Study of New Therapeutic Strategies to Combat Breast Cancer

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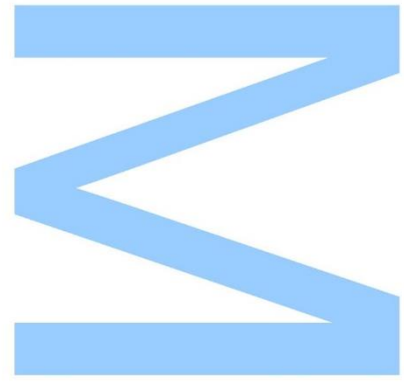

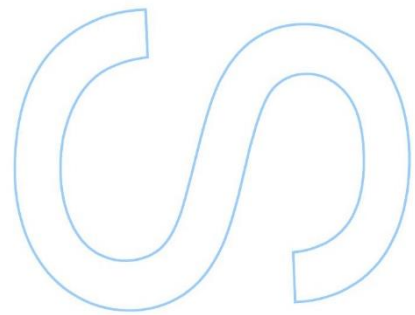

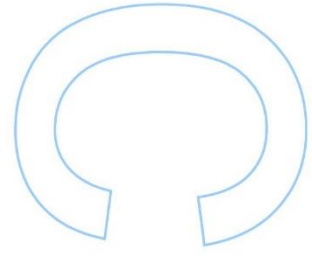

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Resumo

O cancro é uma doença com cada vez mais realce nos dias de hoje, uma vez que afecta e mata milhões de pessoas em todo o mundo. Nomeadamente, o cancro da mama é, ainda, um cancro com elevada incidência e mortalidade, principalmente nas mulheres. Mesmo apesar das taxas de mortalidade por este tipo de cancro terem diminuído nos últimos anos, este continua a ser desafiante a nível de tratamento, principalmente o tipo metastático. Devido a todo este impacto no sector da saúde, a terapia oncológica é alvo de uma intensa e muito dispendiosa investigação. Com o objetivo de melhorar e reduzir os elevados custos esta terapêutica, estratégias como o reaproveitamento de fármacos e combinação de fármacos têm sido bastante aprofundadas e abordadas. Reaproveitamento de fármacos significa dar uma nova utilidade a fármacos que, neste caso, estão aprovados para a terapia de diversas doenças (por exemplo, doenças cardiovasculares), mas não estão aprovados em cancro. Assim, o grande objetivo é que estes fármacos possuam uma atividade a nível oncológico. Por sua vez, combinação de fármacos pressupõe a junção de dois ou mais fármacos, de modo a que a resposta que é obtida através da combinação seja superior à resposta obtida pelos fármacos dados individualmente. Posto isto, através da utilização de fármacos com potencial a serem reaproveitados, combinados com um fármaco de referência em cancro da mama (5-FU), o objetivo deste trabalho foi investigar se esta combinação levava a benefícios terapêuticos, perante os fármacos isolados. Começou-se por um *screening* dos fármacos, de modo a trabalhar-se com aqueles que possuíam maior potencial a serem benéficos nesta terapia, tendo sido escolhidos o verapamil, o itraconazole e a tacrina. Vários estudos de viabilidade celular e de avaliação da proliferação e morte celular, principalmente em células MCF-7, foram efectuados. Foram também realizados estudos de modo à compreensão de uma eventual resistência terapêutica, avaliando-se a transição de um estado epitelial para mesenquimal. Reunindo todos os resultados que foram obtidos neste trabalho, é possível a conclusão de que as combinações do verapamil, itraconazole ou tacrina com o 5-FU, possuem um claro benefício a nível da terapia do cancro da mama, nomeadamente a nível de diminuição de proliferação e viabilidade celular. Ainda, a combinação do itraconazole com o 5-FU parece ser a combinação mais eficaz, sendo um interessante foco em próximos estudos.

Palavras-chave: Cancro da mama, Reaproveitamento de fármacos, Combinação de fármacos, 5-Fluorouracil, Viabilidade celular, Proliferação celular, Morte celular, Transição Epitelial-Mesenquimal.

Abstract

Cancer is a set of extremely complex diseases, which are increasingly prominent today, as it affects and kills millions of people worldwide, being the subject of intense study both in its pathophysiology and therapy. Namely, breast cancer is still a cancer with a high incidence and mortality, especially in women. Even though mortality rates for this type of cancer have declined in recent years, it remains challenging at treatment level, especially the metastatic type. Due to all this impact in the health sector, oncological therapy is the subject of an intense and very expensive study. In order to improve this therapy, as well as reduce its subjacent high costs, strategies such as drug repurposing and drug combinations, have been extensively studied and increasingly addressed. As the name implies, drug repurposing means giving new usefulness to drugs which, in this particular case, are approved for the therapy of various diseases (for example, cardiovascular or metabolic diseases), but are not approved for cancer therapy. Thus, the main goal in the study of these drugs is to have some beneficial oncological activity. Combination of drugs, on the other hand, presupposes the combination of two or more drugs, so that the response that is obtained through the combination is more advantageous than the response obtained by the individually given drugs. Therefore, through the use of drugs with potential to be repurposed, combined with a reference drug in breast cancer (5-FU), the aim of this project was to investigate whether this combination led to therapeutic benefits, comparing with the isolated drugs. We have started with a screening of the drugs, in order to work with those who had the greatest potential to be beneficial in this therapy, with Verapamil, Itraconazole and Tacrine being the chosen drugs. Several cellular viability studies, as well as evaluation of cell proliferation and death, mainly in MCF-7 cells, were performed. Additionally, studies were also carried out in order to understand the effect of the drugs at the level of possible therapeutic resistance, evaluating the epitelial-mesenchymal transition. Combining all the obtained results in this project, it was possible to conclude that the combination of verapamil, itraconazole or tacrine with 5-FU has a clear benefit in breast cancer therapy, namely in the level of decreased proliferation and cell viability. Furthermore, the combination of itraconazole and 5-FU seems to be the most effective, being an interesting focus in future studies.

Keywords: Breast cancer, Drug repurposing, Drug combinations, 5-Fluorouracil, Cell viability, Cell proliferation, Cell death, Epithelial-Mesenchymal Transition.

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Abbreviations

5-FU	5-Fluorouracil
ABCC3	ATP Binding Cassette Subfamily C Member 3
AChEI	Acetylcholinesterase Inhibitor
ADH	Atypical Ductal Hyperplasia
ATRA	All-trans Retinoic Acid
BCRP	Breast Cancer Resistance Protein
bHLH	Basic helix-loop-helix
BSA	Bovine Serum Albumin
CAF	Cyclophosphamide, Doxorubicin (Adriamycin), and 5-Fluorouracil
CDK	Cyclin-Dependent Kinase
CMF	Cyclophosphamide, Methotrexate, and 5-Fluorouracil
CFSE	Carboxyfluorescein Succinimidyl Ester
CHOP	Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone
COX-2	Cyclooxygenase-2
DAB	3,3'-Diaminobenzidine
dATP	Deoxyadenosine Triphosphate
DCIS	Ductal Carcinoma *in situ*
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
DHFU	DihydroFluorouracil
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DPD	Dihydropyrimidine Dehydrogenase
DT	Doubling Time
dTMP	Deoxythymidine Monophosphate
dUMP	Deoxyuridine Monophosphate
dUTP	Deoxyuridine Triphosphate
EDTA	Ethylenediamine Tetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EMA	European Medicines Agency
EMT	Epithelial-Mesenchymal Transition
ENT 1/2	Equilibrative Nucleoside Transporter 1/2
ER	Estrogen Receptor
ESMO	European Society for Medical Oncology
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FdUMP	Fluorodeoxyuridine Monophosphate
FdUTP	Fluorodeoxyuridine Triphosphate
FEC	5-Fluorouracil, Epirubicin and Cyclophosphamide
FITC	Fluorescein Isothiocyanate
FMCm	Feline Mammary Carcinoma (cell line), with metastatic capacity.
FOLFIRI - CETUXIMAB	Folinic acid, 5-Fluorouracil, and Irinotecan plus Cetuximab
FUTP	Fluorouracil Triphosphate
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human Epidermal Growth Factor Receptor 2
HSA	Highest Single Agent

IC_{50}	Half Maximal Inhibitory Concentration
IgG1	Immunoglobulin G 1
MBC	Metastatic Breast Cancer
MCF-7	Michigan Cancer Foundation-7 (cell line)
MET	Metastasis
MFI	Mean Fluorescence Intensity
mTOR	mammalian Target Of Rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
N	number of independent experiments
NCCN	National Comprehensive Cancer Network
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
OAT2	Organic Anion Transport 2
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PDE-5	Phosphodiesterase type 5
PI	Propidium Iodide
PR	Progesterone Receptor
PS	Phosphatidylserine
Q(1-4)	Quadrant 1-4
ReDO	Repurposing Drugs in Oncology
SERM	Selective Estrogen Receptor Modulators
SLC	Solute Carrier transporters
SMO	Smoothened (protein)
TBS	Tris-Buffered Saline
TNBC	Triple Negative Breast Cancer
TS	Thymidylate Synthase
vs	Versus
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WHO	World Health Organization

1. Introduction

1.1. Cancer

Cancer, also called malignant neoplasm, consists of a deviation to the coordinated interaction among cells and organs [1]. It is not a single disease, but a complex group of diseases involving abnormal cell growth, in which there is a potential to invade and/or spread to other tissues of the body [2].

1.1.1. Epidemiology

Epidemiologically, cancer is the second leading cause of death behind heart disease [1], accounting for 14.1 million new cases of cancer worldwide in 2012 and for an estimation of 9.6 million deaths in 2018, being more frequent in adults older than 60 years of age. Nearly half of all cancer deaths are due to liver, lung, stomach and bowel cancers, although the most frequent cancers worldwide are lung and bowel, but also female breast and prostate cancers. Particularly, these four cancer types account for around 4 in 10 of all cancers diagnosed in the whole world [3].

Concerning the incidence of cancer, it is known that it depends on various factors: geography, age, race, and genetic background. In particular, the geographic variation is a very important factor for the differences in the exposure to environmental factors implicated in carcinogenesis, such as infectious agents, smoking, alcohol and diet. The fact that more than 4 in 10 cancers occurring worldwide are in countries at a low or medium level of Human Development Index reflects the importance of the environment and geographic variations [3, 4].

Despite the high impact on human's health, the economic impact of cancer is also very relevant and is increasing throughout the years. As an example, in 2010, the total economic cost of cancer was estimated at approximately €1 trillion [5].

1.1.2. Hallmarks of cancer

As mentioned above, cancer can be defined as a complex group of distinct genetic diseases. However, all these diseases are united by common traits, named hallmarks of cancer [6]. Six established hallmarks exist: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. These hallmarks are important to the understanding of all the complex biology of cancer, being defined as capabilities that enable cancer growth and presence of metastasis. In spite of the six fundamental hallmarks, two new hallmarks have emerged from the intense oncological research: reprogramming of energy metabolism and evading immune destruction. Additionally, it is still important to highlight that a key to the acquisition of the above-mentioned hallmarks is genome instability and the presence of inflammation [7].

Malignant neoplasms are not only composed of cancer cells. In addition to these cells, they exhibit another dimension of complexity, the tumor microenvironment, which contains normal cells that have an important contribution to the acquisition of the referred hallmarks [7]. Thus, a tumor contains not only cancer cells, but also various infiltrating endothelial, hematopoietic, stromal, and other cell types that can have a major influence on the function of a tumor as a whole [8]. The intense study and insight of these concepts will increasingly affect the development of new means to treat human cancer [7].

1.1.3. Cancer treatment modalities

When dealing with cancer, there are three important priorities, which are, in order of importance: prevention, early detection and total eradication. However, these three measures are, in the great majority of the times, difficult to achieve. Particularly, the treatment of cancer is a very complex issue. There are three usual modes: surgery (excision of primary tumor), radiotherapy and pharmacotherapy (that includes cytotoxic chemotherapy, endocrine therapy, biological therapy, gene therapy and immunotherapy, which are also a part of biological therapy). Table 1 summarizes the different kinds of therapy used in cancer treatment.

Table 1 - Different treatment modalities in cancer therapy and the respective description.

Treatment modalities	***Description***
Surgery	Treats the part of the body operated on. In this regard, acts on cancer that is completely contained in one area and hasn't spread [9].
Radiotherapy	Damages the genetic material of cells, blocking their ability to divide and further proliferate [10].
Chemotherapy	Damages essentially proliferating cells, mainly by means of interfering with cell division, resulting in cell stress, that may then lead to cell death. Can, also, directly kill cancer cells [11].
Endocrine Therapy	Targets hormone-dependent pathways, decreasing rates of proliferation in cancer cells, modulating pathways to achieve downregulation or deletion of the source of endogenous hormones [12].
Biological Therapy	Involves the use of living organisms, substances derived from them, or laboratory-produced versions of such substances to treat the disease [13].
Gene Therapy	Includes types of treatment that use genetic material to modify cells, in order to cure [14].
Immunotherapy	Involves or uses components of the immune system. Some consist of antibodies that bind to and inhibit the function of proteins expressed by neoplastic cells. Other include vaccines and T cell infusions [15].

The choice of the right anticancer agents, as well as the right doses, ways of administration and management of toxicities, is a hard and extremely complex process. In this regard, a healthcare delivery team very experienced in the use of all of this therapeutic modalities is required. Additionally, choosing and verifying treatment options based on the individual patient is a crucial issue about dealing with the right administration of the diverse anticancer agents [16].

1.1.4. The difficulties in cancer treatment

Concerning pharmacotherapy, it can be said that the ideal drug is the drug that selectively kills the neoplastic cells, minimizing the adverse effects. However, unfortunately, the differences between normal and neoplastic cells reside, usually, in a quantitative way, such as greater or lesser activation of signaling pathways, sensitivity to hormones or growth factors and different growth parameters. Therefore, pharmacotherapy of cancer is difficult to achieve in a totally successful way [17]. Nevertheless, survival of oncological patients has improved significantly in the last years, mainly because of multidisciplinary care, improved chemotherapeutic agents, the introduction of targeted therapy, and the incorporation of palliative care services [18]. However, despite the advances above mentioned, many patients still fail therapy, explained by the presence of an intratumoral heterogeneity and innate or acquired drug resistance mechanisms, all subjacent to the molecular complexity of the great majority of cancers [8] (Table 2). Events contributing to this complexity include genetic mutations, interactions between the microenvironment and the presence of cancer stem cells, that are defined as cells within a tumor that can both self-renew by dividing and give rise to many cell types that constitute the tumor, and can, therefore, form new tumors [18].

Table 2 - Drug resistance mechanisms and the respective description.

Resistance mechanism	***Description***
Drug Efflux	Efflux pumps pump out a variety of substrates from the cell. A well known example of a drug efflux pump is P-glycoprotein, that promotes the elimination of a great variety of cancer chemotherapeutics [19].
Drug Inactivation	Neoplastic cells can develop resistance through decreased drug activation, since a great variety of anticancer drugs require a metabolic activation to exert their activity [20].
Alterations in Drug Targets	Alterations to the drug target, such as mutations, have an essential role in drug response and resistance [19]. An example constitutes the modified enzyme expression levels at drug target sites [20].

DNA Damage Repair	As many chemotherapeutic drugs induce DNA damage, the cell tends to respond in order to repair or die; therefore, DNA damage repair capacity has a major influence on the efficiency of DNA-damaging drugs [19].
Deregulation of Apoptosis	Drug resistance may be a result of several defects in the death pathways. For example, cells overexpressing Bcl-2 are known to be resistant to various chemotherapeutic drugs [21].
Activation of Prosurvival Signalling	The activation of prosurvival pathways, such as the EGFR (Epidermal Growth Factor) pathway, is a known mechanism of resistance to various chemotherapies [19].
Oncogenic Bypass	An oncogenic bypass means that the primary target of the drug remains unaltered and keeps being inhibited, but because of an adaptative feedback loop and/or a genetic mutation selected during the treatment, an alternative pathway becomes activated [19].
Epithelial-Mesenchymal Transition (EMT)	EMT is a process that allows an epithelial cell to assume a mesenchymal phenotype, acquiring increased migratory capacity, more resistance to programmed cell death, invasiveness and higher production of extracellular matrix components [22].
Integrins	Integrin-mediated adhesion to the extracellular matrix can alter responses to chemotherapeutic drugs by diverse mechanisms, including inhibition of apoptosis [19].
Cytokines and Growth Factors	Activation of oncogenic signaling pathways by cytokines and growth factors can have key roles in resistance of drugs by maintaining the activation of various survival signaling pathways [19].
Epigenetics	Epigenetic alterations play an important role in the development of drug resistance. For example, is known that hypermethylation of the P-glycoprotein promoter is associated with chromatin structural changes and transcriptional repression [23].

Neoplastic cells may use several of these mechanisms at the same time, although there are huge variations between tumors. The creation of a drug resistant population by positive selection can help drive resistance, even though is important to note that acquired resistance cannot simply be viewed as an overgrowth of a resistant cancer cell population. Pre-existing genomic and proteomic profiles, as well as new innovative methods with fewer failures, can be used to predict the development of resistance mechanisms and measure them, being important tools to tackle these mechanisms in patients [24].

Intense pharmacological research is being made about oncological therapy, originating an explosion of costs of new cancer drugs. The big drawback about this situation resides in the fact that there is a crescent recognition that the budgets of most national healthcare services will be unable to support these high costs, once the worldwide spend on oncology drugs in 2013, for example, was nearly €80 billion [25]. However, despite this big investment, there is a little output for a huge pharma research and development spending. This existent gap in productivity remains even though the investment of astronomic amounts of money in novel discovery technologies. So, there is an extreme need for creativity, in order to find new uses and improved versions of existing drugs [26]. Another problem in oncologic research is the fact that cancer is a very complex disease, in which exists distinct molecular signatures with differential levels of sensitivity to treatment, leading to resistance to the different therapeutic modalities, through many different mechanisms discussed above [27, 28]. Thus, there is a requirement for more effective cancer drugs. To face this problem, an interesting approach, named drug repurposing, is being increasingly applied [29]. Highlighting the importance of this approach, the global market for drug repurposing reached €20.7 billion in 2015 and is projected to reach €26.6 billion by 2020 [30]. Additionally, another important response to the problems in cancer therapy encompasses the use of drug combination therapies, as highlighted in section 1.3 [31].

1.2. Drug repurposing

Drug repurposing, drug repositioning, drug rescue or also called drug reprofiling, is a methodology to identify a new indication for already existent drugs. It allows lower costs and a shorter time until approval of the drug, than developing a drug *de novo*, because all phases of clinical trials have already been performed for the approved drugs and the information regarding side effects, pharmacokinetics and interaction with other drugs have been collected [32]. There is, therefore, published data on

parameters, such as pharmacokinetics and bioavailability, that are accessible to both the clinician and the researcher [31]. However, in order to establish maximum tolerated doses, for example, for oncologic purposes, phase I trials may still be required. These trials are also needed if the repurposed drug will be tested in untried combinations with other drugs, since must be established that there are no unacceptable toxicities. Anyway, drug repurposing is a shortening of the extensive drug development process. It can contrast a 10–17 year development lifecycle for *de novo* development versus a 3–12 year process for repurposed drugs, once repurposing builds upon previous research and development efforts [31, 33].

Cytostatic and/or cytotoxic activity for compounds within a wide range of drug classes other than cancer has been demonstrated in several studies. Additionally, more than 10.000 clinical trials investigating drugs in oncology are registered at www.clinicaltrials.gov[1], but only a few candidates progress to the next phase in clinical trials, with an approval rate of cancer drugs entering phase I trials lower than 5%. Notably, in 2016, FDA approved only 22 new drugs compared to 45 in 2015. In oncology, only 4 new drugs were approved in 2016, compared to 14 in 2015. Also, the European Medicines Agency (EMA) approved fewer drugs in 2016 than previous years [34]. Therefore, the study of the arsenal of drugs approved for non-cancer indications might offer effective treatment options for cancer patients. However, despite the growing attraction about this methodology, reports of successful repurposing of drugs as anti-cancer agents have been limited [25]. In this regard, it is notorious that drug repurposing is still in early stages and a huge number of barriers exist. Despite many strategies being implemented and tested, none has been described as ideal. Furthermore, there is a big divergence of interests by the stakeholders involved in drug repurposing, making this process hard to harmonize. Another important barrier is the fact that companies may be reticent to release information about the drug with the fear that the repurposing program uncovers safety and efficacy issues. However, for rare or severe diseases, issues about safety are softened. Thus, repositioning is less risky for these indications, even though such drugs also produce lower returns on investment because of their limited target [35].

[1] ClinicalTrials.gov is a resource that is provided by the U.S. National Library of Medicine. It is a database where clinical studies conducted in the whole world are present.

1.2.1. Drug repurposing process

Commonly, ideal drugs for repurposing share a number of common characteristics. They should be well-known drugs, often available as generics, rather than newer agents, the toxicology profile of the drug must be good, there should have a plausible mechanism of action, relevant for the condition in question, and evidence of efficacy at physiological dosing [31].

Ideas for the repositioning process can come from several processes: serendipitous observations, new knowledge about the drugs or from established technology platforms (Figure 1) [26]. Mainly because the fast advances in technology, nowadays, the serendipity responsible for early discoveries of drugs is being replaced by systematic searches for candidates. Now, it is possible to detect molecular similarities between diseases through examining large and varied datasets. Also, computational models are very important in order to study the interaction between the molecules and the targets. In turn, high-throughput screening systems can quickly test many drugs against a great variety of cell lines. Therefore, the tendency in the drug development field is focused on drug repurposing based on knowledge, mainly computer-aided repositioning approaches, replacing serendipitous findings and/or rational exploitation of drug side-effects [36, 37].

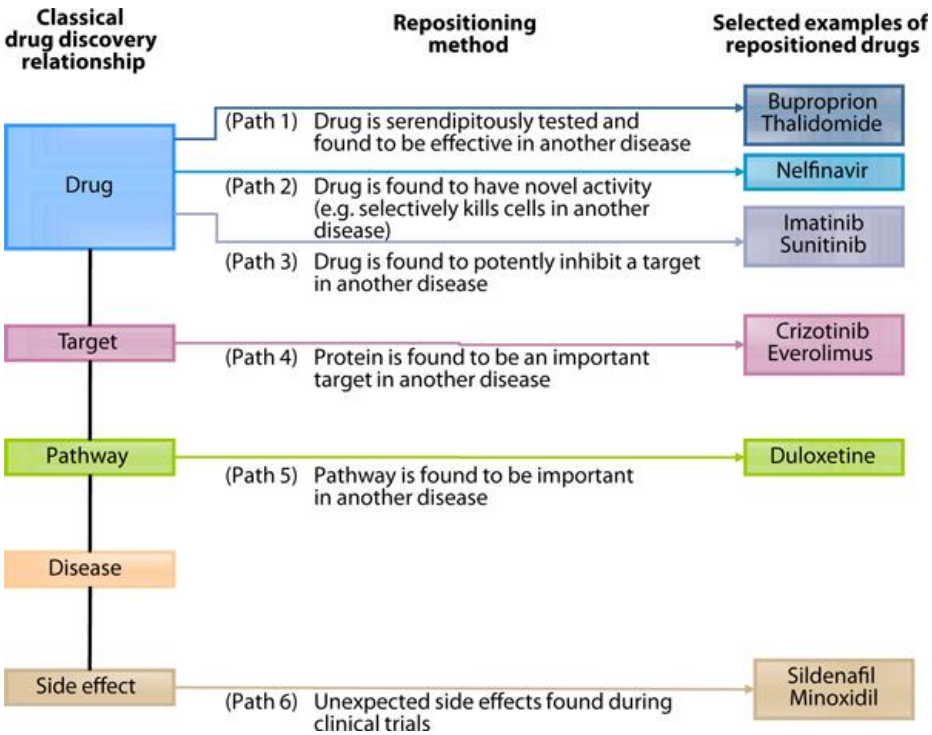


Figure 1 - Potential paths of drug repositioning. New indications for existing drugs can come from various processes, since serendipirous observations, until more rational approaches. Reproduced from Yvonne et al. [38]

1.2.2. Studies and cases of success in drug repurposing

Many currently used drugs have, at least, some actions that may be useful in cancer treatment. Particularly, a lot of off-patent drugs have shown some evidence of anticancer effects, in which about 50% are supported by relevant human data and 16% are supported by data from at least one positive clinical trial. [39]. Figure 2 shows some drug candidates for repurposing and the respective hallmarks of cancer that they may target.

Probably, the best-known example of drug rescue is the use of sildenafil (viagra), originally developed for the treatment of coronary artery disease by Pfizer in 1980s. However, a marked induction of penile erections was serendipitously found during the phase I clinical trials. After Sildenafil failed in Phase II clinical trials for the treatment of angina, it was redirected to the treatment of erectile dysfunctions, initially thought to be a side effect. Another example is thalidomide, originally developed as a sedative by Grünenthal in 1957, used to alleviate morning sickness in pregnant women. Shortly after being introduced, it was found to cause serious birth defects, leading to its withdrawal from the market. However, posterior studies about this drug revealed that thalidomide possesses anticancer activity and this drug is, currently, used for the treatment of multiple myeloma in combination with dexamethasone [40, 41].

There are several studies in the field of repurposing new drugs for oncologic therapy. It is noteworthy a project, named ReDO (The Repurposing Drugs in Oncology), that consists in an international collaboration of diverse research groups and clinicians with the main goal of seeking new and effective cancer treatments, by using existing and well-characterized non-cancer drugs, the potential repurposed cancer drugs. Due to this project, a list of more than 250 non-cancer drugs, for which there is pre-clinical and clinical evidence of anti-cancer action was done. The basis for this list resides in an extensive and active surveillance of the cancer literature [42]. Briefly, for oncologic purposes, drugs like aspirin, itraconazole, verapamil, chloroquine and all-trans retinoic acid (ATRA) have shown anticancer activity in, at least, one randomized clinical trial [25, 43]. Particularly, in the case of itraconazole, that specific mechanisms by which it specifically works are unknown, results of a phase 2 study of this drug with Pemetrexed have shown that this combination has potential as a second-line therapy for metastatic nonsquamous non-small-cell lung cancer [44]. More detailed information about itraconazole will be highlighted in Discussion Section.

Several other drugs induced responses in rare tumors. For instance, tadalafil (PDE-5 inhibitor, primary indicated for erectile dysfunction), inhibits myeloid-derived suppressor cells in cancer patients. Propanolol (beta-blocker, used for hypertension) reduces proliferation and migration in angiosarcoma models, by blocking beta-adrenergic receptors expressed by angiosarcoma cells [45]. Also, metformin has been associated with a favorable response to therapy and increased survival in hepatocellular carcinoma, colorectal, prostate, HER2+ breast cancer, ovarian, pancreas, esophageal and rectal cancer [34]. Other drugs with randomized trial data supporting a survival benefit include cimetidine (colorectal cancer) and progesterone (breast cancer) [46]. However, data suggests that only thalidomide, ATRA, zoledronic acid and Non-Steroidal Anti-Inflammatory Drugs that inhibit COX-2, such as indomethacin and sulindac are currently included in the guidelines of the European Society for Medical Oncology (ESMO) or of the National Comprehensive Cancer Network (NCCN). In the case of the first 3, they were rebranded and reformulated by pharmaceutical companies. In the case of NSAIDs, they are used off-label, being listed in desmoid tumors guidelines [39, 47]. Aspirin, a NSAID, for colorectal cancer, is the only financial orphan drug with positive phase III data, although still not being recommended in clinical guidelines [46].

Figure 2 - Relation between potential drug candidates for repurposing and hallmarks of cancer that they are suggesting to target. Reproduced from Linda et al. [34].

1.3. Drug combination therapy

A disease is increasingly interpreted as a set of molecular pathways that interconnect, having a bigger susceptibility to the simultaneous action of several drugs. This makes possible to study drug combinations in greater depth [48]. Combining drugs has several advantages: decreased toxicity, better efficacy, decreased dosage at an equal or increased level of efficacy, and are very frequently used with the aim of counter drug resistance in cancer therapy [49]. Due to these advantages, drug combinations represent an interesting and increasingly used approach that has become a standard for the treatment of a wide range of diseases, such as cancer and infectious diseases [48].

Chemotherapy can be a very toxic treatment modality to the patient, with several side effects and risks, strongly reducing the immune system by affecting bone marrow cells and increasing susceptibility to host diseases, since it non-selectively targets actively proliferating cells, which ultimately leads to the destruction of not only neoplastic but also neoplastic cells. Thus, despite combination therapy can be toxic if one of the agent of the combinations used is chemotherapeutic, when compared with monotherapy, the toxicity is significantly less because different pathways will be targeted. Also, this conventional method is generally less effective than the combination therapy approach, although being still a very common treatment modality. A big advantage underlying combination therapy is the fact that this works in a synergistic manner, and therefore a lower therapeutic dosage of each individual drug is required. Additionally, combination therapy may produce cytotoxic effects on cancer cells, while simultaneously preventing toxic effects on normal cells. This occurs if one drug in the combination is antagonistic, in terms of cytotoxicity, to another drug in normal cells [50].

Some concerns about using drug combinations include the lack of flexibility in altering the dosing of individual components and the exposure of patients to an eventual unnecessary therapy, as well as difficulties on the understanding of the source of adverse reactions, in case of that [51].

1.3.1. Synergy definition and reference models to detect synergy

When in combination, two drugs producing the same broad therapeutic effect can produce the same effect of various magnitudes compared with the sum of the effects of individual drugs. This effect can be greater than, equal to, or less than this respective sum, being pharmacodynamically synergistic, additive or antagonistic, respectively [52]. Thus, if two or more drugs act synergistically, the possibility of reaching the desired outcome, such as cancer cell death, can be achieved by lower doses of each drug, minimizing their respective adverse effects, associated with higher doses.

The establishment of a reference model to detect and define synergy and antagonism is very important, serving the baseline for quantifying the interaction of two drugs, based on their individual interaction when neither antagonism nor synergy is presented, defined as additivity. Therefore, deviations of this baseline can, then, be seen as synergistic or antagonistic interactions. There are numerous proposed reference models for additive drug interactions, being full of permanent confusions and controversies, as manifested by over 20 definitions of synergy and discrepancies in its determination [53]. In this way, there is still no standardized guideline on how to choose the optimal reference model [54], and to understand these models, complex mathematical and pharmacological concepts are necessary [48].

There are three popular classes of reference models: Highest Single Agent (HSA) model, Loewe Additivity model and Bliss Independence model. These models have been developed based on different assumptions about the expected additive effect of the combination [54]. The HSA model, also called Gaddum's non-interaction model, assumes that the expected effect of the drug combination equals to the higher individual drug effect at the dose in the combination, reflecting the fact that the resulting effect of a drug combination is greater than the effects produced by its individual components [48, 54]. In its turn, the Loewe additivity model is based on the idea that a drug is mixed with itself, being not expected to exist any interaction, once a single drug cannot interact with itself. So, this model defines the expected effect (additivity) as if a drug was combined with itself. On the ther hand, the Bliss Independence model is based on the idea of non-interaction, that each drug is acting independently of one another, but each contributes to a common result. In this way, additivity can be calculated based on the probability of independent events [48, 54]. A brief elucidation of the limitations and advantages of each model will be discussed in the Discussion Section.

Drug combinations may, also, produce pharmacokinetically potentiating or reductive effects. That is, the therapeutic activity of one drug can be enhanced or reduced by another drug via regulation of its absorption, distribution, metabolism, and excretion. A further type of drug combination is named coalistic combination, in which all the drugs are active in combination, but inactive individually [52].

1.3.2. Drug combination studies

Particularly in cancer, numerous clinical trials testing combinations that include chemotherapy drugs, radiation therapy, hormonal therapies, molecularly targeted therapies, and immunotherapies are being carried out [55], with a crescent focus on the combination of cytotoxic chemicals and biotherapies (such as monoclonal or polyclonal antibodies, vaccines, gene therapy, cytokine therapy) [56].

In spite of numerous clinical trials running, there are numerous already approved combinations in cancer treatment. Some examples of combination therapies in cancer are the CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) regimen for Non Hodgkin lymphoma and FOLFIRI-CETUXIMAB (leucovorin (folinic acid), 5-fluorouracil, and irinotecan plus cetuximab), used for the treatment of metastatic colorectal cancer [57].

Combining repurposed pharmaceutical agents with other chemotherapeutic agents has also shown promising results, useful when traditional anti-cancer monotherapy has failed to provide a safe and tolerable treatment for cancer patients [58]. Some examples of these kinds of combinations are nitroglycerin in combination with the chemotherapeutics vinorelbine and cisplatin, and clarithromycin combined with bortezomib. In the first case, one randomized phase II trial demonstrated improved overall survival of patients with non-squamous cell lung cancer. In the case of clarithromycin, it has been shown to induce apoptosis when combined with bortezomib, being only efficacious when administered in a combination regimen in breast cancer and myeloma cells [58]. For instance, other combination of drugs may consist of a repurposed protector agent and a secondary or tertiary agent that kills cancer cells. In this way, the protector drug may be a repurposed agent shown to also display protective roles over normal cells in cancer therapy [58].
In theory, there are a lot of examples of drug combinations that seem to be attractive, based on mechanisms of action that are complementary. However, mainly because of efficacy or safety/tolerability issues, proved to be unsuccessful [59]. A known example

is VEGF and EGFR inhibitors (bevacizumab and erlotinib, respectively) that, as single agents, demonstrated anticancer activities. However, when used in combination, the co-inhibition of the two pathways did not demonstrate an improvement in efficacy in patients with non-small-cell lung cancer, despite promising results from phase I and II studies [59].

Even though notable advantages, many challenges in oncological combination therapies still remain. Notably, this kind of therapy remains a little restricted by toxicity to healthy cells [60]. Furthermore, in order to be effective, molecularly targeted agents and many combinations of these kinds of therapies require a target inhibition in a simultaneous way. Consequently, face problems related pharmacokinetics and toxicities to healthy tissues. A way to solve some of these issues is using nanoparticles or liposomes to the delivery of drugs. Using these approaches, specifically suboptimal exposure and poor target penetration are problems that may be solved [59].

1.4. Breast cancer

1.4.1. Epidemiology

The second most common cancer worldwide and the most common cancer in women is breast cancer, being the fifth most frequent cause of death from cancer overall. Although it is still the most frequent cause of cancer death in women in less developed regions, in more developed regions it is the second most common cause of cancer death in women, after lung cancer [61]. Particularly in Portugal, breast cancer is the cancer with the highest incidence rate. More than 6.000 new cases emerge every year and the incidence of breast cancer is increasing year by year. Also, about 1.600 women in Portugal die every year due to this disease [62]. Breast cancer not only occurs in women, but also occur in men. However, it is a rare condition, and less than 1% of all breast cancers occur in men [63].

In the past two decades, the rates of breast cancer mortality have declined by approximately 30%, with corresponding improvements in 5-year overall survival rates to 90%. However, despite these advances, metastatic breast cancer (MBC) remains a challenge to treat, with an estimated 5-year overall survival rate of only 23% [64]. Particularly, in 2012, nearly 1.7 million new cases of breast cancer were identified and less more than 500.000 cases of death due to breast cancer occurred in the world [65]. Thus, a large portion of the global population is affected by this disease, which

constitutes an important public health issue. As a consequence, it has generated a lot of research interest [66].

1.4.2. Breast cancer classification

Breast cancer is a heterogeneous group of diseases with distinct biological features, leading to huge differences in the response patterns to various treatment modalities and clinical outcomes [67]. So, management of this condition depends on the availability of robust prognostic and predictive factors [68].

The great majority of breast cancer types have their origin in the terminal ductal lobular unit (Figure 3), being ductal carcinoma the most common lesion, a highly heterogeneous group consisting of 80% of all kinds of breast cancer, followed by lobular carcinoma (10-20%). Other subtypes constitute only 5% of all lesions, and include tubular, mucinous, medullary, papillary, micropapillary and metaplastic. In fact, based on WHO classification, there are 17 categories of breast cancer, based on their morphology, but are also linked to particular clinical, epidemiological, and molecular features [69]. However, in the great majority of cases, it is classified as ‘invasive ductal carcinoma not otherwise specified’. Thus, the clinical relevance of this classification is limited [70]. In this regard, as there are several types of breast cancer, there are different ways of classification. Schemes based on physical and anatomical properties, histological grading and TNM staging (a system of classification based on Tumor size, Nearby lymph node involvement and Metastization) quantify the tumor aggressiveness [71]. On the other hand, expression-based schemes rely on techniques, such as microarrays, to achieve expression profiles and, consequently, can be divided into molecular subtyping and gene signatures, that reflect survival outcomes and therapeutic responses [72].

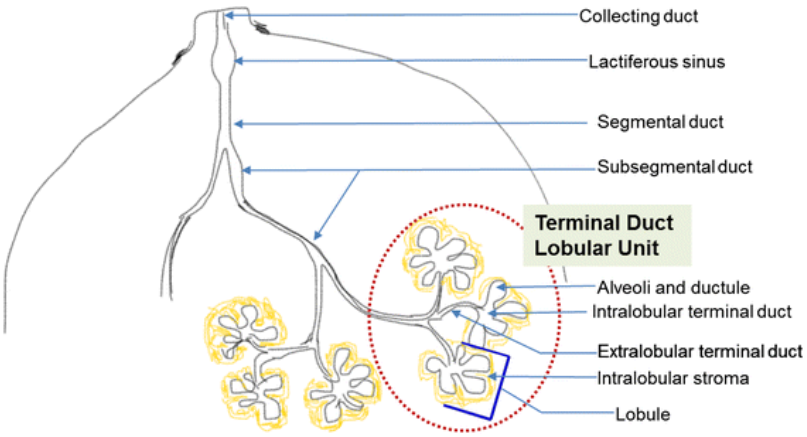


Figure 3 - Segment of breast lobe showing the lobules and the system of ducts. The functional unit of the breast is the terminal duct lobular unit (TDLU), where most types of breast cancer have their origin. Reproduced from Urmila et al. [73].

With special importance, there are three established biomarkers used for breast cancer classification - the expression levels of the Estrogen Receptor (ER), Progesterone Receptor (PR) and HER2 [74] (Table 3). In contrast to the classical histological classification, this classification is based on molecular profiling and gene expression arrays analysis [75]. Based on this, breast cancer can be classified into at least four different subtypes: luminal A, luminal B, HER2-enriched and basal-like breast cancer [66, 74].

Thereby, the histological appearance of the tumors may not be sufficient to establish the biological events involved in cancer development and progression, as well as the underlying complex genetic alterations [67]. Thus, classical biomarkers such as ER, PR and HER2, together with tumor size, tumor grade and nodal involvement, are conventionally used for patient prognosis and respective management [76].

Table 3 - Breast cancer classificantion methods based on specific biomarkers.

Classification method based on biomarkers	Description
Luminal A	Positive expression of the ER and/or PR, with a low pathological grade and low proliferation rates [67].
Luminal B	Positive expression of ER and/or PR, and also HER2 expression. Higher pathological grade and proliferation index than luminal A cancers [67]
HER2 – amplified tumours	Amplification of the HER2 and no expression of ER and PR high pathological grade [77].
Triple Negative (Basal-like)	Do not express hormone receptors (ER, PR) or HER2. Associated with aggressive behaviour and the worst prognosis and responsiveness to treatment [67].

1.4.3. Breast cancer biology

As mentioned above, breast cancer is not a single disease. Instead, it is a collection of breast diseases that have distinct histopathologies, genetic, genomic variations, and clinical outcomes, developing over the time (Figure 4).

In the normal breast terminal ductal lobular unit, exists lobules and ducts that consist of a bi-layered epithelium of luminal and myoepithelial cells [78]. A premalignant lesion, named atypical ductal hyperplasia, is characterized by abnormal cell layers in these ducts or lobules. This condition is thought to be the ductal carcinoma *in situ* (DCIS) precursor lesion, that can be defined as a lesion that contains abnormal cells, but still in a non-invasive way. This condition may give rise to Invasive Breast Cancer (IBC), but it is unclear how to predict which lesions will progress [79]. If cells invade, then the risk for developing metastasis increases exponentially, being the lymph nodes the primary site for breast cancer metastasis. In order to breast epithelial cells give rise to metastatic breast cancer, a number of events based on epigenetic and genetic changes within the microenvironment should occur. Namely, aberrations in the control of proliferation, differentiation, survival, migration and in interactions tumor-stromal cells cause this process to take place. Thus, cells must enter the vasculature (intravasation) and exit the vasculature (extravasation), while surviving in the absence of adhesion and, finally, establish a new tumor in a foreign microenvironment. It is important to notice that cancer cells with stem cell-like characteristics (cancer stem cells) drive breast cancer initiation and progression, as well as recurrence [80]. Luminal or myoepithelial progenitors are affected by different epigenetic and genetic alterations, that consequently give rise to different subtypes of tumors that consist of different cell types [78].

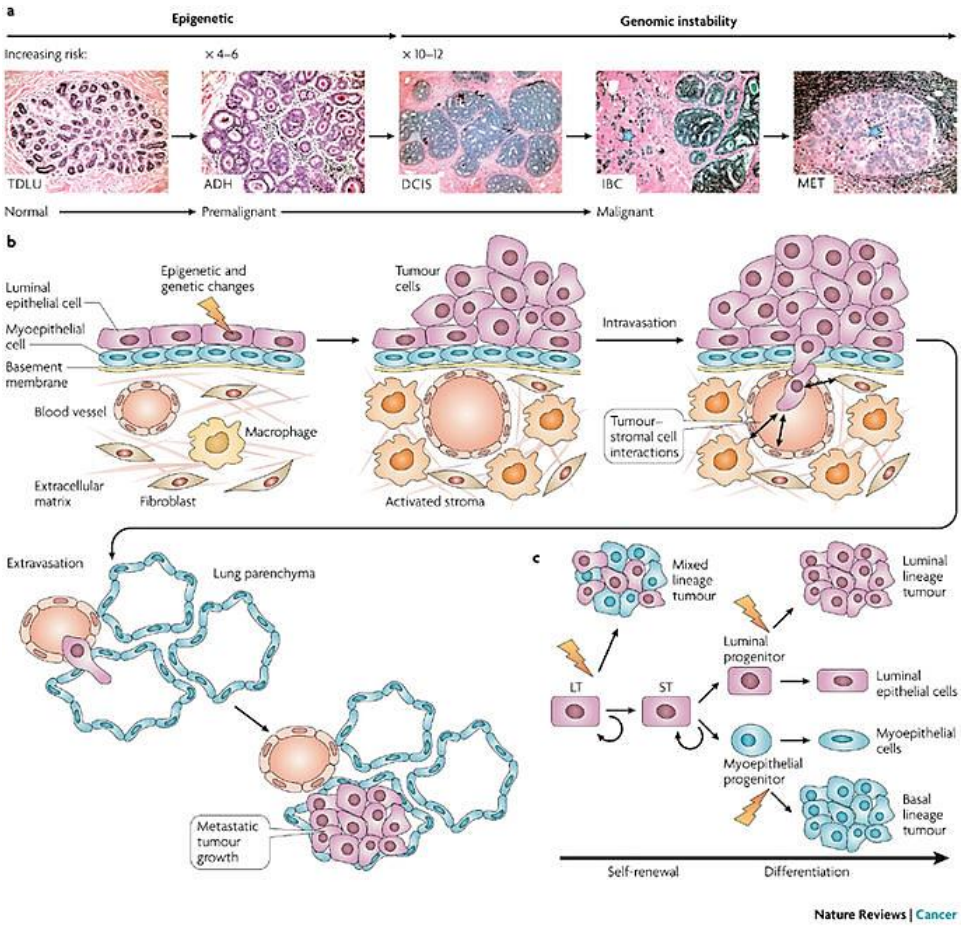


Figure 4 –The biology of breast cancer. **A |** Breast cancer is a disease that develops over the time. It evolves from an ADH to DCIS and IBC, ultimately leading to metastasis (MET) **B |** In order to originate malignant lesions, a number of events based on epigenetic and genetic changes within the microenvironment should occur. To metastize, cells must enter the vasculature (intravasatiion) and exit the vasculature (extravasation). **C |** Cancer Stem Cells drive breast cancer initiation, progression as well as recurrence and therapeutic resistance. Reproduced from Tracy et al. [78].

1.4.4. Breast cancer therapeutics

Even though breast cancer is still very prevalent, there is a drop in mortality over the years, mainly due to improvements in the diagnosis and management, varying widely between diverse geographic areas [81]. However, as discussed in Section 1.4.1, the treatment of metastatic disease remains a major challenge, despite improvements with a better understanding of the use of therapies for early stage breast cancer [82].

The choices of treatment regimens for breast cancer depend on diverse factors, namely the stage and subtype of cancer, the hormone receptor status, if the cancer is HER2 positive or negative, the overall health status of the person (particularly if the person has some other diseases or not, or some historical of cancer), if the woman has gone through menopause or not and, depending on the tolerability to the therapy, it may be adjusted to other treatment options. Given this complexity, it is difficult to have a universally accepted treatment, since each case is a case and the choice of the best

therapy should be tailored to each individual, taking into account the above mentioned factors. Highly qualified healthcare providers are necessary to evaluate the best therapy for each case [83]. The right choice of therapy must consider the benefits over risks. Monitoring the doses, the adherence to therapy, dosing plans and the respective responses to the treatment regimen is a crucial issue [81, 84].

For early-stage breast cancer, surgery is considered the gold-standard treatment. The main objectives of this surgery are the complete resection of the primary tumor, with negative margins, in order to substantially low the probabilities of local recurrences, and also evaluate the status of the tumor and axillary lymph nodes, providing necessary information regarding prognosis [85]. On the other side, the adjuvant treatment's main goal is to treat metastatic disease. This treatment consists of radiation therapy and systemic therapy (including a variety of chemotherapeutic, hormonal and biologic agents) [84]. Table 4 lists the main drugs and drug combinations approved by FDA to prevent and treat breast cancer, as well as a brief descripition of each drug/combination.

Table 4 - Drugs and drug combinations approved by FDA to breast cancer therapeutics.

Drugs approved to prevent breast cancer	***Brief description***
Raloxifene Hydrochloride	Selective Estrogen Receptor Modulator (SERM). Competes with estrogen in the body for binding to the ER [86].
Tamoxifen Citrate	SERM. The active metabolites of this drug compete with estrogen in the body for binding to the ER [86].
Drugs approved to treat breast cancer	
Abemaciclib	Cyclin-Dependent Kinase (CDK) inhibitor that targets the CDK4 (cyclin D1) and CDK6 (cyclin D3) cell cycle pathway [87].
Anastrozole	Nonsteroidal inhibitor of aromatase, which effectively blocks estrogen synthesis [88].
Ado-Trastuzumab Emtansine	Consists of the monoclonal antibody, trastuzumab, linked to emtansine, a cytotoxic agent. Trastuzumab functions by stopping growth of cancer

	cells, binding to the HER2/neu receptor. Emtansine enters cells and destroys them by binding to tubulin [89].
Capecitabine	Fluorouracil prodrug that is used as an antineoplastic antimetabolite, inhibiting DNA and RNA synthesis, cell division and protein synthesis [90].
Cyclophosphamide	Alkylating agent with both antineoplastic and immunosuppressive activities. Forms DNA crosslinks both between and within DNA strands, leading to cell apoptosis [91].
Docetaxel	Antimitotic chemotherapy drug, plant alkaloid. Promotes and stabilizes microtubule assembly [92].
Doxorubicin Hydrochloride	Hydrochloride salt of doxorubicin, that is an anthracycline antibiotic with antineoplastic activity. It intercalates between base pairs in the DNA helix, inhibits topoisomerase II and forms oxygen free radicals [93].
Epirubicin Hydrochloride	Hydrochloride salt of the 4'-epi-isomer of doxorubicin. Intercalates between base pairs in the DNA helix, inhibits topoisomerase II and forms oxygen free radicals [94].
Eribulin Mesylate	Binds to tubulin and inhibits the polymerization of tubulin and the assembly of microtubules [95].
Everolimus	mTOR kinase inhibitor, inhibiting its downstream signaling [96].
Exemestane	Steroidal aromatase inhibitor. Reduce estrogen levels by blocking the action of aromatase in the adrenal glands [97].
5-FU (5-Fluorouracil)	Analog of pyrimidine, being classified as an antimetabolite. Both fluorouracil and its metabolites incorporates into RNA, inhibiting RNA processing. Also, inhibits the synthesis of DNA [98]. More detailed information will be presented in the Discussion Section.
Fulvestrant	Estrogen Receptor antagonist. The results is estrogen receptor deformation and decreased estrogen binding [99].
Gemcitabine Hydrochloride	Converted intracellularly to its active metabolites and the result is a decrease in the deoxynucleotide pool available for DNA synthesis and incorporation in DNA, leading to disruption of DNA synthesis and cell death [100].
Goserelin Acetate	Analog of luteinizing hormone-releasing hormone. Prolonged administration results in a decrease in estradiol production [101].

Ixabepilone	Binds to tubulin and promotes tubulin polymerization and microtubule stabilization, inducing cell cycle arrest and apoptosis [102].
Lapatinib Ditosylate	Reversebly blocks phosphorylation of EGFR and the Erk-1 and-2 and AKT kinases, inhibiting cell proliferation and survival pathways [103].
Letrozole	Nonsteroidal inhibitor of estrogen synthesis. Inhibits aromatase, which results in growth inhibition of estrogen-dependent breast cancer cells [104].
Megestrol Acetate	Derivative of progesterone, with potential anti-estrogenic and antineoplastic activity [105].
Methotrexate	Antimetabolite and antifolate agent with antineoplastic and immunosuppressant activities. Inhibits the synthesis of DNA, RNA and proteins [106].
Neratinib Maleate	Tyrosine kinase inhibitor that exhibits antitumor action against carcinomas that express EGFR, HER2 and Human Epidermal Growth Factor Receptor 4 [107].
Olaparib	Selectively binds to and inhibits PARP (Poly (ADP-ribose) Polymerase), inhibiting PARP-mediated repair of single strand DNA breaks; This inhibition enhances the cytotoxicity of DNA-damaging agents and may reverse tumor cell chemoresistance and radioresistance [108].
Paclitaxel	Inhibits the disassembly of microtubules by binding to tubulin, resulting in the inhibition of cell division. Also, induces apoptosis by binding to and blocking the function of Bcl-2 (antiapoptotic protein) [109].
Palbociclib	CDK inhibitor. Inhibits Cyclin-Dependent Kinase 4 (CDK4) and 6 (CDK6), thereby inhibiting retinoblastoma protein phosphorylation early in the G1 phase, leading to cell cycle arrest [110].
Pertuzumab	Recombinant humanized monoclonal antibody that targets the extracellular dimerization domain of HER2 [111].
Ribociclib	Cyclin-dependent kinase inhibitor that causes cell cycle deregulation by targeting cyclin D1/CDK4 and cyclin D3/CDK6 cell cycle pathway [112].
Tamoxifen Citrate	SERM. The active metabolites of this drug compete with estrogen in the body for binding to the ER [86].
Thiotepa	Alkylating agent that induces crosslinking of alkylated guanine bases in DNA, interferes with both DNA replication, cell division and results in both induction of apoptosis and inhibition of cell growth. [113]

Toremifene	SERM. Binds competitively to estrogen receptors, thereby interfering with estrogen activity [114].
Trastuzumab	IgG1 humanized monoclonal antibody against the extracellular domain of the HER2 receptor [115].
Vinblastine Sulfate	Disrupts microtubule formation and function during mitosis and interferes with glutamic acid metabolism [116].
Drug combinations used in breast cancer	
AC	A chemotherapy regimen consisting of Doxorubicin Hydrochloride (Adriamycin) and Cyclophosphamide [117].
AC-T	A chemotherapy regimen consisting of Doxorubucin Hydrochloride and Cyclophosphamide, followed by Paclitaxel (Taxol) [117].
CAF	A chemotherapy regimen consisting of Cyclophosphamide, Doxorubicin Hydrochloride (Adriamycin), and 5-Fluorouracil [117].
CMF	A chemotherapy regimen consisting of Cyclophosphamide, Methotrexate, and 5-Fluorouracil [117].
FEC	A regimen consisting of 5-Fluorouracil, Epirubicin and Cyclophosphamide [117].
TAC	A combination chemotherapy regimen consisting of Docetaxel (Taxotere), Doxorubicin Hydrochloride and Cyclophosphamide [117].

1.4.5. Drug repurposing and breast cancer

In addition to all the approved drugs, there are drugs that have the potential to be repurposed for breast cancer treatment. These drugs can be very interesting to identify potential biomarkers, to improve longterm surgical outcomes and to be given in association with current treatments in order to improve overall efficacy [118].

To date, no repurposed drugs have been approved for the treatment of breast cancer. However, there are a variety of drugs, which are being studied, with the potential for such. One example is the non-selective beta-blocker propranolol. A lot of studies, including a phase II randomized placebo-controlled trial of propranolol in combination

with another repurposing candidate, etodolac, in women with early stage breast cancer, showed reduced activity of transcription factors that promote both metastasis and inflammation, decreased epithelial-to-mesenchymal transition and, also, decreased tumor-infiltrating monocytes, increasing tumor-infiltrating B cells. Another study showed that that non-selective beta blockade reduced tumor proliferation by 66% in early stage breast cancer, by accessing Ki67 (a marker of proliferation) [119]. Chloroquine is another example, with very promising results in breast cell lines and phase I trials, either alone or in combination with other drugs. Results in mice showed that this drug increased survival time and reduced primary tumor volume, the number and the diameter of lung metastasis [120]. Another potential drug to breast cancer treatment may be the angiotensin receptor blocker losartan, that completely prevented tumor formation in 20% of treated mice and showed a significant reduction in tumor burden in a spontaneous mammary tumor model [121]. Additionally, many retrospective studies suggest that NSAIDs could reduce breast cancer recurrences. With special importance, a phase III study with ketorolac in breast cancer surgery is being performed, suggesting that this NSAID may be another potential drug to repurpose [122]. Concerning to itraconazole, an anti-fungal drug, a pilot trial evaluated its pharmacokinetics, when administered to 13 patients with metastatic breast cancer. This study led to the observations that as the plasma levels of itraconazole increased, higher levels of angiogenesis inhibitors and decreased levels of angiogenic factors were detected [123]. A more detailed information about this particular drug will be presented on the Discussion Section. Statins have also shown promise results. In various breast cancer cell lines (such as MDA-IBC3, Sum149, and Sum190), an increase in apoptosis and in radiosensitivity, as well as inhibition of invasion and proliferation was observed. Clinical trials in breast cancer patients support these laboratory findings, by the demonstration of an improvement in local control and a mortality benefit for the statin users [124]. Computational studies with antivirals, such as ombitasvir (a drug used in hepatitis C) have shown, also, that this drug could be repurposed for the control and prevention of breast cancer [125]. In this line of antivirals, studies with cell lines and mice with nelfinavir have shown promising results in HER2 positive breast cancer treatment trials with the same dosage regimen as that used among HIV patients [126]. Another drug with the potential to be repurposed for breast cancer therapy is aspirin. With regular aspirin use, multiple observational studies reported an improvement in breast cancer survival [127].

Despite a lot of approved drugs and potential drugs to repurpose, breast cancer remains a major healthcare issue. The costs and difficulties in organization of

screening programs make these programs hard to execute. Highly qualified healthcare providers and appropriate conditions in operation rooms are necessary for a proper surgical treatment. Also, advanced treatment approaches that involve radiation are difficult to achieve in developing countries, particularly. Adequate systemic treatments, management of potential severe effects and new targeted therapies are costly and the most sophisticated treatments require advanced and costly pathology, including immunohistochemistry and molecular pathologic analysis. Additionally, the problem of drug resistance, transversal to the majority of cancer types, still remains an important issue of study and combat [128].

2. Aim of this project

This project aimed to investigate the effect of drugs with potential to be repurposed for breast cancer therapy, in combination with an already used drug in this type of therapy (5-FU), so that the combination of these drugs presented advantage in terms of cell viability/proliferation reduction or cell death increase, when compared to individual components of the combination. Three cell lines were used (MCF-7, FMCm and MCF-10A), with the major focus in MCF-7 cells. In order to achieve the obtained results, we pursued the following line of work:

- Screening of a group of potential drugs to repurpose, in MCF-7 and FMCm cell lines, choosing the best drug combinations for the continuity of the work;
- Comparison of the effects of the drugs between MCF-7 and MCF-10A cell lines;
- Cell viability assays in MCF-7 cells;
- Flow cytometry studies in MCF-7 cells;
- Immunocytochemistry studies in MCF-7 cells.

3. Materials and Methods

3.1. Materials

All drugs, reagents and relevant materials used in this project, as well as their respective companies, are listed in the Table 5.

Table 5 - Drugs, reagents and some relevant materials used in this project.

Drugs/ Reagents/ Materials	Companies
5-Fluoruracil	*Sigma - Aldrich*
Verapamil Hydrochloride	*Sigma - Aldrich*
Itraconazole	*Sigma - Aldrich*
Tacrine Hydrochloride	*Sigma - Aldrich*
Isoniazid	*Fluka Analitycal*
Cimetidine	*Sigma - Aldrich*
Pravastatin	*Cayman Chemical Company*
Aspirin	*Sigma - Aldrich*
Chloroquine Diphosphate Salt	*Sigma - Aldrich*
Losartan Potassium	*Sigma – Aldrich*
Dimethyl Sulfoxide (DMSO)	*EMSURE ACS, Merck*
Dulbecco's Modified Eagle's Medium (DMEM)	*Biochrom GmbH*
DMEM/F-12	*Sigma – Aldrich*
Roswell Park Memorial Institute (RPMI) 1640	*Biochrom GmbH*
Phosphate Buffered Saline (PBS)	*Sigma – Aldrich*
Penicilin/Streptomycin Mixture	*Sigma – Aldrich*
Fetal Bovine Serum (FBS)	*Merck Milipore*
Human Insulin	*Actrapid, Novo Nordisk*
Hydrocortisone	*Sigma – Aldrich*
Epidermal Growth Factor	*Sigma – Aldrich*
Trypsin/EDTA	*Gibco, Alfagene*
Trypan Blue	*Sigma - Aldrich*
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)	*Sigma - Aldrich*
bisBenzimide H 33342 trihydrochloride (Hoechst 33342)	*Sigma - Aldrich*
Hank's Balanced Salt Solution (HBSS)	*Sigma - Aldrich*
Neutral Red Solution (0.33%)	*Sigma - Aldrich*
eBioscience ™ Annexin V Apoptosis Detection Kit FITC • 10x Binding Buffer • Annexin V FITC • Propidium Iodide Staining Solution	*Thermo Fisher Scientific*
Invitrogen™ CellTrace™ CFSE Cell Proliferation • CFSE (lyophilized powder)	*Thermo Fisher Scientific*

• DMSO	
Novocastra™ Novolink™ Max Polymer Detection System • Peroxidase Block • Protein Block • Post Primary • Novolink ™ Polymer • DAB Chromogen • Novolink ™ DAB Substrate Buffer (Polymer) • Hematoxylin	*Leica Biosystems*
Target Retrieval Solution	*Agilent Dako*
TBS IHC Wash Buffer + Tween® 20	*Cell Marque, Milipore Sigma*
96-well Plates	*TPP Techno Plastic Products AG*

3.2. Drug solutions

For the treatment of cells with the several drugs under study (5-FU, verapamil, itraconazole, isoniazid, tacrine, aspirin, cimetidine, chloroquine, losartan, pravastatin), all the compounds were dissolved in autoclaved water, except Itraconazole, that was dissolved in DMSO, since it did not present solubility in water. A stock solution of each compound was prepared at a concentration of 10 mM and, with exception of Itraconazole, it was prepared two stock solutions with a concentration of 25 mM and 50 mM, because of the fact that Itraconazole was dissolved in DMSO and it has significant toxicity to cells after a percentage of 0.2%, percentage that was never exceeded in this work. All of these stock solutions were conserved on the freezer at -26ºC. For some experiments, intermediate solutions of 5-FU and Itraconazole were prepared at 1 mM and 2.5 mM, respectively. Depending on the purpose of the assay, test compounds were used in concentrations that range 1 µM to 100 µM, dissolved in culture medium right before contact with cells. The respective concentrations used in each assay are presented in Results Section. It is important to note that the test compounds applied to cells vary with the purpose of the experiment, also specified in the above mentioned Section.

3.3. Cell lines

The experimental work of this dissertation was carried out mainly in the MCF-7 cell line (ATCC - American Type Culture Collection). Additional experiments were carried out in MCF-10A (ATCC - American Type Culture Collection), and FMCm cell lines (IPATIMUP, Porto , Portugal).

MCF-7 is a human breast adenocarcinoma cell line, isolated from a pleural effusion of a 69-year old caucasian woman with invasive ductal carcinoma, in 1970 [129]. It is ER-positive and PR-positive, classified as luminal A molecular subtype and being a poorly-aggressive, with low metastatic potential [130].

MCF-10A cell line is a human mammary, non-tumorigenic, epithelial cell line, established from a 36 years caucasian woman, widely used as an *in vitro* model for studying normal breast cell function and transformation [131, 132].

FMCm is a feline mammary adenocarcinoma cell line, originated from a regional lymph node metastatic lesion of a 12 years old Japanese domestic female cat with a primary mammary adenocarcinoma in stage III, having great metastatic capacity [133].

All of these cell lines are adherent cell lines, being anchorage-dependent and growing as a monolayer.

3.4. Methods

3.4.1. Cell culture

The process of removing cells from animals or plants and their growth in a controlled environment is named cell culture. This technique allows the manipulation of both the physicochemical and the physiological environment in which the cells propagate. Thus, this technique has a huge amount of applications in research studies. In this regard, cell culture is an excellent approach for studying effects of drugs and toxic compounds on cells, being also very useful for investigating its normal biochemistry and physiology [134]. However, this widely used technique has its subjacent limitations, described in the Discussion Section.

- **Culture conditions**

Media, temperature, pH, CO_2 content and the nature of adhesion surface are extremely relevant factors in cell culture.

The culture medium is essential to the success of cell culture, having major influences in the growth of cells, being composed of essential elements for cells, namely salts, carbohydrates, vitamins, amino acids and metabolic precursors. Besides providing essential nutrients for cell growth, the medium is generally supplemented with antibiotics, fungicides, or even both, in order to inhibit contamination, a frequent problem in cell culture techniques. Also, to provide additional growth factors, serum is usually added to the culture medium, being Fetal Bovine Serum the most widely used. Concerning temperature, 37ºC is required for most animal cell lines for an optimal growth. An important aspect about temperature requirements is the fact that the great majority of cultured cells can withstand considerable drops in temperature and survive for several days at 4°C, but few can tolerate 2°C above their optimal temperature, even only during few hours [135]. Relative to pH and CO_2 content, it is known that most normal mammalian cell lines grow well at pH 7.4, and there is very little variability among different cell strains. Therefore, a rigorous control of this value is crucial. This regulation of pH is usually achieved by a buffering system where gaseous CO_2 balances with the CO_3/HCO_3 content that is present in the culture medium. Therefore, cultures need to be maintained in an atmosphere of 5-10% CO_2 in air, supplied in a CO_2 incubator. Additionally, buffering agents like HEPES can maintain pH in a physiological value, despite changes in CO_2, being also frequently used in cell culture. Most commercial culture media include a pH indicator called phenol red, so that the pH status of the medium is constantly indicated by the colour, where yellow and purple colors indicate more acidity and alkalinity, respectively [136].

MCF-7, FMCm and MCF-10A cells were incubated at 37ºC in a humidified atmosphere with 95% of air and 5% of CO_2. Different culture media were used (as described in the literature [137-139]), depending on the cell line. MCF-7 and FMCm cells were cultivated in DMEM (supplemented with stable glutamine) and RPMI 1640, respectively, both supplemented with 10% FBS and 1% of a mixture of penicillin/streptomycin (1000 U/mL; 10 mg/mL). MCF-10A were cultivated in DMEM/F-12, supplemented with the same supplements described above plus 2 µg/mL of human insulin, 20 ng/mL of EGF and 1 µM of hydrocortisone. In all cultures, the pH was 7.4 and the FBS used was inactivated by being submitted to a bath of 56ºC during 30

minutes, in order to decrease its respective cytotoxicity and maintain sterility, reducing the antigenic display of some proteins (which denature under those conditions), mainly the complement cascade, a cascade of enzymes that is part of the immune system [140].

- **Cell maintenance**

For MCF-7, FMCm and MCF-10A cells maintenance, cells were cultured in a monolayer in T75 cm^2 flasks, being subcultured 2-3 days per week, and 1 day per week in the case of MCF-10A, the last cell line growing slowly. To this subculture process, the culture medium was aspired and the cells were washed with 10 mL of PBS. Then, PBS was aspired and cell monolayers of MCF-7 and FMCm were treated with 1 mL of trypsin/EDTA and incubated at 37ºC during 6 minutes to ensure complete cell detachment. In the particular case of MCF-10A cells, 2 mL of trypsin/EDTA was used during 10 minutes of incubation, also at 37ºC. After being trypsinized, cells were resuspended in 9 mL of culture medium and divided according to a split ratio comprised between 1:3 and 1:10 in the fresh medium until a final volume of 20 mL per T75 cm^2 flask. When higher split ratios were performed, culture media were replaced every 2 days.

All the experiments were carried out with cells to 70–80% confluence because adherent, anchorage-dependent cell lines growing in monolayers need to be subcultured at regular intervals to maintain them in exponential growth. Therefore, when the cells are near the end of exponential growth (roughly 70%-80% confluent), they are ready to be subcultured [135].

In this work, cultures with a passage number higher than 50 were not used due to the fact that with a high passage number, the cell viability begins to decline [141].

- **Cell seeding**

The procedure for cell seeding is the same as described above (Cell Maintenance), with the difference that, after trypsinization and posterior resuspension of cells in culture medium, the cell suspensions were centrifuged during 5 minutes at 400g. After that, the supernatant was removed and the pellet was resuspended in 3 mL of culture medium. The cell suspensions were homogenized, and the next step consisted in counting the number of cells using the Neubauer Chamber and Trypan Blue, a cell stain used to assess cell viability, based upon the fact that viable cells do not take up

impermeable dyes like Trypan Blue, but dead cells are permeable and take up the dye. So, viable cells appear white, while dead cells appear blue. Therefore, the number of viable cells/mL in the cell suspension was calculated as follows:

Average of unstained cells count of the 4 areas of the Neubauer chamber x 10^4 (chamber factor) x dilution factor

Then, the cell density in the original suspension, obtained as described above, was adjusted to the pretended seeding densities. For all the experiments (except for the generation of the growth curve, Section 4.2), MCF-7 cells were seeded in 96-well plates with seeding densities within a range of $3x10^4$ cells/mL and $1x10^6$ cells/mL, depending on the purpose of the experiment. An exception occurred in the case of immunocytochemistry experiments, in which MCF-7 cells were seeded in T25 cm^2 flasks with a density of $4.7x10^4$ cells/mL, converted from the density used in 96-well plates ($3.0x10^4$ cells/mL). MCF-10A and FMCm cells were only seeded in 96-well plates, with a seeding density of $3.0x10^4$ cells/mL and $5.0x10^4$ cells/mL, respectively. The justifications for the choices of the different cell densities used will be highlighted in the Discussion Section.

3.4.2. Hoechst 33342 staining

Hoechst 33342 is a bis-benzimide derivative that binds preferentially to AT-rich sequences in the minor groove of double-stranded DNA, being a nucleic acid stain that emits blue fluorescence when bound to double stranded DNA [142]. This dye is often used to distinguish condensed, pycnotic nuclei in apoptotic cells and is also used to make total cells counts, once it stains nuclei [143].

MCF-7 cells were seeded in 96-well plates with a starting seeding density of $1.0x10^4$ cells/cm^2 (approximately 1.6 cells/mL), incubated at 37ºC for 24 hours. After this time, Hoechst 33342 (1 mg/mL in DMSO) was diluted to 5 µg/mL in culture medium, and 200 µL of this solution was added to each well, in a period of incubation of 10 min, in a light-protected manner. After that, images representing each well were taken using an automated microscope (Lionheart™ FX, BioteK Instruments) configured with DAPI light cube. Total cell number was calculated using the software Gen5.0. (version 4), based on Hoechst staining. This procedure was repeated every 24 hours, for 148 hours, in order to obtain a growth curve of MCF-7 cells.

3.4.3. MTT reduction assay

MTT Tetrazolium assay is a widely used methodology for determination of cellular viability. This assay measures the reductive activity of the cell, based on the formation of water insoluble formazan crystals by enzymatic conversion of the tetrazolium compound, by dehydrogenases that exist mainly in the mitochondria of only living cells [144] (Figure 5). Therefore, viable cells with an active metabolism are going to convert MTT into a purple colored formazan product that can be quantified with an absorbance maximum near 570 nm. On the other hand, when cells are not viable, they lose the ability to form formazan crystals [145]. In this way, the amount of formazan crystals formed is generally proportional to the number of metabolically active cells.

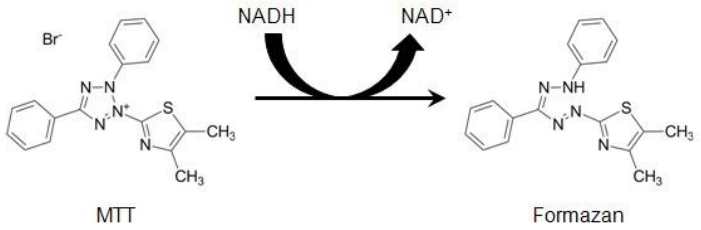


Figure 5 - Formazan crystals are formed by enzymatic conversion of the tetrazolium compound (MTT) by dehydrogenases. Reproduced from Terry et al. [145].

Although widely used, MTT assay has some relevant limitations, that will be reported in the Discussion Section.

MCF-7, MCF-10A and FMCm cells were plated in 96-well plates with a seeding density of $3.0x10^4$ cells/mL for the first two cell lines and $5.0x10^4$ cells/mL for FMCm, maintained at the incubator at 37ºC, for 24 hours. After this time, the different treatments were added to the cells at different concentrations, for 48 or 72 hours, depending on the purpose of the experiment. The cells were, once again, maintained at 37ºC during the referred time and, after that, cell medium was removed and 100 µL of MTT solution (0.5 mg/mL in PBS) was added to each well. Then, the cells were incubated at 37ºC for 3 hours in a light-protected manner, since MTT is light sensitive. At the end of this time, MTT solution was removed and 100 µL/well of DMSO was added, with the purpose of solubilization of formazan crystals formed. The last step consisted in the absorbance readings at 570 nm in an automated microplate reader (Sinergy HT, BioTek Instruments).
Cell viability values were calculated according to the following equation:

$$\textbf{Cell Viability (\%)} = (Abs_{test}/Abs_{control}) \times 100$$

Abs_{test} represents the 570 nm absorbance of cultures exposed to test compounds and $Abs_{control}$ concerns to the 570 nm absorbance of respective control cultures.

3.4.4. Neutral Red uptake assay

The Neutral Red Uptake assay provides an estimation of the number of viable cells in a culture, based on the ability of viable cells to incorporate and bind the neutral red dye in the lysosomes, whereas non-viable cells don't take up the dye. The incorporated dye by viable cells is liberated from them in an acidified ethanol solution. So, this uptake of neutral red rests on the cell's ability to maintain the pH levels. Thus, when the cell can't maintain the pH gradient, the dye cannot be retained. In addition, the uptake of neutral red by viable cells can be modified by alterations in cell surface or lysosomal membranes [146]. Therefore, the amount of dye that the cells can retain is proportional to the number of viable cells [147].

MCF-7 cells were seeded in 96-well plates with a seeding density of $3.0x10^4$ cells/mL. After that, the cells were incubated for 24 hours at 37ºC, and the test compound (only 5-FU) was added to them at concentrations ranging from 1 µM to 100 µM. The treated cells were incubated for 48 hours at 37ºC. Then, cell medium was removed and 100 µL of Neutral Red medium (33 µg/mL in culture medium) was added to each well. Afterwards, the cells were incubated at 37ºC for 3 hours, protected from the light. At the end of this time, Neutral Red Medium was removed and the cells were washed with sterile HBSS. After that, 100 µL of Neutral Red Destain solution was added to each well and the plate was placed in the microtiter plate shaker, protected from light, for at least 10 min., until it formed a homogeneous solution. The final step consisted of the absorbance readings at 540 nm and at 690 nm (reference) in an automated microplate reader (Sinergy HT, BioTek Instruments). Cell viability values were calculated according to the following equation:

$$\textbf{Cell Viability (\%)} = (Abs_{test}/Abs_{control}) \times 100$$

Abs_{test} represents the 540 nm absorbance minus the 690 nm absorbance of cultures exposed to test compound and $Abs_{control}$ concerns to the 540 nm absorbance minus the 690 nm absorbance of respective control cultures.

3.4.5. Annexin V-FITC and PI staining

Propidium Iodide, in conjunction with Annexin V, is widely used to determine if cells are viable, apoptotic, or necrotic, through differences in plasma membrane integrity and permeability [148]. Annexin V, conjugated to green-fluorescent FITC dye, detects the externalization of phosphatidylserine (PS) in apoptotic cells, an event that occurs in early apoptosis. On the other hand, PI stains necrotic cells with red fluorescence. The process is based on the fact that cell membrane integrity excludes PI in viable and apoptotic cells, whereas necrotic and late apoptotic cells are permeable to Propidium Iodide. [149, 150].

MCF-7 cells were seeded in 96-well plates with a seeding density of $1.0x10^6$ cells/mL, incubated at 37ºC for 24 hours. After this time, the different treatments were added to the cells, that were incubated for 3 or 8 hours. Then, cells were trypsinized, washed with HBSS (2%FBS) and centrifuged for 5 min at 400 g. The next step consisted in the addition of Annexin V FITC (1 µL per well) in solution with Binding Buffer (50 µL per well) to each well. This Binding Buffer contains optimal concentration of calcium, required for Annexin V binding to PS on the cell surface [151]. After that, cells were homogenized with this solution and the Annexin was in contact with cells for 15 min at room temperature, in the dark. Then, the cells were placed in cytometer tubes,and 5 min before reading, 2 µL of PI was added to each tube, that represented each different condition. Finally, cell death was determined by flow cytometry (Beckman Coulter Epics XL and BD FACSCanto™ II). The data was analyzed using FlowJo (V10) analysis software.

3.4.6. CFSE labeling of cells

Carboxyfluorescein Succinimidyl Ester (CFSE) is a fluorescent cell staining dye used to assess cell proliferation by flow cytometry. It is cell permeable and covalently labels long-lived intracellular molecules. Thus, when a cell labeled with CFSE divides, the progeny receives half of the number of molecules tagged with the dye, making possible to assess each cell division by measuring the decrease in cell fluorescence via flow cytometry [152]. So, it is used to monitor distinct generations of proliferating cells by dye dilution. Every generation of cells appears as a different peak on a flow cytometry histogram [153].

Cell trypsinization, centrifugation and counting was done as described above in the Cell maintenance Section. In this particular case, after the supernatant has been poured off, MCF-7 cells were resuspended in PBS with 0.1% BSA, in order to obtain a density of $2.0x10^6$cells/mL. A 5 mM CFSE staining stock solution was prepared by adding 18 µL of DMSO to the vial. The CFSE staining stock solution was then diluted in PBS with 0.1% BSA to a concentration of 10 µM. The next step consisted of the addition of the same volume of cells ($2.0x10^6$ cells/mL) to the 10 µM CFSE staining solution, resulting in a final solution of 5 µM CFSE. Next, cells were incubated at 37ºC for 10 min., with the purpose of metabolization of the dye by intracellular esterases, which remove the acetate groups and convert the molecule to the fluorescent ester. After that time, in order to absorb any unbound dye, the solution was neutralized with three volumes of complete medium (DMEM, 10% FBS) and incubated during 5 min on ice to stabilize the staining. Then, cells were centrifuged at 400g for 5 min, the supernatant was removed and the pellet of cells was resuspended in complete medium. After cell count, the suspension was adjusted to a density of $1.0x10^6$ cells/mL. With this density, cells were seeded in 96-well plates for 3 hours. After that, the medium was aspired and test compounds, dissolved in the culture medium, were added to cells, that were incubated at 37ºC for approximately 72 hours. The final step consisted in medium aspiration, washing with PBS, trypsinization of plated cells, resuspension in HBSS (2% FBS) and, finally, cell proliferation was determined by flow cytometry (Beckman Coulter Epics XL) and the data was analyzed using FlowJo (V10) analysis software. 5 min before reading, 2 µL of PI were added to each cytometer tube (that represents each condition), for dead cell exclusion.

3.4.7. Immunocytochemistry

Immunocytochemistry is a methodology used in biomedical research to identify proteins and other macromolecules in cells [154]. It relies on the use of antibodies to test for antigens in a sample of cells. These antibodies are usually linked to an enzyme or a fluorescent dye. When the antibodies bind to the corresponding antigen, the enzyme or dye is activated, and the antigen can then be seen under microscopic observation [155].

MCF-7 cells were seeded in T25 cm^2 flasks with a density of $4.7x10^4$ cells/mL, converted from the density used in 96-well plates ($3.0x10^4$ cells/mL). After that, cells were incubated for 24 hours at 37ºC. Then, the different test compounds were added and acted for 48 hours, with the cells maintained at 37ºC in the incubator. After this

time, the cells were included in cell blocks (one for each different treatment) and different slides were made from each block. Then, the first part of the process consisted in deparaffinization by submerging the slides twice in xylene, for 5 min each time, followed by hydration in alcohol at decreasing concentrations (100%, 95% and 70% alcohol) until rinsing in water. After that, antigen retrieval (also known as unmasking) was performed by using a Retrieval Solution (10% in water), 20 min in a water-bath at 100ºC. The next step consisted in endogenous peroxidase block, by incubation of the slides in a Peroxidase Block solution for 5 minutes. Then, the slides were washed twice in TBS for 5 min. Incubation with Protein Block for 5 minfollowed, and the slides were washed in TBS 2x for 5 min. Afterwards, the slides were incubated with the mouse antibodies anti-Cytokeratin (pan) (1:1200 in BSA 5%), anti-E-Cadherin (1:50 in BSA 5%) and anti-Vimentin (1:500 in BSA 5%), overnight, at 4ºC. Then, the slides were washed twice in TBS, for 5 min and the Post Primary was added, following an incubation time of 30 min. Once again, the slides were washed twice in TBS, for 5 min. After that, they were incubated during 30 min with the Polymer, washed twice in TBS for 5 min. and, to each slide, 150 µL of a solution of 50 µL of DAB Chromogen to 1 mL of DAB Substrate Buffer (Polymer) was added to each slide. Finally, the slides were rinsed in water, counterstained in Hematoxylin during 1 min, washed again for 5 to 10 min, dehydrated (at increasing concentrations of alcohol, 70%, 95% and 2x100%), diaphanized (2x in xylene), and the sections were mounted. The slides were observed on a Nikon Eclipse E600 microscope, coupled to a digital camera (Nikon Digital Sight DS-Fi2). Images were treated with Imaging Software NIS-Elements AR Version 4.30.01.

3.4.8. Statistical analysis

Statistical analysis was performed in all experiments, only in the case of a number of independent experiments equal or bigger than 3 (n≥3). The results are expressed as arithmetic mean±SEM, except in one case, where results are expressed as arithmetic mean±SD, explicit in the subtitles of the graphs. Differences between treated cells and corresponding untreated control were tested using One-Way ANOVA followed by Dunnett's test. Differences between the drug combination and the respective individual drug of that combination that produces more advantageous effects in terms of cell viability reduction were tested by Student’s t-test. Differences were considered to be significant when p value <0.05. One-Way ANOVA followed by Dunnett’s test and Student’s t-test were performed by using SigmaPlot 12.0 and GraphPad Prism 7,

respectively. It is important to note that, for all experiments, no differences were observed between control with/ without DMSO.

4. Results and Discussion

In the present study, we intended to investigate a combination of a reference chemotherapeutic drug (5-FU) and drugs not indicated for cancer, potential drugs to be repurposed drugs. The main goal was to prove that the drug combination was more effective than the respective individual drugs. To conduct these studies, a human breast cancer cell line (MCF-7) was mainly used, as well as a human breast cell line (MCF-10A) and a feline mammary carcinoma cell line (FMCm), the last two used to complementary studies.

MCF-7 is a cell line widely used for the study of breast cancer, being the most studied human breast cancer cell line in the world [156]. Thus, there are a lot of studies with this cell line. However, regarding to studies of 5-FU in combination with the drugs used in this project, in this particular cell line, there are no studies until the date of delivery of this dissertation.

Following the addition of the drugs to cells, several methodologies were used in this work. In order to assess cell viability, MTT and, in much less extension, Neutral Red assay was performed. To evaluate cell death, cell staining with Annexin-V-FITC/PI and the respective analysis by Flow Cytometry was conducted. Flow Cytometry was also performed to evaluate cell proliferation, in which cells were stained with the CFSE label. Finally, to evaluate if resistant cells to therapy evolved from an epithelial to a mesenchymal state, immunocytochemistry was performed. The procedure of each technique was described in the Materials and Methods Section.

4.1. Reference chemotheraputic drug chosen for this project

5-FU was chosen as the reference drug of this study, mainly because it is a potential drug to be used in combination regimens in breast cancer therapeutics, with the major aim of improving its efficacy, as well as its known toxicological profile.

4.1.1. Structure and metabolites of 5-FU

5-Fluorouracil is a heterocyclic aromatic organic compound (Figure 6) with a structure similar to that of the pyrimidine molecules of DNA and RNA. It has a fluorine atom at the C-5 position in place of hydrogen, being an analog of uracil, classified as an

antimetabolite drug [157]. This drug is widely used in the treatment of a range of cancers, including colorectal, liver, ovary, pancreas, stomach, breast and aerodigestive tract cancers [158].

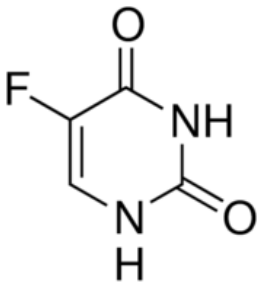


Figure 6 – 5-FU chemical structure.

This drug requires cellular uptake before can be intracellularly converted to active metabolites. Within the superfamily of SLC transporters, members of two families are being discussed to mediate uptake of 5-FU: Organic Anion Transporter 2 (OAT2) that mediates uptake of 5-FU with high affinity, and the two Equilibrative Nucleoside Transporters (ENT) 1 and 2, that transport 5-FU with lower affinity [159, 160]. Studies also report an entrance of 5-FU in the cells by passive diffusion [161]. However, more than 80% of 5-FU is catabolyzed to form the catabolite Dihydrofluorouracil (DHFU) in the liver, where the rate-limiting enzyme in its catabolism (Dihydropyrimidine dehydrogenase, DPD) is expressed in an abundant way. When inside the cell, 5-FU is converted to active metabolites: Fluorodeoxyuridine Monophosphate (FdUMP), Fuorodeoxyuridine Triphosphate (FdUTP) and Fluorouridine Triphosphate (FUTP), that have the main action of this drug: disruption of the RNA synthesis and disruption of the action of Thymidylate Synthase (TS), an enzyme that catalyzes the conversion of Deoxyuridine Monophosphate (dUMP) to Deoxythymidine Monophosphate (dTMP), a step that is absolutely crucial on the formation of thymidylate from uracil and, thus, important for DNA synthesis (Figure 7) [158, 162]. In this regard, this drug is responsible for RNA and DNA damage, acting on S phase of cell cycle [163].

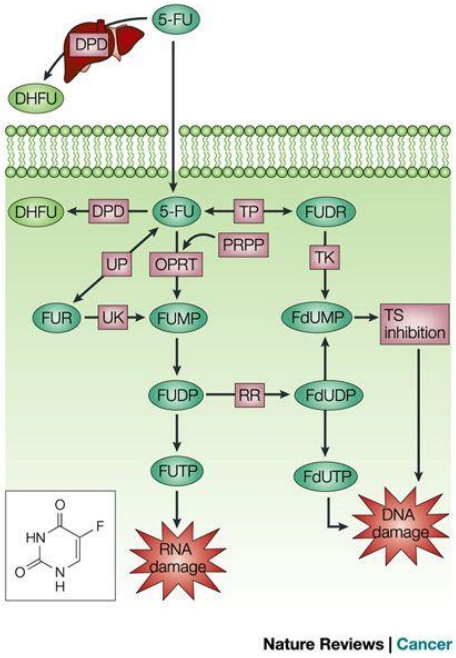


Figure 7 – Pathways of 5-FU conversion to its active metabolites and respective functions. Reproduced from Daniel et al. [164].

4.1.2. Mechanism of action of 5-FU

- **TS inhibition – Interference with DNA synthesis**

As explained above, TS catalyzes a reaction that is crucial for DNA replication and repair. This enzyme functions as a dimer, in which both subunits contain a nucleotide-binding site. FdUMP binds to the nucleotide-binding site of TS, forming a complex with the enzyme, thereby blocking binding of dUMP and, consequently, inhibiting dTMP synthesis [158]. This leads to the inhibition of the formation of thymidylate from uracil, causing depletion of dTMP and an accumulation of dUMP, creating an imbalance in the proportion of intracellular deoxynucleotides (dATP, dGTP and dCTP) [165]. As a result of these actions, 5-FU is responsible for the inhibition of synthesis and DNA repair, leading to DNA damage. Another important factor to have in consideration is the accumulation of dUMP, which subsequently leads to an increase in the levels of deoxyuridine triphosphate (dUTP). In this regard, dUTP and also FdUTP can be misincorporated into DNA, event that, consequently, is responsible to promote the repair of uracil and 5-FU-containing DNA through mechanisms of nucleotide excision repair. The problem of this mechanism is that in the presence of high (F)dUTP/dTTP ratios, there is the formation of a futile cycle, resulting in further false nucleotide incorporation. Collectively, all of these events, promote DNA strand breaks and, ultimately, cell death [158].

- **RNA misincorporation and p53 effects**

In addition to the DNA effects of 5-FU, Fluorouracil can also be incorporated into RNA in place of uridine triphosphate, producing, in this way, a fraudulent RNA and interfering with RNA function [166, 167]. FUTP is extensively incorporated into various species of RNA, disrupting normal RNA processing and function [164, 168]. Specifically, this misincorporation is responsible for inhibition of the processing of pre-rRNA into mature rRNA, disruption of post-transcriptional modification of tRNAs and, also, disruption of the assembly and activity of snRNA/protein complexes, thereby causing an inhibition of the splicing of pre-mRNA [164]. In this way, 5-FU interferes with many aspects of RNA function, leading to disruptive effects on cellular metabolism and cell viability [168].

In addition to interfering with nucleic acid processing, there are evidence that 5-FU also exert effects on p53, increasing its expression, while mutations in p53 may result in 5-FU resistance. This suggests that p53 is also involved in cell-cycle arrest and apoptosis in cells treated with 5-FU [168]. However, further investigation would be required to determine the precise relationship between p53 in the mechanism of action of 5-FU.

4.1.3. 5-FU resistance

Clinical applications of 5-FU have been greatly limited due to a diversity of drug resistance problems, despite many advantages and indications. High level expression of TS, increased activity of deoxyuridine triphosphatase, methylation of the MLH1 gene (essential role in DNA repair) and overexpression of Bcl-2, Bcl-XL and Mcl-1 proteins (antiapoptotic proteins), suggests that multiple factors might contribute to resistance to this drug [157]. Also, there are studies that reveal that the acquisition of 5-Fluorouracil resistance induces epithelial-mesenchymal transition (Section 4.4) [169]. Other data suggest that BCRP (Breast Cancer Resistance Protein), an ATP-binding cassette half transporter that causes resistance to a variety of cancer chemotherapeutics, is also responsible for 5-FU resistance [170]. Another major signaling pathway responsible for chemoresistance induced by various chemotherapeutics, in which 5-FU is included, is NF-κB. Several studies have shown that downregulation of NF-κB could enhance therapeutic efficacy of 5-FU [171] The existence of several transporters that have been implicated in 5-FU resistance, such as ABCC3, constitute another way of resistance [172].

Despite the mechanisms of resistance above mentioned, the limited application of 5-FU in the clinical practice is also related to the huge associated adverse effects, like myelosuppression, photosensitivity, vomiting, diarrhea and, less frequently, angina, coronary arteriosclerosis and thrombophlebitis [173]. For example, for colorectal cancer, the overall response rate with 5-FU alone is still only 10–15%, and the combination of 5-FU with other anticancer drugs has merely improved the response rates to 40–50% [157].

4.1.4. 5-FU and breast cancer

5-FU is an old but effective chemotherapeutic agent for breast cancer, namely for treatment of early staged breast cancer patients, as well as palliative treatments for advanced and metastatic breast cancer. It is used as a single agent or in combination with other drugs, particularly in the CAF, CMF and FEC regimen [174]. Additionally, a lot of research was performed to reduce toxicities, enhance controlled release activity and localize the drug delivery of 5-FU, existing 5-FU prodrugs, namely capecitabine [175].

4.2. Determination of the most suitable cell densities

As the cells grow and divide in a monolayer or in suspension, they usually follow a sigmoid pattern of proliferation, composed of four phases: lag, log or exponential, stationary or plateau and decline [176] (Table 6).

Table 6 – Phases of cell growth and the respective description of each phase.

Phases of cell growth	***Description***
Lag phase	Cells grow slowly, recovering from the stress of sub-culturing [177]. During this period, the cells adapt to the culture conditions [176].
Log or exponential phase	Cells grow fast. This phase lasts until the entire growth surface is occupied, or until the capacity of the medium is exceeded [177].
Stationary phase	Cell proliferation slows and stops. The number of cells in the active cell cycle drops to 0-10%. Cells are most susceptible to injury [176, 177].
Decline phase	The cells lose viability and their number decreases. Cell death occurs as the natural path of the cellular cycle [176, 177].

An important issue to address was to ensure that in the course of all experiments, the cultures were maintained in the exponential phase of growth, like it is observed in tumoral cells *in vivo*. The maintenance of the exponential phase is important to ensure viability, genetic stability, and phenotypic stability [176]. This means that cells need to be subcultured in order to don't enter the stationary phase of growth. It is, therefore, important to generate a growth curve for the cell line to determine its growth characteristics [177].

Based on scientific articles [178], a density of $3.0x10^4$ cells/mL (approximately $1.9x10^4$ cells/cm^2), for MCF-7 cells, was chosen. However, in order to confirm if with this cell density, the cells remained in the exponential phase in the course of all experiments (72 or 96 hours), a growth curve of MCF-7 cells was performed, plating the cells in 96-well plates (0.32 cm^2) with an initial density of 1.0 $x10^4$ cells/cm^2 ($1.6x10^4$ cells/mL), staining the cells with Hoechst 33342 and acquiring the total cell number per well, recorded every 24 hours, during 148 hours. The results are presented below.

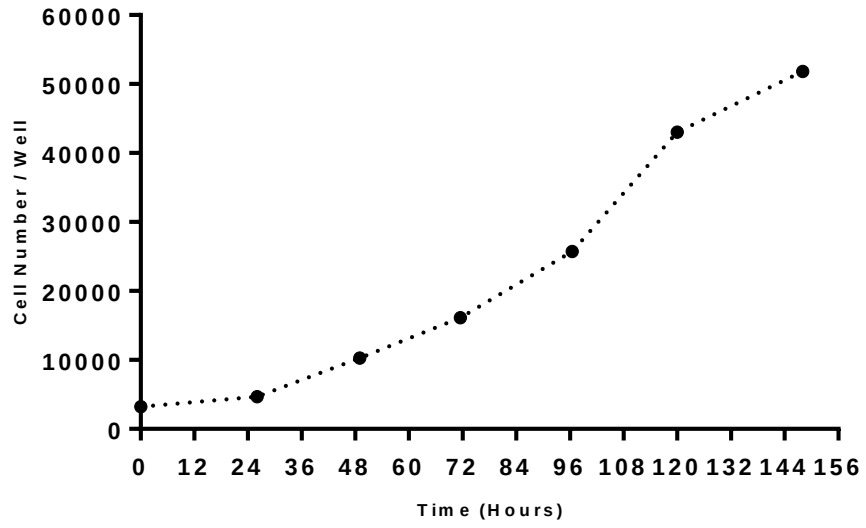


Figure 8 – Growth curve of MCF-7 cells, obtained by staining the cells with Hoechst 33342 staining (procedure described in the Materials and Methods Section) and counting the total cell number, per well (triplicates), for 148 hours.

Analysing the obtained growth curve, it was possible to conclude that the chosen density (corresponding to 6000 cells/well) was suitable for all the course of experiments, since the cells have remained in the exponential phase of growth. An exception occurred in the case of flow cytometry experiments, where extremely high densities were used, in order to possibilitate efficient readings by the cytometer.

By using this data, is also possible to calculate the Population Doubling Time (period required for a culture to double in number [135]), by using the following formula:

$$DT = T \cdot \frac{\ln 2}{\ln(\frac{Xe}{Xb})}$$

Xe is the cell number at the end of the incubation time, *Xb* is the cell number at the beginning of the incubation time and T is the incubation time (any units) [135]. Therefore, applying the above mentioned formula, it was possible to conclude that MCF-7 presents a Doubling Time (DT) of approximately 37 hours.

Concerning to MCF-10A cell line, only one comparative experiment with MCF-7 was carried on. So, in order to obtain a more reliable comparison, the same cell density in both cell lines was used, that is, 3.0×10^4 cells/ mL. Concerning with FMCm cell line, also only one experiment was carried on. In this case, the cell density used was 5.0×10^4 cells/mL, indicated by our research group [137].

4.3. Results

4.3.1. Drug screening

Based on several studies about potential drugs to repurpose [25, 123, 179, 180] and interests of the investigational group, 6 and 9 drugs, each in combination with the reference drug (5-FU) were tested in FMCm and MCF-7 cell lines, respectively, in order to make an initial screening of potential drugs to be used in combination with 5-FU in this project. These drugs were verapamil, aspirin, losartan, chloroquine, cimetidine, itraconazole, tacrine, isoniazid and pravastatin. Each drug was used in a concentration of 50 μM, thus, in a ratio of 1:1 when combined with 5-FU, being in contact with cells for 72 hours. The results were obtained by MTT methodology (procedure described in the Material and Methods Section), and are presented below, for each cell line.

4.3.1.1. FMCm cell line

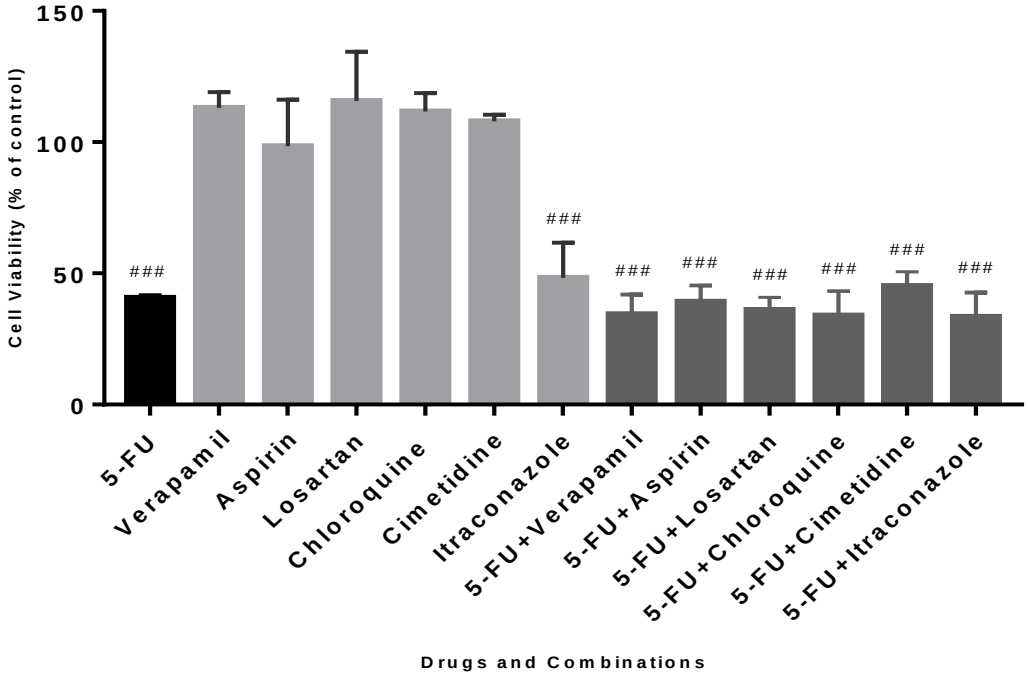


Figure 9 – Effect of drugs and respective combinations with 5-FU on the viability of FMCm cells. Each drug and each combination was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM, and represent the viability of cells (% of control) of 3 independent experiments (n=3). ###p<0.001 vs control.

Analyzing the obtained results, it was possible to conclude that none of the drug combinations were advantageous in terms of reduction of cell viability, relative to the respective drug with more effect on viability reduction of each combination. The lowest cell viability value obtained with drug combinations was obtained with 5-FU combined with chloroquine (33.9±5.4%), not shown to be advantageous relative to the drug of that combination with more effect on cell viability (5-FU), with values of 40.6±1.1%. Clearly, the drug combinations produce an effect almost equal to that of 5-FU alone, showing that the effects of the combination on cell viability are only due to the action of 5-FU and not from the combination of both drugs.

4.3.1.2. MCF-7 cell line

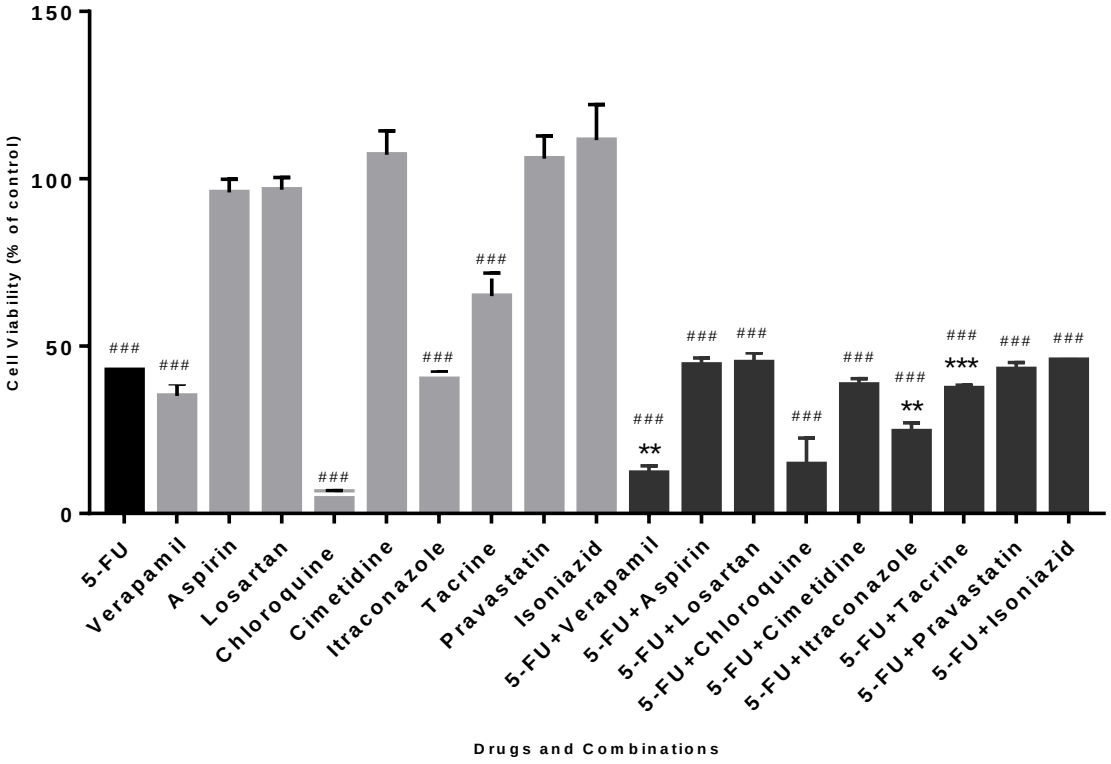


Figure 10 - Effect of drugs and respective drug combinations on the viability of MCF-7 cells. Each drug and each combination was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM, and represent the viability of cells (% of control) of 3-4 independent experiments (n=3,4). ###p<0.001 vs control; **p<0.01 and ***p<0.001 vs single drug of the combination with more effect on cell viability reduction.

For MCF-7 cells, analyzing the obtained results, it was possible to observe that chloroquine was, clearly, more effective in terms of cell viability reduction than all the other drugs and drug combinations (6.5±0.4% of cellular viability). Thus, as the aim of this project was to study a beneficial drug combination in comparison with individual drugs of the combination, chloroquine was excluded from this study. Another important observations that were possible to obtain were that the combinations of 5-FU with aspirin, losartan, cimetidine, pravastatin and isoniazid did not show advantage in terms of reduction of cell viability, relative to the respective drug with more effect on viability reduction of that combination (5-FU), being also excluded from this study. However, three drug combinations were advantageous, in comparison with the drug with more effect on viability reduction of that combination. These drugs were Verapamil, Itraconazole and Tacrine, chosen for the continuity of this project.

The exposure of MCF-7 cells to 5-FU combined with verapamil, itraconazole and tacrine, for 72 hours of contact with cells, resulted in a cell viability reduction (in

comparison with the drug with more effect on viability reduction of that combination) of 23%,17% and 6%, respectively. With 5-FU+verapamil, cell viability was 12.1±4.4%, whereas with 5-FU+itraconazole and 5-FU+tacrine, was 24.5±5.2% and 37.3±0.9%, respectively. In all cases, the differences between drug combinations and the single drug of that combination with more effect on cell viability reduction were considered statistically significant.

4.3.2. Comparison of cellular viability between MCF-7 and MCF-10A cell lines

In order to compare the effects of the chosen drug combinations in a tumoral cell line (MCF-7) and a non-tumoral cell line (MCF-10A), both cell lines were exposed to 50 μM of each drug, for 72 hours. The results, for each cell line, were obtained by MTT methodology (described in the Materials and Methods Section) and are presented below.

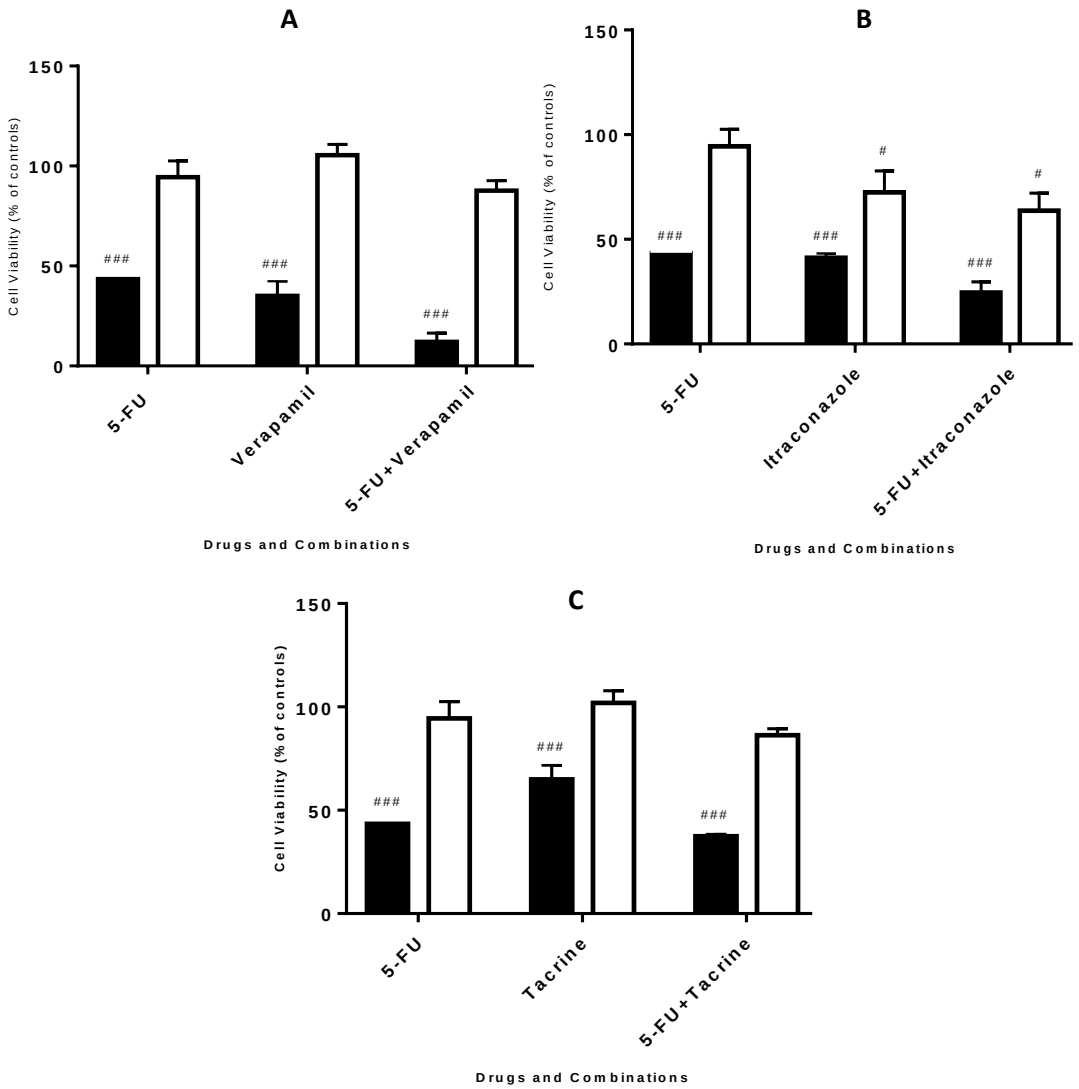
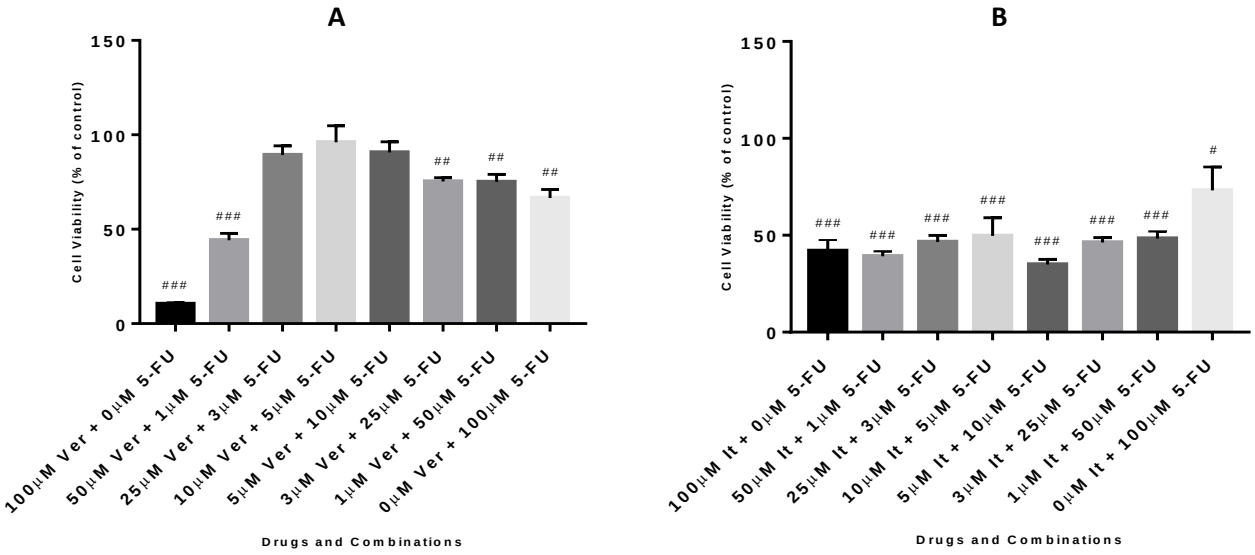


Figure 11 - Effect of 5-FU, verapamil (A), itraconazole (B), tacrine (C) and respective combinations with 5-FU on the viability of MCF-7 cells (left, black bars) and MCF-10A cells (right, white bars). Each drug and each combination was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM, and represent the viability of cells (% of control) of 3-4 independent experiments (n=3,4). ###p<0.001 and #p<0.05 vs respective controls.

Proceeding to an analysis of the results, it was possible to observe that, in general, the drugs had no or little effects on the viability of MCF-10A cells, contrasting with the effects on viability of MCF-7 cells. Taking into account all the three combinations, 5-FU combined with Intraconazole led to the lowest values of cell viability in MCF-10A, 63.6±8.5%, whereas 5-FU combined with verapamil and tacrine led to similar values of cellular viability: 87.6±4.9% and 86.3±3.2%, respectively. All the single drugs and combinations led to effects on cell viability reduction of MCF-7 cells much more pronounced, compared with MCF-10A cell line.

4.3.3. Effect of inversely variable concentrations of each drug of the drug combination, on viability of MCF-7 cells

The next step in this project consisted of the test of inversely variable concentrations of each drug of each drug combination. Thus, one drug of the combination was used in increasing concentrations and the other was used in decreasing concentrations (0, 1, 3, 5, 10, 25, 50 and 100 μM, and vice-versa), acting on cells during a time of 48 hours. The purpose of this experiment was to determine which of the drugs, when in combination, was more preponderant in terms of reduction of cell viability, as well as to perceive a relationship between the two drugs of each combination, varying the concentrations of each. The results were obtained by MTT methodology and are presented below.



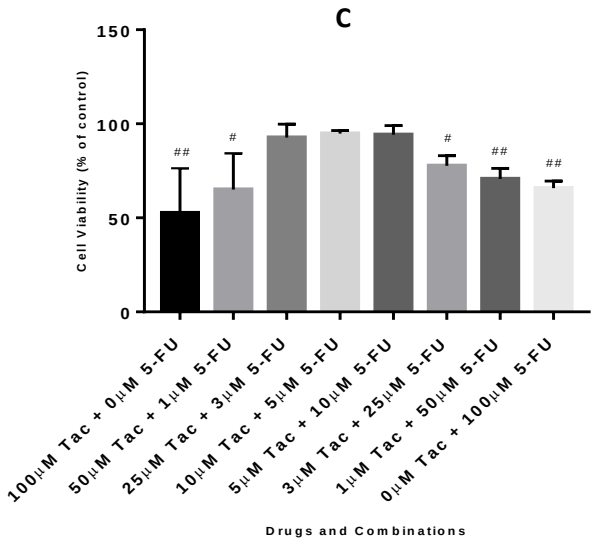


Figure 12 - Effect of verapamil (A), itraconazole (B) and tacrine (C) combined with 5-FU, on the viability of MCF-7 cells. Each drug combination was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM, and represent the viability of cells (% of control) of 3 experiments (n=3). ###p<0.001, ##p<0.01 and #p<0.05 vs control.

Analyzing these results, the lowest values of cell viability were obtained with 100 μM of verapamil and 0 μM of 5-FU (10.4±0.6%), 5 μM of itraconazole and 10 μM of 5-FU (34.9±2.7%), and 100 μM of tacrine and 0 μM of 5-FU (52.6±24%). Overall, with increased concentrations of the verapamil, itraconazole or tacrine, there was an increased reduction on cellular viability, rather than with the increase of 5-FU concentration, as it was notorious when the values that were obtained with 100 μM of the repurposed drugs and 0 μM of 5-FU (and vice-versa) were confronted. However, it was also notorious that changes in the concentrations of both drugs to values lower than 50 μM did not produce changes in the cellular viability that were very different from each other.

4.3.4. Concentration-effect curves and IC_{50} determination of the drugs, in MCF-7 cells

In order to determine the IC_{50} (half maximal inhibitory concentration) values of 5-FU, verapamil, itraconazole and tacrine on the viability of MCF-7 cells, cells were exposed to increasing concentrations of these drugs (1, 3, 5, 10, 25, 50 and 100 μM), converted in logarithm of concentrations, once this scale allows a better interpretation of the results, changing the curve from hyperbolic to a more sigmoid shape [181]. The drugs were in contact with cells for a time of 48 hours, and the cellular viability was determined by MTT reduction assay and, only for 5-FU, by Neutral Red uptake assay, in order to compare to the obtained results by MTT reduction assay. The obtained values allowed the creation of concentration-effect curves (Figures 13-16),

possibilitating the calculation of IC_{50} values (Table 7). The obtained results are presented below.

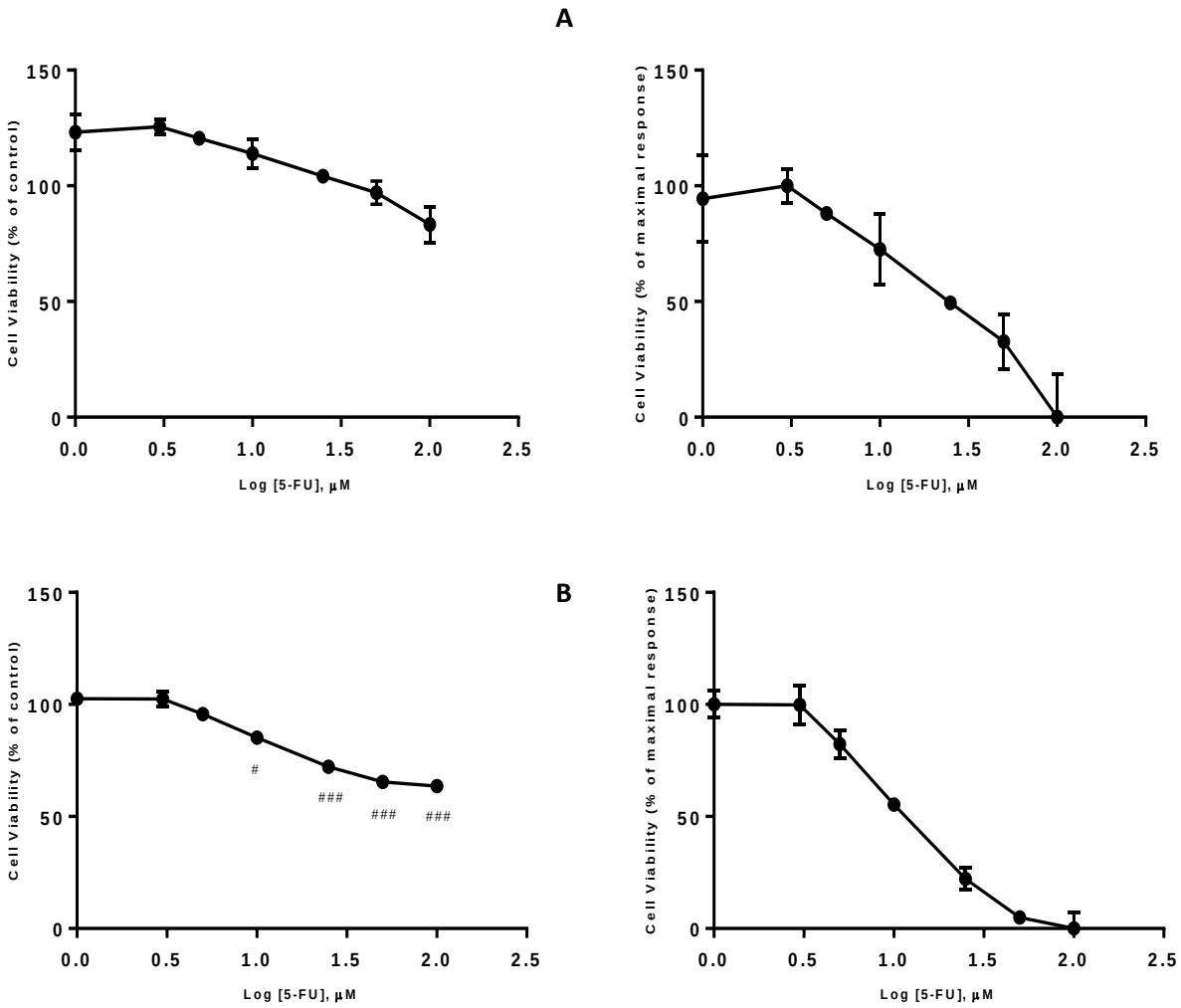


Figure 13 – Concentration-effect curves obtained for 5-FU. The drug was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM of 2-3 independent experiments, obtained by Neutral Red methodology (A) and MTT methodology (B). The left curves represents the viability of cells (% of control), whereas the right curves represents a normalization between 0 and 1 (0 and 100%), where 100% was defined as the concentration of the drug that less affected the cell viability, and all other data points were normalized to this value, being 0% defined as the concentration that affected more the cell viability. ###p<0.001 and #p<0.05 vs control.

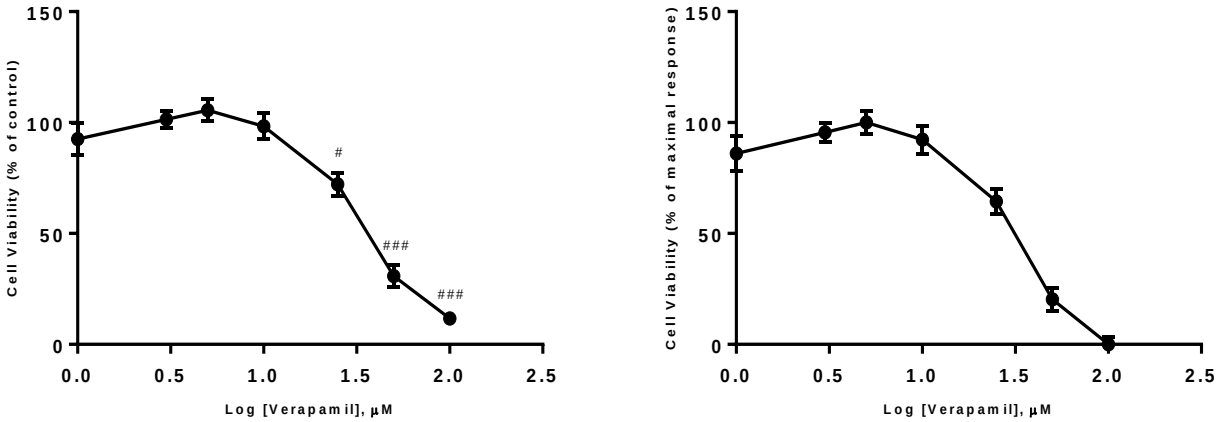


Figure 14 - Concentration-effect curves obtained for verapamil. The drug was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM of 3 independent experiments. The left curve represents the viability of cells (% of control), whereas the right curve represents a normalization between 0 and 1 (0 and 100%), where 100% was defined as the concentration of the drug that less affected the cell viability, and all other data points were normalized to this value, being 0% defined as the concentration that affected more the cell viability. ###p<0.001 and #p<0.05 vs control.

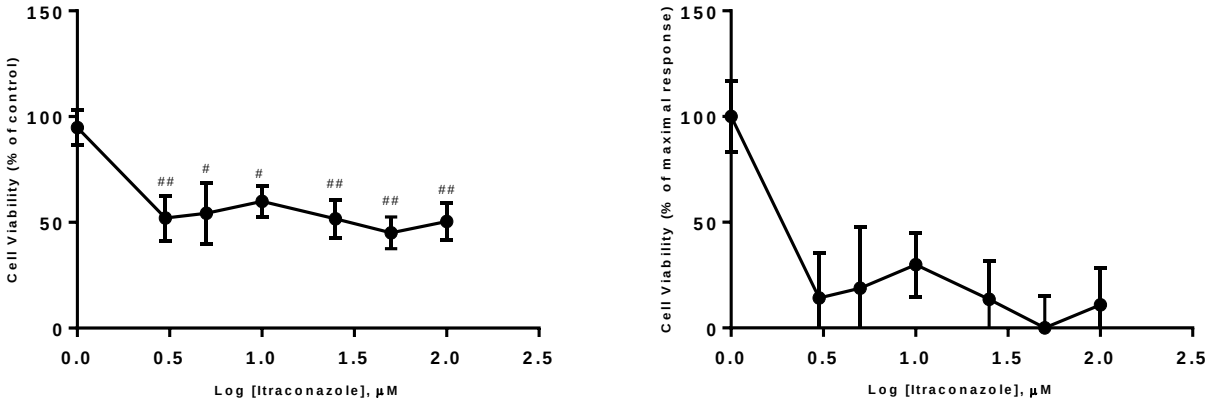


Figure 15 - Concentration-effect curves obtained for itraconazole. The drug was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM of 5 independent experiments. The left curve represents the viability of cells (% of control), whereas the right curve represents a normalization between 0 and 1 (0 and 100%), where 100% was defined as the concentration of the drug that less affected the cell viability, and all other data points were normalized to this value, being 0% defined as the concentration that affected more the cell viability. ##p<0.01 and #p<0.05 vs control.

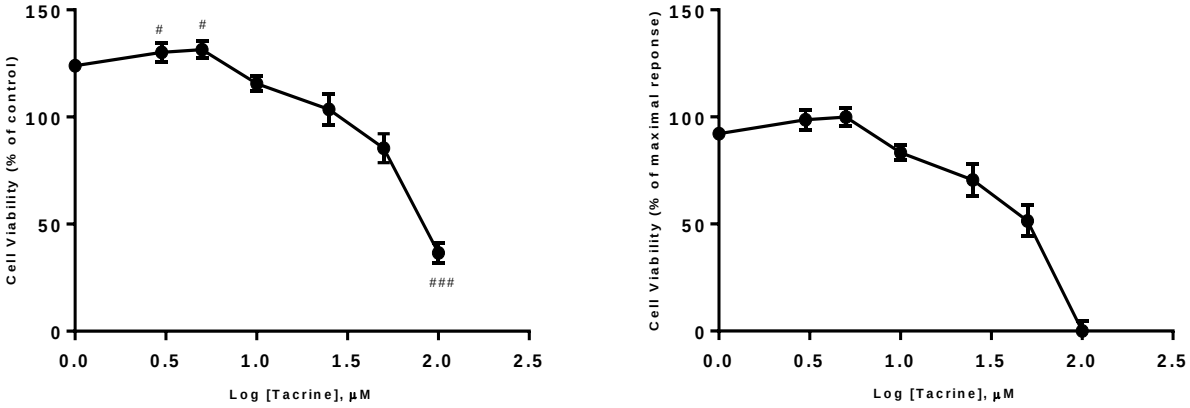


Figure 16 – Concentration-effect curves obtained for tacrine. The drug was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM of 3 independent experiments. The left curve represents the viability of cells (% of control), whereas the right curve represents a normalization between 0 and 1 (0 and 100%), where 100% was defined as the concentration of the drug that less affected the cell viability, and all other data points were normalized to this value, being 0% defined as the concentration that affected more the cell viability. ###p<0.001 and #p<0.05 vs control.

Table 7 – Obtained IC_{50} values for 5-FU, verapamil, itraconazole and tacrine on the viability of MCF-7 cells, with the corresponding 95% confidence intervals.

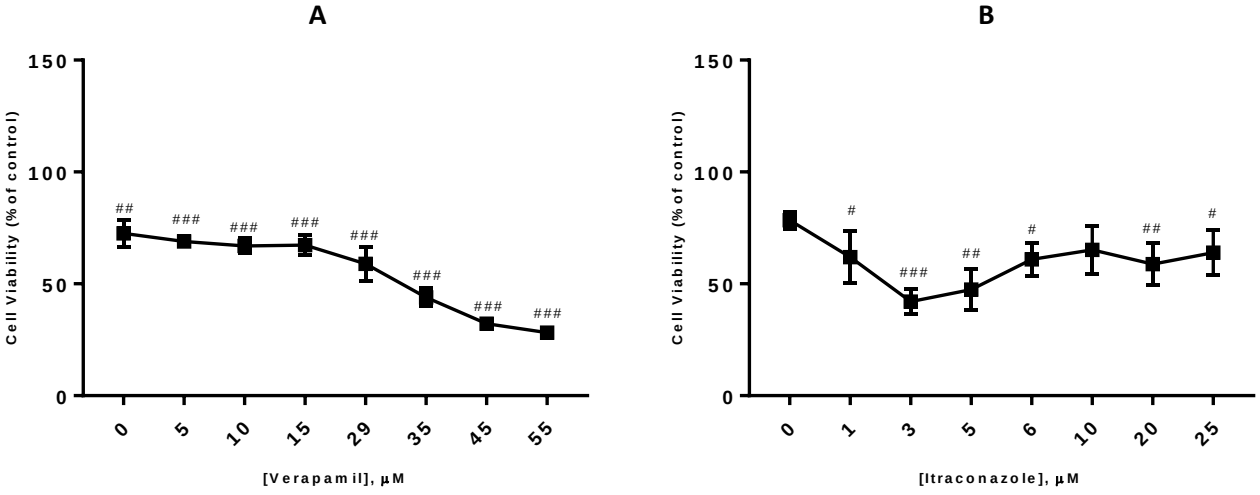
Drug	IC_{50} (µM)	95% Confidence Interval
5-FU (Neutral Red)	23.34	16.61 – 32.54
5-FU (MTT)	11.79	10.18 – 13.72
Verapamil	29.49	23.95 – 38.00
Itraconazole	2.08	1.098 – 4.64
Tacrine	37.79	25.91 – 59.79

The IC_{50} of the drugs was calculated by using the normalized data (between 0 and 100%). So, it was defined as the concentration of the drug that inhibits 50% of cellular viability. However, this method of calculation of IC_{50} has several limitations, reported in

the Discussion Section. This value was calculated using GraphPad Prism 7, more properly using the option “Analyze Data, nonlinear regression (Curve fit)”.

4.3.5. Effect of 5-FU fixed in Its IC_{50} value and variation of the repurposed drugs concentration in values around their IC_{50} values, on viability of MCF-7 cells

After the estimation of the IC_{50} values of 5-FU, verapamil, itraconazole and tacrine on cellular viability, the IC_{50} value obtained for 5-FU by MTT methodology (approximately 11.8 μM) was fixed, and the concentration of the other three drugs was varied by values around the obtained IC_{50} value for each drug (approximately 29, 6[2] and 37 μM for verapamil, itraconazole and tacrine, respectively). It is important to refer that, in this assay, 5-FU was chosen as the fixed concentration drug of the combination since, as observed in the Section 4.3.3, varying the concentration of the repurposed drugs had, in general, more pronounced effects on cell viability, rather than varying the concentration of 5-FU. The drugs were in contact with cells for a time of 48 hours and the cellular viability was determined by MTT reduction assay. The results are presented below.



[2] The IC_{50} value obtained for Itraconazole was, initially, 6 μM and this was the value of IC_{50} considered to this drug. However, in a final phase of the work, the experiment of generating a concentration-effect curve for Itraconazole was repeated and a new value of IC_{50} (2.08 μM) was obtained.

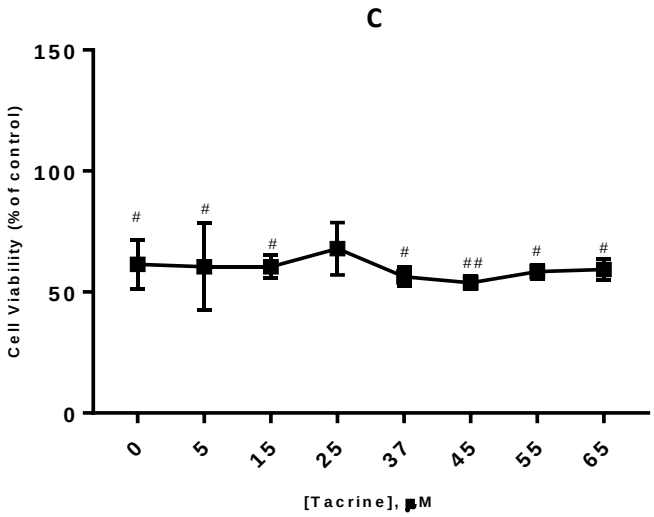
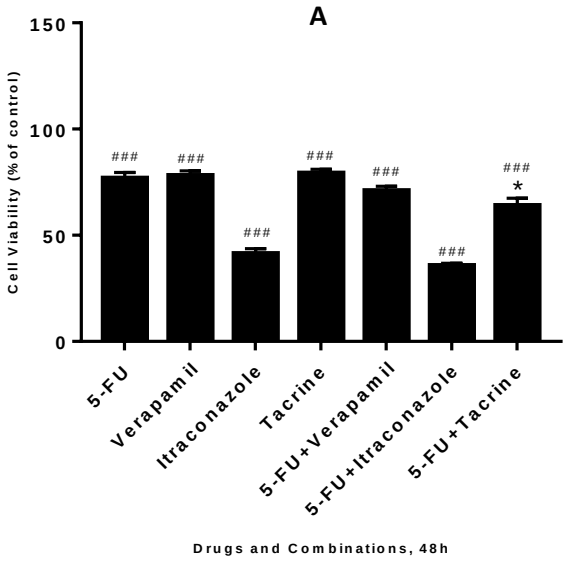


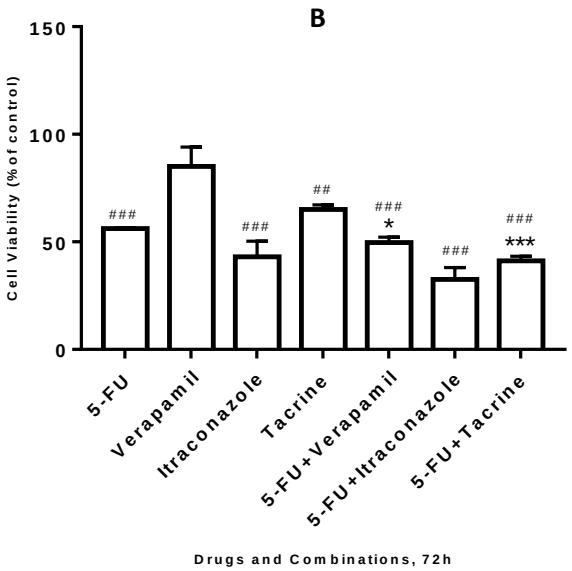
Figure 17 - Effect of verapamil (A), itraconazole (B) and tacrine (C) combined with 5-FU on the viability of MCF-7 cells. Each drug and drug combination was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM, and represent the viability of cells (% of control) of 3 independent experiments. ###p<0.001, ##p<0.01 and #p<0.05 vs control.

Observing the results, it was possible to conclude that the lowest values of cellular viability were obtained for concentrations of 55 and 3 μM, in the case of verapamil and itraconazole, (10.4±0,6%; 34,9±2.7%) respectively. However, in the case of tacrine, there was no obvious differences in cellular viability between the different values of concentrations used. Additionally, important findings were that there was a tendency of lower values of cellular viability with an increase of verapamil concentration, whereas with itraconazole, it seemed that from a certain value of concentration (3 μM), the concentration of the drug had little or no effect on reduction of the cellular viability. Clearly, verapamil appeared to be the drug which was more affected by differences in concentration.

4.3.6. Effect of concentrations of 11.8, 55, 3 and 37 μM of 5-FU, verapamil, itraconazole and tacrine, respectively, on the viability of MCF-7 cells, for 48 and 72 hours

Further, we tested the effect of 5-FU, verapamil, itraconazole, tacrine, and the respective combinations of the last three drugs with 5-FU on the viability of MCF-7 cells, using 5-FU in a concentration of 11.8 μM (IC_{50} value) and verapamil, itraconazole and tacrine in a concentration of 55, 3 and 37 μM, respectively, acting on cells for a time of 48 (A) and 72 (B) hours. The concentrations used for the repurposed drugs were selected taking into account the experiment described on Section 4.3.5, being the concentrations that gave lower values of cellular viability in combination with 5-FU fixed on its IC_{50} value. The results are presented below.





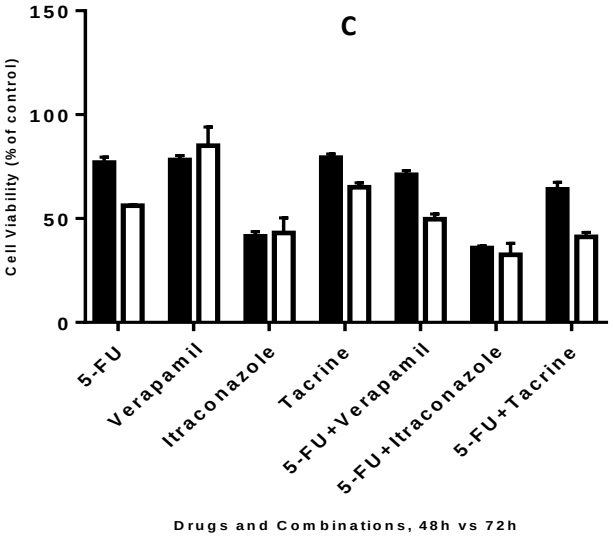


Figure 18 - Effect of 5-FU, verapamil, itraconazole, tacrine, and respective combinations of the last three drugs with 5-FU on the viability of MCF-7 cells, for 48 hours (A), 72 hours (B) and a comparison of both times (C). Each drug and drug combination was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM, and represent the viability of cells (% of control) of 4 independent experiments. ###p<0.001 and ##p<0.01 vs control; *p<0.05 and ***p<0.001 vs single drug of the combination with more effect on cell viability reduction.

Analyzing the obtained results, for 48 hours of actuation of the drugs and drug combinations, it was possible to conclude that the drug combinations seemed to be only slightly advantageous, compared with the drug with more effect on the reduction of cell viability of that combination. In the case of verapamil combined with 5-FU, the single drug with more effect on cellular viability reduction was 5-FU, with values of cell viability of 71.0±2.1% (combination) and 76.9±2.6% (5-FU). In the case of itraconazole combined with 5-FU, values of 35.8±1.1% of cellular viability contrasted with values of 41.4±2.2% of itraconazole, the single drug of the combination with more effect on cellular viability reduction. Lastly, tacrine combined with 5-FU and 5-FU alone (the more efficient drug of the combination) resulted in values of cellular viability of 64.1±3.5% and 76.9±2.6%, respectively. Thus, even though there were no notable differences, there was a tendency of the combination to be more effective than the single drug with more effect on the reduction of cell viability of that combination.

For 72 hours of drugs actuation, it was possible to observe that the combinations of 5-FU+verapamil (49.7±2.5% of cellular viability), 5-FU+itraconazole (32.5±5.5% of cellular viability) and 5-FU+tacrine (41.2±2.1% of cellular viability), compared with the single drug of each combination with more effect on the reduction of cell viability, reduced the cellular viability in 7%, 11% and 15%, respectively, keeping the same tendency above mentioned.

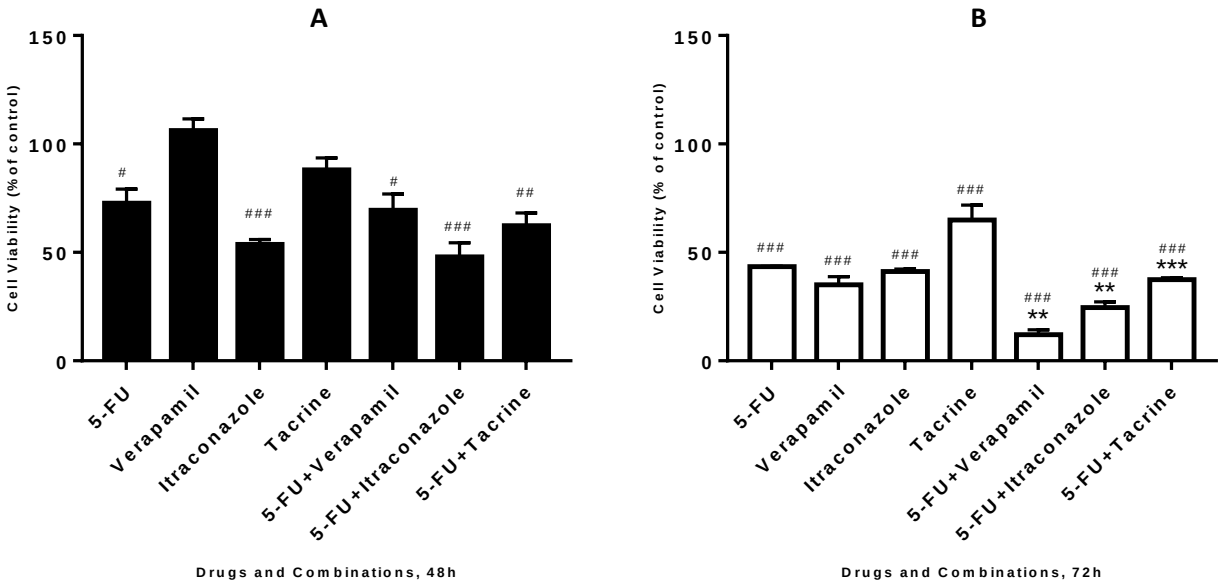
It was also notorious that when comparing different times (48 vs 72 hours), all the drugs and combinations, except verapamil and itraconazole, reduced the cell viability more markedly at 72 hours.

At both 48 and 72 hours, higher differences on cellular viability values were achieved between 5-FU+tacrine and 5-FU (single drug with more effect on this combination). However, the lowest values of cellular viability were obtained with 5-FU+itraconazole.

Even though all drug combinations have not shown statistically significant differences relative to the more effective drug of the combination, with an increase of independent experiments, there is a strong probability of observation of statistically significant differences, since there is a clear tendency of advantageous effects of drug combinations (in both 48 and 72 hours), relative to individual drugs.

4.3.7. Effect of 50 µM of each drug on the viability of MCF-7 cells, for 48 and 72h

Another important step in this work consisted in the test of the effect of 5-FU, verapamil, itraconazole, tacrine, and the respective combinations of the last three drugs with 5-FU on the viability of MCF-7 cells, using all the drugs in a concentration of 50 µM, the same concentration used in the initial screening assay. The drugs were in contact with cells during a time of 48 (A) and 72 (B) hours. The results are presented below.



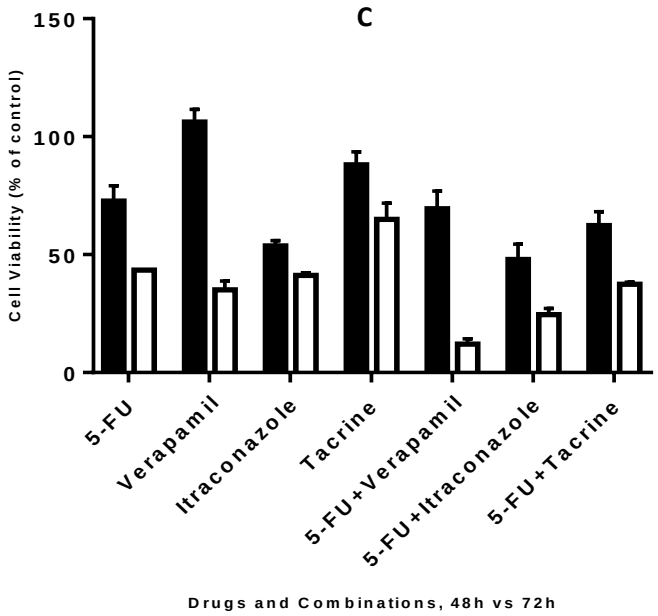


Figure 19 - Effect of 5-FU, verapamil, itraconazole, tacrine, and respective combinations of the last three drugs with 5-FU on the viability of MCF-7 cells, for 48 hours (A), 72 hours (B) and a comparison of both times (C). Each drug and drug combination was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM, and represent the viability of cells (% of control) of 3-4 independent experiments. ###p<0.001, ##p<0.01 and #p<0.05 vs control; **p<0.01 and ***p<0.001 vs single drug of the combination with more effect on cell viability reduction.

Proceeding to the analysis of the obtained results, for 48 hours of actuation of the drugs and combinations, it was possible to conclude that the combinations of 5-FU+verapamil, 5-FU+itraconazole and 5-FU+tacrine, compared with the single drug of the combination with more effect on the reduction of cell viability of that combination, reduced the cell viability in 3%, 6% and 10%, respectively. For 72 hours, this reduction was of 23%, 17% and 6%, respectively.

At 48h of drug actuation, higher differences on cellular viability values were achieved between 5-FU+tacrine and 5-FU (single drug with more effect on this combination). However, the lowest values of cellular viability were obtained with 5-FU+itraconazole (47.9±6.5% of cell viability). On the other hand, at 72h, not only higher differences between the most effective single drug of the combination and the respective combination, but also the lowest values of cellular viability were achieved with the 5-FU+verapamil (12.1±2.2% of cell viability).

It was also notorious that, when comparing different times (48 vs 72 hours), all the drugs combinations and single drugs reduced the cell viability more markedly at 72 hours than at 48 hours.

4.3.8. Comparison of different concentrations of the drugs on the viability of MCF-7 cells, at 48 and 72 hours

The results presented below represent the results obtained in section 4.3.6 and 4.3.7, with the purpose of comparing the different concentrations tested in the different time points (48h and 72 hours).

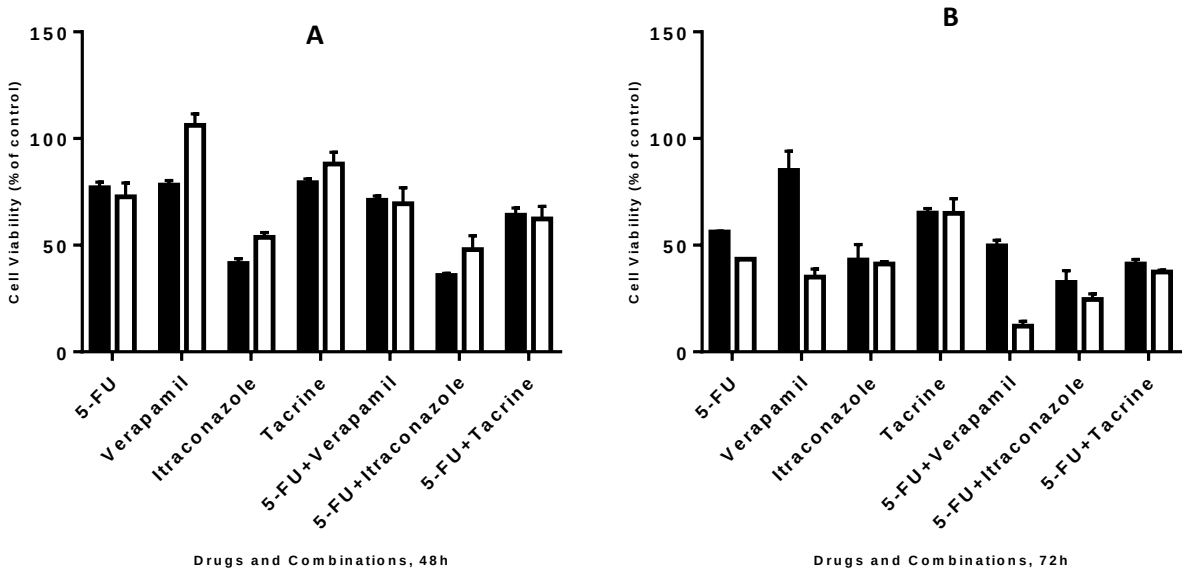


Figure 20 - Effect of 5-FU, verapamil, itraconazole, tacrine, and respective combinations of the last three drugs with 5-FU on the viability of MCF-7 cells, for 48 hours (A) and 72 hours (B). In the left, black bars, 5-FU was used on a concentration of 11.9 μM and Verapamil, Itraconazole and Tacrine were used in a concentration of 55, 3 and 37 μM, respectively. In the right, white bars, all drugs were used in a concentration of 50 μM. Each drug and drug combination was added in fresh medium, in sextaplicates. Results are presented as mean ± SEM, and represent the viability of cells (% of control) of 3-4 experiments.

Analyzing the results obtained, for 48h, it was possible to observe that the different concentrations did not produce very different effects in the case of 5-FU, 5-FU combined with verapamil, 5-FU combined with tacrine and tacrine. On the other side, in the case of verapamil, itraconazole and 5-FU combined with itraconazole, with concentrations of 50 μM, it was possible to observe that cell viability was less reduced.

For 72h, in general, there was marked reduction on cellular viability, compared with 48 hours. Once again, it was possible to observe that the different concentrations did not produce very different effects, except in the case of verapamil and 5-FU combined with verapamil, in which concentrations of 50 μM led to an increase on cell viability reduction.

Taking into account all the different concentrations and time points, the drug combination that appeared to be more effective in terms of cell viability reduction was

5-FU+verapamil in a concentration of 50 μM of each drugs, at 72 hours (12.1±2.2%). However, in all other conditions, 5-FU+itraconazole seemed to be the more effective drug combination, leading to the reduced cellular viability values.

4.3.9. Analysis of proliferation in MCF-7 cells, by CFSE staining

In order to understand the mechanism by which drug combinations act, it was further tested the effect of 5-FU, verapamil, itraconazole, tacrine, and the respective combinations of the last three drugs with 5-FU, on the proliferation of MCF-7 cells, using flow cytometry, namely CFSE staining, as described in Materials and Methods Section. 5-FU was used in a concentration of 11.8 μM (IC_{50} value) and verapamil, itraconazole and tacrine were used in a concentration of 55, 3 and 37 μM, respectively, acting on cells for a time of 72 hours, in order to allow enough time for cells to proliferate (doubling time of 37h). The results (Figures 22 and 23), as well as the performed gating strategies (Figure 21), are presented below. The results are expressed as Mean Fluorescence Intensity (MFI). Higher values of fluorescence intensity mean less cell proliferation.

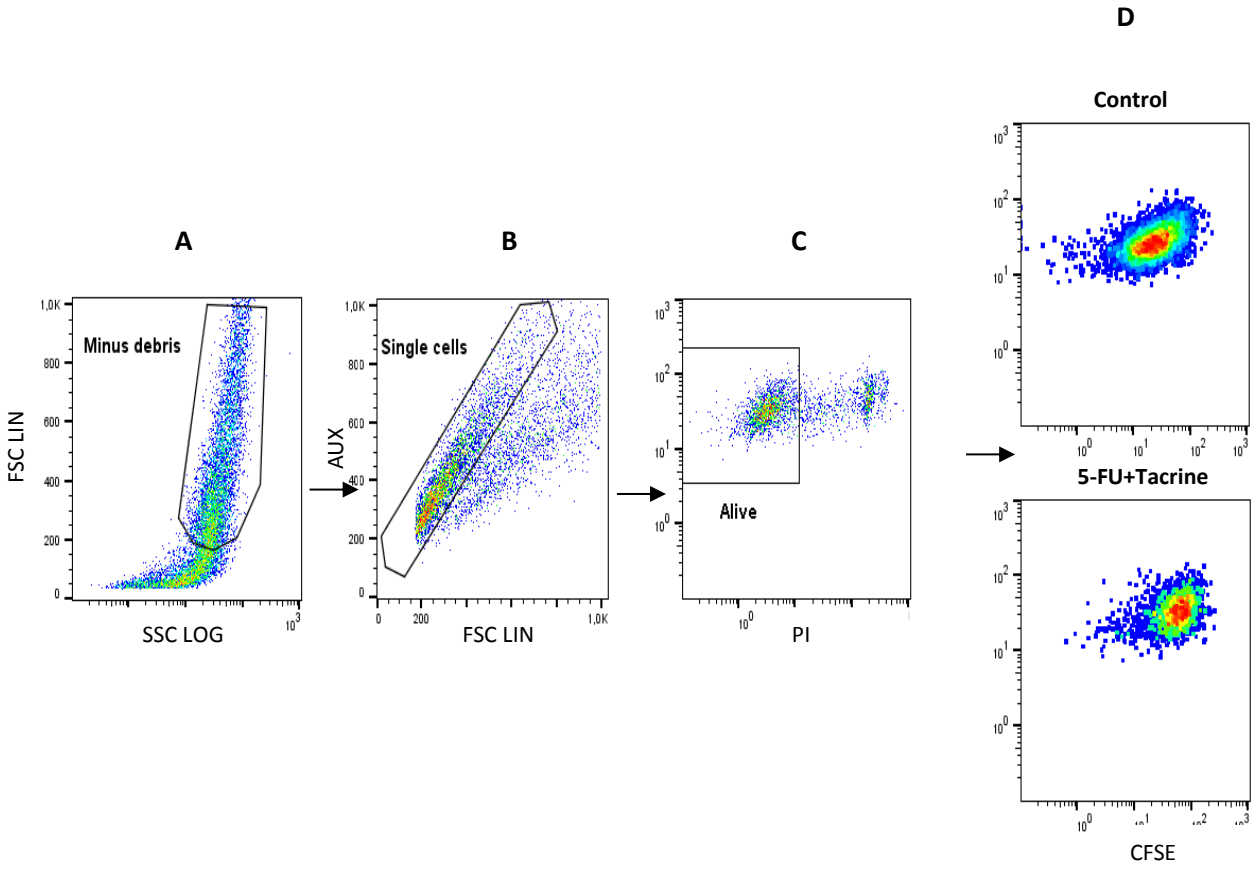


Figure 21 – Flow cytometry gating strategy used in the CFSE proliferation assay of MCF-7 cells. (A) The gate separated MCF-7 cells from cellular debris, whereas in (B) single cells are gated, thus excluding aggregates. (C) Dead cells were excluded by PI incorporation, and (D) represents cell fluorescence due to CFSE staining in non-treated (control) or 5-FU+Tacrine (treated cells), as indicated.

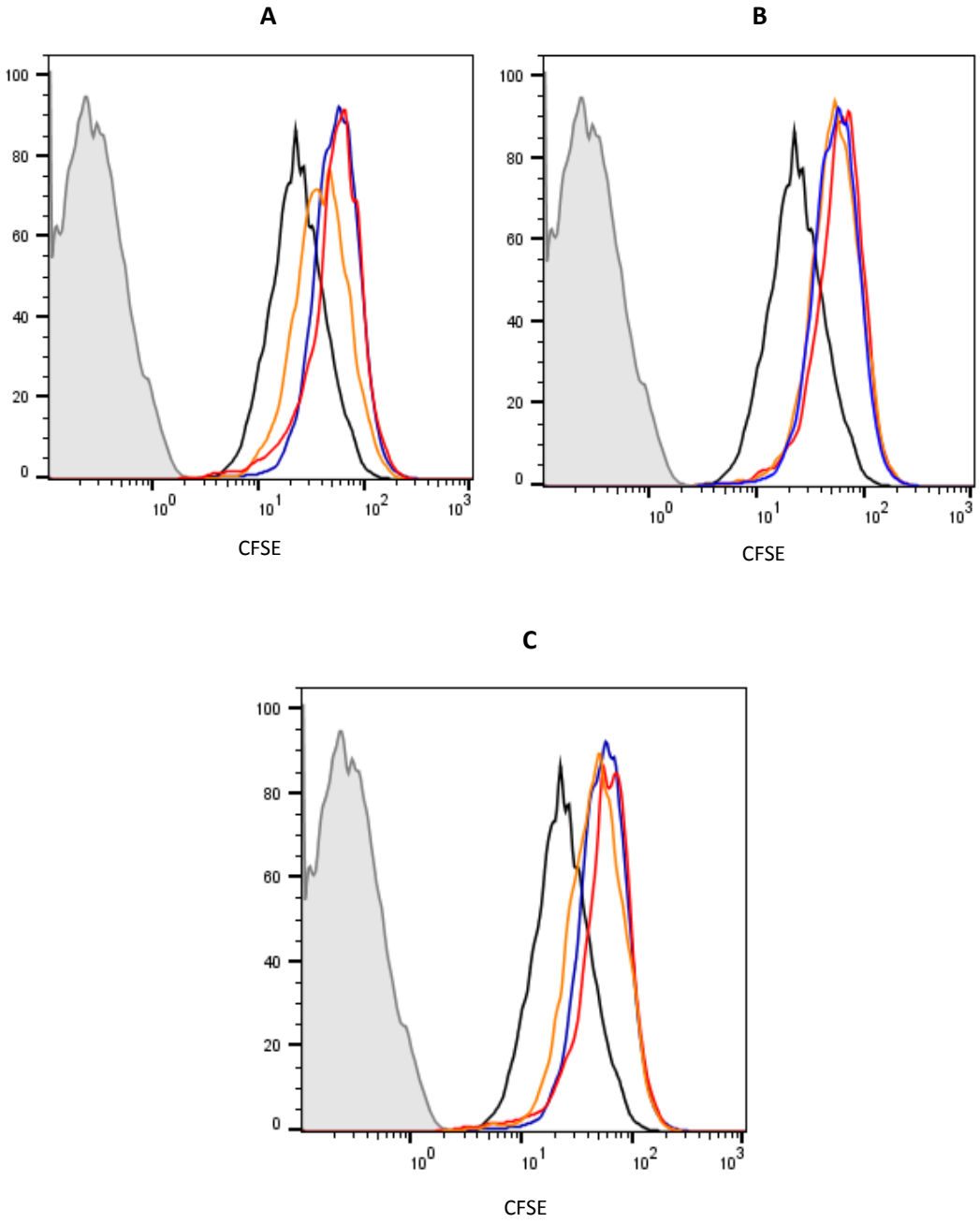


Figure 22 - Cell fluorescence due to CFSE staining. In all graphs, the black line indicates control (stained), the grey line indicates unstained control and the blue line represents 5-FU. (A) The red line indicates the combination of 5-FU+verapamil and the orange line indicates verapamil. (B) Red and orange lines represent 5-FU+itraconazole and itraconazole, respectively. (C) 5-FU+tacrine is represented by the red line, whereas tacrine is represented by the orange line.

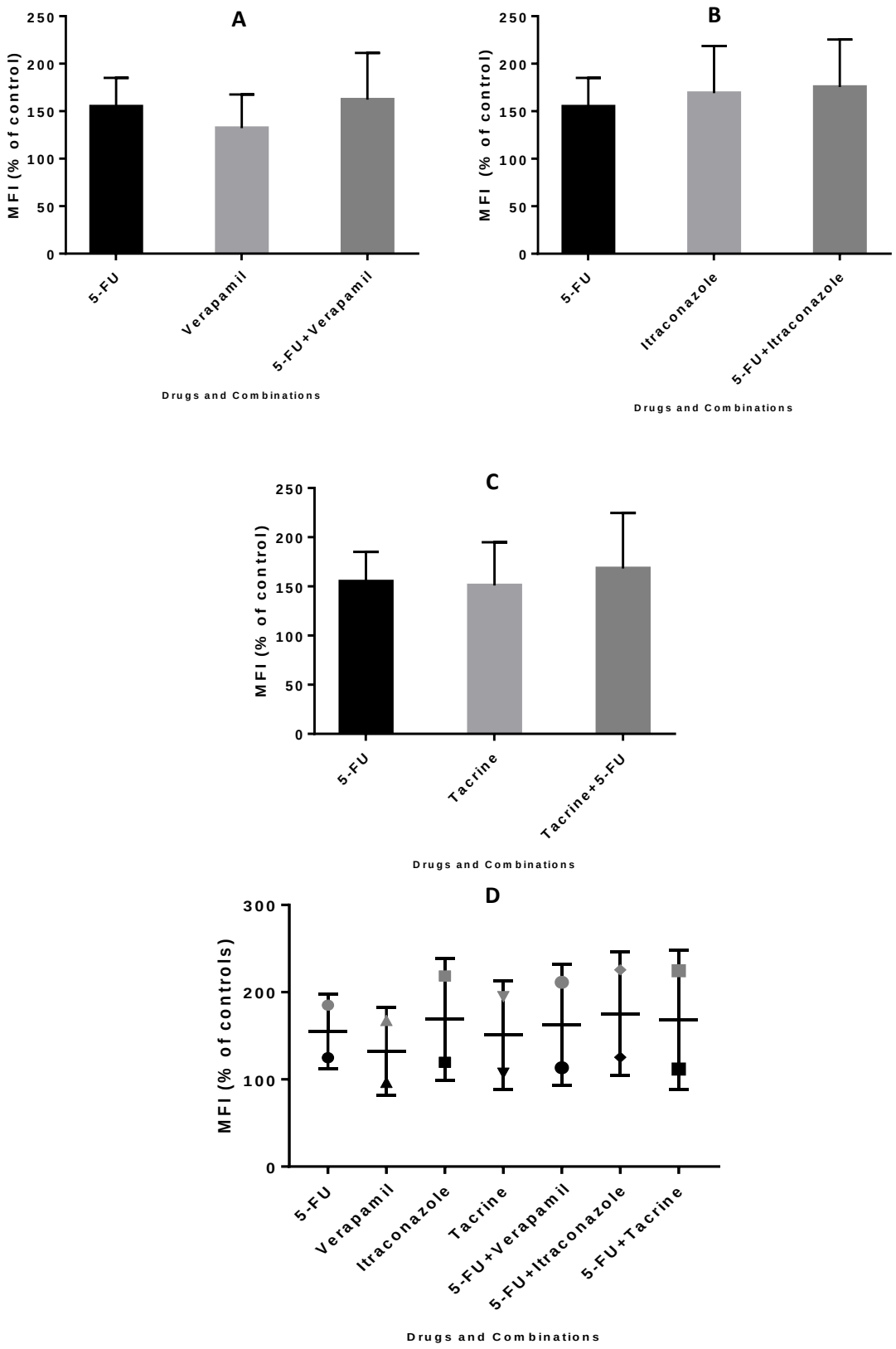


Figure 23 - Effect of 5-FU, verapamil (A), itraconazole (B), tacrine (C), and respective combinations of the last three drugs with 5-FU on the proliferation of MCF-7 cells, and a comparison of both experiments (D). Each drug and drug combination was added in fresh medium, in duplicates. Results are presented as mean ± SEM, and represent MFI (% of control) of 2 independent experiments.

Analyzing the obtained results, it was possible to conclude that all the three drug combinations promoted a more pronounced arrest on cell division, compared to the individual drugs of each combination. Particularly, with 5-FU+verapamil, MFI values were 162.3±48.9%, with 5-FU+itraconazole these values were 175.4±50.1%, and with 5-FU+tacrine, the obtained values were 168.2±56.4%, compared with values of 154.9±30.2% (5-FU) and 169.16±49.5% (itraconazole) obtained with the individual drugs with more effect on cell proliferation arrest of each combination, respectively. Higher differences in MFI values were obtained between 5-FU+tacrine and 5-FU (the most effective single drug of the combination, in this case). However, 5-FU+itraconazole was the drug combination that led to the highest MFI values. It is also important to note that high SEM values were obtained, since the two experiments resulted in very different values. However, analyzing graph D, it was possible to conclude that both experiments showed the same tendency of results, in which drug combinations are advantageous relative to the isolated drugs.

4.3.10. Analysis of cell death in MCF-7 cells, by Annexin V/PI staining

Still in order to understand the mechanism by which drug combinations act, we further tested the effect of 5-FU, verapamil, itraconazole, tacrine, and the respective combinations of the last three drugs with 5-FU on the death of MCF-7 cells, using flow cytometry, namely Annexin V/PI staining, as described in Materials and Methods Section. 5-FU was used in a concentration of 11.8 μM (IC_{50} value) and verapamil, itraconazole and tacrine were used in a concentration of 55, 3 and 37 μM, respectively, acting on cells for a time of 3 or 8 hours. The performed gating strategies (Figure 24), as well as the results (Figures 25 and 26) are presented below. Results are expressed4as percentage of living cells, apoptotic cells and cells in late apoptosis/ necrosis, compared to untreated control.

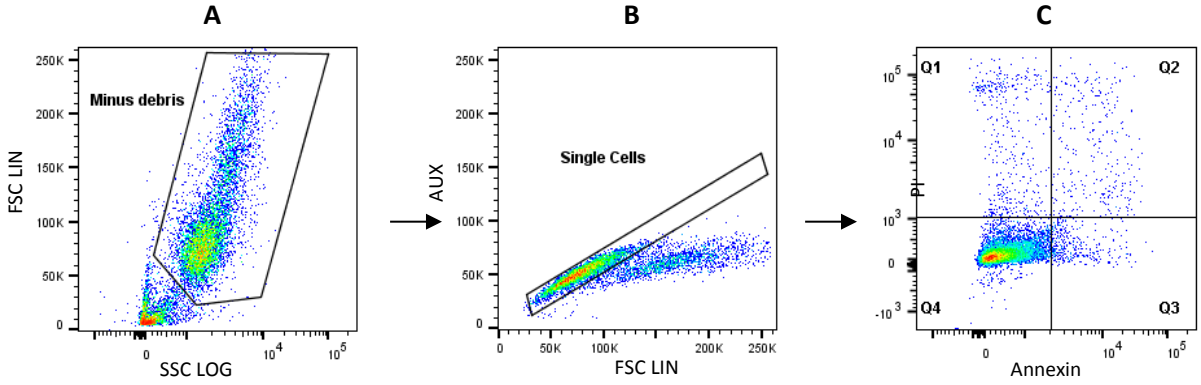


Figure 24 - Flow cytometry gating strategy used to evaluate apoptosis/necrosis in MCF-7 cells. (A) The gate separated MCF-7 cells from cellular debris, whereas in (B) single cells are gated, thus excluding aggregates. (C) Target cells were distinguished based on PI/ Annexin V staining. In Q1+Q2 are represented cells in necrosis/ late apoptosis, in Q3 are represented cells that are in early apoptosis and living cells are represented in Q4.

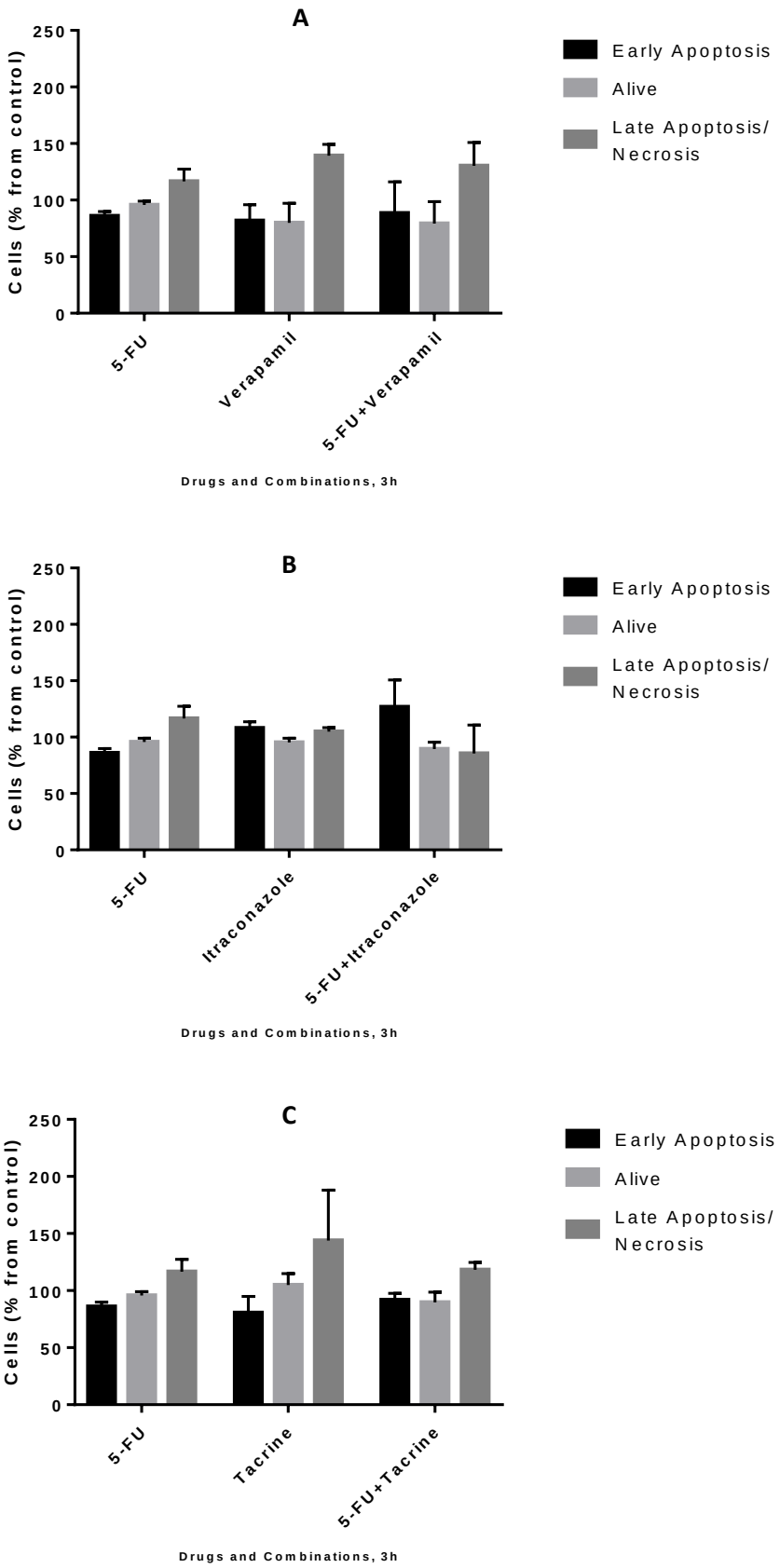


Figure 25 - Effect of 5-FU, verapamil (A), itraconazole (B), tacrine (C), and respective combinations of the last three drugs with 5-FU on the death of MCF-7 cells, for 3 hours. Each drug and drug combination was added in fresh medium, in duplicates. Results are presented as mean ± SEM of 2 independent experiments.

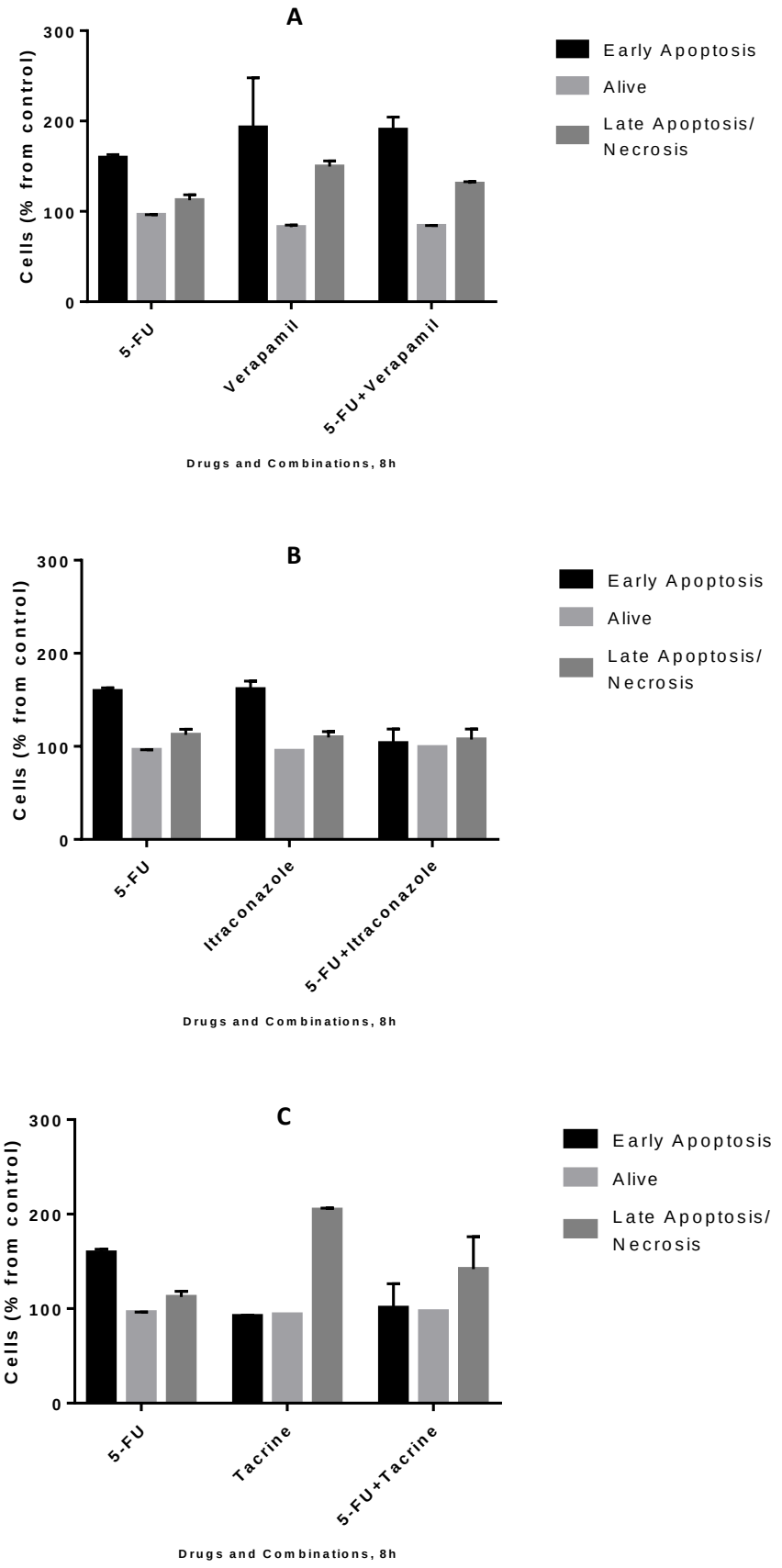


Figure 26 - Effect of 5-FU, verapamil (A), itraconazole (B), tacrine (C), and respective combinations of the last three drugs with 5-FU on the death of MCF-7 cells, for 8 hours. Each drug and drug combination was added in fresh medium, in duplicates. Results are presented as mean ± SD of 1 independent experiment.

The analysis of the obtained results possibilited the conclusion that, at 3 hours of drug exposure, with all the single drugs and combinations, the percentage of living cells were not very different from the control (100%). However, concerning to the percentage of early apoptotic cells, the higher value was obtained with 5-FU+itraconazole (126.8±23.9%), while with all the other drugs and drug combinations, the values were not very different to control values. More pronounced differences were obtained concerning late apoptosis/necrosis: verapamil (139.2±10.2%), 5-FU+verapamil (130.2±20.7%) and tacrine (143.9±44.1%) led to the highest values, compared to control values.

At 8 hours of treatment, the percentage of living cells also remained at values near to control values with all the treatments. Early apoptosis was verified in more extension with the treatment with 5-FU (159.4±3.5%), verapamil (192.9±55.1%), 5-FU+verapamil (190.4±13.9%) and itraconazole (161.4±8.7%), while with rest of conditions, similar values with the control were obtained. Concerning to late apoptosis/necrosis, the higher values were obtained with verapamil (149.6±6.16%), 5-FU+verapamil (130.7±2.3%), tacrine (204.5±1.9%) and 5-FU+tacrine (141.9±34.2%), whereas with the rest of conditions, similar values with the control value were obtained.

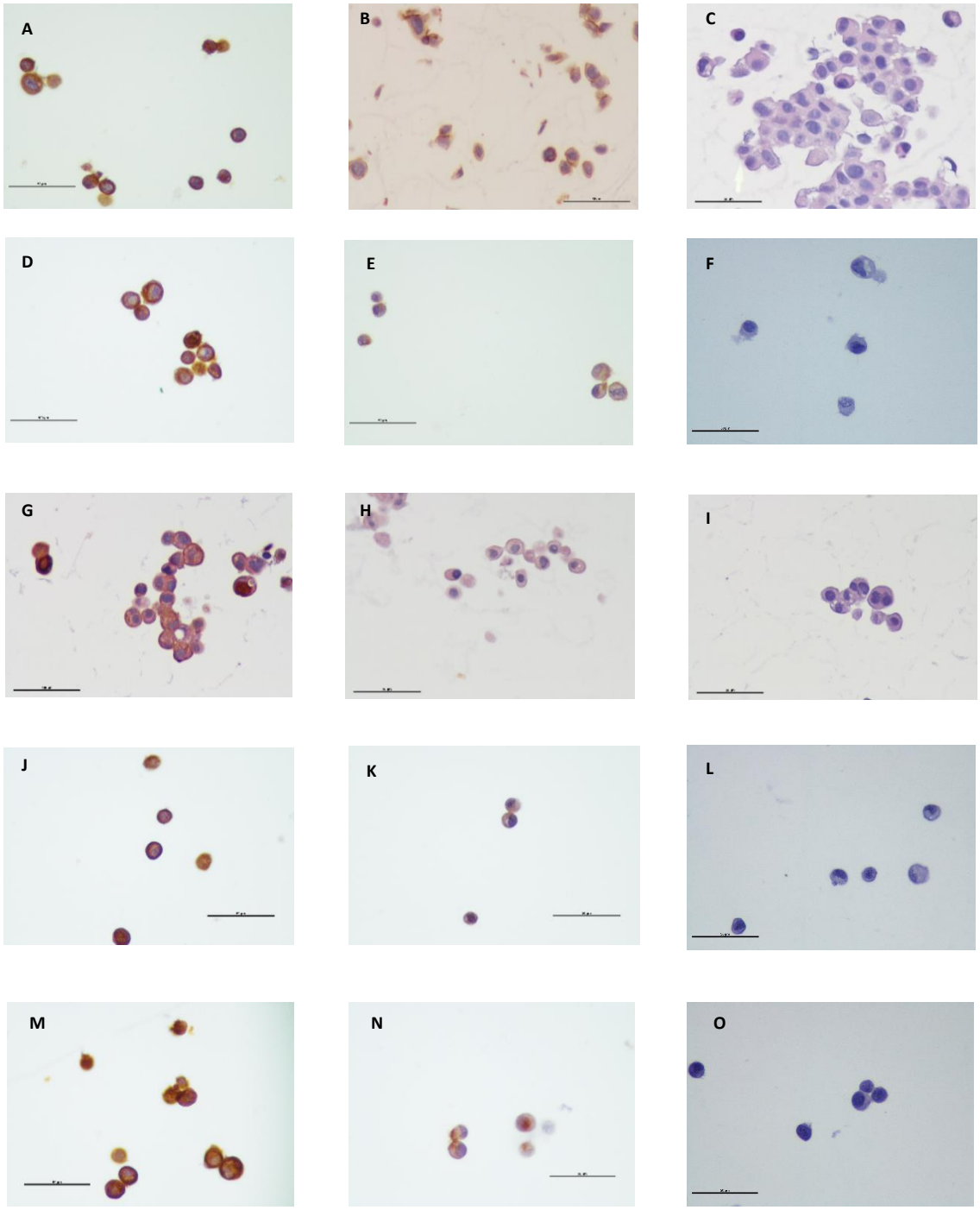
Comparing the obtained results for 3 and 8 hours, in general an increase of early apoptosis, late apoptosis and necrosis was observed. Regarding specifically with the drug combinations, it is relevant to highlight the increase of approximately 102% in early apoptosis with 5-FU+verapamil at 8 hours (vs 3h), the increase in late apoptosis/necrosis of 23.5% with 5-FU+tacrine and the decrease of approximately 20% in apoptosis, with 5-FU+itraconazole.

4.3.11. Evaluation of the transition from an epithelial to a mesenchymal phenotype, in MCF-7 cells

Other performed experiment was an immunocytochemistry assay, testing the epithelial markers cytokeratins AE1/AE3 and e-cadherin, and the mesenchymal marker, vimentin. The aim of this study was an evaluation of whether the resistant cells evolved from an epithelial to a mesenchymal state.

MCF-7 cells were treated with 5-FU, verapamil, itraconazole, tacrine, and the respective combinations of the last three drugs with 5-FU, acting on cells for a time of 48 hours. 5-FU was used in a concentration of 11.8 μM and verapamil, itraconazole

and tacrine were used in a concentration of 55, 3 and 37 μM, respectively. The obtained images are presented below.



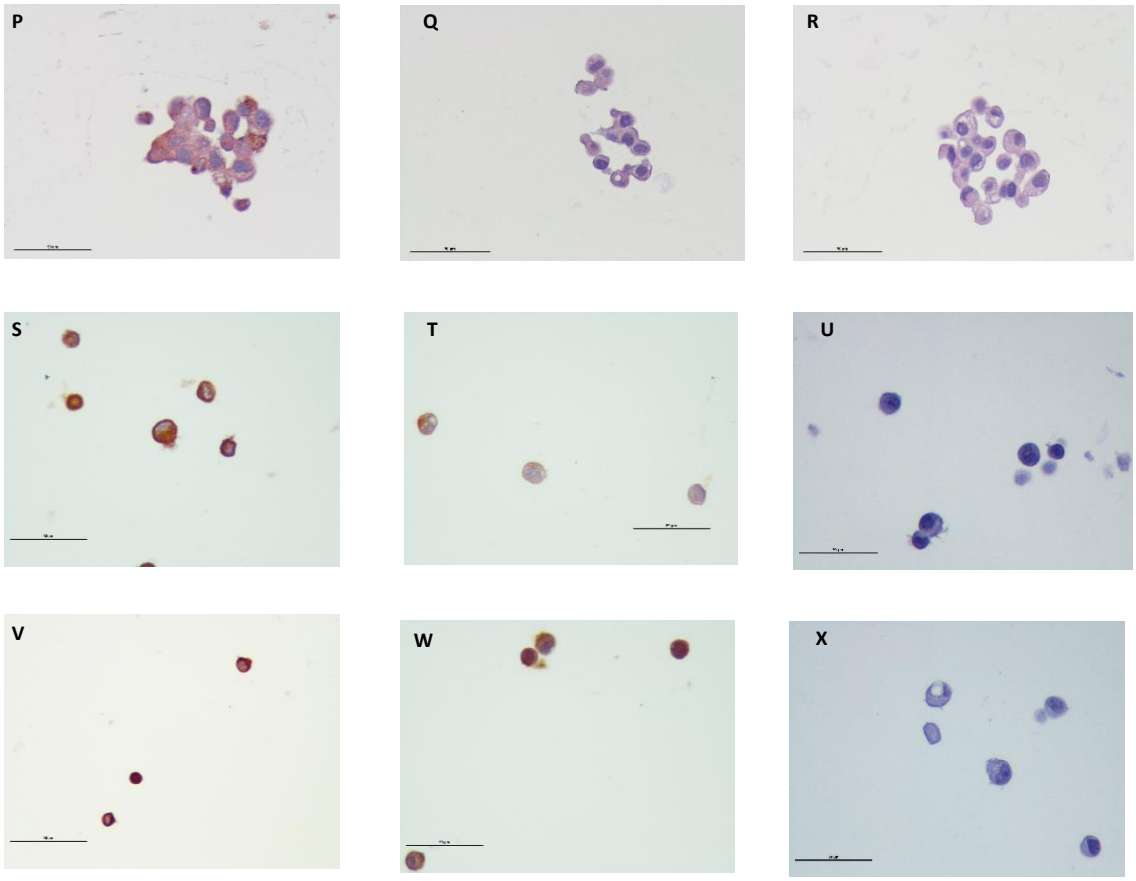


Figure 27 – Expression of EMT markers, AE1/AE3, e-cadherin and vimentin in MCF-7 cells, by immunocytochemistry (procedure explained in the Material and Methods Section). All images were obtained through the same pool of cells, but in different cell blocks, in a magnification of 400x. Images A, B and C represent the positive controls for AE1/AE3, e-cadherin and vimentin, respectively. D, E and F represent images of cells treated with 5-FU and tested for the presence of AE1/AE3, e-cadherin and vimentin, respectively. G, H and I represent images of cells treated with verapamil and tested for the presence of AE1/AE3, e-cadherin and vimentin, respectively. J, K, L and M, N, O represent images of cells treated with itraconazole and tacrine and tested for the presence of AE1/AE3, e-cadherin and vimentin, respectively. P,Q, R and S, T, U represent images of cells treated with 5-FU combined with verapamil and 5-FU combined with itraconazole, respectively and tested for the presence of AE1/AE3, e-cadherin and vimentin. Lastly, cells treated with 5-FU combined with tacrine and tested for the presence of AE1/AE3, e-cadherin and vimentin, respectively, are represented by images V, W and X.

Through the observation of the obtained images, it was possible to observe that, in all conditions, the cells were positively marked for the epithelial markers, AE1/AE3 and E-Cadherin, and negatively marked for the mesenchymal marker, Vimentin. Other findings were the presence of fewer cells, damaged cells, cells with vacuoles and dead cells. These findings will be discussed in the next Section.

4.4. Discussion

Drug repurposing and drug combinations are major approaches applied in order to improve cancer therapy, reducing its subjacent toxicological profile and improving its efficacy. However, despite the growing investigation and interest in these methodologies, there are still subjacent limitations, namely financial and toxicological issues [56, 182].

Combining drug combination and drug repurposing approaches in breast cancer therapy can be an important focus of studies, as it can greatly improves its treatment, namely the metastatic type, that has still many barriers to effective treatment. Thus, this project aimed to study the potential of repurposing drugs, in combination with an already used drug in chemotherapy, in the treatment of breast cancer. The ideal situation is that in which the combination of drugs is advantageous in relation to the individual drugs of that combination, always with the main focus of reducing the toxicological profile and increasing the effectiveness.

Several studies addressing combinations of drugs, one of which is used in cancer and another in non-cancer indications, have shown very positive results in relation to cancer therapy, not exclusively for breast cancer, but also for other kinds of cancer. For example, studies report that the administration of metformin and gemcitabine increase the pro-apoptotic and anti-proliferative activity that has been verified with metformin in a context of pancreatic cancer treatment [183]. Other studies have also shown that when in combination with 5-FU, itraconazole significantly reduced the proliferation rate of gastric cancer cells [184].

Before the in-depth discussion of the obtained results, it is important to mention the limitations of the two most used methodologies along this work, which can always have influence on almost any obtained result: Cell culture and MTT assay.

Cell culture is a major tool used in cellular and molecular biology. The major advantage of using technique is the consistency and reproducibility of results that can be obtained, being easier to handle than animal models, as well as being more easy to control the environment of all the experiments [134]. However, there are subjacent limitations in this widely used methodology. One major limitation is the fact that the chances of microbial or chemical contaminations are very high. Also, it is required a lot of training

to work with cell culture. The cell culture medium, as well as supplements, need to be careffuly chosen, as they have extreme influence on cells and, consequently, on results. Also, the microenvironment in the culture vessel can induce many physical, chemical or physiological changes in the cells. Cell passage number is also an important factor to have in consideration: higher passage numbers may result in alterations in morphology, response to stimuli, growth rates, protein expression and transfection efficiency, compared to lower passage cells [177, 185, 186]. Additionally, and very important, is the fact the working with cells does not exactly replicate the complex environment existent in the whole organism.

Concerning to MTT assay, it measures cell viability in terms of reductive activity, once enzymatic conversion of the tetrazolium compound to formazan crystals are conducted by dehydrogenases occurring in the mitochondria and in other organelles (such as the endoplasmic reticulum), of living cells [187]. MTT assay has long been regarded as the gold standard of cytotoxicity assays, once it has several advantages, such as being a cheap, fast and easy to execute method [144, 188]. However, it addresses several limitations. First of all, it is important to note that this methodology measures cell viability and not directly cell proliferation or cell death, as sometimes erroneously described [187]. As it measures cell viability, it depends on the cell metabolic state, being is notorious that cells with low metabolism or in a stationary phase of growth reduce very little MTT, and vice-versa for cells with accelerated metabolism. This can lead to a misunderstanding of the results obtained [189]. Additionally, MTT has a cytotoxic nature that can, in some way, contribute to cell death, being extremely important optimize its concentration to cells, in order to minimize toxicity. Given the cytotoxic nature of MTT, this method must be considered as an endpoint assay [187]. Therefore, it is important to keep in mind that all the subjacent limitations of these techniques may have some influence on all the results that were obtained.

This project started with drug screening assays in FMCm and MCF-7 cells. Relative to FMCm cell line, taking into account the obtained results, it appears that no combination of drugs had any advantage over the respective individual drugs. This may be most likely due to the fact that this is a particularly aggressive cell line and is, therefore, probably more difficult to deal with [133]. However, with the study of the drugs in MCF-7 cells, the results were more conclusive, and there were combinations of drugs that stood out in this screening and, therefore, were studied throughout this work. The three chosen drugs in the drug screening assay were verapamil, itraconazole and tacrine, all combined with 5-FU, since they were those that showed better responses (increased

reduction of cellular viability), when compared to the individual drugs of each combination.

In particular, verapamil is a calcium channel blocker, mainly a L-type calcium channel blocker, but also a blocker of other calcium channels (namely T- and P- Ca^{2+} channels) [190]. In other words, this drug inhibits the transmembrane flux of calcium ions, being utilized clinically to treat cardiac arrhythmias, angina and cardiomyopathies [191, 192]. Although the known role of ion channels in specialized excitable cells, like neurons and cardiac myocytes, these channels also play critical roles in cancer pathophysiology by several mechanisms, controling cancer cell proliferation by regulating several key survival signaling pathways and membrane potential [193]. Verapamil is also known to be a first generation inhibitor of P-glycoprotein, an important protein present in the cell membrane, that pumps a wide variety of substances out of cells, being an ATP-dependent efflux pump [194]. Thus, when combined with chemotherapeutic agents, this drug may help to promote intracellular drug accumulation. This has been demonstrated in non-small cell lung cancer, colorectal carcinoma, leukemia and neuroblastoma cell lines [180].

Particularly in breast cancer, several studies reported beneficial effects of verapamil. For example, this drug has shown anti-proliferative effect in breast cancer in a mouse model [195]. Also, verapamil increases the survival of patients with anthracycline-resistant metastatic breast carcinoma, in a prospective study of 99 patients [196]. Potentiation of tamoxifen activity by verapamil, in a human breast cancer cell line (MCF-7), has also been reported [197].

However, there are contradictory reports about the anticancer properties of verapamil. For example, in human epidermoid carcinoma cells, verapamil did not inhibit the growth of cells. In a phase III clinical trial, the addition of this drug to vincristine, doxorubicin and dexamethasone (standard therapy) for multiple myeloma did not have any beneficial effects [193]. Additionally, current use of calcium-channel blockers, such as verapamil, for 10 or more years was associated with higher risks of ductal and lobular breast cancer. Thus, more research is needed to confirm these findings and to evaluate potential underlying biological mechanisms of verapamil in cancer [198].

Concerning to itraconazole, it is a broad-spectrum anti-fungal agent that inhibits lanosterol 14-α-demethylase, an enzyme that produces ergosterol in fungi and cholesterol in mammals, being used to treat fungal infections and for prophylaxis in

immunosuppressive disorders [123, 199]. Studies have shown that this drug possesses antineoplastic activity and, also, has a synergistic action when combined with chemotherapeutic agents. Although showed promising anticancer activity in several types of cancer, its precise anticancer mechanism has remained elusive [123, 200]. However, it is known that it acts via several mechanisms to prevent tumor growth, including inhibition of the Hedgehog pathway, prevention of angiogenesis, decreased endothelial cell proliferation and cell cycle arrest. Also, in ovarian and breast cancer, *in vitro* studies confirm that Itraconazole inhibits P-glycoprotein, thus reversing resistance conferred by this efflux pump [123]. Figure 28 summarizes some proposed mechanisms of action of Itraconazole in cancer therapy.

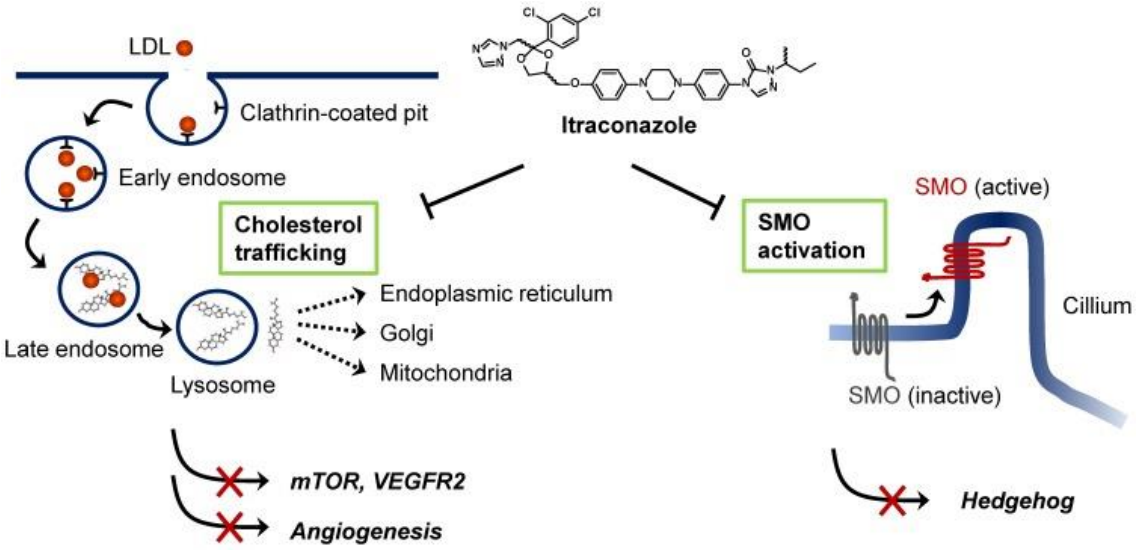


Figure 28 - Itraconazole blocks the cholesterol release from the late endosome/lysosomes, which leads to an accumulation of cholesterols, inhibiting the activity of mTOR and promoting VEGFR2 glycosylation. This prevents angiogenesis and promotes autophagy. Also, itraconazole inhibits Smoothened (SMO) activation in Hedgehog signaling, supressing Sonic hedgehog induced accumulation of SMO, leading to decreased cell proliferation and differentiation. Reproduced from Joong et al. [200].

In breast cancer, a pilot trial evaluated the pharmacokinetics of this drug when administered to 13 patients with metastatic breast cancer. The conclusions were that as the plasma levels of itraconazole increased, higher levels of thrombospondin-1, which inhibits angiogenesis, were detected [201]. Also, in another study, itraconazole inhibited MCF-7 and SKBR-3 cells proliferation via induction of cell death and G0/G1 cell cycle arrest [202].

Lastly, tacrine is a reversible inhibitor of acetylcholinesterase (AchEI), indicated to mild to moderate dementia of the Alzheimer's type [203]. Other pharmacological properties have been associated with this drug: blockade of potassium channels, inhibition of monoamine uptake, and inhibition of the monoamine oxidase. Since second-generation

AChEI have entered the market, tacrine is no longer in clinical use since 2013, due to severe adverse effects [204-206]. In cancer therapy, there are few studies with tacrine. However, there are reports that this drug, linked by a ten carbon chain to a melatonin part (tacrine-melatonin heterodimer C10), has an antiproliferative effect on MCF-7 breast cancer cells, causing autophagy enhancement accompanied by inhibition of mTOR and AKT pathways [207]. Also, other studies with tacrine derivatives reported that they can induce apoptosis and mitochondrial membrane depolarization in the human leukemic cancer cell line HL60 [208].

Despite these drugs have at least minimal evidence of some cancer activity, their mechanisms of action for this condition is not yet fully defined. It is also important to note that in this project, because drugs have been studied in the context of combination with 5-FU, the mechanism by which they act in the cell may be different from that which would be observed with the individual drugs, since the combination may act together in a different way of each of its individual components. Thus, a deep study of these mechanisms may represent an important step in breast cancer therapy, as well as in other types of cancer.

The data obtained in this work allowed to conclude that the three selected drug combinations have potential beneficial effects on breast cancer therapy. However, as referred above, the precise mechanisms of action of the drug combinations are not known. Nevertheless, with the obtained results along this research, it was clear that cellular viability was reduced in comparison with individual drugs of the combination, in the great majority of cases. This reduced cellular viability observed by MTT assay, as well as the cellular damage observed in cells treated with drug combinations, namely presence of damage in cell membranes and cellular vacuoles in the cytoplasm (Figure 27) may be a reflection of events such as cell death or cell proliferation arrest.

Dysregulated cell death is a common feature of many human diseases, including cancer [209]. There are different types of cell death, often defined by morphological criteria [210]. Nomenclature Committee on Cell Death proposes that, in order to be considered death, a cell should have, at least, one of the following molecular or morphological features: lost of the integrity of its plasma membrane; the cell, including its nucleus, has undergone complete fragmentation into discrete bodies, and/or its fragments have been engulfed by an adjacent cell *in vivo*. [210] There are two main distinct modalities of cell death: apoptosis and necrosis. Morphological features of each modality of cell death are presented in Table 8 [210, 211].

Table 8 - Morphological features of apoptosis and necrosis.

Apoptosis	***Necrosis***
Single cells or small clusters of cells	Often contiguous cells
Cell shrinkage and convolution	Cell swelling
Reduction of cellular and nuclear volume; nuclear fragmentation	Dissolution of the cell nucleus, reduction of cellular and nuclear volume; nuclear fragmentation
Intact cell membrane	Disrupted cell membrane
Cytoplasm retained in apoptotic bodies	Cytoplasm released
No inflammation	Inflammation usually present

Both forms of these cell death modalities are involved not only in several physiological but also in pathological conditions, as well as in the elimination of cancer cells following chemotherapy. A frequent phenomenon that occurs in most of the resistant cancers to chemotherapy is a loss of apoptosis. Thus, a therapeutic goal for cancer therapy is the induction of apoptosis in cancer cells. On the other hand, necrosis is not preferred as an anticancer strategy because this kind of cell death leads to the release of toxic components into the extracellular environment, triggering an inflammatory response and, further, necrotic cells are not efficiently cleared by macrophages. So, drugs that eliminate cancer cells primarily through apoptosis, without the involvement of necrosis, are good cancer drugs, mainly due to the lack of inflammatory responses subjacent to necrosis [212]. Consequently, assays that identify apoptosis-inducing agents are important in anticancer drug discovery.

An important aspect to mention is that MCF-7 cell line, the cell line mainly used in this work, lacks a functional caspase-3 gene product, being a caspase-3 deficient cell line. This caspase is important for many aspects in apoptosis, mainly chromatin condensation and DNA fragmentation [213]. However, these cells are still sensitive to apoptosis induction by several stimuli, including various DNA damaging agents, although death occurs in the absence of DNA fragmentation. Also, the distinct morphological features typical of apoptotic cells, such as shrinkage, are not evident in these cells [214, 215]. Despite being required for the acquisition of apoptotic morphology and DNA degradation, caspase-3 is dispensable for phosphatidylserine exposure, allowing the application of Annexin V/PI staining technique to these cells [216].

Besides acting primarly on cell death, drugs can also act on cell proliferation, being called by cytostatic drugs. These kinds of drugs do not kill neoplastic cells, but instead stop them from proliferating and growing, preventing the development of metastasis,

while cytotoxic agents directly lead to cell death. DNA damage, DNA polymerase inhibition, increased oncogenic signaling, oxidative stress and cytoskeletal inhibition comprise mechanisms by which cytostasis occur [217, 218]. However, a separation of these two classes of drugs does not always make sense, since cytotoxicity and cytostasis are not totally independent mechanisms. Frequently, prolonged cytostasis can induce necrosis or apoptosis, leading, subsequently, to cell death. Thus, it can be the initial step for the different mechanisms of cell death [218]. Another important aspect to consider is that the differences between these concepts also depend on drug's dose, phase of the cell cycle upon compound administration, and cellular context. Thus, exclusive cytostatic agents may not exist, once cytostasis is usually followed by either cytotoxicity or cellular escape from the stasis [217].

The results obtained by CFSE staining of cells indicated that all drug combinations showed a tendency to stop cell proliferation, whereas the results obtained by Annexin V/PI staining were not very conclusive. Possibly, the three drug combinations may act primarily in the arrest of cell proliferation, being 5-FU+itraconazole the combination of drugs that most affects cell proliferation. Besides the data obtained by CFSE staining of cells, the data obtained by the comparison of MCF-7 and MCF-10A cell lines also supported this conclusion, once the drugs had a much more pronounced effect on MCF-7 cells than MCF-10A cells, the last cells proliferating less and, thus, dividing less. Additionally, when comparing the results obtained for 48 and 72 hours, the cell viability is more reduced at 72 hours, sustaining the hypothesis that the drug combinations act on cell proliferation, once in 72 hours they have more time to proliferate (and, maybe, incorporate the drug), rather than in 48 hours (doubling time of 37 hours). As mentioned in the section 4.1, 5-FU acts mainly on S phase of cell cycle. So, an explanation about this tendency of drug combinations to stop proliferation may be, for example, a potentiation of the 5-FU action by the repurposed drugs or a promotion of the accumulation of 5-FU inside the cells. Further studies to test this hypothesis would be very relevant.

Even though not very conclusive (mainly because the small number of experiments), Annexin V/PI staining of cells showed that apoptosis was not observed at 3 hours of treatment, except in the case of 5-FU+itraconazole (126.8±23.9%), but was observed in the majority of cases (single drugs and combinations) at 8 hours, suggesting that with later times, the observation of apoptotic events is more clear. The same happened concerning to late apoptosis/necrosis, where an increase in these values was observed at 8 hours of treatment, comparing to 3 hours. Also, it was expected that in the conditions where apoptosis was verified at 3 hours (only verified with 5-

FU+itraconazole), the 8h trend would be an increase in late apoptosis, which was not verified, probably because this process took even longer than 8 hours to occur. The timeline of apoptotic-related biochemical events depends on a huge number of factors, such as cell culture conditions, the cell line used, drug concentration/ stimulus Intensity and exposure time [215]. In cell culture, apoptotic signs may be accomplished in less than two hours [219]. However, there are also studies that report that, in a general way, cultured cells induced to undergo apoptosis exhibit signs of apoptotic events within 5 to 10 hours [215].

Combining all results obtained by this technique, it was notorious that there was a greater tendency to cell death at 8 hours than at 3 hours, either by apoptosis or by necrosis. However, in this moment, it is not possible to conclude that a certain combination acts via apoptosis and/or necrosis, being absolutely crucial to increase the number of independent experiments in order to obtain more sustainable results that can allow better conclusions, namely in the case of Annexin/PI staining of cells. Also, concerning to this particular assay, it would be useful to test in different time points, namely later times. Because of the short duration of this dissertation, it was not possible to do, keeping in view for the near future. Additionally, it is important to refer that this assay was attempted more times throughout this work, but always with several precautions that made it impossible to obtain results, suggesting the need to optimize this technique in this particular case. Particularly, different flow cytometers were used. In some cases, the cells aggregated a lot, which did not allow their analysis. This aggregation was most likely due to the calcium present in the annexin buffer, crucial for annexin, which depends on it, so that it can not be removed [220]. In other cases, in order to don't use trypsin for cell detachment from the monolayer, mechanical detachment with successive and vigorous *up and down's* was used, which killed the cells and, once again, did not allow its analysis. Lately, trypsin was used, situation that allowed the detachment of the cells without killing them.

Crossing data from the proliferation and cell death assays, the seemingly more plausible conclusions are that drug combinations act essentially on pathways related to cell proliferation arrest. After this proliferation arrest, the cells tend to accumulate damage (Figure 27) and, consequently, to die. Hence, it is plausible that the highest cell death values were obtained for 8h. This increase is likely to continue with increasing drug exposure time.

Another factor that should be considered in this work is the concentration of the drugs used in the majority of the experiments, which was based on the concentration-effect

curves and, more specifically, on the IC_{50} values obtained. A main goal of fitting the dose-response curve is to determine the value of the IC_{50}: the concentration of an inhibitor, where the response is reduced by half. Two definitions of IC_{50} exist, namely the absolute and the relative IC_{50} (Figure 29). Relative IC_{50} is the most common and relevant definition, defined as the concentration of inhibitor that is required to bring the curve down to point half way between the top and bottom plateaus of the curve. On the other hand, the absolute IC_{50} is defined as the inhibitor's concentration that provokes a response halfway between a compound that is known to no inhibition of the response, and the maximal concentration of a standard inhibitor that provokes 100% inhibition of that response [221]. This concept correlates with drug potency, an expression of the activity of that drug in terms of the concentration required to produce a defined effect. Thus, higher the potency, the lower the concentration required to produce the effect and the lower the IC_{50} value [222].

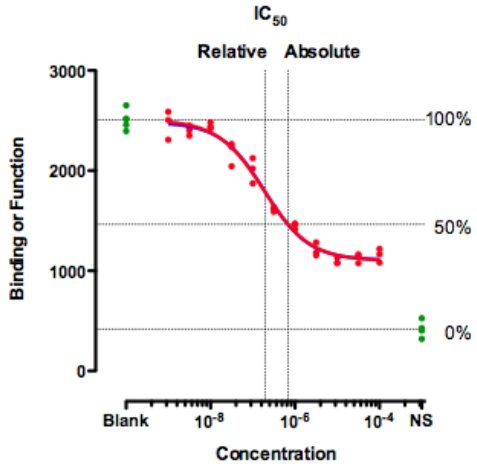


Figure 29 - IC_{50} can be defined by two ways: relative IC50 and absolute IC50. Blank represents the situation where there is no inhibitor, having no response, and NS represents the situation where there is 100% inhibition. Reproduced from www.graphpad.com [221].

The IC_{50} concept is not so clear. For example, it would be ambiguous if the values that define 100% (top plateau of the curve) and 0% (bottom plateau of the curve) are not clearly defined. Thus, if those plateaus are not well defined, the IC_{50} will be very uncertain [221, 223]. An alternative to solve this problem may be normalizing the data in order that responses vary between 0 and 100, forcing the bottom and top plateaus to equal 0 and 100, respectively. However, a normalized model should be applied only when the values that define 0 and 100 are correctly determined. Thus, when the data is normalized between 0 and 100, the relative IC_{50} corresponds to 50% on the Y axis [221, 223]. In this study, to possibilitate the calculation of IC_{50} values of the four drugs, this value was calculated taking into account this type of normalization, since no perfectly defined dose-response curves with defined plateaus, where both maximal and minimal activity were very explicit, were obtained for all the drugs. It would be

necessary to study a broader range of concentrations, as well as repeat the number of experiments, in order to perfectly define the maximum and minimum activity values of each drug, which was not possible due to the short duration of this dissertation. Anyway, the obtained value for the IC_{50} of the reference drug, 5-FU, was consistent with that obtained in studies of this drug in MCF-7 using the same methodology (MTT), justifying the exclusion of the obtained values using Neutral Red methodology [224]. Thus, with the obtained IC_{50} values, Itraconazole appeared to be the most potent drug and Tacrine appears to be the less potent drug, since it has the lowest and highest IC_{50} value, respectively. However, only with these data is it not entirely correct to state which drug is the most potent because of the above mentioned aspects. It is also important to note that, in general, the IC_{50} value that is obtained for a given drug greatly varies depending on the methodology by which it is determined (Neutral Red or MTT, as exemplified), the type of cells in which it is studied and the conditions of culture [225].

Another study carried out in the scope of this work was the evaluation of the transition from an epithelial to a mesenchymal state (EMT) of possible drug resistant cells. EMT is defined as the process by which epithelial cells acquire a mesenchymal phenotype, with fibroblast-like properties and reduced intercellular adhesion and increased motility. This is an important process in the normal development but, also, plays a critical role during tumor progression and malignant transformation, leading to the acquisition of invasive and metastatic properties in cancer cells [226].

Studies have reported that the acquisition of 5-Fluorouracil resistance induces epithelial-mesenchymal transitions through the Hedgehog signaling pathway in HCT-8 colon cancer cells [169]. Also, EMT was associated with acquired resistance to 5-FU in HT-29 colon cancer cells [227]. Additional studies also proved that chemoresistance to 5-FU induces epithelial-mesenchymal transition via up-regulation of Snail in MCF7 human breast cancer cells [228]. Thus, in order to study this transition of state, the presence of e-cadherin, cytokeratins AE1/AE3 and vimentin was studied.

The downregulation of E-cadherin is a molecular signature of EMT. This molecule is an adhesion molecule that is present in the plasma membrane of most epithelial cells. It is a calcium dependent cell-cell adhesion molecule with important roles in epithelial cell behavior, tissue formation, and suppression of cancer, inhibiting invasion and metastasis, frequently repressed or degraded during transformation [226, 229].

Cytokeratins are, also, markers of an epithelial phenotype, found in the intracytoplasmic cytoskeleton of epithelial tissue. This is a protein with an intermediate filament made of keratin, having important roles in cell differentiation [230, 231]. Particularly, cytokeratin AE1/AE3 is a mixture of two different clones of anti-cytokeratin monoclonal antibodies, AE1 and AE3, that detect high and low molecular weight keratins. AE1 detects the high molecular weight cytokeratins 10, 14, 15, and 16 and the low molecular weight cytokeratin 19. On the other hand, AE3 detects the high molecular weight cytokeratins 1, 2, 3, 4, 5, and 6, and the low molecular weight cytokeratins 7 and 8. By combining these two reagents, a single reagent with a broad spectrum of reactivity against a huge diversity of cytokeratins is obtained [231].

Relative to vimentin, it is ubiquitously expressed in normal mesenchymal cells, being known to maintain cellular integrity and providing resistance against stress. Overexpression of vimentin in cancer correlates well with accelerated tumor growth, invasion, and poor prognosis [232].

E-cadherin's downregulation is an important leading event for EMT and is considered a hallmark of this transition, being one of the most reliable markers of EMT. This molecule has an essential role in epithelial homeostasis, thus, its downregulation can lead to decreased expression and/or organization of additional epithelial markers. Concomitantly, increased expression of mesenchymal proteins (such as vimentin), as well as of extracellular matrix remodeling enzymes occurs together with actin cytoskeleton reorganization [233]. Thus, this seems to be a process in which several events occur concomitantly.

Analyzing the obtained images by immunocytochemistry, it was clear that no EMT was observed, in none of the cases. However, in the particular case of 5-FU combined with verapamil, there seemed to be a very week staining of e-cadherin. The reason for this finding is unknown, since there are no studies with this drug combination regarding EMT in cancer cells, particularly in breast cancer cells. This may be related with the mechanism of action underlying the combination, since with isolated drugs this loss of e-cadherin expression was not observed. Thus, this combination may have some action on e-cadherin or even produce a transition from epithelial to mesenchymal state, first observed by a loss of e-cadherin, maybe not fully observed due to a short time of drug action. It could be thought that 48 hours was not enough time for the transition to the mesenchymal state to occur. However, studies show that this transition begins to be observed in earlier times. For example, in a study using the MCF-7 cell line to study

whether EGF induced EMT, it was found that the loss of e-cadherin, as well as increased expression of vimentin, began to be observed about 4-8 hours after the stimulus [234]. Also, another study with MCF-7 cells reported that EMT was observed when analyzed after 48 hours of the induction of the stimulus, fibronectin [235]. However, in another study, EMT occurred on day 7 after the stimulus, in this case with granulocyte macrophage colony-stimulating factor, in colon cancer [236]. Thus, it is notorious that the observation of EMT depends on the stimulus, as well as on the cell lines in which the study is taking place. In this work, the analysis of the eventual transition to a mesenchymal state was in a time of 48 hours after the treatment with the drugs. However, the analysis 48 hours after the stimulus, may not be the ideal time to observe the transition to a mesenchymal state. An interesting future approach could be the study of this transition at different times.

Another important finding is the existence of two types of EMT induction: transient and stable. For example, growth factors usually induce a transient EMT after short treatment (few hours to several days). On the other hand, stable EMT can be induced by, for example, transcription factors of the Snail zinc finger family, double zinc fingers Zeb or bHLH factors, that act as a direct transcriptional repressor of E-cadherin and other epithelial genes and mediate a complete EMT gene program [233]. These mechanisms are still not fully understood. In future work, it would be interesting to see if the used drugs induce EMT in a transient or stable way, evaluating this condition at different times and trying to understand the mechanisms by which these drugs (and, in particular, combinations) induce a mesenchymal state, if this occurs.

To finish all this work, a conclusion about the relationship between the two drugs of each combination would be important: synergy, antagonism or addictivity. When the combined effect of two drugs is greater than that predicted by their individual potencies, the combination is said to be synergistic [237]. As referred in the Introduction section, there are three popular classes of models that define additivity and, thus, synergy and antagonism: Highest Single Agent model, Loewe Additivity model and Bliss Independence model. These models have been developed based on different assumptions about the expected additive effect of the combination and, each, have limitations and advantages [54]. Table 9 presents a summary of the limitations and advantages of each of these three methodologies.

Table 9 - Limitations and advantages of three popular classes of models that define additivity: Highest Single Agent model, Loewe Additivity model and Bliss Independence model.

Methodology	*Advantages*	*Limitations*
Highest Single Agent	More adapted to practical limitations [54]	A drug combined with itself can produce an excess over highest single agent [54]
Loewe Additivity	Potentially the most accurate model [238]. Enables to complement the algebraic analysis with a graphical approach, isobologram analysis [238]	Accurately estimated dose-response curves [239]. Unusable when a dose-response curve is not available or difficult to obtain [239]. Computationally challenging [239], and large amount of data required [240]
Bliss Independence	Maintains high accuracy with an increased number of drugs [241]. Fewer restrictions than Loewe additivity [239]	Assumes that the drugs have exponential dose–effect curves [240]. Depend upon knowledge of mechanisms of action [240]

Thus, analyzing each presented methodology, the Highest Single Agent approach appears to be the more adapted to practical difficulties. In spite of having its limitations, it can provide credible and enough evidence of positive drug combination effect. However, for more rigorous classifications, the Loewe additivity and Bliss independence models are more convenient [240]. It is important to highlight that analysis of drug combinations should be adapted to each level of the whole research process: for the discovery step, the combined application of methods, such as the methods described, may be useful to identify good candidates for research. For more advanced studies, such as preclinical studies, the more precise determination of the effects of the drug combination should be performed. Here, dose–effect curves should be well characterized and a dose–effect approach based on Loewe Additivity appears the more suitable. However, in situations where defined dose–effect curves are not available, the Highest Single Agent approach is appropriate [240]. Even applying more rigorous methods, such as the Loewe additivity model, is important to note that is extremely difficult to conclude about synergism and find optimum ratios of drugs in the combination. For example, in vitro experiments identified a strong antagonism between irinotecan and cisplatin when they were administered at a 1:1 ratio, but in a ratio of 4:1, synergistic effect was observed. Also, the combination of irinotecan and floxuridine was synergistic at an equimolar ratio but was strongly antagonistic at a 10:1 ratio [242].

In this work, since were used drugs in which the concentration-effect curves were not precisely defined and, in some cases, this difficulted the precise calculation of IC_{50} values (only achieved after normalization of the values between 0 and 100%, as referred above), it was chosen the use of the simpler synergy detection method mentioned above: HSA. Thus, it was considered that the drug combination was benefecial (synergic), when presented more advantageous effects comparing with the more effective individual drug of that combination (the highest single agent). Additionally, another factor that made unused the more robust models, such as Bliss independence, was the fact that the repurposed drugs did not have the mechanism of action in cancer fully known and, therefore, it is not known whether these drugs act in the same or different pathways as 5-FU. Thus, for all these reasons, it would not make sense to apply more complex methods of synergy detection. For that purpose, further studies would have to be done.

To conclude, by integrating all the obtained results, it was notorious that all drug combinations showed a tendency to be more effective than the most effective drug of each combination and that, probably, with an increase in the number of independent experiments, this observed tendency would be even more marked and statistically significant in all the cases. The best results were obtained for the 72 hours of treatment and, in the majority of cases, there were no accented differences between the different concentrations tested, as described in the Results Section.
Although the combination of 5-FU and verapamil was the one that led to the lowest cell viability values (approximately 12%, at 72 hours in concentrations of 50 μM of each drug in the combination), the collective of all results pointed out that the combination of 5-FU and itraconazole was the most promising combination. The results obtained by MTT reduction assay support this evidence (both at 48 hours and at 72 hours, regardless of the concentration used, except at 72 hours, at concentrations of 50 μM of each drug in the combination, in which case 5-FU+verapamil was highlighted). Furthermore, analyzing the IC_{50} values obtained, itraconazole was the drug that presented the lowest value, being apparently the most potent. Additionally, CFSE staining of cells showed that 5-FU combined with itraconazole was the drug combination that promoted more elevated values of MFI, indicating a more pronounced proliferation arrest. Studies in MCF-10A cells also supported this hypothesis, since itraconazole was the only drug in which both combined with 5-FU and used individually, showed significantly different effects relative to the control, which may indicate increased toxicity. In addition to all this, it is still important to mention that concerning the three repurposed drugs used in this work, itraconazole is the one that is most

studied in the field of cancer, showing more promising results. Additionally, itraconazole was the drug that was used in a lower concentration and, as known, a successful therapy is a therapy in which efficacy is achieved with the lowest possible drug concentration.

Thus, all the three drug combinations seemed to be promising in breast cancer therapy, even though evidences pointed itraconazole, and particularly its combination with 5-FU, a very relevant object of study for the therapy of breast cancer and, possibly, other types of cancer.

5. Final Conclusions and Future Perspectives

Cancer is a complex group of diseases that involves several pathways and different molecules, being very challenging to treat. Oncological therapy, which includes breast cancer therapy, is increasingly being studied, with therapeutic strategies, such as drug repurposing and drug combination, being largely adopted.

In this project, the combination of drugs with potential to be repurposed (verapamil, itraconazole, tacrine) with a reference chemoterapeutic drug (5-FU) showed advantages over the individual drugs, mainly by arresting cell proliferation and decreasing cellular viability. Thus, the drug combinations showed evidence to be promising for the treatment of breast cancer. However, in order to understand the mechanisms by which these combinations act, additional studies are needed.

In the future, it is crucial to study different and longer times of drug exposure, to increase the range of concentrations applied and the number of independent experiments. It would be interesting to apply more advanced methods of synergy detection, such as the Loewe method, as well as a future study on more resistant breast cancer cell lines (such as the triple negative line MDA-MB-231) and, also, on different cancer cell lines corresponding to other types of cancer. *In vivo* studies for application in human therapy would be extremely important, in a more advanced line of work. However, for all of this to be achieved, it is absolutely necessary a better knowledge about the mechanisms of action underlying these drug combinations, with a special focus on 5-FU combined with Itraconazole, since it was, globally, the most promising drug combination.

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