational and experimental approaches are critical for the implementation of new drug uses [53, 56]. In fact, the average time for a repurposed drug to be approved for treatment of other diseases can be shortened by 5 to 7 years, compared to novel drug development, as experimental data is already available, including safety, toxicity, pharmacokinetics and pharmacodynamics studies [58, 59]. Additionally, the risk of failure is relatively low, as the drug has already undergone clinical trials with an extensive evaluation of its safety. Nevertheless, it is necessary to have in mind that the drug still requires validation in a new clinical trial, since it is possible to observe new side effects as the drug is being tested for a new indication [53, 60]. However, drug repurposing requires less investment, with an estimated cost of 10 times less than that for a novel drug [53, 56, 61].

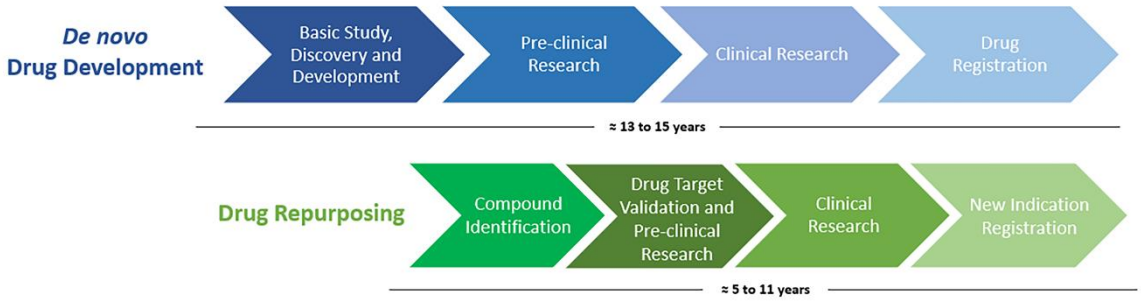

Figure 4 - Schematic comparison of the steps and time involved in developing de novo drugs and drug repurposing. Adapted from [57].

Despite all the advantages mentioned above, drug repurposing has some limitations as well. For the successful translation of a repurposed drug, it is necessary to obtain a sufficient level of evidence demonstrating treatment efficacy, a favourable risk-benefit ratio, and a better therapeutic value [54]. The potential profit predicted from the repurposed drug may also be significantly impacted by additional constraints that are specific to the repurposing of drugs, such as the absence of commercial sponsorship during clinical trials, patent and regulatory considerations and organizational challenges [56]. Thus, some improvements are still needed in this area, namely more integrated data analysis systems, better industry access to introduce the drugs into clinical trials, funding opportunities for drug repurposing initiatives and minimized restrictions imposed by patent and regulation [54, 61].

In the oncology field, the successful development of cancer drugs has proven to be complex, and only a limited number of drug candidates progress to clinical trials, with drug approval to enter in phase I trial being below 5%. In addition to the long time it takes for a drug to receive clinical approval, it also comes with a substantial price tag once it hits the market in order to pay for all the investments [60]. Therefore, researchers and clinicians are considering drug repurposing as an alternative approach in the oncology field as well. Such strategy, however, may be hampered by the great heterogeneity of neoplastic disorders [62]. There are, however, interesting examples of repurposed drugs for cancer treatment. For instance, as inflammation and oxidative stress in the tumour microenvironment (TME) are key factors in tumour progression and metastasis, non-steroidal anti-inflammatory drugs (NSAIDs), such as celecoxib and aspirin, have been found to reduce tumour development. Other targets of drug repurposing may include inhibition of proliferative signalling pathways, induction of apoptosis, regulation of cellular metabolism and activation of antitumor immunity. [53, 60, 62]. Moreover, by targeting different signalling pathways, combination therapies of non-anticancer drugs with the conventional anticancer chemotherapeutic regimens could be a good strategy to tackle MDR. In fact, this strategy is described as a safer and cheaper therapeutic option compared to conventional combination therapy of different anticancer drugs [62-64].

2.1 Drug repurposing in NSCLC

Even though the results are still preliminary, there are already some repurposed drug candidates that have been purposed for NSCLC therapy. Examples include: 1) anti-hypertensives and anti-arrhythmic drugs, such as beta-blockers, which have been shown to decrease tumour angiogenesis and stimulate the immune system , 2) NSAIDs, such as

celecoxib, which inhibit cyclooxygenase (COX) enzymes, namely COX-2 that is known to be upregulated in lung adenocarcinomas and related to enhanced cell proliferation and reduced apoptosis, 3) anti-diabetic drugs such as metformin, by inducing apoptosis, and 4) anti-microbial drugs, such as tigecycline, which were shown to inhibit proliferation and induce apoptosis in NSCLC cell lines [65].

Importantly, our research group identified pirfenidone, an anti-fibrotic, anti-inflammatory and antioxidant drug, which is approved for the treatment of idiopathic pulmonary fibrosis, as a chemosensitizer of NCI-H460 NSCLC cell line to paclitaxel and to a combination of paclitaxel plus carboplatin (CBP) [66].

To sum up, drug repurposing is an effective approach that allows to speed up the drug discovery process. Greater efficiency can still be achieved, but there are still some limitations that must be overcome. Nevertheless, more recent studies have demonstrated the value of repurposing drugs in the field of oncology.

2.2 Pentoxifylline: a drug candidate for cancer

Pentoxifylline is a methylxanthine derivative that has anti-fibrotic, anti-inflammatory and antioxidative properties. Pentoxifylline is currently approved by FDA for the treatment of vascular diseases, namely intermittent claudication [67, 68]. Due to its anticancer potential in several types of cancers, pentoxifylline has become increasingly interesting as a drug-repurposing candidate in oncology. In fact, several studies have been reporting the ability of pentoxifylline to affect cancer cell growth, proliferation, and invasiveness, as well as in reducing the metastatic potential.

Indeed, the first research was performed in a melanoma cell line, B16F10, by Gude *et al.* (1999), who verified that pentoxifylline reduced the growth of cancer cells. Moreover, a reduction in the expression of matrix metalloproteinase (MMP) 9 was observed, suggesting that pentoxifylline impairs cell migration capacity [69]. In fact, a reduction in MMP9 expression was also reported by Reis *et al.* (2018) in HL-60 leukaemia cell lines [70]. Subsequently, pentoxifylline was described as a negative regulator of the STAT3 pathway in a human melanoma cell line, A375, which in turn led to a decrease in MMP2 and MMP9 [71]. Another study reported that pentoxifylline induced an inhibition of Rho GTPases, which led to a reduction of the metastatic potential [72]. Furthermore, pentoxifylline was found to downregulate focal adhesion kinase (FAK), in both melanoma and breast cancer cell lines, via modulation of MAPK/ERK kinases and Akt pathway, leading to a reduction in tumorigenesis and cell proliferation [70]. Moreover, pentoxifylline altered the expression of

integrins, leading to their reduction, as well as impaired the NFκB signalling pathway, resulting in a reduction of the metastatic potential, in mouse melanoma cell lines, B16F1 and B16F10, as well as mouse melanoma models [70, 73].

The effect of pentoxifylline in altering the cell cycle profile or inducing apoptosis was also reported in several cancer types. For instance, pentoxifylline induced cell cycle arrest in G0/G1 phase in the triple negative breast cancer (MDA-MB-231) [74] and liver cancer (HepG2) cell lines [75], resulting in an increase of apoptosis. Furthermore, recent studies also showed that pentoxifylline induced the intrinsic apoptotic pathway, mainly affecting caspase 3 and caspase 9, in human colorectal cancer cell lines, HCT 116 and SW480 [76].

Furthermore, pentoxifylline was shown to interfere with P-gp, a drug efflux pump associated with MDR phenotype of resistant cancer cells, by different manners [70]. For instance, pentoxifylline reduced the expression of the *mdr1* gene in a mouse leukemia multidrug-resistant cell line, L1210/VCR, leading to a decrease in P-gp activity and thus, increasing drug uptake. The *mdr1* decreased expression resulted in the sensitisation of the MDR cell line (L1210/VCR) to vincristine, with an increase in apoptosis having been verified [77, 78].

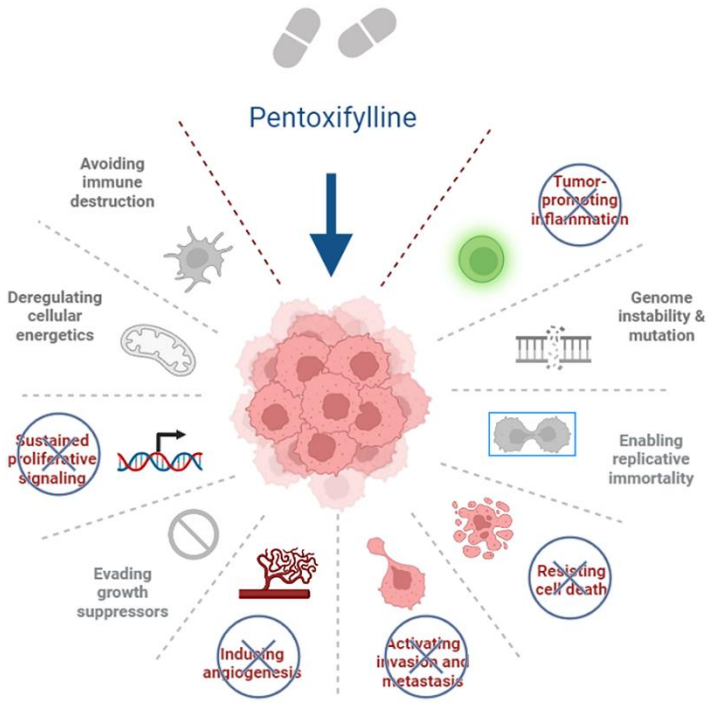

Figure 5 - Effects of pentoxifylline on the cancer hallmarks. Created with Biorender (app.biorender.com)

Importantly, several studies have also demonstrated the effect of pentoxifylline in combination therapies. For instance, pentoxifylline improved radiotherapy effectiveness, and lessen the long-term negative effects of radiation [70]. Moreover, pentoxifylline showed to have chemosensitizing effect in several human cancer cell lines [70]. For instance, Bravo-Cuellar *et al.* (2010) demonstrated that pentoxifylline sensitized cervical cancer cells to adryamicin by increasing apoptosis and decreasing senescence [79]. Moreover, pentoxifylline sensitized cisplatin resistant cervical cells to cisplatin by disrupting the NF-kB pathway, thus preventing the activation of cell survival mechanisms [80]. Pentoxifylline also sensitized prostate cancer cells to docetaxel through cell cycle arrest in G1 phase and increase of apoptosis [81]. Interestingly, our research group demonstrated that pentoxifylline sensitized pancreatic cancer cells to gemcitabine through inhibition of Chitinase 3-like-1 (CHI3L1), an activator of the MAPK and Akt pathways [82]. In the same line, Kim *et al.* (2017) demonstrated an improvement in gemcitabine efficacy, when combined with pentoxifylline, through a decrease in collagen matrix in the stroma and subsequently improving drug delivery to tumour cells, in human pancreatic tumour xenografts [83].

Regarding the effect of pentoxifylline on lung cancer, Ohsaki *et al.* (1996) demonstrated the chemosensitizing effect of pentoxifylline to cisplatin and etoposide treatments, in two different NSCLC cell lines, PC-9 and PC-14 [84]. As far as we know, this is the only report of combined therapy using pentoxifylline with conventional therapy in lung cancer, and still the mechanism of action underlying this effect was not disclosed. Interestingly, pentoxifylline improved and reversed fibrosis in several diseases, including pulmonary sarcoidosis and idiopathic pulmonary fibrosis. This effect was mainly due to pentoxifylline capacity to increase capillary blood flow, along with its anti-inflammatory and antioxidant properties [68, 85]. Therefore, this work suggested a possible effect of pentoxifylline in reduce fibrosis, which has been shown to hamper lung cancer treatment [50]

3. Chitinase 3-like-1 (CHI3L1): a promising therapeutic target in cancer

CHI3L1, also known as YKL-40, is a mammalian glycoprotein that belongs to the glycoside hydrolase 18 gene family. This protein is produced by a variety of cells including immune cells, such as macrophages and neutrophils, fibroblast-like cells, hepatic stellate cells, endothelial cells and cancer cells [86]. CHI3L1 does not present an enzymatic activity, however its carbohydrate-binding domain is greatly preserved conferring its ability to bind

to heparin, chitin and collagen [87]. CHI3L1 is implicated in both physiological and pathological conditions, although its physiological role is not completely understood. CHI3L1 is known to be a potential therapeutic target in inflammatory and neurodegenerative diseases, as well as in oncological diseases, since under these conditions CHI3L1 has a higher expression level [86-88].

Several studies have associated high serum levels of CHI3L1 to poor survival rate and poor prognostic in patients with different types of cancer, including breast cancer [89], urothelial carcinoma [90] and hepatocellular carcinoma [91]. Moreover, studies have associated CHI3L1 overexpression with chemoresistance in epithelial ovarian carcinoma [92] and resistance to immunotherapy in glioblastomas [93]. Our research group demonstrated that CHI3L1, present in the cargo of the extracellular vesicles released by human macrophages, reduced the cellular response of pancreatic cancer cells to gemcitabine [82]. Importantly, The Cancer Genome Atlas (TCGA) analysis demonstrated that high levels of CHI3L1 was associated with low overall survival, as well as with low response to gemcitabine treatment [82].

Mechanistically, CHI3L1 increases the production of pro-tumorigenic and pro-angiogenic factors, as well as activates pro-survival pathways, leading to an increase in tumour cell proliferation, evasion of apoptosis, invasion and suppression of host immune responses [87, 88, 94]. For instance, CHI3L1 promotes a favourable tumour microenvironment, encouraging breast cancer cells to infiltrate the lungs, forming metastases [94]. CHI3L1 induces tumour progression by promoting: 1) angiogenesis, through recruitment of human vascular endothelial cells and increasing vascular endothelial growth factor (VEGF) production [86] 2) invasion, by increasing MMP9 and decreasing cadherin expression levels, allowing to enhance cell motility [95]; and 3) immune suppression, by inducing macrophage differentiation into an anti-inflammatory tumour associated macrophages (TAM) profile, and an accumulation of dendritic cells, which induces anti-tumour immune responses [86]. Furthermore, CHI3L1 activates pro-survival pathways, such as MAPK/ERK, Akt/PKB, and Wnt/β-catenin signallings, by forming a multimeric complex with IL13Rα2 and IL-13, or through interaction of CHI3L1 with CD44 and IL13Rα2, a highly expressed receptor in several types of cancer [96, 97] (**Figure 6**). Several studies have reported that the synergistic activation of these pathways is associated with epithelial to mesenchymal transition (EMT), which favours invasion and metastasis by epithelial cancer cells, and evasion of apoptosis [86]. For instance, Geng *et al.* (2018) demonstrated that CHI3L1, through interaction with CD44, promotes the activation of β-catenin, Erk and Akt signalling pathways, leading to EMT transition as well as metastasis promotion in gastric cancer mouse models [98].

In lung cancer, CHI3L1 was also demonstrated to be highly expressed [86-88, 94]. For instance, in SCLC, CHI3L1 expressed by tumour circulating cells is known to positively regulate the expression of VEGF and MMP9, leading to tumour progression [95]. In NSCLC, studies have reported that CHI3L1 regulates the expression of various EMT factors, conferring an invasive phenotype to cancer cells [99]. Moreover, CHI3L1 affects p53 expression, leading to its downregulation and ubiquitinization, resulting in lung tumorigenesis promotion [100]. Importantly, CHI3L1 has been also related to tissue remodelling through matrix deposition and fibroblasts growth stimulation, leading to the formation of a highly fibrotic stroma in NSCLC [86, 101].

Therefore, taking all these studies into account, CHI3L1 may be considered a promising therapeutic target in several types of cancer [86, 87], including NSCLC, suggesting that therapies targeting this protein could be promising strategies.

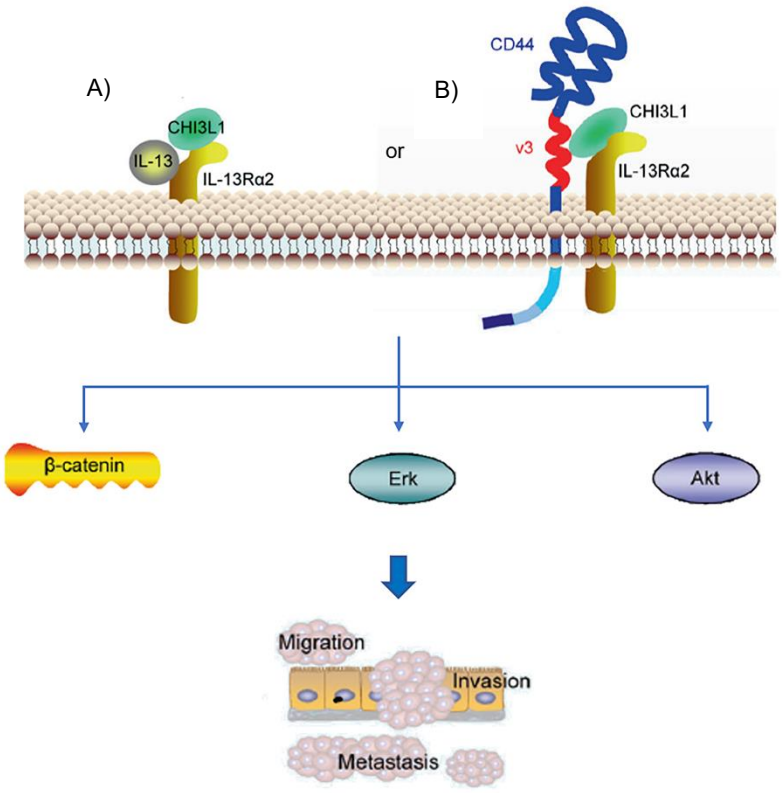

Figure 6 - CHI3L1 interacts with A) IL-13 and IL-13Rα2 to form a multimeric complex or with B) CD44 and IL-13Rα2 to activate the MAPK/Erk, Akt/PKB, and Wnt/β-catenin pro-survival signalling pathways. Adapted from [86]

Aims

Preliminary results by our research group, analysed by The Cancer Genome Atlas Program (TCGA), demonstrated that high levels of CHI3L1 are associated with poor prognosis and reduced survival of NSCLC patients under different drug treatments. Moreover, preliminary data demonstrated that pentoxifylline, a known inhibitor of CHI3L1 and an anti-fibrotic approved drug [82], sensitized the NSCLC cell line (NCI-H460) and its counterpart MDR cell line (RH460) to paclitaxel and carboplatin treatments.

Therefore, the first main aim of this master dissertation was to evaluate the sensitizing effect of pentoxifylline, a non-anticancer drug and known inhibitor of CHI3L1, to different chemotherapeutic regimens, in pairs of sensitive and MDR NSCLC cell lines. Our hypothesis was that the combination of pentoxifylline with conventional chemotherapy might improve treatment in this highly resistant fibrotic cancer.

The specific aims were to:

1) Confirm the preliminary results obtained by the research group regarding the sensitizing effect of pentoxifylline to carboplatin and paclitaxel treatments, in another pair of NSCLC cell lines (A549/A549-CDR2);

2) Evaluate the sensitizing effect of pentoxifylline to vinorelbine and to vinorelbine plus carboplatin treatments, in two pairs of sensitive and MDR NSCLC cell lines (NCI-H460/RH460 and A549/A549-CDR2);

3) Study the effect of the combined drug treatments on a non-tumorigenic cell line (MCF-10A);

The second main aim was to explore the mechanism of action of pentoxifylline. Thus, another specific aims were to:

4) Evaluate the expression of P-gp, CHI3L1 and its downstream proteins (such as Akt, ERK and β-catenin) in the pairs of sensitive and MDR counterpart NSCLC cell lines, in the presence/ absence of pentoxifylline;

5) Study the effect of pentoxifylline on cell cycle profile (using flow cytometry following PI staining), cell death (using Annexin V-FITC detection assay analysed by flow cytometry) and on P-gp activity (using the Rhodamine-123 assay).

Materials and Methods

1. Cell lines and Cell culture conditions

The NSCLC pairs of sensitive and MDR counterpart NCI-H460 / NCI-H460/R (here referred to as RH460 for abbreviation purposes) and A549 / A549-CDR2 cell lines were used. The pair NCI-H460 / RH460 was kindly provided by Dr. Milica Pešić (from Department of Neurobiology, Institute for Biological Research, University of Belgrade) [102, 103]. NCI-H460 / RH460 are large cell carcinoma cell lines, being the RH460 MDR cell line resistant to several drugs including doxorubicin and cross-resistant to etoposide, paclitaxel, vinblastine and epirubicin [102]. The A549 cell line was purchased from American Type Culture Collection (ATCC), and its MDR counterpart cell line, A549-CDR2 cell line, was established by our research group, after gradual and long exposure to increase concentrations of paclitaxel (unpublished work). The A549-CDR2 cell line is currently being characterized by our research group. The non-tumorigenic breast cell line MCF-10A was purchased from ATCC.

The NSCLC NCI-H460 / RH460 cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium with Ultraglutamine I and 25 mM 4-(2-hydroxyethyl) piperazine-1- ethanesulfonic acid, HEPES (Lonza, Basel, Switzerland). The medium was supplemented with 10% fetal bovine serum (FBS) (S181BH-500 biowest) for maintenance and with 5% FBS during the performance of the Sulforhodamine B (SRB) assay. The A549 / A549-CDR2 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L Glucose with UltraGlutamine™ w/ sodium pyruvate (L0103-500 biowest). The MCF-10A cells were cultured in DMEM/F12 Medium (11320-074 Gibco) enriched with inactive horse serum (HS; Biowest), supplemented with 5 mg/mL of hydrocortisone (Sigma Aldrich), 100 mg/mL of human epidermal growth factor (R&D systems), 1 mg/mL of cholera toxin (Sigma Aldrich) 10 mg/mL of insulin (Sigma Aldrich), 1% of penicillin/streptomycin (P/S) (100X, Corning).

All the cell lines were cultured in 25 cm^2 tissue culture flasks and maintained at 37 ºC in a humidified incubator containing 5 % CO_2. The cells were subcultured whenever 80 % confluency was reached. For this, the culture medium was removed and discarded, and the cells in monolayer were washed with pre-warmed phosphate buffered saline (PBS). Then, the triple express was added for harvesting the cells, which consist in detach the cells from the flask by breaking the intercellular and cell-to-substrate bonds, as gently as possible. This trypsinization process is done with cells at 37ºC, for about 4 to 5 minutes. Then, pre-warmed medium was added to inhibit the action of the triple express. From this

cell suspension, some cells were transferred to a new flask, already with culture medium (allowing 30 % of confluence in the new culture flask). The cells in the new culture flask were kept at the incubator.

To ensure the maintenance of the resistant phenotype of MDR cell lines, doxorubicin and paclitaxel were added every three weeks to R-H460 and A549-CDR2, respectively, or six days before any assay in case of R-H460 and three days in case of A549-CDR2.

The morphology of the cell lines was frequently monitored using the inverted light microscopy from Leica DMI1 (Leica Microsystems).

2. Sulforhodamine B (SRB) assay

SRB is a bright pink aminoxanthene dye that under mildly acidic conditions binds to all the protein components of the cells, previously fixed with trichloroacetic acid (TCA), via their basic amino acid residues [104]. Since SRB is directly proportional to the cell mass, as the binding of the dye is stoichiometric, it allows to determine the GI_{50} of a drug [105], meaning the concentration of a drug that inhibits 50% of cellular growth, or to evaluate the cell growth inhibition that a certain concentration of a drug induces.

To perform the SRB assay, 100 µl of cell suspension must be cultured in each well of a 96-well plate, being the number of cells per well dependent on each cell line. The optimal concentration for the two pairs of NSCLC cell lines and for the non-tumorigenic MCF-10A cell line is 5.0 x 10^4 cells/ml, previously determined by our research group. Cells were incubated at 37 °C for 24 hours to allow attachment. Then, cells were treated with different concentrations of drugs. To note that, two plates were used: one to be analysed immediately before cells being treated (T0h) and another plate to be analysed 48 hours after cells being treated (T48h). Upon 48 hours of treatment, cells were fixed with 50 µL ice-cold 10 % (w/v) TCA (Sigma Aldrich) for at least 1 hour at 4 °C, washed 3x with distilled water and air-dried. After that, cells were stained with 50 µL of 1% SRB in 1% (v/v) acetic acid (Merck) for 30 minutes at room temperature (RT) in the dark and washed 3x with 1 % v/v acid acetic. Finally, the air-dried cells were solubilized with 100 µL of 10 mM Tris-Base (Sigma Aldrich) for 5 minutes in an orbital shaker and the optical density was then measured at 510 nm in a microplate reader (Synergy TM BioTek Instruments, Inc.) using Gen5TM software.

The SRB assay was performed to: 1) obtain the GI_{50} of PTX in all NSCLC cell lines under study (NCI-H460, RH460, A549 and A549-CDR2); 2) evaluate the chemosensitizing effect of PTX when combined with different drug regimens (paclitaxel (PAC), vinorelbine

(VR) or carboplatin (CBP) individually, and VR plus CBP) in the two pairs of sensitive and MDR counterpart NSCLC cell lines; and 3) study the cytotoxic effect of the combined therapies on a non-tumorigenic cell lines, MCF-10A.

2.1 Determination of the GI_{50} of Pentoxifylline in NSCLC cell lines

The dose-response curves for PTX on both sensitive and their MDR counterpart cell lines (NCI-H460 / RH460 and A549 / A549-CDR2) were determined using the SRB assay. The GI_{50} concentration values were obtained for each cell line.

Briefly, cells were treated with 5 serial dilutions, PTX at 4 mM, 2mM, 1mM, 0.5 mM and 0.25 mM, as well as, with H_2O (vehicle, control) and with culture medium only (blank).

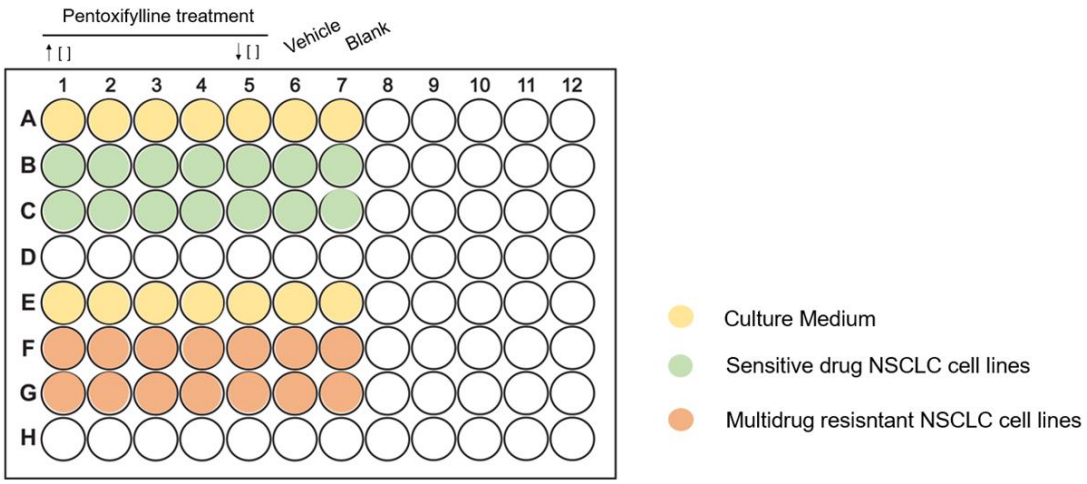

Figure 7 - Schematic representation of a 96-well plate for the determination of GI50 concentrations of pentoxifylline in the sensitive and MDR NSCLC cell lines. The yellow colour represents the medium alone with the serial dilutions of pentoxifylline and H_2O (vehicle), whereas the other colours represent the presence of cells. The green colour represents the wells with NCI-H460 or A549 cells (row B and C) and the orange colour represents the wells with RH460 or A549-CDR2 cells (row F and G). Cells were treated with different concentrations of pentoxifylline (from columns 1 to 5, starting with the higher concentration), with H_2O (vehicle, control) (column 6) and with culture medium only (blank) column 8.

2.2 Study the effect of the combined therapies in NSCLC cell lines

SRB assay was also performed to evaluate the chemosensitizing effect of PTX when combined with different anticancer drugs, on the sensitive and their counterpart MDR NSCLC cell lines. To note that, the combined therapies studied were based on the most

used treatment regimens in the clinic. Thus, the combination of PTX with PAC, VR or CBP individually, and VR plus CBP, were tested.

As previously mentioned, our research group had already studied the combination of PTX with PAC and PTX with CBP on the NCI-H460 / R-H460 pair of cell lines. Consequently, in NCI-H460 / R-H460, the only combination therapies studied were PTX with VR and PTX with VR plus CBP. Regarding the pair of cell lines A549 / A549-CDR2, all the combined therapies mentioned before were tested.

Briefly, cells were plated in a 96-well plate as already described, and after 24 hours they were treated with the combined therapies under study, as well as with each drug individually, and with H_2O and/or DMSO (vehicle, control). The concentrations tested for each drug in each pair of sensitive and MDR cell line are presented in **Table 2**. For this assay, only the plate designed as T48h was used, since specific concentrations were being tested.

Table 2 - Concentrations (μM) tested for each drug in each pair of sensitive and MDR NSCLC cell lines.

		Pairs of sensitive and MDR NSCLC cell lines	
		NCI-H460 / RH460	**A549 / A549-CDR2**
Drug Concentrations (μM)	**Pentoxifylline**	1.0×10^{3}	1.0×10^{3}
	Paclitaxel	* 5.7×10^{-3}	2.7×10^{-3}
	Carboplatin	1.77×10^{1}	8.37×10^{1}
	Vinorelbine	3.5×10^{-2}	2.5×10^{-2}

* , not evaluated in the framework of this dissertation

2.3 Determination of the Fold Change of the combined therapies *vs* conventional chemotherapies

The Fold Change of the percentage of cell growth inhibition of the combined therapies under studied *vs* conventional chemotherapies (currently applied in the clinical practice, including paclitaxel, vinorelbine, carboplatin, vinorelbine plus carboplatin) was determined for all NSCLC cell lines (NCI-H460, RH460, A549 and A549-CDR2), through the following mathematical formula:

$$\textbf{Fold Change} = \frac{\text{\% Cell growth inhibition of combined Therapy (with } \textbf{Pentoxifylline}\text{)}}{\text{\% Cell growth inhibition of Conventional Chemotherapies}}$$

This formula will allow to understand which of the treatments, combined therapies or conventional therapies, presents better results in terms of cell growth inhibition.

2.4 Study the effect of the Combined Therapies on a non-tumorigenic cell line

To study the cytotoxic effect of the combined therapies on the non-tumorigenic cell line, MCF-10A, SRB assay was used. For this study, only the combination of PTX with PAC and PTX with VR plus CBP was tested. In the case of the combination of PTX with PAC, the concentration used of PTX was 1 mM and of PAC was 5.7 nM, since it was the highest concentration of this drug that was tested between the two pairs of sensitive and MDR NSCLC cell lines. For the combination of PTX with VR plus CBP, the concentration of PTX tested was 1 mM, for VR was 25 and 35nM and for CBP was 17.7μM and 83.7 μM (**Table 3**). For all the combined therapies tested, it was included each drug individually, the combination, and H_2O and/or DMSO (vehicle, control).

Table 3 - Concentrations (μM) tested for each drug in both Non-Tumorigenic Cell Lines

		Non-Tumorigenic Cell Lines
Drug Concentrations (μM)	Pentoxifylline	1.0×10^{3}
	Paclitaxel	5.7×10^{-3}
	Carboplatin	a) 1.77×10^{1} and b) 8.37×10^{1}
	Vinorelbine	a) 3.5×10^{-2} and b) 2.5×10^{-2}

3. Western Blotting

Western Blotting (WB), which allows to detect specific proteins from a cell or tissue sample, was carried out to study the effect of pentoxifylline on the levels of P-gp, CHI3L1 and its downstream proteins: β-Catenin, ρ-ERK and ρ-Akt, in both pairs of sensitive and MDR NSCLC cell lines.

3.1 Extraction of total protein

All the NSCLC cell lines (NCI-H460 / R-H460 and A549 / A549-CDR2) were cultured in a 6-well plate at the ideal cell concentration of 5.0×10^{4} cells/mL and were incubated at 37 °C, for 24 hours. After attaching, cells were treated with 1 mM of PTX and H_2O (vehicle, control), as shown in **Figure 8**. After 48 hours, the medium was removed and discarded, and the cells washed with PBS. Then, cells were trypsinized for 4-5 minutes at 37ºC and then resuspended in culture medium. Thereafter, cells were centrifuged at 1200 rpm for 5 minutes at RT, and immediately after, the medium was discarded, and the cells resuspended in 1 ml of PBS. Then cells were centrifuged at 1200 rpm for 5 minutes at 4ºC and the pellet collected. Proteins were then lysed using 50 μL of Winman's Buffer (1% NP-40, 0.1 M Tris HCl pH 8.8, 5 M NaCl and 5 mM EDTA) containing EDTA-free protease and phosphate inhibitors (Roche), for 30 minutes, with agitation, at 4°C. The protein lysates were obtained after centrifugation at 13.000 rpm for 10 minutes at 4°C and stored at -20°C until quantification.

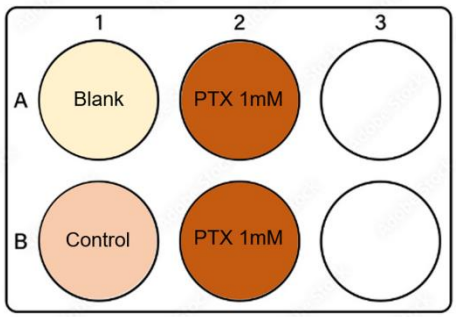

Figure 8 - Schematic representation of a 6-well plate for the treatment of sensitive and MDR NSCLC cell lines. Cells were exposed to culture medium (blank), to H_2O (vehicle, control) and were treated with pentoxifylline at 1 mM, replicated twice.

3.2 Protein Quantification

The quantification of protein lysates was performed using the Bio-Rad DCTM Protein Assay Kit, according to the manufactures instructions. The BSA was used as protein standard for the calibration curve. The absorbance was measured at 655 nm in a microplate reader (SynergyTM Mx, BioTek Instruments Inc.) and analyzed with Gen5TM software.

3.3 Western Blot

After protein quantification, 20 µg of protein samples were prepared. Proteins were denatured at 95°C for 5 minutes using a thermoblock (FALC). Then, the same amount of protein, in the same volume, was loaded in the gel for electrophoresis. The samples were separated on a 10 % SDS-PAGE gel for ± 30 minutes at 70 V followed by ± 1 hour and 30 minutes at 100V. After, the protein lysates were electrotransferred to a nitrocellulose membrane (GE healthcare), in a Mini Trans-Blot® Cell (Bio-Rad) wet transfer system for 2 hours at 100 V. At the end, the membranes were stained with Ponceau to confirm protein transfer. To block the membranes, either 5 % (w/v) non-fat dry milk (Molico) in Tris buffered saline solution with 0.1 % Tween-20 (H5151 Promega) (TBS-T) or 5 % (w/v) bovine serum albumin (BSA) (Sigma Aldrich) in TBS-T, for at least 1 hour in an orbital shaker at RT was used. Note that, BSA was only used when the targeted proteins to be analysed were phosphorylated (the casein present in milk could interfere with these analysis). After blocking, membranes were incubated with the primary antibody for the proteins of interest, using an orbital shaker, overnight, at 4°C. Importantly, β-Actin was used as a loading control. The primary antibodies used were: anti-P-gp (1:100, sc-55510) from Santa Cruz Biotechnology, anti-CHI3L1 (AF2599) from R&D systems, anti-β-Catenin (1:200, sc-7963)

from Santa Cruz Biotechnology, anti-ρ-ERK (1:1000, 9101S), anti-ERK (1:1000, 4695S), anti-ρ-Akt (1:1000, 4051S), anti-Akt (1:1000, 9272S) from Cell Signalling, and anti-β-Actin (1:2000, sc-47778) from Santa Cruz Biotechnology. The membranes were then washed 4x for 7 minutes with TBS-T and incubated for 1 hour in an orbital shaker, at RT with the secondary antibody. The secondary antibodies used were anti-mouse (1:2000, NA931V, ECL™), anti-goat (1:2000, sc-2354) and anti-rabbit (NA934V, ECL™) from Santa Cruz Biotechnology. In the end, the membranes were washed again, and the signal was revealed in Amersham Hyperfilm ECL using the ECL detection reagents (GE Healthcare and Bio-Rad).

4. Cell Cycle Profile by Flow Cytometry

The effect of PTX on the cell cycle profile of both pairs of sensitive and MDR counterpart NSCLC cell lines (NCI-H460 / RH460 and A549 / A549-CDR2) was analysed by flow cytometry. This technique allows to identify the effect of a drug on the cell cycle phases distribution based on the DNA content of the cells. Proliferating cells go through four separate phases: the G1-, S- (DNA synthesis phase), G2-, and M- (mitosis) phase. A sub-G1 peak can be detected, which could be indicative of apoptosis [106].

For the cell cycle analysis, the NSCLC cell lines were seeded at 5.0 x 10^4 cells/mL in 6-well plates for 24 hours. After that, cells were incubated with different conditions: culture medium (blank), medium with H_2O (Vehicle) or with PTX at 1 mM and 2 mM. Upon 48 hours treatment, cells were collected. The medium was removed but not discarded and cells were washed with PBS and detached using triple express. After trypsinization, cells were resuspended in the previously removed medium (so the death cells were included in the assay) and centrifuged at 1200 rpm for 5 minutes at 4 °C. The supernatant was removed, and the pellet fixed with 2 mL of cold 70 % Ethanol (Fisher Scientific), while the samples were being vortexed. After at least 12 hours at 4ºC, cells were centrifuged, under the conditions previously described, and the pellets resuspended in 200 µL of PBS 1x (Sigma Aldrich) containing 5 µg/mL Propidium Iodide (Sigma Aldrich) and 0.1 mg/mL RNase (Invitrogen), for at least 30 min in the dark at 4 ºC. All the samples were analysed by flow cytometry using the BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences). For each analysis, the exclusion of cell debris and aggregates was assured for each analysis, and at least 15 000 events per sample were plotted for all the acquisitions. The % of cells in the sub-G1, G0/G1, S, and G2/M phases of the cell cycle was subsequently determined using the FlowJo 10.8. Software.

5. Cell death by Annexin V-FITC / PI flow cytometry detection assay

The annexin V-FITC / PI detection assay was performed in the sensitive and MDR counterpart NCI-H460 / RH460 cell lines to study the effect of PTX on cell death. The Annexin V labelled with FITC (Annexin V-FITC) interacts with phosphatidylserine (PS), a negatively charged membrane component, through calcium binding, and Propidium iodide (PI), which is a fluorescent probe, penetrates into death cells [107]. In early apoptosis, PS translocate from the internal to external face of the plasma membrane and Annexin V-FITC binds to the Ca^{2+} phospholipid. In late apoptosis / necrosis, occurs nuclear condensation, degradation of cytoskeletal elements and DNA strand breaks, so PI can be used to stain the DNA in the nucleus [107]. Thus, all cells labelled with Annexin V-FITC and / or PI were considered dead [108].

Briefly, NSCLC cell lines were plated at 5.0 x 10^4 cells/mL in 6-well plates for 24 hours to adhere. Then, cells were treated with different conditions: culture medium (blank), medium with H_2O (Vehicle) or medium with PTX at 1mM and 2 mM. Following 48 hours incubation, and 1 hour before collecting the cells, 200 µl of pure ethanol was added to the blank condition well (positive control). Then cells were collected as previously described in section 4. and then centrifuged at 1200 rpm for 5 minutes. The assay was performed according to the Annexin V-FITC Apoptosis Kit Detection (Invitrogen, BMS500FI-300; eBioscienceDX, Bender MedSystems). The supernatant was discarded, and the pellet resuspended in 600 µl of Binding Buffer (4x). Each sample was divided into two other samples: a) auto-fluorescence and b) labelled sample. To the labelled samples, 7.5 µl of Annexin-FITC was added and after 10 minutes of incubation at RT in the dark, PI was added, in ice. The samples were analysed by flow cytometry using the BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences). The exclusion of cell debris and aggregates was performed for each analysis, and at least 15 000 events per sample were plotted for all the acquisitions. The % of death cells was subsequently determined using the FlowJo 10.8. Software.

6. Rhodamine-123 assay by Flow Cytometry

Rhodamine-123 (rh123) assay was used to determine the *in vitro* P-gp inhibitory activity of PTX in the MDR RH460 cell line. Rh123 is a fluorescence dye that is a substrate of P-gp, a MDR related glycoprotein [109, 110]. If cells present high levels of P-gp activity, rhodamine-123 will not be retained inside the cells, so the fluorescent signal will be weak, whereas, if the levels of this protein is reduced, the fluorescent signal will increase.

In order to perform this assay, both sensitive and MDR cell lines NCI-H460 / RH460 were plated at the previously mentioned optimal concentrations. For this assay, after cells attach, two exposing times were performed: a) 1 hour of treatment with the vehicle (medium with H_2O) and/or with PTX at 1 mM, 2 mM, 5 mM and 10 mM; b) 48 hours of treatment with the vehicle and/or PTX at 1 mM and 2 mM. The sensitive drug cell line, NCI-H460, was used as a negative control (since this cell line poorly expresses P-gp). The MDR cell line treated with Verapamil at 20 μM, a known inhibitor of P-gp, was used as a positive control.

Briefly, cells were treated with the vehicle, verapamil or PTX, in the presence/absence of rh-123 at 1 μM, as shown in **Figure 9**. Cells were collected as already mentioned in section 4. and centrifuged at 1200 rpm for 5 min. The pellet was then resuspended with cold PBS and the samples stored in ice, at dark, until being analysed by BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences). The exclusion of cell debris and aggregates was done for each analysis, and at least 15 000 events per sample were plotted for all the acquisitions. The median of FITC was subsequently determined using the FlowJo 10.8. Software.

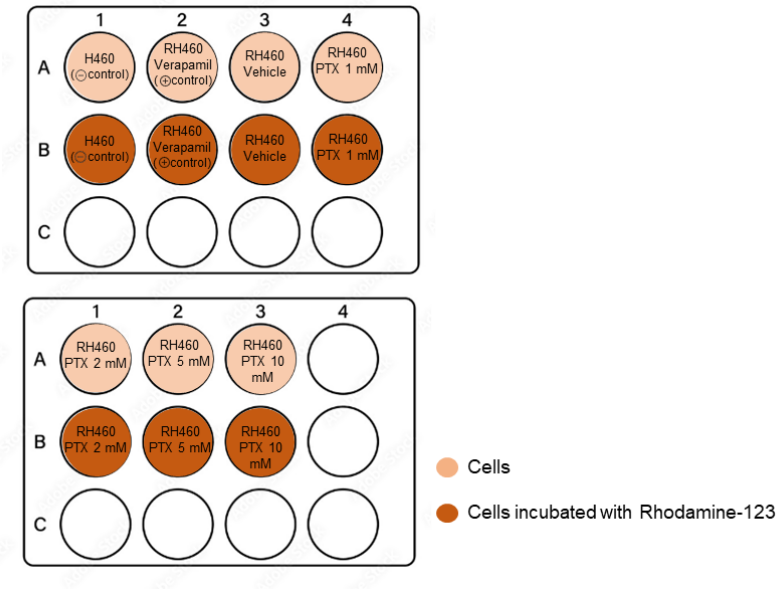

Figure 9 - Schematic representation of 12-well plates for the Rhodamine-123 assay in the sensitive and counterpart MDR NCI-H460 and RH460 NSCLC cell lines. RH460 cells were treated with the vehicle (medium with H_2O) and with pentoxifylline at 1 mM, 2 mM, 5 mM or 10 mM. The sensitive drug cell line, NCI-H460, was used as a negative control. The MDR cell line treated with verapamil was used as a positive control.

7. Statistical analysis

All experiments were carried out at least three times independently and the results are expressed in mean ± standard error of the mean (SEM). The statistical analysis was performed using the two-tailed unpaired t-test, with GraphPad Prism 8.0 software. Statistical significance was considered whenever $p < 0.05$.

Results and Discussion

1. Effect of pentoxifylline on the growth of two pairs of sensitive and MDR NSCLC cell lines

The effect of pentoxifylline (PTX) on the growth of two pairs of sensitive and MDR NSCLC cell lines (NCI-H460 / RH460 and A549 / A549-CDR2) was assessed using the SRB assay. For that, the NSCLC cell lines were treated for 48 hours with five serial dilutions (4 mM, 2 mM, 1mM, 0.5 mM and 0.25 mM) of PTX. The dose-response curves were determined (**Figure 10 and 11**) and the GI_{50} concentrations were subsequently calculated by interpolation on the dose-response curves (**Table 4**).

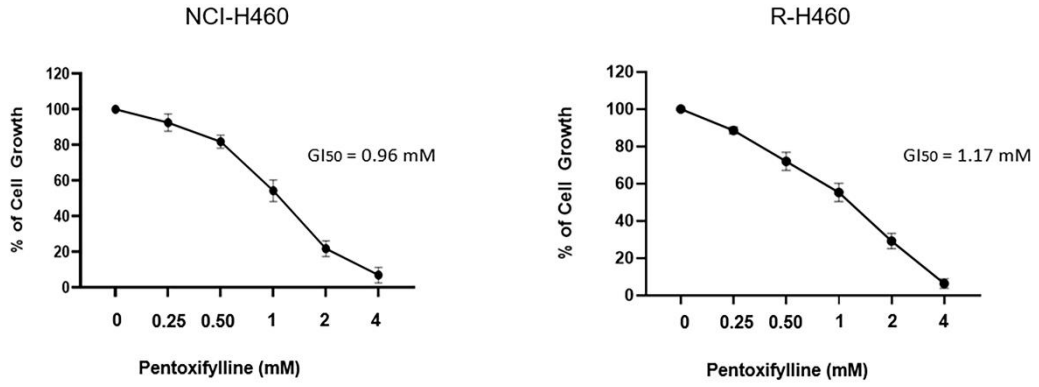

Figure 10 - Dose-response curves of pentoxifylline on the NCI-H460 and RH460 cell lines. Cells were treated with five serial dilutions of this drug for 48 hours and results were obtained by the SRB assay. Results are presented as a percentage (%) of cell growth and are the mean ± SEM of at least three independent experiments.

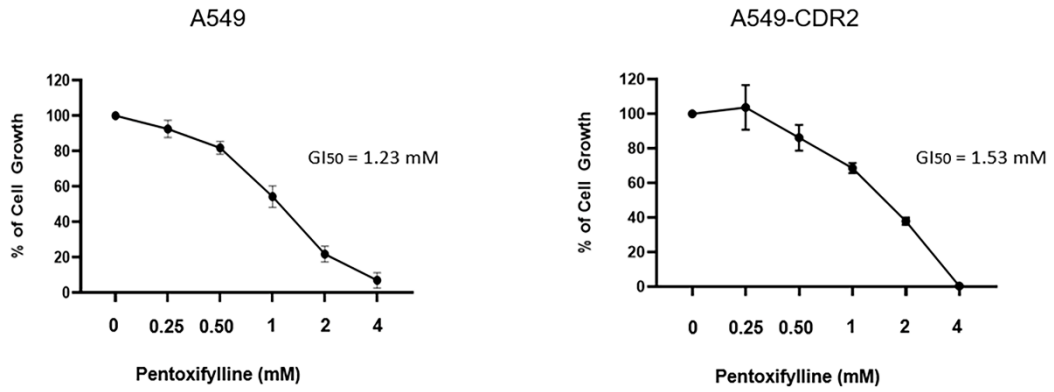

Figure 11 - Dose-response curves of pentoxifylline on the A549 and A549-CDR2 cell lines. Cells were treated with five serial dilutions of this drug for 48 hours and results were obtained by the SRB assay. Results are presented as a percentage (%) of cell growth and are the mean ± SEM of at least three independent experiments.

Table 4 - GI_{50} concentrations of pentoxifylline in the sensitive and MDR counterpart pairs of NSCLC cell lines; NCI-H460 / RH460 and A549 / A549-CDR2. The human NSCLC cell lines were treated with the drug for 48 hours and SRB assay performed. Results are the mean ± SEM of at least three independent experiments.

		GI_{50} * Pentoxifylline (mM)
NSCLC Cell Lines	NCI-H460	0.96 ± 0.08
	RH460	1.17 ± 0.17
	A549	1.23 ± 0.07
	A549-CDR2	1.53 ± 0.04

* GI_{50} means the concentration of drug that causes 50% cell growth inhibition

Our results demonstrated that PTX decreased the cell growth of all the NSCLC cell lines tested, at concentrations bellow 2 mM. Moreover, the GI_{50} concentration of PTX in both drug sensitive cell lines (NCI-H460 and A549) was slightly lower, when compared to their counterpart MDR cell lines (RH460 and A549-CDR2). Thus, our findings suggest that PTX is not a collateral sensitizer compound. Indeed, a drug is consider to have a collateral

sensitivity effect when it has a stronger effect in MDR cells compared to their sensitive parental cells of origin [111]. Interestingly, a study conducted by Bravo-Cuellar *et al.* (2020) also found no significant differences between the IC_{50} (drug concentration that inhibit 50% of cell response) of PTX on two sensitive and their MDR counterpart human cervical cancer cell lines [80]. Moreover, Abdin *et al.* (2021) demonstrated that a resistant breast cancer cell line, MDA-MB-231/ADR was significantly less responsive to PTX treatment, when compared with the sensitive MDA-MB-231 cells [112]. To our knowledge, this is the first report on the effect of PTX in NSCLC cell lines.

It is important to note that the concentration of PTX necessary to inhibit the growth of the NSCLC cell lines is significantly higher (in the mM range) than the concentrations of the anticancer drugs, such as paclitaxel, carboplatin and vinorelbine, to inhibit the same cell lines (in the nM or μM range) [113], **Table 2**. These differences were expected, as PTX is an approved drug for the treatment of vascular diseases and not for cancer treatment.

Therefore, our next goal was to study the sensitizing effect of PTX to different anticancer agents, to identify possible therapeutic combinations able to increase treatment efficacy in NSCLC.

2. Sensitizing effect of pentoxifylline to different drug treatments in pairs of sensitive and MDR NSCLC cell lines

Preliminary results obtained by our research group (unpublished data) demonstrated that PTX at 1 mM sensitized both sensitive and MDR NCI-H460/RH460 cell lines to paclitaxel (PAC) and carboplatin (CBP) treatments. The effect of these drug combinations in inhibiting cell growth was statistically significantly higher, when compared to the effect of each drug alone. Thus, we first validated these results in another sensitive and MDR counterpart cell line (A549 / A549-CDR2). Then, we tested other drug combinations, including the combination of PTX with vinorelbine (VR) and with VR plus CBP, in both pairs of sensitive and MDR cell lines (NCI-H460 / RH460 and A549 / A549-CDR2).

The sensitizing effect of PTX to VR (**Figure 12A**) and to VR plus CBP (**Figure 12B**) in the sensitive NCI-H460 and MDR RH460 cell lines was evaluated, using the SRB assay. The results obtained showed that the combination of PTX at 1 mM with VR at 35 nM significantly decreased the MDR RH460 cell growth, when compared to treatment with each drug individually. Although not statistically significant, this drug combination slight decreased the growth of the sensitive NCI-H460 cell line, when compared to vinorelbine

alone. Interestingly, the combination of PTX at 1 mM with VR at 35 nM plus CBP at 17.7 μM was significantly better in decreasing cell growth, when compared to the treatment of VR plus CBP, in both sensitive NCI-H460 and MDR RH460 cell lines.

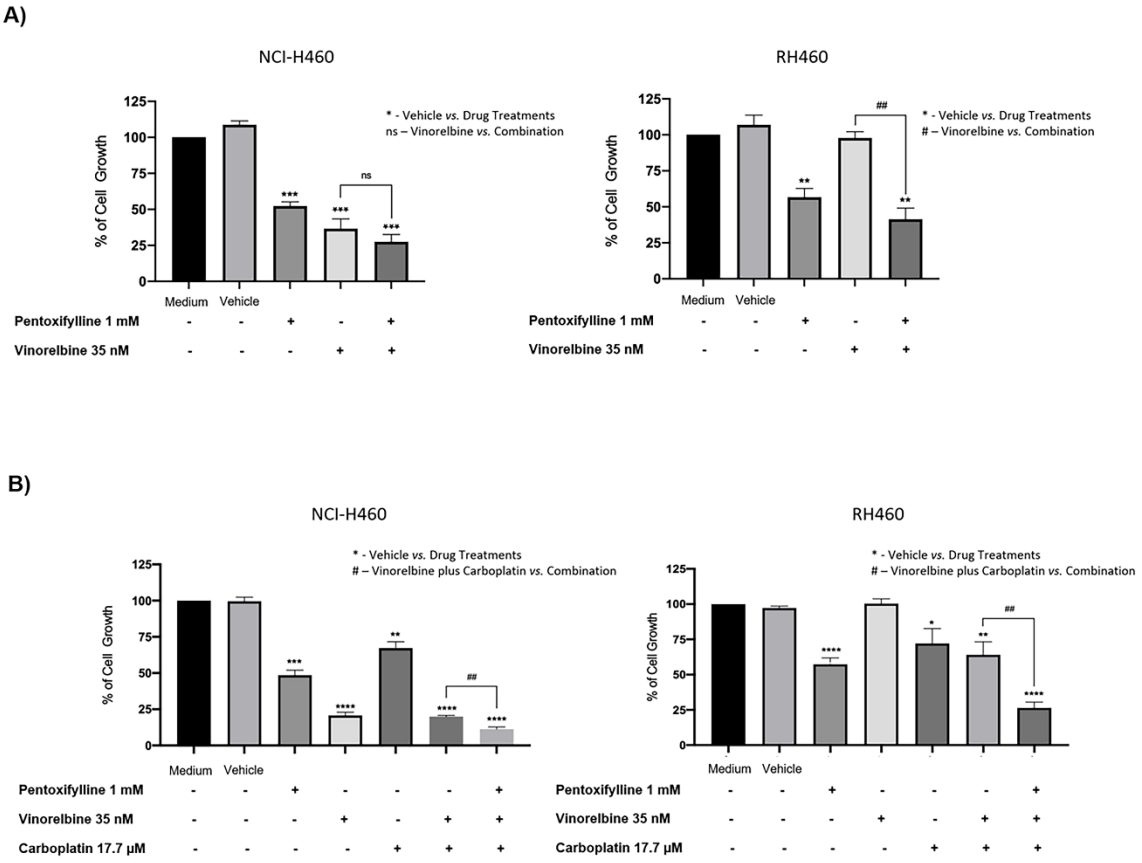

Figure 12 - Effect of the pentoxifylline in combination therapy with vinorelbine or with vinorelbine plus carboplatin, on the % of cell growth of NCI-H460 and RH460 cell lines, assessed by the SRB assay. Cells were treated with drug combinations consisting of pentoxifylline at 1.0 mM with A) vinorelbine at 35 nM; and B) vinorelbine at 35 nM plus carboplatin at 17.7 μM, for 48 hours. The effect of the vehicle at the higher concentration tested was also analysed. Results are presented as a % of cell growth and are the mean ± SEM of at least three independent experiments. * or # indicate $p < 0.05$, ** or ## $p < 0.01$, *** or ### $p < 0.001$, **** or #### < 0.0001; ns (non-significant) indicates $p > 0.05$.

Then, the effectiveness of the different combination therapies was validated in another sensitive and MDR counterpart pair of cell lines, using the SRB assay. For that, the A549 and A549-CDR2 cell lines were incubated with PTX at 1 mM in combination with different drugs, such as PAC at 2.7 nM, CBP at 83.7 μM, VR at 25 nM and VR at 25 nM plus CBP at 83.7 μM (**Figure 13**). The results demonstrated that PTX sensitized both A549 and A549-CDR2 cell lines to PAC treatment, with the drug combination significantly decreasing the cell growth, when compared to each drug individually (**Figure 13A**). The

combination of PTX with CBP was also more effective in inhibiting the cell growth of the sensitive A549 cell line, when compared to the treatment with each drug individually (**Figure 13B**). Although not statistically significant, the combination of PTX with CBP slightly decreased the growth of the MDR A549-CDR2 cell line, when compared with each drug alone. With regard to the combination of PTX with VR, a statistically significant inhibition of cell growth was observed, when compared to each drug individually, in both sensitive and MDR cell lines (**Figure 13C**). The same statistic effect was observed when both pairs of sensitive and MDR cell lines were treated with PTX combined with VR plus CBP (**Figure 13D**).

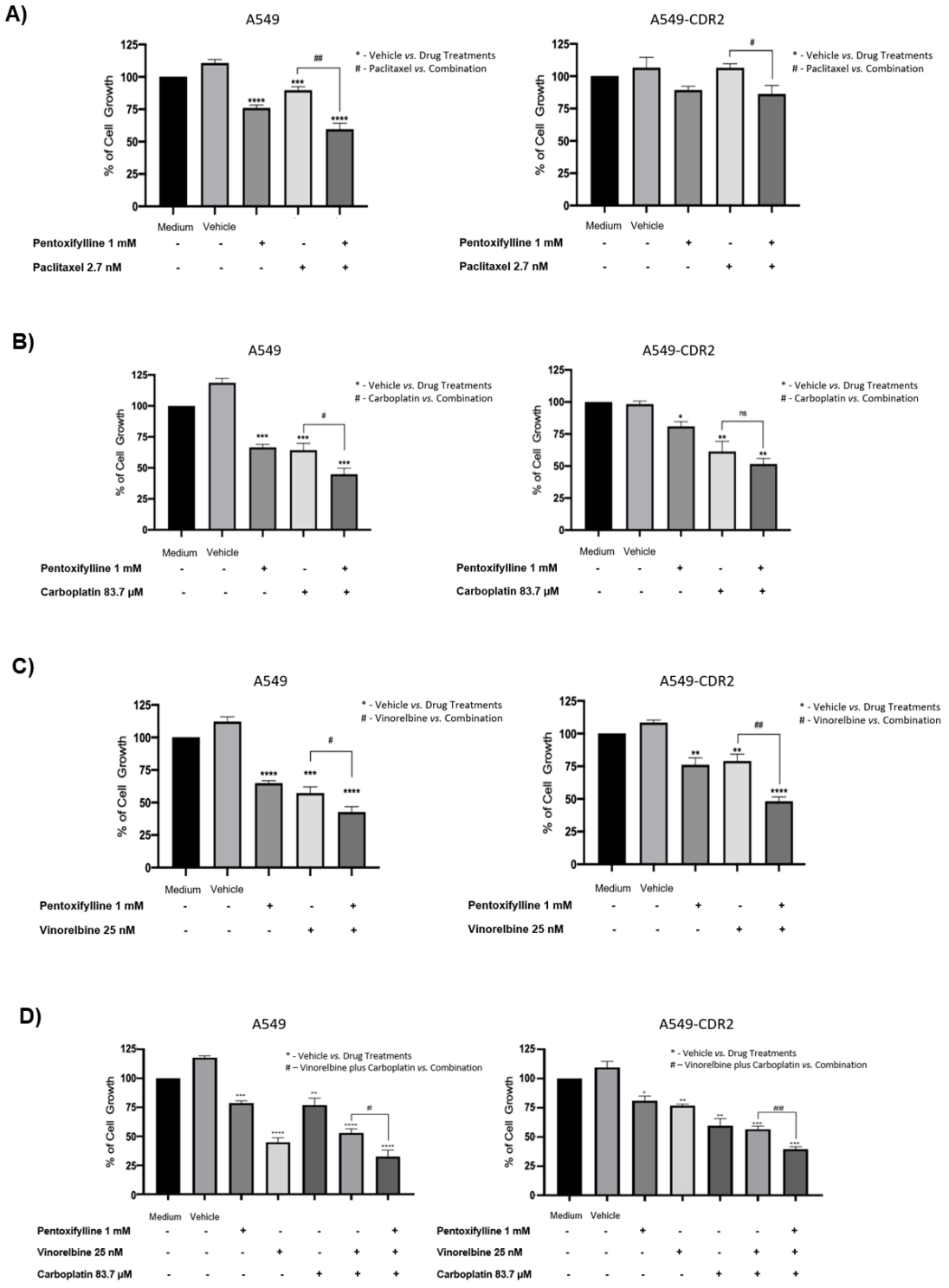

Figure 13 - Effect of the pentoxifylline in combination therapy with different anticancer drugs on the % of cell growth of A549 and A549-CDR2 cell lines, assessed by the SRB assay. Cells were treated with drug combinations consisting of pentoxifylline at 1.0 mM with A) paclitaxel at 2.7 nM; B) carboplatin at 83.7 µM; C) vinorelbine at 25 nM and D) vinorelbine at 25 nM plus carboplatin at 83.7 µM, for 48 hours. The effect of the vehicle at the higher concentration tested was also analysed. Results are presented as a % of cell growth and are the mean ± SEM of at least three independent experiments. * or # indicate $p < 0.05$, ** or ## $p < 0.01$, *** or ### $p < 0.001$, **** or #### < 0.0001 and ns (non-significant) indicates $p > 0.05$.

Importantly, the Fold Change of the percentage of cell growth inhibition of the combined therapies (anticancer drugs combined with PTX) *vs* conventional chemotherapies (anticancer drugs, including paclitaxel, vinorelbine, carboplatin, and vinorelbine plus carboplatin) was evaluated in both pairs of NSCLC cell lines under study (NCI-H460/RH460 and A549/A549-CDR2). The results, presented in **Table 5,** showed that the percentage of cell growth inhibition was higher in all the combined therapies (consisting of anticancer drugs combined with PTX), when compared with the conventional chemotherapies (with only anticancer drugs), in both sensitive and MDR NSCLC cell lines. Interestingly, the best Fold Change was observed for the combination therapies consisting of PTX with VR and PTX with VR plus CBP, in the MDR RH460 cell line (with values higher than 2.0).

Table 5 - Fold Change of the combined therapies with pentoxifylline versus conventional chemotherapies in the pairs of sensitive and MDR NSCLC cell lines (NCI-H460 / RH460 and A549 / A549-CDR2).

	Combination Therapy with **Pentoxifylline PLUS**	**Paclitaxel**	**Vinorelbine**	**Carboplatin**	**Vinorelbine *plus* Carboplatin**
Cell Lines	**NCI-H460**	2.14	1.33	1.86	1.78
	RH460	1.64	2.36	1.74	2.42
	A549	1.5	1.25	1.41	1.62
	A549-CDR2	1.23	1.53	1.19	1.43

Fold change > 1: Combined Therapy with pentoxifylline is better than Conventional Chemotherapy.

Taken together, our data demonstrated that PTX sensitizes both sensitive and MDR NSCLC cell lines to different anticancer regimens. Interestingly, Ohsaki *et al.* (1996) have also demonstrated that PTX at 0.5 and 1 mM sensitized two sensitive NSCLC cell lines (PC-9 and PC-14) to etoposide and cisplatin treatments [84]. Moreover, other studies reported the chemosensitizing effect of PTX in different cancer cell lines. For instance, Bravo-Cuellar *et al.* (2010; 2020) showed that PTX sensitized cervical cancer cells to adryamicin [79], as well as to cisplatin in resistant cervical cancer cells[80]. Moreover, Cancino-Marentes *et al.* (2021) showed that PTX sensitized prostate cancer cells to docetaxel [81]. Importantly, our research group demonstrated that PTX sensitized pancreatic cancer cells to gemcitabine [82]. In the same line, Kim *et al.* (2017) also demonstrated an improvement in gemcitabine

efficacy when combined with PTX, in human pancreatic tumour xenografts [83]. As far as we known, our results were the first to report the chemosensitizing effect of PTX to different drug regimens (PAC, VR, CBP and VR plus CBP) not only in sensitive NCSLC cell lines, but also in MDR NSCLC cell lines. Therefore, our data suggests that combination therapies of PTX, a non-anticancer approved drug, with the most used chemotherapeutic agents in the clinical practice, could be a promising strategy to improve NCSLC treatment [15, 17, 21].

3. Effect of the drug combinations on the growth of MCF-10A non-tumorigenic cell line

To study the cytotoxic effect of the combined drug treatments, the non-tumorigenic cell line MCF-10A was incubated with PTX plus PAC (**Figure 14A**) and with PTX plus VR and/or CBP (**Figure 14B**), at the higher concentrations tested in the NSCLC cell lines. Our results demonstrated that the combined treatment consisting of PTX at 1 mM and PAC at 5.7 nM did not cause a statistically significant increase in toxicity in the MCF-10A non-tumorigenic cell line, when compared with PAC alone. Moreover, PTX at 1 mM combined with VR 35 nM plus CBP 17.7 μM, as well as, with VR 25 nM plus CBP 83.7 μM, did not induce significant toxicity in the MCF-10A non-tumorigenic cell line, when compared to the combination therapy consisting of only VR plus CBP. Therefore, our data suggests that the combination of PTX with the conventional anticancer agents did not cause significant additional cytotoxicity, at least at the concentrations tested.

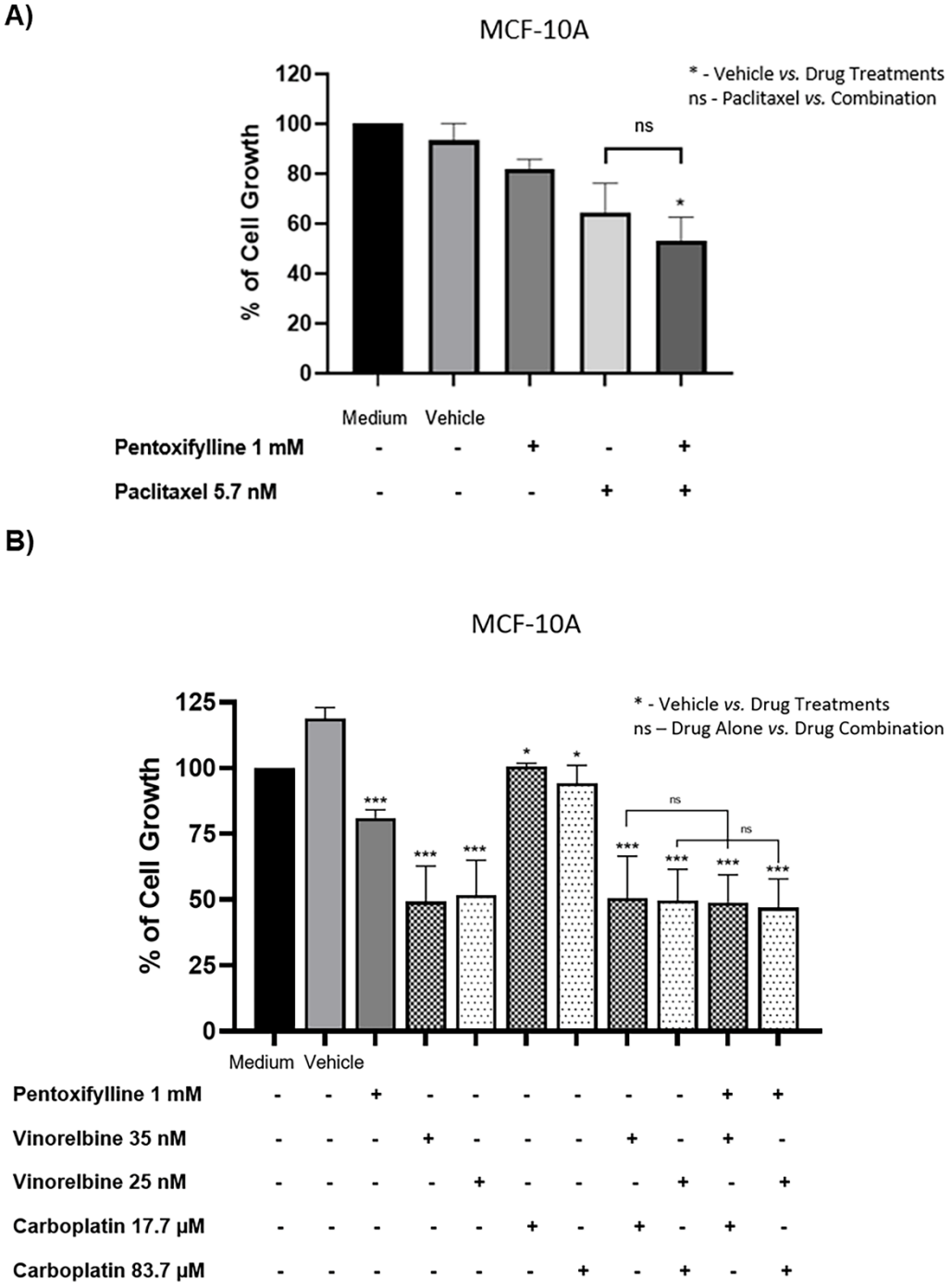

Figure 14 - Effect of the combined treatment with Pentoxifylline plus Paclitaxel and with Pentoxifylline plus Vinorelbine and Carboplatin in the non-tumorigenic cell line MCF-10A, assessed by the SRB assay. Cells were treated with the combined therapies consisting of A) Pentoxifylline at 1.0 mM and Paclitaxel at 5.7 nM; and B) Pentoxifylline at 1 mM combined with VR 35 nM plus CBP 17.7 µM, as well as, with VR 25 nM plus CBP 83.7 µM, for 48 hours. The effect of the vehicle (H_2O plus DMSO) at the highest concentration tested was also analyzed. Results are presented as a % of cell growth and are the mean ± SEM of at least three independent experiments. * indicates $p < 0.05$, *** $p < 0.001$ and ns (non-significant) indicates $p > 0.05$.

4. Effect of pentoxifylline on the expression levels of P-gp, Chitinase 3-like-1 and its downstream proteins in pairs of sensitive and MDR NSCLC cell lines

Studies have demonstrated that PTX reduced the expression of the mdr1 gene, which encodes for P-gp, in a L1210 mouse leukemic cell line resistant to vincristine [114] and in multidrug resistant mouse leukemic L1210/VCR cells [78]. P-gp is a drug efflux pump and its high expression levels have been associated with a MDR phenotype [70]. Indeed, our research group showed that the MDR RH460 cell line overexpresses P-gp [115]. Furthermore, preliminary data from our research group demonstrated that the MDR A549-CDR2 has high expression levels of P-gp (unpublished data). Thus, our next step was to verify the effect of PTX on the expression levels of P-gp, in both sensitive and MDR pairs of NSCLC cells, after 48 hours treatment, by Western Blotting.

Our results confirm the higher expression levels of P-gp in the RH460 and A549-CDR2 MDR cell lines, when compared to their sensitive NCI-H460 and A549 counterparts (**Figure 15**). In addition, although not statistically significant, PTX at 1 mM decreased the expression levels of P-gp in both MDR NSCLC cell lines (RH460 and A549-CDR-2).

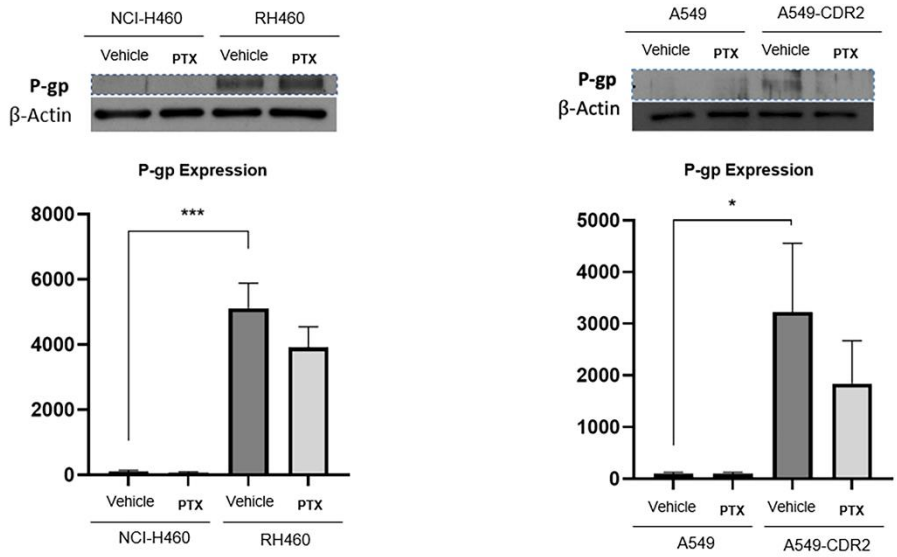

Figure 15 - Effect of pentoxifylline (PTX) on the expression levels of P-gp, determined by Western Blot. P-gp expression levels on NCI-H460 / RH460 (A) and A549 / A549-CDR2 (B) cell lines treated with pentoxifylline at 1.0 mM after 48 hours. The effect of the vehicle (H_2O) at the highest concentration tested was also analysed. β-Actin was used as loading control. Results are presented as a % of expression and are the mean ± SEM of at least three independent experiments. ** $p < 0.01$

As far as we know, this is the first report of the effect of PTX on the expression levels of P-gp in NSCLC cells. In fact, our data is in agreement with Breier *et al.* (1994), who showed that PTX, at a range from 0.1 to 0.5 mM, reduced P-gp expression on a mouse leukemic cell line resistant to vincristine [114]. A possible mechanism of action for the chemosensitizing effect of PTX on MDR cell lines could be the decrease on the expression of P-gp, which in turn leads to an increase in the intracellular drug concentration, thus improving drug treatment efficacy.

Further, the effect of PTX on Chitinase 3-like-1 (CHI3L1) expression levels and its downstream proteins, namely β-catenin, ρ-Akt, and ρ-ERK was also analysed in both sensitive and MDR counterpart pairs of cell lines, NCI-H460 / RH460 (**Figure 16 A-D**) and A549 / A549-CDR2 (**Figure 16 E-H**). Our results showed that PTX decreased the expression levels of CHI3L1 in both sensitive and MDR NSCLC cell lines, with statistically significance on the RH460 cell line. A decrease in the expression levels of β-catenin in both MDR cell lines was also observed, although not statistically significant. Regarding the expression levels of ρ-ERK and ρ-Akt, PTX does not alter their expression levels, in neither the sensitive or the MDR cell lines.

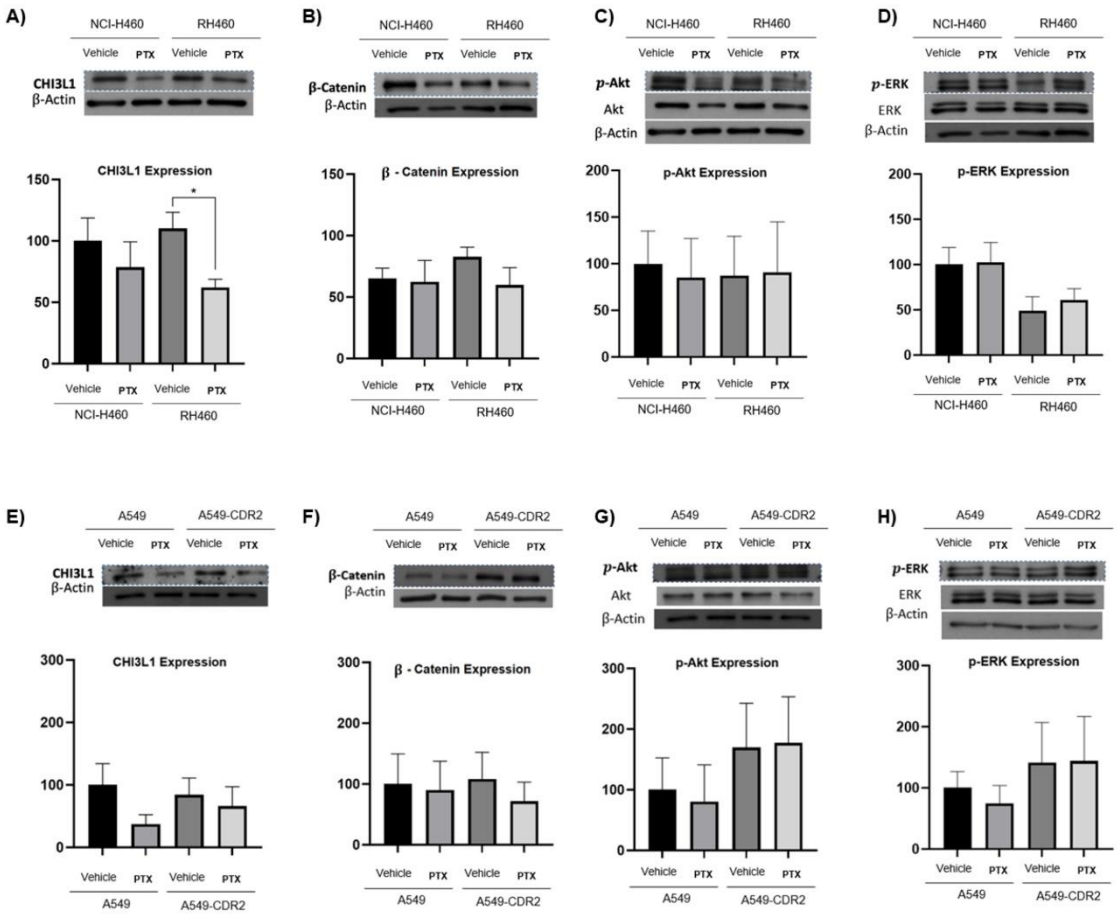

Figure 16 - Effect of pentoxifylline on the expression levels of Chitinase 3-like-1 (CHI3L1) and its downstream proteins, determined by Western Blot. CHI3L1, β-catenin, p-Akt, and p-ERK expression levels on NCI-H460/RH460 (A-D) and A549/A549-CDR2 (E-H) cell lines treated with pentoxifylline at 1.0 mM after 48 hours. The effect of the vehicle (H_2O) at the highest concentration tested was also analysed. β-Actin was used as loading control. Results are presented as a % of expression and are the mean ± SEM of at least three independent experiments.

Table 6 presents the summary of the effect of PTX on the expression levels of P-gp, CHI3L1, β-catenin, ρ-Akt and ρ-ERK in both pairs of sensitive and MDR cell lines, NCI-H460 / RH460 and A549 / A549-CDR2 (analysed by Western Blot).

Table 6 - Summary of the results of the effect of pentoxifylline on the expression leves of P-gp, CHI3L1, β-catenin, ρ -Akt, and ρ-ERK in the two pairs of sensitive and MDR cell lines, analysed by Western Blot. The only significant effect was on CHI3L1 on the RH460 cells, the remaining effects were not significant.

	[PTX]	P-gp	CHI3L1	β-catenin	ρ -Akt	ρ-ERK
Cell Lines	NCI-H460	-	↓	-	-	-
	RH460	↓	↓	↓	-	-
	A549	-	↓	-	-	-
	A549-CDR2	↓	↓	↓	-	-

Several studies demonstrated that methylxanthine drugs, which include PTX, are chitinase inhibitors [116]. Indeed, it has been reported that PTX decreases CHI3L1 levels, having impact in inflammatory diseases, such as chronic intestinal inflammatory diseases [117] and invasive pulmonary aspergillosis [118]. Importantly, our research group has recently demonstrated the effect of PTX in decreasing CHI3L1 expression levels in pancreatic cancer cells, leading to an increase in drug response [82]. The results presented here also demonstrated the potential of PTX in decreasing the expression levels of CHI3L1, in both sensitive and MDR NSCLC cell lines.

Furthermore, it has been reported that CHI3L1 inhibition leads to a decrease in β-catenin expression levels, namely in gastric cancer and Glioblastoma [98, 119]. Other studies have demonstrated that Akt and ERK signalling pathways are downregulated when CHI3L1 is decreased in human pancreatic cancer cells [82], human embryonic kidney and human glioblastoma-astrocytoma cells [120] and in lung cancer cells [121]. Indeed, Wnt/ β-catenin, PI3K/AKT and Raf/MAPK signalling pathways are highly activated in several human cancers [122-124]. Unexpectedly, our data is not in agreement with this literature. With exception of β-catenin expression levels in both MDR cell lines, PTX did not affect the expression levels of the main CHI3L1 downstream proteins, p-Akt and p-Erk, in neither of the cancer cell lines and at the concentrations tested. Although PTX decreased CHI3L1 expression levels, this drug did not affect the main downstream pathways, such as β-catenin, Akt and ERK. One possible reason is that the PTX concentration used in our work, 1 mM, was not enough to see such an effect. For instance, Lee *et al,* (2014) demonstrated that PTX decreased CHI3L1 mRNA expression of a human colon cancer cell line, SW480, and significantly reduced p-Akt expression levels when used at 10 mM [117].

Another possible explanation is that PTX could have effect through other downstream pathways activated by CHI3L1 in NSCLC. CHI3L1 activates other signalling pathways, namely TGF-β through upregulation of SMAD2 and SMAD3 phosphorylation. For example, Qiu *et al.* (2018) showed that CHI3L1 activates the TGF-β signalling pathway in both Bel7404 and HepG2 liver cancer cells, regulating liver fibrosis [125]. Fichtner *et al*. (2006) showed that IL-13Rα2, the CHI3L1 receptor, is responsible for activating the TGF-β signalling pathway through induction of the synthesis of TGF-β1 [126]. Other CHI3L1 downstream pathways have also been described, such as the JAK/STAT signalling pathway. In fact, the activation of STAT3 transcription factor affects several cellular processes, including cell cycle, cell proliferation, cellular death and tumorigenesis [127]. For instance, the downregulation of STAT3 by CHI3L1 in a Swedish amyloid precursor protein (SwAPP) transgenic mice model, led to the reduction of lung tumour development [128]. Park *et al.* (2020) also demonstrated a correlation between high expression levels of CHI3L1 and p-STAT3 levels in human bone tumour tissues [129].

Therefore, our data suggest that the chemosensitizing effect of PTX, in sensitive and MDR NSCLC cells, could be through inhibition of CHI3L1, however without affecting the Akt and the Erk pathways at the concentrations tested.

5. Effect of pentoxifylline on the Cell Cycle Profile of NSCLC Cell Lines

In order to study the effect of PTX on the cell cycle profile in both pairs of sensitive and MDR counterpart NSCLC cell lines, flow cytometry analysis was performed. **Figure 18** shows the results of the effect of PTX at 1 mM and 2 mM on the cell cycle profile of NCI-H460 / RH460 (**Figure 17A**) and A549 / A549-CDR2 (**Figure 17B**) cell lines. PTX, at both concentrations, statistically significantly increased the % of cells in the G0/G1 phase of the cell cycle in the sensitive NCI-H460 cells. When increasing the PTX concentration to 2 mM, the % of cells in the G0/G1 phase of the cell cycle was statistically significantly increased in the sensitive A549 cells and in both MDR cell lines (RH460, A549-CDR2). Furthermore, PTX statistically significantly decreased the % of cells in G2/M phase of the cell cycle of the MDR RH460 cells. Although not statistically significant, our data also showed a slight increase in the % of cells in the sub-G1 peak in both sensitive and MDR pairs of cell lines, which is suggestive of apoptosis [130]. Taking together, PTX affects the cell cycle profile of both sensitive and MDR cells by increasing the number of cells in the G0/G1 phase of the cell cycle.

Drugs that have an effect on the cell cycle are crucial dysregulators of cell cycle progression, thus affecting cancer cell proliferation [131, 132]. Several studies have reported the effect of PTX on cell cycle arrest in different cancer cell models, including in triple negative breast cancer [74, 133], liver cancer [75] and melanoma [134] cell lines. Our results are in agreement with those studies. As far as we known, this is the first report on the effect of PTX on the cell cycle profile on lung cancer cells.

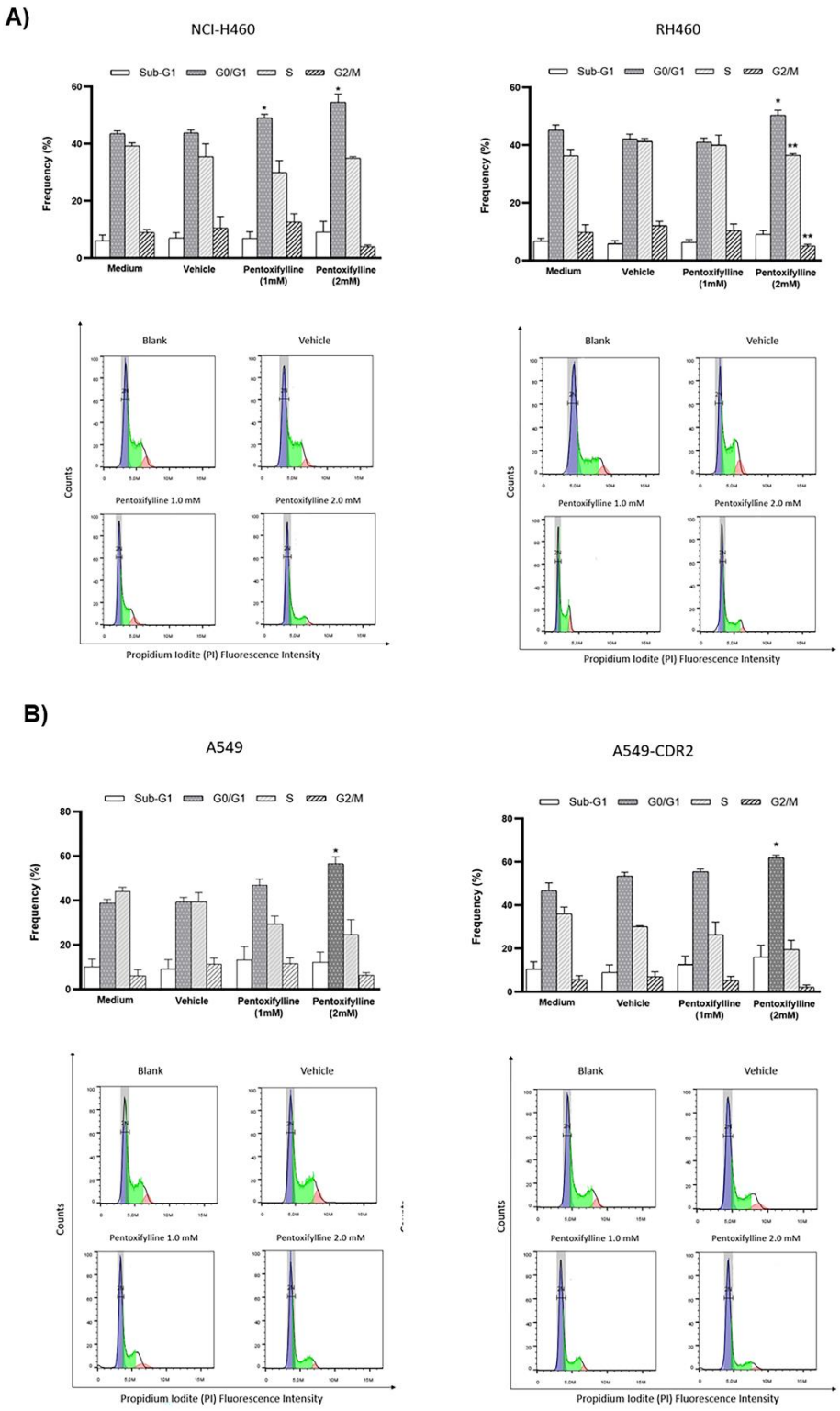

Figure 17 - Effect of pentoxifylline on the cell cycle profile of sensitive and MDR NSCLC cells analysed by flow cytometry following PI staining. (A) NCI-H460 / RH460 and (B) A549 / A549-CDR2 were treated with pentoxifylline at 1.0 or 2.0 mM, for 48 hours. The frequency of the cell cycle phases for each condition, as well as the representative cell cycle histograms are presented. Results are the mean ± SEM of at least 3 independent experiments. * indicates $p < 0.05$ and ** $p < 0.01$ and *** $p < 0.001$

6. Effect of pentoxifylline on Cell Death of NSCLC Cell Lines

As previously mentioned, our results showed that PTX slightly increase the % of cells in sub-G1 peak, being suggestive of apoptosis. Thus, we further analysed the effect of PTX at 1 mM and 2 mM on the levels of cell death, in the sensitive and its MDR counterpart cell lines, NCI-H460 / RH460 (**Figure 18**), by flow cytometry following Annexin V-FITC/PI staining.

As expected, our results showed that PTX at 2.0 mM statistically significantly increased the % of dead sensitive NCI-H460 cells. This effect, however, was not observed in the MDR RH460 cells. Our results in the sensitive cells are in agreement with those of other authors, such as Wang *et al.* (2017) and Al-Husein *et al.* (2022), who demonstrated that PTX at 1 mM (or higher) induced apoptosis in hepatocellular carcinoma (HepG2) [75] and colorectal cancer (HCT116 and SW480) [76] cells. Importantly, other studies reported that the chemosensitizing effect of PTX might be through an increase of apoptosis. For instance, Bravo-Cuellar *et al.* (2010 and 2020) showed that PTX sensitized cervix cancer cells to adriamycin [79] and human cervical cancer cells resistant to cisplatin to cisplatin treatment [80] through an increase in apoptosis. Moreover, another study showed that PTX sensitized human cervical tumour cells to cisplatin through apoptosis induction [135]. Also, Castellanos-Esparza *et al.* (2018) described that the synergistic effect of PTX with simvastatin, in the triple-negative breast cancer cell line MDA-MB-231, was through an induction of cell death [133].

Therefore, our results and the scientific literature suggest that PTX induces apoptosis in several cancer cell models, therefore its capability to sensitize those cells to several anticancer drugs might be due to this increase in apoptosis.

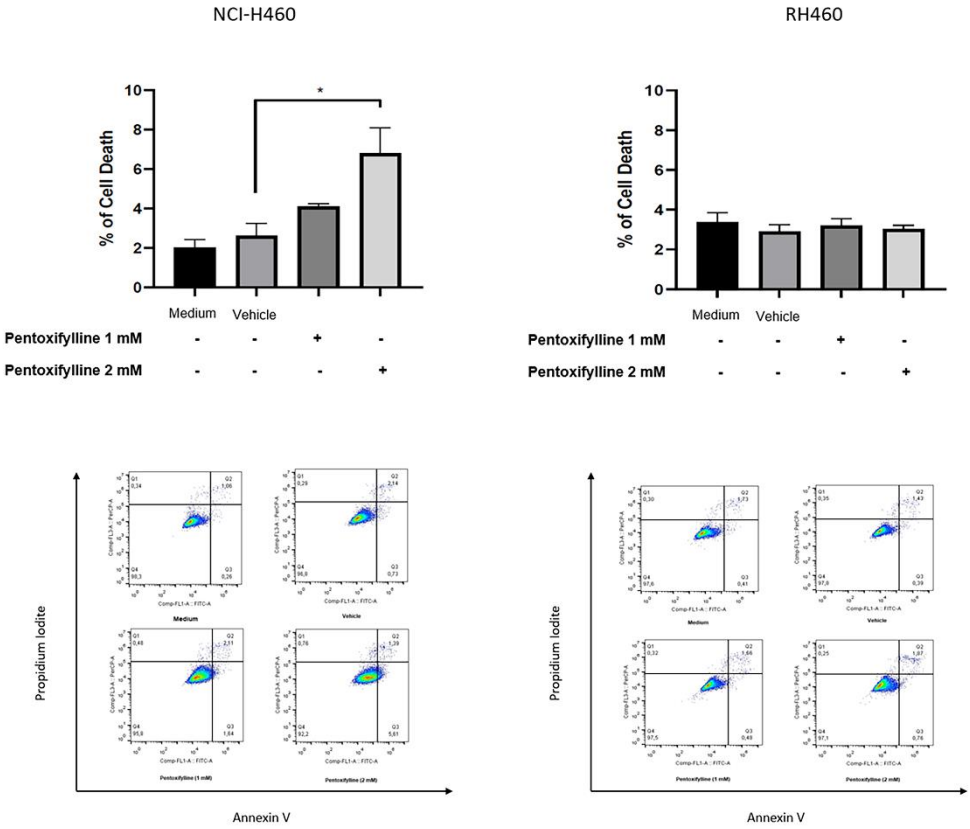

Figure 18 - Effect of pentoxifylline on the % of cell death of NCI-H460 and RH460 cells determined by flow cytometry following Annexin V-FITC/PI staining. Cells were treated with pentoxifylline at 1.0 or 2.0 mM, for 48 hours. The effect of the vehicle at the highest concentration tested was also analyzed. The frequency of the levels of dead cells for each condition and the representative dot plot cytograms showing the % of cell death (Annexin V+ and/ or PI+) and viable cells (Annexin V-PI-) are presented. Results are the mean ± SEM of at least three independent experiments. * indicates $p < 0.05$.

7. Effect of pentoxifylline on the inhibition of P-gp activity

The above presented results (Figure 15) showed that PTX decreased the expression levels of P-gp after 48 hours of treatment, in both MDR NSCLC cell lines, RH460 and A549-CDR2. Thus, we further analysed the effect of PTX on the activity of P-gp, in the MDR RH460 cells, using the Rhodamine-123 (rh123) assay (**Figure 19**). The sensitive cell line NCI-H460 was used as a negative control, as this cell line has low expression of the drug efflux pump P-gp. The RH460 cells (with high P-gp activity) treated with verapamil (a known P-gp inhibitor) at 20 μM was used as a positive control.

As expected, a higher rh123 accumulation was detected when RH460 cells were treated with verapamil, when compared with the same cells treated with the vehicle of the

drug. The obtained results also demonstrated that RH460 cells treated with different concentrations of PTX for 1 hour (from 1 mM to 10 mM) or 48 hours (1 mM and 2 mM) did not have alterations in P-gp activity, as observed by the same rh123 accumulation as in the RH460 cells treated with the vehicle (**Figure 19A, B**). These results are not in agreement with the ones published by Drobná *et al.* (2002), who described that PTX influenced the activity of P-gp in resistant mouse leukemic L1210/VCR cells. Moreover, this study also suggested that PTX sensitized leukemic cells to vincristine through inhibition of P-gp activity [78].

Taken together, our data showed that PTX decreased the expression levels of P-gp, but did not affect its activity at the concentrations tested.

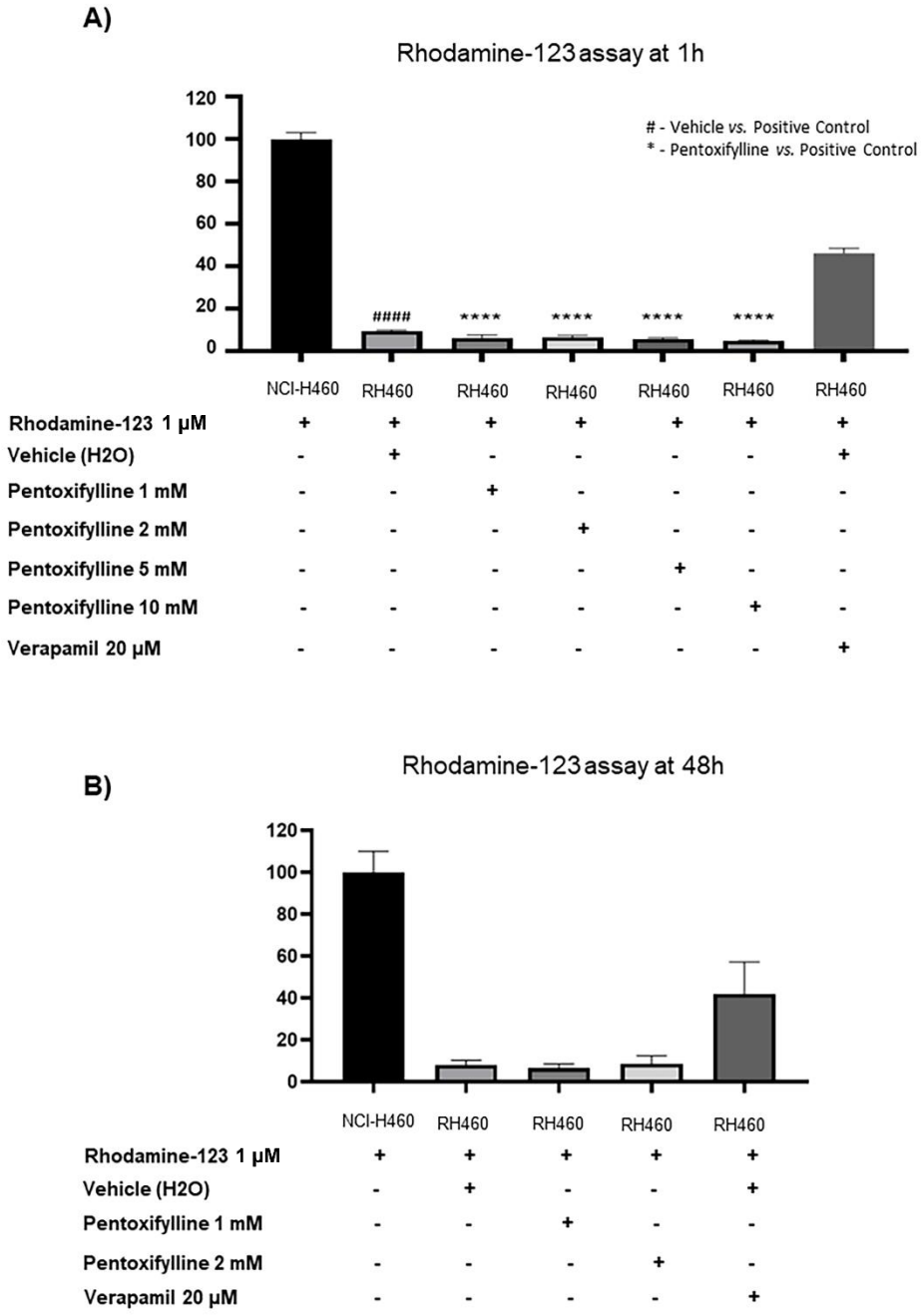

Figure 19 - Effect of pentoxifylline on the activity of P-gp in the MDR RH460 cells, determined by the Rhodamine-123 assay. Cells were treated with A) Pentoxifylline at 1.0, 2.0, 5.0 and 10.0 mM, for 1 hour; and B) Pentoxifylline at 1.0 and 2.0 mM, for 48 hours. NCI-H460 cells were used as a negative control and RH460 cells treated with verapamil at 20 µM were used as a positive control. The effect of the vehicle at the highest concentration tested was also analysed. Results are the mean ± SEM of at least three independent experiments. #### or **** indicate $p < 0.0001$

Conclusion and Future Perspectives

Non-small cell lung cancer (NSCLC) accounts for the majority of lung cancer cases, with a relative survival rate of approximately 16%. Chemotherapy is still the main treatment option for patients with NSCLC, including either patients in the early stages of the disease or those with advanced stages [15, 22]. However, drug resistance is frequently observed in patients with NSCLC, mainly due to its highly fibrotic stroma [50]. Therefore, the development of new therapeutic approaches is of extreme importance.

Pentoxifylline is an anti-fibrotic drug whose antitumour potential has been already described [70]. Moreover, pentoxifylline has sensitized several tumour models, such as human cervical cancer [79], cisplatin resistant cervical cancer [80], prostate cancer [81] and pancreatic cancer [82] cells to different chemotherapeutic agents, including adryamicin, cisplatin, docetaxel and gemcitabine, respectively. Importantly, pentoxifylline is known to be an inhibitor of Chitinase 3-like-1 (CHI3L1) [82, 116]. CHI3L1 is a glycoprotein highly expressed in several tumours, such as breast cancer [89], urothelial carcinoma [90], hepatocellular carcinoma [91] , as well as pancreatic cancer [136], and its expression has been associated with poor prognosis and worse overall survival [87]. Moreover, CHI3L1 activates important pro-survival pathways, such as the Wnt/β-catenin, Akt and MAPK signalling pathways [86].

Preliminary results from our research group, resulting from an evaluation of “The Cancer Genome Atlas” (TCGA) database, demonstrated that high levels of CHI3L1 are associated with low overall survival of NSCLC patients under different drug treatments,. Thus, this data suggests for the first time that CHI3L1 may be a potential molecular target in NSCLC. Moreover, our research group demonstrated that pentoxifylline was able to sensitize both sensitive NCI-H460 and multidrug resistant (MDR) RH460 NSCLC cell lines to paclitaxel and carboplatin treatments. Therefore, the aim of this dissertation was to study the sensitizing effect of pentoxifylline, an inhibitor of CHI3L1, to different chemotherapeutic regimens that are currently used in the clinic for the treatment of NSCLC. In addition, another aim was to explore the mechanism of action of pentoxifylline as a chemosensitizer.

Our results demonstrated that pentoxifylline sensitized both sensitive NCI-H460 and MDR counterpart RH460 cell lines to treatment with vinorelbine or vinorelbine plus carboplatin. These drug combinations caused a reduction in cell growth, when compared to the treatment with each drug individually. Moreover, pentoxifylline sensitized another pair of sensitive A549 and its counterpart MDR A549-CDR2 cell line to the treatment with paclitaxel, carboplatin, vinorelbine, and vinorelbine plus carboplatin, causing an increase in

the inhibition of cell growth, when compared to the treatment with each of drug alone. The Fold Change of the percentage of cell growth inhibition of the combined therapies *vs* conventional chemotherapies confirmed the advantage of combining pentoxifylline with chemotherapy, in both pairs of sensitive and MDR cell lines. Importantly, none of the combined therapies demonstrated a statistically significant increase in cytotoxicity in the MCF-10 non-tumorigenic cell line, when compared to each drug alone.

Regarding the mechanism of action of pentoxifylline, our results showed that pentoxifylline downregulates the expression levels of P-gp (measured by Western Blot), without having an impact on its activity (measured by the rhodamine assay). Thus, pentoxifylline does not have a P-gp inhibitory activity, suggesting that the chemosensitizing effect of this drug is not due to an impairment of this drug efflux pump activity in these NSCLC cells.

Furthermore, pentoxifylline decreased the expression levels of CHI3L1, in both sensitive and MDR counterpart cell lines, without altering the expression levels of the downstream proteins, Akt and Erk. Therefore, the chemosensitizing effect of pentoxifylline, in sensitive and MDR NSCLC cells, could be through other CHI3L1 downstream pathways, such as TGF-β and/or JAK/STAT signalling.

Importantly, pentoxifylline increased the number of cells in the G0/G1 phase of the cell cycle, in both pairs of sensitive and MDR cells. This result might explain the effect of pentoxifylline in NCSLC cell growth, when in combination with different anticancer drugs. Moreover, pentoxifylline also induced cell death in the sensitive NCI-H460 cells (but not in its MDR counterpart cell line). In fact, pentoxifylline had been more effective in inhibiting the growth of the sensitive NSCLC cells, than that of the MDR NSCLC cells. Therefore, our results suggest that pentoxifylline exerts its chemosensitizing effect by interfering with the cell cycle and by causing cell death in the sensitive NSCLC cells. In MDR cells however, pentoxifylline exerts its chemosensitizing effect only through interference with the cell cycle.

As far as we know, our work is the first to report the anticancer effect of pentoxifylline in NSCLC cells as well as the sensitizing effect of pentoxifylline to several chemotherapeutic regimens, in both sensitive and MDR NSCLC cell lines. Although our results suggest the advantage of combining pentoxifylline with conventional chemotherapy, several other pre-clinical studies are still required in order to fully understand the potential of therapeutical combination of pentoxifylline with chemotherapy. In the future, the chemosensitizing effect of pentoxifylline should be further validated in 3D cell models, that better mimic the *in vivo* conditions (than 2D cell models) while avoiding the use of animals. In fact, and since pentoxifylline is an anti-fibrotic drug, it is of extremely importance to test the studied drug

combinations under the influence of cells from the tumour microenvironment (such as macrophages and fibroblasts). Finally, the best drug combinations should be further validated using *in vivo* models. In addition, the mechanism of pentoxifylline should be further explored, for example by evaluating the effect of this drug on TGF-β and/or JAK/STAT signalling pathways.

Overall, the results presented in this dissertation (together with the planned future work), could provide pre-clinical evidence to support the possibility of repurposing pentoxifylline for combination therapies with chemotherapeutic drugs currently used in the clinic, to improve the treatment of NSCLC patients.

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