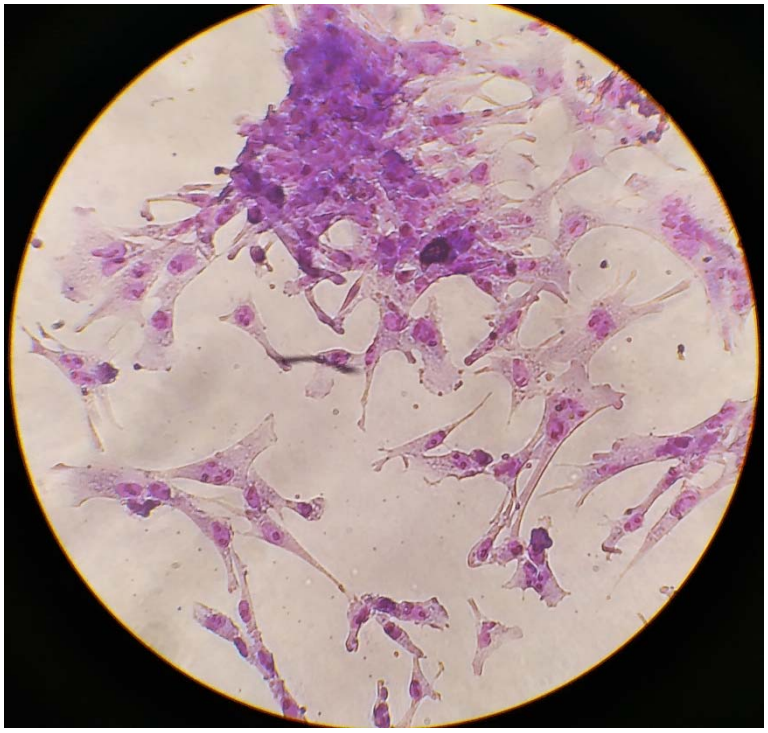
Instituto Universitario de Bio-Orgánica Antonio González
Universidad de La Laguna

SECCIÓN DE BIOLOGÍA | FACULTAD DE CIENCIAS

DEVELOPMENT OF PHENOTYPIC SCREENING METHOD BASED ON CELL STAINING

DESARROLLO DE MÉTODOS DE CRIBADO FENOTÍPICO BASADOS EN TINCIÓN CELULAR



Trabajo de Fin de Máster

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BioLab

La presente memoria de investigación ha sido realizada por la alumna A. Vaitiare Estupiñán Díaz durante el curso académico 2019-2020 en las instalaciones del Instituto Universitario de Bio-Orgánica "Antonio González" (IUBO-AG) y bajo la dirección de los Dres. José M. Padrón Carrillo y Ricardo Reyes Rodríguez. El trabajo forma parte de la línea de investigación *Diseño, descubrimiento y evaluación de fármacos anticancerígenos.*

La Laguna, a 5 de julio de 2020.

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ABSTRACT

The traditional drug discovery process continues to be tedious, costly, time consuming and very risky. One of the hurdles that science faces is that reproducibility varies highly, or in some cases, the experimental results are non-reproducible at all. This drawback is even worse when considering drug development.

Our research focuses on drug development evaluation at the early stages. We seek to revalue the traditional protocols in order to enrich our studies. Our hypothesis is that tissue-staining protocols can be adapted to monolayer cell culture to assess early pharmacological profiling of investigational compounds. The general objective is to adapt tissue-staining protocols to assess the early pharmacological profiling of investigational compounds.

Some staining techniques such as eosin B, erythrosine and hematoxylin, alone or in combination with eosin B/ erythrosine, have been studied in previous works. In this work we will also explore the potential of other staining methods of interest, such as Sudan dyes for the visualization of lipids.

In this work, we will explore the application of some of the existing cell and tissue staining protocols to our phenotypic drug discovery program directed at the discovery of small molecules for cancer treatment.

RESUMEN

El desarrollo de fármacos es un proceso que continúa siendo tedioso, costoso, lento y muy arriesgado. Uno de los obstáculos a los que se enfrenta la ciencia es la falta de reproducibilidad de muchos de los resultados experimentales que se obtienen. Este inconveniente es aún peor cuando hablamos del desarrollo de fármacos, por lo que es de vital importancia ser muy rigurosos en las primeras fases de este proceso.

Nuestra investigación se enfoca en la evaluación del desarrollo de fármacos en las etapas tempranas, y buscamos revalorizar los protocolos tradicionales conocidos para poder así enriquecer nuestros estudios. Nuestra hipótesis de partida es que los protocolos de tinción de tejidos se pueden adaptar al cultivo celular en monocapa para evaluar el perfil farmacológico de compuestos en investigación. El objetivo general es adaptar dichos protocolos para evaluar de forma preliminar el perfil farmacológico de compuestos en investigación.

Algunas técnicas de tinción como la eosina B, la eritrosina y la hematoxilina, sola o en combinación con eosina B/eritrosina, ya han sido ensayadas en estudios anteriores. En este trabajo también exploraremos el potencial de otros métodos de tinción de interés, tales como tintes de Sudan para visualización de lípidos.

La finalidad de este trabajo es explorar la aplicación de algunos de los métodos habituales de tinción de tejidos para nuestro programa de descubrimiento de fármacos orientados al tratamiento del cáncer.

INTRODUCTION

The traditional drug discovery process itself continues to be tedious, costly, time consuming and very risky. Significant investments in drug discovery have failed to be accompanied by a parallel increase in the number of new molecular and biopharmaceutical entities (NMEs, NBEs) gaining regulatory approval by the regulatory agencies (FDA and EMA) [1].

One of the hurdles that science faces is that reproducibility varies highly, or in some case, results are non-reproducible at all. This drawback is even worse when considering drug development. Therefore, it is important to pay attention to the existing protocols and eventually revise them. The term Good Laboratory Practice (GLP) can be considered a quality program related to organizational processes and conditions where non-clinical health and environmental safety are planned, performed, monitored, recorded, reported, and archived. Although not a requirement, the basic concepts of the GLP principles encourage the appropriate application of science, since the need to prepare a study plan with detailed arguments about the reasons for performing the study, as well as producing the proposal. Certainly, this could lead to a more rational execution of the study [2].

Successful drugs pass through five stages of development: discovery, pre-clinical research, clinical trials, regulatory agencies approval (e.g. FDA and EMA) and post-market monitoring. On average, obtaining a potential drug candidate from the laboratory to the pharmacy takes about 14 years, costs more than one billion dollars, and has a low success rate (Figure 1) [3][4]. Thus, new chemical entities (NCE) are needed with diverse mechanisms of action, higher efficacy rates and reduced toxicity profiles. In addition, reductions in drug development timelines could benefit from a better

understanding of the mechanisms responsible for the development of the disease.

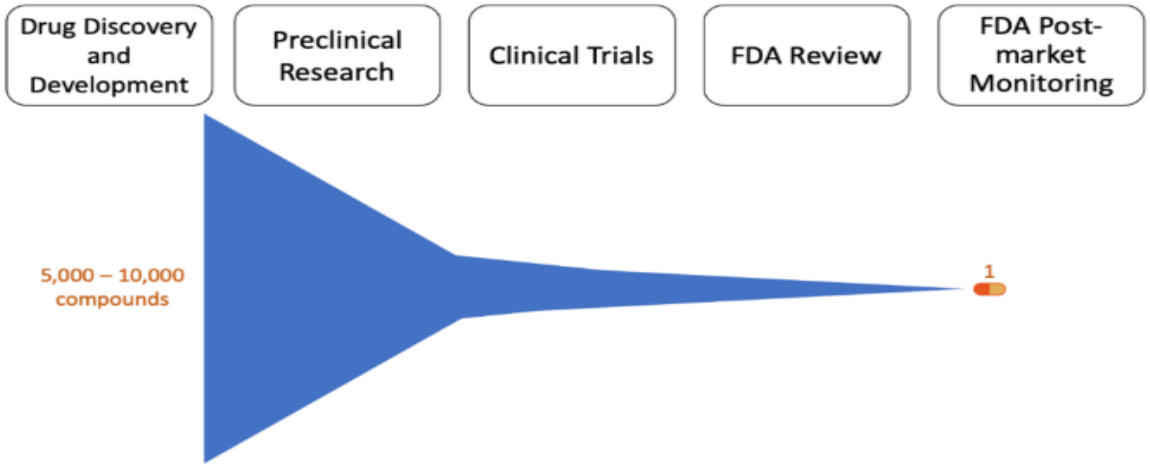

Figure 1. Stages of Drug Development. The height of the blue figure represents the number of compounds that progress from the first to the last stage of drug development over time. *Adapted from [4]*.

Every drug development pathway begins with non-clinical or preclinical assays. These assays should be rapid, sensitive, and inexpensive [5]. Each potential drug is filtered through a system of a dozen progressively more expensive and involved tests/trials to determine if the potential drug is both safe and effective. The preclinical development phase primarily aims to identify which candidate therapy has the greatest probability of success, assess its safety, and build solid scientific foundations before transition to the clinical development phase. Also, during the non-clinical development phase, the candidate compound should meet non-medical objectives, including defining the intellectual property rights and making enough medicinal product available for clinical trials. The non-clinical development of a medicine is complex and regulatory-driven [6].

The basic goals of the non-clinical assays are the early identification of the pharmacological properties and to anticipate the toxicological profile of the new developed drugs. Those can be achieved by studying their mode of

action (pharmacodynamics, PD) and ADME properties (pharmacokinetics, PK). In this study, we will focus on the initial stages of the preclinical studies and, thus, we will pay attention to the PD at the molecular level, i.e. the molecular pharmacology of the NCEs.

The International Council on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use (S7A) defines three types of pharmacological studies (Figure 2) [7][8].

- Primary pharmacodynamic studies are those in which the mode of action of a substance is investigated in relation to its desired (therapeutic) effect.

- Secondary pharmacodynamic studies investigate the mode of action and/or effects of a substance not related to its desired therapeutic target.

- Safety pharmacological studies are those that "investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic range and above" [9].

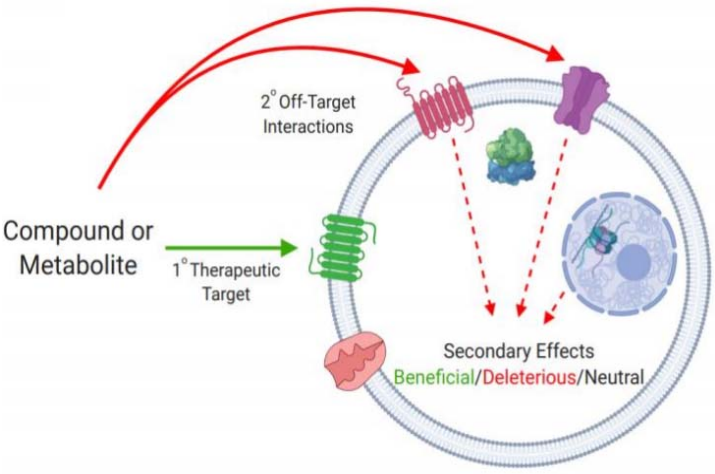

Figure 2. Mechanism of drug action *Adapted from [8]*.

The need to reduce and eventually replace the use of animals in toxicology testing is driving the development of Integrated Testing Strategies

(ITS), which aim to predict human in vivo toxic doses from concentrations that cause effects in vitro, with a minimum of intermediate animal testing. This implies the need to consider both toxicokinetics (TK) and toxicodynamics (TD) as important, if not essential, parts in the risk assessment strategy. For in vitro experiments, we can construct a model comprising the fate of a compound in the cell-based assay, that is, its partitioning between the plastic wall, serum proteins, and lipids, and potentially the compounds dynamics within the cell; combined with a cell growth model and a toxic effects model [10]. Cell-based assays are powerful tools to explore and to anticipate the mechanism of action of NCEs, even when those effects are not being seen on the target tissues.

Our research is framed in assessing drug development, no matter the type of assay endpoint cell line, the number of cells, the concentration of the therapeutic drug, the concentration of the ligand, the incubation time, the desirable assay sensitivity, etc. More knowledge of these key assay parameters needs to be generated through additional experimentation [11].

Staining techniques can be used in almost all categories of pharmacological studies, applying the most appropriate one depending on the parameters of interest that need to be evaluated. Staining techniques remain important tools for many pathologists, providing a powerful complement to immunohistochemistry, flow cytometry, in situ hybridization and other diagnostic technologies that ultimately define a patient's medical profile. They play an important role in diagnosing and monitoring cancer [12]. One of our objectives is the adaptation of fast and low-cost staining protocols to cell cultures, in order to standardize screening methods in drug development.

The basic mechanism of staining techniques is based on acid-base or oxide-reduction reactions, in which one or more dyes interact with different cellular components depending on their chemical affinity. In addition,

visualization is not based on specific binding as in immunochemical or in situ hybridization techniques. Staining techniques rely on two major points: the fixation and the staining protocols.

In cell biology, fixation is the preservation of tissues from autolysis or the action of external factors, by physical or chemical methods. The procedure stops any ongoing biochemical reactions and increases the mechanical stability of treated tissues. Fixation, therefore, is a previous and critical step for cytological processing of cells in monolayer cultures. Although there are standard fixation methods, the correct fixation requires optimization. This implies that the optimal method must be determined empirically and it needs to be adapted to the sample under analysis. Freezing is the physical method of choice for cell and tissue fixation, while chemical fixation uses different fixatives, mainly aldehydes, acids and alcohols, alone or in combination. Common fixation methods and techniques include freezing, immersion and drying (Table 1) [13].

Table 1. Common fixation methods and techniques.

Freezing	Samples with antigens that are too labile for chemical fixation or exposure to the organic solvents used for paraffin-embedding can be embedded in a cryoprotective medium, such as optimal cutting temperature (OCT) compound, and then snap-frozen and stored in liquid nitrogen.
Immersion	Samples are immersed in fixative which then diffuses into and through the tissue or cell sample. Immersion is often combined with perfusion to ensure thorough fixation throughout the tissue.
Drying	Blood smears for staining are air-dried and waved across a flame to heat-fix the cells to the slide.

Fixation is always a compromise, and the requirements of a fixative vary according to the different techniques employed in visualizing the structure of the cells and tissues. Thus, techniques for cytology and histology differ from those for electron microscopy. In addition, the application of different staining methods requires variations in the fixation protocol, such as

air-drying in Giemsa staining of blood smears, and wet fixation for Papanicolaou's method [12].

Staining techniques are used to enhance contrast in tissue and cell samples, generally at the microscopic level. Dyes are frequently used in medicine for the histological and histopathological study of diseases and in the development of new drugs [14]. Table 2 shows a broad classification of staining techniques.

Table 2. Classification of staining techniques. *Adapted from [14]*.

Simple	Allows determining the shape, size, and cell arrangement. It is a quick and simple method to carry out. To perform this staining, the use of a single stain alone is required. There are of two types, namely direct and indirect staining.
Differential	*Topographic*: Difference between the physical and chemical properties of different parts of the cell. To perform this type of staining, the use of more than one dye is required. An example is H-E/Er staining.
	Histochemical: This type of staining identifies specific components in cells and tissues. To perform this type of staining, the use of more than one dye, generally three (trichrome stains), is also required. Examples of these are Masson's staining for connective tissues or Alcian blue staining for the identification of mucoplolysaccharides.

Cell viability is one of the most used parameters in drug development. A cell viability assay is often based on analyzing ongoing cellular metabolism and enzyme activity, measuring factors that reflect the number of living cells in a population. Morphologic diagnosis is based on predominant lesions in the cells or tissue. It may be macroscopic or microscopic and describes the severity, duration, distribution, nature of the lesion and localization. It is a powerful tool for gathering information on the effects that cells experience after the administration of a drug [15].

For routine diagnosis, the use of hematoxylin and eosin (H-E) is the technique of choice for pathologists for viewing details of cellular and tissue structure. The variation of stain intensity is often driven by the pathologist's

learning experience and personal preference. Because this stain demonstrates such a broad range of cytoplasmic, nuclear, and extracellular matrix features, nearly all teaching texts use H-E images. We continue to use this simple and essential stain today, which has remained unchanged for well over a century.

In drug discovery, we expect cancer cells to be killed or injured rather than normal or healthy cells. Regarding their morphology, we seek for changes that indicate how cells are dying. The most common types are necrosis and apoptosis. Necrosis is characterized by the relatively slow disintegration of the cell without the features indicative of active cell death. This process debuts with changes at the cytosolic level, which can be detected with a topographic stain such as H-E. Observed effects include a hydropic or fatty change that is evidenced by the presence of vacuolization and which can be confirmed with other staining techniques such as Sudan black stain for lipid detection. Finally, necrosis is confirmed by condensation (picnosis), nuclear fragmentation (karyorrhexis) and nuclear break (caryiolisis). In contrast, apoptosis is used to describe the morphological mechanism underlying a cell-autonomous active process in which a specialized signaling pathway is active in killing the cell and organizing its disposal. In most situations, cells dying by apoptosis display a very similar pattern of morphological changes. Dying cells start to split attachments to other cells and the extracellular matrix, round up, and protrusions from the plasma membrane (blebs) start to form. The condensed nucleus can be seen to disassemble into several fragments. The entire cell condenses and is reorganized into apoptotic bodies [16].

As part of our interest in the discovery of NCEs for cancer treatment, we will explore the application of some of the existing cell and tissue staining protocols to our phenotypic drug discovery program.

HYPOTHESIS

In the preclinical evaluation of anticancer drugs, there are several methods to assess phenotypic effects. Those protocols get more sophisticated over time and we want to revalue traditional known protocols.

Our hypothesis is that tissue-staining protocols can be adapted to monolayer cell culture to assess early pharmacological profiling of investigational compounds.

OBJECTIVES

The general objective is to adapt tissue-staining protocols to assess the early pharmacological profiling of investigational compounds.

In order to achieve the proposed objective, we will:

1. Run a bibliographic search to look for tissue and cell staining protocols
2. Adapt the staining protocols to monolayer cell cultures
3. Run cell-based assays to assess the phenotypic changes induced by the drugs
4. Classify dyes and cell lines according to the phenotypic parameters to study

MATERIALS AND METHODS

1. Bibliographic search

We will search in three accessible and well-known databases: PubMed, Scopus, and Google Scholar. Article selection process will be based on how close the title and abstract were to the field.

2. Cell lines and culture

2.1. Cell lines

Initially, we will select a panel of human solid tumor cell lines comprising breast (HBL-100, T-47D and MCF-7), lung (A549, and SW1573), colon (WiDr), and cervix (HeLa) cancer cell lines. The cell lines were kindly provided by Prof. G. J. Peters (VUMC, Amsterdam), Prof. A. Pandiella (CIC, Salamanca), Dr. R. Freire (HUC, Tenerife) and CEAMED S.A. (Tenerife).

2.2. Cell passage and maintenance

Cells will be grown in RPMI 1640 supplemented with 1 mM glutamine, 5% fetal bovine serum and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin.). Cells will be kept at 37ºC in a humidified atmosphere of 5% CO_2 and maintained at low passage.

2.3. Cell seeding

Exponentially growing cells will be trypsinized and resuspended in medium. Single cell suspensions will be counted (Moxi Z, Orflo, IN, USA) and diluted to reach the appropriate cell densities for inoculation onto six-well plates for each cell line. Cells will be grown on cover slips by placing them inside each well before cell inoculation. This allows easy staining of cells and visualizing the results.

2.4. Throughput screening

For throughput screening, cell chamber slides will be used. A chamber slide consists of a removable polystyrene media chamber attached to a glass slide treated for adherent cell culture. The sterile system provides optimal binding sites for complex cells. The removal tool allows us to separate the camera from the glass slide. Cell seeding densities and medium volumes will be adapted.

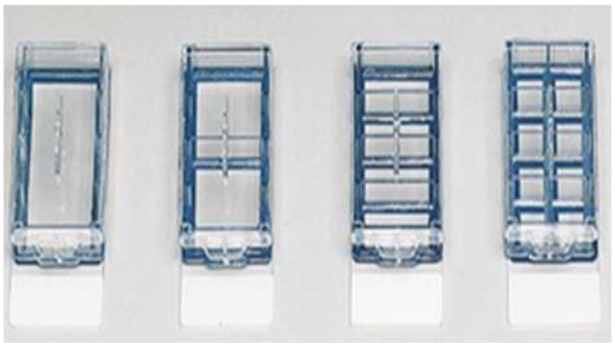

Figure 3. Commercially available chamber slides.

3. Drug exposure assays

3.1. Drugs

Commercially available drugs to be tested will be dissolved in DMSO at an initial concentration of 40 mM. If needed, intermediate dilutions will be prepared in fresh culture medium prior to inoculation onto plates.

3.2. Drug addition and incubation

On the next day after seeding, pure compounds will be added at diverse doses according to their GI_{50} values against the corresponding cell line under study. Cells will be exposed to the drugs for 48 hours, after which time the fixation and staining protocols will be run. Control cells will be treated with an equivalent concentration of DMSO (negative control).

4. Fixation

At the end of drug incubation, medium will be aspirated, and cover slips will be washed with PBS at least twice to remove all the medium. Cells will be fixed with a 3.7% formaldehyde solution of PBS for 30 minutes. Then, the fixing solution will be removed and cells will be washed with PBS. If staining protocols are not performed immediately after fixation, cover slips will be covered with PBS and kept at 4ºC until the staining protocol could be performed.

5. Staining

Cell staining will be carried out incubating for the appropriate time (2–4 minutes) the coverslips with the corresponding dye according to each of the protocols. The excess of dye will be rinsed with water (deionized or tap) or alkaline solution as indicated. After running the corresponding staining protocol, all cover slips were placed in slides glasses to protect samples and improve microscope visualization. A drop of buffered glycerin was placed on the coverslips and mounted on slide for viewing under the light microscope.

5.1. Eosin B

To prepare a 0.5% (w/v) solution, dissolve at room temperature 2.5 g of eosin B in a mixture of 495 mL of water and 0.5 mL of acetic acid. This solution is stable for several months and may be used repeatedly. Molds often grow in it and need to be removed by filtration. The addition of a crystal of thymol to the solution helps to retard the growth of molds. After staining cells, rinse with 96º ethanol.

5.2. Erythrosine

To prepare a 0.5% (w/v) solution, dissolve at room temperature 2.5 g of erythrosine in 500 mL of distilled water. After staining cells, rinse with deionized water.

5.3. Hematoxylin

Stain cells directly with commercially available Harris hematoxylin (0.1% w/v) without modification. Rinse the excess of dye with alkaline solution (0.1% NaOH) or running tap water (pH ~ 9).

5.4. Hematoxylin mixtures

Double staining is achieved by the sequential addition of two dyes. Stain cells with hematoxylin as indicated above. Then, rinse with deionized water and stain with erythrosine or eosin B, as indicated.

5.5.Oil red O

Cover slides with the commercially available solution for 12 minutes, rinse with tap water for 1 minute, cover with hematoxylin solution for 1 minute, wash a second time in running tap water for 3 minutes, and mount [17].

5.6. Sudan black B

Add 100 % propylene glycol to dehydrate for 5 minutes. Decant propylene glycol and add commercially available Sudan black B stain for a minimum of 2 hours (overnight is preferred). Wash with three exchanges of tap or deionized water. Wipe the backs of the coverslips clean using cotton tipped swabs. Differentiate in 85% propylene glycol for 3 minutes. Rinse several times with deionized water and then gently mount the coverslips with the aqueous mounting medium onto a labeled glass slide. If air bubbles are present after cover slipping, they should not be pressed out, since this process will displace stained lipid. Instead, re-immerse the slide in warm water until the coverslip falls off; re-wipe excess water from the slide and re-coverslip [18].

6. Microscope visualization

Stained cells will be observed under the microscope (Axiovert 40 CFL, Zeiss, Germany). For each sample, the pictures will be taken at three different magnifications (100x, 200x, 400x) using the software recommended by the manufacturer (ZEN 2012 blue edition v1.1.0.0). For comparison purposes, the most representative aspects of cancer cell morphology will be used. We will observe phenotypic characteristics such as cell culture organization, cell and nucleus shape, nucleus position in relation to the center of the cell and its size proportion to the cytoplasm [2]. Also, more specific details are considered, such as changes in membrane permeability or in cellular organization, presence of granules or inclusions, protrusion, etc.

IMPLEMENTATION

1. Bibliographic search

We started with a primary literature search in publicly available databases. The terms chosen for the search were: “drug development process”, “cell staining techniques, “topography stain”. We found more than 50 scientific papers related to drug discovery and drug staining techniques. Not all of them were useful for our objectives. Around 23 papers or books were used in this study.

2. Adaption of the staining protocols to monolayer cell cultures

Initially, we studied the effect of cell seeding density. We found that the appropriate cell densities for inoculation onto six-well plates for each cell line were 15,000 (A549 and SW1573), 20,000 (WiDr), 25,000 (HBL-100 and MCF-7), 30,000 (HeLa); and 50,000 cells/well (T-47D).

Next, we explored the time needed for staining. In the first experiments, we used the most common cancer cell line HeLa. This cell line was selected because the cell boundaries can be seen vividly. The results are shown in Table 3.

Table 3. Optimized staining conditions for monolayer cultures.

	Dye concentration (% w/v)	*Staining time (minutes)*
Eosin B	0.5	2
Erythrosine	0.5	2
Hematoxylin	0.1	3–4
Hematoxylin-Eosin B [a]	0.1 + 0.5	4 + 2
Hematoxylin-Erythrosine [a]	0.1 + 0.5	4 + 2
Oil red O	Not tested	
Sudan black B	Not tested	

[a] Values for first and second dye.

The staining with a single dye worked well with the recommended staining times. However, for dual staining cells were exposed to hematoxylin for longer periods (4 minutes) in order to increase contrast and visualize morphological changes or alterations in cell nuclei with more detail.

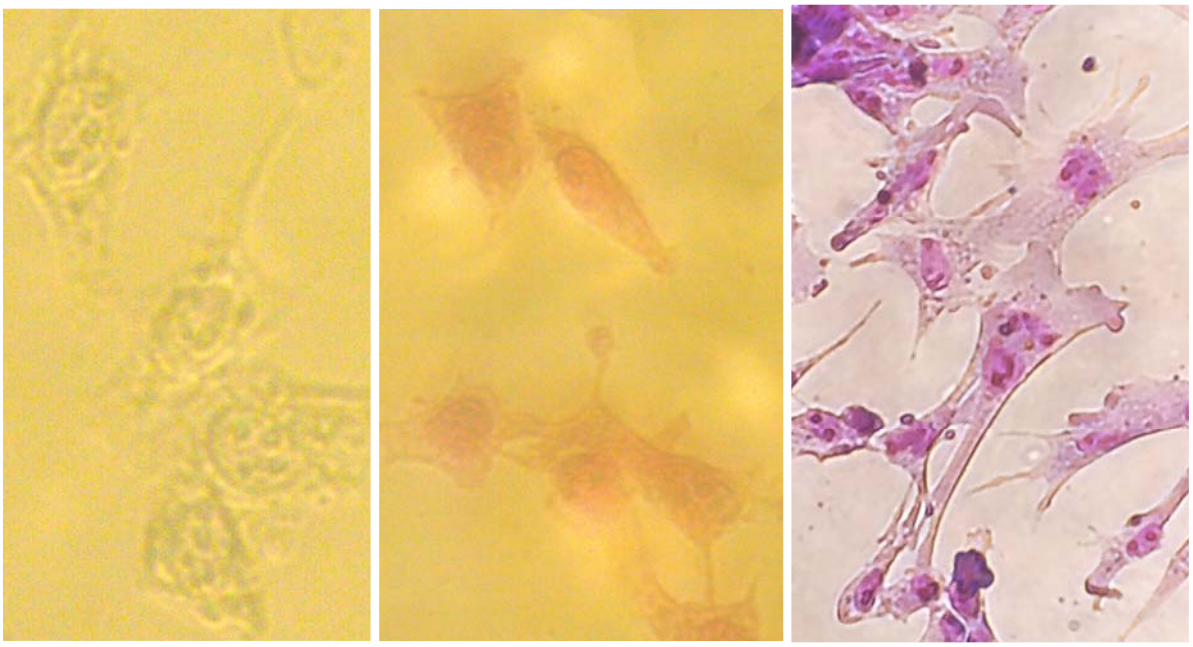

Figure 4. Untreated HeLa cells. Left: non- stained. Center: stained erythrosine. Right: stained with hematoxylin. All pictures were taken at 400x.

Once the staining conditions were established, we studied the shape, size and properties of untreated cells. This must be accomplished before any drug is assayed. Figure 4 shows a representative staining of untreated HeLa cells. The usual appearance of HeLa cells is the shape of a "fried egg". The nucleus is almost 60% of the volume of the cell and looks granulated as the chromatin were very condensed. Other organelles can be vaguely seen as dark spots in the middle of the cytosol. Cell limits are clearly defined, and cells are almost star shaped.

Notice to the reader: At this point of the work, we suffered COVID-19 lockdown and we were unable to continue with the programmed experiments. In addition, we could not take the pictures of the slides already prepared.

3. Phenotypic changes induced by the drugs

After 24 hours of incubation, drug should be added to cells at the corresponding doses. We set a total exposure time of 48 hours. For the preliminary tests, we considered two standard anticancer drugs widely studied and with different mechanisms of action. We selected the microtubule stabilizing agent paclitaxel and the DNA binding drug cisplatin. Paclitaxel induces apoptosis by stabilizing the microtubules, thus inhibiting cellular mitosis, and ultimately inducing apoptosis. We expect treated cells to be rounded because of cell cycle arrest at the G_2/M phase [19]. Cisplatin produces DNA damage and activates a number of cellular pathways, which induce a transient S-phase arrest [20].

4. Classification based on phenotypic effects

Overall, anticancer drugs induce cell death. Thus, the phenotypic effects that could commonly take place are related to the nucleus and cell membrane because the drugs commonly affect cell cycle checkpoints. Morphological changes in the cytoplasm also occur. In a previous work, we defined some preliminary characteristics to be considered (Table 4) [21].

Table 4. Morphological changes in cultured cells and detection by cell staining.

	Nuclear changes		***Cytoplasmic changes***	
Necrosis	Picnosis[a]	Hematoxylin	↓pH (Eosinophilia)	Eosin B, Erythrosine
	Karyorrhexis[b]	Hematoxylin	Hydropic change[d]	Eosin B, Erythrosine
	Cariolysis[c]	Hematoxilin	Fatty change[e]	Eosin B, Erythrosine Oil red, Sudan black
Apoptosis	Picnosis	Hematoxylin		
	Karyorrhexis	Hematoxylin		
	Apoptotic bodies by cellular fragmentation		Hematoxylin- Eosin B/Erythrosine	

[a] Chromatin condensation, [b] Nuclear fragmentation, [c] Nuclear break, [d,e]. Formation of aqueous or fatty vacuoles in the cytoplasm.

EXPECTED IMPACT

Tissue staining is being used already in the study of drug effects. In fact, hematoxylin and eosin potential was investigated as counterstaining to allow for visualization of a marker while confirming the diagnosis on the same slide [25]. However, the authors did not explain the specific morphological changes that the cancer cells underwent. Having staining techniques as a vital part of the study of morphological changes in treated cells, to the best of our knowledge, the literature consulted shows a lack of reports about using cell staining techniques for drug profiling. Furthermore, we could not find evidence of the use of cell staining in screening programs for drug discovery.

Our work was inspired by the results obtained during the study of the morphological changes induced by CK1ε inhibitors in the breast cancer cell lines HBL-100, MCF-7, MDA-MB-453 and T-47D [21]. In that study, we observed that diverse morphological effects were identified in stained cell lines, which were related both to the cell line and the drug used. We also found that some cell lines were more difficult to work with.

We envisioned that tissue-staining protocols could be adapted to monolayer cell cultures to assess early pharmacological profiling of investigational compounds in a cheap, fast and reliable fashion. Moreover, taking the advantage of the differential staining that dyes can provide, we should be able to study the entire cell morphology more clearly (Table 4). We focus our attention on a set of dyes. Their scope is discussed below.

Eosin B binds to and forms salts with basic, or eosinophilic, compounds like proteins containing amino acid residues such as arginine and lysine. This dye stains them dark red or pink as a result of the actions of bromine on fluorescein [22]. We expect cytosol and basic components of the cells to be pink under eosin B stain. This dye is a promising candidate, because while

allows the visualization of morphologic changes, it also permits identifying pH changes due to the drug effect.

Erythrosine belongs to the family of xanthene derivatives, characterized by strong light absorption in the visible range and ability to initiate photochemical reactions [20]. When it is used in contrast with hematoxylin, it can be used as an analog of eosin B. It colors cell structures that are not stained by hematoxylin. With these considerations, we expect the results of erythrosine to be similar to those of eosin B when it is used alone or in combination with hematoxylin.

Hematoxylin is a naturally derived dye that has been used for histological staining. Hematoxylin produces a dark-blue coloring of acid cellular structures, such as the nucleus, and will also stain rough endoplasmic reticulum, ribosomes, and acid mucins [23]. Hematoxylin is used in combination with other stains to create contrast.

The combination hematoxylin and eosin B (H-E) staining is a well stablished technique in histopathology. The contrast between the basophilic hematoxylin and the acidophilic eosin B allows for a clear distinction of the different parts of the cells and of different cell types [24]. H-E staining will allow the monitorization of the pH while identifying the morphology changes induced by the drugs [25].

Another relevant group of dyes to considering are those that stain lipids. In this group, we include oil red O and Sudan black B. Oil red O stains lipid materials in a red-orange color. It is used for the visualization of neutral triglycerides and lipids on tissue sections [26]. We expect to see the unsaturated lipids in intense red, phospholipids in light red and the nuclei in blue, when used in combination with hematoxylin. Sudan black B is a fat-soluble dye which has very high affinity for neutral fats and lipids such as sterols, neutral fats and phospholipids [27]. It stains intracellular lipids and

phospholipids. On microscopic examination, varying degree of black colored pigments are seen in the positive reaction [28].

The final goal of our research proposal will be the (semi)-automation of the method to test multiple compounds in a throughput screening fashion. Our research proposal is focused on producing a big database of phenotypic changes related to cell exposure to drugs. Chamber slide systems could ease the workflow while reduces processing times eliminating the need for prior coating and drying of glass coverslips before seeding cells. Ultimately, we expect to implement the method to be able to stain and examine the cells in 96-well plates. When feasible, this achievement will boost drug discovery.

CONCLUSIONS

1. The literature consulted lacks of reports in which the cell morphology is the main part of the study.
2. We have explored the adaptation of tissue staining techniques to monolayer cell cultures in order to develop a throughput screening protocol for anticancer drug discovery.
3. HeLa cell line was the most convenient cell line for visualizing the phenotypic changes induced by drug treatment.
4. Erythrosine and eosin B can be used as equivalent dyes due to their acid properties.
5. When compared to tissue staining protocols, staining monolayer cultures with hematoxylin in combination with eosin B or erythrosine, required longer staining times than those reported in the literature.
6. Eosin B, erythrosine and hematoxylin are promising techniques to study pH changes inside cell structures together with morphological changes.
7. Combined staining procedures let us see more details at the same time than simple stain.

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