RESEARCH ARTICLE

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In Vitro Antitumor and Antimetastatic Activity of a New Lapachol Derivative against Metastatic Breast Carcinoma

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Abstract

Objective: Breast cancer represents the most prevalent type of tumor throughout the world. Considering the side effects caused by the available treatments, the resistance acquired by cells to cytotoxic agents, and metastasis, it is necessary to search for new sources of antitumor and antimetastatic therapies. Given the numerous antitumor studies involving the synthesis of substances derived from the naphthoquinone lapachol, we investigated the antineoplastic potential of a new synthetic substance (APO-3) derived from lapachol, alone and in combination with the chemotherapeutic agent paclitaxel (PTX), against 4T1 cells, a murine breast cancer cell line. **Methods/Results:** In MTT assay APO-3 and the APO-3/PTX combination were selectively cytotoxic to 4T1 cells, with APO-3/PTX being approximately 6.5 and 15 times more selective than PTX and APO-3, respectively. After zymography, APO-3/PTX was more effective in decreasing matrix metalloproteinase-9 (MMP-9) activity compared with APO-3 alone. In the clonogenic assay, APO-3/PTX reduced the number of colonies more effectively than APO-3 or PTX alone. APO-3/PTX also inhibited cell migration, as did PTX and APO-3 alone. The combination increased the expression of proteins involved in the intrinsic apoptotic pathway and induced cellular morphological changes characteristic of this type of cell death, acting similarly to PTX alone. APO-3 increased Receptor-interacting serine/threonine-protein kinase 1 (RIP1) and caused morphological changes characteristic of apoptosis and necroptosis in 4T1 cells. **Conclusion:** Taken together, APO-3 presented antitumor action against 4T1 cells, but the APO-3/PTX combination was more effective than either substance alone.

Keywords: Breast cancer- metastasis- naphthoquinones- lapachol- paclitaxel

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Introduction

Metastasis is one of main causes of death of patients with breast cancer [1]. Tumor cells detach from the primary tumor through the blood or lymphatic route and colonize sites far from their place of origin [2]. Matrix metalloproteinases (MMPs) play a key role in the main stages of tumor progression. MMP-2 and MMP-9 degrade extracellular matrix (ECM) and basement membrane components, such as collagen I and IV, fibronectin and laminin, facilitating the spread of tumor cell [3]. In breast cancer, overexpression of MMP-2 and MMP-9 is associated with metastasis and, consequently, a poor prognosis [4–6]. MMPs also perform a crucial role in the main stages of tumor progression such as growth, survival, angiogenesis, invasion, migration, and regulation of the immune system [7, 8]. Therefore, studies aimed at inhibiting the activity of these proteinases are an important alternative approach for the treatment of tumors.

Chemotherapy is the main treatment option for advanced breast cancer; the most used regimens combine multiple drugs [9–11]. Even though patients respond well to chemotherapy, the recurrence and metastasis rates are quite high [12]. In addition, these treatments still have limitations due to resistance to cytotoxic agents and low selectivity, which causes numerous side effects [13, 14] and reinforces the importance of searching for new anticancer drugs that minimize these side effects.

Drug combinations provide a way to overcome tumor resistance and a lack of selectivity [15]. Paclitaxel (PTX)

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is a taxane widely used in adjuvant and neoadjuvant treatment of various tumor types, including breast cancer [16]. Natural naphthoquinone and its derivatives have a variety of biological activities well described, including anticancer properties. The natural naphthoquinone lapachol ((2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone), isolated from Tabebuia impetiginosa, is used in the semisynthesis of new analogues that may have better anticancer activity and selectivity [17–19]. In this study, we evaluated the antitumor activity of a new synthetic analogue derived from lapachol (APO-3), alone and in combination with the chemotherapeutic agent PTX, on 4T1 cells.

Materials and Methods

Preparation of standard solutions

APO-3, PTX, and their combination (APO-3/PTX) were diluted at several concentrations (0, 5, 25, 50, 75, and 100 μM). The nomenclature, identification codes, molar masses, and chemical structures of the compounds used in this study are shown in Table 1. Dimethyl sulfoxide (DMSO, 1%) was used to dilute the substances and was also used as a negative control. The APO-3/PTX combination was prepared in a 1:1 molar ratio.

Cell lines and reagents

4T1 cells were purchased from the Cell Bank of Rio de Janeiro (BCRJ code: 0022). The presence of mycoplasmas was assessed using PCR and Bioluminescence assays. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 culture medium supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin (S/P) at 37°C and 5% CO2. Confluent monolayers were dissociated with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA).

DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640, and S/P were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin and FBS were from Gibco (Carlsbad, CA, USA). PTX was purchased from Chiral Chemistry (Brazil). The Poly (ADP-ribose) polymerase (PARP), caspase-9, cleaved caspase-3, and RIP1 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The caspase-8 and β-actin antibodies were procured from Santa Cruz Biotechnology (Dallas, Texas, USA).

Cell viability assay

The cytotoxicity of APO-3, PTX, and APO-3/PTX was assessed with the MTT assay. First, cells were seeded in 96-well plates (5 × 103 cells/well) and incubated overnight. Subsequently, the cells were treated with increasing concentrations of APO-3, PTX, and APO-3/PTX in DMSO (1%) and RPMI (0% FBS and 1% S/P). After treatment for 24 h, 100 μL of MTT solution was added to each well, and the plates were incubated for 3 h to allow the formation of formazan crystals. Formazan was dissolved in DMSO. The absorbance of the solution at 570 nm was measured with a microplate reader (Versa Max, Molecular Devices). The results are expressed as a percentage of the average viable cells in the treatments relative to the control [20].

Tumor specificity (TS)

To assess TS, spleens from healthy BALB/c mice were collected under aseptic conditions. After maceration of the tissue, healthy splenocytes were obtained, plated in 96-well plates (5 × 105 cells/well) and then incubated with the selected substances for 24 h. Then, the MTT viability test was performed [21, 22]. TS was calculated with the following equation: TS = (mean IC_{50} of normal splenocyte cells/mean IC_{50} of 4T1 tumoral cells), where IC_{50} is the half maximal inhibitory concentration [23].

Zymography

The proteolytic enzyme activity of MMP-9 in the cell supernatant was measured with gelatin zymography. Gelatinases were obtained from the cell supernatant. After lysing the cells, the lysate cell supernatant was centrifuged at 14000 g, for 15 minutes, at 4°C. The supernatant was removed, stored and kept at -80°C until the electrophoresis. The total protein concentration was measured with the Bradford method. Equivalent amounts of cell supernatant (20 μg) were mixed with an equal volume of non-denaturing buffer (2% sodium dodecyl sulfate [SDS], 125 mM Tris-HCl, [pH 6.8], 10% glycerol, and 0.001% bromophenol blue). Electrophoresis mini gels (7% polyacrylamide and, 0.1% gelatin) were used run non-reducing conditions (0.025 M Tris(hydroxymethyl) aminomethane, 0.192 M glycine, and 0.1% sodium dodecyl sulfate (SDS) 0.1%, pH 8.5). After electrophoresis, the gels were washed twice in 2.5% TritonX-100 for 60 min at room temperature and then incubated at 37 °C overnight in activation buffer (10 mM Tris- HCl buffer [(pH 8.0]), containing 5 mM (Tris-CaCl2). The gels were stained (0.25% Coomassie blue G-250, 30% ethanol, and 10% acetic acid) for 1 h and destained (30% ethanol and, 10% acetic acid), for 2 h. The gels were photographed; and MMP proteolytic activity was appeared as clear zone bands on a blue background. The gelatinolytic activity was quantified by densitometric quantification using the ImageJ (NIH) software (National Institutes of Health, Bethesda, MD, USA) [24].

Wound-healing assay

4T1 cells were seeded in a 24-well plate (3.5 × 10^5 cells/well) and incubated until they reached 95% confluence. Then, two parallel wounds were made on the monolayer in each well. The cells were washed with phosphate-buffered saline (PBS) to remove cell debris and then treated with APO-3, PTX, or APO-3/PTX diluted in RPMI-1640 supplemented with 2% FBS. Images of the wounds were captured 0, 24, and 48 h after treatment, using an inverted fluorescence microscope (Axio Vert. A1, Carl Zeiss, Jena Germany) at 100× magnification. The ZEN software (2012) was used to measure the wounds. The results were analyzed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) [25].

Clonogenic assay

The clonogenic assay examined 4T1 cells in anchorage-independent soft agar. The test was performed for 21

days according to a previous protocol [24], with some adaptations. An area corresponding to 80% of each well was photographed using the EVOS® FL Auto Imaging System (Invitrogen, Waltham, Massachusetts, USA) at 100× magnification. The number of colonies content was quantified by using the OpenCFU software [26].

Acridine orange (AO)/propidium iodide (PI) double staining

The morphological changes characteristic of cell death were analyzed with AO/PI double staining. The cells (5 × 10^3 cells/well) were treated with the corresponding IC_{50} of each substance for 24 h. Afterward, the plate was centrifuged at 2,000 rpm for 5 min. In a light-free environment, the supernatant was discarded, the cells were resuspended in PBS, and then 10 µL of AO (10 µg/mL) and 10 µL of PI (10 µg/mL) were added. Twenty images per well were obtained at 200× magnification with an Axio Vert.A1 microscope. Morphological analysis was carried out according to a previous study [27]. Finally, the cells were quantified with the Zen software (Zeiss, Jena, Germany). The data were converted to the mean ± standard deviation percentage and normalized against the control.

Western blotting

The 4T1 cells (5 × 105 cells/well) were treated with the IC_{50} of the compounds for 24 h. Then, they were lysed (50 mM Tris [pH 7.6–8], 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 10 mM Na4P2O7, 1% NP-40, and protease inhibitors) and the protein concentration was determined [28]. Sodium Dodecyl Sulfate-PolyAcrylamide Gel Eletrophoresis (SDS-PAGE) was performed at 140 V for 4 h with 15% acrylamide gels. Then, the proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Chicago, Illinois, USA) by semi-dry transfer (Cytiva Amersham, England, UK). The membranes were incubated in 5% nonfat milk in Tris-Buffered Saline, 0.1 tween (TBS-T) for 1 h at room temperature to block nonspecific protein binding. Then, the membranes were incubated with the appropriate primary antibody: total PARP (116 kDa), total caspase 9 (51 kDa), cleaved caspase 3 (17 kDa) [Cell Signaling Technology], total caspase 8 (55 kDa) and β-actina (43 kDa) [Santa Cruz Biotechnology], according to the manufacturers' recommendations at room temperature. After incubation, the membranes were washed with TBS-T and incubated with the appropriate secondary antibody (anti-mouse ou anti-rabbit) diluted 1:1000. The proteins were visualized by chemiluminescence using two solutions (first solution: 100 mM Tris [pH 8.5], 2.5 mM luminol, and 0.396 mM coumaric acid; second solution 100 mM Tris [pH 8.5], and 0.06% H2O). Digital images of the membranes were obtained with a L-Pix Chemi Molecular Imaging system (Loccus, Cotia, São Paulo, Brazil). The ImageJ software was used for densitometric quantification. β-actin was used to normalize the protein expression. The target protein expression is presented as the ratio of the target protein band for the treatment to the same target protein band for the control [24].

Statistical analyses

All the assays were performed in triplicate. Statistical analyses were performed using GraphPad Prism 7.0. Differences between the groups were compared with analysis of variance followed by the Tukey test, with $p < 0.05$ considered to be a significant difference. The IC_{50} was determined by using a non-linear regression test.

Results

APO-3, PTX, and APO-3/PTX were cytotoxic and selective for 4T1 tumor cells

APO-3 (IC_{50} = 0.86 µM) and APO-3/PTX (IC_{50} = 2.77 µM) were more effective in reducing tumor cell viability than PTX alone (IC_{50} = 6.42 µM). APO-3, APO-3/PTX, and PTX were highly selective for 4T1 cells (Table 2), with an IC_{50} of 34.12, 1,617, and 577 µM, respectively. APO-3/PTX and PTX had a much higher IC_{50} than the maximum concentration established in the curve (100 µM); even so, these values were used to calculate a probable TS. The TS for APO-3, APO-3/PTX, and PTX was 39.67, 583.75, and 89.9, respectively. Besides being less toxic than APO-3 alone, APO-3/PTX was almost 15 times more selective than APO-3 alone and about 6.5 times more selective than PTX alone.

APO-3/PTX inhibited MMP-9 activity

APO-3 alone was not able to reduce MMP-9 activity (Figure 1). However, APO-3/PTX (35% $p = 0.0255$) and PTX (53% $p = 0.0020$) inhibited MMP-9 activity.

APO-3, PTX, and APO-3/PTX inhibited 4T1 tumor cell migration

For the control group, the wounds had completely closed after treatment for 48 h. Treatment with APO-3, APO-3/PTX, and PTX inhibited 4T1 cell migration. After treatment for 48 h, the wound size was 56% for APO-3, 54% for APO-3/PTX, and 68% for PTX, respectively (Figure 2).

APO-3/PT effectively decreased the number of colonies of 4T1 cells

APO-3/PTX inhibited almost 80% of the colonies formed compared with the control ($p < 0.0001$). PTX significantly reduced the number of colonies by approximately 60% ($p < 0.0001$). However, APO-3 was not able to decrease the number of colonies (Figure 3).

APO-3, APO-3/PTX, and PTX caused morphological changes in 4T1 cells

AO/PI double staining (Figure 4A) revealed the occurrence of morphological changes. Figure 4B shows the quantification of viable and dead cells: The control group had significantly more viable cells than the treated groups ($p < 0.0001$). Moreover, all treatments significantly increased the morphological changes characteristic of cell death ($p < 0.0001$), including chromatin condensation (CC), nuclear fragmentation (NF), membrane bubbles (MB), changes in cell volume, and extravasation of the cytoplasmic content.

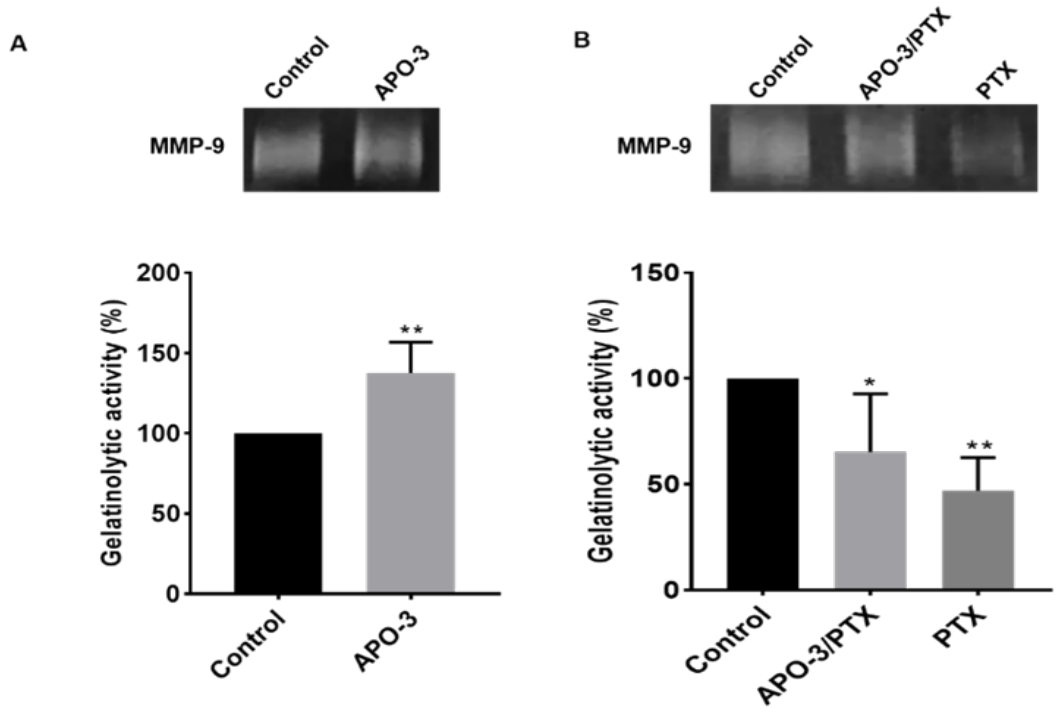


Figure 1. MMP-9 Activity Present in the 4T1 Cell Supernatant after Treatment with (A) APO-3 or (B) APO-3/PTX or PTX. * $p < 0.05$ and ** $p < 0.01$. Control: 1% DMSO.

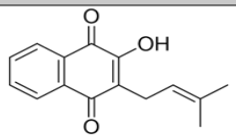
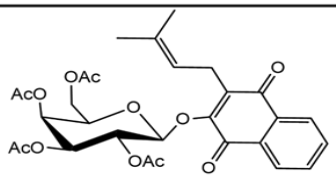
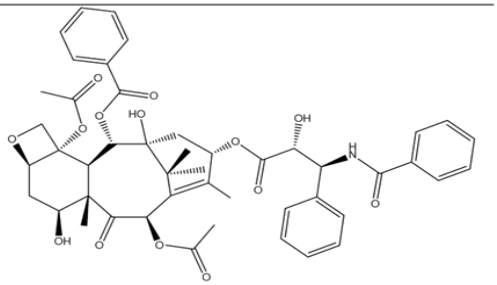
Modulatory effects of APO-3, APO-3/PTX, and PTX on key proteins of cell death pathways

Based on western blotting, APO-3 significantly increased the expression of PARP (129%), caspase-9 (143%), and RIP1 (145%), and decreased the expression of cleaved caspase-3 (51%). On the other hand, APO-3/PTX significantly decreased the expression of PARP (50%), caspase-9 (53%), and RIP1 (48%), and increased the expression of caspase-8 (183%) and cleaved caspase-3 (169%), similarly to PTX (Figure 5).

Discussion

Lapachol has been widely used as a starting point for the synthesis of new bioactive substances with antineoplastic effects against several tumor cells lines, including MDA-MB-231 human breast cancer cells [18, 29, 30]. The chemotherapeutic agent PTX is commonly

Table 1. The Compounds Tested in This Study and Their Nomenclature, Identification Codes, Molar Masses, and Chemical Structures.

Substance	Identification Code	M.M (g/mol)	Chemical Structure
Lapachol	LAP	242.27	
Gal-Lap	APO-3	572.55	
Paclitaxel	PTX	853.91	

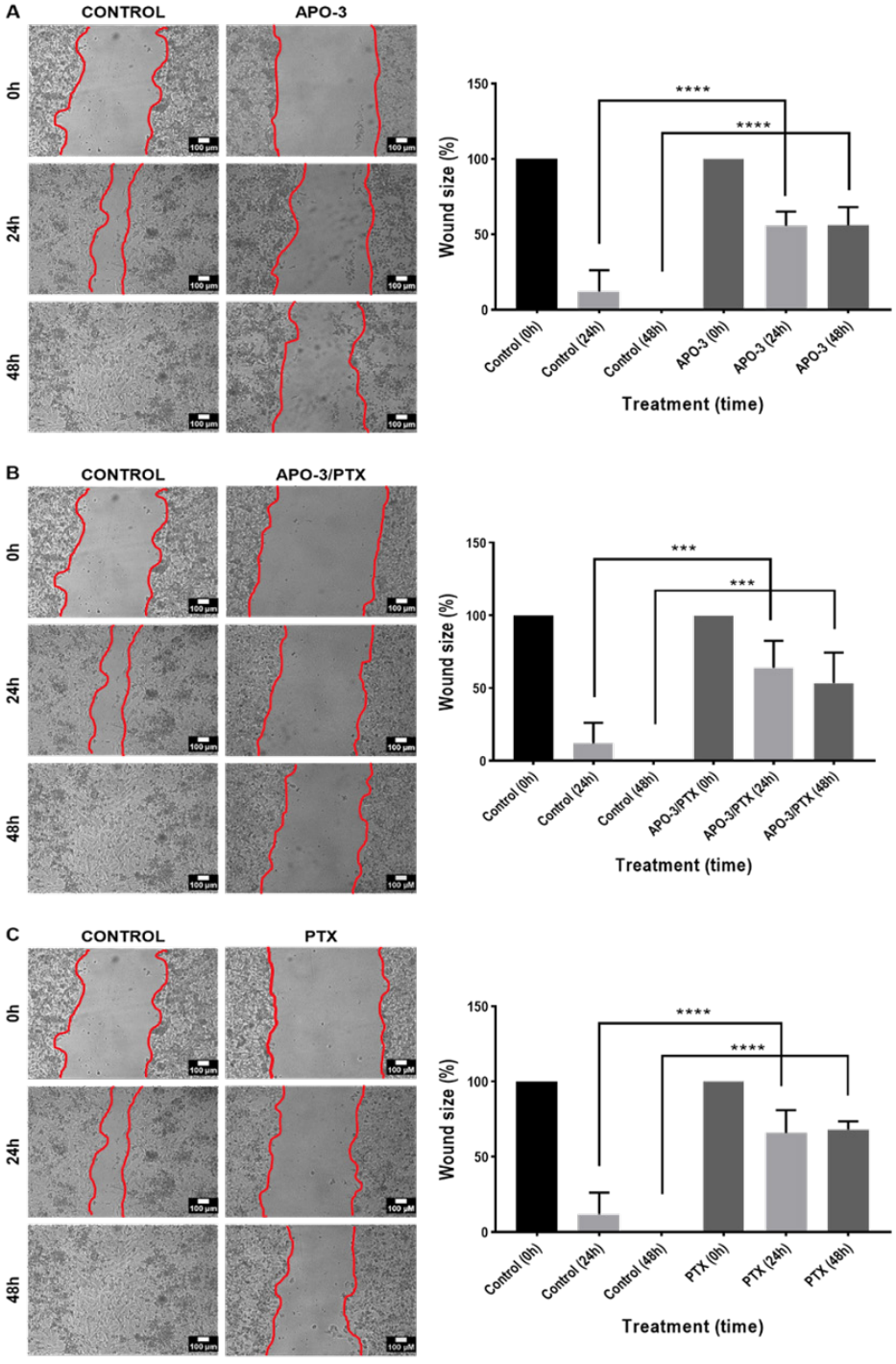


Figure 2. Inhibition of 4T1 Cell Mobility by (A) APO-3, (B) APO-3/PTX, and (C) PTX. The representative images (100× magnification) show the wound at different treatment times. The graphs show the wound size compared with the control group. *** $p < 0.001$ and **** $p < 0.0001$. Control: 1% DMSO.

used clinically, as monotherapy or in combination with other agents, for the treatment of refractory metastatic tumors, such as breast cancer [31]. However, the development of resistance to PTX is the main obstacle faced in treatment [32], requiring the discovery of a new more effective alternative therapy. Thus, we investigated the antitumor potential of a new synthetic substance derived from lapachol (APO-3), alone and in combination

Table 2 APO-3, APO-3/PTX, and PTX IC_{50} and TS for 4T1 Cells and Normal Murine Splenocytes.

Substance	IC_{50} (μM) 4T1	IC_{50} (μM) Splenocytes	TS
APO-3	0.86	34.12	39.67
APO-3/PTX	2.77	1,617	583.75
PTX	6.42	577	89.9

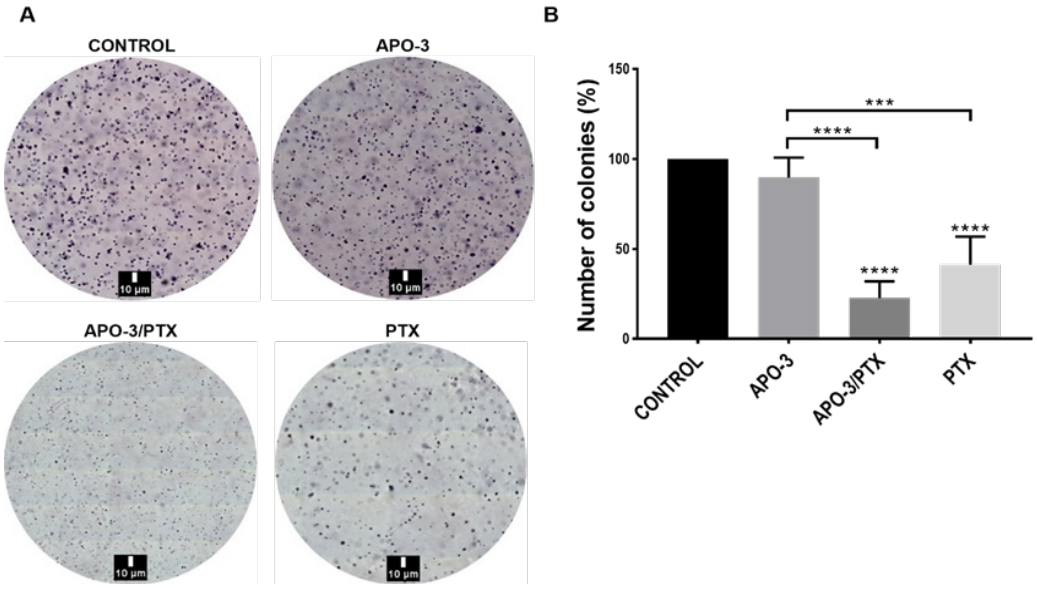


Figure 3. Inhibition of the Colony-Forming Ability of 4T1 Cells after Treatment with APO-3, APO-3/PTX, or PTX. (A) Representative micrographs of the colonies formed. (B) The number of colonies formed as a percentage of the control. *** $p < 0.001$ and **** $p < 0.0001$. Control: 1% DMSO.

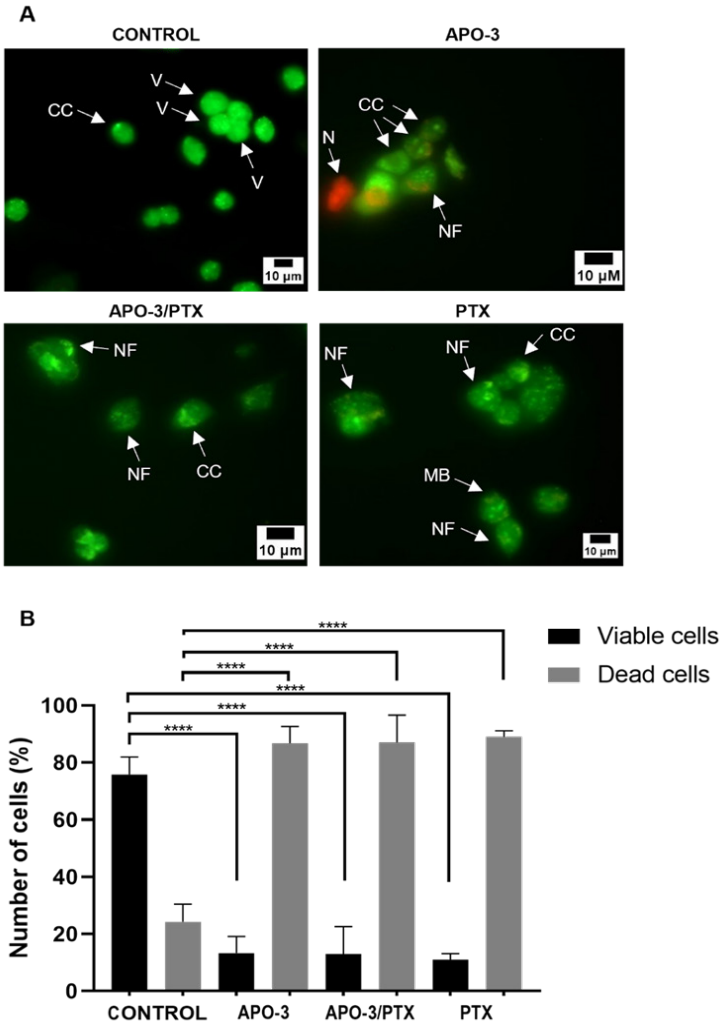


Figure 4. Morphological Changes Characteristic of Cell Ddeath in 4T1 Cells. (A) Morphological changes characteristic of the death of 4T1 cells treated with APO-3, APO-3/PTX, or PTX. (B) Quantification of viable and dead cells. V: viable cells; CC: condensation of chromatin; NF: nuclear fragmentation; MB: membrane bubbles; N: necrotic cells. **** $p < 0.0001$. Control: 1% DMSO.

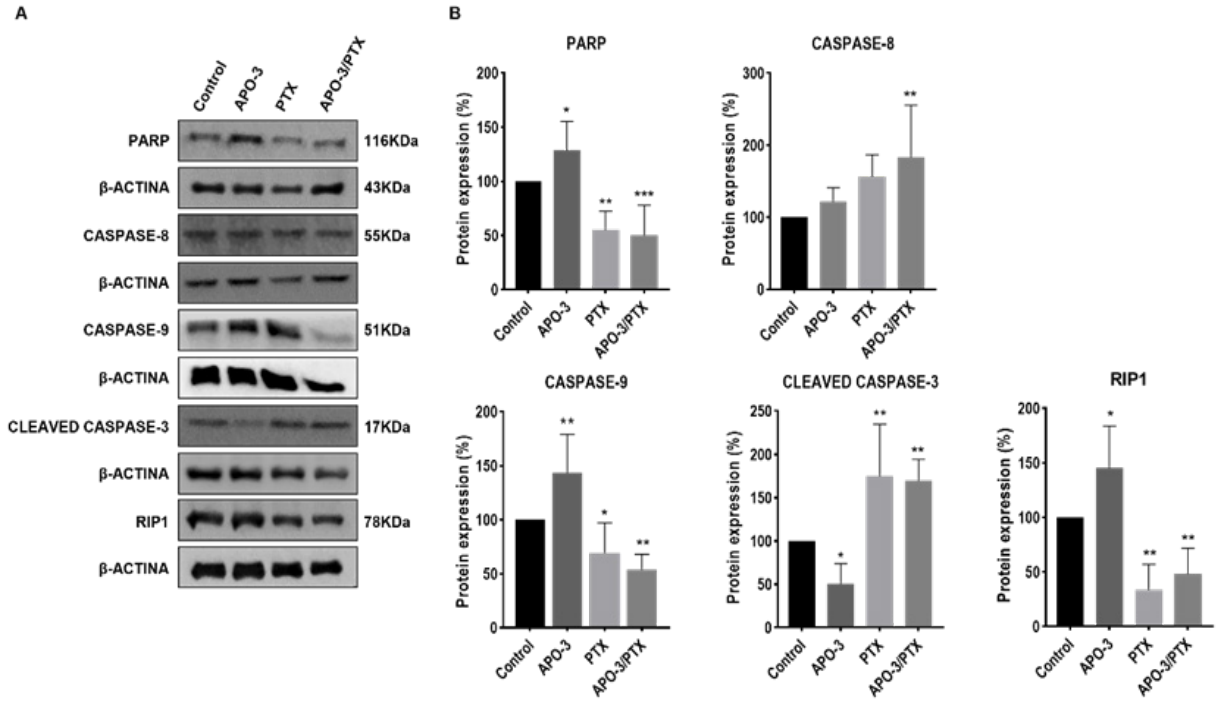


Figure 5. The Expression of Proteins Involved in Cell Death Pathways in 4T1 Cells after Treatment with APO-3, APO-3/PTX, or PTX. (A) Representative western blots showing the expression of PARP, caspase-8, caspase-9, cleaved caspase-3, and RIP1. (B) Quantification of the protein levels. *p < 0.05, **p < 0.01, and ***p < 0.001. Control: 1% DMSO.

with PTX, on a highly metastatic murine breast cancer cell line (4T1), which is widely used in studies investigating stage IV human breast cancer [33, 34]. 4T1 cells represent an excellent model to evaluate the potential of new substances as antitumor agents against human breast cancer.

In this study, APO-3 (IC_{50} = 0.86 μM/ 0.49 μg/mL) and APO-3/PTX (IC_{50} = 2.77 μM / 1.97 μg/mL) were more effective in reducing the viability of 4T1 cells than PTX alone (IC_{50} = 6.42 μM / 5.48 μg/mL). According to the National Cancer Institute (NCI), for an isolated compound to be considered a promising antitumor, it must present IC_{50} values ≤ 4 μg/mL (DEHGHAN-NAYERI et al., 2019). Only APO-3 and APO-3/PTX shown IC_{50} values ≤ 4 μg/mL [35]. Similar results were found in a study using the naphthoquinone thymoquinone and PTX in 4T1 cells: The combination of these compounds also induced stronger cytotoxicity compared with PTX alone [36]. The cytotoxicity results obtained in this work agree with a previous study that showed lapachol analogues had cytotoxic activity against the HBL100 (breast), SW1573 (lung), HeLa (cervical) and WiDr (colon) tumor cell lines [37, 38]. Other studies have also reported the positive effects of the combination of β-lapachone naphthoquinones and plumbagin with PTX on human retinoblastoma and breast tumor cells. They also correlated their results with the cytotoxic synergism between these substances [39, 40]. Because we did not determine the combinatorial index of APO-3 and PTX, we cannot state definitively whether the interaction we observed was synergistic or additive. Because APO-3/PTX had a lower IC_{50} than PTX or APO-3 alone, we speculate that APO-3 adds to PTX cytotoxicity.

The search for new anticancer drugs that are more effective against tumor cells and not harmful to normal cells comprises one of the main challenges of cancer medicine [41]. Regarding selectivity, APO-3, APO-3/PTX, and PTX were not cytotoxic to normal murine splenocytes. Thus, APO-3, APO-3/PTX, and PTX seem to be selective for 4T1 cells, as they presented a TS of 39.67, 583.75, and 89.9, respectively. It is worth mentioning that APO-3/PTX was about 6.5 times more selective than PTX alone and almost 15 times more selective than APO-3 alone. This lower cytotoxicity against normal cells and the TS we calculated are extremely relevant, especially considering that current antineoplastic agents trigger significant adverse effects in patients due to non-specific cytotoxicity [42].

In addition to cytotoxicity and selectivity, a substance must also be effective in preventing tumor cell migration, invasion, and establishment in other sites, a process called metastasis. This process requires degradation of ECM components [43]. MMP-9, a protease produced mainly by tumor cells, can degrade some components of the ECM, which leaves enough room for them to migrate. Consequently, it facilitates spread to distant organs through the circulatory and lymphatic systems [2, 44, 45]. However, the cell migration mechanism is quite complex and depends on the activation of a wide range of intracellular processes, in addition to the action of proteases. The migratory process is mainly associated with dysregulation of signaling pathways that can lead to membrane protrusion and retraction. Furthermore, it is related to regulatory factors associated with the actin cytoskeleton, which is closely linked to cell adhesion dynamics. These parameters can contribute to modify

cell–cell and cell–ECM adhesion necessary for cell invasiveness [46, 47]. We found that APO-3, APO-3/PTX, and PTX inhibited wound closure similarly. However, PTX and APO-3/PTX were more effective in inhibiting MMP-9 secreted by 4T1 cells. Considering that APO-3 alone did not inhibit MMP-9, PTX may have conferred an additional effect when combined with APO-3. Researchers have correlated, among other processes, the migration of tumor cells treated with quinones with the proteolytic action of MMPs [48, 49]. Those studies showed that substances derived from quinone slowed down cell migration in human breast adenocarcinoma lines, and reduced MMP-9 activity. Therefore, we suggest that the inhibition of cell migration demonstrated by APO-3/PTX and PTX alone may be mediated by the reduced MMP-9 proteolytic activity. However, it is important to emphasize that cell migration depends on more than just the activity of this protease, which may explain the fact that APO-3 inhibited cell migration but did not decrease MMP-9 activity.

The APO-3/PTX combination significantly inhibited the ability of 4T1 cells to establish metastases [50]. These results are similar to those reported by authors who also evaluated the action of a quinone derivative and a taxane, alone and in combination, on the clonogenic potential of MDA-MB-231 human breast cancer cells [51]. These authors observed that quinone was more effective in reducing the number of colonies formed by MDA-MB-231 cell line when combined with chemotherapy and suggested that the cytotoxic action of the taxane may be time dependent, as they act specifically on the cell cycle. Taxanes act by stabilizing microtubules, blocking mitosis, and causing cell death. Microtubules are formed by several units of α- and β-tubulin, which make up the cell cytoskeleton and are essential in the development and maintenance of cell shape, as well as in cell division, motility, signaling, and intracellular transport [52]. Taxanes are able to bind specifically to β-tubulin, thus preventing microtubule depolymerization, causing cell cycle arrest in the G2/M phase and, consequently, cell death [53]. Due to the different mechanisms of action of quinones, they may not act in the long term on colony formation. However, when they are combined with taxanes, they can gain an additional effect and inhibit 4T1 colony formation more effectively.

The redox property of naphthoquinone derivatives has also been well described. They increase the production of reactive oxygen species (ROS), due to their reduction of semiquinone, causing irreversible damage to macromolecules, which leads to cell death [54]. The apoptotic potential of this class of substances has been most associated with the production of ROS in several tumor cell lines [55, 56]. However, there has been a growing number of studies reporting that necroptotic cell death is also caused by the increase of ROS caused by naphthoquinones [57, 58].

Treatment with APO-3 alone reduced the expression of cleaved caspase-3, an essential effector protein that triggers apoptosis. Furthermore, it increased the expression of total caspase-9 but did not alter total caspase-8 compared with the control. In addition, we observed an increase in RIP1 protein expression. RIP1 may be involved in two cell death mechanisms investigated in this study, namely apoptosis and necroptosis [59]. The cleavage of caspases is an essential process that leads to their self-activation or the sequential activation of other effectors that trigger apoptosis, and an increase in their total form may indicate a reduction in their active (cleaved) form [60-62]. Based on this, our results suggest that APO-3 may induce necroptosis. Consistently, researchers have shown the induction of necroptosis in human glioma cell lines through upregulation of RIP1 expression and increased production of ROS by naphthoquinone [63, 64]. Furthermore, inhibition of RIP1 by Necrostatin-1 has been shown to prevent necroptosis in addition to significantly decreasing intracellular ROS levels. Moreover, the inhibition of ROS by N-acetyl-l-cysteine (NAC) led to a suppression of RIP1, suggesting that the production of ROS is a determining factor for the induction of necroptosis in glioma cells.

We found that the expression of total PARP, one of the proteins involved in DNA damage repair, was increased, perhaps indicating a reduction in its cleaved form [65]. A limiting factor that can lead to tumor resistance to chemotherapy drugs that induce DNA damage is mainly correlated with increased expression of DNA repair enzymes [66, 67]. Therefore, the possible reduction of cleaved (active) PARP may decrease the probability of resistance to APO-3, because the mechanism of action of this naphthoquinone can lead to DNA damage, mediated by increasing ROS. In addition, cells treated with APO-3 showed some morphological changes characteristic of apoptosis, such as chromatin condensation and nuclear fragmentation [68]. Moreover, we observed other morphological changes, such as increased cell volume, rupture of the plasma membrane, and extravasation of cytoplasmic content, which are typical characteristics of cells undergoing necroptosis [69, 70]. As mentioned previously, PTX acts on the stabilization of microtubules, causing cell cycle arrest in the G2/M phase, and leading to cell death [53]. Previous studies have shown that this mechanism of action induces cell death via apoptosis in several types of tumoral lineages [71, 72]. However, the molecular mechanism by which PTX induces apoptosis has not been fully elucidated [73-76].

In this study, we demonstrated that PTX possibly triggers cell death through the intrinsic apoptotic pathway, given that there were no changes in the expression of total caspase-8, a protein essential for the extrinsic apoptotic pathway. Furthermore, RIP1 expression was decreased, a phenomenon that may indicate that the necroptotic pathway is not activated. PARP, on the other hand, was reduced after treatment with PTX, which may indicate increased expression of its cleaved form. This change in PARP expression may be related to cell cycle arrest caused by PTX, because blocking cycle progression can lead to the induction and recruitment of DNA repair enzymes [77]. Furthermore, several cellular proteins, such as PARP, can be cleaved by active caspase-3, leading to cell death through apoptosis [78, 79]. Consistently, PTX induced apoptosis in human esophageal adenocarcinoma cells through activation of caspase-3 followed by degradation

of PARP [80]. Besides, researchers have demonstrated a decrease in the expression of total caspase-9 in retinoblastoma cells and acute promyelocytic leukemia cells treated with PTX [60, 81], and there was increased expression of cleaved caspase-3 [81]. Although we did not evaluate the expression of cleaved PARP, the increase in cleaved caspase-3 and the decrease in total PARP and caspase-9 may indicate that PTX induced 4T1 cell death via the apoptotic pathway.

Both PTX alone and the APO-3/PTX combination showed similar results. They increased the expression of cleaved caspase-3, decreased the expression of total caspase-9, and increased the expression of total caspase-8, which may be related to the decreased expression of its cleaved form [60, 61]. These results indicate possible activation of the intrinsic apoptotic pathway and not the extrinsic apoptosis pathway. In addition, the decrease in RIP1 expression demonstrated that there was possibly no activation of necroptosis. Furthermore, treatment with APO-3/PTX also reduced the expression of total PARP, which may indicate increased expression of its cleaved form. Various PTX and naphthoquinone combinations have produced similar results. The combination of shikonin with PTX induced apoptosis in human breast cancer cells [82]. In another study, combination of β-lapachone and PTX caused apoptosis in retinoblastoma cells, denoted by increased cleaved caspase-3 and reduced total caspase-9 [39]. These results reinforce the evidence that the APO-3/PTX trigger apoptosis through the intrinsic pathway. Furthermore, APO-3/PTX produced morphological changes characteristic of apoptosis, including chromatin condensation and nuclear fragmentation. Treatment with PTX alone led to other membrane changes, such as blister formation. Taken together, our results strongly suggest the pathway responsible for 4T1 cell death: APO-3 alone may trigger necroptosis, while PTX and APO-3/PTX may trigger apoptosis through the intrinsic pathway.

In conclusion, overall, the results of this study demonstrate that APO-3, a novel synthetic substance derived from lapachol, may have potent antitumor action on 4T1 cells and the combination of APO-3/PTX may be more effective than treatment with these substances alone, especially in controlling the metastatic activity of these cells. Besides that, it is important to highlight that more research is needed to fully understand the all mechanisms involved on the anticancer potential and efficacy observed for APO-3 and APO-3/PTX such as safety, biological activity, specificity and toxicity, especially on in vivo models.

Author Contribution Statement

FMMR: Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. LRS: Methodology, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. FMO: Methodology, Formal Analysis, Investigation, Writing - Original Draft. BK: Validation, Writing - Review & Editing, Visualization. HBS: Validation, Writing - Review & Editing, Visualization. RGT: Validation, Writing - Review & Editing, Visualization. RJA: Conceptualization, Validation, Resources, Writing - Review & Editing, Supervision, Funding acquisition. RIMAR: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. All authors read and approved the final manuscript.

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Ethics approval

Ethical approval was obtained by the Ethics Committee of the Federal University of São João del Rei under protocol number 006/2019.

Data availability

The dataset used and/or analyzed during this study is available from the corresponding author on a reasonable request.

Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

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