Original Article

Topological properties and in vitro identification of essential nodes of the Paclitaxel and Vincristine interactomes in PC-3 cells

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ABSTRACT

Background: Microtubule-targeting agents (MTAs) disrupt microtubule dynamics, thereby inducing apoptosis via mitochondrial pathway activation through the modulation in the expression of the Bcl-2 family.

Methods: To describe topological features of the MTAs networks associated to intrinsic apoptosis induction in p53-null prostate cancer cells, we predicted and compared the interactomes and topological properties of Paclitaxel and Vincristine, and thus, the essential nodes corresponding with the pro- and anti-apoptotic proteins and their kinetics were subjected to experimental analysis in PC-3 cell line.

Results: The essential nodes of the apoptotic pathways, TP53, and CASP3, were identified in both, Paclitaxel and Vincristine networks, but the intrinsic pathway markers BCL2, BAX, and BCL2L1 were identified as hub nodes only in the Paclitaxel network. An in vitro analysis demonstrated an increase in BimEL and the cleaved-caspase-3 proteins in PC-3 cells exposed to both treatments. Immunoprecipitation analysis showed that treatments induced the releasing of Bax from the anti-apoptotic complex with Bcl-2 protein and the role of BimEL as a de-repressor from sequestering complexes, in addition, new protein complexes were identified between BimEL or Bcl-2 and cleaved-caspase-3, contributing data to the Vincristine network for p53-null cells in response to MTAs.

Conclusion: The differences in sensitivities, protein profiles, and protein complex kinetics observed between the drugs confirmed that the selectivity and stimulation of the apoptotic system vary depending on the cell's genotype, the drug used and its exposure period.

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At a glance commentary

Scientific background on the subject

Cancer is a significant cause of morbidity and mortality of the world population, being the first cause of non-violent death. Prostatic cancer has become the most common malignancy among men. Network biology with an experimental approach can be used to describe protein interaction, identify biomarkers and define the topological features of cytostatic drugs among other uses.

What this study adds to the field

Advance and variant forms of prostatic cancer are not susceptible to common treatment which leads to the pursuit of different classes of drugs, such as MTAs, in which the resistance rate and protein interaction between drugs vary. Some protein interactions were previously described by network biology and then verified in this paper, also, new interactions were found.

Network biology has been extensively used to describe how proteins interact and coordinate cellular responses [1]. Protein–protein network approaches have been applied to gain a better understanding of cancer mechanisms [2], to identify cancer subnetworks [3], to discover cancer-related biomarkers [4], and to define the topological features of the interactome of microtubule-targeting agents (MTAs) [5].

High cancer death rates and resistance or over-sensitivity to conventional cancer therapies remain major challenges for researchers [6,7]. Prostate cancer has become the most common malignancy among men and is the second leading cause of cancer death worldwide [8,9]. Prostate cancer is very heterogeneous, often characterized by the presence of AR-dependent and AR-independent cellular clones in the same patient [10], its biological, hormonal, and molecular characteristics are extremely complex [6].

Most prostatic cancers (PCs) are adenocarcinomas characterized by a glandular formation, lack of basal cells, uncontrolled proliferation of malignant tumor cells and the expression of androgen receptor (AR) and prostate-specific antigen (PSA). These adenocarcinomas are indolent and androgen-dependent (AD) [6], although localized PC may be successfully treated with radical prostatectomy and external beam radiation, many patients subsequently develop metastatic disease. Since its growth is driven by androgens, they are primarily treated with hormonal therapy, the androgen ablation therapy (ADT), that aims to block or decrease the expression of the androgen receptor, the hormonal therapy is the standard treatment of hormone-naïve metastatic disease [11–13]. Nearly all patients with metastatic prostate cancer (PC) become resistant to the initial approach with ADT, developing the state known as metastatic castration-resistant prostate cancer (mCRPC) [10].

There are variant forms of prostatic epithelial malignancies, such as the Small-Cell Neuroendocrine Carcinoma (SCNC), which are rare tumors that account for no more than 1% of all carcinomas of the prostate, they lack AR and PSA expression. Although they may arise de novo, they are often seen as recurrent tumors in patients who have received hormonal therapy with conventional prostatic adenocarcinoma [6]. PC-3 is a cell line characteristic of SCNC since PC-3 cells share a lot of traits with prostatic SCNCs, including the lack of expression of the basal markers AR and PSA. PC-3 cells are often referred to as aggressive, androgen-independent (AI), and castration-resistant [6,14]. Nevertheless, the PC-3 line presents a base deletion at codon 138 (GCC→GC) in the TP53 gene, resulting in a frame-shift mutation [15,16] that influences negatively p53 dependent-apoptosis through the mitochondrial pathway, where it directly interacts with different Bcl2 family members, acting as a direct activator of the Bax/Bak effectors, or as a sensitizer/de-repressor of Bcl-x/2 and Mcl-1 [17]. In human cancers, TP53 gene is frequently mutated what not only leads to loss of its tumor suppressive function but also acquires dominant—negative activities and gains new oncogenic properties that increase drug resistance [14,18,19].

Advanced and variants forms of these PCs are only temporary or not susceptible at all to the androgen ablation therapy, leading to the pursuit of different classes of drugs, such as MTAs, which inhibit microtubule dynamics and induce cell death via the mitochondrial intrinsic pathway [12,20].

Microtubule-targeting agents are usually obtained from natural sources, such as Paclitaxel, obtained from the Pacific yew tree (Taxus brevifolia) [8], and Vincristine, obtained from Madagascar periwinkle (Catharanthus roseus) [21]. These agents disrupt microtubules dynamics, thereby inducing apoptosis via mitochondrial pathway activation [19]. Prostate cancer exhibits high levels of anti-apoptotic Bcl-2 and Bcl-xl proteins in refractory and advanced disease, contributing to the defective apoptosis associated with poor prognosis, treatment resistance and disease progression [8,12,22–24].

The mitotic arrest is induced by two kinds of well-known MTAs, including the microtubule-stabilizing agent Paclitaxel [25] and microtubule-destabilizing agents, such as Vincristine and Vinblastine [26], which are associated with the activation of Raf-1 protein kinase and the phosphorylation of the anti-apoptotic protein Bcl-2 [25]. This phosphorylation increases the resistance to many anticancer drugs and may inactivate the Bcl-2 protein, ensuring cell death after microtubule rupture and DNA fragmentation in PC-3 cells [25,26]; additionally, it inhibits the ability of Bcl-2 to interfere with pro-apoptotic proteins, such as Bax, leading to apoptosis [22]. Paclitaxel also induces the accumulation of the pro-apoptotic protein Bim [12], causing a G2/M cell cycle block, mitochondria damage, and p53-independent apoptosis [27].

Activation of the caspase cascade has been correlated with the onset of apoptosis [28]. Caspase inhibition attenuates apoptosis in prostate cancer cells in response to diverse apoptotic stimuli, including androgen ablation. Therefore, reduced caspase expression, which is a frequent event in
prostatic cancer, has been linked with poor prognosis and/or resistance to therapy in several human tumors. Caspases-1, -3, and -9 are the three key caspases implicated in the execution of apoptosis in prostate cancer cells and malignant prostatic tissue, and there is dramatically reduced caspase-1 and -3 immunoreactivity among the tumor cells [29].

The secondary targets of the signaling pathways induced by Paclitaxel and Vincristine in PC-3 cancer cells remain poorly understood. Here, we described the topological properties and identified the essential nodes of Paclitaxel and Vincristine interactomes against the human proteome. Then, experimental validation of those essential nodes, corresponding to pro- and anti-apoptotic proteins and their kinetics in p53-null PC-3 cancer cells in regard to the intrinsic apoptosis induced by Paclitaxel and Vincristine was conducted.

Materials and methods

Paclitaxel and Vincristine network prediction

The chemical information of Paclitaxel and Vincristine was extracted from the PubChem database. After, the interaction between proteins and the MTAs were integrated into a network by using of the Search Tool for Interacting Chemicals (STITCH) 5.0 database [30] (http://stitch.embl.de/), with the following criteria: a confidence score of 0.500, with a maximum of 500 interactions. The prediction methods in the program were active solely against the human proteome. Next, each network was imported into Cytoscape 3.4.0 [31], and the following topological parameters were obtained: Degree (k), which indicates the connection density of each node; Betweenness Centrality (BC), which indicates the number of times a node is visited; Closeness Centrality (CC), which indicates what nodes are closer to the center of the network; and Stress, which indicates how many times a particular node is part of different shortest paths using the Network Analyzer plugin included by default in Cytoscape.

To determine the functional richness of the network, a hypergeometric distribution was used and the false discovery rate (FDR) correction was included, using the Biological Network Gene Ontology (BiNGO) plugin. The FDR correction, with a significance level of 0.05, is shown for two descriptive categories of the clusters. The first represents the biological function, and the second represents node mapping in the Kyoto Encyclopedia of Genes and Genomes (KEGG) [32].

Compounds, cell lines, and culture conditions

The Paclitaxel and Vincristine sulfate (Cayman Chemical) was dissolved in dimethyl sulfoxide (DMSO) and for the experiments, dilutions were made in supplemented Eagle's Minimum Essential Medium (EMEM) with 10% (w/v) Fetal Bovine Serum (Biowest), 2 mM L-glutamine, 5000 IU/ml penicillin and 5 mg/ml streptomycin. The prostate adenocarcinoma PC-3 cells (ATCC® CRL1435™) were grown in supplemented EMEM at 37 °C in a humidified atmosphere containing 5% CO₂.

The 3-(4,5-methyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell viability assay

PC-3 cells were cultured to 80% confluence, then, seven thousand cells approximately were seeded per well in a 96-well plate and were grown at 37 °C, 5% CO₂ for 24 h. Then, MTAs treatments were administered at different concentrations, between 0.01 and 0.15 µg/mL for Vincristine and between 0.005, and 0.0012 µg/mL for Paclitaxel. Incubation was carried out for 48 h. Following the incubation time, 100 µL of 0.5 mg/mL of the MTT solution, dissolved in a medium without phenol red, were added and incubated for 4 h. Formazan crystals were dissolved with 100 µL of DMSO. The results were determined by the optical density (OD) determined by the absorbance at 570 nm [33]. Estimation of the MTAs concentrations required to reduce the 50% of cell viability (IC₅₀) was done using nonlinear regression from plotting cell survival (%) versus drug concentration [µg/mL].

Immunofluorescence analysis

Seven thousand PC-3 cells approximately were seeded per well in a 96-well plate and were grown for 24 h before treatment. The cells were treated with Paclitaxel and Vincristine at the IC₅₀ and incubated for 24 h. Later the cells were fixed in methanol (−20 °C), and then with acetone at −20 °C for 20 s. The microtubules were stained with anti-α-tubulin monoclonal antibody DM1A (Sigma–Aldrich) and goat anti-mouse Alexa Fluor 488 (Molecular Probes), both diluted in 5% BSA/TTBS (w/v) blocking solution. DNA staining was done with 1.0 µg/mL of DAPI (Invitrogen) [22,33]. Fluorescence was monitored using an epifluorescence microscope (Motic AE31), and the images were captured with the MoticCamPro 282A and analyzed with Motic Image plus 2.0 software.

Electrophoresis & western blotting

Protein extraction was carried out from approximately 6 million cells treated for 6, 16, 24 and 48 h with the MTAs at the corresponding IC₅₀ using a lysis buffer (Tris HCl pH 8.0 20 mM, NaCl 137 mM, Glycerol 10%, Np40 1%, EDTA 10 mM) [34], and the protein concentration was determined by Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce). Fifteen µg of protein were subjected to an 11% SDS-PAGE gel electrophoresis using a mini-gel system (Protein II; Bio-Rad Laboratories). Proteins were blotted using polyvinylidene fluoride (PVDF) membranes [35,36]. The transferred membranes were blocked in 5% (w/v) BSA/TTBS followed by overnight incubation with the following primary antibodies: anti-α-Tubulin (Sigma–Aldrich); anti-Bim; anti-Bax; anti-Bcl-2 (Gene-Tex); anti-procaspase-3 (Novus) or anti-caspase-3 (Thermo-Fisher Scientific). Next, the membranes were incubated with the correspondent secondary antibody, either anti-mouse IgG (Sigma–Aldrich) or anti-rabbit IgG (Cell Signaling), for 1 h at room temperature. The bands were visualized using a Chemiluminescent Detection System (Thermo-Fisher Scientific) [22]. Image J software was used to quantify and compare the density of bands [37].
**Co-immunoprecipitation**

One-hundred and fifty µg of protein per treatment were mixed with 2 ug of primary antibody, in a dilution buffer –NaCl (Tris HCl pH 7.4, EDTA 10 nM, NP40 1%, NaF 50 mM, PMSF 1 mM, and protease inhibitors cocktail (Pierce) (Thermo Fisher Scientific) 1:100), and the samples were incubated at 4 °C for 4 h. Later, the protein mix was incubated with the washed resin (Protein G) (Thermo-Fisher Scientific) at 4 °C overnight. The next day, the samples were washed with a +NaCl wash buffer (Tris HCl pH 7.4 1 M, NaCl, EDTA 10 mM, NP40 1%, NaF 50 mM, PMSF 1 mM and protease inhibitors cocktail (Pierce) (Thermo Fisher Scientific) 1:100) and were reduced with 35 µl of a 1x loading buffer and boiling for 5 min. Subsequently, the immunoprecipitated complexes were subjected to electrophoresis and western blotting with primary antibodies against the corresponding essential nodes from intrinsic apoptosis pathway, the detection of the proteins in the complex was conducted as previously described and densitometry analyses were performed using ImageJ software [37].

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**Results**

**Topological nodes that regulate the Paclitaxel and Vincristine interactomes**

The Paclitaxel and Vincristine interactomes showed several secondary targets [Fig. 1]. The Paclitaxel network presented 166 nodes and 1497 edges. The evaluated topological features showed positive correlations, indicative of highly connected nodes, as follows: nodes/k (R: 0.857, R²: 0.657); neighbors/BC (R: 0.972, R²: 0.871) and neighbors/CC (R: 0.767, R²: 0.592) [Fig. 1A]. Then, to choose the essential nodes, the k values were reordered in a decreasing fashion, and the top 30 nodes with the highest values are showed [Table 1]. To determine the importance of the essential nodes during the process the information flow in the network, a linear correlation analysis between the BC and stress parameters was conducted. The essential nodes TP53, AKT1, VEGFA, JUN, and CASP3 seemed to control the information processing in the paclitaxel

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Fig. 1 Topological nodes that regulate the interactome information process derived from Paclitaxel and Vincristine. (A, C) Stress and BC values were used to identify the nodes that connect the most significant shortest in the interactomes and thus, define how the information flow is distributed. (B, D) The essential interactomes were built based of the K parameter and shows the most connected nodes in both interactomes.
network [Fig. 1B], but also in the top the BCL2, BAX and BCL2L1 nodes were identified confirming the mitochondrial-mediated pathway apoptosis induction.

The Vincristine network presented 19 nodes and 42 edges. This network also reveals essential nodes and highly significant correlations were detected among their basic topological parameters as follows: nodes/k (R: 0.913, R²: 0.770); neighbors/BC (R: 0.999, R²: 0.926) and neighbors/CC (R: 0.807, R²: 0.695) [Fig. 1C], and only two essential nodes, TP53 and CASP3, associated to the intrinsic apoptosis, seems to be involved in the information processing of the network [Fig. 1D].

Paclitaxel and Vincristine cause irreversible morphologic damage in PC-3 cells

Vincristine and Paclitaxel inhibitory effect on PC-3 cells proliferation was confirmed by MTT assay. However, the IC_{50} values demonstrated that the cells were significantly more sensitive to Paclitaxel [2.81 nM] than to Vincristine [44.8 nM] [Fig. 2]. Those IC_{50} data were used for all others in vitro analyses.

Morphological analysis was performed to examine the cellular damage that occurred after exposing PC-3 cells to MTAs using immunofluorescence microscopy [Fig. 3A]. There were variations in the cells on the morphological scale, such as microtubule and nucleus integrity and cell shape and size variations. Paclitaxel mainly affects the microtubules, causing microtubule destabilization, and increase in cytosol size, the appearance of some apoptotic bodies (marked with a red arrow), and the emergence of different nuclear morphologies [Fig. 3A]. On the other hand, the Vincristine effect on cell proliferation was more evident than paclitaxel and the cells had dense and compact damaged microtubules around the nuclei, and, thus some apoptotic cells (marked with a red arrowhead). The nuclei of PC-3 cells exposed to Vincristine had irregular and small nuclei compared to the control but not as quite as paclitaxel. Further, Paclitaxel-induced the condensation and fragmentation of the nuclear material (in blue) as well as a reduction of its perimeter [Fig. 3B].

**Table 1 Essential topological nodes from Paclitaxel and Vincristine networks based on the k parameter.**

| Node      | k  | BC     | CC     |
|-----------|----|--------|--------|
| Paclitaxel| 165| 0.621  | 1.000  |
| TP53      | 74 | 0.022  | 0.645  |
| AKT1      | 71 | 0.024  | 0.637  |
| VEGFA     | 68 | 0.021  | 0.630  |
| JUN       | 66 | 0.013  | 0.625  |
| CASP3     | 60 | 0.010  | 0.611  |
| EGFR      | 58 | 0.015  | 0.607  |
| PTGS2     | 55 | 0.010  | 0.600  |
| MAPK1     | 52 | 0.007  | 0.594  |
| STA13     | 50 | 0.008  | 0.589  |
| CASP8     | 49 | 0.004  | 0.587  |
| ERBB2     | 49 | 0.007  | 0.587  |
| CDKN1A    | 47 | 0.003  | 0.583  |
| CYCS      | 46 | 0.004  | 0.581  |
| BIRC5     | 45 | 0.004  | 0.579  |
| MAPK8     | 45 | 0.006  | 0.579  |
| PARP1     | 45 | 0.005  | 0.579  |
| PCNA      | 44 | 0.009  | 0.577  |
| FASLG     | 44 | 0.005  | 0.577  |
| BCL2      | 43 | 0.003  | 0.575  |
| PIK3CA    | 43 | 0.006  | 0.575  |
| PTEN      | 42 | 0.004  | 0.573  |
| BAX       | 41 | 0.002  | 0.571  |
| CDH1      | 40 | 0.008  | 0.569  |
| MAPK14    | 39 | 0.004  | 0.567  |
| MDM2      | 39 | 0.003  | 0.567  |
| SOD2      | 37 | 0.005  | 0.563  |
| BCL2L1    | 37 | 0.003  | 0.563  |
| IL8       | 36 | 0.005  | 0.561  |
| Vincristine| 18 | 0.735  | 1.000  |
| TP53      | 8  | 0.030  | 0.643  |
| PTGS2     | 7  | 0.011  | 0.621  |
| ABCB1     | 7  | 0.015  | 0.621  |
| CASP3     | 6  | 0.009  | 0.600  |
| CYP3A4    | 6  | 0.014  | 0.600  |
| MAPK8     | 5  | 0.004  | 0.581  |
| MAPK7     | 5  | 0.016  | 0.581  |
| CYP3A5    | 5  | 0.007  | 0.581  |
| ABCG2     | 3  | 0.000  | 0.545  |
| CYP3A7    | 3  | 0.001  | 0.545  |
| MAPK10    | 2  | 0.000  | 0.529  |
| TUBB2C    | 2  | 0.000  | 0.529  |
| TUBB2A    | 2  | 0.000  | 0.529  |
| BUB1B     | 1  | 0.000  | 0.514  |
| ASRGL1    | 1  | 0.000  | 0.514  |
| ACP1      | 1  | 0.000  | 0.514  |
| MIXL1     | 1  | 0.000  | 0.514  |
| TWF1      | 1  | 0.000  | 0.514  |

**Fig. 2** Percentage of cell viability of PC-3 cells exposed to different concentrations of Paclitaxel and Vincristine. The inhibitory concentration of cell viability (IC_{50}) was calculated by non-linear regression using Graph Pad 6.0 software.
were divergent between the two drugs. Paclitaxel-induced a slight decrease in the levels of Bax protein, which was dependent on time, while Vincristine caused an increase through a distinct period of the drug incubation. Paclitaxel did not provoke considerable changes in the levels of the procaspase-3 form, but the active form was slightly augmented. Furthermore, in the cells treated with Vincristine, there was a decrease in the levels of procaspase-3 at 48 h, which was related with the decrease in the expression of the activated form that was observed at the same time [Fig. 4B].

In vitro protein–protein interactions in PC-3 cells after Paclitaxel and Vincristine treatment

Immunoprecipitation analyses showed that Bim protein interacted with the essential nodes Bcl-2, Bax and cleaved-caspase-3. However, the intensity of the bands indicated that the function of Bim was dependent on the type of drug [Fig. 5A]. The Bim/Bcl-2 interaction was similar in both treatments, decreasing slightly at 48 h. Bim/Bax complex levels increased significantly at 16 h only in the cells exposed to Paclitaxel. Interestingly, new interactions have been identified in cells that were exposed to vincristine, since there was an augmentation in the interaction between Bim/cleaved-caspase-3 in a time-dependent manner [Fig. 5B].

The Bcl-2/Bax interaction was significantly decreased at 48 h treatment in cells exposed to Paclitaxel [Fig. 5A and B]. In contrast, in cells exposed to Vincristine, the Bax protein has not been completely released at 48 h from the anti-apoptotic complex, elucidating the PC-3 mechanism to Vincristine resistance. Another new protein–protein interaction was revealed between cleaved-caspase-3 and Bcl-2.
where the levels of this complex decreased in the cells treated with Paclitaxel and increased after Vincristine treatment, much like the levels of the Bim/cleaved-caspase-3 complex [Fig. 5B].

**Discussion**

Paclitaxel and Vincristine bind preferentially and reversibly to the β-tubulin microtubule subunit, promoting the polymerization or depolymerization of microtubules, respectively; both drugs disrupt microtubule dynamics [38]. However, the networks here predicted show several secondary targets, indicating their lack of specificity. The Paclitaxel interactome was more highly expressed than the Vincristine interactome and presented more pro- and anti-apoptotic proteins, suggesting that this compound has pleiotropic moieties possibly related to unspecific cellular activation and the regulation of cell death and toxicity. The Vincristine network was more specific towards activated proteins that regulate the induction of cell death. According to preliminary computational results, we observed that Paclitaxel has significant druggability towards Bax, Bcl-2, and cleaved-caspase-3, indicating that this molecule can induce non-regulated biochemical signaling, and stimulate an apoptotic catastrophe [39] and even induce paraptosis [Fig. 1] [40].

The prostate cancer cell line PC-3 is an appropriate model to study the possible causes of the ineffectiveness of the common
drugs used in the treatment of apoptosis-resistant phenotypes cells with p53-null status, which alter the normal function of the network determinants for each drug [Fig. 1]. Previously, it was showed that PC-3 cells exposed to Paclitaxel had an abnormal network of microtubules around the nucleus at 4–8 h after treatment, which induced the formation of multipolar spindles. Nevertheless, those cells entered G2/M to form a defective spindle–chromosome complex that then caused cycle arrest [22]. Cell proliferation assays indicated that a lower concentration of Paclitaxel was sufficient to induce cell death in comparison to Vincristine [Fig. 2], perhaps, because Paclitaxel did target a higher number of nodes. Fluorescent images from PC-3 cells treated with paclitaxel [Fig. 3A] showed enlarged cells with damaged microtubules and irregular and multiple nuclei suggesting mitotic catastrophe [39]. On the other hand, Vincristine effect on cells was more evident, it resulted in enlarged cells with enlarged and irregular nuclei, and aggregation of compact and denser microtubules around a large nucleus [Fig. 3A and B]. In addition, there was the appearance of some apoptotic cells marked with a red arrowhead [Fig. 3A]; these results correspond with previous findings in several resistant cells lines, which can be ascribed to drug efflux pumps, mutations in tubulin that abrogates drug binding, ineffective interaction with the target, deficient induction of apoptosis and overexpression of prosurvival Bcl-2 family proteins [39,41].

During apoptosis, it is expected the decrease of the anti-apoptotic proteins Bcl-2 like [8,23,24]. However, the overexpression of Bcl-2 and Bcl-xl in PC-3 cells might contribute to the apoptosis-resistant phenotype [42,43]. In this respect, our data indicate that the anti-apoptotic protein Bcl-2 decrease occurred after both treatments, but a portion of the Bcl-2-protein remains in the cells, even after 48 h, contributing to the resistance [Fig. 4A and B]. MTAs initiate the translocation of Bim from microtubules to the mitochondria, allowing the Bax/Bak activation and then inducing apoptosis [12,43]. Nonetheless, the increasing of the pro-apoptotic Bim protein found after both drug treatments, corresponded with several studies in which Bim is reported as a tumor suppressor and is essential for apoptosis induction [26,27,37,44]. Literature reports that Bim level increases dramatically after paclitaxel treatment [12], according to our findings, the level of Bim was higher in vincristine treatment than the level induced by paclitaxel.

The forced expression of Bim increases Paclitaxel-mediated killing of cells expressing an undetectable level of Bim. Conversely, the knockdown of Bim, but not Bcl-2 expression, decreases the susceptibility of tumor cells to Paclitaxel-mediated killing [27]. Similar observations were made using a panel of breast and prostate cancer cell lines [27,43]. Furthermore, it was proven that the depletion of Bim levels prevented paclitaxel-induced Bax and Bak activation [39]. Paclitaxel impairs microtubule function, causes G2/M cell cycle blockade, mitochondria damage, and p53-independent apoptosis. These results established Bim as a critical molecular link between the microtubule toxic agent, Paclitaxel, and apoptosis [27].

Prior to any antineoplastic treatment, Bim is bound by Bcl-2, and there has proven to be a displacement of Bim from apoptotic proteins consistent with the Paclitaxel treatment at 48 h in four breast cancer cell lines (MCF-7, T47D, MDA-MB-468 and BT20), 3 p53 mutant [43], similar to our findings, where the Bim/Bcl-2 complex level started diminishing at 48 h for both treatments, releasing Bim to initiate apoptosis via Bax/Bak activation [Fig. 5B]. Likewise, the Bim/Bax complex level decreased, indicating that Bim might act as a tumor suppressor that dissociates the anti-apoptotic interaction and promotes Bax release, replacing the p53 function absent in PC-3 cells [37].

Additionally, the levels of the procaspase and the activated form, cleaved-caspase-3 varied significantly indicating that caspase-3 activation during cell death induction in PC-3 cells depends on the drug mechanism. It was shown that Paclitaxel and Vincristine drive the activation of caspase-3 in two breast cells lines, MCF-7 and MDA-MB231, and as it’s suggested that those microtubules disrupting agents provoke a signaling cascade and a cellular response to DNA damage [40,45]. The activation of caspase-3 has been considered a remarkable end-point of apoptosis independently of the activated pathway, therefore, the activation of caspase-3 for both treatments is concluded as the induction of apoptosis [Fig. 4B] [46].

Furthermore, new interactions between the cleaved caspase-3 and Bcl-2 or Bim were found, in which, Vincristine interactions seems to increase in a time-dependent manner in both treatments. Contrary to Paclitaxel interactions which decrease also in a time-dependent manner [Fig. 5].

**Conclusions**

In this study, we reported secondary targets predicted to microtubule-targeting agents, Paclitaxel and Vincristine and we show differences in their effects on PC-3 androgen-independent prostate cancer cells. These are anticancer drugs with a high clinical value that demonstrated noticeable differences in their sensitivities, protein profiles and the protein complex kinetics, confirming that selectivity in the stimulation of the apoptotic system varies depending on the cell's genotype, and the drug exposure period. Thus, they might differ in the formation and quantification of new protein immunocomplexes, such as the one between cleaved-caspase-3 and the pro-apoptotic proteins Bim and Bcl-2.

**Conflicts of interest**

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2019.04.003.

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