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Modelos tumorais 2D e 3D para a avaliação de fármacos

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“Nunca te esqueças de onde vens.”
“Faz o bem e não olhes a quem.”

- Ensinamentos dos meus avós.

“It is not the strongest of the species that survive, nor the most intelligent, but the most responsive to change.”

- Charles Darwin

Dedication

Aos meus pais, irmã e avós, por tudo.

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Resumo

A combinação de fármacos constitui uma abordagem terapêutica que tem sido usada no tratamento do cancro, uma vez que permite ultrapassar a resistência a fármacos das células cancerígenas e, simultaneamente, erradicar o tumor.

A avaliação da combinação de fármacos é, habitualmente, realizada em modelos de cultura 2D. No entanto, estes modelos são incapazes de representar o perfil de resistência a fármacos que as células do cancro do pâncreas exibem. Deste modo, estes modelos podem sobrestimar o potencial terapêutico da combinação de fármacos, levando a um fraco desempenho terapêutico destes fármacos, nos ensaios *in vivo*. Recentemente, os modelos de cultura 3D, nomeadamente os esferóides, surgiram como plataformas promissoras para avaliação de combinação de fármacos anticancerígenos, uma vez que mimetizam eficazmente os mecanismos dos tumores *in vivo*.

No presente estudo, analisou-se e comparou-se, pela primeira vez, o efeito terapêutico e o potencial sinergético da combinação de fármacos em culturas celulares 2D e 3D. Para tal, estudou-se o efeito da combinação de Doxorrubicina:Resveratrol (DOX:RES), com diferente rácios molares de 5:1 até 1:5, na viabilidade de células cancerígenas do pâncreas (PANC-1).

Os resultados obtidos mostraram que a viabilidade das células PANC-1 foi mais afetada quando as combinações DOX:RES continham maior concentração de RES (rácios molares de 1:2 a 1:5). Estes resultados podem ser explicados pelo facto do RES ter capacidade de reduzir o efluxo da DOX para o exterior da célula, mediado pela glicoproteína-P (P-gp). Os dados revelaram também que o efeito sinérgico da combinação DOX:RES em culturas 2D e 3D foi diferente. De facto, apesar das proporções 1:4 e 1:5 DOX:RES apresentarem ambas um efeito sinérgico para os dois tipos de culturas, os seus valores de Índice de Combinação (CI) foram inferiores (mais sinérgicos) nas culturas 2D. Deste modo, os resultados obtidos revelaram que a combinação DOX:RES é uma abordagem promissora para o tratamento do cancro do pâncreas e corroboram a necessidade de avaliar a combinação de fármacos em culturas 3D.

Palavras-chave

Cancro do Pâncreas, Culturas celulares 2D, Doxorrubicina, Esferóides, Resveratrol.

Resumo alargado

O cancro do pâncreas é uma doença que tem uma elevada taxa de mortalidade associada. Apenas 3-5 % dos pacientes sobrevive após o diagnóstico. A elevada mortalidade associada ao cancro do pâncreas deve-se à falta de sintomas precoces que permitam detetar a doença nos seus estádios iniciais e à existência de mecanismos de resistência a fármacos que tornam a quimioterapia pouco eficaz. Esta resistência é frequentemente associada à sobre-expressão de bombas de efluxo, tais como a P-gp (proteína membranar, codificada pelo gene *MDR1*) e a mudanças na capacidade das células em metabolizar os fármacos. Adicionalmente, a resistência das células à apoptose contribui também para a ineficácia das terapêuticas.

De forma a combater o cancro do pâncreas e a sua resistência a fármacos, têm sido estudadas novas abordagens. Uma destas abordagens envolve a combinação de fármacos, que consiste na utilização de dois ou mais agentes terapêuticos que atuam por diferentes mecanismos, de forma aditiva ou sinérgica, providenciando uma melhor e mais precisa ação terapêutica.

Na atualidade, a principal forma de avaliar a eficácia de combinações de fármacos tem por base, os modelos de cultura celular em 2D. No entanto, estes modelos não conseguem mimetizar as características dos tumores *in vivo,* como sejam os seus mecanismos de resistência a fármacos. Deste modo, combinações terapêuticas que se revelam eficazes em modelos celulares 2D, podem ter um fraco desempenho terapêutico nos ensaios *in vivo*. Os modelos de cultura celular 3D, nomeadamente os esferóides, aparecem como uma alternativa viável para a avaliação do potencial terapêutico de novas terapêuticas, uma vez que estes têm a capacidade de representar o microambiente tumoral e a resistência a fármacos exibida pelos tumores *in vivo*.

De acordo com os artigos que foram por nós consultados, a comparação dos efeitos resultantes da combinação de fármacos em modelos de cultura celular 2D e 3D do cancro do pâncreas ainda não foi efetuada até à data. Desta forma, neste trabalho pretendeu-se investigar e comparar, pela primeira vez, o efeito terapêutico e o potencial sinérgico da combinação de DOX:RES (com rácios molares que variam de 5:1 a 1:5) em culturas celulares 2D (monocamadas) e 3D (esferóides) do cancro do pâncreas usando as células PANC-1. Esta combinação de fármacos nunca foi previamente testada em células cancerígenas do cancro do pâncreas. Deste modo, este trabalho divulga uma nova combinação de fármacos para o tratamento do cancro do pâncreas e permite estudar de que forma a ação combinada dos fármacos é influenciada pelo tipo de cultura celular usada.

Numa primeira fase do estudo, realizou-se uma análise do efeito individual da DOX e do RES na viabilidade das células cultivadas em 2D e 3D. Posteriormente, o efeito da terapia combinada

de DOX:RES foi avaliado em ambos os tipos de cultura celular. Os resultados obtidos demonstraram que a combinação dos fármacos é mais eficaz do que a sua administração isolada. Por outro lado, também se verificou que o uso de combinações de fármacos com maior conteúdo de RES do que DOX permitiu obter uma eficácia terapêutica mais significativa. Estes resultados são promissores, uma vez que foi possível obter um maior efeito terapêutico quando foram usadas elevadas concentrações de RES, que é um produto natural, barato e que diminui os efeitos nefastos consequentes da DOX.

Após a análise do efeito do RES no efluxo e na acumulação da DOX no interior das células por espectroscopia e microscopia de fluorescência, foi possível comprovar que o RES permite uma maior acumulação de DOX no interior das células, através da inibição do seu efluxo para o exterior destas pela P-gp.

Por último, foi avaliada a capacidade das diferentes combinações de DOX:RES reduzirem a viabilidade das células PANC-1, nomeadamente 1DOX:4RES e 1DOX:5RES. Após a determinação dos valores de CI destas combinações em células cultivadas em monocamada ou em esferóides, os resultados obtidos demonstraram que, apesar das proporções 1:4 e 1:5 DOX:RES serem ambas sinérgicas para os dois tipos de culturas, os seus valores CI foram inferiores (mais sinérgicos) nas culturas 2D.

Com base nos dados obtidos é possível verificar que a combinação de DOX e RES pode ser uma mais valia para o tratamento do cancro do pâncreas. Por outro lado, foi também possível evidenciar a necessidade de avaliar a combinação de fármacos em modelos 3D de células, uma vez que o efeito sinérgico das combinações de fármacos é influenciado pelo tipo de cultura usado e os esferóides ao serem mais resistentes a fármacos, poderão prever com uma maior eficácia o efeito destes em modelos tumorais *in vivo*.

Abstract

Drug combination emerged as a solution for the treatment of cancer, once this therapeutic approach allows to surpass the drug resistance of cancer cells and, simultaneously, eradicate the tumor.

The assessment of drug-combination for pancreatic cancer treatment is usually performed in 2D cell cultures. However, these models are unable to mimic the drug resistance profiles found in pancreatic cancer. Thus, they may overestimate the therapeutic potential of the drug combination, leading to poor therapeutic performance in *in vivo* assays. Therefore, 3D culture models, especially spheroids, appear as a promising method for screening anticancer drugs, since they are able to mimic the structural and functional features of solid tumors.

In the present study, the therapeutic effect and the synergistic potential of a particular combination of drugs in 2D and 3D cell cultures were analyzed and compared for the first time. In this way, the effect of the combination of Doxorubicin:Resveratrol (DOX:RES) (at molar ratios ranging from 5: 1 to 1: 5), in the viability of the pancreatic cancer cell line, PANC-1, was studied.

The results showed that the viability of PANC-1 cells was more affected when the DOX:RES combinations contained a higher content of RES (molar ratios of 1:2 to 1:5). These results can be explained, by the ability of RES to reduce the efflux of DOX, mediated by P-glycoprotein (P-gp). Furthermore, these data also revealed that the synergistic effect of the DOX:RES combination was different in both 2D and 3D cell cultures. In fact, although 1:4 and 1:5 DOX: RES ratios were synergistic for these types of cell cultures, their values Combination Index (CI) were lower (more synergistic) in 2D cultures, when compared to spheroids. Overall, the results obtained revealed that the combination DOX:RES is promising approach for the treatment of pancreatic cancer and corroborate the need to perform drug screening.

Keywords

2D cell cultures, Doxorubicin, Pancreatic cancer, Resveratrol, Spheroids.

List of Publications

Articles in peer reviewed international journals:

Barros, A. S., Costa, E. C., Nunes, A. S., de-Melo, D. and Correia, I. J., Comparative study of the therapeutic effect of Doxorubicin and Resveratrol combination on 2D and 3D (spheroids) cell cultures models. *International Journal of Pharmaceutics*, submitted.

Nunes, A. S., Barros, A. S., Costa, E. C., Moreira, A. F. and Correia, I. J., 3D tumor spheroids as *in vitro* models to mimic *in vivo* human solid tumors resistance to therapeutics. *Biotechnology & Bioengineering*, submitted.

Nunes, A. S., Costa, E. C., Barros, A. S., de-Melo, D. and Correia, I. J., Gathering 2D cell cultures derived from 3D MCF-7 spheroids displaying a Doxorubicin resistant profile. *Biotechnology Journal*, submitted.

Table of Contents

List of Figures

Chapter I

Chapter III

List of Tables

Chapter I

Chapter II

List of Acronyms

5-FU	Fluorouracil
ABRAXANE	Albumin-bound Paclitaxel
ABCB1	ATP-binding cassette sub-family B member 1
BT-474	Breast cancer cell line (ATTC®HTB-20™)
BT-549	Breast cancer cell line (ATTC®HTB-122™)
Capan-2	Pancreatic cancer cell line (ATTC®HTB-80™)
CI	Combination Index
CLSM	Confocal scanning electron microscopy
COL1A1	Gene that encodes the major component of collagen I
Colo357	Pancreatic cancer cell line
DMEM-HG	Dulbecco's Modified Eagle medium-high glucose
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DOX:RES	Doxorubicin:Resveratrol
DU-145	Prostate cancer cell line (ATTC®HTB-81™)
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FAK	Focal adhesion kinase
FDA	Food and Drug Administration
FN1	Gene that encodes fibronectic
Gemzar ®	Gemcitabine
Gli36	Glioma cell line (RRID: CVCL_RL88)
GLUT-1	Glucose transporter 1
HeLa	Cervical cancer cell line (ATTC®CCL-2™)
HepG2	Liver cancer cell line (ATTC®HB-8065™)
HIF	Hypoxia-inducible factors
HOSS1	Osteosarcoma cell line 1
HT1080	Sarcoma cell line (ATTC®CCL-121™)
HTH-7	Thyroid cancer cell line (ATTC®CVCL_6289™)
Huh7	Differentiated hepatocytes (ECACC)
IC_{50}	Half maximal inhibitory concentration
IFP	Interstitial fluid pressure
INER-37	Lung cancer cell line (ATTC®CVCL_5621™)
INER-51	Lung cancer cell line (ATTC®CVCL_5531™)

K^-	Negative control
LDH	Lactate dehydrogenase
MCF-7	Breast cancer cell line (ATTC®HTB-22™)
MDA-MB-231	Breast cancer cell line (ATTC®HTB-26™)
MDR1	Multidrug resistance protein 1
MIAPaCa-2	Pancreatic cancer cell line (ATTC®CRL-1420™)
n.s.	Non significant
ONIVYDE ®	Irinotecan liposome injection
P-gp	P-glycoprotein
PANC-1	Pancreatic cancer cell line (ATTC®CRL-1469™)
PBS	Phosphate-buffered saline solution
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
RD	Sarcoma cell line (ATTC®CCL-136™)
RES	Resveratrol
S.D.	Standard deviation
SH-SY5Y	Neuroblastoma cell line (ATTC®CRL-2266™)
SW872	Sarcoma cell line (ATTC®HTB-92™)
T-47D	Breast cancer cell line (ATTC®HTB-133™)
U-118 MG	Glioma cell line (ATTC®HTB-15™)

Chapter I

1. Introduction

1.1. Pancreatic cancer

Cancer is a major worldwide health problem. The number of patients with cancer tend to increase from 12.4 million reported in 2008 to 26.4 million in 2030 [1]. Among the different types of cancer, pancreatic cancer exhibits one of the highest incidence and it is one of the most fatal (Figure 1) [2].

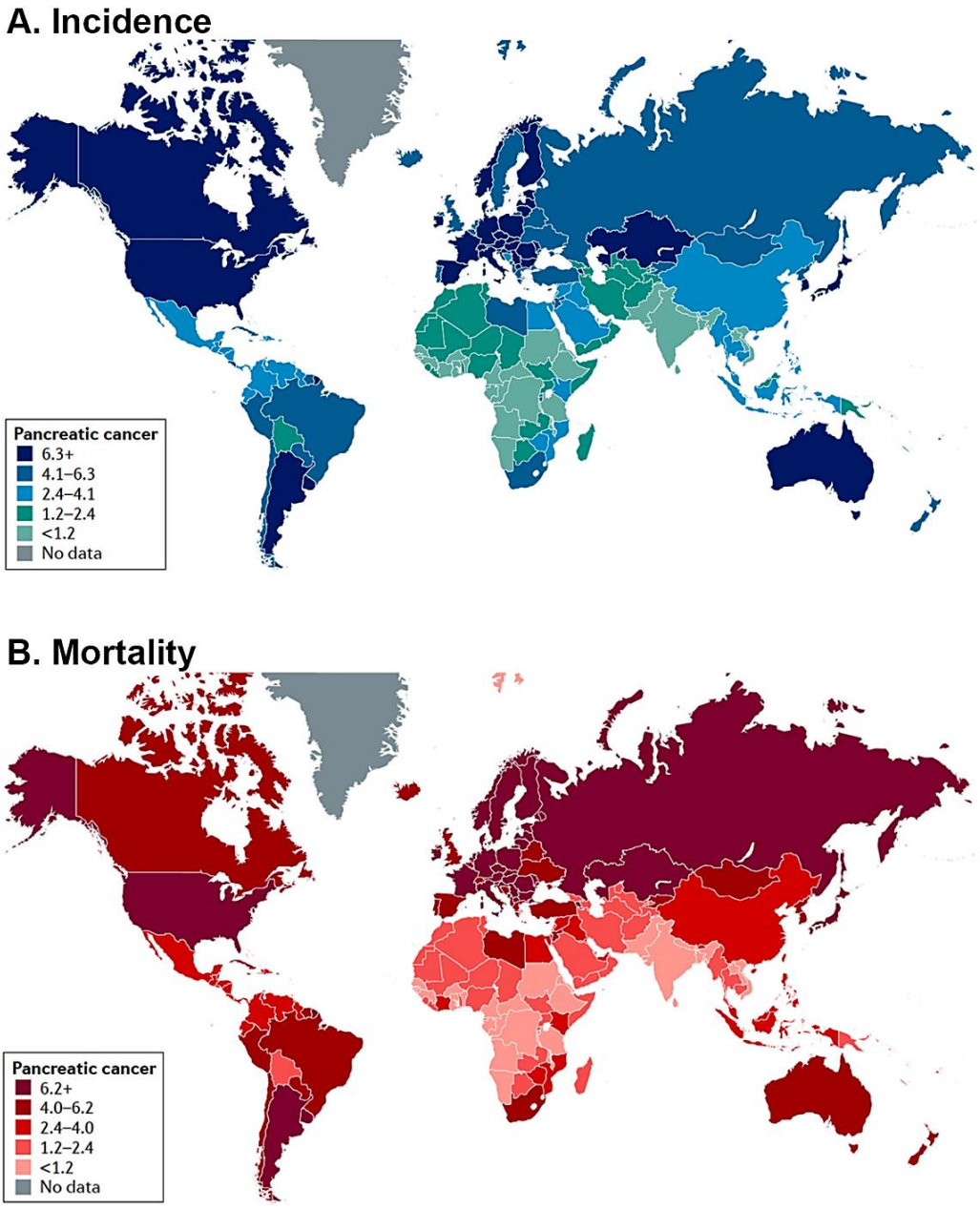

Figure 1. Worldwide estimated rates of incidence (A) and mortality (B) of pancreatic cancer for both sexes (per 100,000 persons) in 2012 (adapted from [2]).

In the United States of America, pancreatic cancer is the fourth leading cause of death [3, 4], having a survival rate of only 3 % after diagnosis [5]. In fact, despite of the progress in the methods used for diagnostic and treatment of this disease, the survival rate of pancreatic cancer patients has not been increased [4].

Pancreatic cancer arises when exocrine and endocrine cells of the pancreas begin to proliferate without any control and form a cancerous mass of cells. This uncontrolled cellular proliferation appears when the DNA of the cells is damaged and therefore mutated [6]. Such DNA damages or variations occur as result of: i) Demographic factors (age, sex, ethnic origin); ii) Genetic factors (family history) and iii) Environmental/lifestyle factors (cigarette smoking, occupational exposures to carcinogens, diet) [6]. Among these factors, the age, smoking and family history are the most important in pancreatic cancer development. In particular, cigarette smoking accounts for 25–29 % of pancreatic cancer incidence [6, 7].

1.2. Pancreatic cancer treatment

The treatment applied to patients suffering from pancreatic cancer depends on several factors, such as the pathological and molecular characteristics of the cancer, as well as the location and the state of the cancer. The main treatments applied to pancreatic cancer comprise: i) surgery, ii) radiotherapy and iii) chemotherapy [8]. Surgery (removal of cancer tissue from the body) appears as the first treatment option and can effectively eradicate the cancer mass when the cancer was diagnosticated in the early years [8, 9]. However, in some cases, due to the stage of cancer development and metastasis, and general health state of the person, not all patients are candidates for surgery [8, 10]. Radiotherapy includes the use of ionizing radiation in the region of the tumor mass in order to kill the cells, or to reduce the size of the tumor mass before its removal by surgery [8, 10]. On the other hand, Chemotherapy uses drugs to kill cancer cells by avoiding cell division and proliferation [11]. This type of treatment is usually prescribed to the patients with any stage of the pancreatic cancer, *i.e.*: i) before surgery in order to shrink the tumor mass (neoadjuvant treatment); ii) after surgery in order to prevent cancer from recurring (adjuvant treatment); and iii) as advanced treatment of those pancreatic cancers that cannot be removed by surgery [8-10].

Currently, the Food and Drug Administration (FDA) approved chemotherapeutic drugs for the treatment of pancreatic cancer are the ABRAXANE (Albumin-bound Paclitaxel), Fluorouracil (5-FU), Gemzar® (Gemcitabine) and ONIVYDE® (Irinotecan liposome injection) [11]. Yet, despite of the large amount of drugs available for pancreatic cancer treatment, these are not specific to pancreatic cancer cells, and after their administration in the bloodstream, they will kill the cancer cells but also damage the healthy tissues [11]. Consequently, Chemotherapy is associated with several side effects, such as vomiting, hair loss and weakness [12]. Furthermore, a major disadvantage of Chemotherapy is its poor therapeutic efficacy after

several treatments due to the cells acquired resistance towards the drugs administered [8, 13, 14].

1.3. Pancreatic cancer drug resistance

Pancreatic cancer cells drug resistance is the main cause of Chemotherapy failure [4]. In fact, chemotherapeutic treatment, a reduction in the tumor mass can be induced but after several administrations of the drug, cancer cells become resistant to them leading to a process called cancer recurrence or relapse, *i.e.* cancer cells are able to circumvent drugs cytotoxicity and rebuild the tumor mass [13]. In case of pancreatic cancer, Ficher *et al.* showed that 37 % of the 35 patients analyzed had recurrent tumors during adjuvant chemotherapy [15]. Further, the survival of patients with tumor recurrence was only 9.3 months, while the median overall survival of patients without early relapse was 26.3 months [15].

The causes of pancreatic cancer cells resistance towards the cytotoxic effect of the drugs are prompted by their: i) reduced drug uptake due to altered surface receptors/carriers; ii) overexpression of drug efflux pumps; iii) reduced ability to undergo apoptosis; iv) increased DNA repair capacity – Figure 2, (reviewed in detail [16, 17]).

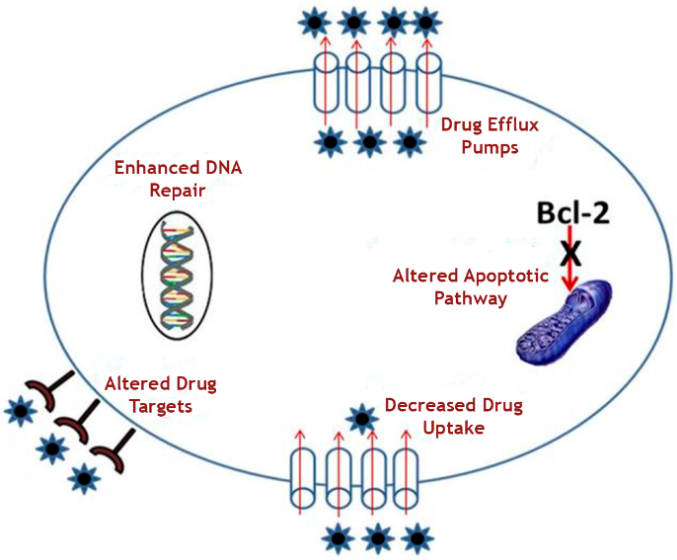

Figure 2. Mechanisms of multidrug resistance exhibited by pancreatic cancer cells (adapted from [16]).

Among the mechanisms adopted by cancer cells to acquire resistance to drugs, the overexpression of genes that codify efflux pumps, such as P-glycoprotein (P-gp), are the most outstanding in pancreatic cancer. P-gp, also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1), is a transmembrane glycoprotein that transports various substances such as ions, toxins, amino acids and drugs, through the cellular membrane [18]. In pancreatic cancer, 93.3 % of the tumors overexpress P-gp [19]. This overexpression of P-gp leads to an increased efflux of drugs, such as taxanes (Paclitaxel), vinca

alkaloids (Vinblastine) and anthracyclines (Doxorubicin (DOX)), from the cells. Consequently, occurs a reduced accumulation of the drugs inside the cells preventing them from performing a significant anticancer effect [20, 21]. In fact, Hoffmann *et al*. found that the P-gp levels increase in pancreatic cancer cells after their treatment with some cytotoxic drugs both in *in vitro* and *in vivo* experiments, showing that the increase of the drugs efflux after chemotherapy is a resistance mechanism [22].

1.4. Combination therapy for surpassing pancreatic cancer cells resistance to drugs

The single administration of drugs (monotherapy) has not accomplished the expected therapeutic outcome in pancreatic cancer. Therefore, combination therapy, a treatment modality that combines two or more therapeutic agents, emerged as a possible therapeutic solution in 60s [23]. Usually, the drugs used in the combination therapy act on different pathways or work by different mechanisms in an additive or synergistic manner, demonstrating a higher anticancer potential than the single administration of these drugs with significantly less toxic effects, since lower doses of drugs are needed to obtain a high therapeutic effect [24-27].

Researchers believe that drug combinations can be used to block a particular resistance mechanism of drug resistance presented by tumor cells by administrating a cytotoxic drug with a drug that reverse the mechanism of drug resistance [28]. Having this in mind, the use of P-gp inhibitors in combination with cytotoxic drugs (that are expelled from the cancer cells by the P-gp) have been investigated [29-31]. Such combinatorial approach will lead to a reduced efflux of the cytotoxic drug from the cells. Until now, a large number of P-gp inhibitors have been tested in clinical trials [32]. The obtained results revealed that these inhibitors act by blocking the drug binding site to the P-gp (either competitively, non-competitively or allosterically) or by inhibiting the P-gp expression (Table 1) [33].

Table 1. Examples of P-gp inhibitors grouped accordingly to their mechanisms of action.

Mechanism of action	Drug/Compound	Ref(s).
Block the drug binding site to the P-gp	Crizotinib	[34]
	Itraconazol	[35]
	Lapatinib	[36]
	Motesanib (AMG-706)	[36]
	Quinidine	[37]
	Tamoxifen	[37]
	Verapimil	[37]
Inhibition of the P-gp expression	Curcumin	[38]
	Nilotinib (AMN-107)	[36]
	Quercetin	[39, 40]
	Resveratrol (RES)	[31, 41]
	Trifluoperazine	[42]
	Trythanthrin	[42]

Borska *et al*. observed that the administration of Quercitin (P-gp inhibitor) and Daunorubicin (cytotoxic drug) on pancreatic cancer cell lines reduced their expression of P-gp and consequently sensitizes the cells to Daunorubicin [39]. In another study, it was showed that the Verapamil targets P-gp and it improves the Gemcitabine cytotoxic effect on pancreatic cancer cells [43].

1.4.1. Resveratrol as an inhibitor of P-gp function

Resveratrol (RES) is a natural polyphenolic molecule that was first isolated by Takaoka from the roots of white hellebore in 1939 [44]. Furthermore, RES has been also found in grapes, wine, peanuts, mulberries, pines, berries, and other flora (Figure 3) [31, 45, 46].

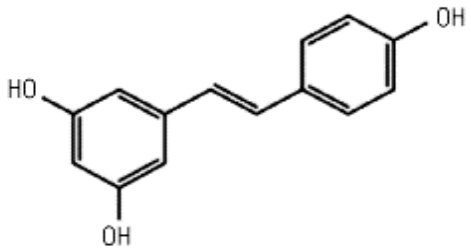

Figure 3. Chemical structure of RES (adapted from [47])

This compound acts in the prevention of cancer due to its antioxidant and anti-inflammatory/immunoregulatory capacities [48, 49]. Jang *et al*. demonstrated the cancer-chemopreventive activity of resveratrol and suggested that this compound possesses the

ability to inhibit all phases of carcinogenesis, such as initiation and progression [50]. Additionally, RES has been shown to induce growth inhibition, cell cycle arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines [51]. Particularly, in pancreatic cancer cells, RES has been shown to directly inhibit the proliferation and viability of the cells *in vitro* in a dose- and time-dependent manner [52-57]. For instance, Zhou *et al*. demonstrated that Capan-2 and Colo357 pancreatic cancer cells viability was affected by RES [57]. Moreover, RES has the ability to inhibit efflux pumps such as P-gp [31, 38, 41, 45, 58, 59]. For instance, Huang *et al*. [60] and Kim *et al*. [41] demonstrated that RES is able to decrease expression of the *MDR-1* gene (gene encoding P-gp) in different types of breast cancer cells (MCF-7, MDA-MB-231). Furthermore, Al-Abd *et al*. also reported that the administration of RES allowed to improve the accumulation of DOX in several cancer cell lines (MCF-7 (breast cancer), HepG2 (liver cancer), HeLa (cervical cancer)), and therefore potentiate the cytotoxicity of the DOX [31]. Still, from the best of our knowledge, the effect of RES in pancreatic cancer cells P-gp activity was not investigated up to now.

1.5. *In vitro* investigation and screening of drug combinations

Before drug combinations be used in the clinic, different studies must be conducted to evaluate a possible synergistic effect of the drugs. For that purpose, prior to the drug combination analysis in humans (clinical trials), preclinical drug combination studies must be performed *in vitro* and/or in animals [61]. For this purpose, 2D cell cultures remain as the most commonly used *in vitro* model to characterize drugs combination synergism due to its simplicity, reproducibility and low cost [62-64]. Nevertheless, flat 2D cell culture models are unable to reproduce the properties of solid tumors as well as their resistance to therapeutics [65, 66]. Consequently, as discussed previously by Ocana *et al.*, there is no correlation between the observed clinical activity of drug combinations and the synergetic data obtained in preclinical models, due to the limited number of preclinical studies that used appropriate methods to study the synergy of drug combinations [67].

Therefore, new and improved *in vitro* models, that are able to reproduce more closely the features *in vivo* human tumors and their resistance to therapeutics, have been investigated to better predict the synergism of drug combinations [64, 68, 69]. Having this in mind, *in vitro* 3D cell culture methodologies namely spheroids, emerged as a viable *in vitro* platform for the investigation of the synergistic effect of drug combinations, once they mimic several properties of real human tumors, as well as their drug resistance mechanisms, namely the up-regulated expression of P-gp (discussed hereafter).

1.5.1. Spheroids' tumors properties and resistance against therapeutics

Spheroids are 3D cellular aggregates that mimic most of the characteristics of *in vivo* tumors. There characteristics comprise: i) hypoxia; ii) altered energy metabolism; iii) low pH; iv) cell cycle arrest; v) extracellular matrix (ECM) proteins deposition, and vi) cell-ECM and cell-cell

interactions, gathering to spheroids a drug resistance profile similar to that demonstrated in different tumors, such as pancreatic cancer (Figure 4).

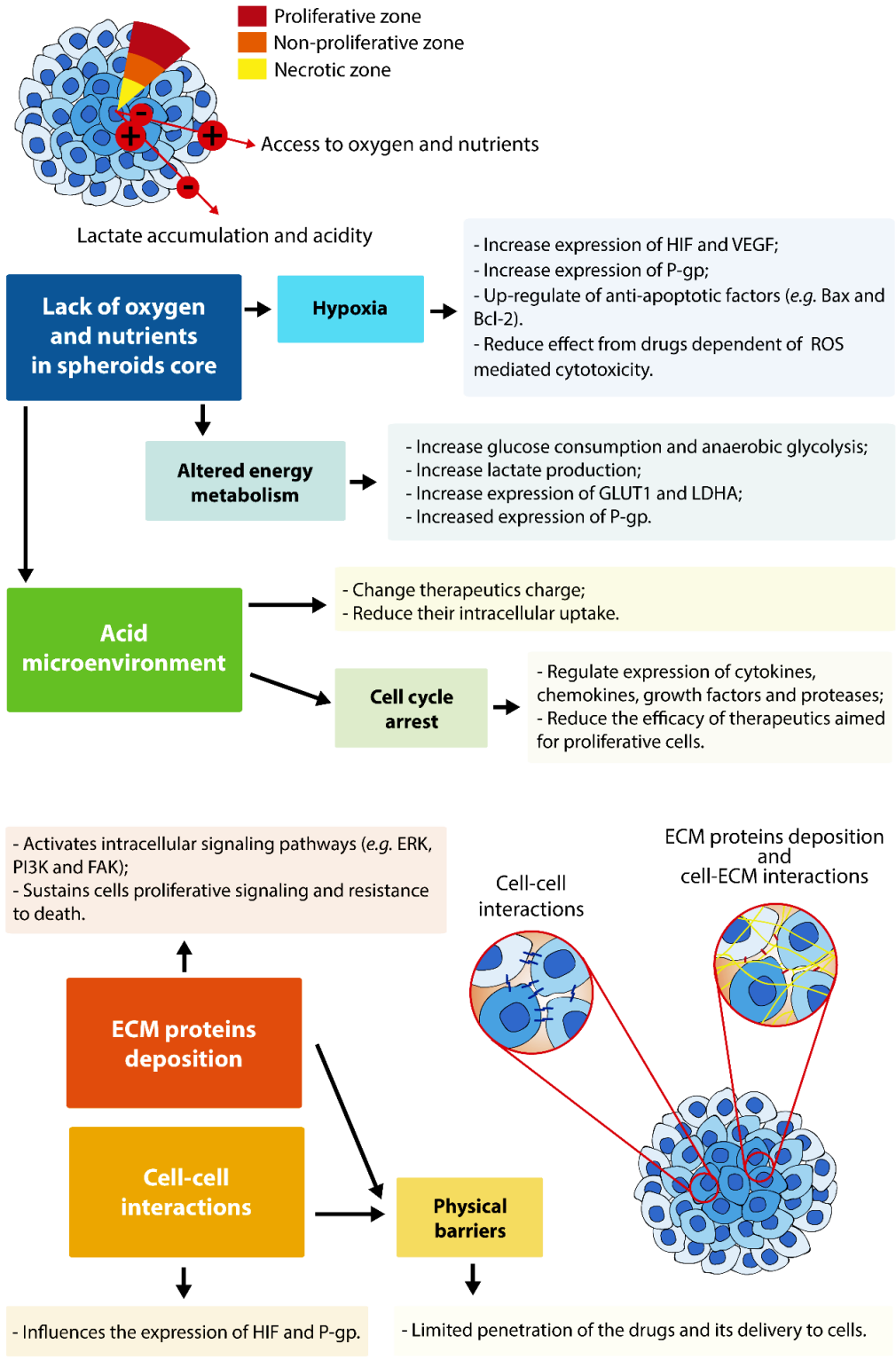

Figure 4. Overview of the spheroids properties which are similar to those found on human tumors (hypoxia, altered energy metabolism, acidic environment, cell cycle arrest, ECM proteins deposition, cell-ECM and cell-cell interactions) as well as their drug resistance mechanisms.

1.5.1.1. Lack of oxygen

Cells in the interior of the human tumors have limited access to the tumor vessels [70]. This impaired blood supply to tumor cells leads to the establishment of a gradient of oxygen, *i.e.* cells in the external layer of the tumor have high access to oxygen while those within the tumor (apart 100 μm of tumor vessels) are in hypoxia [71]. Spheroids due to their 3D cellular organization also create an oxygen gradient, that leads to formation of a hypoxic environment in its inner regions (Figure 4). A study performed by Arai *et al.* analyzed the oxygen content in spheroid cultures of Capan-2, PANC-1 and MIA PaCa-2 pancreatic cancer spheroids demonstrating that hypoxic areas occur in all the spheroids [72].

As consequence of the hypoxic environment found in spheroids, cells display an up-regulated expression of hypoxia-inducible family factors (HIF), as well as *MDR1* gene that codifies the P-gp. Wartenberg *et al.* reported that the expression of both HIF-1α and P-gp was up-regulated in DU-145 prostate tumor spheroids (994 and 388 %, respectively) [73]. In another study, Doublier *et al.* verified that the HIF-1 activation also occurs in MCF-7 breast cancer spheroids, whereas no significant alterations were noticed in MCF-7 cells cultured in monolayer [74]. Additionally, these authors also concluded that the expression of HIF-1α was essential for the P-gp production, which was correlated with the reduced DOX accumulation in the cells that form the spheroids [74].

1.5.1.2. Variations in cells' energy metabolism

As in human tumors, spheroids also have an altered energy metabolism. In *in vivo* tumors, the reduced oxygen content available in the inner regions of the tissue leads to the production of lactate as a result of the anaerobic degradation of glucose [75]. Longati *et al.* observed that the mRNA expression ratio of glucose transporter 1 (GLUT-1; predominant glucose transporter in many types of cancer cells) and lactate dehydrogenase (LDH; enzyme responsible for the lactate production) on the PANC-1 3D/2D cultures was approximately 7.5 and 3.5, respectively (Figure 4) [76]. In the literature, it has been reported that the increase of GLUT-1 and lactate production due to the high glycolytic rate of the cancer cells can lead to drug resistance through the altered expression of P-gp [77]. Additionally, Wartenberg and colleagues demonstrated that the downregulation of glycolysis by the administration of Iodoacetate or 2-Deoxyglucose reduced the P-gp expression in DU-145 and Gli36 glioma spheroids [78].

1.5.1.3. Acidic microenvironment

In human tumors the increased production of lactate by oxygen deprived cells, which have a high glycolytic rate originates an acidic environment (pH of 6.5–7.2) [70, 79]. In spheroids, the lactate production also promotes the acidification of its core (Figure 4) [80]. Carlsson *et al.* observed that spheroids (*e.g.* HT29 colon carcinoma, U-251 MG glioma and HTH-7 thyroid carcinoma spheroids) pH decreased within their structure, *i.e.* the spheroids deepest regions presented lower pH values [81]. These low pH values have an impact on drugs efficiency by

affecting their cellular uptake [82, 83]. For instance, weak basic drugs with a dissociation constant of 7.5–9.5 (*e.g.* DOX, Mitoxantrone, Vincristine, Vinblastine, anthraquinones and vinca alkaloids) are protonated in acidic environments. As result, the cellular uptake of these drugs is reduced, since charged drugs are less internalized by cancer cells [82, 84].

This influence of spheroids pH in drugs uptake was initially verified by Swietach *et al.* [85]. These authors showed that the DOX uptake was proportional to the HCT116 colon cancer spheroid-depth, *i.e.* the deepest and acidic regions of the spheroids presented the lowest cellular drug uptake (1.7-fold lower at pH =6.4 than at pH = 7.4) [85]. Consequently, the half maximal inhibitory concentration (IC_{50}) of DOX was higher at pH = 6.4 [85].

1.5.1.4. Cell cycle arrest

Another feature of the *in vivo* tumors that the spheroids can mimic is the cell cycle arrest. The acidic pH, in association with the lack of oxygen and nutrients induces a dormant state on cells, *i.e.* quiescence or senescence, to the cells in the tumors and in the spheroids (Figure 4) [86]. Despite that, these cells are able to express cytokines, chemokines and growth factors involved in tumorigenesis (reviewed in [86]). Therefore, the cells dormancy in the inner region of the spheroids can contribute for their therapeutics resistance profile.

Barrera-Rodríguez and Fuentes stated that INER-37 and INER-51 lung cancer spheroids are more resistance towards Etoposide, Teniposide, DOX or Camptothecin than 2D cell cultures. A result that was explained by the quiescent cell subpopulation found in the spheroids [87]. In a different study, Gong *et al.* analyzed the cell cycle of MCF-7 cells cultured in monolayer or spheroids through flow cytometry [88]. The obtained data revealed that spheroids possess an increased number of cells in quiescence, *i.e.* spheroids presented 58.48 % of the cells trapped in G0-G1 phase of cell cycle, contrasting with the 40.76 % of the cells cultured in 2D [88]. Accordingly, when incubated with DOX, the MCF-7 cells cultured in monolayers presented an increased cellular death, in fact the IC_{50} of this drug was approximately 50-, 60-, and 80-fold higher for spheroids with 300, 400 and 500 μm diameter, respectively [88].

The non-proliferative state of cells within spheroids can also be responsible for a poor therapeutic efficacy of drugs that are more efficient in proliferative cells, such as Carboplatin, Cisplatin, DOX, Oxaliplatin, Methotrexate and Paclitaxel [89]. Usually, the cytotoxic effect mediated by these types of drugs is dependent on their covalent or noncovalent interaction with the DNA during the process of cellular replication [89]. Having this in mind, Imamura *et al.* evaluated the expression of Ki-67 (a proliferative biomarker) and the effect of Paclitaxel on 2D cell cultures and spheroids of BT-549, BT-474 and T-47D cells (breast cancer cell lines) [66]. The results demonstrated that, for instance, the BT-549 cells presented 84 % of Ki-67 positive cells when cultured in 2D, whereas this value decreased to 46.5 % when they were cultured in spheroids, suggesting that spheroids have greater G0-dormant subpopulation that is responsible for its resistance to Paclitaxel [66].

1.5.1.5. ECM proteins deposition

Spheroids are also able to mimic the ECM protein expression profile that occurs in tumors (Figure 4). Nederman *et al*. studied the presence and expression of ECM proteins in U-118 MG glioma and HTH-7 thyroid cancer spheroids and reported that spheroids were able to produce ECM components such as collagens (type I, III and V), fibronectin and laminin [90]. Subsequently, the same research group observed that the expression of ECM proteins (*e.g.* fibronectin) was more pronounced when glioma cells were cultured in spheroids than when the cells were maintained in 2D cultures [91].

The produced ECM proteins in spheroids not only provide support to cancer cells, but also influence cells sensitivity towards the therapeutics. The ECM molecules (*e.g.* fibronectin, collagen, laminin, hyaluronate, heparan sulfate, elastin, among others) interaction with cell surface receptors (*e.g.* discoidin receptors, syndecans, and mainly integrins) can activate intracellular signaling pathways, like PI3K (phosphoinositide 3-kinase) and FAK (focal adhesion kinase) involved in cancer cells proliferation and survival (reviewed in [92]).

Bai *et al*. verified that the increased expression of ECM proteins (*e.g.* collagen and fibronectin-1) in 3D cultures of soft sarcoma (HT1080, RD, SW872) and osteosarcoma (HOSS1) cell lines contributed for the establishment of a chemoresistant environment to DOX, Gemcitabine and Docetaxel [93]. In brief, the mRNA expression of *COL1A1* (gene that encodes the major component of collagen I) and *FN1* (gene that encodes fibronectin) in HOSS1 cells were 2 and 4-folds higher in the spheroids than in 2D cell cultures, respectively [93]. Accordantly, the IC_{50} values of DOX, Gemcitabine and Docetaxel in HOSS1 cells were superior in spheroids (4.61, 23.55 and 103.2 μM, respectively) than in 2D cell cultures (0.078, 6.23 and 6.72 μM, respectively) [93].

1.5.1.6. Cell-cell interactions

The cell-cell interactions are influenced by the cellular arrangement of the cells, *i.e.* the cell-cell interactions are more pronounced in 3D cellular structures, than in the 2D cells cultures (Figure 4). The increased number of cell-cell interactions established in spheroids can control the behavior of cancer cells, namely in their cell signaling, survival, proliferation and drug sensitivity. Among the cell–cell adhesion receptors, E-cadherins play an important role on tumor cells behavior [94]. The expression levels of E-cadherin influence the therapeutic response of cells to the drugs [95]. Several studies already revealed that the expression of E-cadherins is higher in spheroids than in 2D cultures [95-97]. For instance, E-cadherins expression was more than 5-folds higher in 14 days old spheroids formed with carcinoma cells derived from differentiated hepatocytes (Huh7) than in their 2D cell cultures [96].

Xu *et al*. also demonstrated that the 3D cultures of ovarian cancer cells display increased E-cadherin expression in spheroids with larger volumes, tighter cellular connections, and longer

survival times [95]. Additionally, authors also reported that the expression levels of E-cadherin influence the therapeutic response of cells to drugs [95]. In fact, Cisplatin was less effective in SK-H spheroids than in SK-N or OV-L spheroids (47.5, 60.3 and 58.0 % of death, respectively), a fact attributed to the higher expression of E-cadherins in SK-H spheroids [95].

1.5.1.7 Physical barriers

In tumors, the deposition of ECM proteins, the cell-ECM and cell-cell interactions increase the tissue density and form a physical barrier that limits the penetration of the compounds and their delivery to cells - also known as limited mass transport effect [71, 98]. The deposition of ECM proteins and the close physical interactions among the cells also lead to an increased interstitial fluid pressure (IFP). This IFP contributes for the impaired penetration of pharmaceuticals by convection [71, 98].

The limited drug penetration prompted by the physical barrier was already demonstrated in spheroids (Figure 4) [76, 99]. Longati *et al*. demonstrated that PANC-1 spheroids create a matrix-rich environment composed of various proteins (*e.g.* collagen I, fibronectin I and lumican) that limits the drugs penetration thus increasing its resistance towards different therapeutics, like Gemcitabine [76]. In other study, Wang and colleagues demonstrated that DOX have a poor perfusion in SH-SY5Y neuroblastoma spheroids. The drug penetration strictly limited by the cell layers present at the surface of the spheroids (≈ 70 µm from the periphery of the spheroids) [99].

1.6 Aims

The main aim of this dissertation work plan was the investigation of drug combination (DOX and RES) therapeutic effect on 2D (monolayers) and 3D (spheroids) cell cultures of PANC-1 models. Furthermore, the influence of the cell culture type in the efficacy and synergistic potential of a drug combination was also evaluated on 2D and 3D cell cultures.

The specific aims of this dissertation include:

- Determination of the drug-response curves of DOX and RES (administrated separately) in 2D and 3D *in vitro* PANC-1 models;
- Calculation of the 50 % IC_{50} of DOX and RES in the 2D and 3D *in vitro* PANC-1 models;
- Analysis of the DOX:RES combinations influence on 2D and 3D *in vitro* PANC-1 models cell viability;
- Evaluation of the influence of RES on DOX efflux from the cells and its accumulation;
- Determination of the drug-response curves of 1DOX:4RES and 1DOX:5RES in 2D and 3D *in vitro* PANC-1 models;
- Calculation of the 50 % IC_{50} and CI of 1DOX:4RES and 1DOX:5RES in the 2D and 3D *in vitro* PANC-1 models;
- Comparison of the effect of DOX, RES and DOX:RES in PANC-1 cells viability when cultured as 2D monolayers and 3D spheroids.

Chapter II

2. Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle medium-high glucose (DMEM-HG), gentamycin, PANC-1, paraformaldehyde (PFA), phosphate-buffered saline solution (PBS), resazurin, streptomycin, and trypsin were acquired from Sigma-Aldrich (Sintra, Portugal). Cell culture plates and T-flasks were obtained from Thermo Fisher Scientific (Porto, Portugal). Agarose was bought from Grisp (Porto, Portugal). Fetal bovine serum (FBS) was supplied by Biochrom AG (Berlin, Germany). DOX and RES were purchased from Carbosynth (Berkshire, UK). Cell imaging plates were acquired from Ibidi GmbH (Ibidi, Munich, Germany). The stock solutions of DOX and RES were prepared in methanol obtained from VWR International (Portugal).

2.2 Methods

2.2.1 Cells maintenance and 3D PANC-1 spheroids formation

PANC-1 cells were cultured in DMEM-HG supplemented with FBS (10 % (v/v)) and streptomycin and gentamycin (1 % (v/v)) in 75 cm^2 T-flasks, inside an incubator with a humidified atmosphere at 37 °C and 5 % CO_2 [100]. Spheroids formation was performed as previously described by our group [101]. In brief, agarose structures with spherical microwells were obtained by placing agarose 2 % ((w/v) in H_2O) in micromolds (Microtissues Inc., Providence RI, US). Then, after the sterilization of the agarose structures (UV radiation, 60 min), PANC-1 cells were seeded on the agarose structure (1 x 10^6 cells/agarose structure). After some hours, cells start to aggregate spontaneously in the microwells, allowing the assembly of 81 spheroids/agarose structure. Spheroids used in the following experiments grew during 10 days until they reach a mean diameter of 662.6 ± 70.0 µm (analysis performed by using ImageJ software (National Institutes of Health) [102, 103]). During this period, the medium was changed every 2 days.

2.2.2 Assessment of the cytotoxic activity of DOX and RES in 2D PANC-1 cell cultures

The cytotoxicity of DOX or RES towards PANC-1 cells was evaluated through the resazurin method [104]. In brief, cells were seeded in 96-well culture plates at a density of 10 x 10^3 cells/well. After 24 h, cells were incubated with DOX (0.1-200 µM) and RES (100-600 µM) for 24 h. Non-treated cells were used as negative control (K-). Afterwards, the medium was removed and replaced with medium containing resazurin (10 % (v/v)) for 4 h (37 °C, 5 % CO_2). Then, PANC-1 cells viability was determined by analyzing the fluorescence of resorufin ($\lambda_{ex}/\lambda_{em}$ = 560/590 nm) in a Spectramax Gemini EM spectroflorometer (Molecular Devices LLC, CA, USA). Subsequently, the drugs' dose-response curves were traced in order to determine the DOX and

RES 50 % IC_{50} using OriginLab software (trial version, OriginPro, OriginLab Corporation, MA, USA) [104, 105].

2.2.3 Assessment of cytotoxic activity of DOX and RES in 3D PANC-1 spheroids

3D PANC-1 spheroids were incubated during 24 h with fresh medium containing DOX and RES. Non-treated spheroids were used as negative control (K-). For each condition, a total of 45 spheroids were used. After 24 h of drugs being incubated with spheroids, the resazurin assay was performed to determine cells' viability and the 50 % inhibitory concentration of DOX and RES (as described in section 2.2.2.).

2.2.4 Screening of DOX:RES combinations in 2D and 3D PANC-1 cell cultures

For an initial screening of the DOX:RES combinations effect on PANC-1 cells viability, PANC-1 cells seeded in 2D (as described in 2.2.2.) and 3D PANC-1 spheroids (as described in 2.2.3.) were incubated with three different concentrations of DOX:RES (10.1, 46.3 and 106.3 μM) at several molar ratios (ranging from 5:1 to 1:5) during 24 h (Table 2).

Table 2. DOX and RES molar concentrations used in each DOX:RES ratio.

DOX:RES ratio	10.1 μM of DOX:RES		46.3 μM of DOX:RES		106.3 μM of DOX:RES	
	DOX (μM)	RES (μM)	DOX (μM)	RES (μM)	DOX (μM)	RES (μM)
5:1	8.42	1.68	38.58	7.72	88.58	17.72
4:1	8.08	2.02	37.04	9.26	85.04	21.26
3:1	7.58	2.53	34.73	11.58	79.73	26.58
2:1	6.73	3.37	30.87	15.43	70.87	35.43
1:1	5.05	5.05	23.15	23.15	53.15	53.15
1:2	3.37	6.73	15.43	30.87	35.43	70.87
1:3	2.53	7.58	11.58	34.73	26.58	79.73
1:4	2.02	8.08	9.26	37.04	21.26	85.04
1:5	1.68	8.42	7.72	38.58	17.72	88.58

These concentrations correspond to the 20, 50 and 80 % inhibitory concentrations of DOX (IC_{20}, IC_{50} and IC_{80}, respectively) and were selected in order to be possible to compare the effect of DOX:RES combinations with the single administration of DOX. Non-treated cells were used as negative control (K-). Then, cells' viability was then evaluated through the resazurin method as described above (section 2.2.2.).

For a more detailed comparison of the DOX:RES combination potential in 2D and 3D cell cultures of PANC-1 cells, the synergism of the most effective ratios (1:4 and 1:5 DOX:RES) was compared in PANC-1 monolayers and spheroids. For this end, both models were incubated with the 1:4 (1-150 μM) and 1:5 (1-200 μM) DOX:RES combinations (as described above). Subsequently, the

1DOX:4RES and 1DOX:5RES dose-response curves and IC_{50} values were determined (see section 2.2.2.). Additionally, the Combination Index (CI) of the 1DOX:4RES and 1DOX:5RES combinations were determined for both 2D PANC-1 cell cultures and 3D PANC-1 spheroids at inhibition levels of 50 %, by using the Chou-Talalay method [106], following the Equation 1:

$$\mathrm{CI} = DOX_C / DOX_M + RES_C / RES_M \qquad \textbf{(1)}$$

where DOX_c and RES_c are molar concentrations of DOX and RES that in combination produce a cytotoxicity level of 50 %, while DOX_M and RES_M are the concentrations of the single drugs (monotherapy) which produce the same effect. CI values lower than 1, equal to 1 and higher than 1 indicate that the combination of drugs is synergistic, additive and antagonist, respectively [106].

2.2.5 Efflux of DOX from PANC-1 cells cultured in 2D

The DOX efflux assay was performed following a protocol previously reported in the literature with some modifications [107]. In brief, PANC-1 cells (seeded as described in 2.2.2.) were treated with DOX:RES combinations (46.3 μM) at the different molar ratios (5:1 to 1:5). Afterwards, the fluorescence of the medium in the wells (containing the DOX that is not inside the cells) was measured after 12 h of incubation on a Spectramax Gemini EM spectrofluorometer (Molecular Devices LLC. CA. USA) using an $\lambda_{ex}/\lambda_{em}$ = 470/585 nm. For comparison purposes, the DOX fluorescence intensity values for a specific ratio (DOX fluorescence_C) was normalized to the intensity of cells treated only with DOX, at the same DOX molar concentration that is present on the specific ratio (DOX fluorescence_M), following the Equation 2:

$$\text{Normalized effluxed DOX}\ (\%) = (DOX\ fluorescence\ _C / DOX\ fluorescence_M) \times 100 \qquad \textbf{(2)}$$

2.2.6 Accumulation of DOX in PANC-1 cells cultured in 2D and 3D

The analysis of the DOX intercellular accumulation in PANC-1 cells was performed by adapting protocols available in literature [41, 88, 108, 109]. For this study, 2D cell cultures (seeded as described in 2.2.2.) and 3D PANC-1 spheroids (prepared as described in 2.2.3.) were used. In brief, these cell culture models were incubated with medium containing DOX:RES combinations (46.3 μM) at different molar ratios (5:1 to 1:5). For comparative purposes, cell cultures were also incubated with DOX at the same molar concentrations found in each specific DOX:RES combination. After 24 h, the medium in the wells was removed, cells were chemically fixed with PFA 4 % (during 15 min for 2D cultures and overnight for spheroids) and washed with PBS [102]. Then, samples were imaged by Confocal Scanning Electron Microscopy (CLSM) to observe the accumulation of DOX inside de PANC-1 cells by using a Zeiss LSM 710 confocal microscope (Carl Zeiss AG. Oberkochen. Germany). DOX was visualized by using an $\lambda_{ex}/\lambda_{em}$ = 488/535 – 674 nm.

2.2.7 Statistical Analysis

Data was expressed as mean values ± standard deviation (S.D.). The statistical analysis was performed by using one-way ANOVA test. A *P* value lower than 0.05 (*$P < 0.05$) was considered statistically significant. Data analysis was performed in GraphPad Prism v.6.0 software (Trial version, GraphPad Software, CA, USA).

Chapter III

3. Results and Discussion

In an effort to overcome the lower survival rate of the patients suffering from pancreatic cancer and to fight the drug resistance mechanisms, clinicians have been using drug-combinations to treat this disease instead of administering a single-drug [110-112], once drug combinations can have a synergistic effect that further improves the therapeutic outcome, leading to higher patient's survival rates [113-115]. Drug combinations aimed for pancreatic cancer treatment have been mostly screened in 2D *in vitro* cell cultures [116-119]. However, 2D cell cultures are unable to mimic the resistance profiles displayed by *in vivo* pancreatic tumors [76, 120, 121]. Due to that, 2D monolayers can overestimate the therapeutic potential of drug combinations, leading to a disappointing therapeutic performance in *in vivo* assays [122]. Therefore, it is of major interest to investigate the therapeutic potential of drug-combinations aimed to treat pancreatic cancer in *in vitro* models that better mimic the drug resistance mechanisms presented by *in vivo* pancreatic tumors.

Herein, the therapeutic effect and the synergistic potential of a particular drug-combination towards 2D and 3D cell cultures of pancreatic cancer were compared for the first time. For this purpose, the effect of DOX:RES combinations (at molar ratios ranging from 5:1 to 1:5) on the PANC-1 cells mobility, cultured as 2D monolayers and as 3D spheroids, was analyzed. This drug-combination was selected since it demonstrated a promising anticancer activity towards breast, cervical and liver cancer models [31, 41, 123]. Although, their effect on pancreatic cancer cells was not yet reported. Additionally, RES is a natural polyphenolic molecule found in red grapes that can reduce the efflux of DOX from the cancer cells by down-regulating the *MDR1* gene expression, *i.e.* genes encoding the P-gp (a protein responsible for DOX efflux from the cells) [31].

In order to study different effects of DOX:RES combinations towards PANC-1 cells, an initial screening of the therapeutic capacity of DOX, RES and DOX:RES combinations was first performed. Additionally, the influence of RES in DOX efflux was also screened by fluorescence spectroscopy and microscopy. Then, the DOX:RES combinations with the highest therapeutic potential were thoroughly analyzed in 2D monolayers and 3D spheroids of PANC-1 cells to disclose their IC_{50} and CI values, and thus disclose differences in combinations' synergism.

3.1 DOX and RES cytotoxicity towards 2D and 3D PANC-1 cell cultures

Before studying the therapeutic capacity of DOX:RES towards the 2D and 3D cultures of PANC-1 cells, the effect of DOX and RES (each drug was evaluated separately) in these models was analyzed. In general, DOX display a higher cytotoxic profile towards cancer cells than RES

(Figure 5). In fact, the IC_{50} of DOX towards the 2D PANC-1 cultures was about 6.9-times lower than that of RES (DOX IC_{50} = 46.3 μM; RES IC_{50} = 317.7 μM) (Figure 5 A and C). In other studies, DOX also produced a greater cytotoxic effect on MDA-MB-231 [41], MCF-7, HeLa and HepG2 [31] cells than RES.

Furthermore, comparing the drugs' dose-response curves of cells cultured monolayers and in 3D spheroids, it can be verified that spheroids exhibit a higher resistance to both drugs (Figure 5). Particularly, the IC_{50} of DOX in spheroids was 2.3-times higher to that determined for 2D cell cultures (Figure 5 A and B). This higher resistance to therapeutics can be explained by pancreatic spheroids' higher number of cell-cell and cell-ECM interactions [121], that improve cells' proliferation and, simultaneously, limit the diffusion of the drugs throughout the microtissue [98, 124]. Additionally, spheroids present an up-regulation of the anti-apoptotic agents (*e.g.* Bcl-2 and survivin) [125, 126] and multidrug resistance machinery (*e.g.* drug efflux pumps, such as P-gp [125]), which further increase their resistance towards therapeutics. In other studies, pancreatic cancer spheroids (MIAPaCa-2 and PANC-1 cells) were also more resistant to several drugs (*e.g.* 5-FU, AXP-107-11, DOX, Gemcitabine, H107, among others), when compared to 2D cell cultures [76, 121, 127].

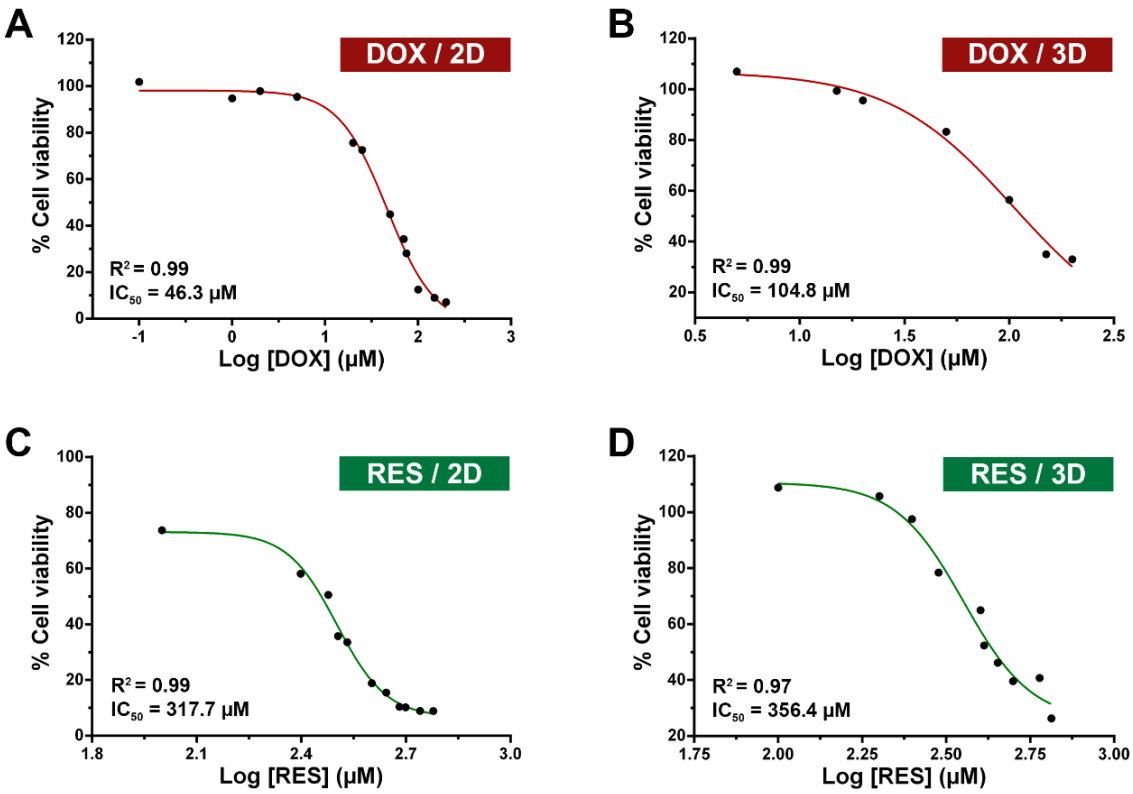

Figure 5. Evaluation of the DOX and RES (monotherapy) effect on PANC-1 cells' viability. Dose-response curves of 2D and 3D (spheroids) cell cultures to DOX (A and B) and RES (C and D) and respective IC_{50} values. Data are presented as mean (n=5).

3.2 Influence of DOX to RES molar ratio on 2D and 3D PANC-1 cell cultures viability

After confirming the higher resistance of 3D spheroids to DOX and RES, a rapid screening of the therapeutic capacity of the different DOX:RES ratios (5:1 to 1:5) was performed. To accomplish that, 2D and 3D PANC-1 models were incubated with the DOX:RES combinations at the concentrations of 20.1, 46.3 and 106.3 μM (these concentrations were selected based on the IC_{20}, IC_{50} and IC_{80} of DOX in 2D cultures, and were selected in order to be possible to compare the effect of DOX:RES combination with the single administration of DOX).

In general, the DOX:RES combinations containing a higher DOX content (2:1, 3:1, 4:1 and 5:1) did not induce a greater reduction in the viability of 2D and 3D cell cultures when compared to the single DOX administration (Figure 6).

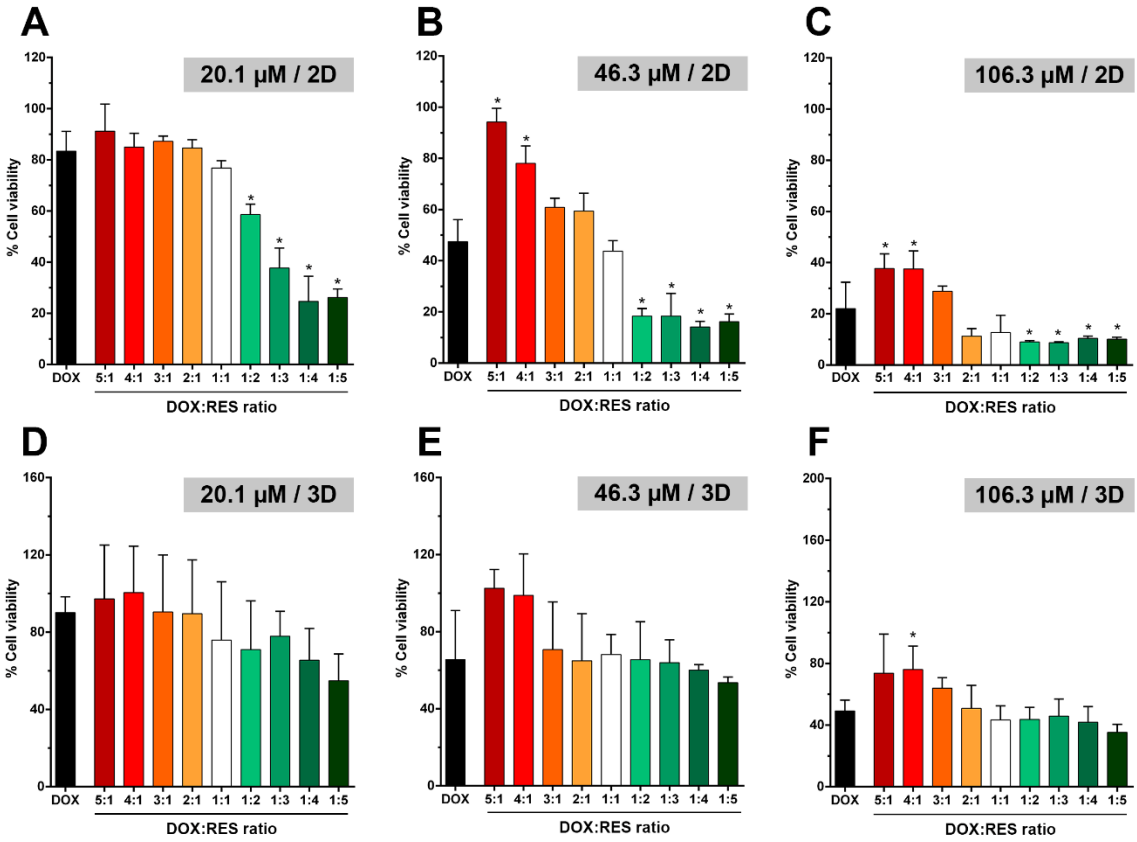

Figure 6. Evaluation of the different DOX to RES molar ratios influence PANC-1 cells' viability. Cell viability of the 2D (A-C) and 3D spheroids (D-F) cell cultures after the administrations of several concentrations (20.1, 46.3 and 106.3 μM) of DOX and DOX:RES (5:1 to 1:5); data are presented as mean ± S.D. (n=5); *p<0.05.

In stark contrast, DOX:RES at 1:1 to 1:5 molar ratios were more effective than free DOX for both types of PANC-1 cell culture models (Figure 6). In particular, the 1DOX:4RES and 1DOX:5RES combinations were, in general, the most effective in the reduction of 2D and 3D cell cultures' viability (Figure 6). Therefore, the DOX:RES combination therapies with a higher RES content are more appealing since these are more effective in the reduction of cancer cells

viability, but also due to the fact that RES is a natural and inexpensive compound. Additionally, DOX:RES combinations with a higher RES content employ a lower DOX dose, which is also appealing since DOX has a high cost and induces severe side effects when used at high concentrations (*e.g.* cardiotoxicity, hepatotoxicity, nephrotoxicity, typhlitis, and other toxicities) [123, 128]. Additionally, previous studies have also demonstrated that RES can also have a significant protective role on the DOX related heart toxicity [128].

3.3 Influence of the DOX:RES ratio on the efflux and intracellular accumulation of DOX

Taking into account that previous reports in the literature showed that RES can improve the accumulation of DOX in MCF-7, MDA-MB-231, HeLa and HepG2 cells' cytoplasm [31, 41, 123], this phenomenon was also investigated for the different DOX:RES ratios administered to PANC-1 cell models (Figure 7 A).

For this end, the DOX efflux from the cells when these were incubated with DOX:RES and only DOX were compared (Figure 7 A - C). The efflux studies revealed that the 2D cultures treated with DOX:RES combinations with a higher content of RES (1:2 to 1:5), for 12 h, displayed the lowest DOX efflux, and this efflux was inversely proportional to the RES content administrated to the cells (Figure 7 B and C). This data is in agreement with the cell viability studies, since the DOX:RES ratios with a higher RES content were the most cytotoxic (Figure 7 A and B).

Motivated by these findings, the accumulation of DOX inside the 2D PANC-1 cell cultures and 3D PANC-1 spheroids were also studied through CLSM, after 12 h of cells being incubated with drugs. For these assays, cells were incubated with DOX:RES combinations and the intracellular DOX accumulation was compared to that occurring when DOX monotherapies were used (controls that contains the same DOX dose used in each drug combination).

In general, for both 2D and 3D models, all the DOX:RES combinations improved the intracellular accumulation of DOX when compared to the single administration of DOX (Figure 7 D and E). Interestingly, even though the 1DOX:4RES and 1DOX:5RES ratios contain a lower amount of DOX, the accumulation of DOX when these ratios were administered to cells was similar or higher to that occurring when the 4DOX:1RES and 5DOX:1RES ratio, that contain higher amounts of DOX, were administrated (Figure 7 D and E). These results further corroborate the improved potential of the 1DOX:4RES and 1DOX:5RES combinations for being applied in the treatment of pancreatic cancer, which it is related with the RES mediated decrease of DOX efflux and increase of DOX accumulation.

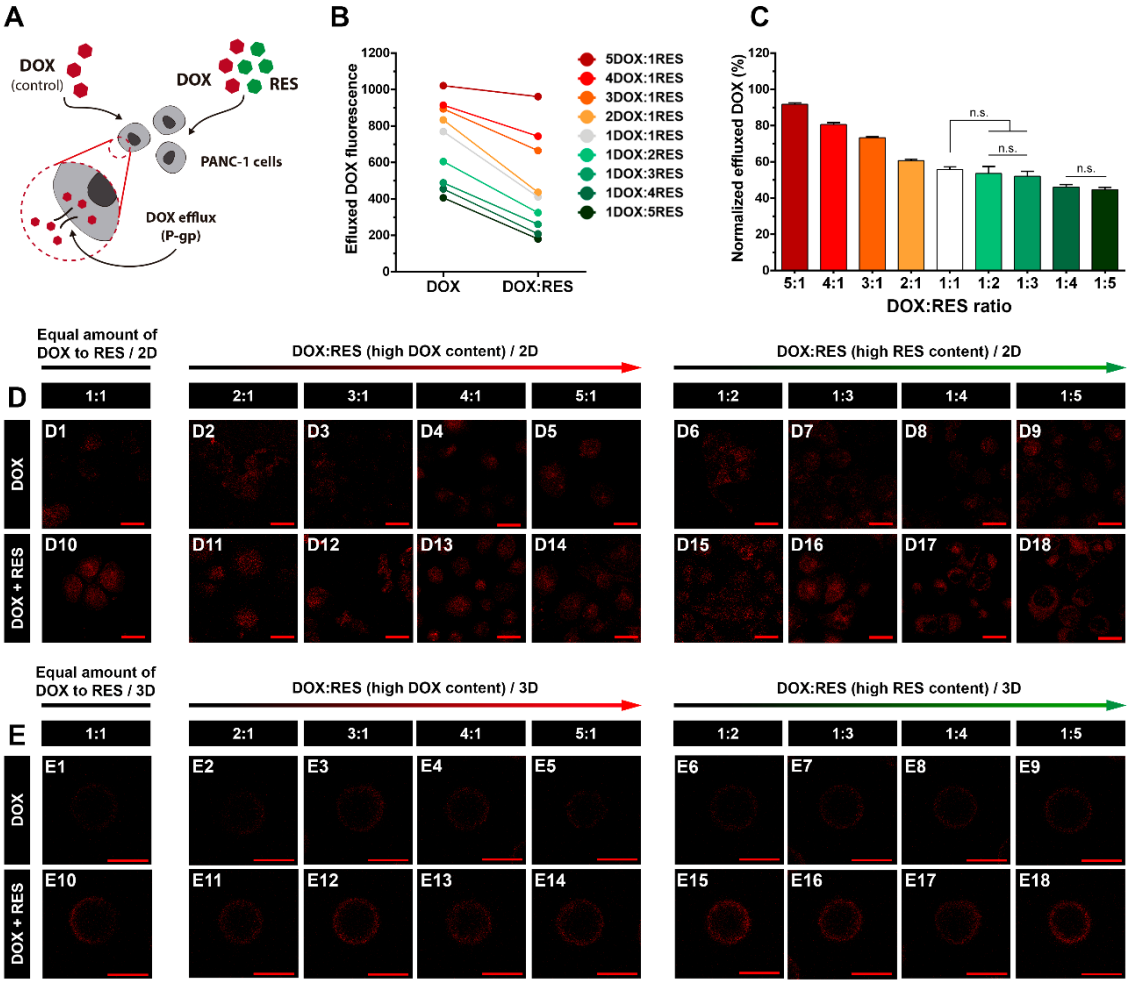

Figure 7. Evaluation of DOX:RES combinations' effect on DOX efflux and accumulation in PANC-1 cells. Schematic representation of the DOX efflux after DOX and DOX:RES administration to cells (A). Comparison and normalization of the DOX effluxed fluorescence in 2D cell cultures, after the incubation of DOX:RES (5:1 - 1:5) during 12 h (B and C); data are presented as mean ± S.D., (n=5); n.s. – non-significant (the differences between the bars not signed with n.s. were statistically significant, $p<0.05$). CLSM images of the intracellular accumulation of DOX in 2D (D) and 3D (spheroids) (E) cell cultures after the incubation of DOX:RES combinations (5:1 - 1:5) during 12 h; DOX accumulation in 2D cell cultures and 3D spheroids incubated solely with the molar concentration of DOX used in each combination were also imaged; red channel: DOX; scale bars correspond to 50 μm.

3.4 Comparison of the DOX to RES combinations at 1:4 and 1:5 molar ratios in 2D vs 3D cell cultures of PANC-1 cells

After confirming that the 1:4 and 1:5 DOX:RES combinations are the most promising, their action towards 2D and 3D (spheroids) PANC-1 cell cultures were evaluated in detail. Initially, the dose-response curves of the combination ratios were traced to determine their IC_{50} (Figure 8 A-D). As expected, the 1:4 and 1:5 DOX:RES combinations demonstrated a higher anticancer effect on 2D cell cultures than the solely incubation of DOX (IC_{50} of DOX was ≈ 6.0- and 6.6-times higher than those of 1DOX:4RES and 1DOX:5RES combinations, respectively). Further investigation revealed that these combinations induce high synergistic effect towards PANC-1

cells monolayers (CI was 0.052 and 0.043 for 1DOX:4RES and 1DOX:5RES combinations, respectively) (Figure 8 F).

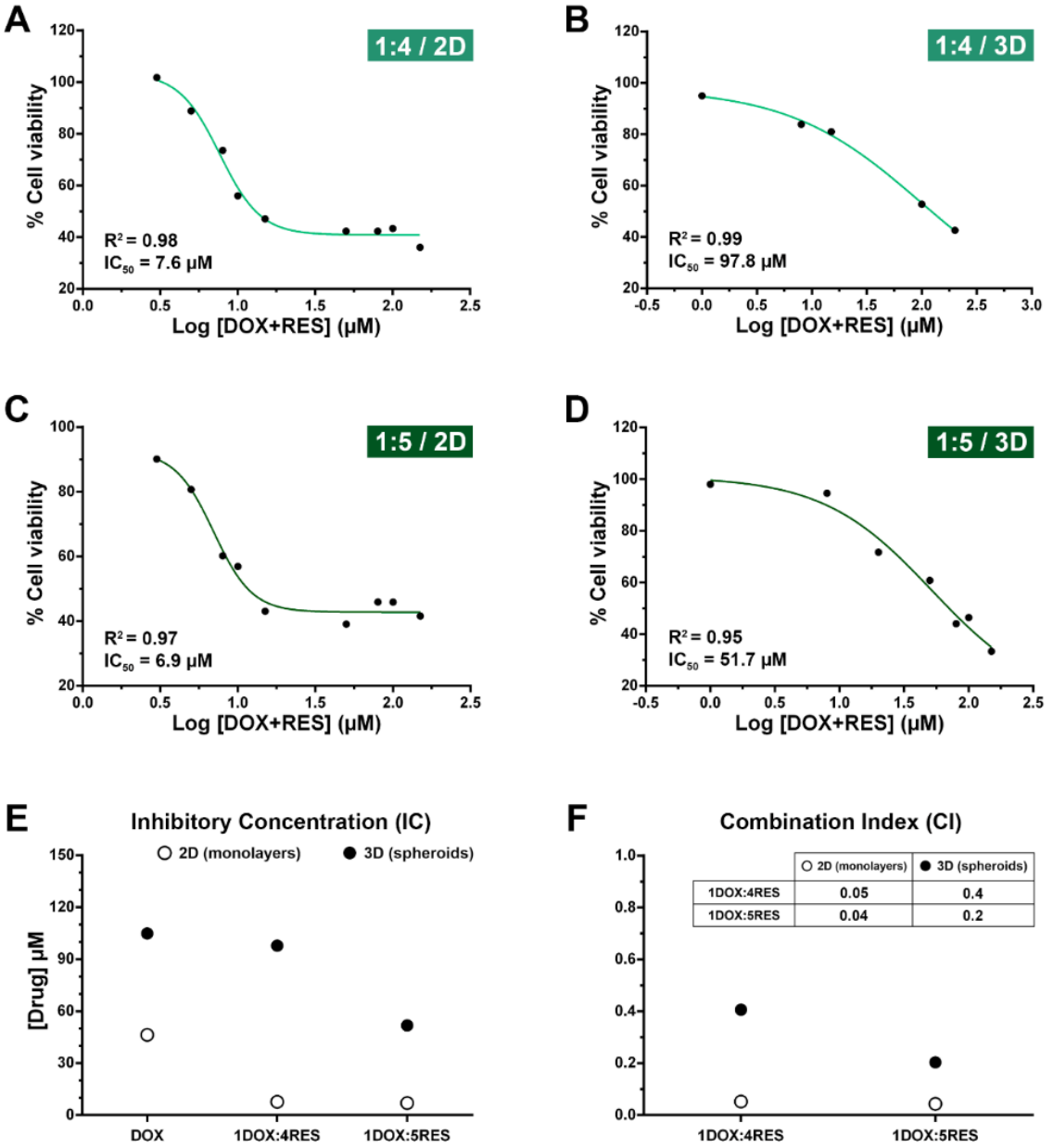

Figure 8. Comparison of the DOX:RES combinations (1:4 and 1:5) effect in PANC-1 models (monolayers *vs*. spheroids). Dose-response curves of 1DOX:4RES (A and B) and 1DOX:5RES (C and D) and respective IC_{50} values; data are presented as mean (n=5). Comparison of the 50 % Inhibitory Concentrations of DOX, 1DOX:4RES and 1DOX:5RES (E). CI of the 1DOX:4RES and 1DOX:5RES (F).

Interestingly, when compared to the effect attained in 2D cultures, the action of the drug combinations in the 3D spheroids was not so effective. In fact, the IC_{50} of 1DOX:4RES combination was only 1.1-times lower than that of DOX (Figure 8 B), and the 1DOX:5RES combination demonstrated a moderately higher therapeutic efficacy, displaying an $IC_{50} \approx$ 2.0-times lower than that of DOX (Figure 8 D). Due to the differences in the inhibitory capacity,

the CI of the two combinations in 3D was also different, revealing that the 1DOX:5RES can achieve a higher synergism, *i.e.* lower CI value (0.203 *vs.* 0.406) (Figure 8 F).

Comparing the CI values obtained in 2D and 3D for both combinations, it is clear that although these DOX:RES ratios had a very similar therapeutic performance when incubated in 2D cultures, their synergistic capacity in 3D spheroids is lower (Figure 8 F). In fact, the 1DOX:4RES and 1DOX:5RES combination had a CI 7.8- and 4.7-times lower in the 2D models than in spheroids (Figure 8 F). These results clearly highlight the utility of using 3D spheroids in the screening of drug-combinations aimed for pancreatic cancer treatment.

Chapter IV

4. Conclusion and Future Perspectives

Pancreatic cancer has the highest death rates associated among all cancer types. In this way, it is urgent to develop effective and accurate therapies, in order to increase patients' survival rates (which are, currently, only 3-5 % after diagnosis).

The single administration of drugs has not been obtaining a successful therapeutic outcome for this type of cancer. Thus, combination therapy, a treatment modality that combines two or more therapeutic agents, emerged as a solution. Each drug used in combination, acts in different metabolic pathways, in an additive or synergistic way thus enhancing their anticancer potential using smaller doses of drugs.

Up to now, the evaluation of the therapeutic effect of drug combination was, in the majority of the studies, performed on 2D culture models. However, this models are unable to reproduce the properties of *in vivo* tumors and may lead to therapeutic failures in *in vivo* clinical trials. In this way, 3D culture models, namely spheroids are more reliable, since they are able to mimic the tumor microenvironment founded *in vivo*.

In this study, it was analyzed and compared, for the first time, the effect of different molar ratios of DOX:RES combinations towards 2D cultures and 3D spheroids of PANC-1 cells. The data obtained revealed that DOX:RES combinations with a higher RES content induced the highest reduction on PANC-1 cells' viability, in both 2D and 3D models, suggesting their improved therapeutic potential. Further analysis showed that this enhanced therapeutic effect is mediated by a RES-concentration dependent on the accumulation of DOX in cells' cytoplasm. A more detailed analysis of the 1DOX:4RES and 1DOX:5RES combinations demonstrated that these have a similar inhibitory capacity (CI_{50} values) towards the 2D PANC-1 models and therefore a comparable synergistic capacity. In stark contrast, the IC_{50} values of the 1:4 DOX:RES combination in 3D spheroids was only slightly lower than that of DOX. On the other hand, the 1DOX:5RES combination was more effective and revealed the higher synergistic potential towards 3D PANC-1 spheroids.

Overall, the 1DOX:5RES combination seems a promising treatment approach for pancreatic cancer, once this combination uses lower doses of DOX and higher doses of a natural compound allowing the reduction of the therapeutic cost, as well as the side effects associated with DOX. Additionally, this study depicts the differential effects of drug-combinations in 2D and 3D, highlighting the utility of using 3D spheroids in the screening of drug-combinations aimed for pancreatic cancer treatment.

In a future perspective, this combination therapy modality should be study for other cancer types and should be implemented in *in vivo* trials, in order to be put into practice. On the other hand, other combinations using natural, easily accessible and inexpensive products and cytotoxic drugs can be tested in order to decrease the amount of cytotoxic drug to be used and also, to decrease treatment costs.

Chapter V

5. References

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