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Inhibitors of CHI3LI for the treatment of pancreatic adenocarcinoma: virtual screening and preliminary studies on inhibition of cell growth

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**INHIBITORS OF CHI3L1 FOR THE TREATMENT OF PANCREATIC
ADENOCARCINOMA: VIRTUAL SCREENING AND PRELIMINARY STUDIES ON
INHIBITION OF CELL GROWTH**

Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Química
Farmacêutica

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Resumo

O cancro pancreático representa a sétima causa principal de morte por cancro a nível mundial. A falta de tratamentos alternativos e a resistência aos fármacos torna este tipo de cancro, um dos cancros mais agressivos. O grupo de investigação em que estive integrada sugeriu que a proteína chitinase 3 like-1 (CHI3L1), que é sobreexpressa no adenocarcinoma pancreático (PDAC), é um alvo terapêutico para este cancro. De facto, o grupo descobriu que esta proteína está associada à resistência à gemcitabina. Além disso, altos níveis de expressão do gene que codifica para a CHI3L1 estão associados a baixa sobrevivência global do doente e baixa resposta ao tratamento com gemcitabina. Assim, é possível que inibidores da CHI3L1 possam inibir o crescimento de células tumorais do pâncreas e sensibilizá-las à gemcitabina.

O objetivo desta dissertação foi realizar um “screening” virtual de milhares de compostos conhecidos, da base de dados DrugBank, para encontrar potenciais ligandos da CHI3L1. Adicionalmente, realizar o “screening” de alguns desses compostos em linhas de células BxPC3 e PANC-1 de forma a avaliar a sua atividade antitumoral.

Utilizando uma estratégia de “screening” virtual, identificamos potenciais moléculas com a capacidade de se ligarem ao CHI3L1. Em primeiro lugar, foi realizado um estudo de docking entre os ligandos conhecidos da CHI3L1 encontrados na literatura (controles positivos), 11.741 moléculas presentes na base de dados DrugBank e o alvo CHI3L1 (código pdb: 1NWU). O rastreio virtual foi realizado utilizando o programa AutoDock Vina, que considera a conformação do alvo como uma unidade rígida enquanto os ligandos podem ser flexíveis e adaptáveis ao alvo. O Vina está programado para procurar a melhor conformação de ligação e o melhor valor de afinidade de ligação para cada ligando.

Um total de 568 moléculas foram identificadas como ligandos potenciais para a CHI3L1, mostrando uma maior afinidade em relação ao alvo comparando com outro ligando conhecido, prednisolone (usado como limiar para “screening” virtual) e os outros controles positivos. As moléculas identificadas foram fármacos já conhecidos, com um fim terapêutico bem estabelecido. Por fim, foram selecionadas duas moléculas com bons “docking scores” e foi feita uma avaliação do seu efeito na inibição do crescimento das linhas de celulares tumorais BxPC3 e PANC-1. A diidroergotamina inibiu o crescimento de células tumorais, contudo precipitou-se em concentrações próximas do GI50. A darifenacina inibiu o crescimento de células tumorais com concentrações de GI50 de 26 μ M para células BxPC3 e 13,6 μ M para células PANC-1. Futuro trabalho confirmará o

efeito dos fármacos identificados na CHI3L1, utilizando para esse efeito uma proteína recombinante humana da CHI3L1.

Caso o seu interesse antitumoral venha a ser confirmado em trabalhos futuros, com ensaios antitumorais adicionais e em modelos tumorais adicionais, seria interessante considerar a hipótese de reposicionar estes fármacos para o tratamento do PDAC.

Palavras-chave: Cancro pancreático, chitinase 3-like-1, reposicionamento de fármacos, “screening” virtual

Abstract

Pancreatic cancer represents the 7th leading cause of cancer death worldwide. The lack of alternative treatments and drug resistance makes this type of cancer one of the most aggressive cancers. The research group in which I am integrated suggested that chitinase 3 like-1 (CHI3L1) protein, which is overexpressed in pancreatic ductal adenocarcinoma (PDAC), is a therapeutic target for this cancer. Indeed, the group found that this protein is associated with resistance to gemcitabine. In addition, high expression levels of the gene that codes for CHI3L1 are associated with low patient overall survival and low response to gemcitabine. Thus, CHI3L1 inhibitors may inhibit the growth of pancreatic cancer cells and sensitize them to gemcitabine.

The aim of this dissertation was to perform a virtual screening of thousands of known compounds, from the DrugBank database, to find potential ligands of CHI3L1. Additionally, perform the screening of some of these compounds in BxPC3 and PANC-1 cell lines in order to evaluate their antitumor activity.

Using a virtual screening strategy, we identified potential molecules with the ability to bind to CHI3L1. Firstly, a docking study was performed between the known CHI3L1 ligands found in the literature (positive controls), 11,741 molecules present in the DrugBank database and the target CHI3L1 (pdb code: 1NWU). The virtual screening was then carried out by using the program AutoDock Vina, which considers the target conformation as a rigid unit while the ligands can be flexible and adaptable to the target. Vina is programmed to search for the best binding conformation and binding affinity value for each ligand.

A total of 568 molecules were identified as potential ligands for CHI3L1, showing a higher affinity towards the target when compared to the ligand prednisolone (used as the threshold for virtual screening) and the other positive controls. The identified molecules were known drugs, with a well-established therapeutical use. Finally, two molecules with good docking scores were selected and an evaluation was made of its effect on growth inhibition of the BxPC3 and PANC-1 cell lines. Dihydroergotamine inhibited tumor cell growth, however it precipitated at concentrations near the GI50. Darifenacin inhibited tumor cell growth with GI50 concentrations of 26 μ M for BxPC3 cells and 13.6 μ M for PANC-1 cells. Future work will confirm the effect of the identified drugs on CHI3L1, by using a human recombinant protein CHI3L1.

In case their antitumor interest is confirmed in future work, with additional antitumor assays and in additional tumor models, it would be interesting to consider repositioning these drugs for the treatment of PDAC.

Key words: Pancreatic cancer, chitinase 3-like-1, repurposing drugs, virtual screening

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List of abbreviations and symbols

3D	Three-dimensional
5-HT	Serotonin receptors

A

ALA	Alanine
AMCase	Acidic Mammalian Chitinase
AML	Acute Myeloid Leukemia
ARG	Arginine
ASN	Asparagine
ASP	Aspartic Acid
ATCC	American Type Culture Collection

B

BCL2	B-cell Lymphoma 2
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C

CCL2	C-chemokine Ligand 2
CHI3L1 (or YKL-40)	Chitinase 3-like-1
CHI3L2 (or YKL-39)	Chitinase 3-like-2
CHID1	Chitinase Domain-containing 1
CHIT1	Chitotriosidase
CLPs	Chitinase-like Proteins
CXCL2	Chemokine CX Motif Ligand 2

D

DF	Darifenacin
DHE	Dihydroergotamine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide

E

EMA	European Medicines Agency
Erk	Extracellular Signal-regulated Kinase

F

FBS	Fetal Bovine Serum
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FDA	Food and Drug Administration
G	
Gal-3	Galectin-3
GEM	Gemcitabine
GH18	Glycoside Hydrolase Family 18
GI ₅₀	Value of 50% cell growth inhibition
GlcNAc	N-acetylglucosamine
GLU	Glutamic Acid
GLY	Glycine
H	
HC-gp39	Human Cartilage Glycoprotein-39
HIS	Histidine
I	
ICAM-1	Intercellular Adhesion Molecule 1
IFN- γ	Interferon Gamma
IL-13	Interleukin-13
IL-13R α 2	Interleukin-13 Receptor α 2
IL-1 β	Interleukin-1 Beta
IL-6	Interleukin-6
K	
KRAS	Kirsten Rat Sarcoma Virus
L	
LEU	Leucine
LFA-1	Lymphocyte Function–associated Antigen-1
LYS	Lysine
M	
MAPK	Mitogen-activated Protein Kinase
mAY	Monoclonal Anti-CHI3L1 Antibody
MET	Methionine
MMP-9	Matrix Metalloproteinase-9

N

NF- κ B	Nuclear Factor- κ b
NS5A	Non-structural protein 5A

O

OVGP1	Oviductal Glycoprotein 1
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P

PBS	Phosphate-buffered Saline
PDAC	Pancreatic Ductal Adenocarcinoma
PDB	Protein Data Bank
PHE	Phenylalanine
PKB or Akt	Protein Kinase B
PLIP	Protein-Ligand Interaction Profiler
PRO	Proline
PTX	Pentoxifylline

R

rhCHI3L1	Recombinant human protein Chitinase 3-like-1
RMSD	Root Mean Square Deviation

S

SBDD	Structure-based Drug Design
SEM	Standard Error of the Mean
SER	Serine
SI-CLP	Stabilin-1 Interacting Chitinase-like Protein
SOSA	Selective Optimisation of Side Activities
SRB	Sulforhodamine B

T

TAMs	Tumor-associated Macrophages
TCA	Trichloroacetic Acid
Th2	T helper Type 2
THR	Threonine
TIM	Triosephosphate Isomerase
TMEM219	Transmembrane Protein 219
TNF- α	Tumor Necrosis Factor Alpha

TRP	Tryptophan
TYR	Tyrosine
V	
VAL	Valine
VEGF	Vascular Endothelial Growth Factor

1.Introduction

1.1 Pancreatic Cancer

Pancreatic cancer is the 12th most commonly occurring cancers and the 7th leading cause of cancer-associated deaths worldwide¹. The incidence and mortality of this type of cancer varies across regions and population ages². In United States, it was estimated that there will be 60,430 new cases (31,950 men and 28,480 women) of pancreatic cancer and 48,220 deaths (25,270 men and 22,950 women) in 2021, making pancreatic cancer the 4th most common cause of cancer-associated death in both genders (8% of all deaths caused by cancer)³. Despite the advances in newly available tools for early diagnosis, its incidence is estimated to increase in approximately 355,000 new cases within 2040⁴.

In the United States, pancreatic cancer is considered a highly fatal disease with a 5-year survival rate of approximately 10% at the time of diagnosis, where 80-85% of patients present either metastatic or unresectable disease^{3,5}. Even for patients diagnosed with a localised and resectable tumor, the prognosis remains low with a 5-year survival rate of only 39%. The location of the tumor dictates if a radical surgery procedure would be effective to eradicate the tumor. Unfortunately, this treatment is only possible in around 20% (or less) of patients at an early stage⁵. Most patients are diagnosed later in life with a median age of 71 years, where surgery can be considered a risk factor. Obesity, type 2 diabetes and tobacco use (20% of pancreatic cancers are caused by smoking) are also associated with pancreatic cancer development. Furthermore, only 10% of all pancreatic cancers are estimated to be attributed to inherit risk factors including family history, Peutz-Jeghers syndrome (increases risk by 35%⁶), Lynch syndrome, chronic pancreatitis, Von Hippel-Lindau syndrome and Multiple Endocrine Neoplasia type 1⁷.

This cancer is characterized for causing almost no symptoms at an early stage. Thus, it is considered a notoriously silent growing tumor, where symptoms develop mainly at an advanced stage⁸. Generalized symptoms make it difficult to identify the presence of cancer, delaying diagnosis of patients. Nausea, abdominal fullness, epigastric or back pain, bloating or changes in stool consistency are considered the most common symptoms⁹. Moreover, other symptoms related with organ function, which indicates an advanced stage of the tumor, include loss of appetite, light-coloured stools or dark urine, fatigue, jaundice, pain in the upper or middle abdomen and back, and weight loss⁸.

There are several types of pancreatic cancer, depending on whether the cancer began in the endocrine or exocrine portion. Pancreatic neuroendocrine tumors developed in the endocrine gland and are considered a rare type (less than 5%) compared to all pancreatic cancers, in which can be divided into functioning (which produce hormones) or nonfunctioning tumor (which do not produce hormones)^{10, 11}. The exocrine cancers are by

far the most common types of pancreatic cancer (more than 95%), including the squamous cell carcinoma, adenosquamous carcinoma, colloid carcinoma and pancreatic ductal adenocarcinoma (PDAC)¹¹. PDAC is considered one of the deadliest solid malignancies, representing more than 90% of all pancreatic cancers diagnosed¹². In addition, pancreatic cancer can also be divided into four categories based on the seriousness of the situation including: resectable, borderline resectable, locally advanced and metastatic¹³.

Pancreatic cancer is considered one of the most challenged cancers due to a unique desmoplastic stroma that comprises a dense extracellular matrix, abnormal vasculature, cancer-associated fibroblasts and inflammatory cells¹⁴. The stroma is also composed by high levels of pancreatic stellate cells, which are responsible for the production of collagen, laminin and fibronectin. These stromal elements lead to desmoplasia, a process that results in a resistance to chemotherapy owing to a hypovascular microenvironment¹⁵. Despite the efforts in the past decades to understand the tumor microenvironment, targeted therapies and immunotherapy have not yielded substantial improvement in clinical outcomes for pancreatic cancer. The microenvironment is enriched by several types of immunosuppressive cells (such as regulatory T cells and myeloid derived suppressor cells) that are responsible for immune evasion reducing the effector function of cytotoxic T cells, leading to an ineffectiveness of immunotherapies^{15, 16}.

Regarding pancreatic cancer treatment, gemcitabine is considered the standard treatment since 1997, when it was shown to be more efficient than the treatment used until that time, the 5-fluorouracil. Over the years, other agents have been developed to improve the overall survival of cancer patients, including FOLFIRINOX, a combination regiment of 5-fluorouracil/leucovorin with irinotecan and oxaliplatin. However, its use is limited by the high toxicity expressed in patients, and the improvement in the overall survival was not substantial^{17, 18}.

Through the years, new potential molecular targets have been emerged with the attempt to overcome the lack of effective treatments and the absence of biomarkers to predict pancreatic cancer. One promising molecular target, proposed by some of the supervisors of this dissertation, is chitinase 3-like-1 protein (CHI3L1)¹⁹, also known to be overexpressed in pancreatic cancer and associated with poor survival²⁰.

1.2. Chitinase 3-like-1 protein (CHI3L1)

1.2.1 Chitinase-like proteins

Glycoside hydrolase family 18 (GH18) incorporates two types of proteins including the chitinases and chitinase-like proteins (CLPs). CLPs are described as highly homologous mammalian proteins with no enzymatic activity due to amino acids substitutions on the catalytic active-site. Besides this change in the binding pocket, the active-site carbohydrate-binding still holds. Interestingly, even without the characteristic chitin-binding domain (with six cysteine residues responsible for the binding to chitinases), the CLPs, such as the proteins that belong to GH18 family, can bind to chitin with high affinity. Both chitinases and CLPs are present in mammals, even though these organisms do not have the capability to contain endogenous chitin²¹⁻²³.

Chitin, a biopolymer responsible for rigidity and structural integrity of cells, tissues and body surfaces, is found in the internal structures of some invertebrates and the exoskeleton of yeasts, fungi, crabs, lobsters, shrimps, algae and insects²⁴. Chitin is considered the second most abundant polysaccharide on the planet after cellulose, and is characterized for being a linear polymer of β -1,4-N-acetylglucosamine (GlcNAc)²⁵. This biopolymer is not accumulated in the environment due to the existence of chitinases²⁶. These types of chitinolytic enzymes, with sizes between approximately 20 and 90 kDa²⁴, depend on a glutamic acid and an aspartic acid residue to hydrolyse chitin directly to low molecular weight chitooligomers²⁷.

Chitinases are present in a wide range of organisms, for multiple physiological processes (including yeasts, bacteria, fungi, insects, humans and plants), with each one containing a variety of proteins (**Figure 1**)²⁶. These proteins are involved in tissue degradation and remodelling, nutrition uptake and immune response regulation²⁸. In the first physiological process (i.e. tissue degradation), the chitin-containing organisms, which are characterized by the presence of a tough layer of chitin, produce chitinases that degrade and remodel the organic matrix during specific stages of development, contributing to changes in the shape and body size in organisms such as insects. Secondly, some microorganisms produce chitinases to help in the digestion processes by using other chitin-containing organisms (exogenous chitin) as a source of food and to aid in the invasion processes in non-chitin containing organisms. Therefore, chitinases are involved in several processes in organisms either with or without chitin. Moreover, hosts easily infected by invading pathogens containing chitin, such as mammals and plants, use chitinases as a defense mechanism, through the capability to degrade the protective cell wall of pathogens and create chitin fragments to indirectly activate the immune response²⁹.

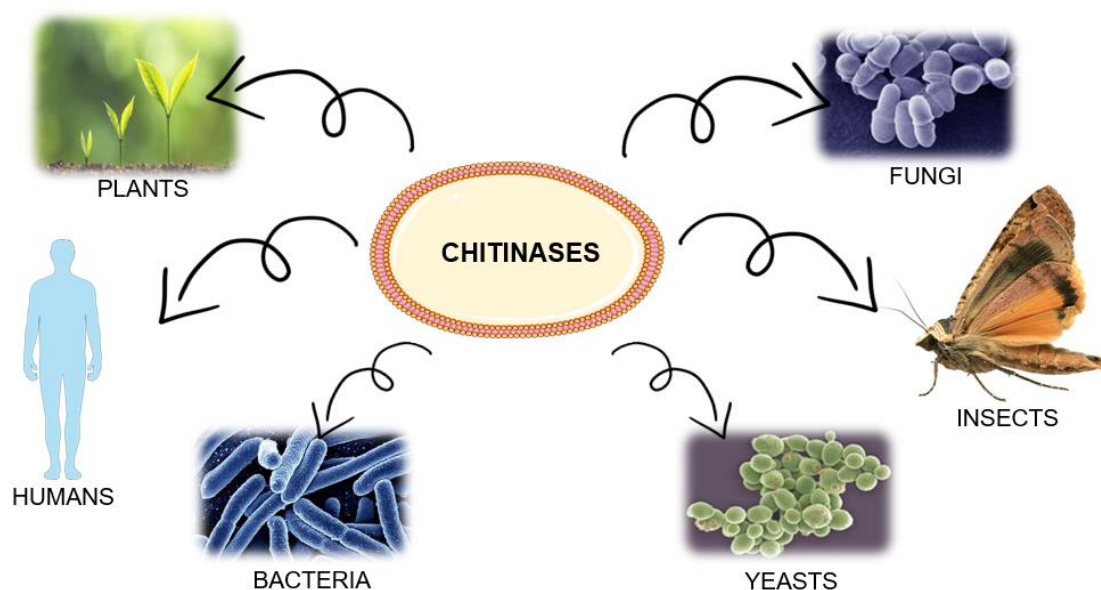


Figure 1. Distribution of chitinases. These chitinolytic enzymes are present in a wide variety of organisms including plants, humans, bacteria, fungi, insects and yeasts.

Chitinases are glycosyl hydrolases responsible for the production of shorter fragments, $(\text{GlcNAc})_2$ and $(\text{GlcNAc})_3$, through the hydrolysis of the β -(1-4)-linkage of N-acetyl-D-glucosamine units present in chitin polymers³⁰. There are two active chitinases capable of processing chitin hydrolysing activity, the chitotriosidase (CHIT1), which was the first mammalian chitinase identified³¹, and the acidic mammalian chitinase (AMCase)³⁰. Both enzymes have highly conserved structural features and contain a catalytic domain and a chitin-binding domain (cysteine-rich carbohydrate) (**Figure 2**)^{32, 33}. The catalytic domain is normally located at the N-terminus, whereas the chitin-binding domain lies at C-terminus. The first adopts a triosephosphate isomerase (TIM) fold, which consists of a $(\beta/\alpha)_8$ -barrel structure, where β -sheets are surrounded by α -helices. Inside the barrel, a conserved sequence motif (DXXDXDXE, where D = aspartic acid, E = glutamic acid, and X = any amino acid) exists in the β_4 strand, forming the active site pocket of the enzyme^{34, 35}. Glutamic acid and central aspartic acid are the key amino acid residues in substrate binding that occur in the centre of the $(\beta/\alpha)_8$ -barrel. Thus, the glutamic acid is responsible for donating a proton required for hydrolysing the β -(1-4) glycosidic bond in chitin, whereas the second aspartate determines the stabilization of the substrate during catalysis^{32, 34, 36}.

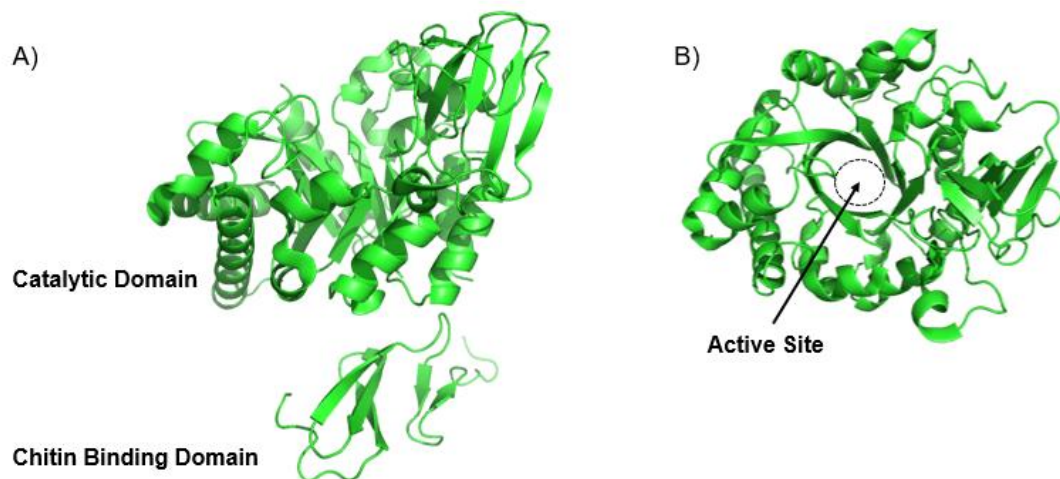


Figure 2. 3D structure of chitotriosidase (CHIT1). A) Cartoon representation of CHIT1 contain a catalytic domain and a chitin-binding domain. B) The active side pocket present inside the $(\beta/\alpha)_8$ -barrel structure of catalytic domain. Adapted from ³³.

Molecular phylogenetic analyses and genomic synteny suggest that both active chitinases (CHIT1 and AMCase) have the same evolutionary profile, which resulted from an early gene duplication event. Additional duplication events were followed by mutations leading to the loss of chitinase activity, allowing the evolution of a broad spectrum of CLPs^{21, 23}. Structurally CLPs consists of a 39 kDa TIM barrel structure, one of the most versatile folds in nature, similar to the catalytic domain of active chitinases³⁶. Even with the lack of chitinolytic activity by the substitution of a key glutamic acid residue in the DXXDXDXE motif, to either isoleucine, tryptophan or leucine, CLPs retain the ability to bind chitin oligosaccharides with great affinity due to the retained active-site carbohydrate binding^{37, 38}. Studies have shown that if there is a substitution back to the one in active chitinases, CLPs recover the chitinase activity³⁹.

A set of enzymatically inactive CLPs lacking the catalytic motif has been identified, belonging to the GH18 family, including chitinase 3-like-1 (CHI3L1 or YKL-40 or human cartilage glycoprotein 39), chitinase 3-like-2 (CHI3L2 or YKL-39), stabilin-1 interacting chitinase-like protein (SI-CLP), oviductal glycoprotein 1 (OVGP1), chitinase domain-containing 1 (CHID1), Ym1 and Ym2. The last two, only found in mouse, are produced by macrophages after a fungal or parasitic infection. Besides, Ym1 has also been associated with allergic airway inflammation^{32, 40}. Nowadays, the physiological roles of these proteins are not yet fully understood, although it appears that they are expressed in a huge variety of T helper type 2 (Th2) inflammatory pathologies such as fibrosis, asthma and helminth infection²⁹.

Interestingly, CLPs are lectins that combine properties of growth factors and cytokines. Multiple cell types were found to be responsible for producing them, including macrophages, neutrophils, epithelial cells, dendritic cells, chondrocytes, synoviocytes (fibroblast-like synovial cells) and cancer cells. Moreover, the high presence of CLPs in circulation is found in several types of cancer, being considered attractive targets for cancer therapy⁴¹. The work of this dissertation will focus on CHI3L1 as a possible therapeutic target for PDAC.

1.2.2. CHI3L1

In 1997, the human *CHI3L1* gene encoding for the secretory factor CHI3L1 was isolated. This gene is located in a highly conserved area on chromosome 1q31-1q32, comprising 10 exons distributed over an 8-kb region and 7498 base pairs, and also covers ~8 kbp of genomic DNA⁴². This gene is considered an interesting model due to the strict correlation shown in late macrophage differentiation, where the transcriptional regulation of CHI3L1 was already described. Evidence has shown the probable existence of two independent transcription start sites and a promotor sequence, containing several known factors and specific binding of nuclear proteins such as PU.1, Sp1, Sp3, USF, AML-1 and C/EBP. The Sp1 transcription factors family has a predominant role in controlling CHI3L1 promoter activity for macrophage differentiation⁴³.

CHI3L1 is a phylogenetically highly conserved protein with a high sequence homology with chitinases (approximately 53%). It is considered an indispensable member of the GH18 family, able to bind chitin without enzymatic activity^{22, 44}. Originally, CHI3L1 was discovered in the culture supernatant of MG63 cell lines⁴⁵. However, it has already been found to be present in human, cow, goat, chimpanzee, pig, guinea pig, rat and mouse⁴⁶.

The CHI3L1 protein can also be known as human cartilage glycoprotein-39 (HC-gp39) due to its presence in the monolayer or explant culture of human articular chondrocytes⁴⁷. Moreover, this protein is also named YKL-40, given the molecular weight of 40 kDa and the three N-terminal amino acid residues present in the secreted form: tyrosine (Y), lysine (K) and leucine (L)²³. CHI3L1 protein consists in a single polypeptide chain of 383 amino acids⁴⁷ and is characterized by an isoelectric point of 7.6⁴⁸. Studies of the three-dimensional (3D) structure of CHI3L1 showed a division into two globular domains⁴⁹. The structure revealed the presence of an eight-stranded β/α -barrel domain with a TIM fold and a second domain composed of six antiparallel β -strands, with one α -helix ($\alpha + \beta$) domain inserted after strand $\beta 7$ (**Figure 3**). Furthermore, a 43-Å long

carbohydrate-binding groove was discovered at the C-terminal side of the β -strands in the $(\beta/\alpha)_8$ -barrel, where the protein-carbohydrate interactions are dominated by stacking of aromatic amino acid residues side chains^{22, 50}.

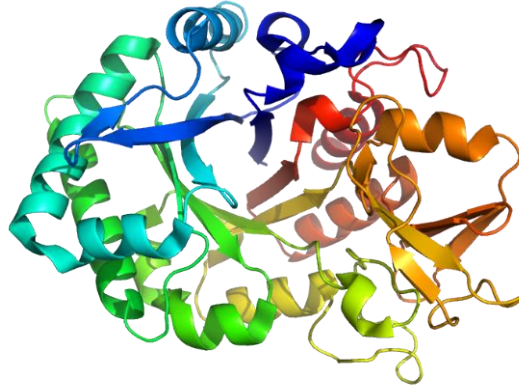


Figure 3. Crystal structure of CHI3L1. The 3D structure of the protein exhibits a $(\beta/\alpha)_8$ -barrel structure with an insertion of an $\beta + \alpha$ domain.

As already mentioned, CHI3L1 is a secreted glycoprotein that has the same ability to bind to chitin-like oligosaccharides of different lengths. Contrarily to active chitinases, CHI3L1 has no chitinase activity due to the substitution of an essential glutamic acid with leucine in the catalytic domain. Eventually, this protein interacts differently with short and long chitin fragments: chitin disaccharides tend to occupy the distal subsites, whereas longer chains have preference in binding to the central subsites in the groove. Consequently, the binding of the fragments contributed to the identification of nine sugar-binding subsites in the groove. In addition, the presence of chitin fragments in the binding pocket of CHI3L1 has also demonstrated that the conformational structure does not change dramatically when bound to a ligand^{47, 48, 50}. Furthermore, the protein also interacts with other molecules in addition to chitin-like oligosaccharides, including heparin, hyaluronic acid and all three forms of collagen (types I, II and III)⁵¹.

Studies addressing the possibility of CHI3L1 mediating its effects through cell membrane receptors have been conducted. To date, interleukin-13 receptor $\alpha 2$ (IL-13R $\alpha 2$) and its interacting complexes with transmembrane protein 219 (TMEM219), galectin-3 (Gal-3) and CD44 have been recognized as receptors of CHI3L1⁵²⁻⁵⁵. Studies demonstrated that CHI3L1 binds to IL-13R $\alpha 2$, a glycosylated transmembrane protein⁵⁶ expressed in a variety of cancers including glioblastoma multiforme⁵⁷, colorectal cancer⁵⁸, pancreatic cancer⁵⁹, breast cancer^{60, 61}, among others. Thereby, CHI3L1 forms a multimeric complex with IL-13R $\alpha 2$ and interleukin-13 (IL-13), leading to the activation of mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/Erk), protein kinase B (PKB)/Akt and Wnt/ β -catenin cell signalling pathways, regulating several

processes such as apoptosis, inflammation, cell death, antibacterial response, invasion and metastasis (**Figure 4**). IL-13R α 2 was found to be a GH18 receptor with a crucial role in CHI3L1 effector responses. Moreover, when IL-13R α 2 was eliminated from an experimental system, the response was partially abrogated, suggesting the presence of other CHI3L1 receptors⁵².

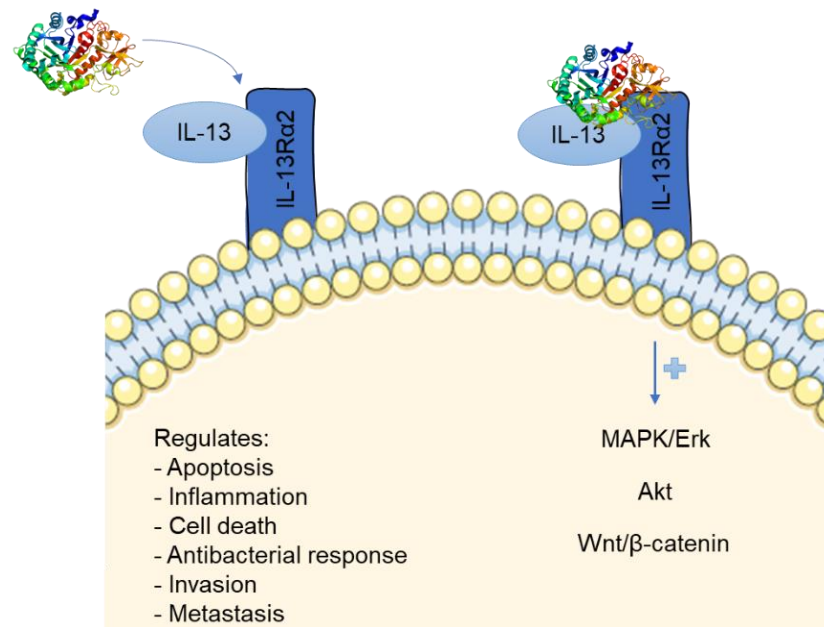


Figure 4. Interaction of CHI3L1 with IL-13R α 2 (interleukin-13 receptor α 2) and IL-13 (interleukin-13). Formation of a multimeric complex that leads to activation of cell signalling pathways including MAPK/Erk (mitogen-activated protein kinase/ extracellular signal-regulated kinase), Akt (protein kinase B) and Wnt/β-catenin. Regulation of several cellular mechanisms such as apoptosis, inflammation, cell death, antibacterial response, invasion and metastasis. Adapted from ^{51, 52}.

The interaction between IL-13R α 2 and TMEM219 demonstrated an ability to stimulate CHI3L1 by enhancing the expression of heparin binding EGF-like growth factor on murine peritoneal macrophages and the activation of MAPK/Erk and PKB/Akt, but not Wnt/β-catenin signalling pathway in epithelial cells. TMEM219 has been identified as a binding partner for IL-13R α 2, where the CHI3L1-IL-13-IL-13R α 2-TMEM219 complex is responsible for enhancing the anti-apoptotic response induced by CHI3L1 stimulation⁵³. Another interaction relies on the binding of IL-13R α 2 with Gal-3, a β-galactoside-binding protein expressed in the nucleus, cytoplasm and extracellular milieu of cells including lung epithelial cells. Gal-3 physically interacts with CHI3L1-IL-13-IL-13R α 2 complex to block IL-13R α 2-TMEM219 binding, which reduces the ability of CHI3L1 to activate anti-apoptotic effects and increase the Wnt/β-catenin signalling⁵⁴. Lastly, the physical interaction between CHI3L1 and CD44, a cell-surface transmembrane glycoprotein, activates Erk and

Akt, as well as the β -catenin signalling pathways (**Figure 5**). Particularly, CD44 and CD44v3 peptide, but neither CD44v6 peptide nor CD44s protein, bound to IL-13R α 2 and CHI3L1 to form a complex. Then, through CHI3L1/CD44 interaction, studies showed that CHI3L1 can promote gastric cancer cell growth, proliferation, migration and metastasis⁵⁵.

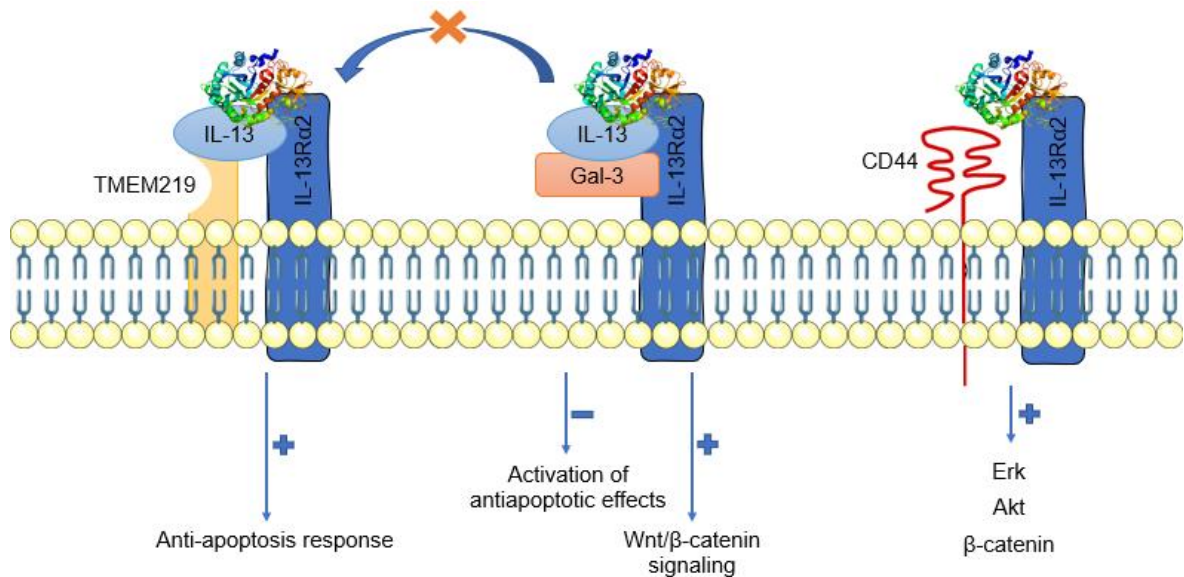


Figure 5. Interaction of IL-13R α 2 (interleukin-13 receptor α 2) with TMEM219 (transmembrane protein 219), Gal-3 (galectin-3) and CD44. The multimeric complex CHI3L1-IL-13-IL-13R α 2-TMEM219 is capable of enhancing the anti-apoptotic response due to CHI3L1 stimulation, where it is blocked by the interaction of Gal-3 with CHI3L1-IL-13-IL-13R α 2. This interaction diminishes the ability of CHI3L1 to activate anti-apoptotic effects and increase Wnt/ β -catenin signalling. The CHI3L1/CD44 interaction activates Erk (extracellular signal-regulated kinase), Akt (protein kinase B) and β -catenin signalling pathways. Adapted from ⁵¹.

Therefore, CHI3L1 mediates different biological activities, depending on the cell type and environment. CHI3L1 is normally synthesized and secreted by a multitude of different cell types including bone cells, chondrocytes, endothelial cells, fibroblast-like cells, hepatic stellate cells, liver cells, macrophages, mammary epithelial cells, neutrophils, osteoclasts, stem cells, synoviocytes, vascular smooth muscle cells and cancer cells^{44, 62, 63}. For example, in chondrocytes, CHI3L1 is regulated by tumor necrosis factor alpha (TNF- α) and activated by nuclear factor- κ B (NF- κ B), a protein responsible for controlling cell survival through the regulation of cell growth, proliferation and apoptosis⁴⁶. In addition, CHI3L1 also plays a critical role in tissue repair and remodelling, by mediating a variety of essential tissue regenerative processes, including the synthesis and degradation of the extracellular matrix, immune cell activation, the balance between type Th1 cells and Th2 cells, macrophage differentiation and dendritic cell accumulation^{52, 64}. Furthermore,

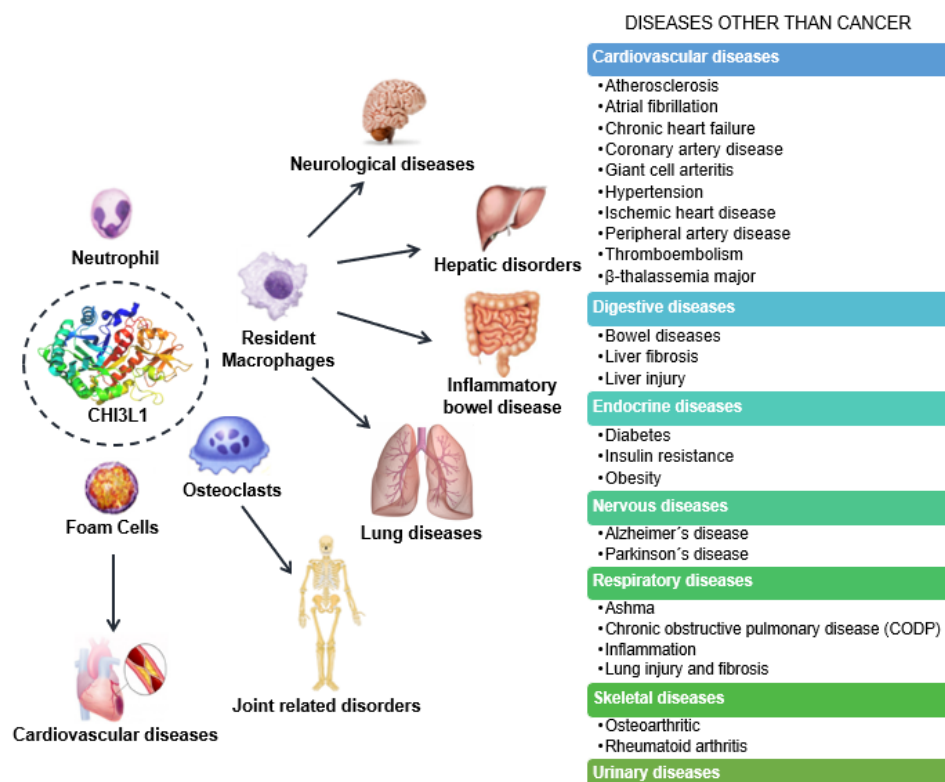
several types of cells (macrophages, chondrocytes, epithelial cells and osteoclasts) can regulated CHI3L1 expression by a number of inflammation and immune response mediators including IL-13, IL-6 (interleukin-6), IL-1 β (interleukin-1 beta), and interferon gamma (IFN- γ), in cancer and inflammatory diseases⁶⁵. Overexpression of CHI3L1 has been observed in normal and unhealthy human tissues. Through an immunohistochemical staining procedure, it is possible to map CHI3L1 expression in normal human tissues. A widespread cellular distribution of CHI3L1 was found in a series of cells including polymorph nuclear granulocytes, exocrine epithelial cells, neurons, mast cells, endothelial cells, endocrine cells and basal layer squamous epithelial cells, where an intense granular cytoplasmic staining was observed. This suggests that CHI3L1 may be related to increased metabolic activity or proliferation/turnover rate because it not only participates in normal cell functions but also in neoplastic and inflammatory processes. Further support to this hypothesis is the absence of protein staining in cells with no nucleus-mediated metabolism such as erythrocytes and superficial cells of squamous epithelia⁶⁶.

CHI3L1 has also shown to play a critical role in the pathogenesis of a vast array of diseases (**Figure 6**)⁵¹. Indeed, high levels of CHI3L1 expression have been detected in different types of diseases, such as inflammatory, chronic and neurodegenerative diseases including liver fibrosis⁶⁷, rheumatoid arthritis⁶⁸, asthma⁶⁹, coronary artery disease⁷⁰, chronic obstructive pulmonary disease⁷¹, obesity⁷², diabetes⁷³, inflammatory bowel disease⁷⁴, osteoarthritis⁷⁵, Parkinson's disease⁷⁶, Alzheimer's disease⁷⁷, among others.

Figure 6. CHI3L1 expression in various diseases other than cancer. A variety of diseases in different organ systems have already been related with the presence of high levels of CHI3L1 expression, a protein expressed in several cell types, including cardiovascular, digestive, endocrine, nervous, respiratory, skeletal and urinary diseases. Adapted from ^{23, 51}.

Therefore, considering, the different diseases with high expression of CHI3L1, this protein may possibly be considered as a biomarker for diseases characterized by inflammation, increased extracellular tissue remodelling and ongoing fibroses⁶². In fact, CHI3L1 was found to be a biomarker of inflammation⁷⁸ also in the brain, where the protein is expressed in activated microglia⁷⁹ and in astrocytes⁸⁰. CHI3L1 was also found in high concentrations in Alzheimer's disease patients, when compared with cognitively normal individuals, and its levels were demonstrated to be correlated with other dementia markers such as amyloid beta and tau proteins. Thus, it is possible that CHI3L1 might eventually contribute to the diagnosis and prognosis of Alzheimer's disease^{81, 82}.

1.2.3. CHI3L1 and Cancer



As already mentioned, a variety of advanced human cancers are associated with high serum levels of CHI3L1, including breast cancer⁸³, hepatic carcinoma⁸⁴, ovarian cancer⁸⁵,

non-small cell lung cancer⁸⁶, urothelial carcinoma⁸⁷, thyroid cancer⁸⁸, pancreatic cancer²⁰, colorectal cancer⁸⁹, among others. Furthermore, an increase levels of CHI3L1 has been correlated with low survival rates of cancer patients^{90, 91}. All these evidence suggest that CHI3L1 might be a potential prognostic and diagnostic cancer biomarker⁴⁶.

For instance, surrounding tumor cells, such as infiltrating macrophages, also express CHI3L1. Tumor-associated macrophages (TAMs) are responsible for the production of several tumor-promoting factors including chemokines, cytokines and angiogenic factors such as basic fibroblastic growth factor, platelet-derived growth factor, epidermal growth factor and vascular endothelial growth factor (VEGF), being involved in solid tumor development, mainly through tumor angiogenesis, a process responsible for new vasculature formation⁹²⁻⁹⁵.

TAMs produce all the three human CLPs (CHI3L1, CHI3L2 and SI-CLP), being antagonistically regulated by cytokines. Since CHI3L1 was described as a promoter for adhesion and migration of vascular endothelial cells, its role in angiogenesis has been well explored⁹⁶. CHI3L1 acts as an angiogenic factor promoting tumor growth and vessel formation⁹⁷. The development of antiangiogenic therapy might be considered a promising strategy to target CHI3L1 in a variety of human cancers. Synthetic molecular inhibitors against intracellular mediators or membrane receptor kinase, as well as monoclonal antibodies specific for angiogenic factors are therapeutic agents to suppress cancer growth and development^{93, 98}.

Additionally, a neutralizing antibody from mice, the monoclonal anti-CHI3L1 antibody (mAY), was developed to control CHI3L1 functions by blocking tumor angiogenesis and metastasis. This approach demonstrated the potential of anti-CHI3L1 strategies in cancer types which overexpressed CHI3L1. Studies also found that this antibody effectively inhibits angiogenesis induced by CHI3L1, both in cultured cells and xenografted animal models. The CHI3L1 neutralizing antibody in combination with ionizing radiation also promotes therapeutically benefits on cancer progression⁹⁹. Interestingly, in U87 tumor cells, researchers found that mAY induced death response to γ -irradiation via down-regulation of phosphorylated Akt expression¹⁰⁰.

Other approaches have been used to study CHI3L1 in cancer. The blockage of CHI3L1 by a siRNA gene downregulation approach decreased the levels of the protein in cancer cells, leading to total suppression of tumor growth¹⁰¹. Alternative approaches to prevent CHI3L1 activity were based on the interaction of small molecules or neutralizing antibodies in signalling pathways, including the MAPK inhibitor SU1498, Akt inhibitor GSK690693, monoclonal anti-integrin $\alpha_v\beta_3$ (LM609) and $\alpha_v\beta_5$ (P1F6) antibodies. Some of

these approaches have shown results *in vitro*, although their therapeutic benefits have not yet been validated *in vivo*¹⁰².

Furthermore, chitin, which is known for binding proteins of the GH18 family, also blocked the effects of CHI3L1 induced cancers. The strong binding of this biopolymer induced a shift from a pro-tumorigenic Th2 type to an anti-tumorigenic Th2 type immune response with increased IFN- γ . CHI3L1, whose functions contribute to tumor growth and metastasis, demonstrated an inhibitory role in IFN- γ production and caused an up-regulated expression of proinflammatory mediators such as C-chemokine ligand 2 (CCL2), chemokine CX motif ligand 2 (CXCL2) and matrix metalloproteinase-9 (MMP-9). In a mammary tumor-bearing mice model, chitin treatment promoted an immune response by causing a decrease in serum levels of CCL2, CXCL2 and MMP-9 expression in circulation and consequently, a decrease in pulmonary metastasis^{97, 103}. Moreover, resveratrol, a natural phenol, can repress the growth, proliferation and invasion of human glioma U87 cells *in vitro* by decreasing the activity of CHI3L1 promoter and reducing both mRNA transcription and protein expression¹⁰⁴. Resveratrol and prednisolone were shown to be repressors of CHI3L1 expression by decreasing transcriptional mRNA and the level of protein expression, thereby reducing the proliferation of acute lymphoblastic leukemia cells (CCRF-CEM)¹⁰⁵. Recently, another small molecule, pentoxifylline, has also been shown to inhibit CHI3L1 in PDAC¹⁹. CHI3L1 expression can also be regulated by miRNAs, such as miR-125a-3p. The miR-125a-3p regulates upstream stimulatory factor 1 to suppress CHI3L1 and eventually, to inhibit cancer metastasis¹⁰⁶. A representation of anti-CHI3L1 therapeutic strategies is shown in **Figure 7**.



Figure 7. Strategies to target overexpressed CHI3L1. Neutralizing antibodies such as mAY (monoclonal anti-CHI3L1 antibody), monoclonal anti-integrins $\alpha_v\beta_3$ (LM609) and $\alpha_v\beta_5$ (P1F6)

antibodies, siRNA gene knockdown, miRNAs and small molecules such as MAPK inhibitor SU1498, Akt inhibitor GSK690693, chitin, resveratrol, prednisolone and pentoxifylline are examples of strategies able to interfere with CHI3L1 expression.

Therefore, the development of new potential strategies to overcome CHI3L1 overexpression in several human cancers is of great importance. Drug repurposing might be a useful approach to accomplish that purpose.

1.3. Drug Repurposing

Developing new drugs is considered a time-consuming, complicated and expensive process, the journey to success associated with a high degree of failure¹⁰⁷. The methodology known as drug repurposing (also referred as drug repositioning, reprofiling, switching, among others) is a concept relatively recent that may have emerged in 2004 by the authors Ashburn and Thor, who possibly mentioned it for the first time¹⁰⁸. The concept reflects a great potential in discovering new therapeutic uses for marketed drugs and identifying therapies for untreated diseases^{108, 109}. Therefore, repurposing requires the use of the side properties responsible for adverse effects or the biological properties already approved by changing the formulation, the dose or the route of administration¹¹⁰. From 2010 to the present day, the use of drug repurposing to develop new drugs has increased exponentially, in studies conducted both by the scientific academy and the pharmaceutical industry¹⁰⁹.

Since the release of the first definition of drug repurposing, the concept was extended to include drugs withdrawn from the market due to safety problems and active substances that failed the clinical phase because of lack of efficacy or presence of toxicity in their development¹¹⁰. However, drug repurposing excludes any structural modification in drugs to obtain a new indication, which can include the selective optimisation of side activities (SOSA) approach¹¹¹. The approach relies on finding and isolating the biological property responsible for the adverse effect of a drug in a particular disease, where through a structural modification, the modified drug can be used to treat a new disease. Additionally, it also excludes substances that have not pass through a clinical investigation such as the ones held in chemical libraries, to identify those with new biological properties involving a hit-to-lead chemistry, eventually to optimise the new effects¹¹⁰.

Based in several aspects, drug repurposing revealed huge differences when compared with the traditional approach that involves *de novo* development and of new molecular entities in drug discovery. Drug repurposing is characterized by a strategy of four stages including: i) compound identification; ii) compound validation; iii) development;

and iv) post-market safety monitorization by the Food and Drug Administration (FDA) in United States, the European Medicines Agency (EMA) in Europe or the Infarmed in Portugal (**Figure 8**). In contrast, traditional drug discovery involves five stages: i) discovery and preclinical; ii) safety review; iii) clinical research; iv) FDA review; and v) monitorization of the post-market safety by the organizations already mentioned. In drug development, drug repurposing represents a strategy associated with a decreased risk of failure and is also responsible for the reduction in time and costs of drug development, given the structural database availability and advances in bioinformatics/cheminformatics tools, including the application of structure-based drug design (SBDD) and artificial intelligence technology¹¹²⁻¹¹⁴.



Figure 8. Drug repurposing strategy. Stages to approve already marketed drugs for a new indication.

Drug repurposing relies on two fundamental principles: a) drugs specific for a disease may have complementarity to other targets in different diseases; and b) a drug can be associated with several targets and pathways¹⁰⁸. Developing new drugs through this strategy requires approximately 3-12 years, to be approved by FDA and/or EMA, while in traditional approaches the estimated time is between 10 and 16 years. A repurposed drug can reduce development time and cost by entering directly into preclinical studies and clinical trials, avoiding the initial 6 to 9 years normally required by *de novo* drug development. Moreover, on average, researchers only spend 1 to 2 years in the identification of new drug targets and about 8 years to fully establish a new condition to the repurposed drug^{115, 116}. Since the potential candidate has already passed through the original preclinical and phase I studies of drug development, information such as pharmacodynamic, pharmacokinetic and toxicity profiles, clinical efficacy and safety profile are available, leading to reduction in the risks associated with the early stages of drug development, as well as a decrease in costs¹¹⁷⁻¹¹⁹.

The traditional discovery program is normally directed to establish drugs to treat chronic and complex diseases, while with the drug repurposing strategy, drugs are developed to treat diseases to which finding treatment is hard, such as infectious diseases and neglected diseases. Eventually, the purpose is achieved by the availability of a diverse set of approaches including omics data (transcriptomics, genomics, proteomics and metabolomics, among others), database resources, bioinformatics/cheminformatics

tools and disease targeted-based repositioning methods. The last approach provides the exploration of unknown mechanisms of action of existing drugs, including for example, finding new biomarkers for diseases, the understanding of drug-drug similarities and the knowledge of all targets¹¹⁷. However, it is noteworthy that when the formulation, dosage or route of administration of a known drug is changed, a re-examination of the safety profile is required to achieve a new medical product for a new indication different from the existing one¹¹⁰.

1.3.1. Drug Repurposing Strategies

There are two main alternatives and complementary approaches to obtain a potential repurposed drug: experiment-based approach and *in silico*-based approach. Then, the screening of already known drugs for new pharmaceutical indications can be accomplished by experimental assays which involve cell/organism-based and protein target-based screens, *in vitro* and *in vivo* models, without the necessity for structural information of the targets. Thus, the experimental repositioning includes several approaches such as target screening approach, cell assay approach, animal model approach and clinical approach¹²⁰.

Through the years, several research laboratories and pharmaceutical companies have successfully incorporated the *in silico* technique and tool for drug discovery¹²¹. In this approach, the complementarity between a drug and a target is carried out by virtual screening of public databases of chemical libraries, which requires precise structural information about drug targets^{121, 122}. Several databases and high throughput screening libraries emerged to provide that type of information due to the ability to evaluate 3D structures of drugs and targets, search for biological networks and evaluate ligand similarity¹²³. Advances in computer sciences led to improvements in drug repurposing methods such as data mining¹²⁴ and molecular modeling¹²⁵ approaches, which are usually divided into three categories. The first, is the machine learning, which predicts drug-target interactions by using machines to generate models such as support vector, artificial neural network and decision tree, among others^{126, 127}. Secondly, the network-based approaches, which models biological data (protein-protein interactions, metabolic pathways and drug-target interactions) as a network to find new therapeutic properties of drugs and achieve information about the target¹²⁸. Finally, the text mining, relies on mined and organized raw data of different sources to extract information through efficient algorithms¹²⁹. Consequently, through the *in silico* approaches, drug repurposing can take advantages of

their benefits including the low risk of failure, decreased time-consumed and reduced costs¹¹⁵.

One of the most used drug repurposing methods to accelerate the drug discovery process is molecular docking, which is also involved in the understanding and prediction of information about novel indications, side effects and therapeutic mechanisms. Docking has the ability to virtually screen several potential molecules to identify new drug-target interactions¹³⁰. The process can be done relatively fast by docking marketed drugs towards a target or by using a computational approach that can dock a specific drug on a series of targets known as reverse docking¹³¹. In contrast to docking, reverse docking allows the identification of targets for a given active ligand among a set of targets, which can be known as the “one-ligand many-targets” concept. Thus, like docking, it is an approach that needs preparation of a data set, search for ligand poses and scoring complex structures, which can be achieved by the several algorithms and docking methods that are available¹³². To summarize, both approaches only require structural information, where one of them designs compounds for a specific target (docking) and the other is responsible for discovering targets for a drug of interest based on ligand-receptor complementarity (reverse docking)¹³³.

After the merge of knowledge miming with molecular modeling tools to recognize a potential target, fulfilled by the computational approaches already mentioned, results should be validated by pre-clinical experiments (*in vitro* and *in vivo* tests) and clinical studies¹³⁴. Selected compounds can be evaluated in complex phenotypic assays, typically in 96-well formats, by using pharmacologically achievable concentrations. Also, several compounds with different modes of action can be tested simultaneously by performing a high-throughput screening. Furthermore, many whole-organism phenotypic assays have been improved for drug discovery and repurposing, including larval zebrafish *Danio rerio* and the nematode worm *C. elegans*¹³⁵. Simultaneous application of *in silico* and experimental methodologies offers logical opportunities to discover new indications for known drugs more successfully and rapidly¹³⁶.

To sum up, in drug repurposing integration of several scientific data from different disciplines is required including, *in silico* approaches, *in vitro* and *in vivo* assays and clinical studies¹³⁷.

1.3.2. Drug Repurposing Limitations

Even with the opportunities offered to repurpose a drug, the strategy features many challenges since it involves multiple factors such as investment and market demands,

commercial models, technology and patents. The main challenge relies on the identification of a new therapeutic indication for an already marketed drug, which consists in the main purpose of drug repurposing¹³⁸. From an economic perspective, three major types of compounds show great potential: i) drugs that are no longer manufactured due to commercial problems; ii) drugs not present in big markets but in the developing country markets; and iii) marketed drugs with patents near the expiration deadline¹³⁹. So, before initiating the process, elements such as intellectual property protection, economic considerations and data availability about the compound must be evaluated, as they are considered to be the limitations of drug repurposing^{140, 141}.

1.4. Molecular Docking

Computer-based strategies are usually used to identify compounds with desirable characteristics that will interact with a particular target¹⁴². Thus, they are considered cost-effective tools for the recognition of the basis in drug-receptor interactions and design of new therapeutic drugs¹⁴³. Docking represents one of the most used approaches in SBDD, which depends on possessing knowledge on a given protein structure¹⁴⁴. Through the years, several investigations of biological, chemical and structural data integrate molecular docking as a drug discovery tool applied in academia and industry, whose popularity has been increasing slowly¹⁴⁵.

Molecular docking was developed during the early 1980s and initiated with the obtention of target structures usually from the Protein Data Bank (PDB), providing access to structural features¹⁴⁶. After recognition of the 3D structure of the receptor, the ligand can be docked inside the binding site and several characteristics such as hydrophobic, electrostatic and steric interactions are explored to better fit a ligand^{147, 148}. The interactions between small-molecular ligands and the appropriate receptor are characterized at the atomic level, to understand the behaviour of ligands in the binding site and elucidate fundamental biochemical processes^{149, 150}. Then, studies to know how ligand binds to the target (position and orientation) are carried out to find all possible conformations, followed by the prediction of the interaction energy values, to identify the most favourable binding conformation for the respective ligand (**Figure 9**)^{148, 151}.

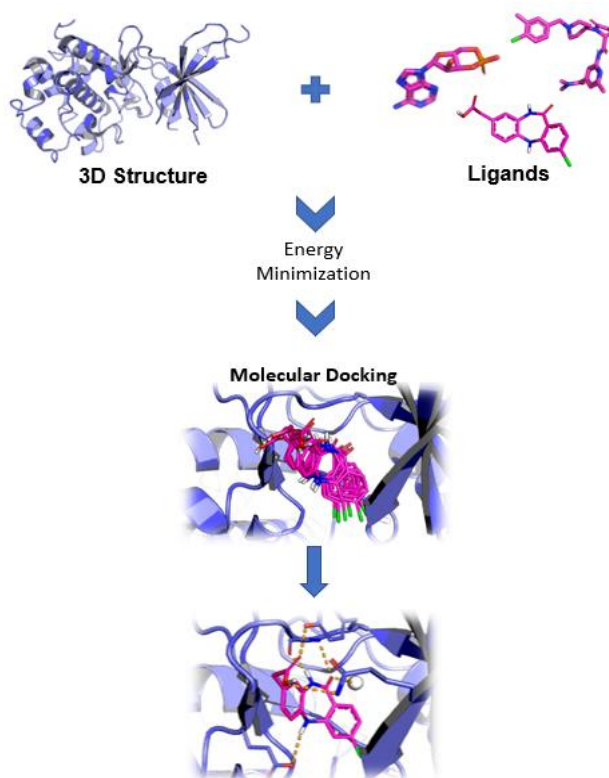


Figure 9. Workflow of molecular docking process. The strategy starts with the obtention of 3D structures of the target and ligands, followed by energy minimization. Molecular docking is then carried out inside the binding pocket of the target, leading to the identification of the most favourable binding conformation and the respective interactions. Adapted from ^{148, 152}.

Over the years, molecular docking programs have been emerging to evaluate the binding modes and calculate the values of binding affinity¹⁵³. These types of computational programs, whose popularity has been increasing slowly in the pharmaceutical industry, have been considered a key role in the discovery of new drugs. The time involved in the development and the costs of bringing drugs to the market are considered the main factors for the use of molecular docking. Researchers are increasingly inclined to these methods for being faster than performance of experiments in the laboratory and are also low cost methods. Furthermore, computational methods can also be used as a starting point for *in vitro* experiments or to validate *in vitro* studies¹⁵⁴. To distinguish docking programs, two crucial aspects appeared: search algorithm and scoring function. Understanding the characteristics of each of them is relevant in order to perform more accurate binding studies¹⁵⁵.

Search algorithms have the capability to analyse the pose of small molecules into the binding site of the receptor, where all possible modes of binding are generated. Consequently, it has been useful to discover valuable conformations between the molecules¹⁵⁶. Search strategies are normally divided into three main classes: systematic,

stochastic or random and deterministic methods (**Figure 10**). Systematic search algorithms explore incrementally a grid of values for each degree of freedom in a ligand, where the class can be subdivided in exhaustive search, databases, incremental construction and conformational ensemble¹⁵⁷⁻¹⁵⁹. The second class, stochastic search algorithms, perform random changes to the degrees of freedom of a ligand¹⁵⁷. Genetic algorithms, Monte Carlo, swarm optimization and tabu search methods are some of the most common algorithms of this class¹⁶⁰. Finally, deterministic search algorithms determinate the orientation and conformation of the ligand by the initial state that influence the movement to the next state, which usually has equal or lower energy value than the initial one. Approaches such as molecular dynamics and energy minimization methods are examples presented in this class^{157, 161}.

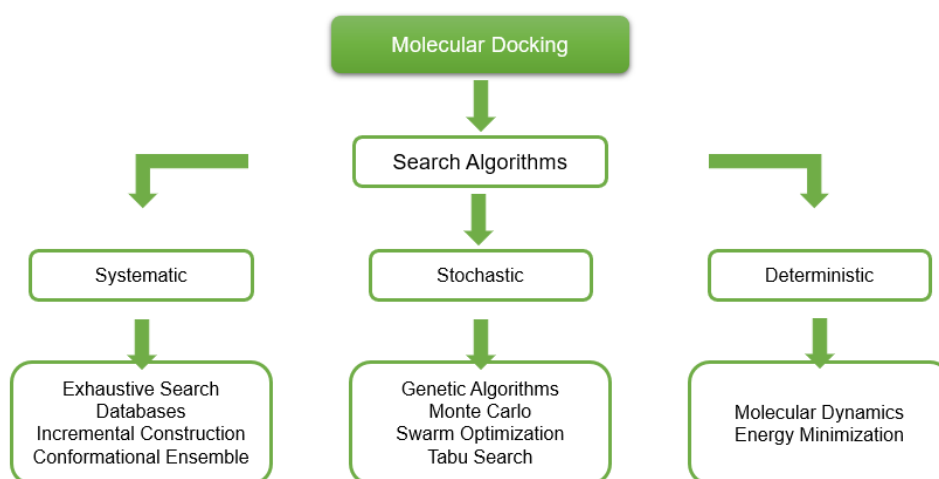


Figure 10. Main classes of search algorithms. The strategy is classified as systematic, stochastic and deterministic. Then, each class can be subdivided in more specific approaches.

Scoring functions were developed to predict the strength of the bond between a ligand and a receptor, acting as complement for search algorithms. They allow evaluation of the quality of relevant poses and determination of the best possible ligand conformation^{162, 163}. Currently, scoring functions are divided into four major categories: force field, empirical, knowledge-based and machine learning; allowing the prediction of the conformation between a ligand and the respective receptor (**Figure 11**)¹⁶⁴. However, when the interaction value is compared with experimental results, it may not be compatible due to challenges such as desolvation and entropic effects¹⁶⁵⁻¹⁶⁷. To overcome this limitation, a phenomenon called consensus scoring emerged to improve molecular docking accuracy. It consists in the combination of two or more scoring functions to balance errors existing in single scoring function¹⁵².

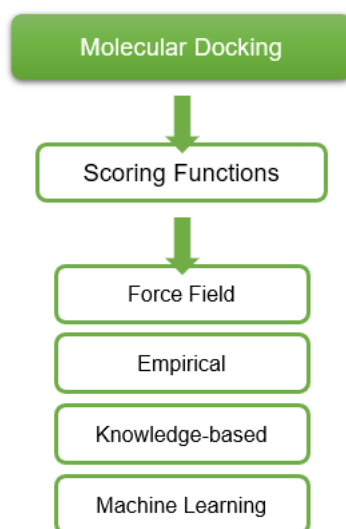


Figure 11. Scoring functions. A methodology divided into four major classes including force field, empirical, knowledge-based and machine learning.

Molecular docking programs have been developed through the years. In fact, over the last two decades, more than 60 different programs emerged to perform a search algorithm (pose evaluation) and binding affinity calculation¹⁵³. The development of new algorithms tends to focus on different aspects and stages of the docking system, including characteristics such as posing, flexibility, sampling, scoring, and optimization, among others. Studies involving new programs are often compared with already existing programs, which normally require improvements, including efficiency in predicting binding affinity, increase in speed and greater accuracy in docking pose prediction¹⁶⁸. Understanding the characteristics of each docking program is relevant in order to perform more accurate binding studies and docking-based virtual screening. Both commercial (such as LigandFit, GOLD, Surflex-Dock and Glide) and academic (such as AutoDock and AutoDock Vina) programs are available for the scientific community¹⁵⁵.

AutoDock is used since the year of 1990, and it searches a huge conformational space around a protein involving a ligand, predicting the best bound conformation and the values of binding energies with accuracy and speed. The docking is conducted by a grid-based method, where a grid embedded in the target protein allows the quick determination of the interaction between a probe atom and the target. The probe is placed in the formed grid box. Then, the interaction value is stored and it is used as reference for docking^{169, 170}.

The AutoDock relies on an evaluation of several potential positions of the ligand, which require a specific “search space” in the receptor¹⁷¹. Another program that also depends on the concept already described is AutoDock Vina, the program used in this

work. It was developed and implemented in the Molecular Graphics Lab by Dr. Oleg Trott at The Scripps Research Institute. Compared to AutoDock, the AutoDock Vina reveals improvement relatively to the accuracy behind the binding mode predictions and it also speeds up the execution by using multithreading on multi-core machines. These results were obtained during flexible redocking of 190 ligand-receptor complexes in both softwares¹⁷²⁻¹⁷⁴.

Since 2010, AutoDock Vina is responsible to automatically calculating the grid maps and the results are effectively grouped in a user-friendly way. The docking programs considers the receptor as rigid and the ligands as flexible¹⁷³. The scoring function involved in this program derives from the PDBbind data set¹⁷⁵. It consists in a combination between empirical and knowledge-based scoring functions, extracting information from experimental affinity values and the preferential conformation of the formed ligand-receptor complexes. To optimize the procedure, several stochastic optimization approaches were investigated, such as simulated annealing, genetic algorithms, particle swarm optimization, among others. Throughout development, the Iterated Local Search global optimizer was the chosen algorithm, a set of successive steps involving a mutation and a local optimization (Broyden-Fletcher-Goldfarb-Shanno method)¹⁷³.

Docking appeared for the first time to assist molecular recognition in ligands towards a target of interest and to investigate the relationship between several targets in a given disease. In drug discovery, several applications relied on docking to achieve their functionalities. One consists in the prediction of a target pool for compounds based on ligand-receptor complementarity (target fishing and profiling), where some of the targets are responsible for drug off-target effects (prediction of adverse drug reactions). Moreover, the identification of compounds that simultaneously modulate a series of selected targets involved in the same disease, at the same time, are also employed by docking (polypharmacology), as well as the discovery of new purposes for already known drugs (drug repurposing)¹⁷⁶.

The described computational tool may be applied in a variety of applications, despite the presence of intrinsic limitations. One of the major complications involves the scoring of compound conformation, where approximated values are used, leading to the lack of correlation with experimental binding values^{177, 178}. Another limitation during this process comprises the participation of water molecules that can form hydrogen bonding interactions inside the receptor pocket¹⁷⁹. The problem relies on the identification of the molecules in crystal structures, due to not knowing the correct position of hydrogen and how water molecules are affected by ligands in the binding pocket¹⁸⁰. Additionally, the flexibility of the receptor is still considered a concern in this field, where the different

conformational states of a receptor must be taken into consideration since the ligand can be active¹⁸¹. Beyond the limitations already presented, docking requires known crystal structures. When the structure is not known, a computational approach like homology modeling can be used to predict the 3D structure of the unknown target, through the building of homology models from amino acid sequence of templates¹⁸².

Overcoming the emerging limitations has been considered a necessity to improve docking accuracy. The combination of other computational methods with docking can be a strategy to fulfil these tasks, including the improvement of drug discovery applications. Therefore, docking has gained popularity among researchers and has become a great tool for pharmaceutical industry, leading to the discovery of new drugs or refinement of existing ones with the help of computational tools¹⁷⁶.

2.Aims

The aim of this dissertation was to:

i) perform a virtual screening of thousands of known compounds, from the DrugBank database, to find potential ligands of CHI3L1.

ii) perform the screening of some of these compounds, which had good docking scores in the *in silico* screen, in BxPC3 and PANC-1 cell lines in order to evaluate their antitumor activity.

3. Materials and Methods

3.1. Molecular Docking Virtual Screening

3.1.1. Preparation of Receptor and Ligands

The 3D structure of CHI3L1 was obtained from the PDB (pdb code: 1NWU) at 2.20 Å resolution. The protein target was prepared using AutoDock Tools 1.5.6 (Scripps Research Institute, USA)¹⁸³. The 3D structures of the controls were drawn using ChemDraw 15.1 and minimized by the MM2 force field method at a root mean square deviation (RMSD) gradient of 0.01 kcal/mol.Å using Chem3D 15.1 (PerkinElmer, USA). 11,741 Molecules (approved and experimental drugs, compounds under investigation, nutraceuticals, illicit and withdrawn compounds) were obtained from the DrugBank database (Version 5.1.8)¹⁸⁴. The energy minimization of all database was performed by the MM2 force field method at a RMSD gradient of 0.01 kcal/mol.Å. The existing chirality was preserved and partial charges were calculated according to the standard parameters of the force field.

3.1.2. Molecular Docking

Docking simulations between the CHI3L1 (pdb code: 1NWU) and the controls and database molecules were undertaken in AutoDock Vina (Scripps Research Institute, USA) by using the program PyRx 0.8, allowing an automated screen¹⁷³. AutoDock Vina considered the target conformation as a rigid unit while the ligands were allowed to be flexible and adaptable to the target. Vina searched for the lowest binding energy conformations and returned nine different conformations for each ligand. AutoDock Vina was run using an exhaustiveness of 8 and a grid box with the dimensions of 19.35, 27.08, and 24.07 Å built around the crystallographic ligand. Conformations and interactions were visualized using PyMOL version 2.4 (Schrödinger, USA)^{185, 186}.

3.2. Biological evaluation

3.2.1. Drugs

Darifenacin hydrobromide (DF; SML1102), dihydroergotamine mesilate CRS (DHE; D1950000), gemcitabine (GEM; G6423) and pentoxifylline (PTX; P1784) were purchased from Sigma-Aldrich. DF and GEM were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at concentrations of stock solutions of 40 mM and 60 mM, respectively. PTX and DHE was dissolved in H₂O for Cell Biology (Sigma-Aldrich) at concentration of stock

solution of 100 mM and 7.35 mM, respectively. The compounds DF, GEM and PTX were stored at -20 °C. The compound DHE was stored at 4 °C, according to manufacture.

3.2.2. Cell culture

The human pancreatic cancer cell lines BxPC3 and PANC-1 were acquired from the American Type Culture Collection (ATCC). These cell lines were genotyped and routinely tested for *Mycoplasma* contamination, which was performed at the Core Facility of i3S, Porto. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, with 4.5 g/L glucose and with UltraGlutamine, Lonza) complemented with 10% fetal bovine serum (FBS, Biowest) at 37°C in a humidified 5% CO₂ incubator.

To maintain the cells, twice a week, the medium was change. After removing the medium, cells were washed with phosphate-buffered saline (PBS) and incubated for a short period with tripLE express to trypsinize the cells. Then, cells were resuspended with new culture medium and a certain volume (depending on the desired dilution) was added in a new flask with new medium. The cell number and viability were assessed with the Trypan Blue exclusion assay and all the experiments were carried out with cells at the exponential phase of growth and with more than 90% of viability. For the cell growth inhibition assay (sulforhodamine B assay), cells were grown in medium supplemented with 5% FBS.

3.2.3. Trypan Blue Exclusion Assay

The Trypan Blue Exclusion assay was carried out to maintain cells at an appropriate density in the culture flasks, to confirm the cell viability and to plate the same number of cells in each well for the drug treatments. The dye Trypan Blue penetrates into dead cells, which have disrupted membranes, allowing to distinguish between alive (bright) and dead (blue) cells. Cell suspensions were mixed with 0.2% (v/v) trypan blue dye (ratio 1:1) and added into a Neubauer chamber for cell counting.

3.2.4. Cell plating

For cell plating, after trypsinization of the cells, cell suspension was transferred to a falcon and the number of cells was counted by trypan blue exclusion assay, allowing the determination of cells/mL on the initial suspension. Then, cells were plated in 96 well plates, at a previously determined optimal cell concentration: 7.5×10^4 cells/mL for BxPC3

cells and 5×10^4 cells/mL for PANC-1 cells, in a total volume of 100 μ L/well¹⁹. Then cells were let to adhere for 24 h at 37°C in a humidified incubator with 5% CO₂.

3.2.5. Drug treatment

BxPC3 and PANC-1 cells were treated with different conditions for 48 h: five different serial dilutions of the tested drugs; DMSO or H₂O (vehicle as Control, as in the higher concentration used to dilute the tested compounds); and medium only (Blank). GEM was added at concentrations between 0.062 μ M - 1 μ M in BxPC3 cells and 0.375 μ M - 5 μ M in PANC-1 cells. PTX was added at concentrations of 0.156 mM – 2.5 mM in BxPC3 and 0.375 mM – 6 mM in PANC-1 cells. DHE was added at concentrations between 2.81 μ M – 100 μ M to both cell lines. DF was added at concentrations between 6.25 μ M – 100 μ M to both cell lines.

3.2.6. Sulforhodamine B assay

The sulforhodamine B (SRB) assay has been used to study cell growth inhibitory activity by measuring cellular protein content. This method relies on the binding of a bright pink aminoxanthene dye, SRB, with protein components (basic amino acids residues) under mild acid conditions. SRB binding is then dissociated and extracted through a basic condition, where the amount of dye can be used to measure the cell mass, allowing an indirectly determination of cell number¹⁸⁷. The SRB assay also allows to determine the GI₅₀ concentration, which causes 50% of cell growth inhibition, for each drug/compound being tested.

After 48 h of treatment, cells were fixed with 50 μ L of 10% (w/v) ice-cold trichloroacetic acid (TCA, Sigma) and left 1 h or overnight at 4°C. Then, cells were washed with distilled water and let to air-dry at room temperature. Cells were then stained with 50 μ L of 0.4% (w/v) SRB (Sigma), prepared in 1% acetic acid, followed by incubation in the dark for 30 minutes. Then, cells were washed with 100 μ L of 1% (v/v) acetic acid to remove unbound dye. Once again, cells were let to air-dry and the bound SRB solubilised with 100 μ L of 10 mM Tris base (Sigma), followed by 5 minutes in the mechanical agitator (300 rpm). The absorbance was measured at 510 nm in a microplate reader (Synergym Biotek, Gen51.09, RTL-510 Program). The GI₅₀ concentrations of each drug tested were determined by interpolation of the dose-response curves.

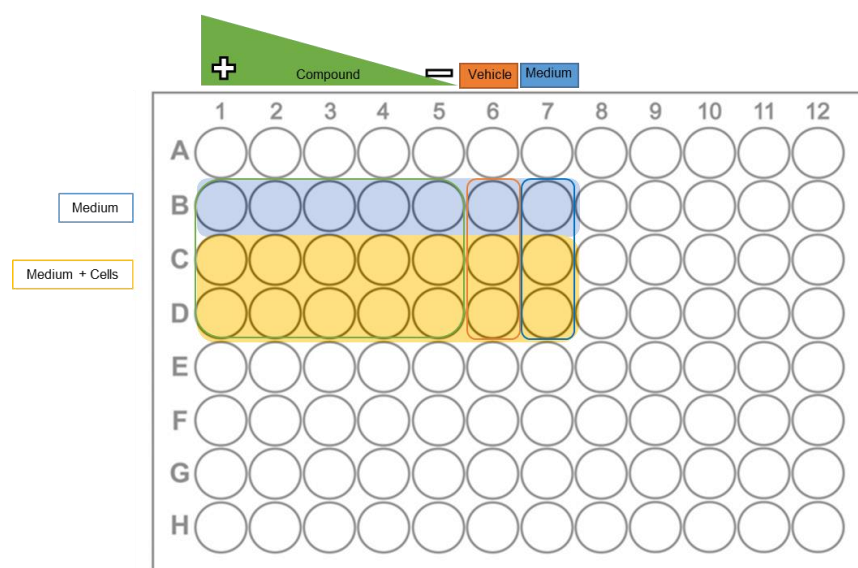


Figure 12. Layout of the SRB experimental plate. Different serial dilutions of the tested drugs/compounds, which were tested in duplicate (in each independent experiment), from high concentration (wells in column 1) to low concentration (wells in column 5) were added to the cells. The Control consisted in the vehicle (DMSO or H₂O), tested at the same concentration as in the higher concentration used to dilute the tested drugs/compounds. The Blank (medium only) and the background (medium plus each drug/compound concentration) were also included.

4.Results and Discussion

4.1. Virtual Screening

As described in the literature, CHI3L1 is a CLPs without chitinase activity, due to the substitution of an essential glutamic acid with leucine in the active site¹⁸⁸. The protein consists in a $(\beta/\alpha)_8$ -barrel domain with one α -helix ($\alpha + \beta$) domain inserted after strand $\beta 7$ ⁵⁰. The 2.20 Å resolution structure of homo sapiens CHI3L1 (pdb code: 1NWU) was selected to perform a virtual screening on a database of known compounds in search of potential new CHI3L1 inhibitors for the treatment of pancreatic cancer⁴⁹.

The structure of 1NWU presents a crystallographic ligand - chitotetraose - thus creating a reference for this study. Through the knowledge of how and where this ligand binds to the protein, a grid box with the dimensions of 19.35, 27.08, and 24.07 Å was created around the crystallographic ligand in the program AutoDock Vina. The grid box was then used to perform the docking studies in the known binding site. The studies allowed the evaluation of possible poses within the binding pocket of CHI3L1, and also the obtention of the binding affinity values, which allowed the prediction of binding strength of thousands of known compounds (approved and experimental drugs, compounds under investigation, nutraceuticals, illicit and withdrawn compounds) from the DrugBank database.

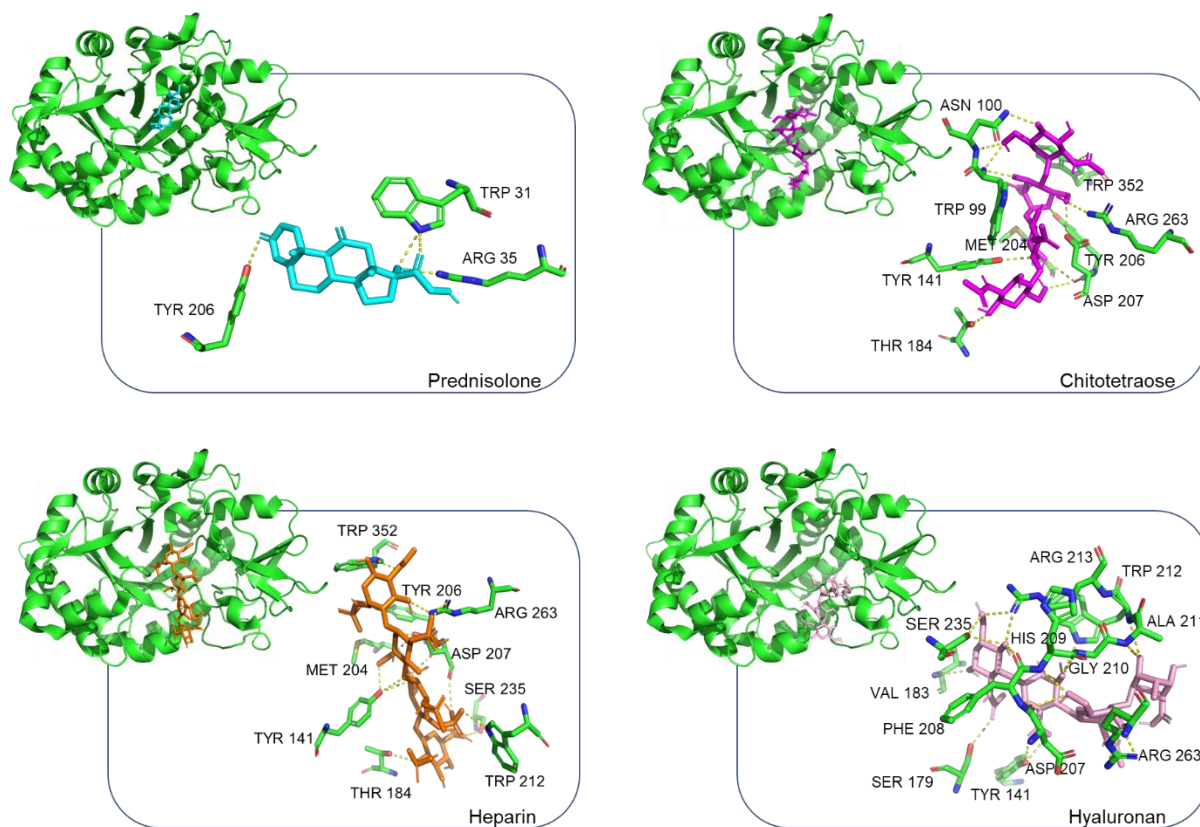
Firstly, docking studies were performed using the known compounds described in the literature, which includes ligands (chitotetraose⁴⁹, chitohexaose¹⁸⁹, heparin^{51, 189}, collagen^{51, 189} and hyaluronan¹⁸⁹) and inhibitors (caffeine¹⁹⁰, pentoxifylline¹⁹, prednisolone¹⁰⁵, resveratrol^{104, 105}, theophylline¹⁹⁰) of CHI3L1. These positive controls revealed scoring values between -5.9 to -10.1 kcal/mol (**Table 1**).

Table 1. Docking scores for the positive controls of CHI3L1.

Name	Score (kcal/mol)	Molecular Weight	logP	Therapeutic class
Prednisolone	-10.1	360.4	1.66	Glucocorticoid
Chitotetraose	-9.1	662.7	0.22	Chitin-like oligosaccharides
Heparin	-8.8	1134.9	-13.20	Anticoagulant
Hyaluronan	-8.5	776.6	-1.80	Glycosaminoglycan
Collagen	-8.4	1471.6	-7.00	Protein
Chitohexaose	-7.9	985	-0.56	Chitin-like oligosaccharides
Resveratrol	-7.5	228	2.57	Stilbenoid

Pentoxifylline	-7.1	278	0.08	Hemorheologic Agent
Caffeine	-5.9	194	-0.24	Stimulant
Theophylline	-5.9	180	-0.26	Phosphodiesterase inhibitor

Amongst the positive controls, the inhibitor prednisolone was shown to bind CHI3L1 with the highest affinity, presenting therefore the most negative score value (-10.1 kcal/mol). A visual interpretation of the conformations and interactions of prednisolone and the other positive controls with CHI3L1 was performed to analyse the binding mechanism (**Figure 13**). The compounds are ordered according to the degree of affinity to CHI3L1, i.e from the most negative value of score to the less negative value of score (-10.1 to -5.9 kcal/mol).



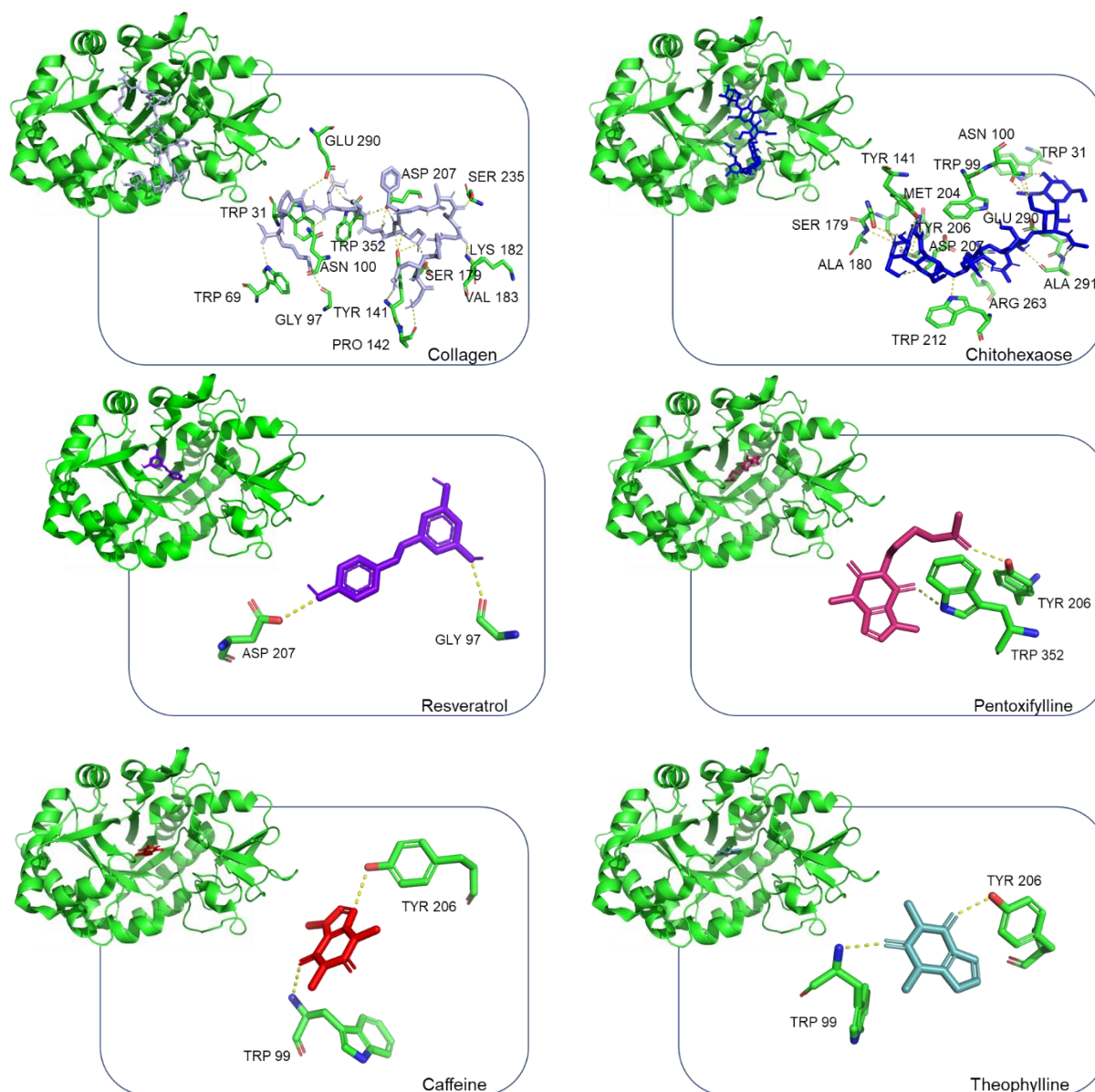


Figure 13. Visual representation of conformations and interaction with CHI3L1 through PyMOL. Crystal structure of CHI3L1 with the positive controls, showing its binding to the active site by hydrogen bond interactions (yellow broken lines).

A study of the crystal structure with a long chitin fragment demonstrated that TRP 99, ASN 100, TRP 352, ARG 263 and TYR 141 participate in ligand recognition⁵⁰. Some ligands have been shown to be able to interact with the described residues of the target. TRP 99 interacts with chitotetraose, chitohexaose, caffeine and theophylline. ASN 100 interacts with chitotetraose, collagen and chitohexaose. TRP 352 interacts with chitotetraose, heparin, collagen and pentoxifylline. ARG 263 interacts with chitotetraose, heparin, hyaluronan and chitohexaose. TYR 141 interacts with chitotetraose, heparin, hyaluronan, collagen and chitohexaose. It is possible to verify that chitotetraose and

chitohexaose interact with almost all the residues already described since they are chitin-like oligosaccharides. Additionally, through the analysis of these representations, TYR 141, TYR 206 and ASP 207 are the residues that most intervene in the binding of positive controls, whereas the residues that least intervene in the binding are ARG 35, TRP 69, PRO 142, ALA 180, LYS 182, PHE 208, HIS 209, GLY 210, ALA 211, ARG 213 and ALA 291.

After the analysis of the positive controls, 11,741 molecules from the DrugBank database were docked by using the same methodology used for the positive controls. A total of 568 molecules (4.8 % of the screened molecules) showed higher affinity for CHI3L1 compared with the inhibitor prednisolone, the positive control with the best score value. The top ranked approved drugs were then selected for a more thoughtful *in silico* analysis, and amongst those molecules, the most suitable ones were selected for *in vitro* testing (**Table 2**).

Table 2. Characteristics of the top ranked approved drugs.

Name	Score	Therapeutic class	Mechanism of action	Anticancer activity previous shown in some types of cancer	Ref.
Dactinomycin Or Actinomycin D Or Actinomycin C1	-14.4	Anticancer agent	- One of the first antibiotics isolated from the bacterium <i>Streptomyces parvulus</i> - Administered intravenously - Binds to DNA and inhibits RNA synthesis (transcription) - Blocks the transcription of DNA by RNA polymerase through intercalation between adjacent guanine-cytosine bases pairs	- Embryonal rhabdomyosarcoma - Wilms Tumors - Ewing sarcoma - Choriocarcinoma - Testicular tumors - Pancreatic cancer (PANC-1 cells)	191-197
Dihydro-ergotamine	-13.0	Serotonin agonist (5-HT)	- Derivate of ergotamine -Used for the treatment migraines - Antimigraine effects due to the high binding of 5-HT1B, 5-HT1D, and 5-HT1F receptors - Interacts with adrenergic, cholinergic and dopaminergic receptors	- Acute Myeloid Leukemia - Lung cancer	198-204

Plicamycin Or Mithramycin A	-12.2	Anticancer agent	- First isolated by <i>Streptomyces plicatus</i> - DNA/RNA polymerase inhibitor - DNA-binding transcriptional inhibitor - Forms a complex with DNA in the presence of divalent cations - Binds to guanine-cytosine-rich regions of DNA - Downregulates RNA synthesis - Inhibits cancer cell growth - Facilitates tumor necrosis factor α and Fas ligand-induced apoptosis	- Chronic myeloid leukemia - Acute myeloid leukemia - Testicular cancer - Pancreatic cancer	205-212
Venetoclax Or ABT-199	-12.0	Anticancer agent	- First selective B-cell lymphoma 2 (BCL2) inhibitor - First of a new class of anticancer drugs (BH3-mimetics) - Induce apoptosis of chronic lymphocytic leukemia cells via TP53-independent mechanism - Treatment of patients with 17p gene deletion	- Chronic lymphocytic leukemia - Acute myeloid leukemia - Multiple myeloma - Pancreatic cancer (enhance sensitivity to gemcitabine)	213-220
Temoporfin Or m-THPC	-12.0	Photo-sensitizing agent	- Activates with red light (652nm), producing reactive species, leading to cell destruction	- Head and neck squamous cell carcinoma	193, 221, 222
Dirlotapide	-11.9	Microsomal triglyceride transfer protein inhibitor	- Used in the management of obesity in dogs - Inhibits the assembly and release of lipoprotein into the bloodstream	-----	223-226
Nafarelin	-11.9	Gonadotropin releasing hormone receptor agonist	- Binds with high affinity to the gonadotropin releasing hormone receptor on anterior pituitary cells - Stimulates the secretion of luteinizing hormone and follicle-stimulating hormone - Used for the treatment of endometriosis and in assisted reproduction protocols	- Leiomyomas (benign tumor) - Prostate cancer	227-230

Velpatasvir	-11.8	Antiviral	- Inhibits non-structural protein 5A (NS5A), leading to a prevention of viral replication - Used in the treatment of hepatitis C virus infection in combination with sofosbuvir	----	231-233
Lifitegrast	-11.8	Integrin antagonist	- Used for the treatment of dry eye disease (also known as keratoconjunctivitis sicca) - A direct competitive antagonist developed to target lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) interaction - Inhibits T-cell recruitment and activation - Inhibits proinflammatory cytokines release - Inhibits the formation of immunological synapse - Decreases the ocular inflammatory cycle - Suppresses tumor metastasis via targeting ICAM3-LFA-1 interaction	- Breast and lung tumor tissues (treatment of tumor metastasis)	234-237
Darifenacin	-11.8	Muscarinic receptor antagonist	- Anticholinergic and antispasmodic agent - Used for the treatment of urinary incontinence and overactive bladder syndrome - A selective muscarinic M3 receptor antagonist that reduces the urgency to urinate	- Gastric cancer - Lung cancer - Colorectal cancer	238-243

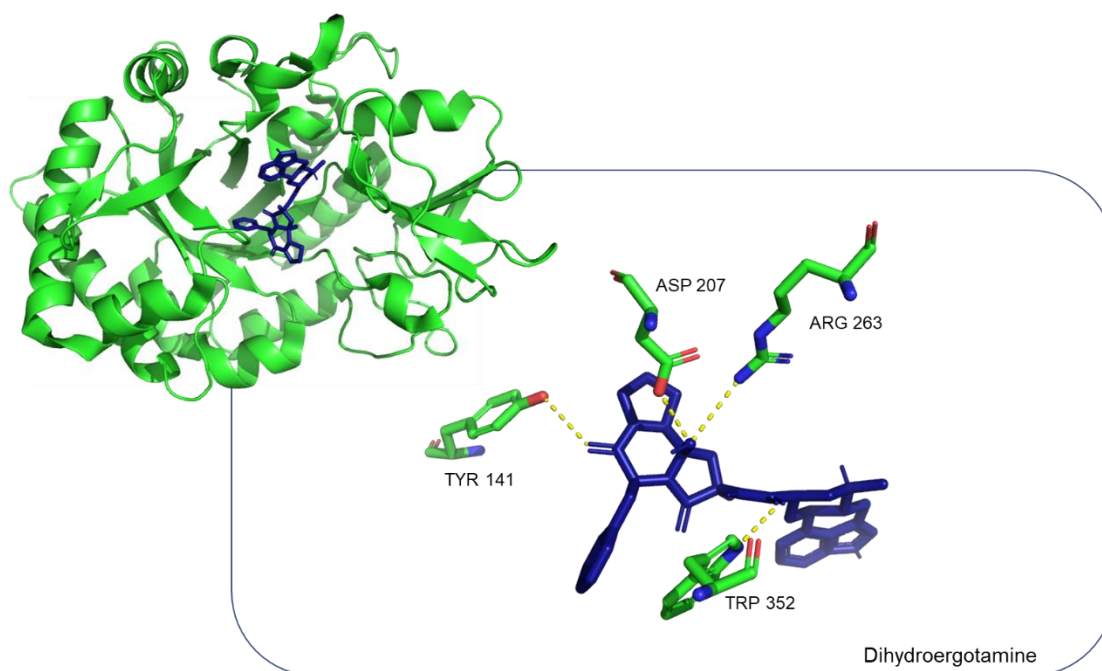
Dihydroergotamine, velpatasvir and darifenacin were the three selected compounds for further analysis of the interactions with the binding pocket of CHI3L1. To make this selection, some parameters had to be considered, including whether the compound had already been used in the area of cancer, whether it had been used for human or veterinary use, whether it is able to enter the cell, whether it was available for immediate purchase, among others. Dihydroergotamine, a serotonin agonist, showed a score value of -13.0 kcal/mol. Velpatasvir, an antiviral, presented a lower score compared with

dihydroergotamine, -11.8 kcal/mol, but still a value higher than prednisolone; and the same happened with darifenacin, a muscarinic receptor antagonist (**Table 3**).

Table 3. Score values of the three selected compounds.

Name	Score (kcal/mol)	Molecular Weight	logP	Therapeutic class
Dihydroergotamine	-13.0	583.7	3.04	Serotonin agonist
Velpatasvir	-11.8	883	5.39	Antiviral
Darifenacin	-11.8	426.6	4.35	Muscarinic receptor antagonist

A visual interpretation was also performed for the three selected compounds (dihydroergotamine, velpatasvir and darifenacin), to analyse in more detail the binding mechanism with CHI3L1 (**Figure 14**). The compounds were then ordered by the values of binding affinity (score). Through this analysis carried out for the three compounds, ASP 207 was found to be the residue that is implied in the binding of all the compounds. This residue was also detected as being important in the binding of the positive controls. Comparing with the study involving the chitin fragment⁵⁰, residues such as ASN 100, TYR 141, ARG 263 and TRP 352 are present in at least one of the three compounds.



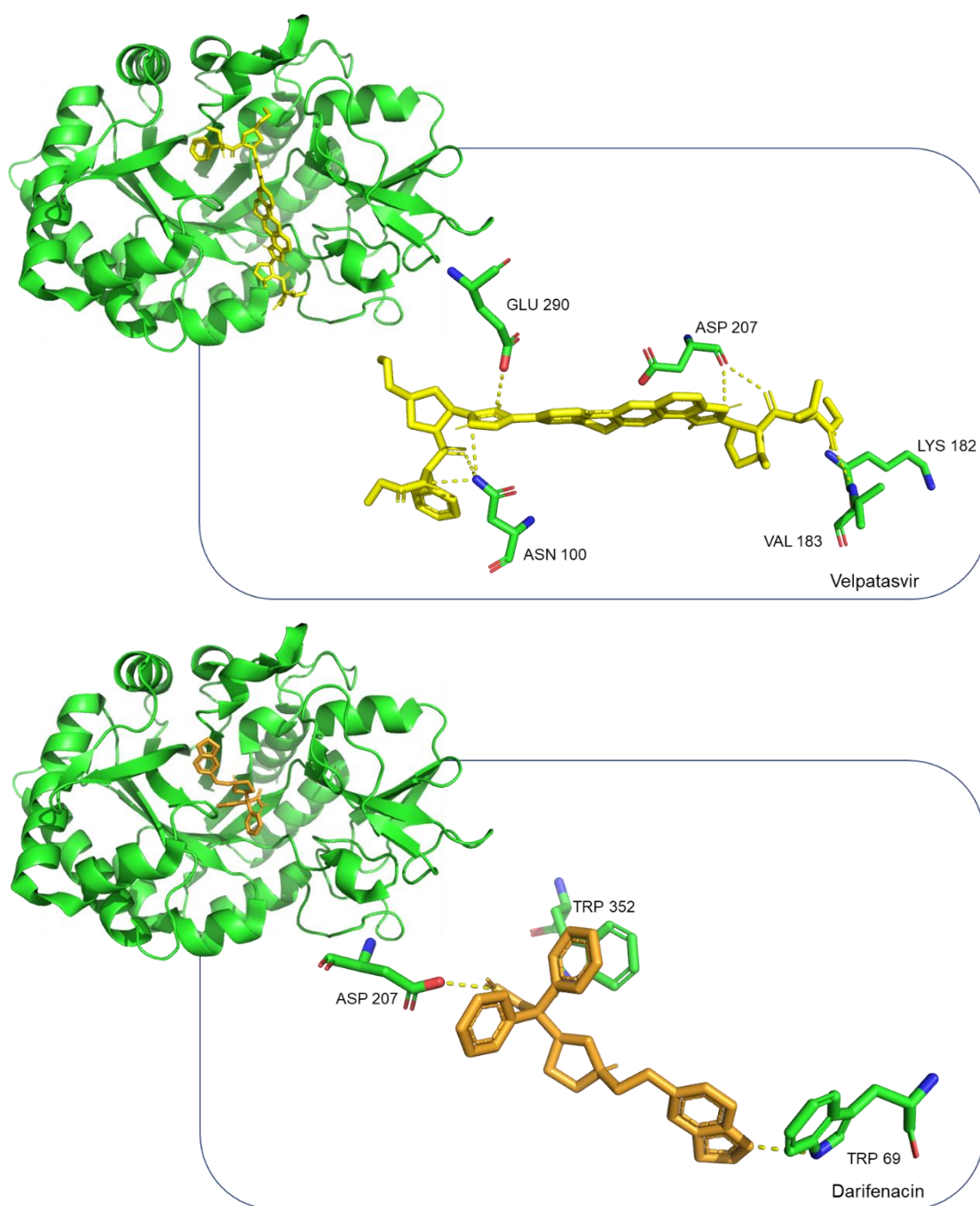


Figure 14. Visual representation of conformations and interaction with CHI3L1 through PyMOL. Crystal structure of CHI3L1 with the three selected compounds, showing its binding to the active site by hydrogen bond interactions (yellow broken lines).

Then, further analysis was performed using the web tool Protein-Ligand Interaction Profiler (PLIP), a tool able to identify non-covalent interactions between the CHI3L1 and the ligands (positive controls and selected compounds). The tool was able to identify interactions such as hydrophobic interactions, hydrogen bonds, π -cation interactions, salt bridges and π -stacking (**Table 4**).

Table 4. Interactions between test ligands and controls, and the target CHI3L1, as predicted by the docking study.

Compound	Hydrophobic Interactions	Hydrogen Bonds	π -Cation Interactions	Salt Bridges	π -Stacking
Dihydro-ergotamine	PHE 58; TRP 99 ALA 138; LEU 140 ALA 177; TRP 352	ASP 207; TYR 141 ARG 263; TRP 352	---	ARG 263 GLU 290	TYR 27 TRP 31
Velpatasvir	TRP 31; PHE 58 TRP 69; TRP 99 ASN 100; TRP 212	ASN 100; LYS 182 VAL 183; ASP 207 GLU 290	ARG 263	---	TRP 69 TRP 99
Darifenacin	TRP 31; PHE 58 TRP 69; TRP 99 ASN 100; LEU 140	TRP 69; ASP 207 TRP 352	---	---	TRP 31 TRP 352
Caffeine	---	TRP 99; TYR 206	TRP 352	---	---
Chitohexaose	TYR 141 MET 204 PRO 281	TRP 31; TRP 99 ASN 100; TYR 141 SER 179; ALA 180 MET 204; TYR 206 ASP 207; TRP 212 ARG 263; GLU 290 ALA 291	---	---	---
Chitotetraose	TYR 27; TRP 99 ALA 177; PHE 261 THR 293; TRP 352	TRP 99; ASN 100 TYR 141; THR 184 MET 204; TYR 206 ASP 207; ARG 263 TRP 352	---	---	---
Collagen	TRP 31; PHE 58 TRP 69; TRP 99 TYR 141; PHE 208 TRP 212	TRP 31; TRP 69 GLY 97; ASN 100 TYR 141; PRO 142 SER 179; LYS 182 VAL 183; ASP 207 SER 235; GLU 290 TRP 352	ARG 263	---	ARG 263
Heparin	PHE 261 THR 293 LEU 356	TYR 141; THR 184 MET 204; TYR 206 ASP 207; TRP 212 SER 235; ARG 263 TRP 352	---	ARG 263	---
Hyaluronan	---	TYR 141; SER 179 VAL 183; ASP 207 PHE 208; HIS 209 GLY 210; ALA 211 TRP 212; ARG 213 SER 235; ARG 263	---	---	---
Pentoxifylline	PHE 58; TRP 99 TRP 352	TYR 206; TRP 352	---	GLU 290	---

Prednisolone	TRP 31; TRP 99 TYR 206; PHE 261 THR 293; TRP 352	TRP 31; ARG 35 TYR 206	---	---	TRP 352
Resveratrol	TRP 31; PHE 58 TRP 69; TRP 99 ASN 100; TRP 352	GLY 97; ASP 207	---	---	---
Theophylline	---	TRP 99; TYR 206	---	---	TRP 352

By pooling the data obtained by the PLIP, it was possible to compare the non-covalent interactions present in the binding of all compounds with the target CHI3L1 through the construction of an histogram (**Figure 15**). The histogram also allows to evaluate the number of ligands contacting a residue of the target and the number of residues that contact the ligands on the binding pocket.

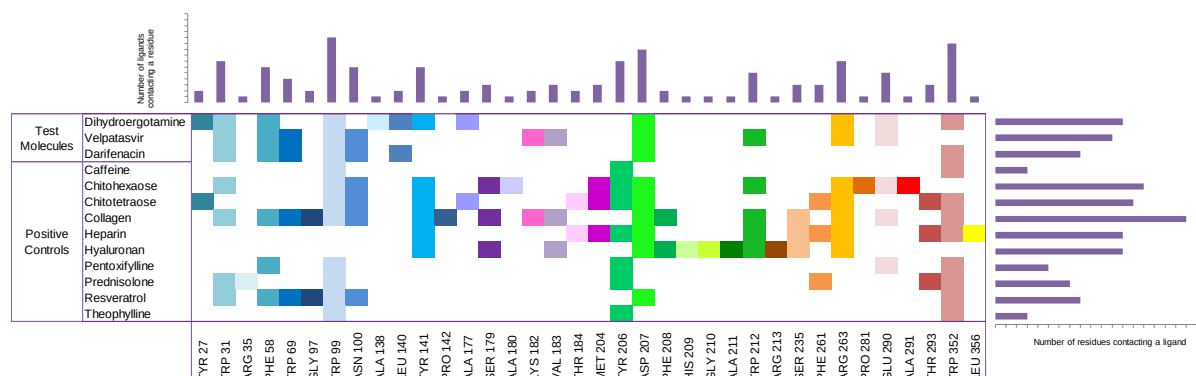


Figure 15. Interaction histogram. Contact details of the positive controls and the selected compounds with the binding pocket of CHI3L1 represented by a color.

After evaluation of the histogram, the residues that most intervene in the binding of positive controls and the selected compounds with CHI3L1 are TRP 31, TRP 99, ASP 207, ARG 263 and TRP 352; and the residues that less intervene are TYR 27, TRP 69, ALA 177, LYS 182, VAL 183 and TRP 212. Despite the presence of several residues in the interactions, most of the residues that intervene in the binding of positive controls are not involved in the binding of the selected compounds. However, test compounds are able to establish interactions with different residues, such as ALA 138 and LEU 140, which may allow to strengthen the binding of those molecules to the target.

The ligands that interact with more residues on the binding pocket of CHI3L1 are collagen, chitohexaose and hyaluronan, and the ones that interact with less residues are caffeine and theophylline.

4.2. *In vitro* experiments

Pancreatic cancer is known to be one of the most fatal malignant tumors worldwide, characterized by the lack of effective drug treatments. Normally, this type of cancer is detected in an advanced stage and finding available treatments can be challenging²⁴⁴. Today and since the 1980s, cytotoxic chemotherapy remains the only choice to fight this disease²⁴⁵. Thus, new treatments need to be developed. Gemcitabine (GEM) is a cytidine analogue originally developed as an antiviral agent. Nowadays, GEM, approved by the FDA, is used alone or in combination with nab-paclitaxel as a first-line treatment for pancreatic cancer. GEM improved the one-year survival rate, when compared to the existing treatment up to that date (5-fluorouracil), from 2% to 18%^{14, 245-247}. The mechanism of action of GEM relies on inhibition of DNA synthesis since, after intracellular phosphorylation, this agent is incorporated into the cellular DNA, leading to blockage of the G1/S phases in the cell cycle and inhibition of cell growth^{248, 249}.

In this study, GEM was used for the inhibition of the two PDAC cell lines. Thus, to evaluate the PDAC cell growth inhibition effect of GEM, the SRB assay was performed. For that, indirectly determination of cell number was assessed by measuring cell protein levels¹⁸⁷. BxPC3 and PANC-1 cell lines were treated with different concentrations of GEM during 48 h, and dose-response curves were determined. In addition, the concentration of drug that caused 50% cell growth inhibition (GI_{50}) was determined, by interpolation of the determined curve (**Figure 16**). GEM presented a GI_{50} concentration of $0.11 \pm 0.02 \mu\text{M}$ in BxPC3 and $1.01 \pm 0.42 \mu\text{M}$ in PANC-1 cells. These results are in agreement with other studies¹⁹. Indeed, BxPC3 cells, which are wild-type cells for KRAS (Kirsten rat sarcoma virus), are more sensitive to GEM treatment, when compared with the more resistant PANC1 cells, which have mutated KRAS. Thus, a higher GI_{50} concentration of GEM was found in the more resistant cell line, which is also characterized by more aggressive features²⁵⁰.

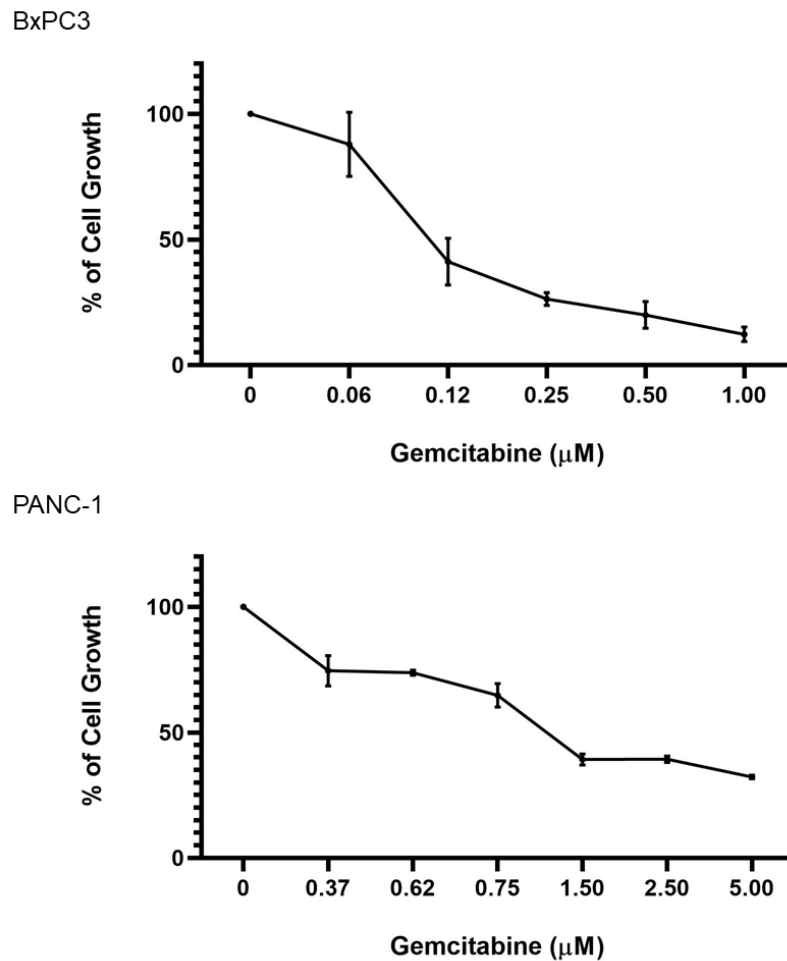


Figure 16. Effect of gemcitabine in two PDAC cell lines. BxPC3 and PANC-1 cells were treated with increasing concentrations of gemcitabine (0.06 μM – 1.00 μM and 0.37 μM – 5.00 μM , respectively). After 48 h treatment, the inhibition of cell growth was determined with the SRB assay. Data shows the mean $\pm$ the standard error of the mean (SEM), from three independent experiments.

Despite the efficacy of GEM treatment in some patients with pancreatic cancer, chemoresistance eventually appears, limiting the use of this chemotherapeutic drug. The ability of cancer cells (with the help of the surrounding microenvironment) to gain resistance to this cytotoxic drug, after repetitive few weeks of treatment, restricts the survival rate of patients to six months^{251, 252}. The mechanisms involved in GEM resistance include pathways that influence the survival of cancer cells and drug metabolism, deregulation of miRNAs and alterations in the tumor microenvironment²⁵².

Recent studies have shown that combination treatments can be a useful strategy to overcome drug resistance. In fact, the combination of GEM with pentoxifylline (PTX) has already been suggested. Studies performed in PDAC cell lines, by some members of our research team, demonstrated that PTX is able to revert GEM resistance induced by the recombinant human protein CHI3L1 (rhCHI3L1)¹⁹. This was in agreement with another

study with a human pancreatic tumor xenograft model, which showed a synergetic effect when GEM was administrated with PTX (leading to 50% inhibition of tumor growth), thus improving GEM efficacy²⁵³. PTX is a known CHI3L1 inhibitor (also confirmed by us through molecular docking for CHI3L1), and a FDA-approved drug to treat diseases other than cancer²⁵⁴. PTX is a methylxanthine derivate firstly approved for peripheral vascular disease and eventually, it was selected as a drug repurposing candidate for the treatment of PDAC^{255, 256}.

Therefore, in this study, PTX was used as a positive control for inhibition of PDAC cell lines growth. The dose-response curves were obtained for PTX and the GI₅₀ concentrations were determined: 1.13 ± 0.07 mM in BxPC3 and 1.45 ± 0.17 mM in PANC-1 (**Figure 17**). These results are in agreement with previous work from our research group, which demonstrated similar antitumor activity of PTX in both PDAC cancer cell lines¹⁹, suggesting that probably KRAS is not involved in the response to this drug.

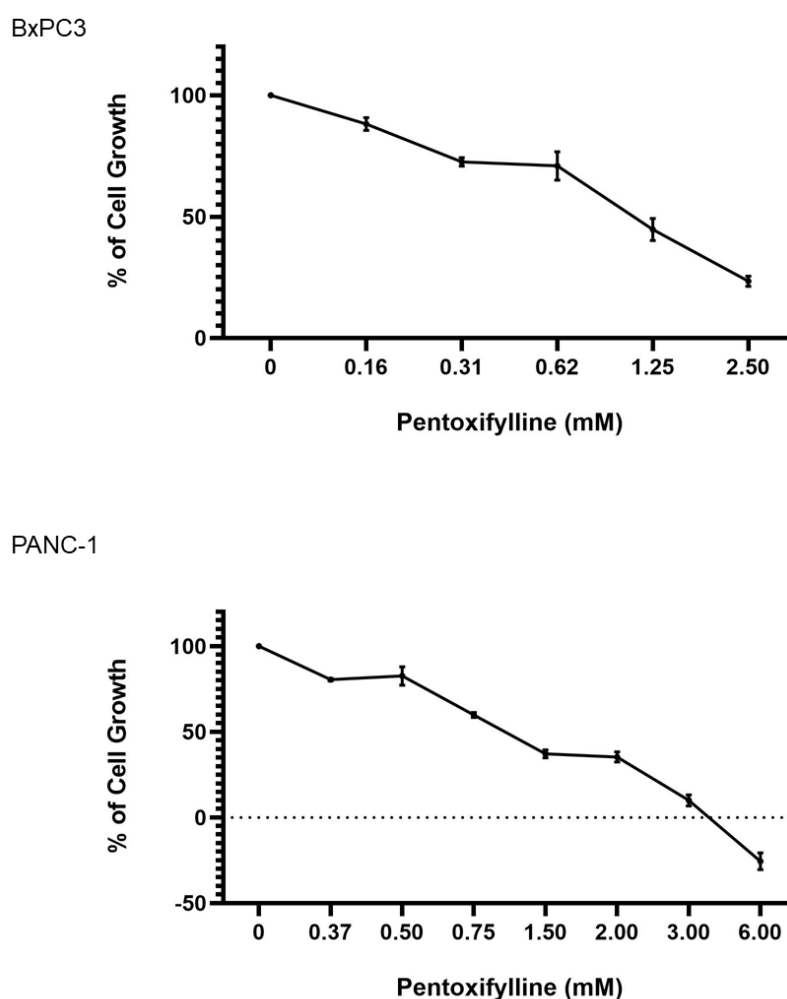


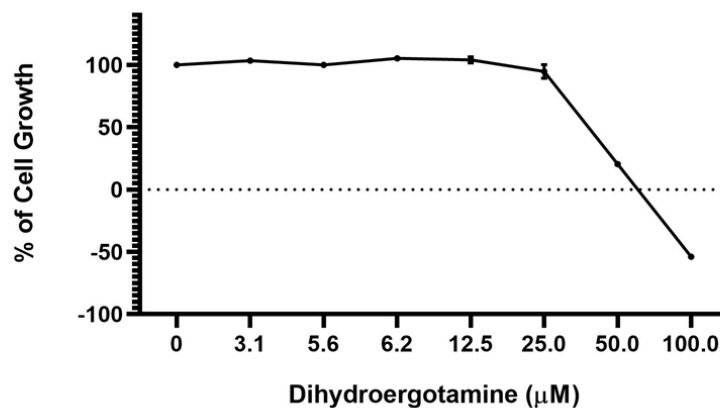
Figure 17. Effect of pentoxifylline in pancreatic cancer cells. BxPC3 and PANC-1 were treated with increasing concentrations of pentoxifylline (0.16 mM – 2.50 mM and 0.37 mM – 6.00 mM,

respectively). After 48 h, the cell growth inhibition was measured using the SRB assay. Data shows the mean $\pm$ SEM of three independent experiments.

Next, the SRB cell growth inhibition assay was performed with two of the selected compounds, namely dihydroergotamine and darifenacin. Unfortunately, it was not possible to conduct the SRB assay for the third selected compound, velpatasvir, due to problems related to obtaining the sterile compound within the timeframe allowed to conclude this dissertation. This compound will be tested in future work. Therefore, BxPC3 and PANC-1 cell lines were treated with dihydroergotamine or darifenacin, during 48 h, in order to confirm if they could inhibit PDAC cells growth.

Regarding the results obtained for dihydroergotamine, the GI_{50} concentrations were: $35.7 \pm 2.4 \mu\text{M}$ in BxPC3 and $41.0 \pm 0.6 \mu\text{M}$ in PANC-1 cells (**Figure 18**). Therefore, similar GI_{50} concentrations were obtained for the two PDAC cell lines, suggesting that KRAS may not be involved in the response to this drug. However, by microscopic observation of the cells treated with this compound, it was possible to verify that, at concentrations near the GI_{50} (at around $45 \mu\text{M}$) or higher, a precipitate was present in the culture medium (**Figure 19**). This evidence suggests that dihydroergotamine may not be a good compound for further *in vitro* experiments, meaning it was decided not to proceed with further testing.

BxPC3



PANC-1

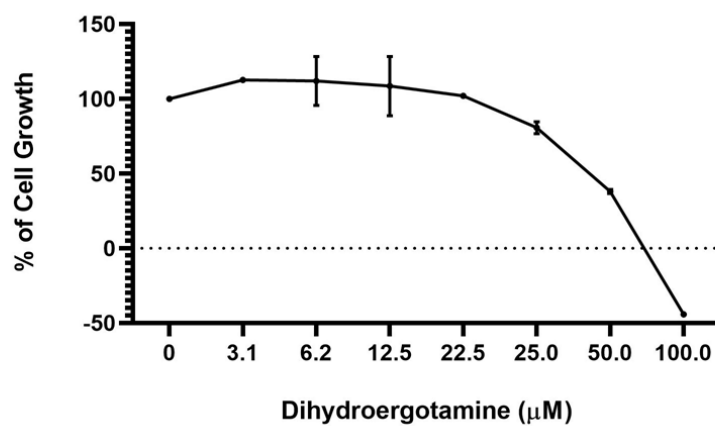


Figure 18. Effect of dihydroergotamine in PDAC cells. BxPC3 and PANC-1 cells were treated with increasing concentrations of dihydroergotamine (2.8 μM – 100.0 μM). After 48 h, the cell growth inhibition was determined using the SRB assay. Data shows the mean $\pm$ SEM of three independent experiments.

As demonstrated in **Figure 19**, as the concentration of dihydroergotamine increases, the cell growth of both cell lines decreases. However, some precipitates were observed at 45 μM (indicated with arrows in **Figure 19**) and also with higher concentrations (data not shown).

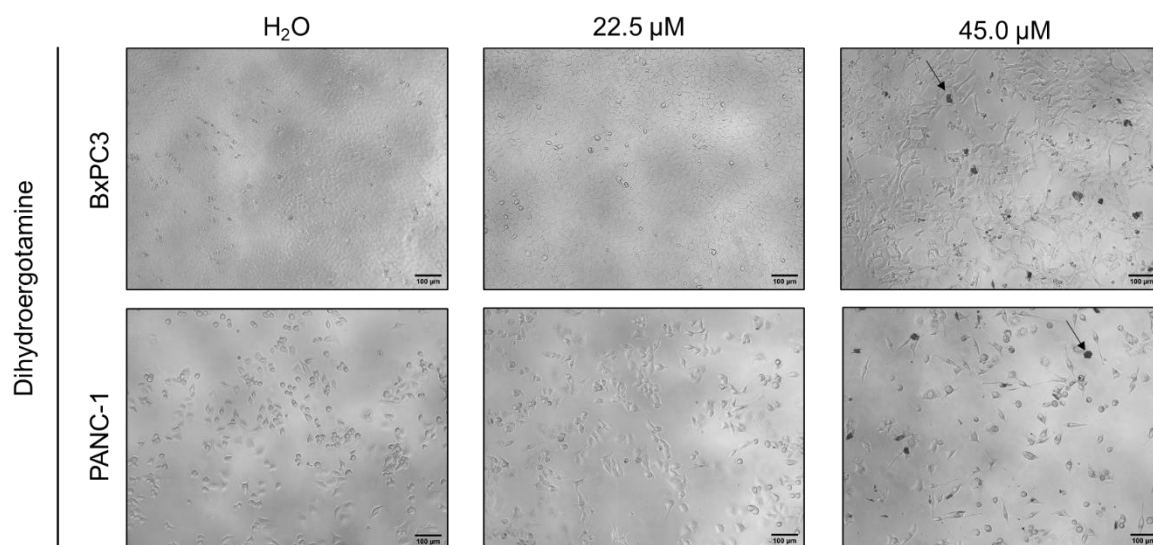


Figure 19. Visualization of the effects of dihydroergotamine in the BxPC3 and PANC-1 cell lines. Cell growth in BxPC3 and PANC-1 cell lines treated with two concentrations of the compound dihydroergotamine (22.5 μM and 45.0 μM) or with the vehicle (H_2O). Microscopic observation of precipitate formation at a concentration of 45 μM (arrow). Bar = 100 micrometres.

Dihydroergotamine is a FDA-approved drug for the treatment of migraine²⁰⁴. In our study, this drug was tested with a new therapeutical purpose, antitumor activity against pancreatic cancer. Other studies had previously demonstrated that dihydroergotamine has antitumor activity in a variety of cancers, including acute myeloid leukemia (AML)^{201, 202} and lung cancer²⁰³. Indeed, an antileukemic activity was observed across human AML cells both *in vitro* and in a mouse model, in which dihydroergotamine exhibited cell growth inhibition, a delay in AML progression and a promotion of myeloid differentiation^{201, 202}. Additionally, the compound also induced apoptosis in lung cancer cells at concentrations of 5 μM and 10 μM ²⁰³. Interestingly, to our knowledge the effect of dihydroergotamine in pancreatic cancer had never been previously reported.

Regarding the results obtained for darifenacin, the GI_{50} concentrations were: $26.0 \pm 2.1 \mu\text{M}$ in BxPC3 and $13.6 \pm 2.7 \mu\text{M}$ in PANC-1 cells (**Figure 20**). Interestingly, this compound was more effective in inhibiting the more resistant PANC-1 cells, when compared with the more sensitive BxPC3 cells.

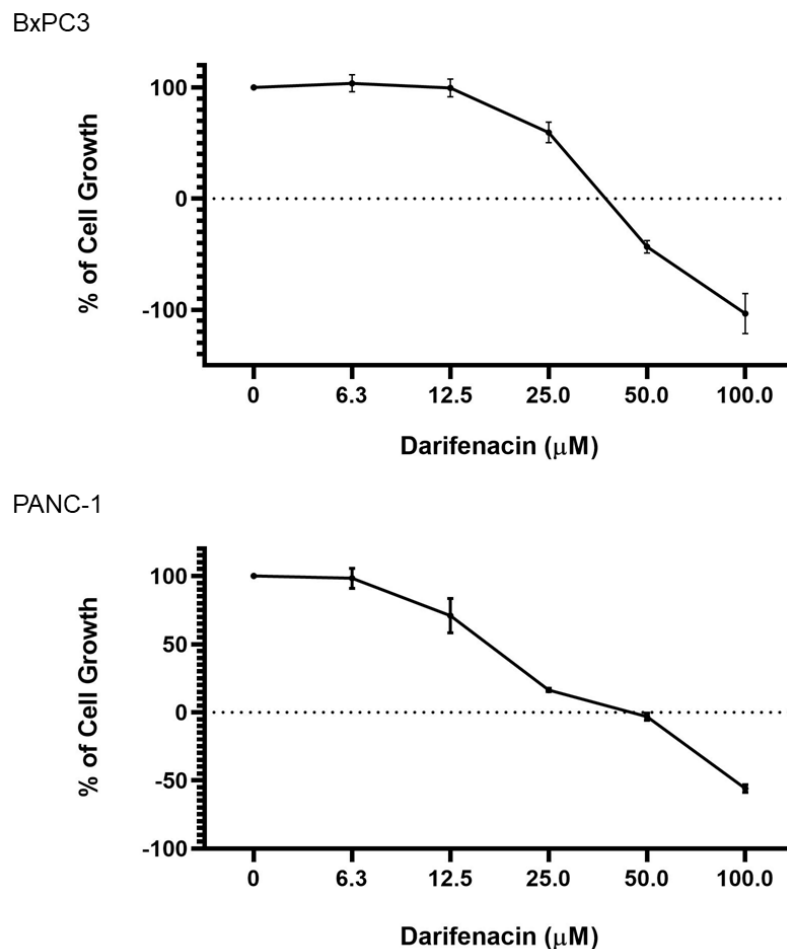


Figure 20. Effect of darifenacin in pancreatic cancer cells. BxPC3 and PANC-1 were treated with increasing concentrations of darifenacin (6.3 μM – 100.0 μM , on both cell lines). After 48 h, the cell growth inhibition was measured using the SRB assay. The data are the mean $\pm$ SEM of three independent experiments.

Additionally, the cell growth inhibition effects of darifenacin were also documented by taking photographs of BxPC3 and PANC1 cells treated with 100 μM or 25 μM of this drug, or with the vehicle of the drug (DMSO) as a control. As shown in **Figure 21**, the cell growth was highly inhibited when comparing with control cells (treated with DMSO). Moreover, this was a dose-dependent effect.

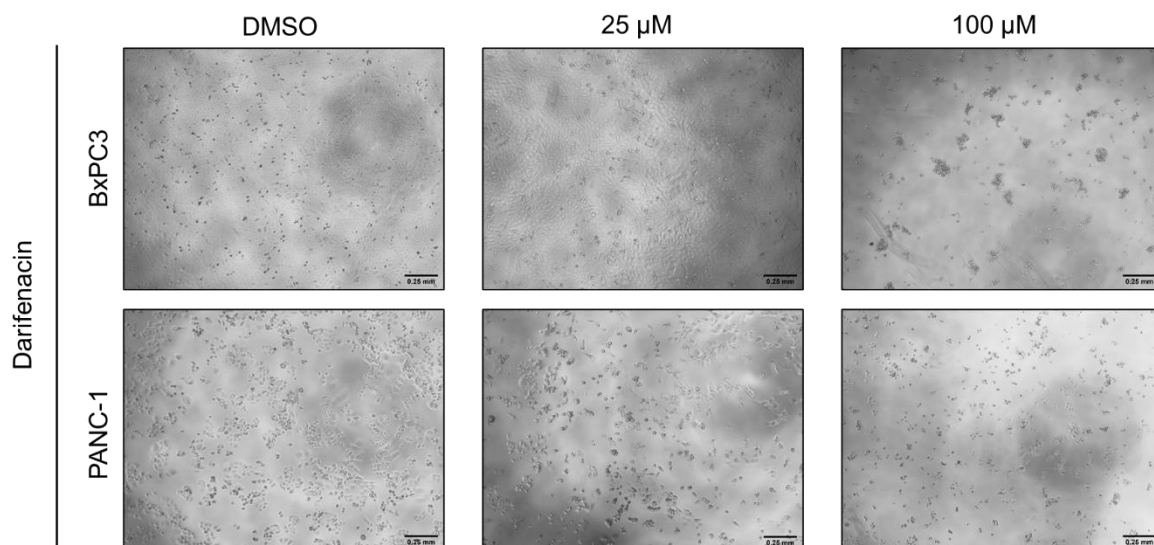


Figure 21. Visualization of the effects of darifenacin in the BxPC3 and PANC-1 cell lines. Cell growth inhibition of BxPC3 and PANC-1 cell lines treated with two concentrations of the compound darifenacin (25 μ M and 100 μ M) or with the compound vehicle (DMSO) as control. Bar = 0.25 millimetre.

Our data revealed that darifenacin was able to significantly inhibit the growth of both PDAC cell lines studied. Darifenacin is an approved drug for the treatment of overactive bladder and urinary incontinence through the blockage of the muscarinic M3 receptor²⁵⁷. This selective antagonist acts as an anticholinergic and antispasmodic agent. Thus, darifenacin contributes to a decrease in the urgency to urinate and bladder contraction, and additionally, increases the volumetric capacity of the bladder^{258, 259}. Due to the use of darifenacin in the clinic, the safety profile, clinical efficacy and toxicity data have already been well-established²⁵⁸. Moreover, darifenacin was previously shown to have antitumor activity in a variety of cancers including colorectal cancer²⁴³, gastric cancer^{241, 260} and lung cancer^{242, 261}. Indeed, Hering *et al.* (2021) demonstrated that darifenacin has antiproliferative and apoptotic effects on colorectal cancer. *In vitro*, the compound inhibited cell proliferation, viability and invasion of human colon adenocarcinoma cells. Additionally, darifenacin was able to reduce tumor growth and the ability of the cancer to metastasise in a xenograft mouse model²⁴³. The same effects were also observed in human gastric cancer cells and lung cancer cells. Darifenacin inhibited gastric cells invasion and migration, as well as tumor growth *in vivo*^{241, 260}. Furthermore, an antiproliferative activity of darifenacin was also observed in small cell lung cancer and non-small cell lung cancer, both *in vitro* and *in vivo*^{242, 261}. The findings presented in this dissertation suggest that darifenacin can also be a potential compound for the treatment of pancreatic cancer. To

our knowledge, this is the first study to show tumor cell growth inhibitory activity of darifenacin in pancreatic cancer.

5. Conclusion

Previous results from our research group revealed that CHI3L1 could be a potential therapeutic target for the treatment of pancreatic cancer. The identification of CHI3L1 inhibitors could be a strategy to find alternative drugs to treat patients with pancreatic cancer. Therefore, this work aimed to identify drugs that could show potential as inhibitors of CHI3L1 and inhibitors of PDAC cell growth.

By using the docking program (AutoDock Vina) in 11,741 molecules from the DrugBank database towards the target CHI3L1, 568 of these molecules (4.8% of the screened molecules) presented higher affinity for CHI3L1 than the known inhibitor prednisolone (-10.1 kcal/mol), which is the positive control with the best score value. Thus, any of the identified molecules could be a potential inhibitor of the CHI3L1 protein.

To investigate the possibility of some of these drugs to inhibit PDAC cell growth, two top ranked compounds (dihydroergotamine and darifenacin) from different therapeutic classes, neither of which had been approved for cancer treatment, were selected for PDAC cell growth inhibition studies. Both of the selected compounds had been previously documented to have antitumor activity in a variety of cancers, but not in pancreatic cancer. In the present work, both compounds were tested in two different PDAC cell lines (BxPC3 and PANC-1 cells). Dihydroergotamine inhibited tumor cell growth, however it precipitated at concentrations near the GI_{50} . Darifenacin inhibited tumor cell growth with GI_{50} concentrations of 26 μ M for BxPC3 cells and 13.6 μ M for PANC-1 cells, demonstrating to be a promising drug inhibitor of PDAC cell growth.

In conclusion, since darifenacin has already been approved for the treatment of a disease other than cancer and thus has a safety profile in humans already established, and in our work, it also showed *in silico* capacity to bind to CHI3L1 and decreased cell growth of PDAC cell lines, further experiments with this drug should be considered. Future work will also confirm the effect of darifenacin on CHI3L1, for example by using a recombinant CHI3L1 protein, and evaluate if at least part of the observed tumor cell growth inhibition effect of this drug is due to an inhibition of CHI3L1.

6.References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R. L.; Torre, L. A.; Jemal, A., Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians* **2018**, *68* (6), 394-424.
2. Global cancer observatory: cancer today. <https://gco.iarc.fr/today/home> (accessed January 25, 2021).
3. Siegel, R. L.; Miller, K. D.; Fuchs, H. E.; Jemal, A., Cancer Statistics, 2021. *CA Cancer J Clin* **2021**, *71* (1), 7-33.
4. Global cancer observatory: cancer tomorrow. <https://gco.iarc.fr/tomorrow/en> (accessed January 25, 2021).
5. Cancer Facts & Figures 2021. *American Cancer Society journal, CA: A Cancer Journal for Clinicians* **2021**.
6. Benzel, J.; Fendrich, V., Familial Pancreatic Cancer. *Oncol Res Treat* **2018**, *41* (10), 611-618.
7. Puckett, Y.; Garfield, K., Pancreatic Cancer. In *StatPearls*, StatPearls Publishing Copyright © 2020, StatPearls Publishing LLC.: Treasure Island (FL), 2020.
8. Adel, N., Current treatment landscape and emerging therapies for pancreatic cancer. *Am J Manag Care* **2019**, *25* (1 Suppl), S3-s10.
9. Walter, F. M.; Mills, K.; Mendonça, S. C.; Abel, G. A.; Basu, B.; Carroll, N.; Ballard, S.; Lancaster, J.; Hamilton, W.; Rubin, G. P.; Emery, J. D., Symptoms and patient factors associated with diagnostic intervals for pancreatic cancer (SYMPTOM pancreatic study): a prospective cohort study. *Lancet Gastroenterol Hepatol* **2016**, *1* (4), 298-306.
10. Hidalgo, M.; Cascinu, S.; Kleeff, J.; Labianca, R.; Löhr, J. M.; Neoptolemos, J.; Real, F. X.; Van Laethem, J. L.; Heinemann, V., Addressing the challenges of pancreatic cancer: future directions for improving outcomes. *Pancreatology* **2015**, *15* (1), 8-18.
11. Pancreatic Cancer Types. <https://www.hopkinsmedicine.org/health/conditions-and-diseases/pancreatic-cancer/pancreatic-cancer-types> (accessed January 25, 2021).
12. Kunovsky, L.; Tesarikova, P.; Kala, Z.; Kroupa, R.; Kysela, P.; Dolina, J.; Trna, J., The Use of Biomarkers in Early Diagnostics of Pancreatic Cancer. *Can J Gastroenterol Hepatol* **2018**, *2018*, 5389820-5389820.
13. Mizrahi, J. D.; Surana, R.; Valle, J. W.; Shroff, R. T., Pancreatic cancer. *The Lancet* **2020**, *395* (10242), 2008-2020.
14. Teague, A.; Lim, K.-H.; Wang-Gillam, A., Advanced pancreatic adenocarcinoma: a review of current treatment strategies and developing therapies. *Ther Adv Med Oncol* **2015**, *7* (2), 68-84.
15. Uzunparmak, B.; Sahin, I. H., Pancreatic cancer microenvironment: a current dilemma. *Clin Transl Med* **2019**, *8* (1), 2-2.
16. Manji, G. A.; Olive, K. P.; Saenger, Y. M.; Oberstein, P., Current and Emerging Therapies in Metastatic Pancreatic Cancer. *Clin Cancer Res* **2017**, *23* (7), 1670-1678.
17. Mohammed, S.; Van Buren, G., 2nd; Fisher, W. E., Pancreatic cancer: advances in treatment. *World J Gastroenterol* **2014**, *20* (28), 9354-9360.
18. Vogl, U. M.; Andalibi, H.; Klaus, A.; Vormittag, L.; Schima, W.; Heinrich, B.; Kafka, A.; Winkler, T.; Öhler, L., Nab-paclitaxel and gemcitabine or FOLFIRINOX as first-line treatment in patients with unresectable adenocarcinoma of the pancreas: does sequence matter? *BMC cancer* **2019**, *19* (1), 28-28.
19. Xavier, C. P. R.; Castro, I.; Caires, H. R.; Ferreira, D.; Cavadas, B.; Pereira, L.; Santos, L. L.; Oliveira, M. J.; Vasconcelos, M. H., Chitinase 3-like-1 and fibronectin in the cargo of extracellular vesicles shed by human macrophages influence pancreatic cancer cellular response to gemcitabine. *Cancer Lett* **2021**, *501*, 210-223.
20. Chen, H. T.; Zheng, J. M.; Zhang, Y. Z.; Yang, M.; Wang, Y. L.; Man, X. H.; Chen, Y.; Cai, Q. C.; Li, Z. S., Overexpression of YKL-40 Predicts Poor Prognosis in Patients Undergoing Curative Resection of Pancreatic Cancer. *Pancreas* **2017**, *46* (3), 323-334.
21. Bussink, A. P.; Speijer, D.; Aerts, J. M. F. G.; Boot, R. G., Evolution of mammalian chitinase(-like) members of family 18 glycosyl hydrolases. *Genetics* **2007**, *177* (2), 959-970.

22. Houston, D. R.; Recklies, A. D.; Krupa, J. C.; van Aalten, D. M., Structure and ligand-induced conformational change of the 39-kDa glycoprotein from human articular chondrocytes. *J Biol Chem* **2003**, 278 (32), 30206-12.
23. Di Rosa, M.; Distefano, G.; Zorena, K.; Malaguarnera, L., Chitinases and immunity: Ancestral molecules with new functions. *Immunobiology* **2016**, 221 (3), 399-411.
24. Bhattacharya, D.; Nagpure, A.; Gupta, R. K., Bacterial chitinases: properties and potential. *Crit Rev Biotechnol* **2007**, 27 (1), 21-8.
25. Shahidi, F.; Abuzaytoun, R., Chitin, chitosan, and co-products: chemistry, production, applications, and health effects. *Adv Food Nutr Res* **2005**, 49, 93-135.
26. Hamid, R.; Khan, M. A.; Ahmad, M.; Ahmad, M. M.; Abdin, M. Z.; Musarrat, J.; Javed, S., Chitinases: An update. *J Pharm Bioallied Sci* **2013**, 5 (1), 21-29.
27. Watanabe, T.; Kobori, K.; Miyashita, K.; Fujii, T.; Sakai, H.; Uchida, M.; Tanaka, H., Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J Biol Chem* **1993**, 268 (25), 18567-72.
28. Huang, Q. S.; Xie, X. L.; Liang, G.; Gong, F.; Wang, Y.; Wei, X. Q.; Wang, Q.; Ji, Z. L.; Chen, Q. X., The GH18 family of chitinases: their domain architectures, functions and evolutions. *Glycobiology* **2012**, 22 (1), 23-34.
29. Chen, W.; Jiang, X.; Yang, Q., Glycoside hydrolase family 18 chitinases: The known and the unknown. *Biotechnol Adv* **2020**, 43, 107553.
30. Boot, R. G.; Blommaert, E. F.; Swart, E.; Ghauharali-van der Vlugt, K.; Bijl, N.; Moe, C.; Place, A.; Aerts, J. M., Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. *J Biol Chem* **2001**, 276 (9), 6770-8.
31. Bussink, A. P.; van Eijk, M.; Renkema, G. H.; Aerts, J. M.; Boot, R. G., The biology of the Gaucher cell: the cradle of human chitinases. *Int Rev Cytol* **2006**, 252, 71-128.
32. Kumar, A.; Zhang, K. Y. J., Human Chitinases: Structure, Function, and Inhibitor Discovery. *Adv Exp Med Biol* **2019**, 1142, 221-251.
33. Fadel, F.; Zhao, Y.; Cousido-Siah, A.; Ruiz, F. X.; Mitschler, A.; Podjarny, A., X-Ray Crystal Structure of the Full Length Human Chitotriosidase (CHIT1) Reveals Features of Its Chitin Binding Domain. *PLoS One* **2016**, 11 (4), e0154190.
34. Chou, Y. T.; Yao, S.; Czerwinski, R.; Fleming, M.; Krykbaev, R.; Xuan, D.; Zhou, H.; Brooks, J.; Fitz, L.; Strand, J.; Presman, E.; Lin, L.; Aulabaugh, A.; Huang, X., Kinetic characterization of recombinant human acidic mammalian chitinase. *Biochemistry* **2006**, 45 (14), 4444-54.
35. Stam, M. R.; Blanc, E.; Coutinho, P. M.; Henrissat, B., Evolutionary and mechanistic relationships between glycosidases acting on alpha- and beta-bonds. *Carbohydr Res* **2005**, 340 (18), 2728-34.
36. Fusetti, F.; von Moeller, H.; Houston, D.; Rozeboom, H. J.; Dijkstra, B. W.; Boot, R. G.; Aerts, J. M.; van Aalten, D. M., Structure of human chitotriosidase. Implications for specific inhibitor design and function of mammalian chitinase-like lectins. *J Biol Chem* **2002**, 277 (28), 25537-44.
37. Lee, C. G.; Da Silva, C. A.; Dela Cruz, C. S.; Ahangari, F.; Ma, B.; Kang, M. J.; He, C. H.; Takyar, S.; Elias, J. A., Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu Rev Physiol* **2011**, 73, 479-501.
38. Vega, K.; Kalkum, M., Chitin, chitinase responses, and invasive fungal infections. *Int J Microbiol* **2012**, 2012, 920459.
39. Schimpl, M.; Rush, C. L.; Betou, M.; Eggleston, I. M.; Recklies, A. D.; van Aalten, D. M. F., Human YKL-39 is a pseudo-chitinase with retained chitooligosaccharide-binding properties. *Biochem J* **2012**, 446 (1), 149-157.
40. Chang, N. C.; Hung, S. I.; Hwa, K. Y.; Kato, I.; Chen, J. E.; Liu, C. H.; Chang, A. C., A macrophage protein, Ym1, transiently expressed during inflammation is a novel mammalian lectin. *J Biol Chem* **2001**, 276 (20), 17497-506.

41. Kzhyshkowska, J.; Yin, S.; Liu, T.; Riabov, V.; Mitrofanova, I., Role of chitinase-like proteins in cancer. *Biol Chem* **2016**, 397 (3), 231-47.
42. Rehli, M.; Krause, S. W.; Andreesen, R., Molecular characterization of the gene for human cartilage gp-39 (CHI3L1), a member of the chitinase protein family and marker for late stages of macrophage differentiation. *Genomics* **1997**, 43 (2), 221-5.
43. Rehli, M.; Niller, H. H.; Ammon, C.; Langmann, S.; Schwarzfischer, L.; Andreesen, R.; Krause, S. W., Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation. *J Biol Chem* **2003**, 278 (45), 44058-67.
44. Kazakova, M. H.; Sarafian, V. S., YKL-40--a novel biomarker in clinical practice? *Folia Med (Plovdiv)* **2009**, 51 (1), 5-14.
45. Johansen, J. S.; Williamson, M. K.; Rice, J. S.; Price, P. A., Identification of proteins secreted by human osteoblastic cells in culture. *J Bone Miner Res* **1992**, 7 (5), 501-12.
46. Johansen, J. S.; Schultz, N. A.; Jensen, B. V., Plasma YKL-40: a potential new cancer biomarker? *Future Oncol* **2009**, 5 (7), 1065-82.
47. Hakala, B. E.; White, C.; Recklies, A. D., Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. *J Biol Chem* **1993**, 268 (34), 25803-10.
48. Renkema, G. H.; Boot, R. G.; Au, F. L.; Donker-Koopman, W. E.; Strijland, A.; Muijsers, A. O.; Hrebicek, M.; Aerts, J. M., Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. *Eur J Biochem* **1998**, 251 (1-2), 504-9.
49. Fusetti, F.; Pijning, T.; Kalk, K.H.; Bos, E.; Dijkstra, B.W. Crystal structure of human cartilage gp39 (HC-gp39) in complex with chitotetraose. <https://www.rcsb.org/structure/1NWU>.
50. Fusetti, F.; Pijning, T.; Kalk, K. H.; Bos, E.; Dijkstra, B. W., Crystal structure and carbohydrate-binding properties of the human cartilage glycoprotein-39. *J Biol Chem* **2003**, 278 (39), 37753-60.
51. Zhao, T.; Su, Z.; Li, Y.; Zhang, X.; You, Q., Chitinase-3 like-protein-1 function and its role in diseases. *Signal Transduct Target Ther* **2020**, 5 (1), 201.
52. He, C. H.; Lee, C. G.; Dela Cruz, C. S.; Lee, C. M.; Zhou, Y.; Ahangari, F.; Ma, B.; Herzog, E. L.; Rosenberg, S. A.; Li, Y.; Nour, A. M.; Parikh, C. R.; Schmidt, I.; Modis, Y.; Cantley, L.; Elias, J. A., Chitinase 3-like 1 regulates cellular and tissue responses via IL-13 receptor $\alpha 2$. *Cell Rep* **2013**, 4 (4), 830-41.
53. Lee, C. M.; He, C. H.; Nour, A. M.; Zhou, Y.; Ma, B.; Park, J. W.; Kim, K. H.; Dela Cruz, C.; Sharma, L.; Nasr, M. L.; Modis, Y.; Lee, C. G.; Elias, J. A., IL-13R $\alpha 2$ uses TMEM219 in chitinase 3-like-1-induced signalling and effector responses. *Nat Commun* **2016**, 7, 12752.
54. Zhou, Y.; He, C. H.; Yang, D. S.; Nguyen, T.; Cao, Y.; Kamle, S.; Lee, C. M.; Gochuico, B. R.; Gahl, W. A.; Shea, B. S.; Lee, C. G.; Elias, J. A., Galectin-3 Interacts with the CHI3L1 Axis and Contributes to Hermansky-Pudlak Syndrome Lung Disease. *J Immunol* **2018**, 200 (6), 2140-2153.
55. Geng, B.; Pan, J.; Zhao, T.; Ji, J.; Zhang, C.; Che, Y.; Yang, J.; Shi, H.; Li, J.; Zhou, H.; Mu, X.; Xu, C.; Wang, C.; Xu, Y.; Liu, Z.; Wen, H.; You, Q., Chitinase 3-like 1-CD44 interaction promotes metastasis and epithelial-to-mesenchymal transition through β -catenin/Erk/Akt signaling in gastric cancer. *Journal of Experimental & Clinical Cancer Research* **2018**, 37 (1), 208.
56. He, C. H.; Lee, C. G.; Ma, B.; Kamle, S.; Choi, A. M. K.; Elias, J. A., N-Glycosylation Regulates Chitinase 3-like-1 and IL-13 Ligand Binding to IL-13 Receptor $\alpha 2$. *Am J Respir Cell Mol Biol* **2020**, 63 (3), 386-395.
57. Bhardwaj, R.; Suzuki, A.; Leland, P.; Joshi, B. H.; Puri, R. K., Identification of a novel role of IL-13R $\alpha 2$ in human Glioblastoma multiforme: interleukin-13 mediates signal transduction through AP-1 pathway. *Journal of Translational Medicine* **2018**, 16 (1), 369.
58. Barderas, R.; Bartolomé, R. A.; Fernandez-Aceñero, M. J.; Torres, S.; Casal, J. I., High Expression of IL-13 Receptor $\alpha 2$ in Colorectal Cancer Is Associated with Invasion, Liver Metastasis, and Poor Prognosis. *Cancer Research* **2012**, 72 (11), 2780-2790.

59. Fujisawa, T.; Joshi, B.; Nakajima, A.; Puri, R. K., A novel role of interleukin-13 receptor alpha2 in pancreatic cancer invasion and metastasis. *Cancer Res* **2009**, *69* (22), 8678-85.
60. Kawakami, K.; Kawakami, M.; Puri, R. K., Specifically targeted killing of interleukin-13 (IL-13) receptor-expressing breast cancer by IL-13 fusion cytotoxin in animal model of human disease. *Mol Cancer Ther* **2004**, *3* (2), 137-47.
61. Zhao, Z.; Wang, L.; Xu, W., IL-13R α 2 mediates PNR-induced migration and metastasis in ER α -negative breast cancer. *Oncogene* **2015**, *34* (12), 1596-1607.
62. Johansen, J. S., Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. *Dan Med Bull* **2006**, *53* (2), 172-209.
63. Schultz, N. A.; Johansen, J. S., YKL-40-A Protein in the Field of Translational Medicine: A Role as a Biomarker in Cancer Patients? *Cancers (Basel)* **2010**, *2* (3), 1453-91.
64. Sutherland, T. E., Chitinase-like proteins as regulators of innate immunity and tissue repair: helpful lessons for asthma? *Biochem Soc Trans* **2018**, *46* (1), 141-151.
65. Yeo, I. J.; Lee, C. K.; Han, S. B.; Yun, J.; Hong, J. T., Roles of chitinase 3-like 1 in the development of cancer, neurodegenerative diseases, and inflammatory diseases. *Pharmacol Ther* **2019**, *203*, 107394.
66. Ringsholt, M.; Høgdall, E. V.; Johansen, J. S.; Price, P. A.; Christensen, L. H., YKL-40 protein expression in normal adult human tissues--an immunohistochemical study. *J Mol Histol* **2007**, *38* (1), 33-43.
67. Johansen, J. S.; Christoffersen, P.; Møller, S.; Price, P. A.; Henriksen, J. H.; Garbarsch, C.; Bendtsen, F., Serum YKL-40 is increased in patients with hepatic fibrosis. *J Hepatol* **2000**, *32* (6), 911-20.
68. Johansen, J. S.; Stoltenberg, M.; Hansen, M.; Florescu, A.; Hørslev-Petersen, K.; Lorenzen, I.; Price, P. A., Serum YKL-40 concentrations in patients with rheumatoid arthritis: relation to disease activity. *Rheumatology (Oxford)* **1999**, *38* (7), 618-26.
69. Elias, J. A.; Homer, R. J.; Hamid, Q.; Lee, C. G., Chitinases and chitinase-like proteins in T(H)2 inflammation and asthma. *J Allergy Clin Immunol* **2005**, *116* (3), 497-500.
70. Wang, Y.; Ripa, R. S.; Johansen, J. S.; Gabrielsen, A.; Steinbruchel, D. A.; Friis, T.; Bindeslev, L.; Haack-Sørensen, M.; Jørgensen, E.; Kastrup, J., YKL-40 a new biomarker in patients with acute coronary syndrome or stable coronary artery disease. *Scand Cardiovasc J* **2008**, *42* (5), 295-302.
71. Sakazaki, Y.; Hoshino, T.; Takei, S.; Sawada, M.; Oda, H.; Takenaka, S.; Imaoka, H.; Matsunaga, K.; Ota, T.; Abe, Y.; Miki, I.; Fujimoto, K.; Kawayama, T.; Kato, S.; Aizawa, H., Overexpression of chitinase 3-like 1/YKL-40 in lung-specific IL-18-transgenic mice, smokers and COPD. *PLoS One* **2011**, *6* (9), e24177.
72. Scherthaner, G. H.; Höbaus, C.; Brix, J., YKL-40 and its complex association with metabolic syndrome, obesity, and cardiovascular disease. *Anatol J Cardiol* **2016**, *16* (12), 959-959.
73. Rathcke, C. N.; Vestergaard, H., YKL-40, a new inflammatory marker with relation to insulin resistance and with a role in endothelial dysfunction and atherosclerosis. *Inflamm Res* **2006**, *55* (6), 221-7.
74. Vind, I.; Johansen, J. S.; Price, P. A.; Munkholm, P., Serum YKL-40, a potential new marker of disease activity in patients with inflammatory bowel disease. *Scand J Gastroenterol* **2003**, *38* (6), 599-605.
75. Volck, B.; Johansen, J. S.; Stoltenberg, M.; Garbarsch, C.; Price, P. A.; Østergaard, M.; Østergaard, K.; Løvgreen-Nielsen, P.; Sonne-Holm, S.; Lorenzen, I., Studies on YKL-40 in knee joints of patients with rheumatoid arthritis and osteoarthritis. Involvement of YKL-40 in the joint pathology. *Osteoarthritis and Cartilage* **2001**, *9* (3), 203-214.
76. Hall, S.; Surova, Y.; Öhrfelt, A.; Study, t. S. B.; Blennow, K.; Zetterberg, H.; Hansson, O., Longitudinal Measurements of Cerebrospinal Fluid Biomarkers in Parkinson's Disease. *Movement Disorders* **2016**, *31* (6), 898-905.

77. Choi, J.; Lee, H. W.; Suk, K., Plasma level of chitinase 3-like 1 protein increases in patients with early Alzheimer's disease. *J Neurol* **2011**, *258* (12), 2181-5.
78. Olsson, B.; Lautner, R.; Andreasson, U.; Öhrfelt, A.; Portelius, E.; Bjerke, M.; Hölttä, M.; Rosén, C.; Olsson, C.; Strobel, G.; Wu, E.; Dakin, K.; Petzold, M.; Blennow, K.; Zetterberg, H., CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis. *Lancet Neurol* **2016**, *15* (7), 673-684.
79. Veerhuis, R.; Van Breemen, M. J.; Hoozemans, J. M.; Morbin, M.; Ouladhadj, J.; Tagliavini, F.; Eikelenboom, P., Amyloid beta plaque-associated proteins C1q and SAP enhance the Abeta1-42 peptide-induced cytokine secretion by adult human microglia in vitro. *Acta Neuropathol* **2003**, *105* (2), 135-44.
80. Craig-Schapiro, R.; Perrin, R. J.; Roe, C. M.; Xiong, C.; Carter, D.; Cairns, N. J.; Mintun, M. A.; Peskind, E. R.; Li, G.; Galasko, D. R.; Clark, C. M.; Quinn, J. F.; D'Angelo, G.; Malone, J. P.; Townsend, R. R.; Morris, J. C.; Fagan, A. M.; Holtzman, D. M., YKL-40: a novel prognostic fluid biomarker for preclinical Alzheimer's disease. *Biol Psychiatry* **2010**, *68* (10), 903-912.
81. Muszyński, P.; Groblewska, M.; Kulczyńska-Przybik, A.; Kułakowska, A.; Mroczko, B., YKL-40 as a Potential Biomarker and a Possible Target in Therapeutic Strategies of Alzheimer's Disease. *Curr Neuropharmacol* **2017**, *15* (6), 906-917.
82. Baldacci, F.; Lista, S.; Palermo, G.; Giorgi, F. S.; Vergallo, A.; Hampel, H., The neuroinflammatory biomarker YKL-40 for neurodegenerative diseases: advances in development. *Expert Rev Proteomics* **2019**, *16* (7), 593-600.
83. Shao, R.; Cao, Q. J.; Arenas, R. B.; Bigelow, C.; Bentley, B.; Yan, W., Breast cancer expression of YKL-40 correlates with tumour grade, poor differentiation, and other cancer markers. *Br J Cancer* **2011**, *105* (8), 1203-9.
84. Qiu, Q.-C.; Wang, L.; Jin, S.-S.; Liu, G.-F.; Liu, J.; Ma, L.; Mao, R.-F.; Ma, Y.-Y.; Zhao, N.; Chen, M.; Lin, B.-Y., CHI3L1 promotes tumor progression by activating TGF- β signaling pathway in hepatocellular carcinoma. *Scientific Reports* **2018**, *8* (1), 15029.
85. Lin, H. W.; Chiang, Y. C.; Sun, N. Y.; Chen, Y. L.; Chang, C. F.; Tai, Y. J.; Chen, C. A.; Cheng, W. F., CHI3L1 results in poor outcome of ovarian cancer by promoting properties of stem-like cells. *Endocr Relat Cancer* **2019**, *26* (1), 73-88.
86. Thörm, I.; Andritzky, B.; Schuch, G.; Burkholder, I.; Edler, L.; Johansen, J. S.; Bokemeyer, C.; Schumacher, U.; Laack, E., Elevated pretreatment serum concentration of YKL-40-An independent prognostic biomarker for poor survival in patients with metastatic nonsmall cell lung cancer. *Cancer* **2010**, *116* (17), 4114-21.
87. Lee, Y. E.; Chan, T. C.; Tian, Y. F.; Liang, P. I.; Shiue, Y. L.; Chen, Y. S.; He, H. L., High expression of Chitinase 3-like-1 is an unfavorable prognostic factor in urothelial carcinoma of upper urinary tract and urinary bladder. *Urol Oncol* **2019**, *37* (5), 299.e7-299.e18.
88. Cheng, S.-P.; Lee, J.-J.; Chang, Y.-C.; Lin, C.-H.; Li, Y.-S.; Liu, C.-L., Overexpression of chitinase-3-like protein 1 is associated with structural recurrence in patients with differentiated thyroid cancer. *The Journal of Pathology* **2020**, *252* (2), e5503.
89. Song, Z.; Chen, E.; Qian, J.; Xu, J.; Cao, G.; Zhou, W.; Wang, F.; Chen, M.; Xu, D.; Wang, X.; Hu, X.; Ju, Z.; Huang, X.; Cai, X., Serum chitinase activity prognosticates metastasis of colorectal cancer. *BMC Cancer* **2019**, *19* (1), 629.
90. Bergmann, O. J.; Johansen, J. S.; Klausen, T. W.; Mylin, A. K.; Kristensen, J. S.; Kjeldsen, E.; Johnsen, H. E., High Serum Concentration of YKL-40 Is Associated with Short Survival in Patients with Acute Myeloid Leukemia. *Clinical Cancer Research* **2005**, *11* (24), 8644-8652.
91. Johansen, J. S.; Christensen, I. J.; Riisbro, R.; Greenall, M.; Han, C.; Price, P. A.; Smith, K.; Brünner, N.; Harris, A. L., High serum YKL-40 levels in patients with primary breast cancer is related to short recurrence free survival. *Breast Cancer Res Treat* **2003**, *80* (1), 15-21.
92. Yancopoulos, G.; Davis, S.; Gale, N.; Rudge, J.; Wiegand, S.; Holash, J., Vascular-specific growth factors and blood vessel formation. *Nature* **2000**, *407*, 242-248.

93. Kim, K. J.; Li, B.; Winer, J.; Armanini, M.; Gillett, N.; Phillips, H. S.; Ferrara, N., Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* **1993**, *362* (6423), 841-4.
94. Ganapathy, V.; Ge, R.; Grazioli, A.; Xie, W.; Banach-Petrosky, W.; Kang, Y.; Lonning, S.; McPherson, J.; Yingling, J. M.; Biswas, S.; Mundy, G. R.; Reiss, M., Targeting the Transforming Growth Factor-beta pathway inhibits human basal-like breast cancer metastasis. *Mol Cancer* **2010**, *9*, 122.
95. Kzhyshkowska, J.; Riabov, V.; Gudima, A.; Wang, N.; Orekhov, A.; Mickle, A., Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. *Frontiers in Physiology* **2014**, *5* (75).
96. Libreros, S.; Garcia-Areas, R.; Iragavarapu-Charyulu, V., CHI3L1 plays a role in cancer through enhanced production of pro-inflammatory/pro-tumorigenic and angiogenic factors. *Immunol Res* **2013**, *57* (1-3), 99-105.
97. Shao, R., YKL-40 acts as an angiogenic factor to promote tumor angiogenesis. *Front Physiol* **2013**, *4*, 122.
98. Fahmy, R. G.; Dass, C. R.; Sun, L. Q.; Chesterman, C. N.; Khachigian, L. M., Transcription factor Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumor growth. *Nat Med* **2003**, *9* (8), 1026-32.
99. Shao, R.; Francescone, R.; Ngernyung, N.; Bentley, B.; Taylor, S. L.; Moral, L.; Yan, W., Anti-YKL-40 antibody and ionizing irradiation synergistically inhibit tumor vascularization and malignancy in glioblastoma. *Carcinogenesis* **2014**, *35* (2), 373-82.
100. Faibish, M.; Francescone, R.; Bentley, B.; Yan, W.; Shao, R., A YKL-40-neutralizing antibody blocks tumor angiogenesis and progression: a potential therapeutic agent in cancers. *Mol Cancer Ther* **2011**, *10* (5), 742-51.
101. Shao, R.; Hamel, K.; Petersen, L.; Cao, Q. J.; Arenas, R. B.; Bigelow, C.; Bentley, B.; Yan, W., YKL-40, a secreted glycoprotein, promotes tumor angiogenesis. *Oncogene* **2009**, *28* (50), 4456-68.
102. Pouyafar, A.; Heydarabad, M. Z.; Mahboob, S.; Mokhtarzadeh, A.; Rahbarghazi, R., Angiogenic potential of YKL-40 in the dynamics of tumor niche. *Biomedicine & Pharmacotherapy* **2018**, *100*, 478-485.
103. Libreros, S.; Garcia-Areas, R.; Shibata, Y.; Carrio, R.; Torroella-Kouri, M.; Iragavarapu-Charyulu, V., Induction of proinflammatory mediators by CHI3L1 is reduced by chitin treatment: Decreased tumor metastasis in a breast cancer model. *International Journal of Cancer* **2012**, *131* (2), 377-386.
104. Zhang, W.; Murao, K.; Zhang, X.; Matsumoto, K.; Diah, S.; Okada, M.; Miyake, K.; Kawai, N.; Fei, Z.; Tamiya, T., Resveratrol represses YKL-40 expression in human glioma U87 cells. *BMC Cancer* **2010**, *10*, 593.
105. Zadi Heydarabad, M.; Baharaghdam, S.; Azimi, A.; Mohammadi, H.; Eivazi Ziaei, J.; Yazdanpanah, B.; Zak, M. S.; Farahani, M. E.; Dohrabpour, A.; Partash, N.; Talebi, M., The role of tumor suppressor of resveratrol and prednisolone by downregulation of YKL-40 expression in CCRF-CEM cell line. *J Cell Biochem* **2019**, *120* (3), 3773-3779.
106. Kim, K. C.; Yun, J.; Son, D. J.; Kim, J. Y.; Jung, J.-K.; Choi, J. S.; Kim, Y. R.; Song, J. K.; Kim, S. Y.; Kang, S. K.; Shin, D. H.; Roh, Y.-S.; Han, S.-B.; Hong, J. T., Suppression of metastasis through inhibition of chitinase 3-like 1 expression by miR-125a-3p-mediated up-regulation of USF1. *Theranostics* **2018**, *8* (16), 4409-4428.
107. Xue, H.; Li, J.; Xie, H.; Wang, Y., Review of Drug Repositioning Approaches and Resources. *Int J Biol Sci* **2018**, *14* (10), 1232-1244.
108. Ashburn, T. T.; Thor, K. B., Drug repositioning: identifying and developing new uses for existing drugs. *Nat Rev Drug Discov* **2004**, *3* (8), 673-83.

109. Langedijk, J.; Mantel-Teeuwisse, A. K.; Slijkerman, D. S.; Schutjens, M. H., Drug repositioning and repurposing: terminology and definitions in literature. *Drug Discov Today* **2015**, *20* (8), 1027-34.
110. Jourdan, J. P.; Bureau, R.; Rochais, C.; Dallemagne, P., Drug repositioning: a brief overview. *J Pharm Pharmacol* **2020**.
111. Wermuth, C. G., Selective optimization of side activities: the SOSA approach. *Drug Discov Today* **2006**, *11* (3-4), 160-4.
112. Agrawal, P., Artificial Intelligence in Drug Discovery and Development. *Journal of Pharmacovigilance* **2018**, *06*.
113. Hughes, J. P.; Rees, S.; Kalindjian, S. B.; Philpott, K. L., Principles of early drug discovery. *Br J Pharmacol* **2011**, *162* (6), 1239-49.
114. Rudrapal, M.; Chetia, D., Endoperoxide antimalarials: development, structural diversity and pharmacodynamic aspects with reference to 1,2,4-trioxane-based structural scaffold. *Drug Des Devel Ther* **2016**, *10*, 3575-3590.
115. Rudrapal, M.; Khairnar, S.; Jadhav, A., Drug Repurposing (DR): An Emerging Approach in Drug Discovery. 2020.
116. Juárez-López, D.; Schcolnik-Cabrera, A., Drug Repurposing: Considerations to Surpass While Re-directing Old Compounds for New Treatments. *Archives of Medical Research* **2020**.
117. Allarakhia, M., Open-source approaches for the repurposing of existing or failed candidate drugs: learning from and applying the lessons across diseases. *Drug Des Devel Ther* **2013**, *7*, 753-66.
118. Jin, G.; Wong, S. T., Toward better drug repositioning: prioritizing and integrating existing methods into efficient pipelines. *Drug Discov Today* **2014**, *19* (5), 637-44.
119. Zhang, Z.; Zhou, L.; Xie, N.; Nice, E. C.; Zhang, T.; Cui, Y.; Huang, C., Overcoming cancer therapeutic bottleneck by drug repurposing. *Signal Transduction and Targeted Therapy* **2020**, *5* (1), 113.
120. Oprea, T. I.; Overington, J. P., Computational and Practical Aspects of Drug Repositioning. *Assay Drug Dev Technol* **2015**, *13* (6), 299-306.
121. Rosa, S. G. V.; Santos, W. C., Clinical trials on drug repositioning for COVID-19 treatment. *Rev Panam Salud Publica* **2020**, *44*, e40-e40.
122. Hurle, M. R.; Yang, L.; Xie, Q.; Rajpal, D. K.; Sanseau, P.; Agarwal, P., Computational drug repositioning: from data to therapeutics. *Clin Pharmacol Ther* **2013**, *93* (4), 335-41.
123. Sam, E.; Athri, P., Web-based drug repurposing tools: a survey. *Briefings in Bioinformatics* **2019**, *20* (1), 299-316.
124. Wang, Y.; Yella, J.; Jegga, A. G., Transcriptomic Data Mining and Repurposing for Computational Drug Discovery. *Methods Mol Biol* **2019**, *1903*, 73-95.
125. Xu, X.; Huang, M.; Zou, X., Docking-based inverse virtual screening: methods, applications, and challenges. *Biophys Rep* **2018**, *4* (1), 1-16.
126. Fukuoka, Y., Machine Learning Approach for Predicting New Uses of Existing Drugs and Evaluation of Their Reliabilities. *Methods Mol Biol* **2019**, *1903*, 269-279.
127. Masoudi-Sobhanzadeh, Y.; Motieghader, H.; Masoudi-Nejad, A., FeatureSelect: a software for feature selection based on machine learning approaches. *BMC Bioinformatics* **2019**, *20* (1), 170.
128. Park, S.; Lee, D.-g.; Shin, H., Network mirroring for drug repositioning. *BMC Medical Informatics and Decision Making* **2017**, *17* (1), 55.
129. Sun, P.; Guo, J.; Winnenburg, R.; Baumbach, J., Drug repurposing by integrated literature mining and drug-gene-disease triangulation. *Drug Discovery Today* **2017**, *22* (4), 615-619.
130. Orecchioni, S.; Roma, S.; Raimondi, S.; Gandini, S.; Bertolini, F., Identifying Drug Repurposing Opportunities in Oncology. *Cancer J* **2019**, *25* (2), 82-87.

131. March-Vila, E.; Pinzi, L.; Sturm, N.; Tinivella, A.; Engkvist, O.; Chen, H.; Rastelli, G., On the Integration of In Silico Drug Design Methods for Drug Repurposing. *Frontiers in pharmacology* **2017**, *8*, 298-298.
132. Lee, A.; Lee, K.; Kim, D., Using reverse docking for target identification and its applications for drug discovery. *Expert Opin Drug Discov* **2016**, *11* (7), 707-15.
133. Luo, H.; Mattes, W.; Mendrick, D. L.; Hong, H., Molecular Docking for Identification of Potential Targets for Drug Repurposing. *Curr Top Med Chem* **2016**, *16* (30), 3636-3645.
134. Tanaji, T. T.; Santosh, A. K.; Alan, C. R., Successful Applications of Computer Aided Drug Discovery: Moving Drugs from Concept to the Clinic. *Current Topics in Medicinal Chemistry* **2010**, *10* (1), 127-141.
135. Wilkinson, G. F.; Pritchard, K., In vitro screening for drug repositioning. *J Biomol Screen* **2015**, *20* (2), 167-79.
136. Turanli, B.; Grøtli, M.; Boren, J.; Nielsen, J.; Uhlen, M.; Arga, K. Y.; Mardinoglu, A., Drug Repositioning for Effective Prostate Cancer Treatment. *Frontiers in physiology* **2018**, *9*, 500-500.
137. Nowak-Sliwinska, P.; Scapozza, L.; Ruiz i Altaba, A., Drug repurposing in oncology: Compounds, pathways, phenotypes and computational approaches for colorectal cancer. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **2019**, *1871* (2), 434-454.
138. Naylor, S.; Schonfeld, J., Therapeutic drug repurposing, repositioning and rescue: Part I—Overview. *Drug Discovery World*. **2014**.
139. Sleight, S. H.; Barton, C. L., Repurposing Strategies for Therapeutics. *Pharmaceutical Medicine* **2010**, *24* (3), 151-159.
140. Murteira, S.; Millier, A.; Toumi, M., Drug repurposing in pharmaceutical industry and its impact on market access: market access implications. *Journal of Market Access & Health Policy* **2014**, *2* (1), 22814.
141. Talevi, A.; Bellera, C. L., Challenges and opportunities with drug repurposing: finding strategies to find alternative uses of therapeutics. *Expert Opin Drug Discov* **2020**, *15* (4), 397-401.
142. Kumar, A.; Jha, A., Chapter 7 - Drug Development Strategies. In *Anticandidal Agents*, Kumar, A.; Jha, A., Eds. Academic Press: 2017; pp 63-71.
143. Marshall, G. R., Computer-Aided Drug Design. *Annual Review of Pharmacology and Toxicology* **1987**, *27* (1), 193-213.
144. Kalyanamoorthy, S.; Chen, Y.-P. P., Structure-based drug design to augment hit discovery. *Drug Discovery Today* **2011**, *16* (17), 831-839.
145. Lima, A. N.; Philot, E. A.; Trossini, G. H. G.; Scott, L. P. B.; Maltarollo, V. G.; Honorio, K. M., Use of machine learning approaches for novel drug discovery. *Expert Opinion on Drug Discovery* **2016**, *11* (3), 225-239.
146. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E., The Protein Data Bank. *Nucleic Acids Research* **2000**, *28* (1), 235-242.
147. Diller, D. J.; Merz, K. M., Jr., High throughput docking for library design and library prioritization. *Proteins* **2001**, *43* (2), 113-24.
148. Ferreira, L. G.; Dos Santos, R. N.; Oliva, G.; Andricopulo, A. D., Molecular docking and structure-based drug design strategies. *Molecules* **2015**, *20* (7), 13384-421.
149. McConkey, B. J.; Sobolev, V.; Edelman, M., The performance of current methods in ligand–protein docking. *Current Science* **2002**, *83* (7), 845-856.
150. Meng, X. Y.; Zhang, H. X.; Mezei, M.; Cui, M., Molecular docking: a powerful approach for structure-based drug discovery. *Curr Comput Aided Drug Des* **2011**, *7* (2), 146-57.
151. Kapetanovic, I. M., Computer-aided drug discovery and development (CADD): in silico-chemico-biological approach. *Chem Biol Interact* **2008**, *171* (2), 165-176.
152. Torres, P. H. M.; Soderro, A. C. R.; Jofily, P.; Silva-Jr, F. P., Key Topics in Molecular Docking for Drug Design. *International journal of molecular sciences* **2019**, *20* (18), 4574.

153. Pagadala, N. S.; Syed, K.; Tuszynski, J., Software for molecular docking: a review. *Biophys Rev* **2017**, *9* (2), 91-102.
154. Phillips, M. A., *Has Molecular Docking Ever Brought Us a Medicine?* IntechOpen: 2018.
155. Wang, Z.; Sun, H.; Yao, X.; Li, D.; Xu, L.; Li, Y.; Tian, S.; Hou, T., Comprehensive evaluation of ten docking programs on a diverse set of protein–ligand complexes: the prediction accuracy of sampling power and scoring power. *Physical Chemistry Chemical Physics* **2016**, *18* (18), 12964-12975.
156. Gioia, D.; Bertazzo, M.; Recanatini, M.; Masetti, M.; Cavalli, A., Dynamic Docking: A Paradigm Shift in Computational Drug Discovery. *Molecules (Basel, Switzerland)* **2017**, *22* (11), 2029.
157. Brooijmans, N.; Kuntz, I. D., Molecular recognition and docking algorithms. *Annu Rev Biophys Biomol Struct* **2003**, *32*, 335-73.
158. Kitchen, D. B.; Decornez, H.; Furr, J. R.; Bajorath, J., Docking and scoring in virtual screening for drug discovery: methods and applications. *Nature Reviews Drug Discovery* **2004**, *3* (11), 935-949.
159. Guedes, I. A.; de Magalhães, C. S.; Dardenne, L. E., Receptor-ligand molecular docking. *Biophys Rev* **2014**, *6* (1), 75-87.
160. Huang, S.-Y.; Zou, X., Advances and challenges in protein-ligand docking. *International journal of molecular sciences* **2010**, *11* (8), 3016-3034.
161. Sousa, S. F.; Fernandes, P. A.; Ramos, M. J., Protein-ligand docking: current status and future challenges. *Proteins* **2006**, *65* (1), 15-26.
162. Jain, A. N., Scoring functions for protein-ligand docking. *Curr Protein Pept Sci* **2006**, *7* (5), 407-20.
163. Guedes, I. A.; Pereira, F. S. S.; Dardenne, L. E., Empirical Scoring Functions for Structure-Based Virtual Screening: Applications, Critical Aspects, and Challenges. *Front Pharmacol* **2018**, *9*, 1089.
164. Batool, M.; Ahmad, B.; Choi, S., A Structure-Based Drug Discovery Paradigm. *International journal of molecular sciences* **2019**, *20* (11), 2783.
165. Wang, G.; Zhu, W., Molecular docking for drug discovery and development: a widely used approach but far from perfect. *Future Med Chem* **2016**, *8* (14), 1707-10.
166. Ruvinsky, A. M., Role of binding entropy in the refinement of protein-ligand docking predictions: analysis based on the use of 11 scoring functions. *J Comput Chem* **2007**, *28* (8), 1364-72.
167. Mysinger, M. M.; Shoichet, B. K., Rapid context-dependent ligand desolvation in molecular docking. *J Chem Inf Model* **2010**, *50* (9), 1561-73.
168. Yuriev, E.; Ramsland, P. A., Latest developments in molecular docking: 2010–2011 in review. *Journal of Molecular Recognition* **2013**, *26* (5), 215-239.
169. Goodsell, D. S.; Morris, G. M.; Olson, A. J., Automated docking of flexible ligands: applications of AutoDock. *J Mol Recognit* **1996**, *9* (1), 1-5.
170. AutoDock. <http://autodock.scripps.edu/> (accessed November 17, 2020).
171. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J., AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of computational chemistry* **2009**, *30* (16), 2785-2791.
172. AutoDock Vina. <http://vina.scripps.edu/> (accessed November 19, 2020).
173. Trott, O.; Olson, A. J., AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of computational chemistry* **2010**, *31* (2), 455-461.
174. Nguyen, N. T.; Nguyen, T. H.; Pham, T. N. H.; Huy, N. T.; Bay, M. V.; Pham, M. Q.; Nam, P. C.; Vu, V. V.; Ngo, S. T., Autodock Vina Adopts More Accurate Binding Poses but Autodock4 Forms Better Binding Affinity. *J Chem Inf Model* **2020**, *60* (1), 204-211.

175. Österberg, F.; Morris, G. M.; Sanner, M. F.; Olson, A. J.; Goodsell, D. S., Automated docking to multiple target structures: Incorporation of protein mobility and structural water heterogeneity in AutoDock. *Proteins: Structure, Function, and Bioinformatics* **2002**, *46* (1), 34-40.
176. Pinzi, L.; Rastelli, G., Molecular Docking: Shifting Paradigms in Drug Discovery. *Int J Mol Sci* **2019**, *20* (18).
177. Yuriev, E.; Agostino, M.; Ramsland, P. A., Challenges and advances in computational docking: 2009 in review. *J Mol Recognit* **2011**, *24* (2), 149-64.
178. Elokely, K. M.; Doerksen, R. J., Docking challenge: protein sampling and molecular docking performance. *J Chem Inf Model* **2013**, *53* (8), 1934-45.
179. Bissantz, C.; Kuhn, B.; Stahl, M., A medicinal chemist's guide to molecular interactions. *Journal of medicinal chemistry* **2010**, *53* (14), 5061-5084.
180. Spyarakis, F.; Cavasotto, C. N., Open challenges in structure-based virtual screening: Receptor modeling, target flexibility consideration and active site water molecules description. *Arch Biochem Biophys* **2015**, *583*, 105-19.
181. Totrov, M.; Abagyan, R., Flexible ligand docking to multiple receptor conformations: a practical alternative. *Current Opinion in Structural Biology* **2008**, *18* (2), 178-184.
182. Muhammed, M. T.; Aki-Yalcin, E., Homology modeling in drug discovery: Overview, current applications, and future perspectives. *Chem Biol Drug Des* **2019**, *93* (1), 12-20.
183. Forli, S.; Huey, R.; Pique, M. E.; Sanner, M. F.; Goodsell, D. S.; Olson, A. J., Computational protein-ligand docking and virtual drug screening with the AutoDock suite. *Nat Protoc* **2016**, *11* (5), 905-919.
184. Wishart, D. S.; Feunang, Y. D.; Guo, A. C.; Lo, E. J.; Marcu, A.; Grant, J. R.; Sajed, T.; Johnson, D.; Li, C.; Sayeeda, Z.; Assempour, N.; Iynkkaran, I.; Liu, Y.; Maciejewski, A.; Gale, N.; Wilson, A.; Chin, L.; Cummings, R.; Le, D.; Pon, A.; Knox, C.; Wilson, M., DrugBank 5.0: a major update to the DrugBank database for 2018. *Nucleic Acids Res* **2018**, *46* (D1), D1074-d1082.
185. Seeliger, D.; de Groot, B. L., Ligand docking and binding site analysis with PyMOL and Autodock/Vina. *J Comput Aided Mol Des* **2010**, *24* (5), 417-22.
186. Lill, M. A.; Danielson, M. L., Computer-aided drug design platform using PyMOL. *J Comput Aided Mol Des* **2011**, *25* (1), 13-9.
187. Vichai, V.; Kirtikara, K., Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc* **2006**, *1* (3), 1112-1116.
188. Eurich, K.; Segawa, M.; Toei-Shimizu, S.; Mizoguchi, E., Potential role of chitinase 3-like-1 in inflammation-associated carcinogenic changes of epithelial cells. *World J Gastroenterol* **2009**, *15* (42), 5249-5259.
189. Kognole, A. A.; Payne, C. M., Inhibition of Mammalian Glycoprotein YKL-40: IDENTIFICATION OF THE PHYSIOLOGICAL LIGAND. *J Biol Chem* **2017**, *292* (7), 2624-2636.
190. Lee, I. A.; Kamba, A.; Low, D.; Mizoguchi, E., Novel methylxanthine derivative-mediated anti-inflammatory effects in inflammatory bowel disease. *World J Gastroenterol* **2014**, *20* (5), 1127-38.
191. PubChem Compound Summary for CID 457193, Dactinomycin. National Center for Biotechnology Information, 2021.
192. Vardanyan, R. S.; Hruby, V. J., 30 - Antineoplastics. In *Synthesis of Essential Drugs*, Vardanyan, R. S.; Hruby, V. J., Eds. Elsevier: Amsterdam, 2006; pp 389-418.
193. Avendaño, C.; Menéndez, J. C., Chapter 4 - Anticancer Drugs Acting via Radical Species: Radiotherapy and Photodynamic Therapy of Cancer. In *Medicinal Chemistry of Anticancer Drugs (Second Edition)*, Avendaño, C.; Menéndez, J. C., Eds. Elsevier: Boston, 2015; pp 133-195.
194. Dactinomycin. In *LiverTox: Clinical and Research Information on Drug-Induced Liver Injury*, National Institute of Diabetes and Digestive and Kidney Diseases: Bethesda (MD), 2012.
195. Sobell, H. M., Actinomycin and DNA transcription. *Proceedings of the National Academy of Sciences* **1985**, *82* (16), 5328-5331.

196. Kwok, K. K.; Vincent, E. C.; Gibson, J. N., 36 - Antineoplastic Drugs. In *Pharmacology and Therapeutics for Dentistry (Seventh Edition)*, Dowd, F. J.; Johnson, B. S.; Mariotti, A. J., Eds. Mosby: 2017; pp 530-562.
197. Kleeff, J.; Kornmann, M.; Sawhney, H.; Korc, M., Actinomycin D induces apoptosis and inhibits growth of pancreatic cancer cells. *Int J Cancer* **2000**, *86* (3), 399-407.
198. Colman, I.; Brown, M. D.; Innes, G. D.; Grafstein, E.; Roberts, T. E.; Rowe, B. H., Parenteral Dihydroergotamine for Acute Migraine Headache: A Systematic Review of the Literature. *Annals of Emergency Medicine* **2005**, *45* (4), 393-401.
199. PubChem Compound Summary for CID 10531, Dihydroergotamine. National Center for Biotechnology Information, 2021.
200. Silberstein, S. D.; McCrory, D. C., Ergotamine and Dihydroergotamine: History, Pharmacology, and Efficacy. *Headache: The Journal of Head and Face Pain* **2003**, *43* (2), 144-166.
201. Boudreaux, S. P.; Duren, R. P.; Call, S. G.; Nguyen, L.; Freire, P. R.; Narayanan, P.; Redell, M. S.; Conneely, O. M., Drug targeting of NR4A nuclear receptors for treatment of acute myeloid leukemia. *Leukemia* **2019**, *33* (1), 52-63.
202. Call, S. G.; Duren, R. P.; Panigrahi, A. K.; Nguyen, L.; Freire, P. R.; Grimm, S. L.; Coarfa, C.; Conneely, O. M., Targeting Oncogenic Super Enhancers in MYC-Dependent AML Using a Small Molecule Activator of NR4A Nuclear Receptors. *Sci Rep* **2020**, *10* (1), 2851.
203. Chang, S. H.; Lee, A. Y.; Yu, K. N.; Park, J.; Kim, K. P.; Cho, M. H., Dihydroergotamine Tartrate Induces Lung Cancer Cell Death through Apoptosis and Mitophagy. *Chemotherapy* **2016**, *61* (6), 304-12.
204. Silberstein, S. D.; Shrewsbury, S. B.; Hoekman, J., Dihydroergotamine (DHE) - Then and Now: A Narrative Review. *Headache* **2020**, *60* (1), 40-57.
205. Plicamycin. In *LiverTox: Clinical and Research Information on Drug-Induced Liver Injury*, National Institute of Diabetes and Digestive and Kidney Diseases: Bethesda (MD), 2012.
206. PubChem Compound Summary for CID 163659, Plicamycin. National Center for Biotechnology Information: 2021.
207. Duverger, V.; Murphy, A. M.; Sheehan, D.; England, K.; Cotter, T. G.; Hayes, I.; Murphy, F. J., The anticancer drug mithramycin A sensitises tumour cells to apoptosis induced by tumour necrosis factor (TNF). *British Journal of Cancer* **2004**, *90* (10), 2025-2031.
208. Furman, B. L., Plicamycin. In *xPharm: The Comprehensive Pharmacology Reference*, Enna, S. J.; Bylund, D. B., Eds. Elsevier: New York, 2007; pp 1-4.
209. Holstege, C. P., Mithramycin. In *Encyclopedia of Toxicology (Third Edition)*, Wexler, P., Ed. Academic Press: Oxford, 2014; pp 347-348.
210. Yuan, P.; Wang, L.; Wei, D.; Zhang, J.; Jia, Z.; Li, Q.; Le, X.; Wang, H.; Yao, J.; Xie, K., Therapeutic inhibition of Sp1 expression in growing tumors by mithramycin a correlates directly with potent antiangiogenic effects on human pancreatic cancer. *Cancer* **2007**, *110* (12), 2682-90.
211. Jia, Z.; Gao, Y.; Wang, L.; Li, Q.; Zhang, J.; Le, X.; Wei, D.; Yao, J. C.; Chang, D. Z.; Huang, S.; Xie, K., Combined treatment of pancreatic cancer with mithramycin A and tolfenamic acid promotes Sp1 degradation and synergistic antitumor activity. *Cancer Res* **2010**, *70* (3), 1111-9.
212. Gao, Y.; Jia, Z.; Kong, X.; Li, Q.; Chang, D. Z.; Wei, D.; Le, X.; Suyun, H.; Huang, S.; Wang, L.; Xie, K., Combining betulinic acid and mithramycin a effectively suppresses pancreatic cancer by inhibiting proliferation, invasion, and angiogenesis. *Cancer Res* **2011**, *71* (15), 5182-93.
213. PubChem Compound Summary for CID 49846579, Venetoclax. National Center for Biotechnology Information, 2021.
214. Roberts, A. W., Therapeutic development and current uses of BCL-2 inhibition. *Hematology* **2020**, *2020* (1), 1-9.
215. Awan, F. T.; Byrd, J. C., 99 - Chronic Lymphocytic Leukemia. In *Abeloff's Clinical Oncology (Sixth Edition)*, Niederhuber, J. E.; Armitage, J. O.; Kastan, M. B.; Doroshow, J. H.; Tepper, J. E., Eds. Elsevier: Philadelphia, 2020; pp 1850-1871.e5.

216. Anderson, M. A.; Deng, J.; Seymour, J. F.; Tam, C.; Kim, S. Y.; Fein, J.; Yu, L.; Brown, J. R.; Westerman, D.; Si, E. G.; Majewski, I. J.; Segal, D.; Heitner Enschede, S. L.; Huang, D. C.; Davids, M. S.; Letai, A.; Roberts, A. W., The BCL2 selective inhibitor venetoclax induces rapid onset apoptosis of CLL cells in patients via a TP53-independent mechanism. *Blood* **2016**, *127* (25), 3215-24.
217. Guerra, V. A.; DiNardo, C.; Konopleva, M., Venetoclax-based therapies for acute myeloid leukemia. *Best Pract Res Clin Haematol* **2019**, *32* (2), 145-153.
218. Gentile, M.; Petrungaro, A.; Uccello, G.; Vigna, E.; Recchia, A. G.; Caruso, N.; Bossio, S.; De Stefano, L.; Palumbo, A.; Storino, F.; Martino, M.; Morabito, F., Venetoclax for the treatment of chronic lymphocytic leukemia. *Expert Opin Investig Drugs* **2017**, *26* (11), 1307-1316.
219. Vaxman, I.; Sidiqi, M. H.; Gertz, M., Venetoclax for the treatment of multiple myeloma. *Expert Review of Hematology* **2018**, *11* (12), 915-920.
220. Zhou, Y.; Liu, H.; Xue, R.; Tang, W.; Zhang, S., BH3 Mimetic ABT-199 Enhances the Sensitivity of Gemcitabine in Pancreatic Cancer in vitro and in vivo. *Dig Dis Sci* **2018**, *63* (12), 3367-3375.
221. Tan, I. B.; Dolivet, G.; Ceruse, P.; Vander Poorten, V.; Roest, G.; Rauschning, W., Temoporfin-mediated photodynamic therapy in patients with advanced, incurable head and neck cancer: A multicenter study. *Head Neck* **2010**, *32* (12), 1597-604.
222. PubChem Compound Summary for CID 60751, Temoporfin. National Center for Biotechnology Information, 2021.
223. Papich, M. G., Dirlotapide. In *Saunders Handbook of Veterinary Drugs (Fourth Edition)*, Papich, M. G., Ed. W.B. Saunders: St. Louis, 2016; pp 255-257.
224. Wren, J. A.; Gossellin, J.; Sunderland, S. J., Dirlotapide: a review of its properties and role in the management of obesity in dogs. *J Vet Pharmacol Ther* **2007**, *30 Suppl 1*, 11-6.
225. PubChem Compound Summary for CID 9917862, Dirlotapide. National Center for Biotechnology Information, 2021.
226. Chapter 30 - Nutritional Assessment and Management. In *Canine and Feline Gastroenterology*, Washabau, R. J.; Day, M. J., Eds. W.B. Saunders: Saint Louis, 2013; pp 386-397.
227. PubChem Compound Summary for CID 25077405, Nafarelin. National Center for Biotechnology Information, 2021.
228. Letassy, N. A.; Thompson, D. F.; Britton, M. L.; Suda, R. R., Sr., Nafarelin acetate: a gonadotropin-releasing hormone agonist for the treatment of endometriosis. *Dicp* **1990**, *24* (12), 1204-9.
229. Minaguchi, H.; Wong, J. M.; Snabes, M. C., Clinical use of nafarelin in the treatment of leiomyomas. A review of the literature. *J Reprod Med* **2000**, *45* (6), 481-9.
230. Barbieri, R. L., Clinical applications of GnRH and its analogues. *Trends Endocrinol Metab* **1992**, *3* (1), 30-4.
231. PubChem Compound Summary for CID 67683363, Velpatasvir. National Center for Biotechnology Information, 2021.
232. Kamal, S. M., Chapter 6 - Hepatitis C Treatment in the Era of Direct-Acting Antiviral Agents: Challenges in Developing Countries. In *Hepatitis C in Developing Countries*, Kamal, S. M., Ed. Academic Press: 2018; pp 209-246.
233. Feld, J. J.; Jacobson, I. M.; Hézode, C.; Asselah, T.; Ruane, P. J.; Gruener, N.; Abergel, A.; Mangia, A.; Lai, C. L.; Chan, H. L.; Mazzotta, F.; Moreno, C.; Yoshida, E.; Shafran, S. D.; Towner, W. J.; Tran, T. T.; McNally, J.; Osinusi, A.; Svarovskaia, E.; Zhu, Y.; Brainard, D. M.; McHutchison, J. G.; Agarwal, K.; Zeuzem, S., Sofosbuvir and Velpatasvir for HCV Genotype 1, 2, 4, 5, and 6 Infection. *N Engl J Med* **2015**, *373* (27), 2599-607.
234. PubChem Compound Summary for CID 11965427, Lifitegrast. National Center for Biotechnology Information, 2021.
235. Abidi, A.; Shukla, P.; Ahmad, A., Lifitegrast: A novel drug for treatment of dry eye disease. *J Pharmacol Pharmacother* **2016**, *7* (4), 194-198.

236. Perez, V. L.; Pflugfelder, S. C.; Zhang, S.; Shojaei, A.; Haque, R., Lifitegrast, a Novel Integrin Antagonist for Treatment of Dry Eye Disease. *Ocul Surf* **2016**, *14* (2), 207-15.
237. Shen, W.; Zhang, X.; Du, R.; Fan, Y.; Luo, D.; Bao, Y.; Yang, W.; Luo, N.; Luo, Y.; Zhao, S., ICAM3 mediates tumor metastasis via a LFA-1-ICAM3-ERM dependent manner. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **2018**, *1864* (8), 2566-2578.
238. PubChem Compound Summary for CID 444031, Darifenacin. National Center for Biotechnology Information, 2021.
239. Steers, W. D., Darifenacin: Pharmacology and clinical usage. *Urol Clin North Am* **2006**, *33* (4), 475-82, viii.
240. Skerjanec, A., The clinical pharmacokinetics of darifenacin. *Clin Pharmacokinet* **2006**, *45* (4), 325-50.
241. Yang, T.; He, W.; Cui, F.; Xia, J.; Zhou, R.; Wu, Z.; Zhao, Y.; Shi, M., MACC1 mediates acetylcholine-induced invasion and migration by human gastric cancer cells. *Oncotarget* **2016**, *7* (14), 18085-18094.
242. Song, P.; Sekhon, H. S.; Fu, X. W.; Maier, M.; Jia, Y.; Duan, J.; Proskosil, B. J.; Gravett, C.; Lindstrom, J.; Mark, G. P.; Saha, S.; Spindel, E. R., Activated cholinergic signaling provides a target in squamous cell lung carcinoma. *Cancer research* **2008**, *68* (12), 4693-4700.
243. Hering, N. A.; Liu, V.; Kim, R.; Weixler, B.; Droeser, R. A.; Arndt, M.; Pozios, I.; Beyer, K.; Kreis, M. E.; Seeliger, H., Blockage of Cholinergic Signaling via Muscarinic Acetylcholine Receptor 3 Inhibits Tumor Growth in Human Colorectal Adenocarcinoma. *Cancers (Basel)* **2021**, *13* (13), 3220.
244. Zhou, B.; Xu, J.-W.; Cheng, Y.-G.; Gao, J.-Y.; Hu, S.-Y.; Wang, L.; Zhan, H.-X., Early detection of pancreatic cancer: Where are we now and where are we going? *International Journal of Cancer* **2017**, *141* (2), 231-241.
245. Miller, A. L.; Garcia, P. L.; Yoon, K. J., Developing effective combination therapy for pancreatic cancer: An overview. *Pharmacol Res* **2020**, *155*, 104740.
246. Springfield, C.; Jäger, D.; Büchler, M. W.; Strobel, O.; Hackert, T.; Palmer, D. H.; Neoptolemos, J. P., Chemotherapy for pancreatic cancer. *Presse Med* **2019**, *48* (3 Pt 2), e159-e174.
247. Burris Iii, H. A.; Moore, M. J.; Andersen, J.; Green, M. R.; Rothenberg, M. L.; Modiano, M. R.; Cripps, M. C.; Portenoy, R. K.; Storniolo, A. M.; Tarassoff, P.; Nelson, R.; Dorr, F. A.; Stephens, C. D.; Von Hoff, D. D., Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: A randomized trial. *Journal of Clinical Oncology* **1997**, *15* (6), 2403-2413.
248. Mini, E.; Nobili, S.; Caciagli, B.; Landini, I.; Mazzei, T., Cellular pharmacology of gemcitabine. *Annals of Oncology* **2006**, *17*, v7-v12.
249. Zeng, S.; Pöttler, M.; Lan, B.; Grützmann, R.; Pilarsky, C.; Yang, H., Chemoresistance in Pancreatic Cancer. *International journal of molecular sciences* **2019**, *20* (18), 4504.
250. Sipos, B.; Möser, S.; Kalthoff, H.; Török, V.; Löhr, M.; Klöppel, G., A comprehensive characterization of pancreatic ductal carcinoma cell lines: towards the establishment of an in vitro research platform. *Virchows Arch* **2003**, *442* (5), 444-52.
251. Ghosn, M.; Ibrahim, T.; Assi, T.; El Rassy, E.; Kourie, H. R.; Kattan, J., Dilemma of first line regimens in metastatic pancreatic adenocarcinoma. *World J Gastroenterol* **2016**, *22* (46), 10124-10130.
252. Binenbaum, Y.; Na'ara, S.; Gil, Z., Gemcitabine resistance in pancreatic ductal adenocarcinoma. *Drug Resistance Updates* **2015**, *23*, 55-68.
253. Kim, J. H.; Shin, B. C.; Park, W. S.; Lee, J.; Kuh, H.-J., Antifibrotic effects of pentoxifylline improve the efficacy of gemcitabine in human pancreatic tumor xenografts. *Cancer Sci* **2017**, *108* (12), 2470-2477.

254. Rao, F. V.; Andersen, O. A.; Vora, K. A.; DeMartino, J. A.; van Aalten, D. M. F., Methylxanthine Drugs Are Chitinase Inhibitors: Investigation of Inhibition and Binding Modes. *Chemistry & Biology* **2005**, *12* (9), 973-980.
255. Samlaska, C. P.; Winfield, E. A., Pentoxifylline. *J Am Acad Dermatol* **1994**, *30* (4), 603-21.
256. Rebelo, R.; Polónia, B.; Santos, L. L.; Vasconcelos, M. H.; Xavier, C. P. R., Drug Repurposing Opportunities in Pancreatic Ductal Adenocarcinoma. *Pharmaceuticals (Basel)* **2021**, *14* (3).
257. Yamada, S.; Ito, Y.; Nishijima, S.; Kadekawa, K.; Sugaya, K., Basic and clinical aspects of antimuscarinic agents used to treat overactive bladder. *Pharmacology & Therapeutics* **2018**, *189*, 130-148.
258. Zinner, N.; Kobashi, K. C.; Ebinger, U.; Viegas, A.; Egermark, M.; Quebe-Fehling, E.; Koochaki, P., Darifenacin treatment for overactive bladder in patients who expressed dissatisfaction with prior extended-release antimuscarinic therapy. *Int J Clin Pract* **2008**, *62* (11), 1664-1674.
259. Darifenacin. In *LiverTox: Clinical and Research Information on Drug-Induced Liver Injury*, National Institute of Diabetes and Digestive and Kidney Diseases: Bethesda (MD), 2012.
260. Yu, H.; Xia, H.; Tang, Q.; Xu, H.; Wei, G.; Chen, Y.; Dai, X.; Gong, Q.; Bi, F., Acetylcholine acts through M3 muscarinic receptor to activate the EGFR signaling and promotes gastric cancer cell proliferation. *Sci Rep* **2017**, *7*, 40802.
261. Song, P.; Sekhon, H. S.; Lu, A.; Arredondo, J.; Sauer, D.; Gravett, C.; Mark, G. P.; Grando, S. A.; Spindel, E. R., M3 muscarinic receptor antagonists inhibit small cell lung carcinoma growth and mitogen-activated protein kinase phosphorylation induced by acetylcholine secretion. *Cancer Res* **2007**, *67* (8), 3936-44.



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