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Ciências

Modelo tumoral *in vitro* para a avaliação terapêutica de fármacos

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"Tudo o que um sonho precisa para ser realizado
é que alguém acredite que ele possa ser realizado."

Roberto Shinyashik

Dedication

À minha mãe, ao meu pai, ao meu namorado e à minha verdadeira família,
por todo o apoio nas alturas mais difíceis e por acreditarem sempre que eu seria capaz.

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Resumo

Na atualidade, as terapias anticancerígenas utilizadas na clínica apresentam uma baixa eficácia. Tal facto é explicado pela capacidade que as células cancerígenas têm em desenvolver resistência a fármacos anticancerígenos, o que contribui para a elevada taxa de mortalidade associada a esta doença. Os investigadores estão presentemente a desenvolver novos fármacos com atividade anticancerígena para melhorar a taxa de sobrevivência dos doentes que padecem desta patologia. As culturas de células cancerígenas *in vitro* em 2D têm sido o principal modelo utilizado numa primeira fase de desenvolvimento destas novas abordagens terapêuticas. No entanto, este tipo de modelo de cultura celulares não mimetiza as principais características dos tumores que afetam o ser humano, assim como os mecanismos de resistência a fármacos que os tumores apresentam. Por outro lado, os modelos *in vitro* 3D, nomeadamente os esferóides produzidos com células cancerígenas, apresentam um perfil de resistência a fármacos semelhante ao encontrado em tumores sólidos humanos (*e.g.* cancro de mama). Devido a esta característica, estes modelos têm sido usados pelos investigadores como ferramentas para a avaliação de fármacos anticancerígenos. No entanto, as técnicas e métodos utilizados para a análise da eficácia terapêutica de fármacos em modelos *in vitro* estão apenas desenvolvidos e padronizados para células cultivadas em 2D. Deste modo, o desenvolvimento de modelos de cultura de células 2D que apresentem um perfil de resistência a fármacos semelhante ao dos esferóides pode ser uma mais valia, uma vez que estes modelos podem ser estudados através de equipamentos, técnicas e metodologias já estabelecidas. Em estudos recentes foi demonstrado que as culturas celulares 2D obtidas por desagregação de esferóides e mantidas em meio de cultura suplementado com glutationa (GSH) apresentavam o mesmo fenótipo que as células presentes nos esferóides 3D. No presente trabalho, avaliou-se a resistência à doxorrubicina (DOX) de culturas 2D de MCF-7 obtidas por desagregação de esferóides em meio de cultura suplementado com GSH, assim como a cultura 2D convencional e em esferóides 3D. Os resultados obtidos demonstraram que este modelo de cultura celular apresenta uma resistência à DOX próxima à determinada para os esferóides. De facto, a concentração inibitória de 50 % (IC_{50}) da DOX em culturas de células MCF-7 derivadas de esferóides e que foram cultivadas em GSH foi cerca de 8 vezes mais elevada do que a obtida para as culturas de células 2D convencionais. Neste trabalho foi também demonstrado que o aumento da resistência das células MCF-7 derivadas de esferóides advém da maior atividade da glicoproteína-P (P-gp) e da redução dos níveis de espécies reativas de oxigénio (ROS) no citoplasma destas células. Em suma, a cultura de células em 2D obtidas a partir da desagregação de esferóides representa uma melhoria para o desenvolvimento futuro de terapias anticancerígenas, devido ao facto de exibirem uma resistência a fármacos semelhante à exibida pelas células cultivadas em modelos 3D.

Palavras-chave

Culturas celulares 2D, Cancro da Mama, Doxorrubicina, Esferóides, Glutationa, Resistência a fármacos.

Resumo Alargado

O cancro é uma das principais causas de morte em todo o mundo, sendo o cancro da mama o que apresenta não só o maior número de novos casos diagnosticados, mas também de mortes registadas. Entre as opções de tratamento desta doença, a quimioterapia é a mais comumente utilizada em ambiente clínico. No entanto, as células tumorais podem adquirir resistência aos fármacos, o que destaca a importância de desenvolver novas formulações de agentes anticancerígenos mais eficazes.

O desenvolvimento de novos fármacos é um processo composto por várias etapas, incluindo uma seleção exaustiva de candidatos durante a fase pré-clínica. Durante esta fase, são efetuados diferentes ensaios *in vitro* (culturas de células) e *in vivo* (animais) para determinar as propriedades farmacológicas dos fármacos, bem como as suas ações terapêuticas e os efeitos secundários que estes possam ter no organismo humano. Para este propósito, os modelos *in vitro* mais usados até aos dias de hoje são aqueles em que a cultura de células é realizada em monocamada (modelos *in vitro* 2D), uma vez que este tipo de cultura de células é simples, fácil de manusear, possui elevada reprodutibilidade e um baixo custo. No entanto, estes modelos não reproduzem as propriedades exibidas pelos tumores que afetam o ser humano e, por conseguinte, estes são incapazes de prever com eficácia a ação do fármaco neste tipo de tumores. Com o objetivo de ultrapassar estas limitações, surgiram os modelos 3D *in vitro* como os esferóides tumorais, que são pequenos agregados celulares com forma esférica. Estes agregados celulares apresentam um microambiente e uma organização celular que lhes confere resistência a fármacos anticancerígenos de forma semelhante ao que acontece nos tumores que afetam os seres humanos.

Apesar do potencial dos esferóides para a avaliação de novos fármacos, estes ainda não são amplamente utilizados e validados durante os ensaios pré-clínicos, devido ao fato das técnicas e métodos usados na análise de fármacos em modelos *in vitro* não estarem otimizados e padronizados para os modelos 3D. Para contornar este problema, os investigadores têm vindo a desenvolver culturas de células 2D que apresentam resistência a fármacos. Estas culturas apresentam um perfil de resistência a fármacos semelhante ao encontrado em tumores *in vivo* e podem ser analisados através de ensaios padronizados e equipamentos utilizados para culturas de células 2D convencionais.

Um dos modelos de cultura 2D resistentes a fármacos atualmente desenvolvido é aquele que são utilizadas as células do cancro da mama com resistência a adriamicinas (MCF-7/ADR). Como o nome indica, estas células são mais resistentes a adriamicinas, tais como a DOX, e são obtidas mantendo as células em cultura durante algumas semanas ou meses em meio suplementado com este fármaco. Apesar destas células serem um modelo resistente do

cancro da mama amplamente descrito na literatura, a obtenção destas células é demorosa e dispendiosa.

Recentemente, verificou-se que culturas 2D do cancro da mama com um fenótipo semelhante aos tumores *in vivo* podem ser obtidas a partir da desagregação de esferóides. Adicionalmente, este fenótipo é mantido quando as células são mantidas em cultura na presença de GSH (agente redutor e antioxidante encontrado em elevados níveis nos tecidos do cancro da mama).

No entanto, até aos dias de hoje, a aplicabilidade das células derivadas de esferóides para avaliação de fármacos ainda não foi investigada. Deste modo, o trabalho de investigação apresentado nesta dissertação pretende demonstrar o efeito da DOX em células do cancro da mama (MCF-7) obtidas de esferóides desagregados que cresceram durante 10 dias (cultivadas na presença e ausência de GSH). O efeito da DOX nestas células foi comparado com aquele obtido em culturas 2D convencionais e esferóides do cancro da mama. Para tal, a viabilidade das células dos diferentes modelos em estudo foi analisada através do ensaio da resazurina e através de espectrometria de fluorescência. Os resultados obtidos demonstraram que a cultura de células derivadas de esferóides apresenta uma resistência superior à DOX do que aquela exibida pela cultura convencional de células em 2D. Por outro lado, a resistência das células derivadas de esferóides foi próxima daquela apresentada pelos esferóides.

De forma a investigar os possíveis mecanismos envolvidos na resistência das células derivadas de esferóides, foi investigado a atividade da P-gp (proteína que é responsável pelo efluxo da DOX para o exterior das células) e os níveis intracelulares de ROS (radicais envolvidos na citotoxicidade promovida pela DOX) nestas células. A atividade da P-gp foi analisada através de espectrometria e microscopia de fluorescência usando um substrato desta proteína (rodamina 123 (Rho 123)). Através dos resultados obtidos foi possível observar que as culturas convencionais 2D apresentam um menor efluxo de Rho 123 e maior acumulação desta no seu interior, quando comparadas com as culturas de células derivadas de esferóides. Por fim, os níveis intracelulares de ROS foram analisados por microscopia de confocal e verificou-se que as células obtidas de esferóides possuem menos radicais no seu interior em comparação com as células dos modelos convencionais 2D. Em conclusão, os modelos 2D compostos por células derivadas de esferóides são provavelmente mais resistentes à DOX devido à sua maior atividade da P-gp, mas também devido aos seus níveis mais reduzidos de ROS.

Com base nos resultados obtidos, espera-se que os modelos de cultura 2D obtidos de células dissociadas de modelos 3D possam ser uma mais valia para o desenvolvimento e investigação de novos fármacos anticancerígenos, uma vez que estes são obtidos em menos de 2 semanas, representam melhor a resistência a fármacos dos tumores *in vivo*, e podem ser analisados através de técnicas e métodos padronizados que são amplamente usados nas culturas tradicionais 2D.

Abstract

The anticancer therapies used nowadays in the clinic display a low efficacy. The ability of cancer cells to develop resistance to drugs used in chemotherapy, contributes to the high mortality associated with this disease. The development of new drug formulations is crucial for improving patient survival rates. *In vitro* 2D cancer cell cultures have been the main model used, in a first phase, for the development of these new therapies. However, these cultures are unable to mimic the main characteristics of *in vivo* tumors, such as their drug resistance mechanisms. On the other hand, *in vitro* 3D cancer models, in particular the cancer cell spheroids, have a drug resistance profile similar to that found in human solid tumors, such as breast cancer. Due to that, these models have been used by researchers as a tool to study anticancer drugs. Nevertheless, the techniques and methods used to analyze the therapeutic efficacy of drugs in *in vitro* models are developed and standardized only for 2D cells in culture. In this way, the develop of 2D cell culture models that display a drug resistant profile similar to the spheroids can be advantageous since these models can be studied through established equipment, techniques and methodologies. Further investigations revealed that 2D cell cultures obtained by spheroid disintegration and maintained in culture medium supplemented with glutathione (GSH) had the same phenotype as the cells present in 3D spheroids. In the present work, the resistance to doxorubicin (DOX) of 2D cultures of MCF-7 obtained by disaggregation of spheroids and then cultured in culture medium supplemented with GSH was evaluated. The results obtained demonstrated that this model of cell culture presents a DOX resistance profile closer to that presented by spheroids. In fact, the 50 % inhibitory concentration (IC_{50}) of DOX in 3D-derived MCF-7 cell cultures supplemented with GSH was about 8-times higher than that obtained for conventional 2D cell cultures. In this work it was also possible to demonstrate that the increase in resistance of spheroid-derived MCF-7 cells results from increased P-glycoprotein (P-gp) activity and from the reduction of intracellular reactive oxygen species (ROS) levels in these cells. In summary, 2D cell culture obtained from spheroid disaggregation represents an improvement for the future development of anticancer therapies, owing to its ability to present in the 2D model a drug resistance similar to that exhibited by 3D models.

Keywords

2D cell cultures, Breast cancer, Doxorubicin, Drug resistance, Glutathione, Spheroids.

List of Publications

Articles in peer reviewed international journals:

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 Diogo, D. and Correia, I. J., Gathering 2D cell cultures derived from 3D MCF-7 spheroids displaying a Doxorubicin resistant profile. *Biotechnology Journal*, submitted.

Barros, A. S., Costa, E. C., Nunes, A. S., de-Melo Diogo, 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.

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Chapter I

List of Acronyms

2D(-GSH)	Traditional 2D cell cultures that grow in the absence of GSH
2D(+GSH)	Traditional 2D cell cultures that grow in the presence of GSH
2.5D(-GSH)	3D-derived cell cultures that grow in the absence of GSH
2.5D(+GSH)	3D-derived cell cultures that grow in the presence of GSH
ADR	Adriamycin-resistant
ANOVA	Analysis of variance
BRCA1	Breast cancer susceptibility genes 1
BRCA2	Breast cancer susceptibility genes 2
BSO	Buthionine sulfoximine
CLSM	Confocal Laser Scanning Microscopy
DCF	2',7'-dichlorofluorescein
DMEM-F12	Dulbecco's Modified Eagle's medium F-12
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicine Agency
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GSH	Glutathione
H_2DCFDA	2',7'-dichlorofluorescein diacetate
HeLa	Human cervical carcinoma
HTS	High throughput screening
IC_{20}	20 % inhibitory concentration
IC_{50}	50 % inhibitory concentration
IC_{80}	80 % inhibitory concentration

K-	Negative control
MCF-7	Oestrogen-dependent human breast adenocarcinoma
P-gp	P-glycoprotein
PBS	Phosphate-buffered saline solution
PFA	Paraformaldehyde
PGA	Polyglycolide
PLA	Polylactide
Rho 123	Rhodamine 123
ROS	Reactive oxygen species
S.D.	Standard deviation
UV	Ultraviolet

Chapter I

1. Introduction

1.1. Breast cancer

Cancer is considered a leading cause of death in both developed and undeveloped countries, and the number of cases are expected to increase due to population growth and aging [1, 2]. This disease is prompted by the activation of oncogenes and/or the deactivation of tumor suppressor genes in the cells that gather malignance properties, known as the Hallmarks of Cancer (Figure 1), such as: i) sustained proliferation and immortality; ii) improved escape to growth suppresser signaling; iii) invasion and metastatic capacities; iv) angiogenesis induction; v) and resistance to cell death mechanisms [2-5].

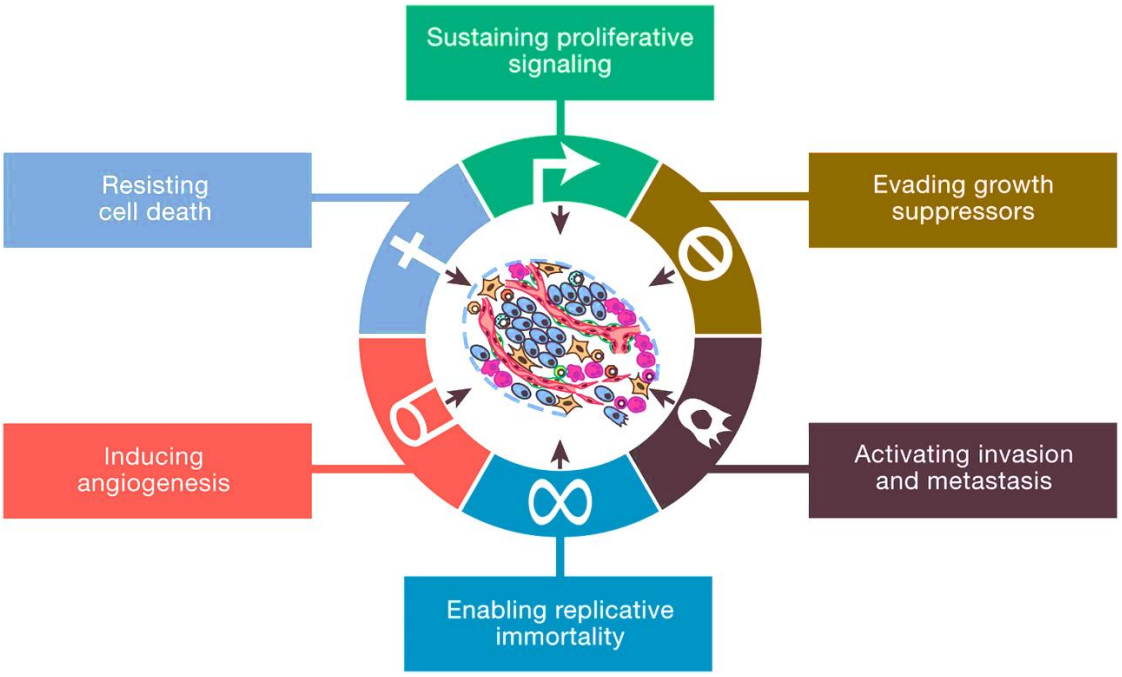

Figure 1. Summary of the Cancer Hallmarks responsible for the development, maintenance and progression of cancer (Adapted from [5]).

Siegel and co-workers estimate that in 2018 over 1.7 million people will be diagnosed with cancer and almost 610 thousand deaths will occur in the United States of America [6]. Within the values previously described, 269 thousand people will be diagnosed with breast cancer and 41 thousand deaths will occur, being this type of cancer the most frequently diagnosed and the leading cause of cancer-related deaths among women worldwide (Figure 2) [6-8]. In fact, once metastases are detected the median survival is in the range of 18–24 months [3, 8, 9].

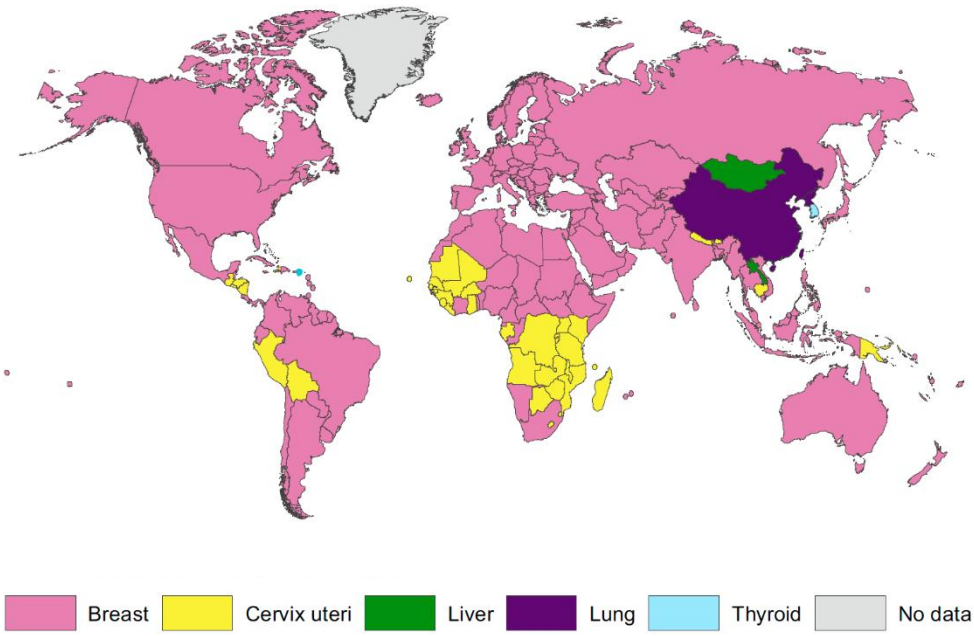

Figure 2. Representation of the most frequently diagnosed cancer types in women worldwide in 2012, according to the International Agency of Research on Cancer (Adapted from [9]).

Breast cancer consists in a heterogeneous group of neoplasms originating from the epithelial cells lining the milk ducts [10]. Its incidence increases after the age of 50 years and some of the risk factors associated to this disease are infertility, age of first fulltime pregnancy, age of menopause, usage of hormones (estrogen and or progestin) in postmenopausal stage and family history [11, 12]. There are also other factors at the genetic level that lead to a greater predisposition for the development of breast cancer, namely mutations at the breast cancer susceptibility genes 1 and 2 (*BRCA1* and *BRCA2*, respectively) [13], which are correlated with nearly 40-80 % chances of developing cancer [11].

1.1.1. Breast cancer treatment

Breast cancer treatment approach depends on the type and stage of the disease. These treatments are usually divided in two main groups: i) local treatment and ii) systemic treatment [14, 15]. The local treatment approach is performed in order to treat the tumor without affecting the other parts of the patient body [14]. These treatments include surgery (removal of cancer tissue from the body) and radiotherapy (administration of ionizing radiation in the region of the tumor mass to control or kill the malignant cells) [2, 14]. On the other hand, the systemic treatments include the oral administration or injection of anticancer agents that can reach all the parts of the human body. These treatments include the chemotherapy (administration of anticancer cytotoxic drugs to kill the malignant cells) and hormone therapy (administration of hormones to control the growth of hormone receptor-positive breast cancers) [14, 15].

Among these treatments, chemotherapy is the clinical treatment approach most used towards cancer related diseases since 1940 [2, 16, 17]. Chemotherapy includes neoadjuvant chemotherapy (to shrink the tumor before the removal of the tumor mass by surgery), adjuvant chemotherapy (after surgery to kill any cancer cells that were not removed during the operation) and for advanced breast cancer treatment, since at this stage the disease has already spread outside the breast and underarm area and cannot be removed by surgery [14, 18].

Still, tumor cells frequently develop resistance against the chemotherapeutic drugs and consequently cancer cells tend to not respond to the chemotherapy. A study performed in 240 patients with metastatic breast cancer who received multiple lines of cytotoxic chemotherapy regimens, demonstrated that of the percentage of patients that responded to the treatment decrease after multiple sessions of administration of chemotherapy. In fact, the response rates of breast cancer patients after the first-, second- and third- line therapy were 60.5, 51.1 and 35.7 %, respectively [19]. Additionally, the median progression-free survival was 7.6 months for first-line *vs.* 5.1 months for second-line *vs.* 3.6 months for third-line chemotherapy [19]. Another study performed in metastatic breast cancer patients showed that 6-month benefit in terms of progression-free survival with first-line therapy was shown by 289 patients (63.5 %) at first line, 128 (40.5 %) at second line, 76 (33.8 %) at third line, and 34 (23.3 %) at fourth line [20].

The main mechanisms of drug resistance displayed by breast cancer cells may include: i) drug target modification; ii) drug degradation by drug-metabolizing enzymes; iii) inactivation of DNA repair mechanisms; iv) escape to cell death; v) decrease of drug uptake by cells; and vi) increased drug efflux from breast cancer cells (reviewed in detail in [21]). Among these mechanisms, the increase of drug efflux mediated by P-glycoprotein (P-gp; efflux membrane transporter) is one of the most widely described mechanisms exhibited by cancer cells resistance towards drugs [22]. In fact, Sanfilippo and co-workers observed that cancer treated with chemotherapy present higher P-gp expression than untreated breast cancers (40 % *vs.* 9 %) [23]. Having this in mind, the development of new anticancer drugs and pharmaceuticals to overcome the mechanisms of resistance is crucial for the effective treatment of breast cancer.

1.2. Discovery and development of anticancer drugs

The validation of new anticancer agents comprises several steps (Table 1). During the first step - preclinical stage - *in vitro* (cell cultures) and *in vivo* (animals) models are essential to determine the pharmacological properties of the drug formulations, as well as their therapeutic action, for further evaluation in humans – clinical stage [2, 24].

Table 1. The drug development process according to the US Food and Drug Administration (FDA) [25].

Step		Aims
1	**Discovery and Development**	• Discover new insights into a disease process that allow researchers to design a drug to stop or reverse the effects of the disease; • Search of existing treatments that have unexpected effects; • Investigate of how the drugs is absorbed, distributed, metabolized and excreted; • Investigate the potential benefits and mechanisms of action of the drug; • Determine of the best way to administer the drug and the best dosage; • Overview of the possible side effects or adverse events (toxicity); • Investigate the possible interaction of the drug with other drugs and treatments; • Comparison of the effectiveness of the drug with similar drugs.
2	**Preclinical Research**	• Screen of therapeutic effectiveness of the drug using *in vitro* and *in vivo* models; • Evaluate of the dosing and toxicity levels of the drug.
3	**Clinical Research**	• Perform trials that are done in people; • Clinical trials comprises 4 phases: ▪ Phase I – Safety and dosage; ▪ Phase II – Efficacy and side effects; ▪ Phase III – Efficacy and monitoring of adverse reactions; ▪ Phase IV – Safety and efficacy.
4	**FDA Drug Review**	• Review thoroughly all submitted data on the drug; • Make a decision to approve (or not) the use of the drug in clinical environment.
5	**FDA post-Market Drug Safety Monitoring**	• Review the reports of problems with prescription and over-the-counter drugs; • Decide the addition of cautions to the dosage or usage information about the drug, as well as other measures for more serious issues.

1.2.1. *In vitro* and *in vivo* models for anticancer drugs screening

The *in vitro* and *in vivo* models are important tools in cancer research, enabling the determination of drugs pharmacological properties, such as absorption, distribution, metabolism and excretion, which is fundamental for determining the therapeutic action of the drug without causing toxicity to the organism [24, 26].

The *in vitro* models include the manipulation of cells outside the human body (Table 2). The first attempt to perform cell culture in laboratory took place at the end of the 19th century by Wilhelm Roux, who cultured chicken embryonic cells in a saline solution for a reduced number of days [27, 28]. Then, in 1950, George Gey cultured for the first a human cancer cell line, obtained from a human cervical carcinoma - HeLa cell line [24, 29]. Since then, different

types of cells started to be cultured in glass or polystyrene flat surfaces as monolayers of cells [24, 30]. Triggering a great revolution in the development of new anticancer therapies and overall knowledge of the cellular mechanisms. The cell monolayers, *i.e.* 2D cell cultures, become the most used model for the analysis of cellular responses to various chemical drugs, enabling a better understanding of the mechanisms of action of those drugs, due to their simplicity, reproducibility and low-cost [4, 31-34].

Table 2. Summary of the *in vitro* and *in vivo* models used for drug screening purposes.

Tumor model	Advantages	Disadvantages	REFs.
***In vitro* MODELS**			
2D cell cultures (monolayers)	• Less expensive • Less time-consuming; • Different variables and parameters can be determined; • Fundamental for further testing in animal models; • Compatible with High Throughput Screening (HTS).	• Different cell lines are required to mimic different pathologies; • Fails to mimic the 3D organization of human tumors; • Cells lose some of their natural functional abilities and shape; • Gene expression of cells is different from that found in human tumors; • Lack of drug resistance mechanisms; • Lack of extracellular matrix (ECM); • Low number of cell-cell and cell-ECM interactions.	[24, 30, 31, 35]
3D scaffold-based cell cultures (cells seeded on artificial 3D structures)	• Wide variety of natural, semi-synthetic and synthetic biomaterials can be used; • Physicochemical and biological properties are customizable; • High number of artificial cell-ECM interactions are established; • High number of cell-cell interactions occur; • Commercial products are available.	• External materials are needed; • ECM is artificial; • Specific equipment and tools are required; • Some biomaterials can interfere with the therapeutics response; • Low reproducibility; • Techniques are laborious, expensive and may be difficult to scale-up; • May not be compatible with HTS.	[36-41]
3D scaffolds-free cell cultures (spheroids)	• No external biomaterials are required for its formation; • ECM is produced by the cells; • High number of cell-ECM and cell-cell interactions are established; • No specific equipment and tools are required to perform this type of cell-culture; • The majority of the techniques are inexpensive; • Compatible with HTS; • Commercial products are available.	• Optimizations are required for some types of cells; • Spheroids may be destroyed during their analysis and manipulation; • Lack of protocols and optimized/standardized tests for this type of models.	[26, 36, 42-45]

In vivo MODELS			
Animals (*e.g.* rats, monkeys, dogs)	• Allow the evolution of the drugs in a complete body system; • Allows the study of tumor growth rate, metastization and angiogenic process.	• Investigation of human tumors requires an immunocompromised host; • Differences between species (animals *vs.* humans); • High costs; • Legal problems; • Ethical issues; • They are not performed without prior *in vitro* testing.	[31, 46-48]

Following the *in vitro* assays, the therapeutics are evaluated in *in vivo* assays, *i.e.* in animal models (*e.g.* dogs, fishes, monkeys, pigs, rats, among others) (Table 2). These models can mimic the overall system of the human body, as well as the cancer tissue microenvironment [31]. Still, animal experimentation is very expensive and several ethics and laws are established to control the overuse and unethical manipulation of the animals [47]. According to Russell and Burch (authors of *The Principles of Humane Experimental Technique*, 1959 [49]), all animal experiments should apply the "3 R's" Rule: i) Replacement of animals with alternative testing methods; ii) Reduction of the number of animals used; and iii) Refinement of methods to minimize animal suffering as a consequence of adverse side-effects of the experimental protocol [47, 50, 51]. Since then, the 3 R's is taking into account in the protocols of survival experimentation submitted to regulatory agencies such as FDA and the European Medicine Agency (EMA) [52]. These agencies certify that the experiments are performed only with de number of animals needed to obtain the results, with minimum repetitions and with limited periods of time [46]. Additionally, another great disadvantage of the *in vivo* experiments is the difference lack of correspondence results found between animals and humans [31]. In fact, different studies have already demonstrated that the effect of a drug on animals (*e.g.* drug bioavailability) is different from that observed in humans [53-55].

Therefore, in order to overcome the legal and ethical issues associated with animal experimentation, different laboratories have been developing 3D cell culture models to fill the gap between the 2D cell culture models and *in vivo* animal models. 3D *in vitro* models are capable of reproducing the properties of the human tumors and their resistance to therapeutics (when compared to the 2D cell cultures). Therefore, these 3D cell culture models can contribute to reduce the number of drug formulation that will further be evaluated in animals and therefore reduce the number of animals used in experimentation [24, 31, 47].

1.2.1.1. 3D cell culture models: tumor spheroids

There are several approaches used to perform cell culture in 3D, separated into two major groups, namely scaffold-based and scaffold-free (Table 2) [36]. Scaffold-based 3D cultures involve cells attachment and grow on artificial 3D platforms (*e.g.* hydrogels, membranes) [36, 37]. The scaffolds can be produced with natural (*e.g.* alginate, collagen, gelatin, alginate) and/or synthetic (*e.g.* polylactide (PLA), polyglycolide (PGA)) biomaterials [39]. The artificial structures are produced in order to obtain a structure that mimics the native ECM formed in human tumors and also to allow cells migration and proliferation to form the 3D microtissue [38, 39].

Despite of the approaches developed for promoting cells grow on artificial structures, this type of culture is laborious and costly once it requires high-tech equipment and techniques to produce the artificial ECM in a high reproducible manner. Additionally, the scaffold-based 3D cell cultures are mostly developed for tissue engineering purposes, *i.e.* to be inserted into a lesion (temporary or permanently) to allow the growth of cells (*e.g.* mesenchymal cells) and promote the restoration of the native structure and biological functions of the tissue [39].

An alternative to scaffold-based 3D cell culture is the scaffold-free 3D culture of cells. Scaffold-free or non-scaffold-based 3D cell cultures gather all the methods that do not use exogenous artificial platforms for cells attachment and grow in a 3D organization [43]. These methods include the formation of 3D microtissues as cellular aggregates that are denominated as spheroids. Spheroids are considered the 3D cell culture models that better represent the characteristics of solid tumors (reviewed in detail in [36, 56, 57]). These scaffold-free microtissues represent the tissue-like cytoarchitecture, nutrients and oxygen gradient, ECM protein deposition, cell-cell and cell-ECM interactions, as well as the drug resistance profile exhibited by human tumors (Table 3) [43, 45].

Table 3. Overview of the spheroids properties that mimic those of human tumors.

Human tumor property	Representation in spheroids	REFs.
Lack of oxygen	• Spheroids due to their 3D cellular organization also display an oxygen gradient, that leads to the formation of a hypoxic environment in its inner regions.	[58]
Altered energy metabolism	• Spheroids demonstrate increased glucose consumption and anaerobic glycolysis, resulting in lactate production.	[59]
Acid microenvironment	• Spheroids demonstrated low pH (6.5–7.2) in the deeper spheroids layers due to the lactate accumulation.	[60]
Cell cycle arrest	• Spheroids interior is composed of cells in quiescence/senescence state.	[61, 62]
ECM proteins deposition	• Spheroids cells express and deposit ECM proteins (*e.g.* collagen, fibronectin and laminin).	[63-65]

Cell-cell interactions	• Spheroids have increased cell-cell contacts and E-cadherins expression.	[66-73]
Physical barriers	• Enriched ECM proteins deposition, number of cell-ECM and cell-cell interactions and therefore microtissue density.	[36]

Spheroids resistance to certain drugs is associated with their up-regulation of P-gp. Walker and co-workers verified that the greater expression of efflux pumps such as P-gp in MCF-7 cells within the spheroid does affect the overall accumulation of drugs and consequently the effectiveness of drug therapy [74]. In another study, Doublier and co-workers also verified that the P-gp production in MCF-7 breast was higher than the MCF-7 cells cultured in monolayer, leading to a reduced doxorubicin (DOX) accumulation within spheroids' cells [75].

Still, despite of the potential of spheroids for drug screening purposes, this model is not yet widely used for validation of drugs during the preclinical phase [4], due to the fact that the routine low-cost techniques and equipment are not optimized/standardized for the analysis of 3D tissues [36, 76]. Additionally, a limited amount of information is provided in the literature about the protocols that can be used to a more harmonized analysis of the effects of new compounds on the 3D spheroids structure, morphology, volume/size, cellular distribution, viability and gene expression patterns [36]. For instance, the analysis of drugs' effects in spheroids cells' viability through colorimetric assays (*e.g.* resazurin assay) is challenging [36, 76]. In spheroids, cell-cell interactions hinder a homogeneous distribution of the resazurin throughout spheroids' cellular population, leading to inaccurate results [76].

To circumvent this problem, researchers are developing drug resistant 2D cell cultures [4]. These models show a drug resistance profile more alike to that found in *in vivo* tumors and can be analyzed through standardized assays and equipment used for conventional 2D cell cultures [4]. The most commonly used resistant 2D cell culture model are the breast cancer cultured cells with resistance to adriamycins (MCF-7/ADR) [77]. These cells are generally obtained by maintaining MCF-7 cells in a culture medium supplemented with adriamycins (*e.g.* DOX) during long periods of time [78, 79]. However, this procedure is quite expensive and time-consuming. Recently, Koshkin *et al.* demonstrated that 3D-derived MCF-7 cells (cells obtained from 3D spheroids disaggregation) are able to preserve their 3D-phenotype when cultured in 2D [4]. Additionally, this phenotype was maintained for long periods by culturing the 3D-derived cells in medium supplemented with 5 mM of glutathione (GSH; reducing and antioxidant agent found in high levels in various cancer (*e.g.* breast cancer) and an influencer of cancer cells resistance [80-88]) [4]. However, so far, the applicability of the 3D-derived cells in drugs screening has not been investigated. Furthermore, the effect of drugs in 3D-derived cells has not yet been compared to that occurring in conventional 2D models and 3D spheroids. Therefore, this work intended to investigate the effect of DOX in 3D-derived MCF-7

cells (cultured in presence and absence of GSH) and compare it to that occurring in MCF-7 cells cultured in 2D and as 3D spheroids. Then, the possible mechanisms that prompted the increased resistance to DOX of 3D-derived cells were investigated, namely the P-gp activity and intracellular reactive oxygen species (ROS) levels.

1.3. Aims

The main aim of this dissertation work plan was the investigation of an alternative resistant breast cancer *in vitro* model that can be used for drug screening using standardized assays and equipment.

The specific aims of this dissertation include:

- Development of a resistance 2D MCF-7 cell culture model obtained from the dissociation of 3D spheroids;
- Analysis of the effect of DOX in the cell viability of traditional 2D cell cultures, resistant 3D-derived cell cultures and 3D spheroids of MCF-7 cells;
- Determination of the drug-response curves of DOX in the different breast cancer *in vitro* models and respective 20, 50 and 80 % inhibitory concentrations (IC_{20}, IC_{50} and IC_{80});
- Comparison of the drug resistance of the different breast cancer cell culture models;
- Investigation of the effect of GSH medium supplementation in MCF-7 cells resistance;
- Evaluation of P-gp activity and ROS intracellular levels in the traditional 2D cell culture models and 3D-derived cell cultures of breast cancer.

Chapter II

2. Materials and Methods

2.1. Materials

Oestrogen-dependent human breast adenocarcinoma (MCF-7) cells were acquired from ATCC (Middlesex, UK). Cell culture plates and T-flasks were obtained from Thermo Fisher Scientific (Porto, Portugal). 2',7'-dichlorofluorescein diacetate (H_2DCFDA), Dulbecco's Modified Eagle's medium F-12 (DMEM-F12), ethylenediaminetetraacetic acid (EDTA), gentamicin, GSH, paraformaldehyde (PFA), phosphate-buffered saline solution (PBS), resazurin, rhodamine 123 (Rho 123), streptomycin and trypsin were purchased from Sigma-Aldrich (Sintra, Portugal). Agarose was brought from Grisp (Porto, Portugal). Fetal bovine serum (FBS) was obtained from Biochrom AG (Berlin, Germany). DOX was acquired from Carbosynth (Berkshire, UK). Cell imaging plates were gotten from Ibidi GmbH (Ibidi, Munich, Germany). The store solution of DOX was prepared in methanol purchased from VWR International (Portugal).

2.2. Methods

2.2.1. Cells maintenance and 3D MCF-7 spheroids formation

MCF-7 cells were cultured in DMEM-F12 supplemented with FBS (10 % (v/v)) and streptomycin and gentamycin (1 % (v/v)) in 75 cm^2 T-flasks. Cells were maintained inside an incubator at 37 °C, in a humidified atmosphere with 5 % CO_2 [89]. For spheroids formation, microwells coated with agarose structures were used to guide the cells self-assembly, as previously described by our group [90]. In brief, these agarose structures were produced by placing agarose 2 % (w/v in H_2O) in micromolds (Microtissues Inc., Providence RI, US). Then, these agarose structures were placed in cell culture plates (12-wells) and sterilized by ultraviolet (UV) radiation during 60 min. Afterwards, cells recovered by using 0.25 % trypsin (1:250) and EDTA 0.1 % (w/v) were seeded on the agarose structures ($1x10^6$ cells/ agarose structure). After some period of time, the MCF-7 cells aggregate spontaneously, allowing the obtention of 81 spheroids per agarose structure. These spheroids were cultured in DMEM-F12 (FBS (10 % (v/v) and streptomycin and gentamycin 1 % (v/v)) inside an incubator with a humidified atmosphere (37 °C, 5 % CO_2). The culture medium of the spheroids was exchanged every 2 days. Spheroids used in all the following experiments were grown during 10 days and displayed a mean diameter of 694.073 ± 62.183 µm. Spheroids' size was determined by using ImageJ software (National Institutes of Health), as previously described by our group [91, 92].

2.2.2. Screening of DOX effect in MCF-7 cells cultured in 2D

MCF-7 cells were seeded in 96-well culture plates at a density of 12400 cells/well and were incubated for 24 h with cell culture medium with and without GSH (5 mM) supplementation [4]. Afterwards, the culture medium was removed and cells were incubated with fresh cell culture medium containing different concentrations of DOX (0.1 – 200 µM). After 24 h, the

medium was replaced with culture medium containing resazurin (10 % (v/v)) for 4 h (37 °C, 5 % CO_2). Then, MCF-7 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). In all of the cell viability assays, cells solely incubated with medium were used as the negative control (K-).

Subsequently, the drugs' dose-response curves were traced using OriginLab software (trial version, OriginPro, OriginLab Corporation, MA, USA), in order to determine the DOX 20, 50 and 80 % inhibitory concentrations (IC_{20}, IC_{50} and IC_{80}, respectively) [93, 94].

2.2.3. Screening of DOX effect in MCF-7 cells cultured in 2.5D

3D MCF-7 spheroids were digested by using 0.25 % trypsin (1:250) and EDTA 0.1 % (w/v). Afterwards, the 3D-derived cells were seeded in a 96-well culture plate at a density of 12400 cells/well and incubated for 24 h with fresh culture medium with and without GSH (5 mM) supplementation. Then, cells were incubated with culture medium containing DOX for 24 h (1-200 µM). Cells' viability and the DOX inhibitory concentrations were determined as described in section 2.2.2..

2.2.4. Screening of DOX effect on 3D MCF-7 spheroids

3D MCF-7 spheroids were incubated during 24 with fresh culture medium containing DOX (25-200 µM). For each condition, a total of 45 spheroids were used (total of five wells, each well with 9 spheroids). After 24 h, MCF-7 cells viability and the DOX inhibitory concentrations were determined as described in section 2.2.2..

The effect of DOX on 3D MCF-7 spheroids was also monitored by acquiring optical microscopy images using an Olympus CX41 inverted optical microscope equipped with an Olympus SP-500 UZ digital camera. Spheroids' diameter was done by using ImageJ software (National Institutes of Health), as previously described in our group [91, 92]).

2.2.5. Analysis of P-gp activity through Rho 123 efflux assay

The Rho 123 efflux assay was performed according to methods previously described in the literature with slight modifications [95]. In brief, MCF-7 cells (obtained from 2D cell cultures or 3D-derived) were cultured in 96-well culture plates at a density of 12400 cells per well and incubated for 24 h with fresh medium (with or without GSH 5 mM). Then, the medium was replaced by fresh medium containing Rho 123 (8 µM). After MCF-7 incubation with the probe during 1 h, the medium containing the probe was removed and fresh medium was added to the wells. Then, the fluorescence of the medium in the wells containing the Rho 123 expelled from the cells through the P-gp was measured at 2, 8, 16 and 24 h ($\lambda_{ex}/\lambda_{em}$ = 507/525 nm) with a Spectramax Gemini EM spectroflorometer (Molecular Devices LLC, CA, USA). For comparison purposes, the obtained fluorescence intensity values were normalized with the fluorescence intensity of the initial Rho 123 solution administrated to the cells, *i.e.* Rho 123 at 8 µM.

2.2.6. Rho 123 and DOX accumulation in MCF-7 cells

The analysis of the Rho 123 and DOX accumulation in the cells of the different models was performed by adapting protocols previously described [95-97]. In brief, MCF-7 cells (obtained from 2D cell cultures or 3D-derived) were cultured in µ-slide 8-well imaging plates (Ibidi GmbH, Munich, Germany) at a density of 12400 cells/well in medium with or without GSH supplementation (5 mM). After 24 h, the medium was replaced with fresh medium containing Rho 123 (8 µM) or DOX (9 µM) and cells were incubated for 1 h. Then, culture medium was replaced by fresh medium and cells incubated for 8 and/or 24 h. After this period, the medium in the wells was removed, cells were chemically fixed (PFA 4 % during 15 min) and washed with PBS. Samples were then observed by Confocal Scanning Electron Microcopy (CLSM) to observe the accumulation of Rho 123 and DOX inside the MCF-7 cells by using a Zeiss LSM 710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany). Rho 123 and DOX were visualized by using $\lambda_{ex}/\lambda_{em}$ = 514/519 - 650 nm and $\lambda_{ex}/\lambda_{em}$ = 488/535 - 674 nm, respectively.

2.2.7. ROS levels analysis in MCF-7 cells through H_2DCFDA assay

Intracellular levels of ROS in MCF-7 cells were analyzed by using H_2DCFDA (a non-fluorescent compound that is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS), following a protocol previously described in literature [98]. In brief, MCF-7 cells (obtained from 2D cell cultures or 3D-derived) at a density of 12400 cells/well were cultured, during 24 h in µ-slide 8-well imaging plates (Ibidi GmbH, Munich, Germany), in cell culture medium with or without GSH supplementation (5 mM). Then, cells were incubated during 30 min with hydrogen peroxide (H_2O_2; 0.3 % (v/v). Afterwards, cells were washed with PBS and then incubated with 10 µM of a H_2DCFDA in PBS for 1 h. Lastly, the ROS levels in the cells were indirectly determined by imaging the DCF fluorescence by CLSM through a Zeiss LSM 710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) and using a λ_{ex} of 488 nm and a λ_{em} of 493–599 nm.

2.2.8. 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, USA).

Chapter III

3. Results and Discussion

Cancer is a leading cause of death in both developed and undeveloped countries [1, 2]. Among the treatments used in the clinic to treat this disease, chemotherapy is the most commonly used [99]. However, tumor cells can acquire resistance to anticancer drugs (mostly due to the up-regulation of multidrug resistance proteins like drug efflux transporters (*e.g.* P-gp)), which highlights the importance of developing more effective therapeutics [2, 100, 101].

The validation of new anticancer agents comprises several steps, including an exhaustive candidate selection during a preclinical stage. During this stage, different *in vitro* (cell cultures) and in *in vivo* (animals) assays are performed to determine the pharmacological properties of the drug formulations, as well as their therapeutic action [2, 24]. For this purpose, the analysis of drugs' performance has been performed using 2D *in vitro* models due to its simplicity, reproducibility and low-cost [4, 31-34]. Still, these 2D *in vitro* models are unrealistic representations of the human solid tumors, leading to unprecise results concerning drug effectiveness [28, 30]. Having this in mind, 3D *in vitro* models, such as tumor spheroids (small cellular aggregates with a spherical-like shape), emerged in the early 70s to serve as an intermediate model between standard 2D cell cultures and animal *in vivo* models [34, 36]. Unlike the 2D *in vitro* models, spheroids present a microenvironment and cellular organization that grant them a great resistant to anticancer drugs [75, 102-105]. Despite of the potential of spheroids for drug screening purposes, this cell culture model is not yet widely used due to the fact that the routine techniques and methods are not optimized/standardized for the analysis of 3D tissues [36, 76].

Therefore, there is an urge need to develop new resistant 2D cell culture models that can be produced using inexpensive and simple processes, and that at the same time, display a drug-resistant profile similar to that found in *in vivo* solid tumors. Therefore, in this study, resistant 2D cell culture models of breast cancer (termed as 2.5D(+GSH) MCF-7 cell culture) were obtained by a simple method that takes less than two weeks (Figure 3). This model was produced by culturing in 2D the MCF-7 cells obtained from 10 days-old 3D spheroids. The reasoning for this approach lays on the fact that several reports have demonstrated that 3D-derived cells present different properties from their equivalents cultured only in 2D [4, 106]. Furthermore, 3D-derived cells can also maintain their 3D phenotype upon their disassociation (*e.g.* proliferation rate, drug expelling capacity) [4, 106]. Herein, the 3D-derived MCF-7 cells were cultured in medium supplemented with GSH (5 mM) since GSH i) is present in high concentrations in breast cancer tissues (in comparison to disease-free breast tissue) [88], ii) can influence cells' resistance to therapeutics [82-87], and iii) can maintain for long periods of time the 3D phenotype of 3D-derived cells [4]. Hereafter, the effect of DOX in the viability of 2.5D(+GSH) cells was determined and compared to that

occurring in conventional 2D cell cultures (2D(-GSH) MCF-7 cells) and in 3D MCF-7 spheroids. The mechanisms that may be involved in 2.5D(+GSH) MCF-7 cells' resistance to DOX were also investigated.

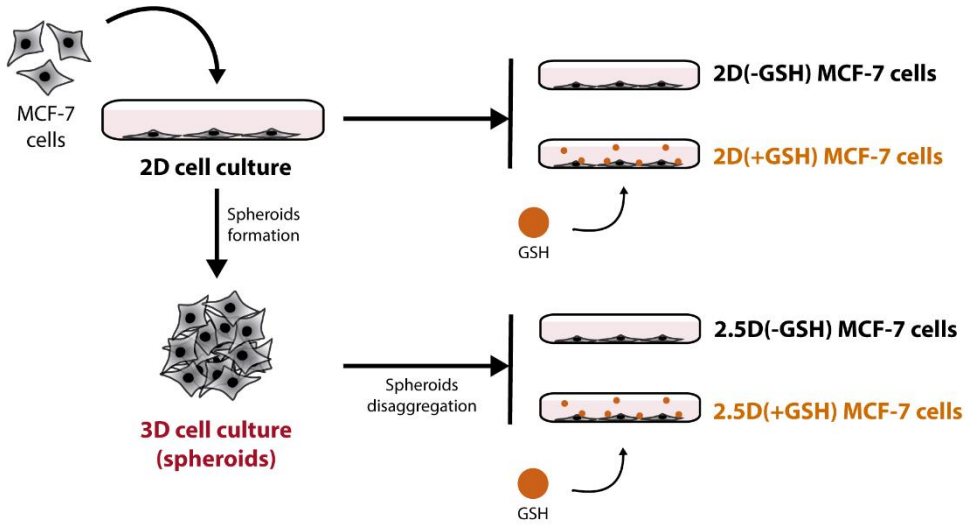

Figure 3. Schematic illustration of the MCF-7 cell cultures used in this work. During the study, it was used 2D(-GSH) cultures (traditional 2D cell cultures that grow in absence of GSH); 2D(+GSH) cultures (traditional 2D cell cultures that grow in presence of GSH); 2.5D(-GSH) cultures (3D-derived cell cultures that grow in absence of GSH); 2.5D(+GSH) cultures (3D-derived cell cultures that grow in presence of GSH); and 3D spheroids.

3.1. 2D, 2.5D and 3D breast cancer cell cultures response to DOX

The effect of DOX in the viability of traditional 2D cell cultures (2D(-GSH) MCF-7 cells) and 2.5D(+GSH) MCF-7 cell cultures was first investigated (Figure 4 A and B). Then, DOX-response curves were traced in order determine the drug's concentrations required to kill 20, 50 and 80 % (IC_{20}, IC_{50}, IC_{80}) of the cells. It was verified that 2.5D(+GSH) MCF-7 cells were less affected by DOX when compared to 2D MCF-7 cells (IC_{20} = 37.486 μM; IC_{50} = 68.679 μM; IC_{80} = 125.828 μM *vs.* IC_{20} = 3.140 μM; IC_{50} = 9.042 μM; IC_{80} = 26.034 μM). In fact, the determined inhibitory concentrations revealed that the IC_{50} of DOX in 2.5D(+GSH) MCF-7 cells was 8-fold higher than the IC_{50} value obtained for the traditional 2D cell cultures.

The resistance to DOX displayed by 2.5D(+GSH) MCF-7 cells is dependent on the GSH supplementation and on the 3D-derivation of these cells (Figure 4 E). DOX had a similar effect on conventional 2D cell cultures and on 2D cell cultures supplemented with GSH (2D(+GSH) (Figure 4 E). On the other hand, 3D-derived cells without GSH supplementation (2.5D(-GSH) could display (at low drug doses) some resistance to the effect of DOX. In stark contrast, 2.5D(+GSH) cells were greatly less affected by DOX (at various concentrations), thereby confirming the improved DOX resistance profile displayed by this model.

To compare the level of resistance of the 2.5D(+GSH) to that displayed by 3D cell cultures, the effect of the DOX in 3D MCF-7 spheroids and its drug-response curves were also determined (Figure 4 C).

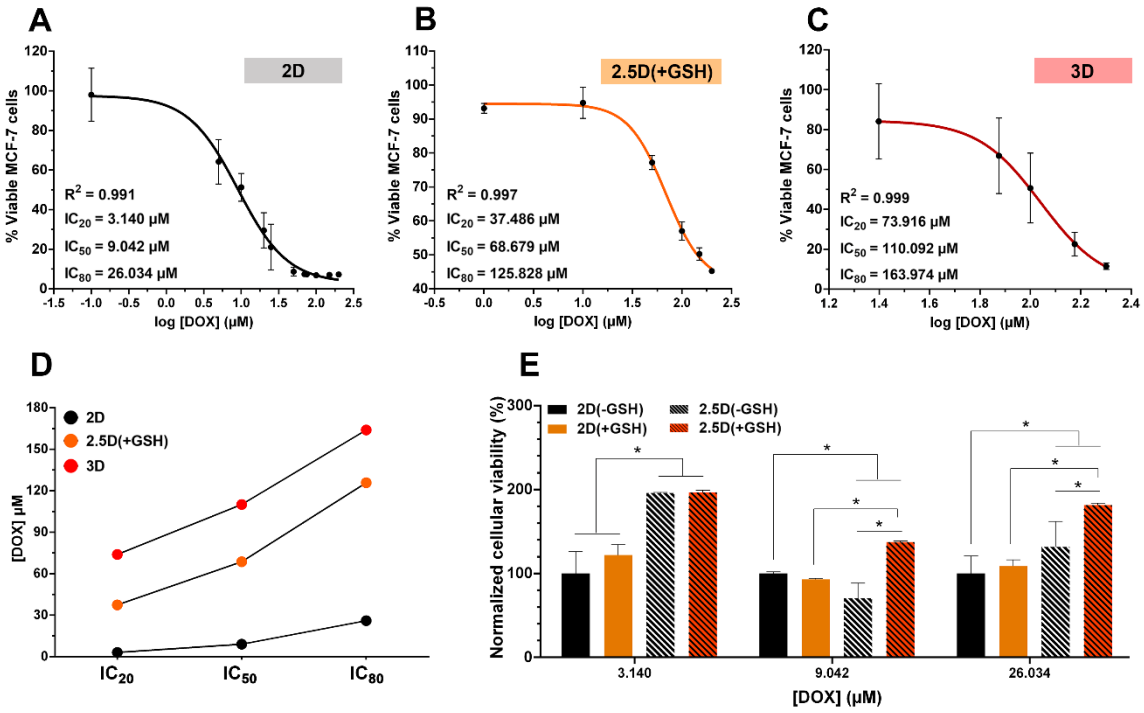

Figure 4. Evaluation of the DOX effect on the cellular viability of 2D, 2.5D and 3D MCF-7 cell cultures. Dose-response curves of 2D(-GSH) (A), 2.5D(+GSH) (B) and 3D (C) MCF-7 cell cultures to DOX. Comparison of 20, 50 and 80 % inhibitory concentrations of DOX (IC_{20}, IC_{50} and IC_{80}) in 2D(-GSH), 2.5D(+GSH) and 3D MCF-7 cell cultures (D). Cell viability of the different models after the administration of several concentrations of DOX (3.140, 9.042 and 26.034 µM) during 24 h (values were normalized towards the cell viability of 2D(-GSH) cells) (E); data are presented as mean ± S.D. (n=5); *P<0.05.

As expected, DOX prompted a decrease on the spheroids' size (Figure 5 A and B) [107, 108]. The IC_{20}, IC_{50} and IC_{80} of DOX in the 3D spheroids were determined to be 73.916, 110.092 and 163.974 µM, respectively (Figure 4 C). As expected spheroids are more resistant to the effect of DOX when compared to 2.5D(+GSH) cells. However, 2.5D(+GSH) cells' DOX resistance profile is closer to that displayed by 3D spheroids when compared to conventional 2D models (Figure 4 D). Together, these results confirm that the 2D culture of MCF-7 cells derived from 3D spheroids in presence of GSH can be an alternative *in vitro* model of breast cancer.

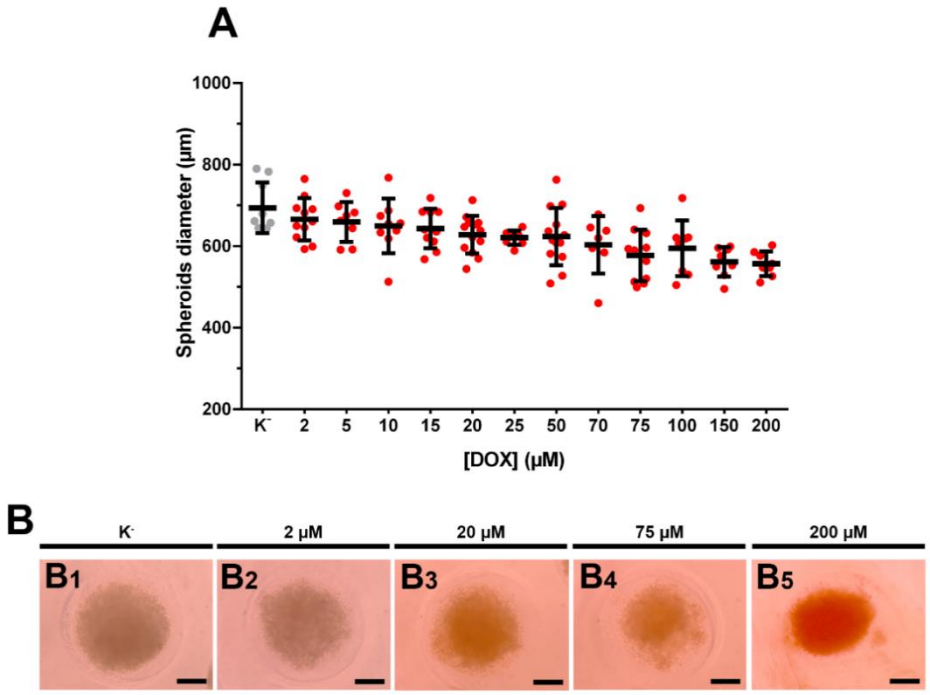

Figure 5. DOX effect on 3D MCF-7 spheroids size. Diameter measurements (A) and optical microscopy images (B) of spheroids treated with DOX (2-200 µM); K- represents the negative control (cells incubated only with medium); scale bars correspond to 200 µm.

3.2. 2.5D(+GSH) breast cancer cell culture resistance mechanisms towards DOX

High expression of efflux pumps is associated with the increased resistance displayed by cancer cells to different drugs. For instance, one of the main DOX resistance mechanisms presented by cancer cells is the up-regulation of P-gp expression [4, 109]. Various studies have demonstrated that GSH can affect the expression of this multidrug resistance transporter [82, 86, 87]. As an example, Hong *et al*. observed that the treatment of cells with a GSH synthesis inhibitor (Buthionine sulfoximine (BSO)) induces the up-regulation of the P-gp expression [82]. Therefore, the increased DOX resistance displayed by 2.5D(+GSH) cells may be mediated by an up-regulation of the P-gp function.

To confirm this hypothesis, the efflux of a P-gp substrate (Rho 123), as well as its accumulation inside the cells cultured in 2D and 2.5D, in presence and absence of GSH, was studied (Figure 6). The data obtained through fluorescence spectroscopy demonstrates that the efflux of Rho 123 occurs in all the types of MCF-7 cultures over time (Figure 6 B). However, the 2.5D(+GSH) MCF-7 cells showed higher Rho 123 efflux compared to the remaining cultures. In particular, after 24 h of Rho 123 incubation, 2.5(+GSH) MCF-7 cells demonstrated 47.367 ± 8.871 % of Rho 123 efflux, while the traditional 2D cell cultures (2D(-GSH) MCF-7 cells) showed only 33.200 ± 6.788 % (Figure 6 B). Furthermore, these results also demonstrate that in both 2D and 2.5D MCF-7 cells, the efflux of the Rho 123 seems to be higher when the cells were maintained in the presence of GSH (Figure 6 B).

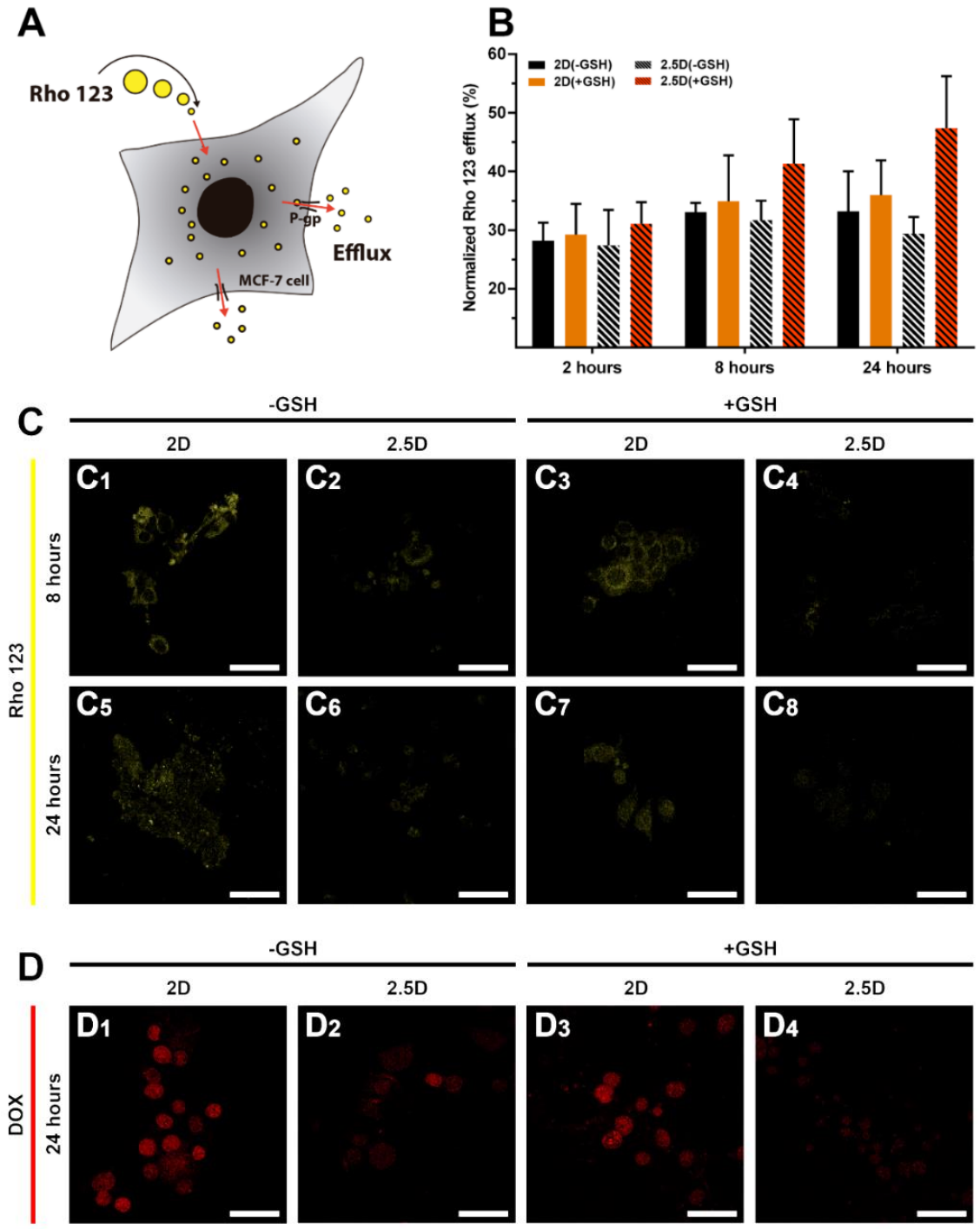

Figure 6. Evaluation of the P-gp function in MCF-7 cells. Schematic representation of the Rho 123 efflux mechanism in MCF-7 cells (A). Normalized Rho 123 efflux in 2D(-GSH), 2D(+GSH), 2.5D(-GSH) and 2.5D(+GSH) MCF-7 cells; data are presented as mean ± S.D. (n=5) (B). CLSM images of the accumulation of Rho 123 (C) and DOX (D) in 2D(-GSH), 2D(+GSH), 2.5D(-GSH) and 2.5D(+GSH) MCF-7 cells; yellow channel: Rho 123; red channel: DOX; scale bars correspond to 50 μm.

To corroborate these observations, the accumulation of Rho 123 and DOX in the cellular compartments of the MCF-7 cells was assessed by CLSM images (Figure 6 C and D). As expected, the lowest accumulation of the Rho 123 at 8 and 24 h occurred in the 2.5D(+GSH) MCF-7 cells (Figure 6 C4 and C8). The same profile was also observed for DOX, *i.e.* the

2.5D(+GSH) group displayed the lowest accumulation of DOX (Figure 6 D4). Interestingly, in this assay, the accumulation of DOX in 2D(+GSH) appeared to be also decreased (Figure 6 D2). Nevertheless, this model does not display an improved DOX-resistance profile (Figure 6 E).

These results disclose that one of the mechanisms that may be involved in 2.5D(+GSH) cells improved resistance to DOX is the increase of P-gp activity, which is influenced by the presence of GSH.

The therapeutic efficacy of DOX can be limited by ROS scavengers, such as GSH. In fact, DOX-induced ROS production is a major mechanism of action of this drug (excessive amount of ROS causes DNA damage and can lead to the activation of signaling pathways involved in cancer cells apoptosis) [110]. As an antioxidant, GSH stabilizes the redox state of the cells by ROS scavenging, as previously demonstrated [111].

A study performed by Armstrong *et al.* demonstrated that the treatment of cells with BSO (inhibitor of GSH synthesis) resulted in an early decline in cellular GSH, followed by an increase of ROS levels, which further led to the induction of various apoptotic signals [84]. Therefore, H_2DCFDA cellular reactive oxygen species detection assays were performed to elucidate if GSH influenced the 2.5D(+GSH) MCF-7 cells resistance to DOX by decreasing the ROS levels in these cells (Figure 7).

In this type of assay, bigger fluorescence signals on cells are directly correlated with a higher presence of ROS. As observed in CLSM images, the 2.5D(+GSH) MCF-7 cells displayed a weaker fluorescence intensity when compared to the other type of MCF-7 cell cultures, indicating that ROS levels are lower in this model, probably due to its higher ROS scavenging capacity (Figure 7 A4). Therefore, decreased ROS levels may be another mechanism responsible for the resistance of 2.5D(+GSH) cells to DOX.

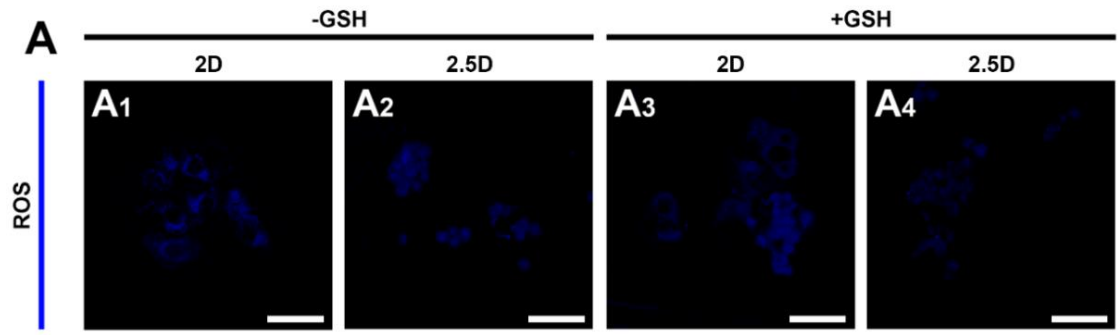

Figure 7. Evaluation of the ROS levels in MCF-7 cells. CLSM images of the ROS generation by 2D(-GSH), 2D(+GSH), 2.5D(-GSH) and 2.5D(+GSH) MCF-7 cells after their treatment with 0.3 % hydrogen peroxide (H_2O_2) through the H_2DCFDA assay; blue channel: H_2DCFDA oxidized (DCF) by ROS; scale bars correspond to 50 µm.

Chapter IV

4. Conclusion and Future Perspectives

Due to the increment of new cases and deaths associated with breast cancer, new therapeutics for treating this disease are highly demanded. To accomplish the development of such therapeutics, all the anticancer therapeutics candidates must be evaluated on *in vitro* and *in vivo* assays (preclinical stage of drug development process) and further in humans (clinical trials).

Up to now, 2D cell cultures remain as the most commonly used *in vitro* method for therapeutics screening due to its simplicity, reproducibility and low cost. Nevertheless, flat 2D cell culture models are unable to reproduce the properties of *in vivo* solid tumors as well as their resistance to therapeutics. On the other hand, the use of *in vivo* models is associated with economical and ethical issues. *In vitro* 3D cell culture emerged as a viable intermediate step between standard 2D cancer cell cultures and *in vivo* animal experimentation for drug screening purposes, since it mimics the main features exhibited by *in vivo* solid tumors. However, there is a lack of equipment and assays optimized and regulated to perform the analysis of therapeutics action in spheroids. Therefore, drug resistant 2D cell cultures can be used as an alternative to investigate the effect of anticancer drugs since this type of culture displays a drug-resistance profile more similar to that found on 3D spheroids.

In this study, it was demonstrated that a drug resistant 2D cell culture model can be obtained in a short-period of time by culturing in 2D the cells obtained from the dissociation of 10 days old spheroids (2.5D MCF-7 cell culture). To increase the resistance of the cells of this model to DOX, it was necessary to culture cells in medium supplemented with GSH. On the 3D-derived cultures, we verified that GSH improved cells survival when they were incubated with DOX. The 2.5D(+GSH) model demonstrated a higher resistance to DOX than that exhibited by conventional 2D cell culture. On the other hand, the resistance of spheroid-derived cells was close to that determined for spheroids. It was revealed that such effect was likely mediated by the increased P-gp function. The cells of the 2.5D(+GSH) model presented a higher efflux of Rho 123 and lower accumulation of this molecule in its interior, when compared with the conventional 2D cell culture. The decreased ROS scavenging levels was also found on the cells of the 2.5D(+GSH) model, unlike the conventional model.

Overall, the 2.5D(+GSH) MCF-7 cell cultures can be a promising simple and inexpensive tool to evaluate drugs and other therapeutics aimed for breast cancer treatment, since this model is compatible with the methodologies and techniques that are already in use for conventional 2D *in vitro* assays.

Additionally, in a near future, the methodology used to obtain the 2.5D(+GSH) MCF-7 cell culture model could be applied to other cell lines and to evaluate other drugs or drug resistance mechanisms found in cancer cells.

Chapter V

5. References

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