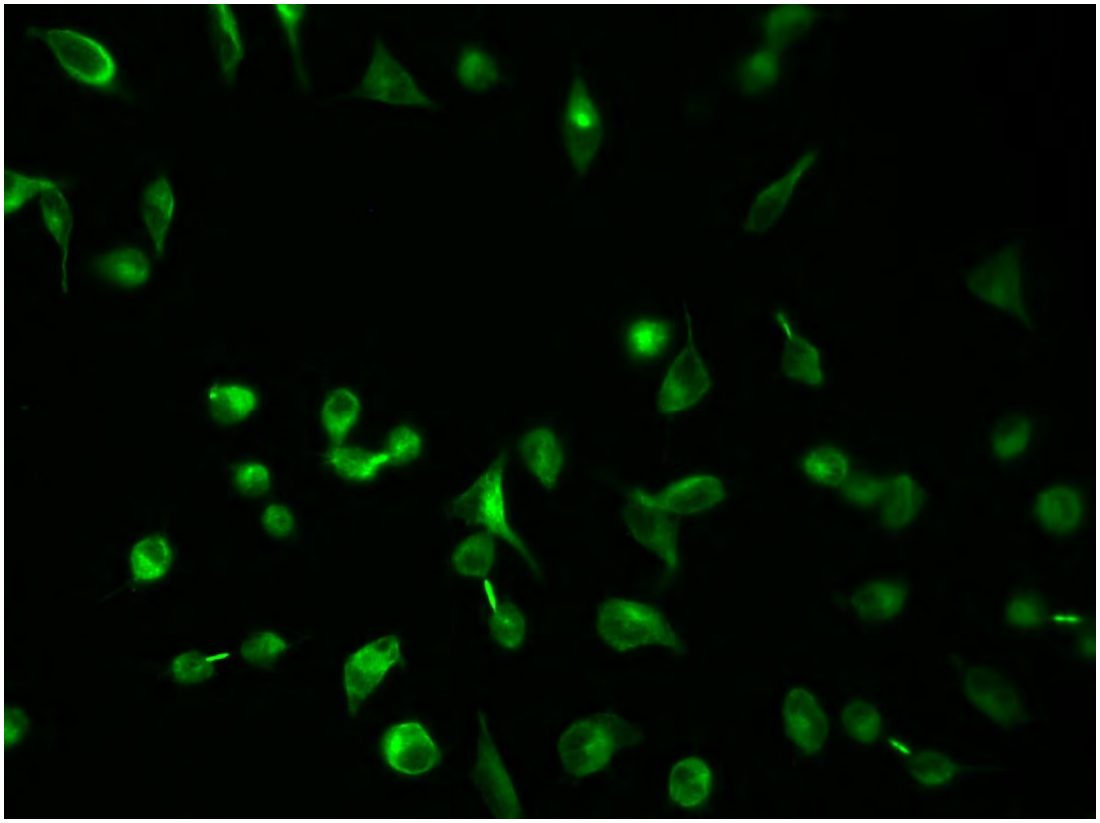
Instituto Universitario de Bio-Orgánica Antonio González
Universidad de La Laguna

SECCIÓN DE BIOLOGÍA | FACULTAD DE CIENCIAS

EARLY PHARMACOLOGICAL PROFILING OF SMALL MOLECULES

Perfil farmacológico temprano de pequeñas moléculas



Trabajo de Fin de Grado

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Grado en Biología

Junio 2021

La presente memoria de investigación ha sido realizada por Aday González Bakker 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.*

La Laguna, a 4 de junio de 2021.

TABLE OF CONTENTS

ABSTRACT 4

RESUMEN 4

INTRODUCTION 5

1. Resistance to antitumor drugs 5
2. Paclitaxel: activity and resistance mechanisms 6
3. Microtubules in cell migration 7

HYPOTHESIS 8

OBJECTIVES 8

MATERIALS AND METHODS 9

1. Cell lines and culture 9
2. Cell migration assay 9
3. β-tubulin expression 10
4. Apoptosis induction 11

RESULTS AND DISCUSSION 12

1. Wound healing assay 12
2. Western blotting 13
3. Indirect immunofluorescence 14
4. Apoptosis induction 15

CONCLUSIONS 16

CONCLUSIONES 16

REFERENCES 17

ABSTRACT

Resistance to antitumor drugs is a major issue in cancer treatment. One of the drugs affected by this phenomenon is paclitaxel (PTX), a cytotoxic alkaloid used against multiple types of tumours. PTX binds to β-tubulin inducing microtubule stabilization altering normal microtubule dynamics during mitosis which ends in cell death. One of the different mechanisms associated to drug resistance is the alteration of β-tubulin. In the case of PTX, changes in microtubule dynamics or β-tubulin could lead to resistant cell types. In this study, we carried out a phenotypic comparison between HeLa cells (sensitive to PTX) and a PTX-resistant variant. The results did not show significant differences in migration dynamics between these cell types, neither in β-tubulin expression and localization. We speculate that another type of mechanism that does not involve microtubules could be responsible for the resistance to PTX, but further experiments will be needed to conclude this.

RESUMEN

La resistencia a fármacos antitumorales es un problema creciente para tratamientos de cáncer. Uno de los fármacos afectados es el paclitaxel (PTX), un alcaloide citotóxico empleado en el tratamiento de diferentes tipos de tumores. Este se une a la β-tubulina e induce la estabilización de microtúbulos alterando su dinámica durante la mitosis lo que finalmente lleva a la muerte celular. Uno de los mecanismos asociados a la resistencia a fármacos son cambios en su diana. En el caso del PTX cambios en la dinámica de microtúbulos o en la β-tubulina podrían ser el mecanismo que otorga resistencia. En este estudio llevamos a cabo una comparación fenotípica entre la línea celular HeLa y su variante resistente a PTX en aspectos que involucran microtúbulos. Los resultados no mostraron diferencias significativas entre ambos tipos celulares en cuanto a la dinámica de migración, tampoco en la expresión ni localización de la β-tubulina. Especulamos que otro tipo de mecanismo que no involucra los microtúbulos es el responsable de esta resistencia al PTX. Sin embargo, más estudios en esta línea son necesarios para determinar el mecanismo de resistencia.

INTRODUCTION

1. Resistance to antitumor drugs

Drug resistance is a phenomenon were diseases become tolerant to a pharmaceutical treatment. For example, bacteria that develop resistance to antibiotics. This circumstance also happens in diseases such as cancer, where an effective treatment can turn useless. In fact, 90% of failures in chemotherapy are related to drug resistance [1]. Drug resistance responds to the intratumour heterogeneity, provoking drug treatments the selection of those cells having a superior growth advantage in a given context [2]. Cancer cells acquire drug resistance through diverse mechanisms [3]. Thus, some resistant tumours overexpress drug efflux pumps, like the ATP-binding cassette (ABC) transporter P-glycoprotein (PGP), the product of the ABCB1 (or MDR1) gen [4]. Drugs affected by this mechanism are the Vinca alkaloids. Other possible mechanisms are drug inactivation or lack of activation, like in cytarabine (AraC) [5]. Drug target alterations such as mutations or changes in the expression, for example thymidylate synthase expression is a key determinant of 5-FU response, also occurs in resistant cell lines [6]. Another possible mechanism is enhancement of DNA damage repair, specially in drugs like cisplatin which induces DNA crosslinks. DNA damage response (DDR) mechanisms can reverse the drug-induced damage. The last resistance mechanism contemplated is cell death inhibition, where in resistant cells, BCL-2 family proteins and other antiapototic proteins are highly expressed [7].

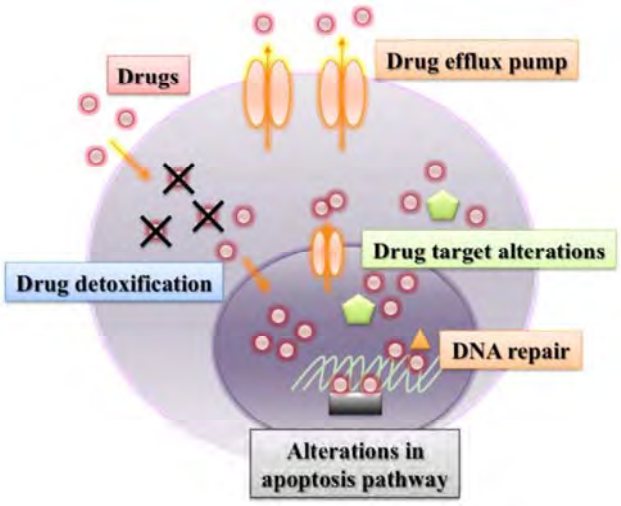

Figure 1. Common mechanisms of drug resistance in cancer cells [3].

These different mechanisms can appear individually or combined in antitumor drugs resistance. This represents a complex challenge where de understanding of the concrete mechanism underlaying resistance is essential to design effective therapies.

2. Paclitaxel: activity and resistance mechanisms

PTX (Taxol®) is a cytotoxic alkaloid, discovered and isolated firstly from *Taxus brevifolia* [8]. It is approved for the treatment of different types of cancer, including breast and ovarian cancer. PTX belongs to the group of antimitotic agents like colchicine or Vinca alkaloids, but shows a different mechanism since it promotes microtubule stabilisation instead of preventing polymerisation. PTX binds to microtubule polymer [9], concretely to β-tubulin enhancing polymerisation of tubulin, even in the absence of GTP. This stabilisation induces mitotic arrest activating the mitotic checkpoint (spindle assembly checkpoint), the cell cycle control mechanism acting during mitosis to ensure that each daughter cell will receive one copy of every chromatid. Chromatids connect to spindle microtubules through their kinetochores. Paclitaxel treatment arrests cells in mitosis due to the presence of a small number of unattached kinetochores that activate the mitotic checkpoint. Cells will either die during mitotic arrest or due to a process called slippage where cells enter directly in G_1 phase resulting in a single tetraploid cell [10]. It has also been reported multipolar spindles induction as PTX mechanism. These multipolar spindles result in multiple aneuploid unviable cells. These different mechanisms are associated to PTX concentration. Higher concentrations induce cell death because of mitotic, whilst at lower concentrations cell death occurs as a consequence of multipolar spindles [11]. In any case, the underlying mechanism of action directly involves microtubules, which are PTX's target. Changes in microtubules could explain the resistance to this drug, which is an increasing problem in chemotherapy treatments.

Furthermore, apoptosis induction through PTX has been reported as a downstream process of microtubule dynamics disruption since microtubule network integrates components of apoptosis [12]. Because of PTX's induction of apoptosis, it has been reported that the overexpression of antiapoptotic protein from BCL2 family, which controls intrinsic apoptosis pathway (or mitochondrial apoptosis pathway), can confer resistance to PTX [13].

The resistance acquired to PTX has been reported related to different mechanisms descripted above, due to overexpression of PGP, changes in the behaviour (microtubule

dynamics) or the composition of his target, or due to the expression of cell death inhibition (antiapoptotic proteins).

3. Microtubules in cell migration

Microtubules are one of the major components of cytoskeleton (including actin and intermediate filaments). They are assembled by GTP mediated union of tubulin heterodimers (constituted by α-tubulin and β-tubulin) forming a tubular structure of ~25 nm in diameter. Once GTP is hydrolysed to GDP depolymerization occurs. The continuous changes from polymerisation (growth) to depolymerisation (shrinkage) is known as microtubule dynamics. This is fundamental to the different functions that perform microtubules [14]. This element of cytoskeleton is fundamental in important processes like cell division or cell migration.

In cell migration, microtubule dynamics has different roles as provide an intracellular transport network for components which are essential to maintain polarity and directionally in cell migration. Microtubules, due to their ability to resist high compressive loads contribute to generate pushing forces [15]. They are important also in the cellular adhesion because they supply and recycle integrins, which mediate union to substrate and transport cadherins that mediate junction between cells. For these to result in efficient directional movement, microtubules need asymmetric organization of the microtubule network. Microtubule dynamics regulation is key to the front/back polarity in the cell. Other characteristics, such as localisation of different kinesins (e.g. kinesin-4Kif4) stabilize microtubules at the cell front, while other types disassemble microtubules at the cell rear [16]. Positive-end tracking proteins (+TIPs) are implicated in capturing microtubules in the front. These (and others) differential regulation of intracellular events at the leading edge versus the cell rear results in efficient directional migration.

In view of the role of microtubules in cell migration, it is anticipated that a change in microtubule stability and dynamics, e.g. as a response to resist to anti-microtubule agents like PTX, could affect the way the cells migrate.

In this work, we compared the migration ability and β-tubulin expression between a cell line sensitive to PTX (HeLa) with its resistant strain HeLa/PTX.

HYPOTHESIS

It is widely known that the treatment of tumours with the anticancer drug PTX induces resistance. Among the multiple possible resistance mechanisms, PTX-induced alterations are likely to occur in the microtubule dynamics of the cells. Thus, resistance to PTX could modify the cell migration properties of cancer cells probably due to changes in the target protein β-tubulin.

OBJECTIVES

The main purpose of this work is to assess differences on the phenotypic profiling of a PTX sensitive and a PTX resistant cancer cell line. To achieve this goal, we define the following objectives.

1. To visualize and quantify the differences on cell migration.
2. To visualize β-tubulin expression.
3. To determine the level of apoptosis induction.

MATERIALS AND METHODS

1. Cell lines and culture

1.1. Cell lines

In this study, we selected the cervical cancer cell line HeLa as a model of PTX sensitive cell line. This cell line was a kind gift from Prof. G. J. Peters (VUMC, Amsterdam, The Netherlands). We also used the PTX resistant cell line HeLa/PTX, which was developed in our group [17].

1.2. Cell passage and maintenance

Cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 5% fetal bovine serum (FBS), and 100 U/mL penicillin and 0.1 mg/mL streptomycin as antibiotics. They were incubated at 37ºC, in humidified atmosphere 5% CO_2 and maintained at low passage.

2. Cell migration assay

To study cell migration, we used the wound healing (scratch) assay, an easy and well-developed method [18]. First, we trypsinised cell cultures to prepare single cell suspensions. After cell counting, we prepared the appropriate dilution for seeding at a density of 30,000 cells/well. We seeded 750 µL onto a 24 well plate and incubate the cells until they reached 80-90% confluence. Afterwards, a mark was drawn on the outside bottom of each well. This will enable us to find the same point (reference point) when taking pictures. For each well, a scratch on the cell culture was made perpendicularly to the mark using a sterile p200 tip. Then medium is replaced for fresh medium without FBS. Pictures were taken with a brightfield microscope (Axiovert 40 CFL, Zeiss, Germany) at one magnification (10X) using the software ZEN 2012 (blue edition v1.1.0.0) at different time intervals (0, 4, 24 and 28 hours from the scratch).

For the quantification of cell migration, we ran a bibliographic search to find a software that enabled us to achieve this task. Free software imageJ (1.53e version) and its plugin *Wound_healing_size_tool* [19] were used. We adjusted the parameters *variance*

window radius and *threshold value* for each image. The algorithm provided the results expressed as scratch area percentage.

3. β-tubulin expression

3.1. Protein extraction

For protein isolation from HeLa and HeLa PTX cells, a cocktail of protease inhibitors (10 µM peptistatin, 20 µM leupeptin, and 800 µM apoprotinin) was prepared in RIPA lysis buffer. The process is carried out in ice to avoid protein degradation. Cells were trypsinised, resuspended in fresh medium and centrifugated at 1000 rpm for 5 minutes (AllegraTM X-12R Centrifuge, Beckam Coulter Inc.). Supernatant (medium) was discarded, and the cell pellet was washed with PBS, passed to a microtube and centrifugated at 200 g for 5 minutes (Eppendorf Centrifuge 5418R). Then, the PBS was removed and RIPA with protease inhibitors was added and incubated 30 minutes in the fridge and 10 minutes in the freezer (–20ºC). Then it was centrifugated at 10000 g for 10 minutes and the supernatant was kept whilst the pellet was discarded.

3.2 Protein quantification

Protein quantification was carried out by bicinchoninic acid assay using PierceTM BCA Protein Assay Kit (Thermo scientific) in 96 well plates. A standard curve of different dilutions of bovine serum albumin (BSA) from a stock solution at 1 mg/mL was prepared in 0.9% NaCl. For protein samples were measured using a 1:5 dilution in 0.9% NaCl solution. Both standard and samples were prepared in triplicates. Thus, 200 µL of a 50/1 mix of reagents A (bicinchoninic acid) and B, prepared in darkness, is added to each well and the plate is incubated at 37ºC for 30 minutes. After that time, the absorbance at 540 nm is measured in a plate reader (BioTek Powerwave XS). The standard curve allowed to calculate, based on their absorbance, the amount of protein in HeLa and HeLa PTX protein extracts.

3.3. Electrophoresis and Western Blotting

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) Laemmli [20] protocol and β-tubulin was detected by Western blotting. Equal amounts of protein (40 µg) were mixed (3:1) with 4X commercial Laemmli buffer solution with 10% β-mercaptoethanol. Next, the mixture samples were denatured by incubation at 96ºC for 7 minutes. Then, SDS-PAGE was performed using a handmade

discontinuous acrylamide/bisacrilamide 10% gel at constant voltage (160V) until dye front reached the bottom of the gel.

After electrophoresis, electro-transfer of the protein in gel was performed. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 280 mA for 1 hour at 4ºC. Afterwards, the membrane was blocked in Tris-buffered saline with Tween (TBST) (100 mM Tris [pH 7.5], 150 nM NaCl, 0.1% Tween) with 5% of BSA for 1 hour. Then, it was incubated with the primary antibody, monoclonal mouse anti-β-tubulin (Sigma-Aldrich®) in a 1:5000 dilution for 2–16 hours. Afterwards, the membrane was washed with TBST three times and incubated with an HRP-conjugated secondary antibody anti-mouse for 1 hour. Bands were visualized using a Chemidoc (Imager Chemi premium, VWR), after incubated with ClarityTM Western ECL substrate (Biorad).

3.4. Indirect immunofluorescence

Cells were cultured on glass coverslips (22 x 22 mm) and incubated for 24 hours. Then, cells were fixed with 4% *p*-formaldehyde for 20 minutes and quenched in 50 mM NH_4Cl. Afterwards, cells were blocked in PBS supplemented with 0.3% Triton X-100 (Sigma-Aldrich®) and 5% BSA. After blocking cells were incubated with primary antibody monoclonal mouse anti-β-tubulin for 1 hour. Then, they were washed three times with PBS and incubated with fluorochrome-conjugated secondary antibody anti-mouse (Invitrogen™) for 1-2 hours. Once mounted, samples were visualized with confocal immunofluorescence microscopy using SP5 Leica apparatus with LAS-AF software (Leica Microsystems) at 20X and 40X magnifications.

4. Apoptosis induction

To analyse apoptosis induction in HeLa and its PTX resistant variant, DNA laddering was assessed through agarose electrophoresis.

DNA extraction was carried out using Tri-reagent DNA isolation protocol (Sigma-Aldrich®). After quantification, agarose electrophoresis was ran using 1% agarose gels in TE buffer (10 mM Tris, 1mM EDTA, pH 8.0) using mini-subcell GT electrophoresis chamber (BioRad) at 70V until dye front reached the end of the gel. For DNA staining, uncommon preloading method was used. DNA stain GelRed™ was diluted 1:500 in loading buffer (0.25% bromophenol blue, 0.25% xylene-cyanole, 30% glycerol), mixed with the samples and loaded to the gel [21].

RESULTS AND DISCUSSION

Drug resistance is one of the main threats in cancer chemotherapy. The early assessment of the phenotypic changes occurring in drug resistance might be useful to design better chemotherapeutic strategies. In this work, we studied potential phenotypical differences between HeLa cells and a PTX resistant variant developed in our group.

1. Wound healing assay

The acquired resistance against microtubule stabilizing drugs could result from a change in microtubule dynamics where resistant cells present fewer stable polymers that confer resistance, since PTX binding only occurs in polymerized microtubules [22]. PTX is a drug that stabilises the microtubules and thus, it might affect cell motility. Therefore, we looked at the migration ability of HeLa and HeLa/PTX cells with the wound healing assay. In this method, a scratch is made in a monolayer culture of cells and the closure of the wound (as a result of cell migration) is quantified. Figure 2 shows the migration of cells after 4 and 24 hours after the scratch was done. The images reveal that migration of cells to close the wound occur in both cell lines.

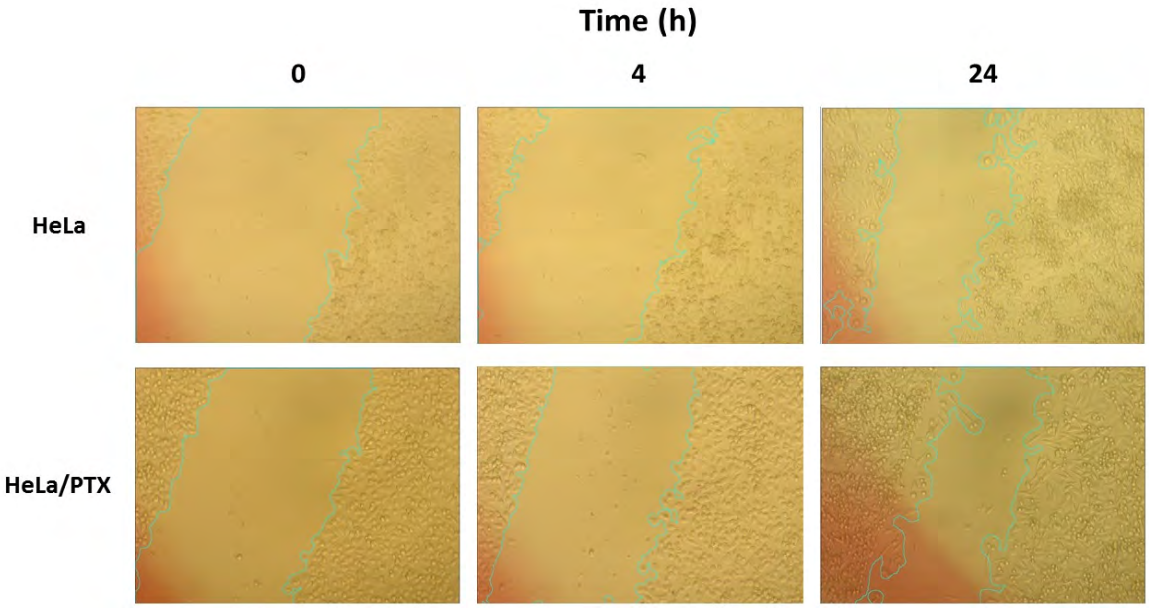

Figure 2. Representative microscopy images of the wound healing assay for HeLa and HeLa/PTX cells at different time intervals. The wounded area is marked in blue. Wound healing assay was replicated five times for each cell type.

The migration of cells was quantified with Equation 1, which compares the wounded area (A) at any time point (t) with the initial value ($t = 0$). The results are shown in Figure 3.

The trend in both cell types is very similar. Both cell lines seem to show the same migration dynamic. To check this, a T-test confirmed the lack of statistically significant difference in microtubule dynamics between these variants (Table 1). Therefore, the PTX-acquired resistance did not induce noticeable changes in cell migration properties. In previous studies, resistant cell lines that developed a resistant mechanism usually grew poorly in the absence of PTX [22]. Those cells seemed to need low drug concentration to have normal microtubule dynamics, which enabled them to grow. In our study, we did not observe this behaviour.

$$Wound\ Closure\% = \left(\frac{A_{t=0} - A_{t=\Delta t}}{A_{t=0}}\right) x100\% \qquad \text{Equation 1}$$

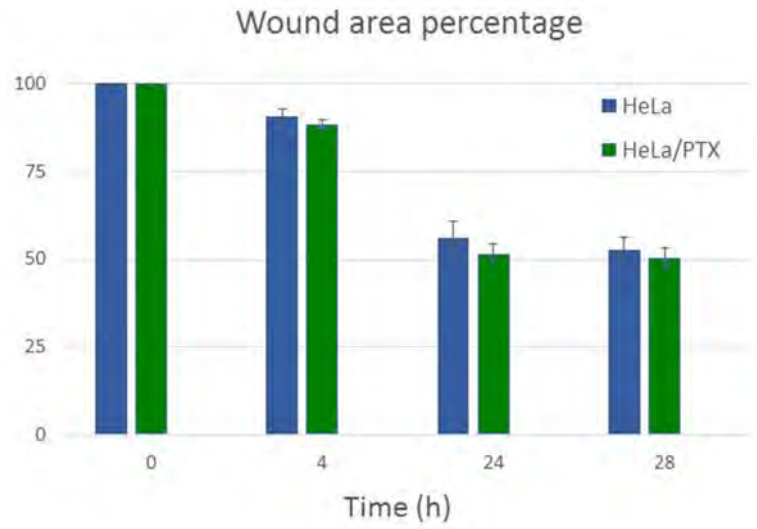

Figure 3. Mean wounded area (%) at the indicated time intervals.

Table 1. Descriptive statistics from wound closure assays after 24 hours.

	Wound Closure (%)			
	Minimum	Maximum	Mean	Standard deviation
HeLa PTX control [a]	42.6	53.8	48.6	5.7
HeLa control [a]	32.9	55.0	44.0	9.4

(a) P-value >0.05

2. Western blotting

Next, we looked to the expression of β-tubulin in HeLa and HeLa/PTX cells. Attending to the bands of β-tubulin in the Western blotting PVDF membrane (Figure 4), the intensity of both bands looked very similar. Therefore, the expression of the protein did not

appear to be different in the resistant variant when compared to the parent cell line. In our study, we did not observe an increase of the expression of β-tubulin that could explain the acquired resistance.

Figure 4. PVDF membrane revealed after incubation with anti-β-tubulin antibody.

Another mechanism described for resistance against this type of drugs, is the overexpression of concrete β-tubulin isotypes. Microtubule dynamics varies depending on the dominant isotype [23]. This could be studied using isotype specific antibodies. Since we did not spot a different microtubule behaviour in HeLa/PTX cells, we speculate that difference in the expression of isotypes is unlikely to occur.

3. Indirect immunofluorescence

In addition, an immunofluorescence assay was carried out to visualize possible differences in microtubule distribution between HeLa and HeLa/PTX cells (Figure 5). In the images, we could not spot significant differences between both cell types. Thus, no differential distribution is conferred by the resistance.

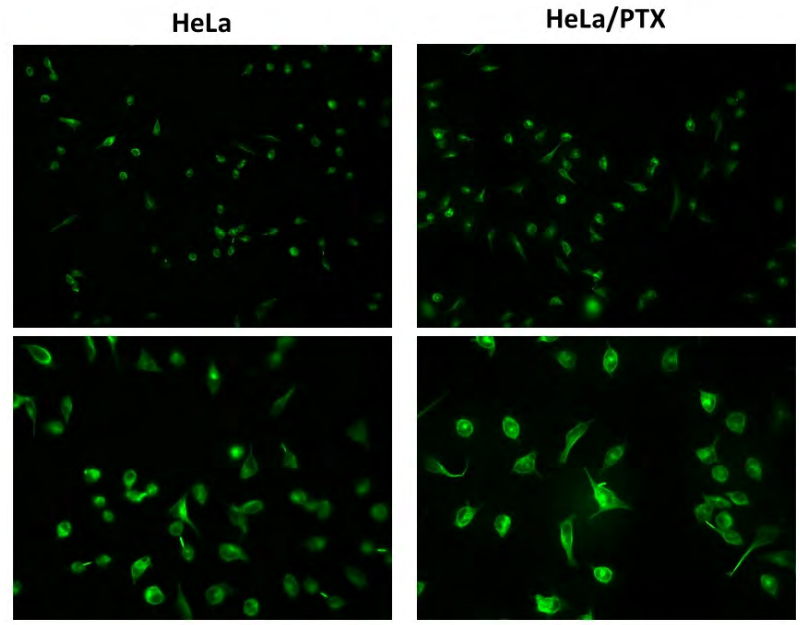

Figure 5. Immunofluorescence images taken of HeLa and HeLa/PTX using anti-β-tubulin as primary antibody. *Magnification: top 20X; bottom 40X*.

4. Apoptosis induction

To evaluate differential apoptosis induction between HeLa and HeLa/PTX cells, agarose electrophoresis of cells exposed to PTX was performed. In order to confirm if PTX induces apoptosis in HeLa cells we expected to observe the typical DNA laddering. This would have suggested that the possible mechanism that confers resistance to HeLa cells could be an inhibition of cell death mechanism possibly involving BCL2 proteins [13]. At present, we are still developing the method to improve DNA ladder assay to detect apoptosis.

It has been reported for 1A9 human ovarian carcinoma cells with resistance to PTX (1A9PTX10 and 1A9PTX22) that mutations near the binding site in β-tubulin (Phe_{270}-to-Val and Ala_{364}-to-Thr, respectively) abrogate PTX action in vitro [24]. Altogether, our assays suggest that the resistance mechanism to PTX developed in HeLa/PTX cells does not involve β-tubulin. However, we cannot discard completely a change in β-tubulin that could abrogate the binding of PTX but without altering microtubule dynamics.

We could consider another type of resistance mechanism, like the overexpression of PGP, which acts as a drug-extracting pump associated with the drugs resistance in tumour cells. This is a common mechanism against PTX and other alkaloids which are substrates of this pump. Yet another possible mechanism of resistance could be the overexpression of any cell death inhibition protein. Since the resistance can be conferred by more than one mechanism some of these could also be happening jointly.

In summary, further experiments are needed to determine the resistance mechanisms to PTX that HeLa/PTX cells have developed.

CONCLUSIONS

1. We did not observe significant differences in cell migration between PTX sensitive (HeLa) and PTX resistant cancer cell lines (HeLa/PTX).

2. We did not observe overexpression of β-tubulin in the resistant variant.

3. We did not observe differences in the cellular distribution of β-tubulin between HeLa and HeLa/PTX cells.

CONCLUSIONES

1. No observamos diferencias significativas de migración celular entre las variantes resistente y sensible a PTX de HeLa.

2. No observamos sobreexpresión de β-tubulina en la variante resistente.

3. No observamos entre HeLa y HeLa/PTX diferencias en la localización celular de β-tubulina.

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