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Universidad de La Laguna

SECCIÓN DE
BIOLOGÍA
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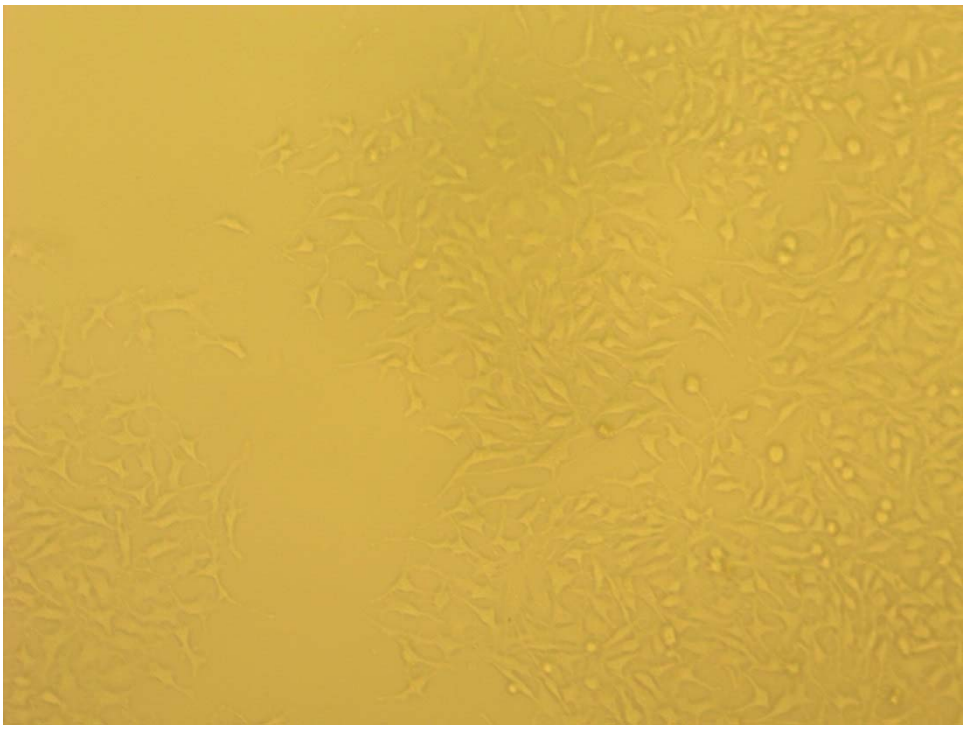
EARLY PHARMACOLOGICAL PROFILING OF SMALL MOLECULES

Perfil farmacológico temprano de pequeñas moléculas



Trabajo de Fin de Grado

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La presente memoria de investigación ha sido realizada por Óscar Tejera de Burgos durante el curso académico 2020-2021 en las instalaciones del Instituto Universitario de Bio-Orgánica "Antonio González" (IUBO-AG) y bajo la dirección del Dr. José M. Padrón y de Adrián Puerta. El trabajo forma parte de la línea de investigación *Diseño, descubrimiento y evaluación de fármacos anticancerígenos.*

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TABLE OF CONTENTS

ABSTRACT

Drug resistance is one of the greatest challenges in cancer therapy. It is common for cancer cells to develop drug resistance after multiple treatment cycles. Diverse mechanisms of resistance have been described. The study of the phenotypical responses of cell lines resistant to anticancer drugs can help to anticipate the outcome of the treatment in patients. Through phenotypic assays, we can observe how a candidate molecule affects a cell line, and from this point, perform tests to understand the role that the candidate molecule plays in the complex mechanism of the pathology. In this study, we have used the clinical drug paclitaxel (PTX) to generate resistant HeLa and T-47D sublines. We were only able to obtain a variant of HeLa cells, which resulted 21 times more resistant to PTX than the parental line. The new subclone was tested with the clonogenic assay and the chemosensitivity assay to compare its biological response to that of the original cell line.

RESUMEN

La resistencia a fármacos es uno de los grandes problemas que existen a la hora de tratar el cáncer. Es común que las células cancerígenas puedan desarrollar resistencias tras varios ciclos de tratamiento. El estudio de las respuestas fenotípicas de las líneas celulares resistentes a los medicamentos anticancerígenos puede ayudar a anticipar el resultado del tratamiento en los pacientes. Mediante ensayos fenotípicos, podemos observar cómo una molécula candidata afecta a una línea celular y, a partir de este punto, realizar pruebas para comprender el papel que juega la molécula candidata en el complejo mecanismo de la patología. En este estudio, hemos utilizado el fármaco clínico paclitaxel (PTX) para generar sublíneas resistentes a HeLa y T-47D. Solamente pudimos obtener la variante de HeLa, que resultó ser 21 veces más resistente a PTX que la línea parental. Este nuevo subclon se evaluó mediante el ensayo clonogénico y el ensayo de quimiosensitividad para comparar su respuesta biológica frente a la línea celular originaria.

INTRODUCTION

The main goal in cancer drug discovery is killing or reprogramming malignant cells while keeping the adverse effects on the non-tumor cells to a minimum. Diverse chemotherapeutic strategies are used to achieve this goal, normally based around the activity or expression of molecular targets in cancer cells different from normal cells. However, drug resistance is one of the great challenges in cancer therapy. Even if cancer cells have a set of intrinsic resistances, it is common for cancer cells to develop drug resistance after multiple treatment cycles [1]. One of the most common mechanisms of resistance to anticancer drugs is the overexpression of ABC transporters, which detect and expel drugs from the cell [2]. Other mechanisms of resistance do exist, such as the reduction of the entry of the therapeutic agent or the overexpression of the target.

We can classify drug discovery into two main strategies, targeted drug discovery (TDD) and phenotypic drug discovery (PDD). TDD relays on the prior knowledge of a molecular interaction in order to select candidate drugs, and is the dominant drug discovery strategy at the moment. However, this method has its challenges and limitations. Despite a large number of targeted agents have being approved and are in clinical development, it is quite rare to find "magic bullets", where a single target is driving the whole cancer phenotype. Normally, the signal mediators targeted in drugs discovered by this method are nodes in complex signal networks, in which other factors can diminish greatly the expected impact of a targeted inhibitor, making them underperform in clinical trials [3]. However, PDD is a discovery process that identifies chemicals that have desirable phenotypic effects on cells, such as changes on their growth rate, morphology or metabolic activities, without needing prior knowledge of their biochemical activity or mode of action against specific molecular targets. Allowing the discovery of novel treatments for diseases for which the root cause is unknown, complex or multifactorial, and for which scientific understanding is insufficient to provide valid molecular targets.

Inhibition of proliferation has been the dominant theme in cancer drug discovery for many decades [4], even pre-dating knowledge of the underlying molecular targets and mechanisms. Such antiproliferative drug discovery has resulted in the development of a core repertoire of chemotherapeutic agents, some of them being first in class drugs [5]. However, most PDD projects are often not target-agnostic, constituting what some authors define as "mechanism-informed phenotypic drug discovery" (MIPDD) [6]. The

experimentation that was conducted in this work would fall into this category of MIPDD, as we knew that paclitaxel (PTX or Taxol®), is a staple in worldwide cancer treatment [7] that functions as a mitotic inhibitor [8].

PTX is a tricyclic diterpenoid that contains a taxane ring and an amide function, occasionally being considered a pseudo alkaloid because of it (Fig. 1). Both the taxane ring and the C-13 side chain were shown to be essential to its cytotoxic activity [9]. Moreover, in order to achieve a correct drug-receptor interaction, the oxetane ring and the homochiral ester chain that it possesses are important features [10]. PTX acts as a chemotherapeutic agent binding selectively to the β subunit of the tubulin proteins, promoting their polymerization and assembly, thereby stabilizing the formation of the microtubules [11]. This causes the formation of a dysfunctional mitotic spindle, which results in a catastrophic mitotic arrest at G_2-M phase and eventually results in cell death through an apoptotic pathway [12]. It has also been informed that PTX restricts tumor angiogenesis and induces the expression of genes and cytokines that lead to the inhibition of cellular growth and apoptosis [13]. The combination of both antiproliferative and cytotoxic properties constitutes a big part of the great antitumor efficacy of PTX [14]. Despite the great antitumor capabilities of PTX, tumor cells eventually will develop taxol resistance after multiple treatment cycles are applied to them [1].

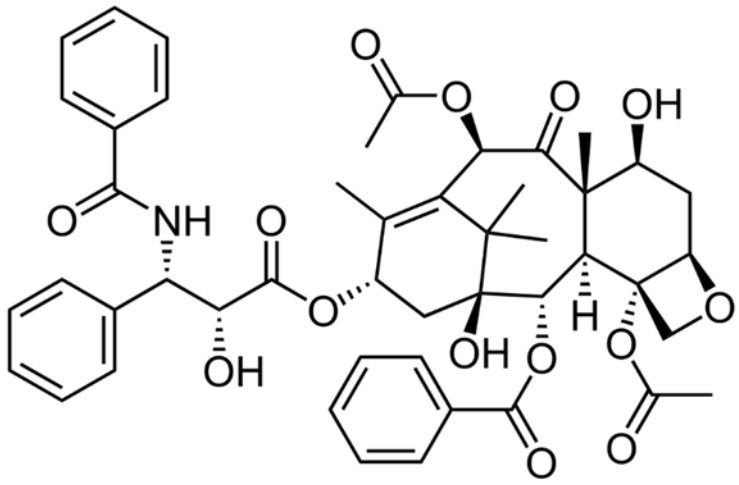

Figure 1. Chemical structure of paclitaxel (PTX).

Chemoresistance is a major problem in cancer treatment associated with poor response, tumor recurrence, and metastasis, being one of the main causes of mortality in cancer patients, and PTX is not exempted from it. PTX-resistant tumor cells have been

isolated and analyzed in the laboratory. Diverse forms of resistance have been described. A series of mutant Chinese hamster ovary cells have altered LX- and/ or β-tubulin subunits that confer resistance to PTX [15]. In other form of resistance described, cells have become dependent on PTX, and actually require the drug for cell replication. The altered tubulin subunits were identified by aberrant migration during electrophoresis on polyacrylamide gels [16]. In PTX-epothilone resistance, isolated human ovarian cancer cells expressed mutant β-tubulin with a diminished capacity for tubulin polymerization by the drugs. The acquired mutations affected the floor of the hydrophobic pocket surrounding the PTX ligand (Phe-270→Val, Ala-364→Thr), modifying this pocket in such a way that PTX is not able to bind correctly to β-tubulin. A smaller taxoid surrogate like epothilone may scape these protein sequence changes and stimulate mutations on the M-loop contiguous to the PTX-binding site, causing resistance to both PTX and epothilone tubulin polymerization [17]. Since PTX resistance is associated with the spindle assembly checkpoint (SAC), proteins such as Mad2, BubR1 and Aurora A are potential markers of PTX resistance. However, suppression of Mad2 and BubR1 in PTX-treated cells eliminated the checkpoint function, leading to PTX resistance correlating with a decrease in the cyclin-dependent kinase-1 activity [18]. Over-expression of Aurora kinase A (Aur-A) and FOXM1 was also observed in PTX-resistant TNBC cells, suggesting that Aur-A may have a part in the protection of tumor cells against PTX [19]. Aberrantly regulated expression of FOXM1 and KIF20A was also associated with PTX resistance in MCF-7 cells [20].

Other mechanism of drug resistance that cells are able to develop is the over-expression of efflux drug proteins, which is associated not only with PTX resistance, but to resistance to multiple chemotherapeutic agents. The ATP-binding cassette (ABC) superfamily of drug efflux proteins includes P-glycoprotein (P-gp), also known as ABCB1 or MDR-1 [21]. The ABCB gene is involved in the resistance to PTX mediated by over-expression of P-gp, which is consequently associated with the efflux of the drug outside of cells [22]. However, sensitivity to PTX in PTX-resistant sublines of SK-BR-3 and MCF-7 cells increased significantly but not completely through silencing of ABCB1. Therefore, a number of mechanisms are suggested to take part in PTX resistance in breast cancer.

Epigenetic modulation may also play a relevant role, representing an important area of molecular regulation mechanisms connected to changes in the expression of miRNAs that play a crucial role in PTX resistance [23]. The acquisition of resistance to PTX was identified in a study focusing on the expression of miR-200c-3p. The over-expression of

miR-200c-3p contributed to resistance of breast cancer cells to PTX therapy [24]. Increased expression of IKBKB also corresponded to resistance to PTX [25]. Additionally, increased expression of the actin-binding protein CapG promoted PTX resistance in cancer cells and was related to PTX resistance in patients through targeting CapG-mediated hyperactivation of the PI3K/Akt pathway [26]. Mutations on the PI3K/Akt pathway have as well been shown to be related to PTX resistance in the HeLa cell line [27]. Autophagy also has been shown to have a positive effect on PTX resistance in cervical cancer cells [28].

In this work, we intend to develop cell lines resistant to PTX by the exposure to the drug at different time intervals. The new cell lines will be characterized through phenotypic assays in an effort to identify the mechanism of resistance to PTX.

HYPOTHESIS

Drug resistance is one of the greatest challenges in cancer treatment. In particular, PTX induce resistance in treated patients. The study of the phenotypical responses of cell lines resistant to PTX can help to anticipate the outcome of the treatment in patients.

OBJECTIVES

The main goal of this work is to evaluate the drug resistance capabilities of our own generated PTX-resistant variants of human solid tumor cell lines. This would permit future studies of the mechanisms behind PTX resistance in this cell lines and foresee methods that could circumvent PTX resistance.

With this goal in mind, we consider the following objectives:

1. Run a literature search to gather information on the mechanism of action of PTX and its drug resistance mechanisms.
2. Perform multiple PTX treatment cycles on the cell lines to make them acquire resistance.
3. Assess the drug resistance capabilities of the generated variants through morphological studies and antiproliferative assays.

MATERIALS AND METHODS

Literature search

We looked for preexisting scientific literature through the well-known and accessible search engines in PubMed and Google Scholar. The terms used for the search were “HeLa PTX resistance”, “HeLa paclitaxel resistance” “T-47D PTX resistance” and “T-47D Paclitaxel resistance”. Article selection process was based around how close the title and abstract were to the field, prioritizing review type articles.

Cell lines and culture

The cancer cell lines used in this study were T-47D and HeLa, both of which are sensitive to PTX. The cell lines were kindly provided by Dr. G. J. Peters (VUmc, Amsterdam, The Netherlands). Cells were cultivated in RPMI medium supplemented with FBS (5%), antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin) and glutamine (2 mM). Cells grow at 37ºC in a humidified atmosphere of 5% CO_2. To prevent cytokines affecting cellular growth, dead cells were removed from the cultures through washing with PBS. Cultures were maintained at low passage.

Generation of a PTX resistant cell line

HeLa and T-47D cells were exposed to multiple treatment cycles with the antitumor drug PTX. Initially, cells were exposed to a high dose of PTX (100 nM) for 24 hours, after which time the cells were observed through the microscope to check for phenotypical responses. Then, the cell culture medium was removed and fresh medium was added to the cells. After 7 days, cells reached confluence and a new 24-hour treatment with PTX (100 nM) was started. Similarly, after 24 hours the cell culture medium was removed and fresh medium was added to the cells. At this point, we run multiple treatment cycles with variable recovery times (4-7 days) starting at an initial concentration of 10 nM of PTX and progressively increasing the dose up to 100 nM for HeLa and 40 nM for T-47D cells (Fig. 2). Drug exposure times were also increased progressively from 24 to 96 hours.

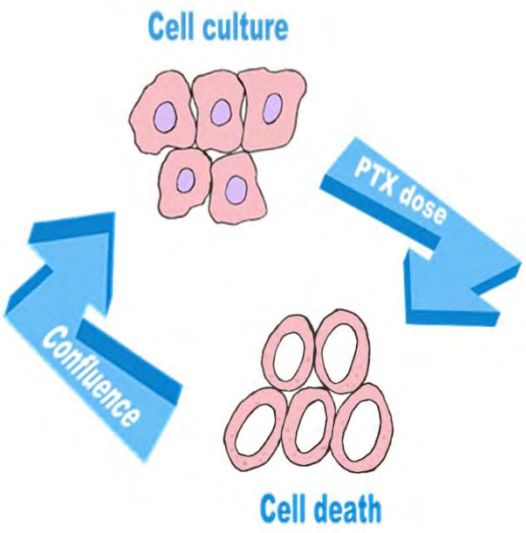

Figure 2. Treatment cycle diagram.

Antiproliferative assays

Clonogenic assay

Cells were initially trypsinized, resuspended in medium and counted with a Moxi Z automated cell counter (Orflo Technologies, Ketchum, ID 83340, USA). Then, cells were seeded in 6 well plates at densities of ~500 cells/well. After 24 hours two doses of PTX (40 and 80 nM) were added to test wells. After seven days of drug exposure, media was removed and wells were washed with 1 mL of PBS for 5 minutes. Immediately afterwards, cells were fixed to the plate using 0.75 mL of absolute methanol, while shaking for 6 minutes. Then methanol was discarded. Cells were then stained for 5 minutes while shaking with 0.75 mL of 1% crystal violet. Then, the dye was rinsed with water, leaving only the stained colonies on the wells. Photos of the wells were taken, and using the image processor software *AutoCellSeg*, colonies were counted in each of them. We defined a colony as a cluster of at least 50 cells.

Chemosensitivity testing

Single cell suspensions were counted (Moxi Z) and diluted to reach the appropriate cell densities to inoculate 2,500 cells/well onto 96 well plates. After 24 hours of equilibration in growth medium, PTX was added at increasing decimal dilution (0.01–1000 nM). The maximal test concentration was set at 1 μM. DMSO (0.25% v/v) was used as negative control. Drug incubation times were 48 hours, following which monolayer cells were fixed onto the wells using of ice-cold trichloracetic acid solution (50% w/v, 25 μL)

for 60 minutes at 4ºC. Then, the plates were rinsed with water, following which sulforhodamine B (SRB) solution (0.4% w/v in 1% acetic acid, 25 µL) was added for 15 minutes. Unbound SRB was rinsed with acetic acid (1%). Cell-bound SRB was dissolved with Tris solution (10 mM, pH 10.5, 150 µL). The optical density of each well was measured at 530 and 620 nm using PowerWave XS microplate reader. This dual wavelength was used to reduce optical interference caused by scratches, fingerprints or other matters that equally absorb light at both wavelengths. The antiproliferative activity, expressed as 50% growth inhibition (GI_{50}), was calculated according to NCI formulas [29].

RESULTS AND DISCUSSION

Literature search

A considerable amount of research was found on HeLa and PTX resistance, with a total of 162 articles. However, much less was found on T-47D resistance to PTX, with less than 30 articles showing up in our literature search. The search results most relevant to us formed the basis of the introduction and are included in the references section of this work.

Cell culture and creation of a drug resistant variant

The mechanism of PTX cytotoxicity has been shown to highly depend on the concentration of the drug in the cell [30]. We initiated the process with high doses, in order to create a great selective pressure, rewarding those mutants with a higher PTX tolerance. As expected, the mortality rate after this first dose was very high (Fig. 3).

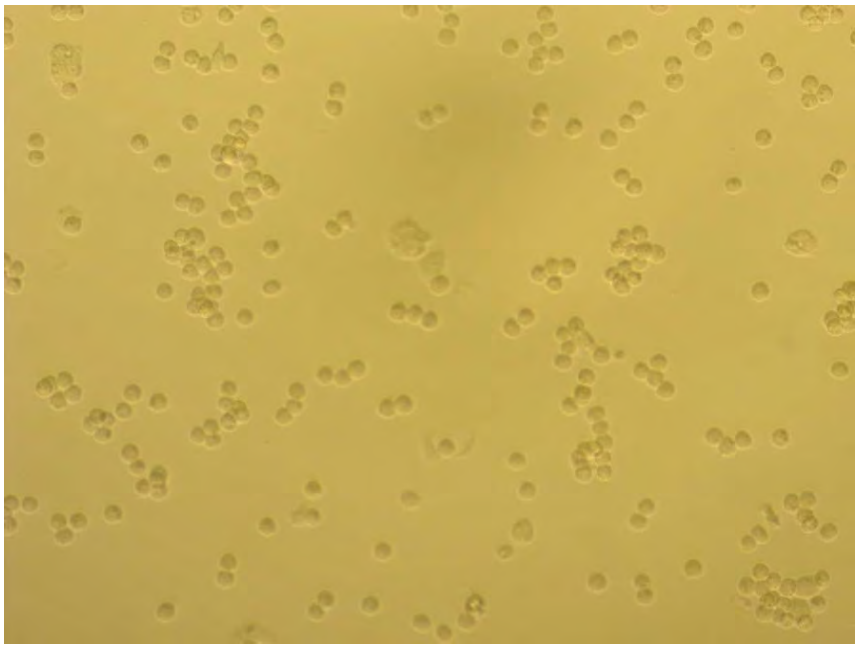

Figure 3. T-47D cells 24 h after the first exposure to 100 nM PTX.

We then added increasingly greater doses for longer periods of time, to generate a pressure that would get us a selection of the most PTX-resistant cells. The reason behind the following relatively lower doses, was to keep the pressure on the already resistant cells without straining them too much. Meanwhile, PTX kept non-resistant cells from succeeding. After successive treatment cycles, a noticeable drop in cell death was observed through the microscope in cells for the HeLa line (Fig. 4). Thus, it was decided to perform the antiproliferative assays on the variant we had generated from the line.

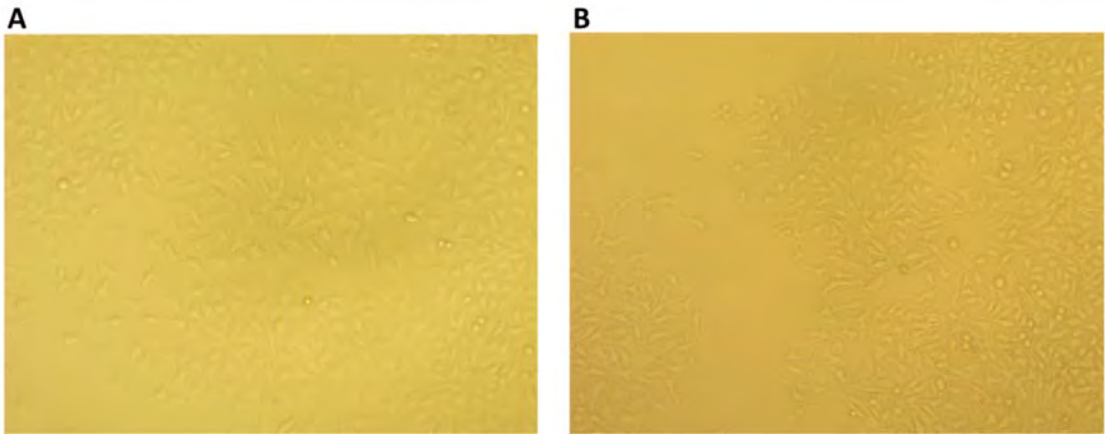

Figura 4. (A) HeLa and (B) HeLa/PTX cultures.

However, we could not achieve the same results with cell cultures from the T-47D line. Even the cells that underwent a morphological change after the low concentration treatments, died shortly after with a slightly bigger dose of PTX (Fig. 5). This may be due to the intrinsic vulnerability of this mammary cancer line to PTX.

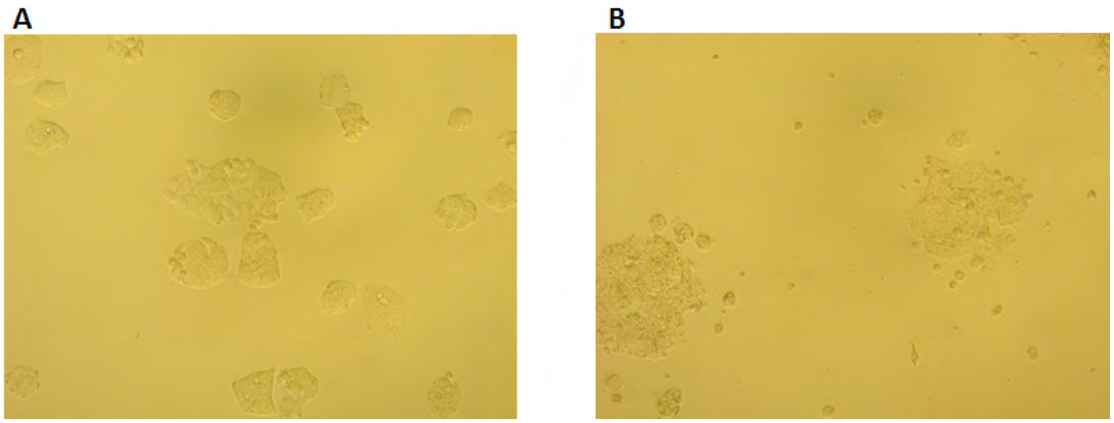

Figura 5. (A) T-47D and (B) T-47D/PTX cultures.

Antiproliferative assays

Clonogenic assay

Cells from the HeLa line grew normally in the control wells. However, no growth was observed in the wells where PTX was present. In the other hand, the HeLa/PTX resistant line did grow adequately in the negative control well, while also presenting a 12% and 0.5% growth at 40 nM and 80 nM PTX doses, respectively (Fig. 6). Overall, the results confirm that the creation of the drug resistant variant HeLa/PTX was a success.

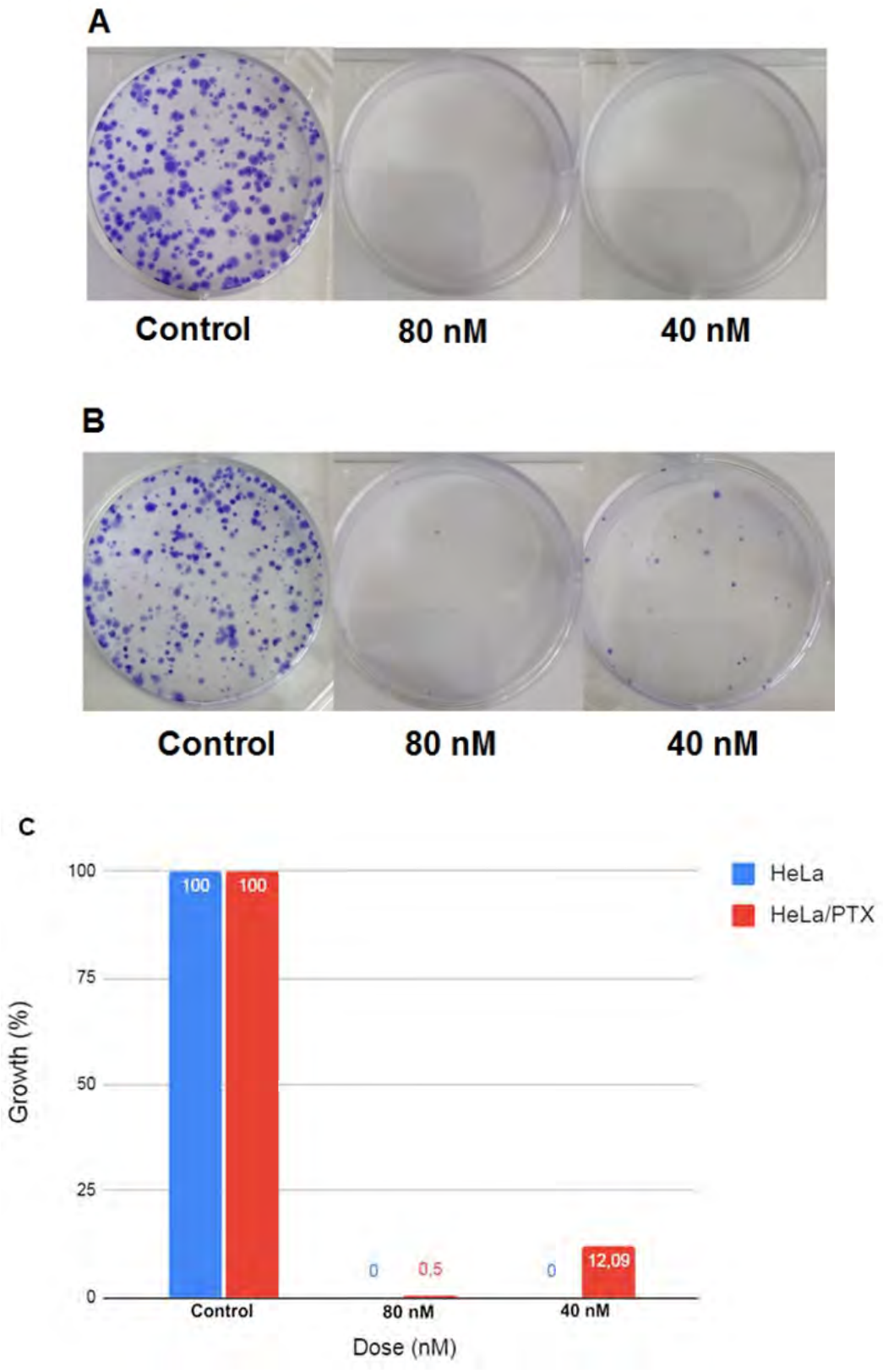

Figure 6. Clonogenic assay on (A) HeLa and (B) HeLa/PTX after 7 days of exposure to PTX. (C) Growth (%) of HeLa and HeLa/PTX after 7 days of exposure to PTX.

Chemosensitivity assay

The experiments revealed that the GI_{50} after 48 hours of exposure to PTX of the HeLa/PTX variant was 21 times higher than the GI_{50} of the wild-type HeLa line (Table 1). As it can be seen in the dose-response curves (Fig. 7), it was also observed that with progressively greater concentrations of PTX, while both lines reduced their growth, the standard HeLa line experimented a much more accelerated and sudden decrease, while the HeLa/PTX variant showed a slower decay in its growth. This further cement the fact that the HeLa/PTX variant has indeed achieved a certain degree of drug resistance to PTX.

Table 1. Antiproliferative activity (GI_{50}) of cell lines exposed to PTX.

Cell line	GI_{50} (nM)	SD
HeLa	2.3	0.3
HeLa/PTX	49	7.8

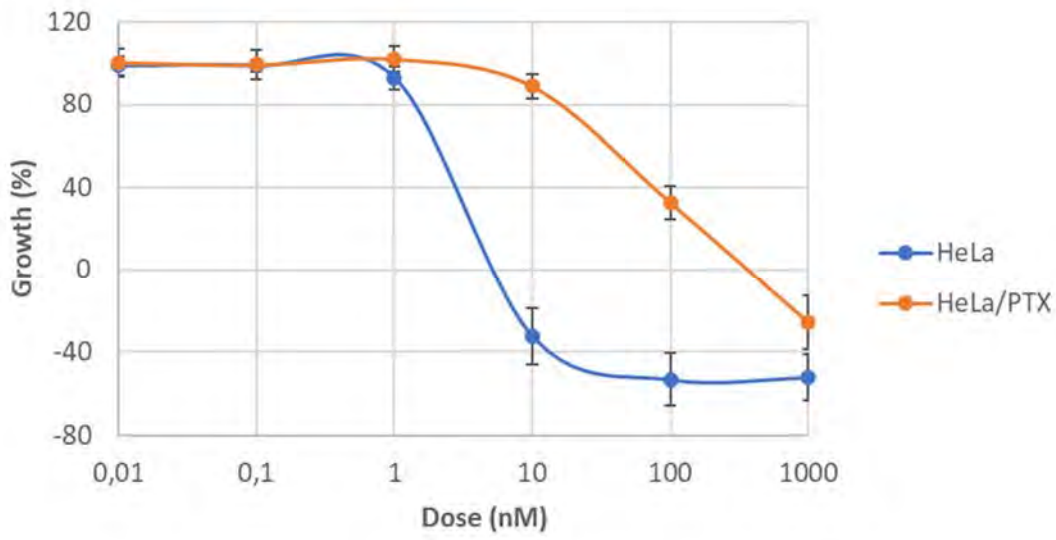

Figure 7. Dose-response curves of HeLa and HeLa/PTX cells exposed to PTX.

In summary, we exposed HeLa and T-47D cells to PTX in order to induce drug resistance. For HeLa cells, we were able to obtain a new variant (HeLa/PTX) that shows more resistance to PTX than the parental line. Further experiments will shed light on the mechanism of the acquired resitance.

CONCLUSIONS

1. HeLa resistance to PTX seems to have been researched a fair amount, as quite some literature was found on our search.
2. T-47D resistance to PTX appears to not be a very researched upon topic, due to the low amount of search results found.
3. The treatment cycles performed seem to be a good way to induce resistance in the HeLa line.
4. Adjustments to the treatment cycles and more time will be needed in order for T-47D to achieve PTX resistance.
5. Having successfully achieved a PTX resistant variant of the HeLa line, further experimentation can now be realized in order to understand the mechanism through which this line achieves its resistance to drugs. This knowledge will be of great value, as it could lead to the development of new therapies to bypass the acquired drug resistances in cancer treatment.

CONCLUSIONES

1. La resistencia de HeLa a PTX parece estar relativamente bien documentada, ya que una buena cantidad de literatura fue encontrada en nuestra búsqueda.
2. La resistencia de T-47D no parece ser un tema muy estudiado, ya que se encontraron pocos resultados en la literatura.
3. Los ciclos de tratamiento realizados parecen una buena manera de inducir resistencia en la línea HeLa.
4. Se requieren ajustes en los ciclos de tratamiento y más tiempo para lograr que T-47D adquiera resistencia a PTX.
5. Habiendo conseguido con éxito crear una variante resistente de la línea HeLa, nueva experimentación podrá ser realizada para desentrañar el mecanismo a través del cual esta línea logra adquirir la resistencia al fármaco. Este conocimiento será de gran valor, ya que podría llevar al desarrollo de nuevas tratamientos capaces de sobrepasar la resistencia adquirida al fármaco en la terapia del cáncer.

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