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Modelos *in vitro* para rastreio de fármacos

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“Out of suffering have emerged the strongest souls;
the most massive characters are seared with scars.”
Khalil Gibran

Dedication

To my parents, sister, and girlfriend.

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Resumo

As doenças do foro oncológico são certamente o principal foco de investigação na área da biologia e da farmacologia. Por conseguinte, todos os anos são publicados milhares de artigos relativos a este tema em revistas da especialidade. O desenvolvimento de novas terapêuticas para tratamento do cancro é também do interesse das empresas farmacêuticas. Contudo, até aos dias de hoje, o custo associado ao desenvolvimento de fármacos continua muito elevado. Deste modo, as instituições académicas e as grandes empresas farmacêuticas têm vindo a estabelecer colaborações que visam a diminuição destes custos, nomeadamente os que incluem as despesas dos ensaios pré-clínicos.

Atualmente existem vários ensaios de viabilidade celular em forma de *kit* que são usados durante os ensaios pré-clínicos. Contudo, a maioria destes *kits* são dispendiosos, o que tem despoletado uma necessidade crescente de desenvolver novos ensaios, que permitam avaliar o efeito terapêutico de novos fármacos com maior celeridade e menor custo.

No presente estudo foi investigada a aplicação do Cristal Violeta (CV), um corante pouco dispendioso, para determinar a viabilidade celular de células cancerígenas do colo do útero e da mama. Uma vez que não existe qualquer ensaio padronizado e/ou otimizado para o uso deste composto para a determinação da viabilidade celular descrito na literatura, o principal foco deste trabalho passou pela otimização de um protocolo utilizando o CV para futura avaliação em larga escala (HTS) de fármacos.

Durante a otimização do protocolo de CV para determinação da viabilidade celular foram considerados os seguintes pontos: fixação ou ausência de fixação das células e a concentração da solução de CV utilizada para marcar as células. Os resultados obtidos demonstraram que há um aumento de absorbância do CV proporcional ao aumento do número de células para todas as variações do ensaio de CV. O protocolo em que se procedeu à fixação das células e em que foi usada uma solução mais concentrada de CV foi escolhido como sendo o protocolo otimizado, *i.e.*, mais adequado para determinar a viabilidade celular, uma vez que este demonstrou maior sensibilidade, precisão e menor variabilidade no rácio sinal/célula nos resultados obtidos.

O ensaio otimizado de CV também permitiu avaliar a eficácia de um fármaco (Doxorubicina (DOX)) de forma semelhante a outros ensaios amplamente descritos na literatura, tal como é o caso do ensaio do brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT) e o da Resazurina. Foi ainda verificado que o ensaio otimizado de CV pode ser aplicado para HTS de novos fármacos.

Em conclusão, sendo o CV um composto de baixo custo, este poderá possivelmente ser usado futuramente na investigação de fármacos para tratamento do cancro.

Palavras-chave

Cristal Violeta, desenvolvimento de fármacos, ensaios *in vitro*, viabilidade celular.

Resumo alargado

Os tratamentos anticancerígenos disponíveis na atualidade, nomeadamente a cirurgia, quimioterapia e radioterapia, não apresentam a eficácia terapêutica desejada. Acrescendo a este facto, a ocorrência de efeitos secundários associados à administração destas terapêuticas é bastante frequente. Estes efeitos incluem fadiga, perda de cabelo, perda de peso, náuseas e vómitos, com consequências nefastas para a já débil saúde dos doentes oncológicos. Estes efeitos adversos contribuem para as elevadas taxas de mortalidade associadas às doenças cancerígenas. Devido a estas limitações, o desenvolvimento de novos fármacos para o tratamento do cancro é fundamental. No entanto, este é um processo extremamente moroso e dispendioso. De facto, os gastos associados à investigação de moléculas terapêuticas e à sua introdução no mercado possui um custo estimado em cerca de 1,395 mil milhões de dólares, dos quais 430 milhões sucedem de gastos associados à fase pré-clínica.

Deste modo, os investigadores têm procurado desenvolver métodos *in vitro* que permitam validar em larga escala – *high-throughput screening* (HTS) - a eficácia de novas moléculas terapêuticas de forma a acelerar o processo de desenvolvimento de fármacos e ainda reduzir os custos associados à fase pré-clínica.

O Cristal Violeta (CV), um composto pertencente à classe dos triarilmetanos, tem sido usado na área da microbiologia há largos anos. Nas últimas décadas, este composto tem sido também utilizado para corar células eucarióticas com o objetivo de avaliar vários parâmetros, tais como a capacidade das células de aderir a biomateriais, formar colónias, migrar e proliferar. Este composto já foi também descrito para determinar a viabilidade de células. Nos ensaios de viabilidade celular, o CV é usado como um corante específico para células vivas. O CV é então extraído destas células e a absorbância da solução resultante medida através de espetroscopia ultravioleta-visível (UV-vis). A absorbância da solução é proporcional ao número de células viáveis.

No entanto, na literatura não está disponível um protocolo otimizado e padronizado para a avaliação da viabilidade celular através do CV. Foi identificada então a necessidade de otimizar um protocolo experimental que permita a utilização do CV na determinação da viabilidade celular. A concretização deste objetivo irá levar a que os investigadores tenham disponível um novo método menos dispendioso, mas que permite a obtenção de resultados tanto ou mais fiáveis que os obtidos através de outros ensaios de viabilidade celular, indo assim de encontro com as necessidades da indústria farmacêutica.

No presente estudo, o protocolo de viabilidade celular usando o CV foi otimizado através da análise de vários fatores que podem influenciar o ensaio, nomeadamente a possível fixação das

células com paraformaldeído (PFA) e a variação da concentração da solução de CV usada para marcar as células. Foram usadas duas linhas celulares cancerígenas como modelo celular, uma do colo do útero (HeLa) e uma da mama (MCF-7). Estas linhas celulares foram escolhidas não só devido à elevada prevalência destes cancros na população feminina em Portugal e no mundo, mas também devido ao facto destas linhas celulares serem das mais recorrentes para a avaliação do potencial de fármacos *in vitro*.

Para determinar qual dos protocolos de CV é o mais indicado para determinar a viabilidade celular, vários parâmetros foram estudados, nomeadamente o declive das retas obtidas a partir da regressão linear (da qual se inferiu a sensibilidade do ensaio), o coeficiente de determinação (r^2) (do qual se deduziu quão bem as retas obtidas se ajustavam aos resultados obtidos - linearidade, resultando num rácio sinal/célula mais constante), e o coeficiente de variação (do qual se retiraram elações quanto à precisão do ensaio). Foi concluído que o ensaio de CV que permite a obtenção de resultados mais precisos e mais sensíveis em ambas as linhas celulares foi aquele que envolveu a fixação das células e a sua coloração com a solução de 0,5% CV. Verificou-se ainda que este protocolo resultava num r^2 mais elevado, significando que existe um rácio sinal/célula mais constante, em comparação com os outros protocolos de CV. Com base nestes dados, foi possível concluir que o ensaio de CV otimizado é aquele no qual as células são fixadas e coradas com uma solução de CV de 0,5%.

O ensaio de CV otimizado foi ainda comparado com dois ensaios amplamente utilizados na determinação da viabilidade celular – o ensaio do brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT) e da Resazurina. Verificou-se que, entre os três ensaios avaliados, os resultados do ensaio da Resazurina possuem uma maior sensibilidade, enquanto que os do ensaio do CV possuem uma maior linearidade. O ensaio de MTT demonstrou baixa sensibilidade em relação aos restantes ensaios, e uma linearidade ligeiramente mais reduzida que o ensaio de CV.

Após a otimização do método de coloração usando o CV, este ensaio foi usado para quantificar a ação citotóxica de um fármaco anticancerígeno (DOX). Os resultados para o IC_{50} da DOX obtidos pelo método otimizado de CV, foram semelhantes aos valores obtidos pelos ensaios de MTT e Resazurina. Posteriormente, procedeu-se à avaliação da utilização do método do CV em HTS de fármacos, tendo sido verificado que o ensaio de CV pode ser usado em HTS.

Os resultados obtidos neste estudo permitiram concluir que o protocolo aqui desenvolvido pode ser usado para avaliar a eficácia terapêutica de fármacos. É esperado que o CV possa ser utilizado pela indústria farmacêutica em estudos de larga escala de desenvolvimento de fármacos num futuro próximo, respeitando os critérios definidos pelas agências nacionais (Infarmed) e internacionais (European Medicine Agency, Food and Drug Administration) que regulamentam o desenvolvimento de novos fármacos.

Abstract

Cancer is the main target of research in the field of biology and pharmacology. Every year, thousands of cancer-related articles are published in specialized scientific journals. The development of new anticancer therapies is also one of the main interests of pharmaceutical companies. However, academia and big pharma have recently set their sights on cutting expenses related to drug development through co-operation protocols. Currently, various cellular viability assays (kits) are available in the market. However, most of these kits are expensive. To fulfill this need, there is a crescent demand for new protocols, such as cellular viability and cytotoxicity assays, that can be used for drug development in a faster and cheaper manner.

In the present study, the application of Crystal Violet (CV), a compound that is relatively cheap, to determine the cellular viability of breast cancer and cervical cancer cells was evaluated. As no uniform and/or optimized CV cellular viability assay has been described in literature, the main focus of this work was the optimization procedure of a CV protocol, and its application for high-throughput screening (HTS) of new therapeutics.

Two points of interest for the optimization of the protocol were considered: the possible fixation of cells and the concentration of the CV solution used for cell staining. The obtained results show there is an increase in absorbance proportional to the number of seeded cells for all CV protocol variations. The optimization procedure was successful, as it was shown that fixing and staining cells with a CV solution of higher concentration increased sensibility and decreased the variance of the signal/cell ratio in comparison with other tested protocols.

It was also shown that the optimized CV assay may also be used as an alternative method for drug efficacy screening to other cellular viability assays widely described in literature, such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Resazurin assays. It was also observed that the optimized CV assay may be applied for HTS of new anticancer drugs.

Overall, as CV is a compound that is cheap to acquire, it may be used during anticancer drug development in the future.

Keywords

Crystal Violet, cellular viability assay, drug development, *in vitro* assays

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List of acronyms

2D	Bi-dimensional
3D	Tri-dimensional
3T3	Mouse fibroblast
ADP	Adenosine diphosphate
APH	Acid Phosphatase
ATP	Adenosine triphosphate
CDC	Centers for Disease Control and Prevention
CV	Crystal Violet
DGS	*Direcção-Geral de Saúde*
DMEM-F12	Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12
DMEM-HG	Dulbecco's Modified Eagle's Medium with high glucose
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin hydrochloride
dsDNA	Double-stranded deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetate
EMA	European Medicines Agency
EU	European Union
FBS	Fetal bovine serum
FDA	Food and Drug Administration
HeLa	Human cervix adenocarcinoma
HTS	High-throughput screening
IC_{50}	Half-maximal inhibitory concentration
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride
KU-7	Human bladder cancer
LDH	Lactate Dehydrogenase
MCF-7	Oestrogen-dependent human breast adenocarcinoma
MG-63	Human osteosarcoma-derived
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide Adenine Dinucleotide
NCHS	National Center for Health Statistics

OIER	Oxidized intermediate electron receptor
PBS	Phosphate-buffered saline solution
PFA	Paraformaldehyde
PI	Propidium Iodide
PPS	Phosphatidylserine
r^2	Coefficient of determination
RIER	Reduced intermediate electron receptor
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TB	Trypan Blue
US	United States
UV-vis	Ultraviolet and visible light
WHO	World Health Organization
WST	Water-soluble tetrazolium
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Chapter I

Introduction

1. Introduction

The currently available anticancer therapies (surgery, chemotherapy and radiotherapy) are known for their limited effectiveness and also by their associated side-effects [1]. Therefore, there is a huge demand for new therapeutics specific for the treatment of this pathology, making it one of the main targets of pharmaceutical companies. However, the development of new pharmacological agents is a long and costly procedure [2]. During the second phase of drug development (Preclinical Research), a large catalog of compounds is tested to check for any beneficial effects on the treatment of cancer [3]. Those compounds showing the most promising results are selected to be further evaluated in other phases of drug development (Clinical Trials).

1.1. Cancer

Cancer is usually defined as a collection of related diseases characterized by cells displaying uncontrolled proliferation [4]. This disease is one of the main health concerns affecting the society of the 21st century [5]. The latest figures published by the GLOBOCAN project of the World Health Organization (WHO) show that approximately 14.1 million new cancer cases and 8.2 million cancer-related deaths took place worldwide, during the year of 2012 [6]. According to the latest statistics published by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC) of the United States (US), over 595 thousand individuals died due to cancer during the year of 2015 [7]. In the European Union (EU), the number of cancer-related deaths is predicted to tally at over 1.3 million deaths during the year of 2017 [8]. The latest statistics show that, in the year of 2012, a staggering 3.4 million new cancer cases were diagnosed in Europe, while almost 1.8 million individuals perished due to oncologic diseases [6]. In 2035, it is expected that nearly 4.9 million people will be diagnosed with cancer, and approximately 2 million cancer-related deaths will occur in Europe (including countries other than those belonging to the EU) [9]. In Portugal, the latest report from the *Direcção-Geral de Saúde* (DGS) estimates that over 50 thousands of new cancer cases were diagnosed in Portugal during the year of 2015, and predicts an increase in the number of new cancer cases during the year of 2035 to over 60 thousand individuals [10].

1.1.1. Breast and cervical cancer: overview and available therapeutics

Breast and cervical cancers are two of the most frequent malignant neoplasms that affect women worldwide. In the year of 2017, 29.6% of total new cancer cases and 14.4% of total cancer-related deaths are estimated to be caused by female breast cancer, in the US [11]. According to the DGS, in Portugal, 1.5 thousand individuals died because of breast cancer in the year of 2014 [10]. Cervical cancer was estimated to be found in 13 thousand new individuals and causing death in 7 thousand women in the US, during the year of 2012 [12]. It is also predicted to reach over 12 thousand new female cases and over 4 thousand deaths for the US,

during the year of 2017 [11]. In the annual report performed by the DGS, it was stated that 210 individuals died due to cervical cancer during the year of 2014 in Portugal [10].

The type of therapy employed for the treatment of breast cancer depends on the size of the tumor, as well as the amount and type of proteins that the tumor cells express. For the treatment of breast cancer, partial or full surgical removal of the affected breast tissue is usually the first-line treatment. Radiation therapy is often deployed after removal of the affected breast, using a dosage usually ranging from 45 to 50Gy [13]. Systemic chemotherapy may be used to diminish the tumor size prior to surgical removal or to decrease the risk of recurrence. The drugs to be used depend on the protein expression profile of the breast tumor, as changes in protein expression modulate the sensitivity of the cancer cell to certain therapies. Common drugs for breast cancer include endocrine modulators (e.g., Tamoxifen), which are often used to treat estrogen receptor-positive breast tumors (cells whose activity is influenced by estrogens), while anthracyclines (e.g., Doxorubicin (DOX)) are more widely used for triple-negative breast tumors (unresponsive to endocrine modulators) [13].

For cervical cancer, the first therapeutic approach used is also the surgical removal of cervical cancer [14]. Radiation or systemic chemotherapy treatments may also be administered in cases where there is a risk of recurrence. The recommended radiation dosage is 80 to 90Gy in early-stage cervical cancer [14]. In the chemotherapeutic treatment approach of cervical cancer several compounds, like Cisplatin (alone or together with Gemcitabine) may be used [14]. Metastatic tumors derived from cervical cancer are normally treated with other chemotherapeutic agents, such as Docetaxel, Gemcitabine, Irinotecan, polyethylene glycol-liposomal DOX and Topotecan [14, 15].

1.2. Drug development

Drug development encompasses a range of biological, biomolecular and biopharmaceutical studies that aim to develop new therapeutic agents that are effective in the treatment of diseases affecting humans. Developing a new pharmaceutical agent is a long and very costly process [16, 17]. In the case of anticancer drugs, latest estimates state that the full process of developing a single new drug takes 8.7 years on average and has an estimated associated cost of approximately 1.4 billion US dollars [2, 16]. The drug development process comprises 4 phases (Figure 1).

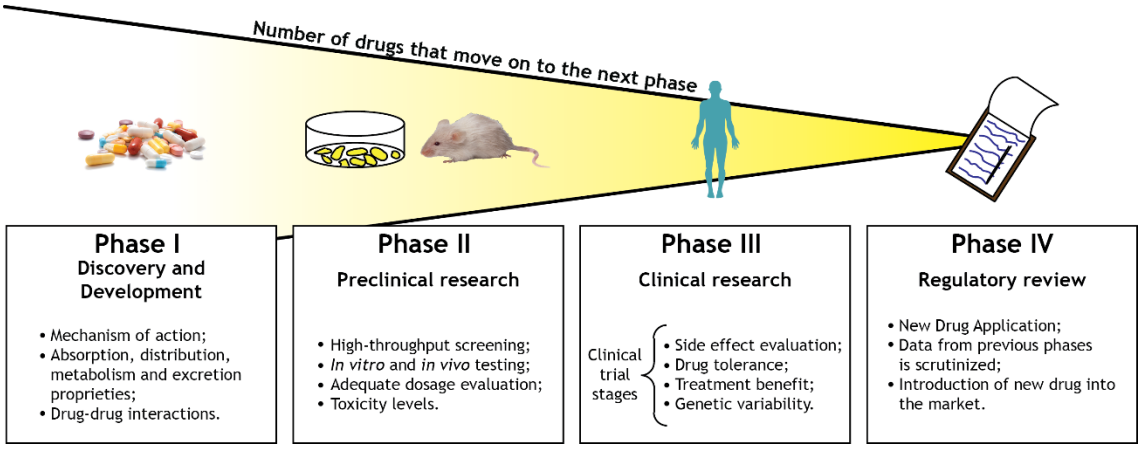


Figure 1. Overview of the phases (I, II, III and IV) of drug development according to the United States' Food and Drug Administration (FDA).

Phase I (Discovery and Development) is focused on compound synthesis and the evaluation of the biomolecular and biopharmaceutical proprieties of various drug candidates to be applied in the treatment of a target disease. Initially, specialized software is used to design new molecules for cancer therapy, in a process known as *in silica* drug development [18]. The prior knowledge of metabolic pathways in the organism as a whole, as well as bio-signaling pathways that are altered in cancer cells and other tumor-associated cells, is of crucial importance for this first phase of drug development [18]. The absorption, distribution, metabolism and excretion characteristics of the drug are also taken into account during this first phase, allowing researchers to optimize the molecular structure of the therapeutic molecules [19, 20]. For instance, drugs used in the field of neuro-oncology must be capable of bypassing the blood-brain barrier, which requires extensive optimization of the design of the molecules [21].

During phase II (Preclinical Research), *in vitro* high-throughput screening (HTS) studies are undertaken to determine which compounds possess the desired effect, focusing on their possible toxicity to cancer cells. *In vitro* studies are usually performed in bi-dimensional (2D) homotypic cell cultures, where one cell line is seeded on a flat surface to form a single layer of cells [22]. However, these models were still not ideal, since monolayer culture of cells cannot represent the tri-dimensional (3D) structure of *in vivo* human tumors and their drug resistance capabilities are diminished in comparison [23]. Due to that, 3D *in vitro* models, such as tumor spheroids, replicate more accurately the structure and physiology of tumor tissue [25, 33, 34]. However, currently available production techniques used to perform cell culture in 3D are still plagued by higher costs, diminished reproducibility and lack of adoption by pharmaceutic businesses [23]. Hence, despite all their advantages, 3D cellular culture models remain as a second-line model for drug testing during the preclinical studies.

After the promising therapeutic molecules are characterized in *in vitro* 2D cultures, researchers perform *in vivo* studies by using animal models (e.g., mice, dogs, pigs and fishes) to investigate the appropriate drug dosage levels and delivery methods [20, 23]. The molecules are only able to enter into clinical trials after *in vivo* validation of their efficacy and safety.

Phase III (Clinical Research) comprises of several stages during which drugs are tested in an increasing number of human individuals [24]. The first stage is usually performed in healthy volunteers, with the objective of gathering as much data as possible about how the drug interacts with the human body. Different dosage regimens are applied to patients, based on data gathered during *in vivo* assays. After administration, the pharmacokinetic and pharmacodynamic profile of the drug is evaluated, and other possible side-effects are evaluated. The drug concentrations administered to patients are also frequently adjusted to find the best formulation and dosage for the intended therapeutic effect. The second stage of the clinical phase is performed on hundreds of patients suffering from the target pathology, to assess if the therapeutic effect of the new drug is better in comparison to currently available anticancer therapies. In the third stage, a few thousand patients are enrolled in drug evaluation to provide further efficacy data. Information about rarer side effects that were not detected during the previous phase due to the small sample size (less genetic variability) or shorter study length is also acquired during this stage.

Phase IV (FDA Review) constitutes a critical step since it is the last stage before the possibility of the drug to enter into the market. A New Drug Application is submitted to the regulatory agency, and all the data obtained in Phases I to III, as well as its proposed label, possible drug abuse information, drug patent information and other study data are evaluated. Ultimately, additional studies may be further requested, prescribing information may be added, or the drug may be accepted as submitted.

After approval, the commercially available drugs are constantly monitored by regulatory agencies such as the FDA (in the USA), European Medicines Agency (EMA) (in the EU), and Infarmed (in Portugal), as all the information obtained during development may not be enough to fully understand possible consequences of the long-term effects of drug administration.

1.2.1. *In vitro* HTS of drugs during the preclinical phase

In vitro HTS is performed for the screening of hundreds or even thousands of compounds in parallel to find the best candidates for the treatment of a disease [25], being a selection approach to check whether compounds that should in theory be effective in killing cancer cells, do possess that ability in practice.

In HTS, a library of compounds is tested all at once in one or several plates previously seeded with the cells of interest [25]. Each compound is tested with a very low number of replicates (n=1 to n=3) to reduce the costs of producing the compounds to be tested. Therefore, the assay results should be highly accurate and sensitive [25, 26]. For this end, a high quality assay must be used in order to identify the compounds of interest [25, 26]. However, HTS assays, such as those that involve cellular viability assays, do not enable researchers to reach any conclusions about the therapeutic efficacy of the tested compounds, since HTS assays are only performed with a very low amount of different drug concentrations [25, 26].

Due to the financial constraints of small corporations such as academic research units, as well as the extraordinary costs associated to preclinical drug development for both small and large pharmaceutical companies, there is a huge demand for quick and inexpensive HTS methods that provide reliable results during this stage of drug development. DiMasi *et al*. stated that better preclinical studies could contribute to reduce the cost of the development of a new drug by over 200 million US dollars [27], roughly half of the then-latest estimated total cost of the preclinical stage [16]. The reduction of the time required for new drugs to move on to the next phases is also a key parameter, as it allows new drugs to enter into the market earlier and to reduce personnel costs over time.

1.3. Cellular viability assays used to perform drug HTS

Cellular viability assays are fundamental during the preclinical evaluation of new potential therapeutic molecules, since they provide data about the potential of a particular molecule to be used as an anticancer agent [28]. *In vitro* cellular viability assays are frequently utilized to determine the half-maximal inhibitory concentration (IC_{50}) of a drug. This value corresponds to the concentration of compound that is required to reduce the cellular viability in half (typically expressed in μM or mg mL^{-1}). The IC_{50} may therefore serve as a measure of drug potency: lower values mean that a compound is more effective for cancer therapy [29].

A IC_{50} assay is performed by incubating cells with various concentrations of a specific drug. The percentage of viable cells for each of the drug concentrations is usually determined with cellular viability assays, namely 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), Resazurin, among others. By fitting the concentrations and resulting cellular viability to a sigmoidal curve described by a modified Hill equation (Equation 1), the IC_{50} can then be calculated [30].

$$\%_{viable\ cells}(\%) = E_{max} \times \frac{1-[drug]^n}{[drug]^n + IC_{50}^n} \times 100 \qquad (1)$$

Where $\%_{viable\ cells}$ is the percentage of viable cells, E_{max} the maximum effect of the drug, $[drug]$ is the concentration of the cytotoxic drug, n is the Hill coefficient (a factor that

characterizes the slope of the curve when $x = IC_{50}$), and IC_{50} is the half-maximal inhibitory concentration [29, 31].

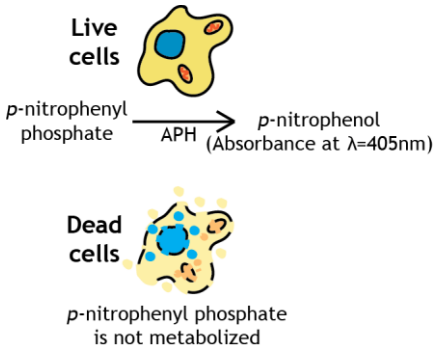
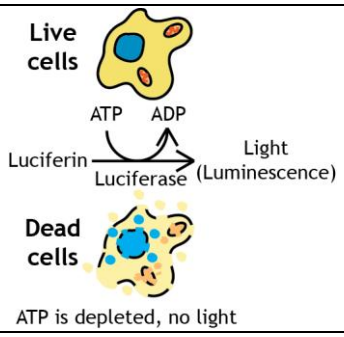
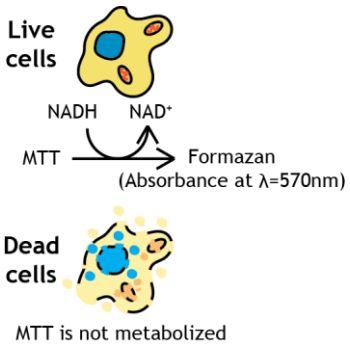
1.3.1. Cellular viability assays

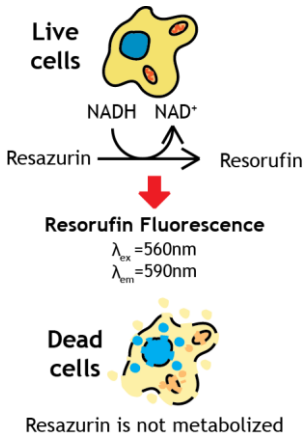
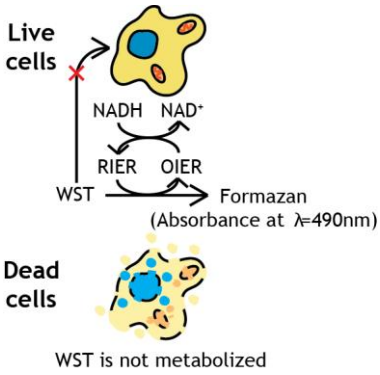
Most of the cellular viability assays are essentially divided in two main groups. The first group is composed of assays based in the analysis of metabolic state of cells (activity of mitochondrial and cytoplasmic enzymes, or metabolite levels). The Acid Phosphatase (APH) is one such assay, based on living cells' ability to metabolize a substrate. The APH enzyme converts *p*-nitrophenyl phosphate into *p*-nitrophenol, a colored compound whose absorbance at a wavelength of 405nm may be subsequently measured by ultraviolet-visible (UV-vis) spectroscopy [32, 33]. The Adenosine Triphosphate (ATP) bioluminescence assay is another assay based on the metabolism of viable cells, that evaluates cellular viability through the use of firefly luciferase. This enzyme produces light as a result of catalyzing the oxidation of luciferin into oxyluciferin in viable cells (only viable cells possess ATP, which is necessary for this reaction) [34]. The resulting light intensity of the reaction can then be quantified with the use of a luminometer. The MTT, MTS and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assays (among others present in Table 1, such as water-soluble tetrazolium salts (WST) assays) are based on the living cells' ability to reduce tetrazolium salts into an intensely colored compound - formazan, whose absorbance is then measured by UV-vis spectroscopy [35, 36]. The Resazurin assay, also known as AlamarBlue assay, involves the reduction of Resazurin into Resorufin, that emits fluorescence at λ=590nm when excited with light with λ=560nm. This reaction is dependent on the cellular levels of NADH, which are greatly diminished after cell death [36–38].

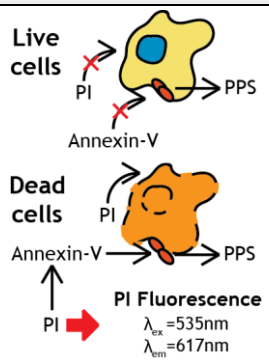
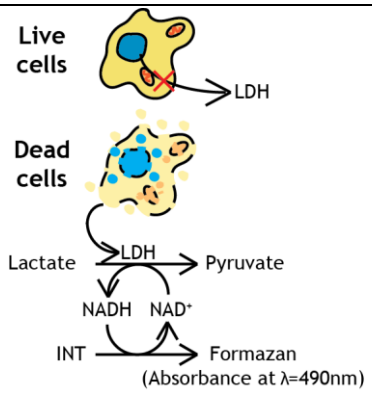
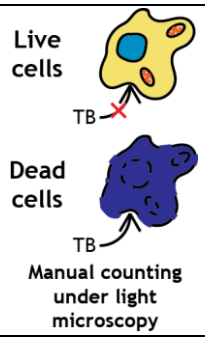
The second group includes assays based on the evaluation of the physical integrity of cellular compartments (e.g., cellular membrane and nuclear membrane). Assays that use Annexin-V, Lactate Dehydrogenase (LDH), Propidium Iodide (PI) and Trypan Blue (TB) are prime examples of assays that discriminate between living and dead cells per their cellular membrane integrity. Annexin-V, PI and TB are not able to bypass the bilipid membrane of viable cells due to their molecular weight and polarity. Consequently, these compounds only stain dead cells. Annexin-V exclusively binds to phosphatidylserine (PPS) residues that are only exposed on the outer surface of the cellular membrane when cells undergo apoptosis [39]. Then, the fluorescence of the dye that is bonded to the Annexin-V (e.g., PI) can be measured [40]. The fluorescence emitted by PI is thus indirectly proportional to the number of viable cells. In LDH release assays, compromised cell membranes allow the release of intracellular LDH to the outside environment of the cell, and the percentage of dead cells may be indirectly quantified by the activity of LDH, which catalyzes the oxidation of lactate to pyruvate, leading to the production of reduced nicotinamide adenine nucleotide (NADH). Next, a tetrazolium salt (normally 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT)), is converted into a colored formazan product after reacting with the reduced NADH. This product can then be quantified by UV-vis

[28, 41–43]. The resulting absorbance of the formazan product is proportional to the percentage of dead cells [42]. The TB dye exclusion assay is one of the most widely used cellular viability assays that analyses cellular viability accordingly to the cellular membrane integrity. When cells die, TB is able to enter the cytoplasm, staining dead cells blue, while living cells appear white. Live and dead cells can then be counted under an optical microscope [44]. In Table 1, a list of commonly used cellular viability assays can be found, categorized according to their underlying mechanisms.

Table 1. Overview of different methods used for quantifying cellular viability in the presence of a particular drug.

General underlying mechanism – METABOLIC			
Assay name	**Mechanism**	**Advantages and disadvantages**	**Refs.**
APH		↑ Adherent and non-adherent cell lines can be used in this assay; ↑ High sensitivity; ↑ Simple and quick to execute. ↓ Requires cells to express APH over a certain threshold.	[32, 33]
ATP bioluminescence		↑ Highest sensitivity; ↑ Lengthy incubation steps are not required; ↑ Simple and quick experimental protocol. ↓ Levels of ATP may be affected by other events that are not correlated with cell death; ↓ Very expensive.	[34, 37]
MTT		↑ High correlation between the obtained results and the number of viable cells; ↑ Simple and fast; ↑ Suitable for HTS; ↑ The substrate and the reaction product do not absorb light at the same wavelength. ↓ Cells can only be used once in the MTT assay; ↓ Conversion of MTT may be affected by other events not correlated with cell death; ↓ Requires extra pipetting steps to solubilize formazan crystals; ↓ Low sensitivity.	[35, 37, 43, 45]

Resazurin		↑ Intermediate electron receptor is not necessary, but may be used to accelerate the reaction; ↑ Samples' absorbance may be determined by UV-vis spectroscopy) or fluorescence, but the latter is preferred due to higher sensitivity; ↑ Some cell lines may be used to monitor drug cytotoxicity over time. ↓ Loss of linearity when high fluorescence intensities are detected due to quenching; ↓ May lead to the overproduction of reactive oxygen species; ↓ Possible physiological interference; ↓ Sensitive to metabolic alterations.	[36–38, 46, 47]
WSTs (MTS, WST-1, WST-8 and XTT)		↑ Cells can be used to monitor drug cytotoxicity over time; ↑ Higher sensitivity than the MTT assay; ↑ One-step procedure, minimizing pipetting-related systematic errors. ↓ Cannot discriminate between cytotoxic and cytostatic drug effects; ↓ Intermediate electron receptors such as Phenazine Ethyl Sulfate or Phenazine Methyl Sulfate may be toxic to cells; ↓ More expensive than the MTT assay; ↓ Much higher background absorbance.	[37, 43, 48, 49]

General underlying mechanism – EVALUATION OF CELL MEMBRANE INTEGRITY			
Assay name	**Mechanism**	**Advantages and disadvantages**	**Refs.**
Annexin-V/PI		↑ High sensitivity; ↑ Useful to detect cells in early stages of apoptosis; ↓ Relatively expensive; ↓ Cellular damage induced during the protocol may lead to staining artifacts.	[40]
LDH release		↑ LDH remains stable up to 48 hours after cell death. ↓ Composition of the majority of commercial kits is unknown; ↓ LDH activity may be altered by the tested drugs; ↓ More appropriate for cells showing necrotic traits.	[28, 41-43]
TB dye exclusion		↑ Cheap assay; ↓ Cells must be counted quickly after staining with TB; ↓ Very low precision and sensitivity.	[44]
ADP (adenosine diphosphate); AO (Acridine Orange); APH (Acid Phosphatase); ATP (adenosine triphosphate); INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride); LDH, Lactate Dehydrogenase; NADH (Nicotinamide Adenine Dinucleotide); OIER (oxidized intermediate electron receptor); PI (Propidium Iodide); RIER (reduced intermediate electron receptor); TB (Trypan Blue); WST (water-soluble tetrazolium).			

All of the assays presented in Table 1 suffer from one or more performance-related disadvantages such as low sensitivity, precision, inconstant signal/cell ratio or unspecific response to cell death. This affects negatively on the reliability of these assays, and as such, new methods for cellular viability assessment are necessary.

1.4. Crystal Violet

Tris(4-(dimethylamino)phenyl)methylium chloride (Figure 2), commonly known as Crystal Violet (CV) or Gentian Violet or Basic Violet 3, has been a staple substance used in the laboratory since it was used for the first time by Hans Christian Gram in 1884 to differentiate bacteria cellular wall composition and structure [50].

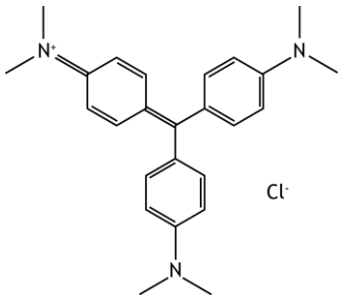


Figure 2. Molecular structure of CV [51].

CV has been also described as a dye capable of entering into eukaryotic cells (through transmembrane proteins present in the cells' plasmatic membrane [52]). Therefore, several uses for crystal violet in the field of biology have been found, such as those that include the clonogenic assays [53, 54], as well as the evaluation of adhesion [55–57], migration and invasion [58–60], morphology [60, 61], proliferation [62–64] and viability [65–68] of the cells. Although CV is not the main choice to perform these assays, since this compound is irritant [69], toxic [69] and is suspected to promote mutagenesis [70, 71], CV remains as a viable option due to the fact that these issues happen at very high dosages, they can be minimized by using basic laboratory safety equipment (e.g., lab coat, protective glasses, gloves and a safety mask) and CV possesses a low cost and its usage is simple [67].

1.4.1. *In vitro* CV-based cellular viability assay

CV can be used to stain only adhered cells and therefore determine the percentage of viable cells [72]. The use of CV to determine the percentage of viable cells and the efficacy of a treatment was already demonstrated by several different authors. For instance, in a study performed by Bosio *et al.*, a CV cellular viability assay was performed to evaluate the cytotoxic effect of $CaCO_3$-biopolymer microparticules loaded with DOX in human osteosarcoma-derived (MG-63) and mouse fibroblastic (3T3) cells [68]. In another investigation conducted by Miyajima *et al.*, the cytotoxicity of cis-dichlorodiammineplatinum together with reactive oxygen species production catalysts in bladder cancer (KU-7) cells was assessed by using a CV cellular viability assay [73]. An overview of these studies, among others [65, 67, 68, 72–80], allowed the definition of the general steps of the protocol used to determine cellular viability using CV, as summarized in Figure 3.

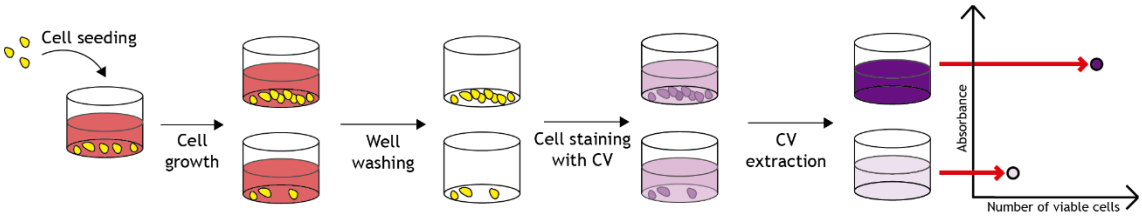


Figure 3. Schematic illustration of the general protocol used to perform of CV-based cellular viability assay. Living cells adhere to the bottom of the wells, while dead cells lose their adhesive capabilities and are washed away from the wells. Therefore, only adherent and healthy cells are stained with CV, which is subsequently extracted from the cells to measure its absorbance at a wavelength of 570nm.

In accordance with Figure 3, in the CV protocol cells are initially stained with CV [72]. Cells may have been previously non-fixed [72] or fixed [68, 81]. Following the staining procedure, the excess dye (CV that is not staining the cells) is discarded by washing the wells with phosphate-buffered saline (PBS) or tap water, in order to quantify only the CV retained by the cells. CV is then extracted from the cells with an extraction solution, usually composed of a detergent, i.e. sodium dodecyl sulfate (SDS) and Triton X-100, or an organic solvent (ethanol or methanol). The wells' contents are then transferred to a clean 96-well microplate, and the CV absorbance determined at 570nm [72]. Higher CV absorbance values are correlated with higher number of cells.

Although the CV-based protocol for the determination of cellular viability has been already described by different authors [65, 67, 68, 72–80], there is no single standardized CV protocol yet described for cellular viability quantification. Additionally, the influence of cell fixation and the concentration of CV used for the staining solution in the sensitivity and efficacy of the CV method, as well as their applicability for HTS, requires further investigation (Figure 3). Such data will allow researchers to use the CV protocol during Phase II of drug development in order to reduce the cost and time required for a new drug to arrive to the clinical assay phase.

1.5. Aims

The main aim of the workplan of this master's dissertation was the optimization of an experimental protocol that can be used for drug HTS. The specific aims of this dissertation are:

- Evaluation of the influence that cell fixation and CV concentration used to stain the cells have on the sensitivity, linearity and precision of the CV-based cellular viability assay;
- Comparison of the sensitivity, linearity and precision of the optimized CV protocol with other commercially available cellular viability assays (namely MTT and Resazurin);
- Determination of the DOX drug-response curve in breast and cervical cancer cells by using the optimized CV, MTT and Resazurin assays;
- Comparison of the IC_{50} values obtained with the optimized CV, MTT and Resazurin assays.

Chapter II

Materials and methods

2. Materials and methods

2.1. Materials

Human cervix adenocarcinoma (HeLa) and oestrogen-dependent breast adenocarcinoma (MCF-7) cell lines were obtained from ATCC (Middlesex, UK). 96-well flat-bottomed cell culture plates and 75cm^2 T-flasks were bought from Thermo Fisher Scientific (Porto, Portugal) and Orange Scientific (Braine-l'Alleud, Belgium). Ultrapure water was obtained using a Merck Millipore Milli-Q Integral Water Purification System (Lisbon, Portugal). Dulbecco's Modified Eagle's Medium with high glucose (DMEM-HG), Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM-F12), antibiotic and antimycotic solution (containing 10,000units mL^{-1} penicillin, 10mg mL^{-1} streptomycin and 25µg mL^{-1} amphotericin B), ethylenediamine tetraacetate (EDTA), PBS, Resazurin, TB and trypsin were acquired from Sigma-Aldritch (Sintra, Portugal). Fetal bovine serum (FBS) was bought from Biochrom AG (Berlin, Germany). Paraformaldehyde (PFA) was obtained from Merck (Lisbon, Portugal). MTT was acquired from Alfa Aesar (Karlsruhe, Germany). SDS was obtained from Panreac AppliChem (Famões, Portugal). CV was bought from Amresco (Solon, US). Absolute methanol and dimethyl sulfoxide (DMSO) were obtained from Fisher Chemical (Porto Salvo, Portugal). DOX was obtained from Carbosynth Ltd (Compton, UK).

2.2. Cell culture

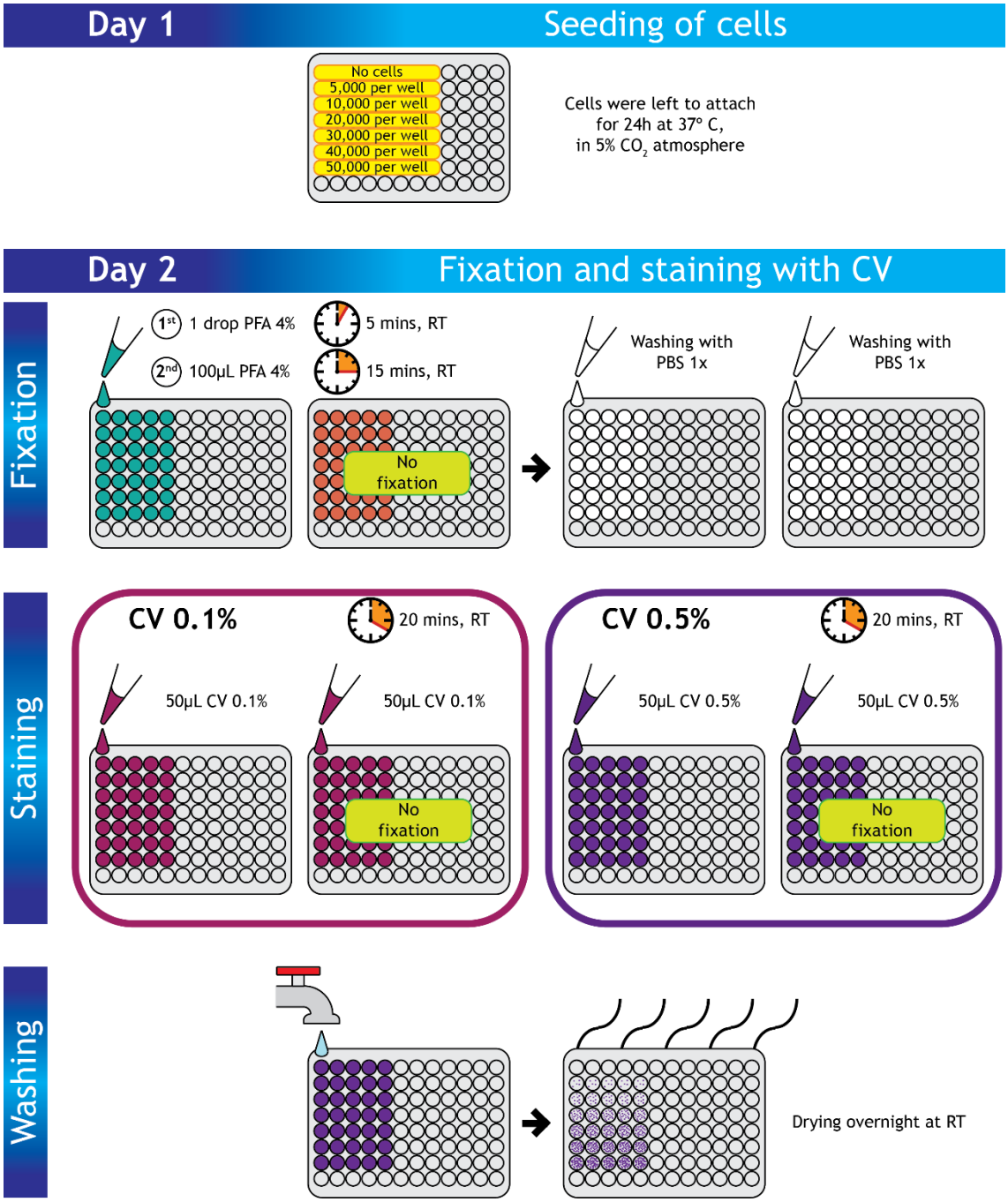
HeLa and MCF-7 cell lines were grown in 75cm^2 T-flasks, in DMEM-HG and DMEM-F12 culture medium respectively, both supplemented with 10% (v/v) FBS and 1% (v/v) of a solution mixture composed by 10,000units mL^{-1} penicillin, 10mg mL^{-1} streptomycin and 25µg mL^{-1} amphotericin B. Cells were grown inside an incubator with a humidified atmosphere of 5% CO_2, at 37ºC. When cells attained confluence, they were harvested using a solution composed of 0.18% trypsin (1:250) and 5mM EDTA.

2.3. Optimization of a CV-based cellular viability assay

The CV assay was performed by adopting a protocol previously developed by Feoktistova *et al*. [72] (Figure 4). Briefly, the CV assay was performed over the course of 3 days: on the first day, HeLa and MCF-7 cells were seeded in 96-well microplates at various densities (5,000, 10,000, 20,000, 30,000, 40,000 and 50,000 cells per well), after harvesting and the number of viable cells was determined through the TB dye exclusion cellular viability assay [44]. Wells without cells were used as controls.

After allowing cells to attach for 24 hours, HeLa and MCF-7 cells were fixed with PFA 4% in two successive steps: initially, one drop of PFA was added to each well and incubated at room temperature (RT) for 5 minutes, and then 100µL of PFA was pipetted into every well, and subsquently incubated for 15 minutes. For comparative purposes, cells were not fixed in certain wells (Figure 4). Afterwards, wells containing either fixed or non-fixed cells were washed by

discarding the supernatant and washing once with PBS. Cells were then stained with either the 0.1% or 0.5% CV (w/v) in 20% methanol (v/v in ultrapure water) solution by incubating samples for 20 minutes at RT. Next, all plates were carefully washed under a stream of tap water until the excess of CV was removed from the wells. Plates were carefully inverted over paper towels to remove most of the water out of the wells, and then left to dry overnight at RT to allow leftover water to fully evaporate. On the third day, CV was extracted from the cells with SDS 10% (w/v) by incubating samples at RT for 30 minutes with gentle manual agitation. Each well's content was then transferred to a 96-well microplate, and diluted 1:2 in SDS 10%. The absorbance of each well was determined at 570 nm using a xMark plate spectrophotometer (Bio-Rad).



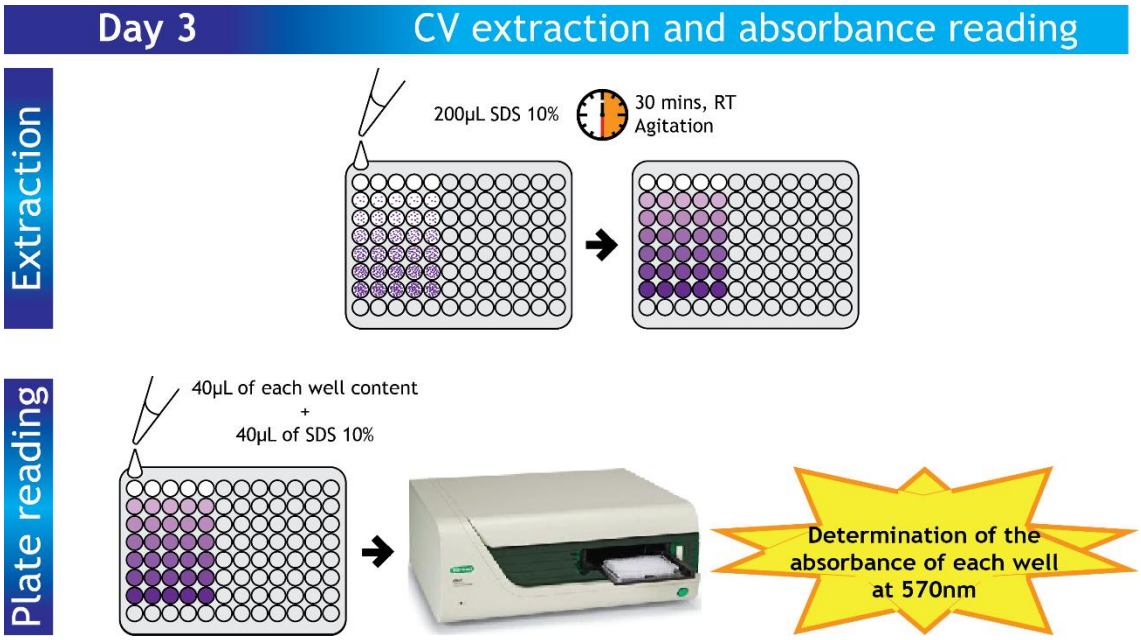


Figure 4. Experimental setup used to evaluate the effect of cell fixation as well as CV solution concentration on the results obtained through the CV assay. This experimental protocol was performed with HeLa and MCF-7 cells.

As controls, the stock 0.1% CV solution was diluted to a final CV concentration of 0.0025% (w/v) in 20% methanol (v/v in ultrapure water) and the ultraviolet-visible (UV-vis) spectra of all reagents used during the CV assay (CV, methanol, PFA 4%, PBS 1x and SDS 10%) was then measured by pipetting 80µL of each solution to the wells of a 96-well plate. Spectra were acquired in the range of 400-700nm using a xMark plate spectrophotometer (Biorad) with a spectral resolution of 10nm. Absorbance of empty wells was determined and used as control.

As a second set of controls, 10,000, 20,000, 30,000 and 40,000 HeLa cells were seeded in 96-well plates and allowed to attach for 24 hours. On the following day, culture medium was removed and cells were incubated with SDS 10% for 30 minutes. Spectrum data was acquired in the range of 400-700nm using a xMark plate spectrophotometer (Biorad) with a spectral resolution of 10nm. Absorbance of empty wells was acquired and used as control.

2.4. MTT and Resazurin assays for cellular viability determination

2.4.1. MTT assay

The protocol used to perform the MTT assay was adapted from Ribeiro *et al.* [82]. Cells were seeded in 96-well cell culture plates (using 5,000, 10,000, 20,000, 30,000, 40,000 and 50,000 cells per well). Wells without cells were used as control. The following day, the medium was removed from the wells and 100µL stock MTT solution (5mg mL^{-1} in PBS 1x) was added. The plates were incubated for 4 hours. Afterwards, 150µL of DMSO were added and the plates were

incubated, at RT, for 30 minutes under mild stirring. Absorbance of each well was then measured at 570nm using a xMark plate spectrophotometer (Bio-Rad).

2.4.2. Resazurin assay

The Resazurin assay was performed accordingly to the protocol described by de Melo-Diogo *et al.* [83] and Moreira *et al.* [84]. Cells were seeded in 96-well cell culture plates (5,000, 10,000, 20,000, 30,000, 40,000 and 50,000 cells were seeded in each well). Wells without cells were used as control. After 24 hours, the culture medium was replaced by a solution of 0.1mg mL^{-1} Resazurin prepared in fresh medium supplemented with 10% FBS and 1% antibiotic and antimycotic solution. The plates were then incubated for 4 hours. Fluorescence of each well was measured on a Molecular Devices Gemini EM microplate reader, using an excitation wavelength of 560nm and an emission wavelength of 590nm.

2.4.3. Evaluation of the CV, MTT and Resazurin cellular viability assays

The cellular viability results attained in the CV, MTT and Resazurin assays were analyzed to compare their sensitivity, linearity and precision. After determining the absorbance (in the CV and MTT assays) and fluorescence (in the Resazurin assay) of the wells with different cellular densities, a linear regression analysis of the results was performed using the GraphPad Prism v6.01 software (Trial version, GraphPad Software Inc., 2012, CA, USA). This type of analysis was chosen as, in theory, the proportion between absorbance or fluorescence and the number of viable cells should be a constant. This constant is often referred to as signal/cell ratio. A linear equation that describes the variation of absorbance or fluorescence as a function of the number of cells was thus obtained (Equation 2). This type of equation is known as a linear function, describing the proportional increase of the absorbance (or fluorescence) as a consequence of the increase of the number of viable cells.

$$y = a \times n_{cells} + b \qquad (2)$$

Where y is the absorbance (or fluorescence, in the case of the Resazurin assay) of each sample, a is the slope (which is often referred as the sensitivity of the assay [85]), n_{cells} the independent variable determined by the number of viable cells and *b* the absorbance (or fluorescence, in the case of the Resazurin assay) when $n_{cells} = 0$. Linear regression of the results was performed by using an iterative algorithm that minimizes the sum of the squares of the residuals (differences between the observed value and the value predicted by the model) so that the equation obtained reflects that absorbance or fluorescence grows proportionally to the number of adherent cells, and therefore the method can be used in order to quantify the number of viable cells up to the maximum number of seeded cells. The coefficient of determination (r^2) was automatically calculated by the GraphPad Prism 6.01 software during linear regression. This parameter serves as a metric for the linearity of the assay (as reflected by the previously obtained results) due to its statistical meaning, i.e. as the r^2 increases, the better the equation

reproduces the absorbance or fluorescence changes provoked by the cells present in the wells. In other words, as the r^2 increases, a lower variation in the signal/cell ratio occurs, which is fundamental for establishing a quality assay to be used in drug development.

Another complementary parameter that was used for assessing the precision of the assays in this work was the coefficient of variation, which was defined by Ivanov *et al*. as the ratio between the standard deviation (SD) and the mean of a dataset [86]. The coefficient of variation for all points obtained in the CV, MTT and Resazurin assays was calculated using Equation 3. The maximal coefficient of variation needed for validation was set to 20% as previously described in literature [86, 87].

$$CVariation\ (\%) = \frac{\sigma_S}{\bar{x}_S} \times 100 \qquad (3)$$

Where σ_S represents the SD of the datapoint and $\bar{x}_S$ the mean of the datapoint.

2.5. Determination of the IC_{50} of DOX

A DOX stock solution (3.34mM) was produced by dissolving DOX in absolute methanol previously filtered using a 20nm pore size membrane. HeLa and MCF-7 cells were seeded separately in flat-bottom 96-well plates (20,000 cells per well), and cultured with DMEM-HG and DMEM-F12 complemented with 10% FBS and 1% antibiotic and antimycotic solution. After allowing cells to adhere for 2 days, the culture medium was removed and the drug was administered. MCF-7 cells were incubated with 0.10, 1.00, 1.50, 2.00, 5.00 and 20.00μM of DOX and HeLa cells with 0.01, 0.05, 0.07, 1.00, 1.50, 2.00, 3.00, 8.00, 10.00 and 15.00μM of DOX. In all conditions, the percentage of methanol was 5% (v/v) in culture medium, as DOX was dissolved in absolute methanol and the concentration of methanol in all wells should be equal, so that the concentration of DOX would be the sole variable in this protocol. 5% methanol (v/v) in culture medium has been previously shown to not influence the cellular viability of HeLa and MCF-7 cells in comparison to wells incubated only with culture medium. After 24 hours of incubation, the medium with DOX was removed and the cellular viability was assessed through CV, MTT or Resazurin assays.

Cells without being in contact with the drug were used as negative control (5% methanol (v/v) in culture medium). Positive control (100% dead cells) corresponds to cells killed by incubation with SDS 10% (w/v) for 24 hours.

To obtain the DOX IC_{50} values for MCF-7 and HeLa cell lines, the dose-response curves were determined using non-linear regression analysis with the Levenberg-Marquardt algorithm of the OriginLab 2017 software (Trial version, OriginLab Corporation, 2017, MA, USA). The percentage of viable cells determined through the CV, MTT and Resazurin assays was related to the concentration of DOX added to each well. After the non-linear regression analysis, a DOX

concentration-cell viability curve was graphed using the data obtained through the CV, MTT and Resazurin assays. The sigmoidal curve obtained is generically defined by the modified Hill equation described in Equation 1 (section 1.3.1.), of which the IC_{50} is an essential parameter of the equation obtained after the non-linear regression analysis performed by the OriginLab 2017 software [29, 31].

2.5.1. Evaluation of CV assay suitability for HTS

HTS of potentially useful anticancer molecules usually relies on the employment of cellular viability assays, namely on the MTT and Resazurin assays. Various metrics that allow researchers to compare the result reliability and the result quality of different cellular viability assays have been described in literature, such as the Assay Signal Window [25] and the Z-factor [26]. To determine the cellular viability protocol, CV, MTT or Resazurin, most suitable for HTS, the Z-factor of the assays was calculated, as previously described by Zhang *et al*. [26] (Equation 4).

$$Z = 1 - 3\frac{\sigma_{C^-} + \sigma_{C^+}}{|\bar{x}_{C^-} - \bar{x}_{C^+}|} \qquad (4)$$

Where Z represents the Z-factor value, σ_{C^-} represents the SD of the negative control, σ_{C^+} the SD of the positive control, $\bar{x}_{C^-}$ the mean of the negative control and $\bar{x}_{C^+}$ the mean of the positive control. As per the recommendations of Zhang *et al.*, the minimal Z-factor required for confirmation of an assay's suitability for HTS was set at 0.5 [26].

2.6. Optical microscopy imaging

HeLa and MCF-7 cultures were visualized using an Olympus CKX41 inverted optical microscope equipped with an Olympus SP-500UZ digital camera at various pre-determined timepoints during the execution of the CV assay (immediately after washing with PBS, overnight drying and extraction of CV) as well as 24 hours past incubation with DOX.

2.7. Statistical analysis

The statistical analysis of the obtained results was performed by using ordinary one-way ANOVA. A *P*-value lower than 0.05 ($p<0.05$) was considered statistically significant. The data was analyzed utilizing the GraphPad Prism 6.01 software (Trial version, GraphPad Software, Inc., 2012, CA, USA).

Chapter III

Results and discussion

3. Results and discussion

3.1. Overview of the parameters optimized in the CV cellular viability assay

The CV-based cellular viability protocols found in literature show several differences between them [65, 88–90]. Through the analysis of these protocols, it was possible to notice that in some of them cells were fixed, i.e. the study previously published by La Monica *et al.* [81], and in other cases the CV concentrations used were different. In theory, fixation ensures that cells stay fixed to the wells during the execution of the CV-based cellular viability protocol, avoiding the loss of cells that would otherwise be regarded as viable by the CV assay. Therefore, only viable cells will be posteriorly stained with CV. However, to the best of my knowledge, there is no study in literature describing that the fixation of cells can in fact influence the sensitivity, linearity and precision of the results obtained through the CV assay.

To study the influence of the concentration of the CV solution used for cell staining, two of the most used concentrations described in literature were used in this work, namely 0.1% [75, 79, 91–93] and 0.5% [68, 74, 77, 90] (w/v) (Step 4, in Figure 5). Since aqueous solutions of CV tend to precipitate after weeks of storage, the CV solution was prepared in methanol 20% (v/v in ultrapure water), as previously described by Feokstistova *et al.* [72] and Limame *et al.* [58]. Methanol is an organic solvent that solubilizes CV [94]. Cells were incubated with CV solution for 20 minutes, as previously performed by Feoktistova *et al.* [72] to allow CV entrance into cell cytoplasm. When longer periods of incubation were used, the CV became adsorbed to the well (non-specific staining). Based on its molecular size and polarity, it is expected that this compound is able to interact with cell membrane proteins and then enter into the cells' cytoplasm [52]. However, to the best of my knowledge, no study in literature exists describing that the CV concentration can impact the sensitivity, linearity and precision of the results attained through the CV assay.

Taking this into account, two questions were focused on when designing the optimization procedure:

- Cell fixation before staining leads to increased sensitivity, linearity and precision of the experimental results?
- The concentration of CV used for staining cells to increased sensitivity, linearity and precision of the experimental result?

To answer these questions, in this study, cell fixation was performed and CV concentration was optimized with the objective of enhancing the sensitivity, linearity and precision of the results (Figure 5). Cellular viability assays with high sensitivity, linearity and precision are extremely useful since they allow the acquisition of very reliable results during anticancer therapeutics

screening. The obtained results can then be used as valuable data towards regulatory approval of the drug.

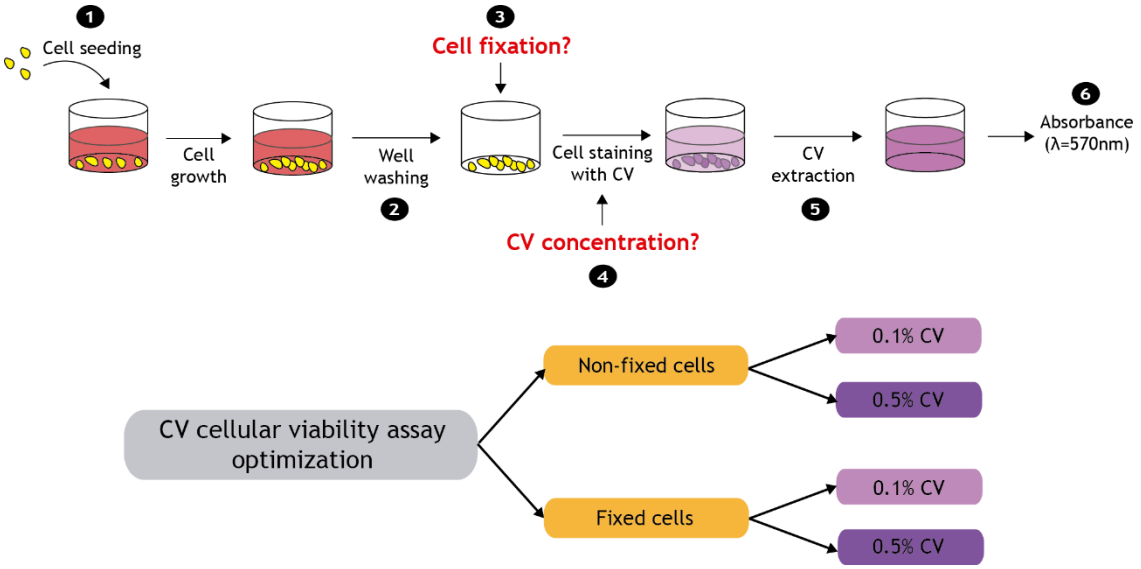


Figure 5. Schematic representation of the CV-based cellular viability assay and the parameters investigated in this work.

Cell fixation was accomplished using PFA 4%, since it is inexpensive and easy to handle, albeit other substances, such as formaldehyde [80], formalin [77], glutaraldehyde [68] or methanol [74] could be used for the same purpose. Additionally, it was verified that PFA does not absorb in the 570nm region (Figure 6A).

As it was used to solubilize CV, the UV-vis spectrum of methanol was acquired using a spectrophotometer. It was shown that methanol does not display any significant absorbance in the wavelength used to read the CV absorbance (λ=570nm) (Figure 6A).

To remove CV from the stained cells, a solution of SDS 10% (w/v) was used (Step 5, Figure 5). SDS does not absorb light at λ=570nm (Figure 6A). SDS is often used for this purpose, since this detergent is able to disrupt the phospholipids found in cell membranes, allowing the successful release of CV (Figure 7).

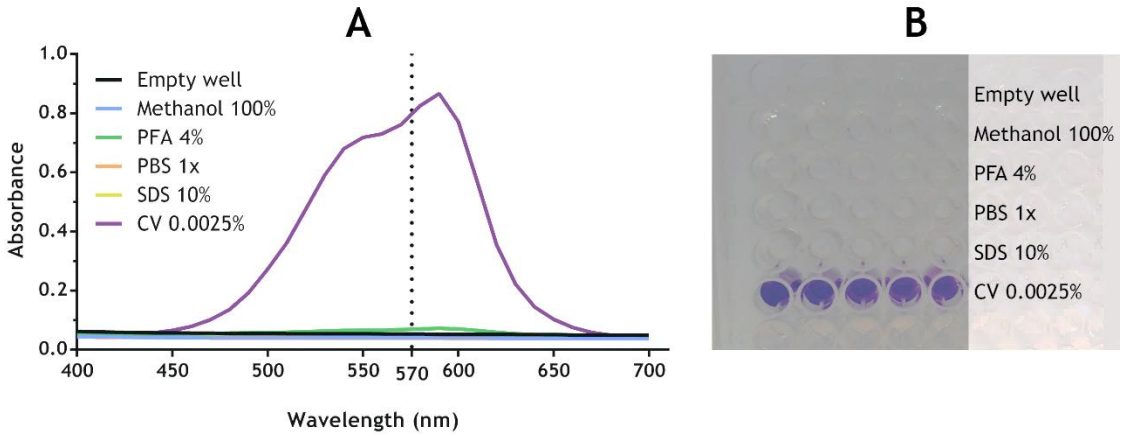


Figure 6. UV-vis spectra of the different reagents used in the CV-based cellular viability assay **(A)** and macroscopic image of the plate and solutions used for plotting the UV-vis spectra **(B)** (*n=5)*.

In Figure 6A it is possible to observe that PBS 1x, methanol, PFA 4% and SDS 10%, do not display any absorbance at λ=570nm. Lastly, Figure 8 also demonstrates that cells do not absorb light in the range of 400 to 700nm even for high cellular densities. These results allow us to conclude that cellular components, such as proteins and small molecules, as well as the other solutions used in the CV assay, do not absorb light at the wavelength used to measure the absorbance of CV.

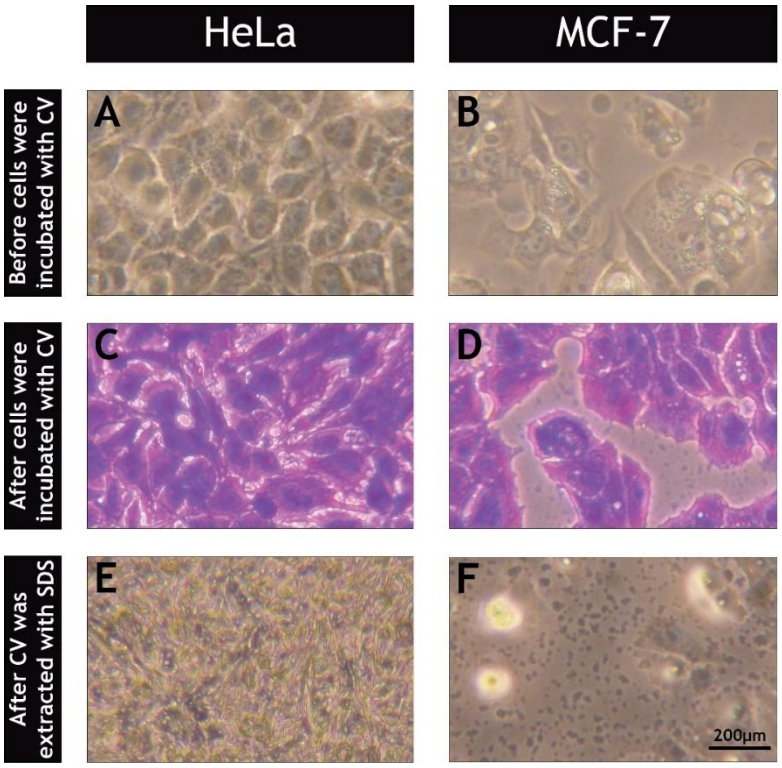


Figure 7. Optical microscopy images of HeLa and MCF-7 cells before **(A, B)** and after **(C, D)** being stained with 0.5% CV, and after CV removal from cell cytoplasm after using 10% SDS **(E, F)**.

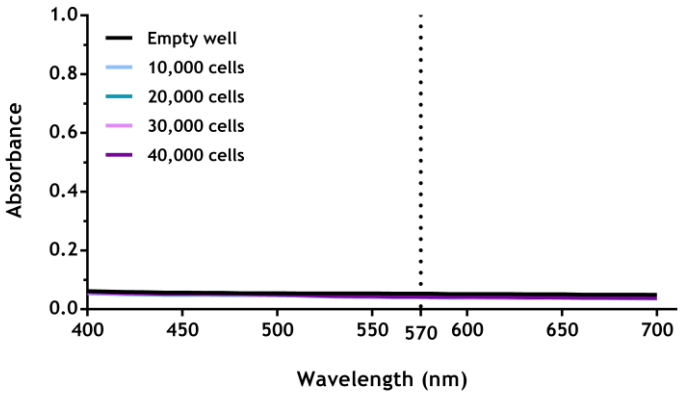


Figure 8. UV-vis spectra of different wells containing different HeLa cell densities incubated in SDS 10% (w/v) (10,000 to 40,000 cells/well) (*n=5)*.

3.2. Influence of cell fixation and of CV concentration in the sensitivity, linearity and precision of the CV assay

The sensitivity of a cellular viability assay is correlated with the capacity of the assay to detect small variations in cell viability. The studies performed with a high number of cells may not require the most sensitive assay, since assays with relatively low sensitivity are able to detect differences between samples with 100,000 and 150,000 viable cells (as an example). Still, as most drug screening studies are performed using a range of 5,000 to 20,000 cells [83, 84, 95], it is crucial to obtain an optimized assay that has a high degree of sensitivity.

To investigate the sensibility of the different CV assay variations that were performed with or without cell fixation, as well as cell staining being performed with a 0.1% or 0.5% CV solution, a linear regression analysis of the CV absorbance in function of the number of cells initial seeded (5,000 to 50,000 cells per well) was obtained. The linear regression results of the different CV-assays are shown in Figure 9.

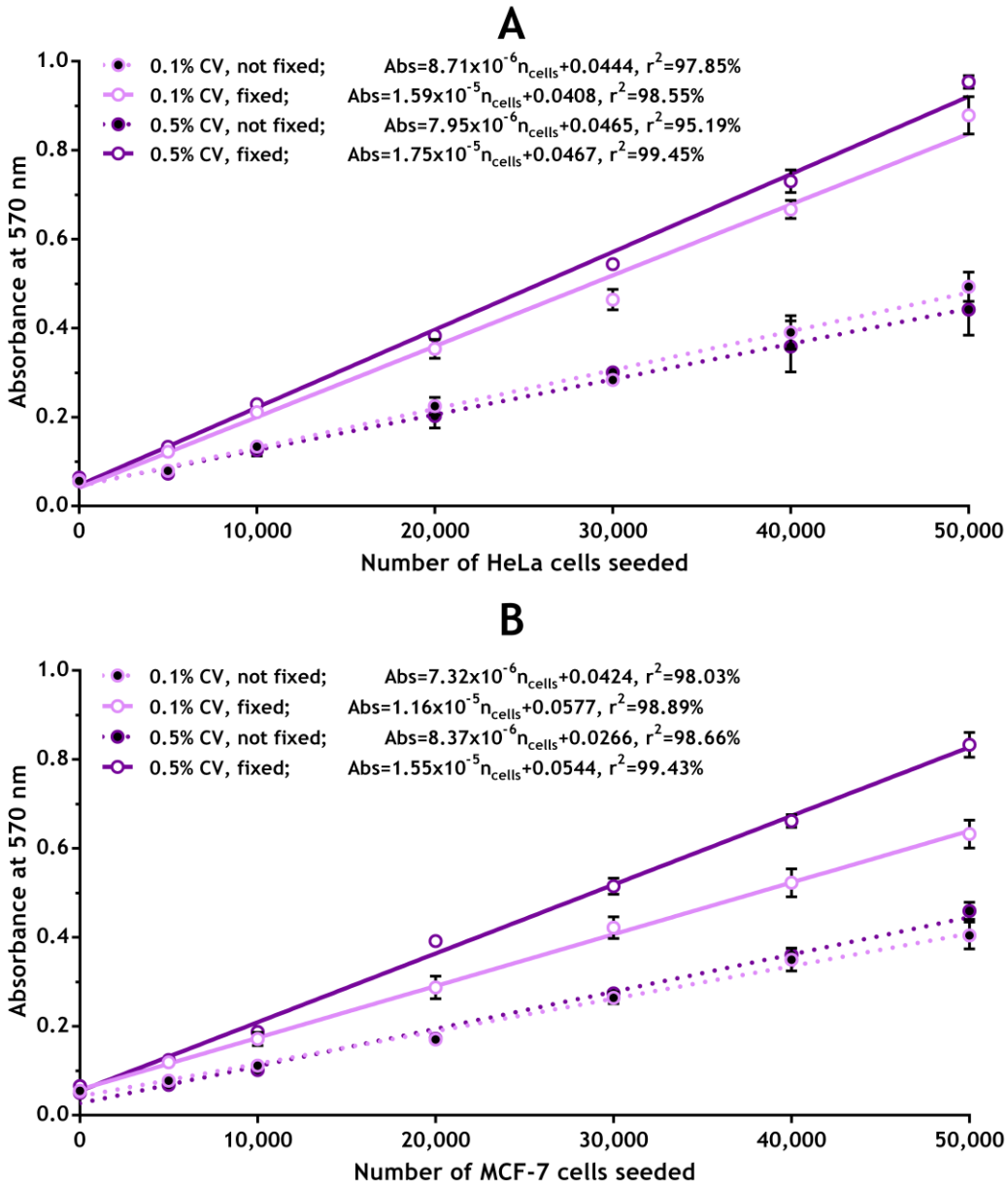


Figure 9. CV assays performed to assess the influence of cell fixation and CV solution concentration on absorbance values obtained, when different densities of HeLa **(A)** and MCF-7 **(B)** cells are used (5,000, 10,000, 20,000, 30,000, 40,000, 50,000 cells per well). CV absorbance was measured at λ=570nm. Linear regression of the results was performed in GraphPad Prism 6.01. Results are represented as the mean±SD (*n=5)*.

The results presented in Figure 9 demonstrate that there is a linear correlation between the number of cells seeded and the CV absorbance, independently of cells being fixed or not and of the CV solution concentration used. The analysis of the obtained results for fixing cells and staining with the 0.5% CV solution had the highest slope (parameter a of Equation 2, seen in section 2.4.3.) for both cell lines ($1.75\text{x}10^{-5}$ for HeLa and $1.55\text{x}10^{-5}$ for MCF-7 cells). As stated in literature, the slope of the straight line is proportional to the sensitivity of the assay [85]. Due to this, it is possible to conclude that cell fixation and the staining of cells with the 0.5%

CV solution has the highest sensitivity among the different conditions used in this study (Figure 9).

The r^2 is a statistical analysis parameter that was also evaluated. This parameter determines how well a geometrical model reflects the amount of variation in the response variable (absorbance, y) explained by the independent variable (number of seeded cells, n_{cells}) in the linear regression model. In other words, the r^2 is an indicator of the variation in the signal/cell ratio, and therefore, an indicator of the linearity of the assay. Larger r^2 values mean that variance in the signal/cell ratio is lower, and linearity is thus higher. An assay with a high degree of linearity is important because wide changes in the signal/cell ratio could lead to mistaken conclusions about drug efficacy, increasing costs for pharmaceutical companies.

The r^2 values of the linear regression analysis (presented in Figure 9) were higher when cells were fixed and stained with the 0.5% CV solution (99.45% for HeLa and 99.43% for MCF-7 cells). These values show the existence of a stronger correlation between the number of seeded cells with the absorbance values determined by UV-vis spectroscopy.

The coefficients of variation were also determined as described by Ivanov *et al.* [86]. This important indicator relativizes the SD of a point to the mean of the same point, enabling researchers to assess if the data has a huge dispersion. As such, it may serve to evaluate the precision of an assay. As the coefficient of variation decreases, the precision of the data increases [96]. In accordance with Iversen *et al.*, a good cellular viability assay must have associated a coefficient of variation that is under 20% [87].

Coefficients of variation for 5,000, 10,000, 20,000, 30,000, 40,000 and 50,000 cells per well, with or without cell fixation and by staining cells with the 0.1% or 0.5% CV solution, in both HeLa and MCF-7 cells, were in accordance with guidelines previously established [87] (Figure 10), indicating that the results obtained are very precise [96]. Therefore, all tested protocols were shown to provide a good evaluation of cellular viability. The protocol where cells were fixed and a concentration of 0.5% CV was used showed the most promising results, since the coefficient values were lower for the majority of cell numbers tested for both cell lines in study (Figure 10).

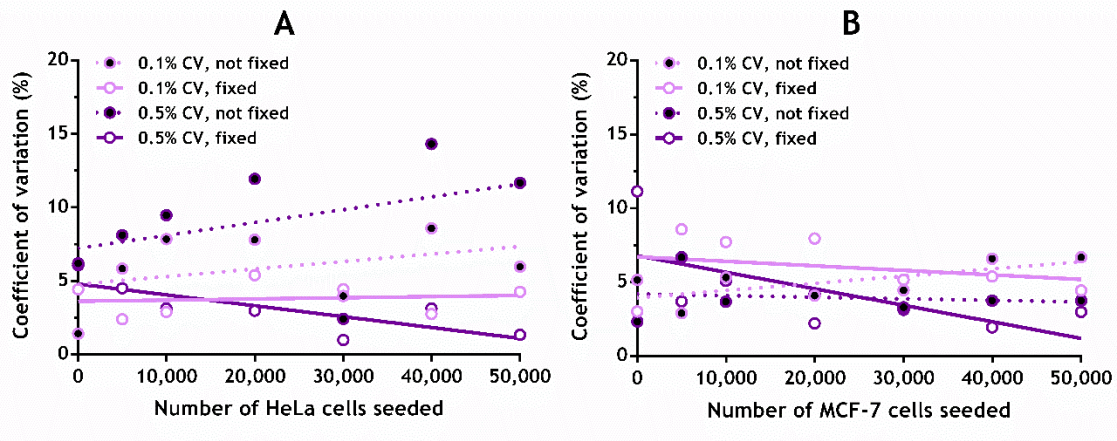


Figure 10. Coefficient of variation of the results obtained through the different CV assays. HeLa **(A)** and MCF-7 **(B)** cells were seeded at different cellular densities (10,000 to 50,000 cells/well).

For both cell lines (HeLa and MCF-7 cells), it was shown that the protocol that involves the fixation of cells with PFA and the staining with 0.5% CV resulted in a stronger correlation between absorbance and the cell number and higher sensitivity. Moreover, the precision of the CV assay appeared to be at its maximum under these conditions. Due to that, it is possible to conclude that cell fixation and their staining with 0.5% CV is the optimal experimental protocol to perform this assay. Hereafter, the CV protocol was done using the solution with 0.5% CV and cells were fixed.

3.3. Comparison of the results obtained in the CV, MTT and Resazurin assays

The CV assay was compared with other assays commonly used for determining the percentage of viable cells, namely the MTT and Resazurin assays [82–84]. These cellular viability assays have been extensively used for drug screening purposes and were therefore chosen due to its widespread use and relatively low cost. In Figure 11, the linear regression analysis of absorbance and fluorescence vs the number of cells is displayed for the optimized CV, MTT and Resazurin assays.

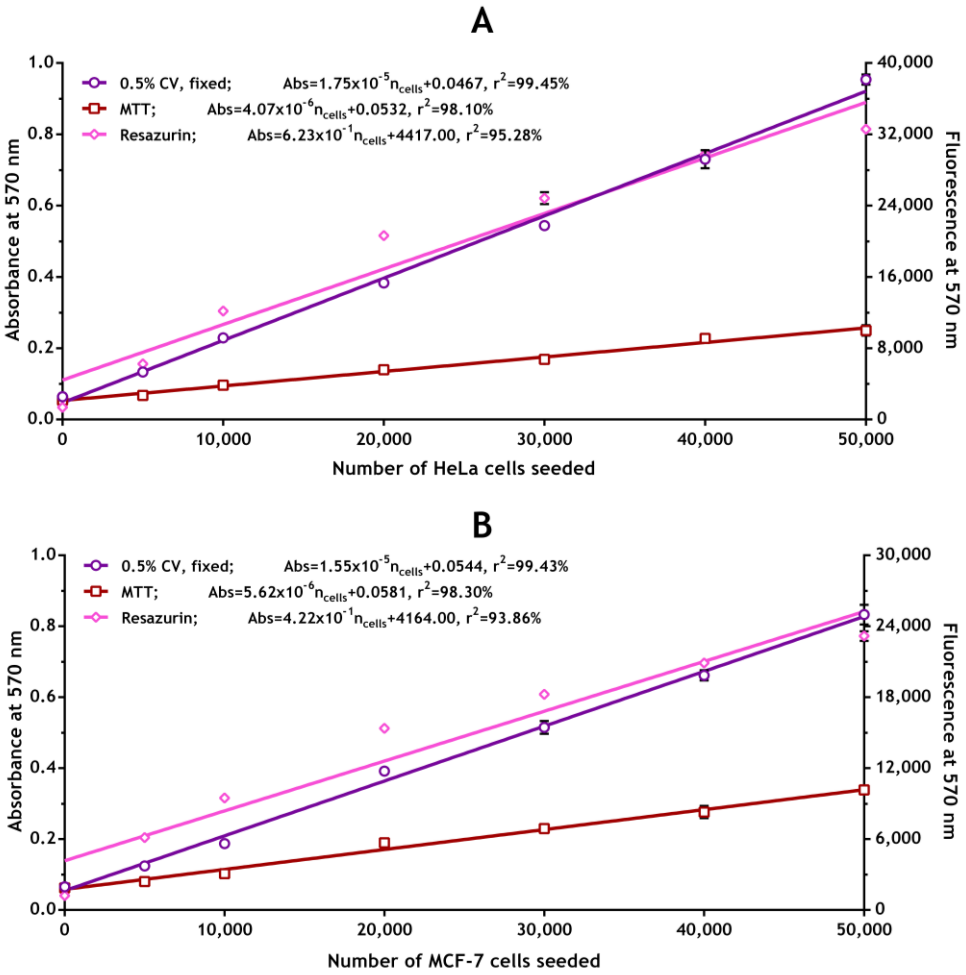


Figure 11. Comparison of the absorbance and fluorescence values obtained through the CV, MTT and Resazurin assays, used to assess the number of viable HeLa **(A)** and MCF-7 **(B)** cells previously seeded. The absorbance readings of the CV and MTT assays are plotted on the left YY axis, while the fluorescence readings of the Resazurin assay are plotted on the right YY axis. Results are represented as the mean±SD (*n=5*).

Through the analysis of Figure 11, it is possible to conclude that all cellular viability assays demonstrate a direct relationship between the number of cells and the absorbance or fluorescence values for both cell lines. When comparing the assays, the Resazurin assay demonstrated the best sensitivity, since the slope of the straight line is higher in comparison to the other assays for both HeLa and MCF-7 cells (0.62 and 0.42, respectively). This is consistent with the literature, as it is stated that because the Resazurin assay was performed by reading the fluorescence of Resorufin (Resazurin that was reduced by the cells) in the wells, this assay is more sensitive than assays where only absorbance was determined [28, 37].

The results obtained revealed that the CV assay is more sensitive than the MTT assay as can be confirmed through the analysis of the slope of the straight line (Figure 11). In the case of the MTT assay the slope values were 4.07×10^{-6} and 5.62×10^{-6} for HeLa and MCF-7 cells, respectively, while for the CV assay they were 1.75×10^{-5} and 1.55×10^{-5} for HeLa and MCF-7, respectively.

The analysis of the r^2 obtained for the linear regressions of the different cellular viability assays (Figure 11) revealed that this parameter was higher for the optimized CV assay than for MTT and Resazurin assays. The r^2 value for CV optimized assay was of 99.45% and 99.43% for HeLa and MCF-7 cells, respectively. These results demonstrate that the CV assay has the lowest variance and therefore the most constant signal/cell ratio, in comparison to the MTT and Resazurin assays. The r^2 obtained from the linear regression of Resazurin assay results was the lowest for both cell lines, meaning that the Resazurin assay has the highest variance and the least constant signal/cell ratio among the three different cellular viability assays tested. This is caused by the loss of linearity of response for higher numbers of cells (>20,000 cells/well), after which the flattering of the linear regression curve is visible (Figure 11). In prior studies performed by Nakayama *et al.*, similar results were obtained for various healthy and cancer cell lines (being MCF-7 one of the cell lines used in the study) [38]. This occurs since higher cell densities lead to a higher rate of reduction of Resazurin into Resorufin. Consequently, the higher concentration of Resorufin leads to fluorescence quenching (decrease of fluorescence intensity) [37]. The loss of linearity for high cell densities limits the maximum number of cells that can be used on this assay. The CV assay circumvents this limitation, as its results demonstrate linearity up to the maximum number of cells capable of adhering simultaneously on the bottom of 96-well microplate wells (according to the instructions of various manufacturers, the maximum is 50,000 cells per well), proving itself to be better than the Resazurin assay.

The coefficient of variation obtained for the optimized CV assay as well as for the MTT and Resazurin assays, was calculated using the means and the SDs of the results, according to Equation 3 (section 2.4.3.). The coefficient of variation was below the maximum limit defined by Iversen *et al.* (20%) [87], for all assays tested for both cell lines (Figure 12), showing that the optimized CV, MTT and Resazurin assays can obtain results with high precision.

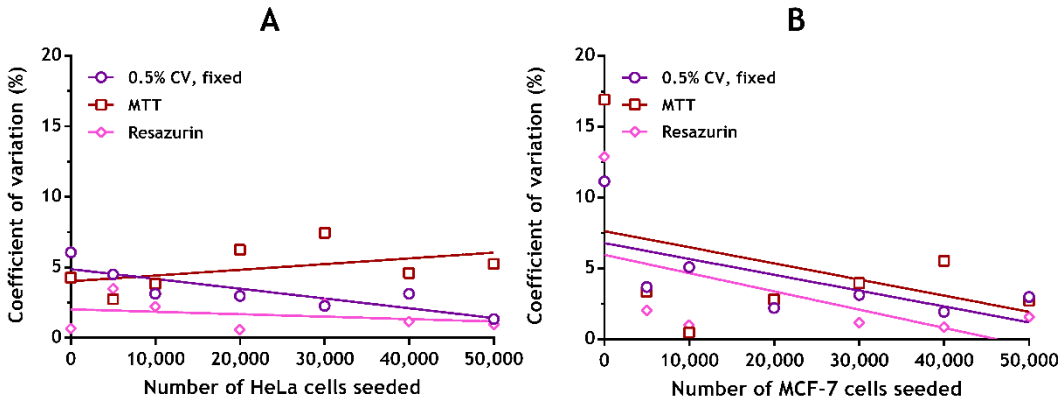


Figure 12. Coefficients of variation of the results obtained through the CV, MTT and Resazurin assay. HeLa and MCF-7 cells were used in **(A)** and **(B)**, respectively.

3.4. Determination of DOX IC_{50} for HeLa and MCF-7 cells through the CV, MTT and Resazurin assays

To evaluate the suitability of the CV assay in drug screening, the DOX IC_{50} was determined with the CV assay and the value was compared with those obtained in the MTT and Resazurin assays. DOX was chosen as a model drug because it is frequently used in the clinic for the treatment of breast [13] and cervical cancer [15]. DOX interacts with nuclear double-stranded deoxyribonucleic acid (dsDNA), inhibiting deoxyribonucleic acid (DNA) topoisomerase II from unwinding supercoiled DNA and stops the transcription process [97]. Furthermore, it stabilizes the topoisomerase II-dsDNA complex, preventing the double helix from resealing. Therefore, DOX inhibits replication and ultimately leads to cell death [97]. It has also been observed that DOX promotes intracellular production of free radicals that may inflict damage to DNA, leading to programmed cell death [98]. Some studies have also shown that besides interacting with nuclear DNA, DOX can also interfere with mitochondrial DNA [99, 100].

The results obtained for HeLa and MCF-7 cellular viability after the administration of different concentrations of DOX are displayed in Figure 13 and Figure 14. The percentage of viable cells obtained for each concentration of DOX were used for the calculation of IC_{50} values, which are displayed in Table 2.

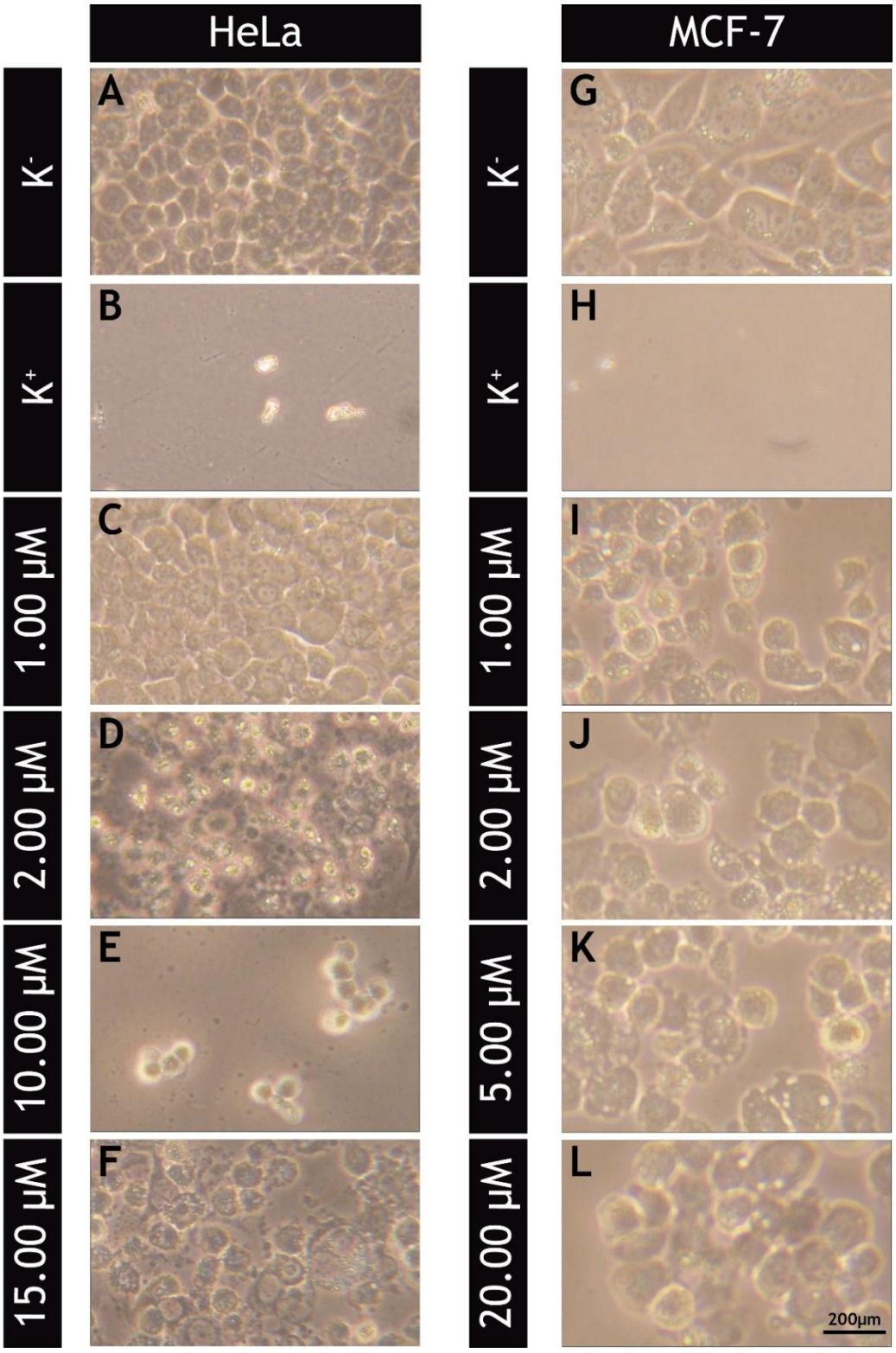


Figure 13. Optical microscopy images of HeLa **(A-F)** and MCF-7 **(G-L)** cells after being incubated with various concentrations of DOX for 24 hours (1.00, 2.00, 10.00 and 15.00µM for HeLa and 1.00, 2.00, 5.00, 20.00µM for MCF-7). K^- represents the negative control (100% live cells), while K^+ represents the positive control (100% dead cells).

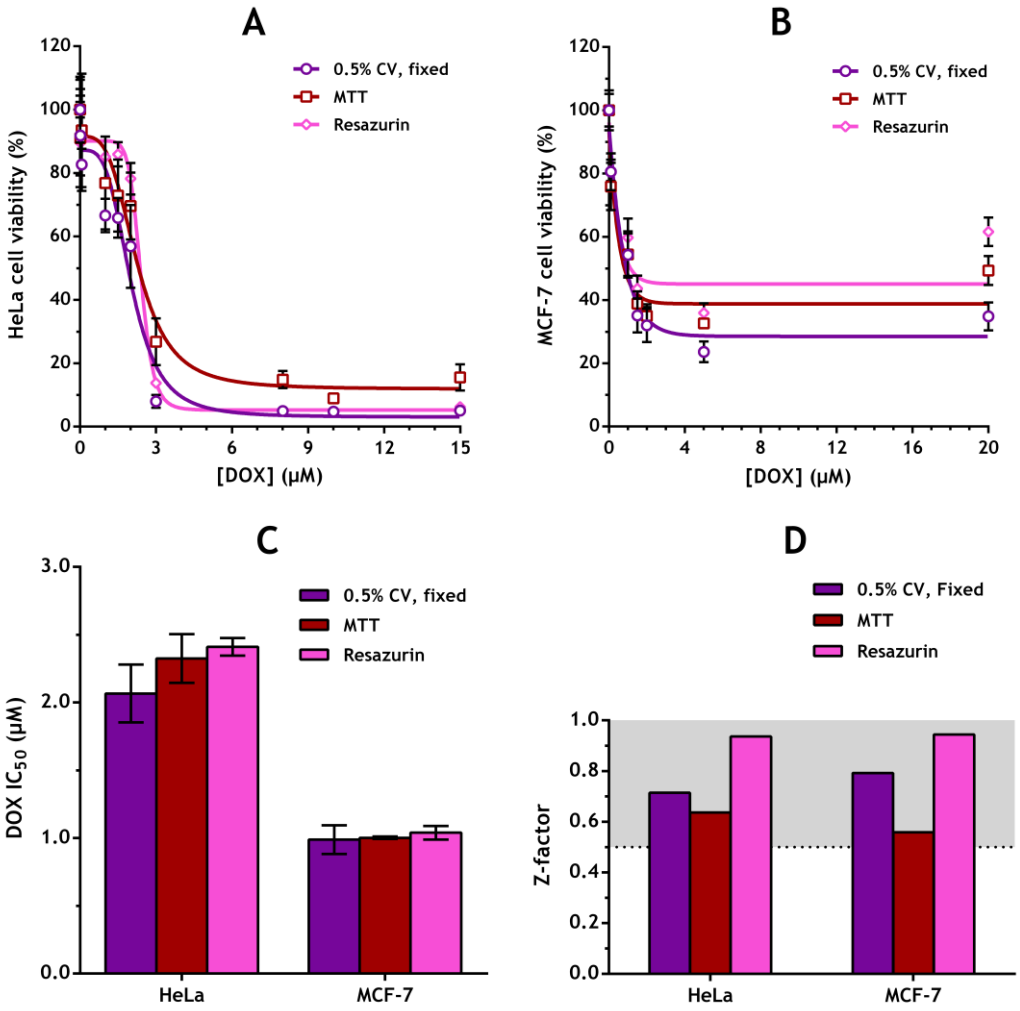


Figure 14. Effect of DOX concentration on HeLa and MCF-7 cells as determined through the CV, MTT and Resazurin assays after 24-hour incubation. Dose-response curves of DOX for HeLa and MCF-7 are plotted in **(A, B)** (*n=5*), respectively. IC_{50} values calculated were plotted in **(C)** for a more comprehensive visualization. The results are presented as the mean±SD. Z-factors calculated for the CV, MTT and Resazurin assays are presented in **(D)**. The gray area represents range of Z-factor values (0.5-1.0) which are suitable for HTS.

Table 2. DOX IC_{50} values determined for HeLa and MCF-7 cells through the CV, MTT and Resazurin assays. Results are represented as the mean±SD.

Cell line	IC_{50} (μM)		
	0.5% CV, fixed	MTT	Resazurin
HeLa	2.07 ± 0.21	2.32 ± 0.18	2.41 ± 0.07
MCF-7	0.99 ± 0.11	1.00 ± 0.01	1.04 ± 0.05

The dose-response curves obtained in the CV, MTT and Resazurin assays, presented in Figures 14A and 14B, show that the viability of HeLa and MCF-7 cells decreased as the concentration of DOX increased, consistently with the results available in literature [101]. IC_{50} values calculated for the same cell line were not significantly different (Figure 14C and Table 2), so it is possible

to conclude that the optimized CV assay is able to predict the effect of DOX in HeLa and MCF-7 cells like other commercially available cellular viability assays (like the MTT and the Resazurin assays).

The Z-factors of the different cell viability assays were also determined to evaluate the suitability of CV assay for drug HTS. The determination of this statistical parameter is fundamental to evaluate if the cellular viability assay can be performed with a very low number of replicates, and if it is suitable for HTS [26, 87]. Since HTS is performed in a large scale, and a great amount of time and money is invested in this stage, the determination of the Z-factor will allow the researchers to avoid the use of unsuitable cellular viability assays. According to previous studies [26], Z-factor values between 0.50 and 1.00 mean that the values of the negative and positive controls are far apart from each other, which means that there is a wide range of results that are noticeably different from the controls. From Equation 4 (section 2.5.1), it is possible to deduce that as the difference between the means of the negative and positive controls ($\bar{x}_{C^-} - \bar{x}_{C^+}$) reaches infinity (the case where the negative and positive control values are farthest apart), the Z-factor will progressively approach 1.00. Due to that, assays that possess Z-factor values between 0.50 and 1.00 are classified as excellent cellular viability assays for HTS [26]. On the other end, as the difference between the values of the positive and negative controls decreases and approaches 0, meaning that the range of results noticeably different from the controls is progressively smaller, the Z-factor decreases accordingly. Because of this, smaller Z-factor values are far from ideal [26]. Z-factors were calculated using the absorbance and fluorescence values of the positive and negative controls of the cellular viability assays, and plotted in Figure 14D for a comprehensive visualization.

As shown in Figure 14D, the Z-factors obtained for all assays were within the acceptance criteria established previously by Zhang *et al.*, i.e., 0.5-1.0 (illustrated as the gray-colored graph area) [26]. Resazurin showed the highest Z-factor values for both cell lines (0.94 for both HeLa and MCF-7 cells), while MTT had the lowest value (0.64 for HeLa, 0.56 for MCF-7). The optimized CV assay had a relatively high Z-factor (0.71 for HeLa, 0.79 for MCF-7), showing to be a very useful solution for a more sensible, accurate and less expensive method for drug HTS applications.

Based on the results obtained during this master's dissertation, it is possible to conclude that the CV assay protocol was successfully optimized taking into account the fixation of cells and the concentration of CV solution. This assay has a high sensitivity, a signal/cell ratio of lower variance and the results attained have a high precision. This study also led to the conclusion that the CV assay has a higher sensitivity than the MTT assay, but lower sensitivity than the Resazurin assay. However, the higher linearity (higher r^2) of the CV assay results led to the conclusion that the results in the CV assay better reflected the actual number of viable cells in each well than the other two methods (MTT and Resazurin). When the effect of DOX in HeLa and MCF-7 was evaluated through the CV, MTT and Resazurin assays, it was concluded that the

CV assay allowed the acquisition of similar results to those attained by the MTT and Resazurin assays, showing that the CV assay (using cell fixation and 0.5% CV) may also be employed to characterize the drug cytotoxic profile during drug development. The advantages and disadvantages of the optimized CV-based cellular viability protocol, in comparison with the MTT and Resazurin reduction assays are summarized in Table 3.

Table 3. Advantages and disadvantages of the optimized CV assay.

Optimized CV-cellular viability based assay	**Advantages**	• Higher sensitivity than the MTT assay; • Higher predictive capacity than the MTT and the Rezasurin reduction assays (high r^2); • High precision (low overall coefficient of variation); • Results obtained through the CV assay are similar to those acquired by the MTT and Resazurin assays; • More suitable for drug HTS (high Z-factor) than the MTT reduction assay; • Less expensive than the MTT and Resazurin assays.
	Disadvantages	• Lower sensitivity than the Resazurin assay; • Less suitable for drug HTS than the Rezasurin reduction assay (lower Z-factor); • Time-consuming; • Potentially toxic, irritant and mutagenic if basic laboratory safety advice is not followed.

Chapter IV

Conclusions and future perspectives

4. Conclusion and future perspectives

Despite the tremendous evolution in the field of oncology, the currently available therapies still fail to reach the desired therapeutic efficacy. Due to that, new potential therapeutics are being developed by pharmaceutical companies and research centers. However, the pharmaceutic industry, and especially academic institutions, face an uphill battle due to the failure of drugs in the early stages of development, namely during the preclinical phase. Henceforth, the development of new *in vitro* cell culture models aimed for drug HTS has been sped up. Furthermore, the increasing pressure from the regulatory entities (Infarmed, EMA, FDA) to reduce the number of animals used in experimentation has also triggered the demand for more effective *in vivo* assays.

Until recently, monolayer cell culture had remained as the main methodology used for screening potential new drugs. Many different assays are described in literature to assess the effect of the cytotoxic drugs in cancer cellular viability (e.g. MTT, Resazurin, MTS, TB dye exclusion, LDH release assays). However, most of these assays are extremely expensive when performed in large scale. Therefore, there is a huge demand for new cell viability quantification methods with high sensitivity, precision and low cost.

In this dissertation, the CV cellular viability assay was optimized considering two key elements: cell fixation and CV solution concentration. The results obtained revealed that performing the fixation of cells before staining them with a highly-concentrated CV solution yielded not only a higher sensitivity, precision and linearity, but also heightened the suitability of this method for HTS. It was also observed that the CV assay can be used for assaying the therapeutic efficacy of a particular drug. The therapeutic effects obtained through the CV assay were comparable to those attained through the MTT and Resazurin assays.

In conclusion, the work developed during this master's dissertation aims to give a contribution to the pharmaceutical industry for the development of new anticancer therapeutics, as it is crucial for drug development that new, quick-to-perform methods that are highly sensitive and precise are established, in order to reduce the number of animals used during *in vivo* testing, before clinical trials take place. The use of this new, optimized and cheap assay for large-scale anticancer drug screening will also allow to decrease the costs associated with the drug development process.

In the future, this assay may also be used to evaluate cellular viability within 3D cell culture models, which have been shown to better mimic *in vivo* tumor behavior and the cells' pharmacological response. This conversion would enable the optimization of the CV cellular viability assay in 3D culture models, a procedure yet to be performed for widespread assays such as the MTT and Resazurin assays. Furthermore, the conjugation of this optimized CV assay with other assays is another possibility up for consideration.

Chapter V

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5. References

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