Pomolic acid exhibits anticancer potential against a docetaxel-resistant PC3 prostate cell line

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Abstract. Prostate cancer (PC) is one of the leading causes of cancer-related death in the male population worldwide. Mortality of PC is dependent on tumor recurrence and its progression to metastatic disease. We examined the effects of pentacyclic triterpene pomolic acid (PA) on docetaxel-resistant PC3 cells. Cell viability was evaluated using the MTT assay. Apoptosis was evaluated by cell cycle analysis using flow cytometry. The activity of multiple drug resistance (MDR) proteins was determined by the accumulation of specific substrates [mitoxantrone, rhodamine 123 and 5-carboxy-fluorescein diacetate (CFDA)]. The evaluation of epithelial to mesenchymal transition (EMT) proteins was conducted by immunocytochemical assays. It was demonstrated that PC3R cells presents multidrug resistance and EMT phenotype and express active P-gp/ABCB1 and MRP1/ABCC1. It was shown that PA strongly reduced the viability and induced apoptosis of both PC3 and PC3R cell lines. Moreover, PA bypassed P-gp/ABCB1, downmodulated MRP1/ABCC1 activities, and partially reverted EMT induced by DTX. Our goal was to evaluate the potential of PA for the development of novel strategies to treat castration-resistant PC.

Introduction

Prostate cancer (PC) is the most common cancer diagnosed in males in more than one-half of all countries and is the main cause of cancer-related death in males in 46 countries (1). At diagnosis, in approximately 90% of cases, PC is organ-confined or only locally advanced (2). Treatment is dependent on multiple parameters including clinical stage and prostate-specific antigen (PSA) levels; the options for localized PC are active surveillance, local radiotherapy or prostatectomy (3). With the spread of the disease outside the prostate, androgen deprivation therapy (ADT) by surgical or chemical castration is the recommended therapeutic strategy (3,4). Unfortunately, the response is only transient and most patients will develop resistance to ADT and progress towards castration-resistant prostate cancer (CRPC) in ~18-36 months (5). The androgen receptor (AR) axis is still active during PC progression and is an essential player in CRPC. Other options to achieve maximal androgen deprivation are the direct blockage of AR function with competitive antagonists or reduction of intra-tumoral androgen synthesis (3,4). Moreover, taxanes appear to be the only class of chemotherapeutic agents capable of acting on aggressive castration-resistant clones which emerge during ADT (6,7) besides being the first therapeutic option to provide a significant overall survival (OS) advantage and improvement of the quality of life in the setting of metastatic CRPC (mCRPC) (6). Despite these benefits, development of resistance to docetaxel is inevitable, and PC will eventually progress, and the treatment for patients progressing during or after docetaxel is limited and the prognosis is poor. Resistance to taxanes involves a multidrug resistance (MDR) phenotype with cross reactivity to unrelated drugs, alterations in the apoptotic signaling pathway and increased expression of transport proteins of the ABC family of transporters (8,9).

Some new agents have been evaluated for mCRPC in phase III clinical trials in the last 5 years. Although some improvement in OS and symptomatic benefits have been obtained, none of these agents is considered curative (10). Therefore, there is an urgent need for ongoing research to accomplish new approaches able to bypass the resistance mechanisms evolving in CRPC or, to block signaling pathways that lead to CRPC (11,12). Recently, pentacyclic triterpenes are emerging as important anti-neoplastic molecules. Increasing evidence shows that triterpenes target important biological events in cancer including cell proliferation, migration, invasion, and alterations of mechanisms involved in apoptotic control (13-15). Moreover, they are also effective against neoplastic cells whose resistance is mediated by ABC transporters (16-18). In addition, triterpenes were shown to inhibit certain signaling pathways important for the development, invasion/progression and metastasis in PC cell lines (19,20) and in experimental models of cancer (21).

The present study examined the effects of the pentacyclic triterpene pomolic acid (PA) on docetaxel-resistant PC3 cells.

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Herein, we showed that PA strongly reduced the viability and induced apoptosis in both parental (PC3) and docetaxel-resistant (PC3R) cell lines. We also showed that PA bypasses the mechanisms of resistance mediated by P-gp/ABCB1 and MRP1/ABCC1 activity. In addition to this effect, PA reverted the alteration of various markers of EMT in docetaxel-resistant PC3 cells.

Materials and methods

Reagents and cell lines. Docetaxel (Taxotere®) and MK571 were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Pomolic acid (PA), provided by BioBioPha Co. Ltd. (Yunnan, China), was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM, stored at -20˚C and diluted in culture medium for use. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Life Technologies, Inc. (Carlsbad, CA, USA). DMSO, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI) and KO 143 were obtained from Sigma Chemical Co./Merck KGaA (Darmstadt, Germany). Rhodamine 123 (Rho123) and 5-carboxyfluorescein diacetate (5-CFDA) were obtained from Calbiochem/EMD/Merck KGaA. Doxorubicin (cat. no. ab120629), vincristin (cat. no. ab120226), and anti-N-cadherin antibody (polyclonal rabbit, cat. no. ab12221) were from Abcam (Cambridge, MA, USA). Verapamil was obtained from Alexis Biochemicals (San Diego, CA, USA). Mitoxantrone (cat. no. sc-207888) and PE-labeled anti-MRP1 (QCRL-2) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-E-cadherin/CDH1 antibody (HECD-1) (monoclonal mouse, cat. no. 13-1700) was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-vimentin antibody, clone SP20 (rabbit monoclonal, cat. no. VPRM17) and Vectashield mounting medium (cat. no. H-1000) were from Vector Laboratories (Burlingame, CA, USA). Anti-N-cadherin, clone SP90 (rabbit monoclonal) was obtained from Sigma-Aldrich/Merck KGaA. Goat anti-rabbit IgG, F(ab')$_2$ fragment conjugated to cyanine Cy™3 (111-166-047), and goat anti-mouse IgG, F(ab')$_2$ conjugated to cyanine Cy™3 (111-586-072) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

The human prostate cell line PC3 (ATCC, CRL-1435™) was grown in DMEM, supplemented with 10% heat-inactivated FBS, 100 U penicillin and 100 mg/ml streptomycin in disposable plastic bottles at 37˚C in 5% CO_2. Cells were sub-cultured using trypsin-EDTA every 3-4 days. The docetaxel-resistant PC-3 cell line was developed by stepwise increased concentrations of docetaxel, essentially as previously described (22). PC3 cells were continuously maintained in docetaxel, with treatments beginning at 4 nM. After treatment, the surviving cells were re-seeded into new flasks and allowed to recover. The procedure was repeated until the cells displayed resistance to treatment as evaluated by cellular viability (MTT assay). New cycles were performed, in which the docetaxel concentration was subsequently increased up to a final concentration of 20 nM. Surviving cells were termed PC3R.

Cell viability assay. Cell viability was assessed using the MTT assay as previously described (15). Briefly, 180 ml of a PC3 or PC3R cell suspension (10^4 cells/well) was distributed in 96-well plates and incubated for 24 h at 37˚C/5% CO_2 to allow the culture to stabilize. The cells were then treated with 20 ml of medium, various concentrations of docetaxel (0.5, 1, 2, 4, 8, 12, 16, 20, 100, 500 and 1,000 nM); PA (1, 2.5, 5, 7.5, 10, 12.5, 15 μg/ml equivalent to 5.29, 10.57, 15.86, 21.15, 26.44, 31.73 μM); DMSO concentrations for each dose were used as controls. After 72 h of incubation, the culture was treated with 20 μl MTT (2.5 mg/ml) and maintained for 4 h at 37˚C in the dark before the supernatant being discarded. The formazan produced by reduction of the MTT by viable cells was dissolved in DMSO and the optical density was measured in a multi-mode microplate reader (Synergy™ HT; BioTek Instruments, Inc., Winooski, VT, USA) at 570 nm (reference filter 630 nm). Experiments were repeated at least 3 times. The results are expressed as the mean ± SD of percent inhibition of cell viability.

DNA fragmentation assay. Apoptosis was assessed by cell cycle analysis using flow cytometry as previously described (15). After 24 h of resting, the plated prostate cells ($2x10^4$/well) were treated with media or various concentrations of pomolic acid (PA) (2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 μg/ml) and incubated for another 48 h. After this time, cells were harvested and re-suspended in a hypotonic fluorescent solution (50 μg/ml PI and 0.1% Triton X-100 in 0.1% Na citrate buffer) for 1 h, at 4˚C in the dark. The cell cycle was analyzed by flow cytometry (FL-2) (FACSCalibur; Becton Dickinson, San Jose, CA, USA) to determine the sub-G0/G1 DNA content. Sub-diploid populations were considered to be apoptotic. Data acquisition and analysis were carried out by BD CellQuest software, version 3.1f (BD Biosciences, San Jose, CA, USA). Each experiment was repeated at least 3 times. The results are presented as representative histograms and as mean ± SD of the percentage of the DNA that was fragmented.

Activity of ABC transporter proteins. The functional activity of the MDR proteins was determined based on the intracellular accumulation of specific substrates as previously described (15). For each experiment, cells ($1x10^5$/well) were seeded into 24-well plates and pre-incubated for 24 h at 37°C/5% CO_2 to allow stabilization of the culture. Plated cells were then incubated for 30 min with substrates specific for P-gp/ABCB1 (200 ng/ml Rho123), MRP1/ABCC1 (5 μM CFDA) or BCRP/ABCG2 (3 μM mitoxantrone) in the presence of medium or the conventional inhibitor of these proteins, verapamil (50 μM), MK571 (50 μM) and KO143 (40 μM). Then the cells were washed in phosphate-buffered saline (PBS), harvested and kept on ice until flow cytometric analysis (FACSCalibur; Beckton-Dickinson cytometer). In this condition, an increase in cellular fluorescence correlates with transport activity. Results are presented as representative histograms or as the mean ± SD of arbitrary units of mean fluorescence intensity (MFI).

To assess the effect of PA on the activity of P-gp/ABCB1 and MRP1/ABCC1, plated cells were incubated for 30 min with medium (autofluorescence), with substrates specific for each protein in the presence or absence of specific inhibitors or, with substrate plus the desired concentration of PA. After harvesting, the cells were analyzed as described above.

Immunocytochemical assays. PC3 or PC3R cells ($2x10^4$/well) were seeded on coverslips in 24-well plates, left to rest for 24 h

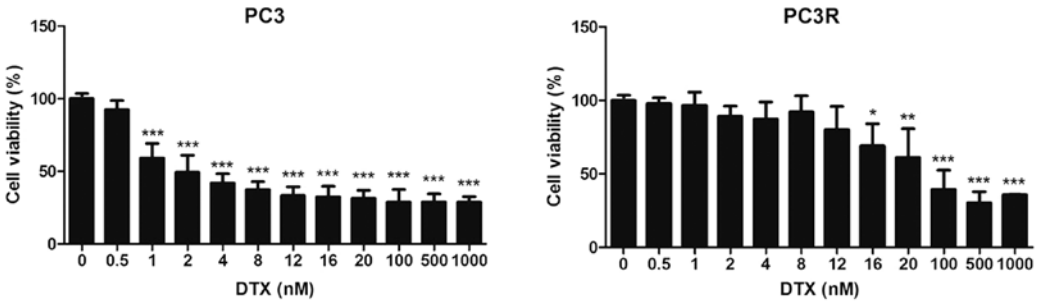

Figure 1. Sensitivity of the cell lines to docetaxel (DTX). PC3 and PC3R cells were plated ($2.5x10^3$/well) for 24 h and then treated with different concentrations of DTX. After 72 h, cellular viability was evaluated by MTT assay. Results are expressed as mean ± SD of at least 3 experiments performed in triplicate. Asterisks indicate significance in relation to the control ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$).

and then treated with medium or 5 μg/ml of PA. After 48 h of incubation, the cells were washed twice with PBS, pH 7.4, and fixed with a 4% buffered paraformaldehyde solution containing 4% sucrose for 40 min at 4˚C. After washing in PBS, the coverslips were permeabilized with PBS-0.5% Triton, pH 7.0 for 15 min, and then blocked with PBS-5% BSA-0.1% Triton-Tween 0.05% for 1 h. After this time, coverslips were incubated with the diluted primary antibodies (E-cadherin 1:50; N-cadherin: 1:100; vimentin 1:50) at 4˚C overnight. Then the coverslips were washed in PBS-0.25% Tween and incubated with secondary antibodies [goat anti-rabbit IgG, $F(ab')_2$ fragment-Cy™3 (1:200), and goat anti-mouse IgG, $F(ab')_2$ -Cy™3 (1:200)] diluted in PBS pH 7.4 for 1 h. Next, the coverslips were washed 2 times with PBS-0.25% Tween and then stained with 0.5 mg/ml DAPI for 5 min. Following washes with PBS and distilled water, the coverslips were mounted with Vectashield medium and observed in an epifluorescence microscope (Eclipse E-800; Nikon Corp., Tokyo, Japan). The quantitative analysis was performed using an image analysis system (Image-Pro Plus 4.5; Media Cybernetics, Inc., Rockville, MD, USA) composed of a digital camera (Evolution; Media Cybernetics, Inc.) coupled to a fluorescence microscope. High quality images of cells were captured (2048x1536 pixels buffer), using a 40x objective lens. At least 20 fields/coverslip were captured. For vimentin quantification, since all cells were labelled, the percentage of immunoreactive cells was calculated from the DAPI-positive cells.

Statistical analysis. The results are presented as mean ± standard deviation (SD) of at least 3 independent experiments. Student's t-test was used to analyze comparisons. One-way analysis of variance (ANOVA) and Dunnett and Tukey multiple comparison test were used to analyze differences among groups. $P<0.05$ was considered to refer to statistically significant differences. Statistical analysis was performed using GraphPad Prism 5 software (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA).

Results

Sensitivity of the cell lines to docetaxel (DTX). The MTT assay was used to evaluate the response of PC3 cells to docetaxel (DTX). Cells were treated with different concentrations of DTX and viability was assessed 72 h later. Fig. 1 (left panel) shows that 2 nM of DTX reduced PC3 viability by approximately 50%, demonstrating the sensitivity of the cell line to

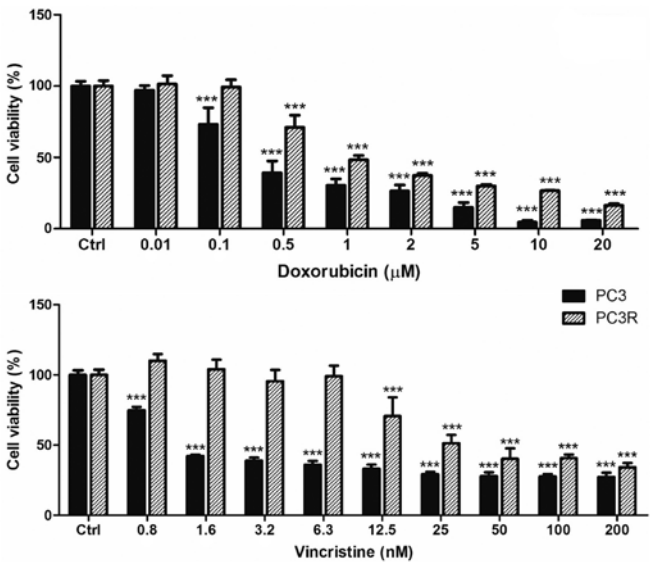

Figure 2. PC3R presents a multidrug resistance phenotype. PC3 and PC3R cells were plated ($2.5x10^3$/well) and 24 h later treated with the indicated concentrations of doxorubicin or vincristine. Cell viability was assessed by MTT assay, 72 h later. Results are expressed as mean ± SD of at least 3 experiments performed in triplicate. Asterisks indicate significance in relation to the control ($^{***}P<0.001$).

DTX. Establishment of a docetaxel-resistant PC3 cell line, PC3R, was accomplished by 4-5 cycles of treatment with stepwise increasing concentrations of the drug from 4 to 20 nM, as described in Materials and methods. Higher concentrations were avoided due to their toxicity. At this point, cells were treated with DTX in the same conditions used for the parental line and the cell viability was measured by MTT assay. Fig. 1 (right panel) shows that the treated cells (PC3R) were resistant to low concentrations of DTX, being responsive only to higher concentrations of the drug.

DTX-resistant cell line PC3R displays a multidrug resistance phenotype. To ascertain whether the PC3R cell line presents with a multidrug resistance phenotype, parental and resistant cells were treated with doxorubicin or vincristin (Fig. 2) and the cell viability was assessed by MTT assay, 72 h later. The results showed an increase in resistance of the PC3R cells to both drugs corroborating the multidrug resistance phenotype of this cell line.

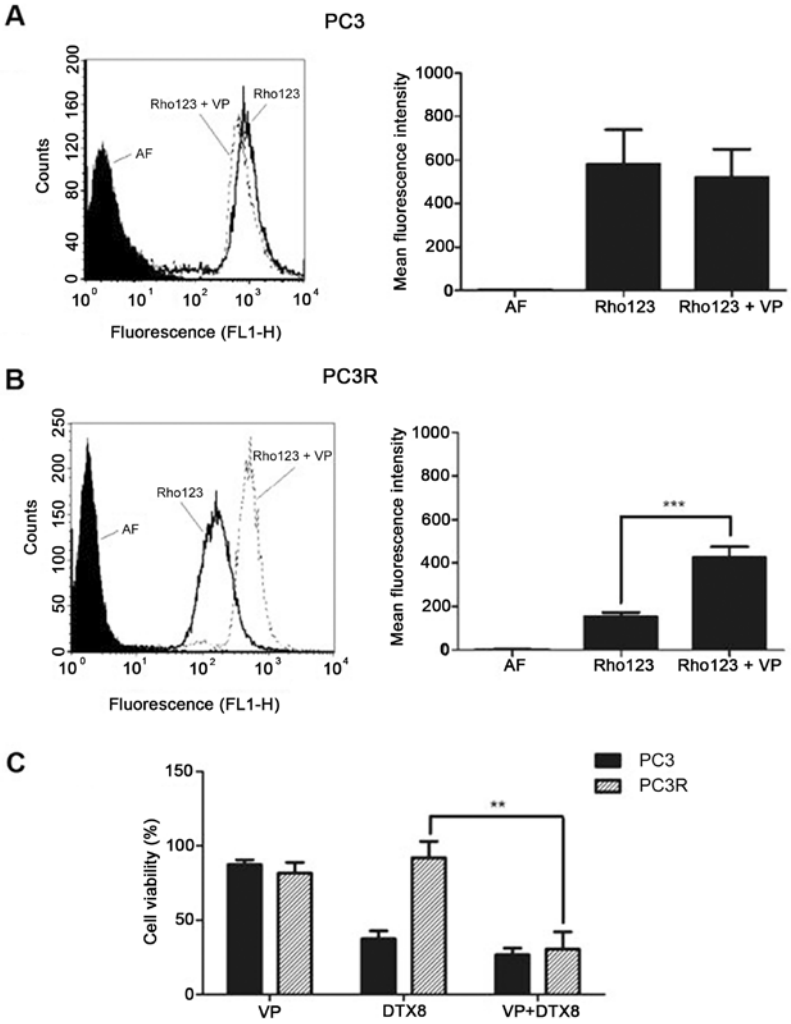

Figure 3. Activity of P-gp/ABCB1 on PC3 lineages. PC3 (A) and PC3R (B) cells were incubated for 30 min in the presence of medium (AF), rhodamine 123 (Rho123) or Rho123 plus verapamil (VP), and intracellular fluorescence was evaluated by flow cytometry as described in Materials and methods. (Left panels) Representative histograms. (Right panels) Graphs of the mean fluorescence intensity (MFI) ± SD of 3 experiments expressed as arbitrary units (a.u.). (C) Inhibiton of P-gp/ABCB1 reverts DTX resistance. PC3 and PC3R cells were plated ($2.5x10^3$ cells/well) and 24 h later treated with either P-gp/ABCB1 inhibitor VP, docetaxel (DTX) (8 nM) or DTX plus VP and cellular viability was assessed by MTT assay, 72 h later. Results are expressed as mean ± SD of at least 3 experiments performed in triplicate. Asterisks indicate significance in relation to the control (**P<0.01; ***P<0.001).

Activity of ABC transporters on PC3 lineages. Extrusion of drugs by transporter proteins of the ABC family is one of the main mechanisms of drug resistance. In order to investigate the involvement of the transporter proteins in DTX resistance, we analyzed the activity of P-gp/ABCB1, MRP1/ABCC1 and BCRP/ABCG2 on both parental and resistant cells. For this, cells were incubated for 30 min with substrates for BCRP/ABCG2, MRP1/ABCC1 and P-gp/ABCB1 (mitoxantrone, CFDA or Rho123) in the presence or absence of inhibitors specific for each transporter (KO143, MK571 or verapamil), and the cell fluorescence was evaluated by flow cytometry. As shown in Fig. 3, while no P-gp/ABCB1 activity was observed in the parental cell line (Fig. 3A), a significant increase in activity (P<0.001) was observed in the DTX-resistant PC3R cell line (Fig. 3B). To evaluate the involvement of P-gp/ABCB1 activity in DTX resistance, cells were treated with 8 nM DTX in the presence or absence of the P-gp inhibitor verapamil (VP) and cell viability was measured by MTT assay 72 h later. The results (Fig. 3C) suggest that inhibition of P-gp activity reverts the resistance of the PC3R cell line to DTX.

Different from P-gp, both PC3 and PC3R cell lines displayed MRP1/ABCC1 activity (Fig. 4A and B). To test the involvement of this protein in DTX response, we measured the viability of the cells after 72 h treatment with 8 nM DTX in the presence or absence of the MRP1/ABCC1 inhibitor MK571. Although the co-treatment induced a significant decrease in cell viability of both lineages this response was more evident in the PC3R cells (Fig. 4C).

Pomolic acid induces cell death of the PC3 lineages. However, even when these transporters were inhibited, approximately 40% of the cells remained alive suggesting the involvement of other resistance mechanisms in PC3 and PC3R cells. These mechanisms are not mediated by BCRP/ABCG2 as a negligible activity of this transporter was observed in the parental and DTX-resistant cell lines (data not shown).

In an attempt to identify options for CRPC treatment, we evaluated the effects of PA on the PC3R cell line. For this, parental and resistant cell lines were incubated with different concentrations of the triterpene and the cell viability was

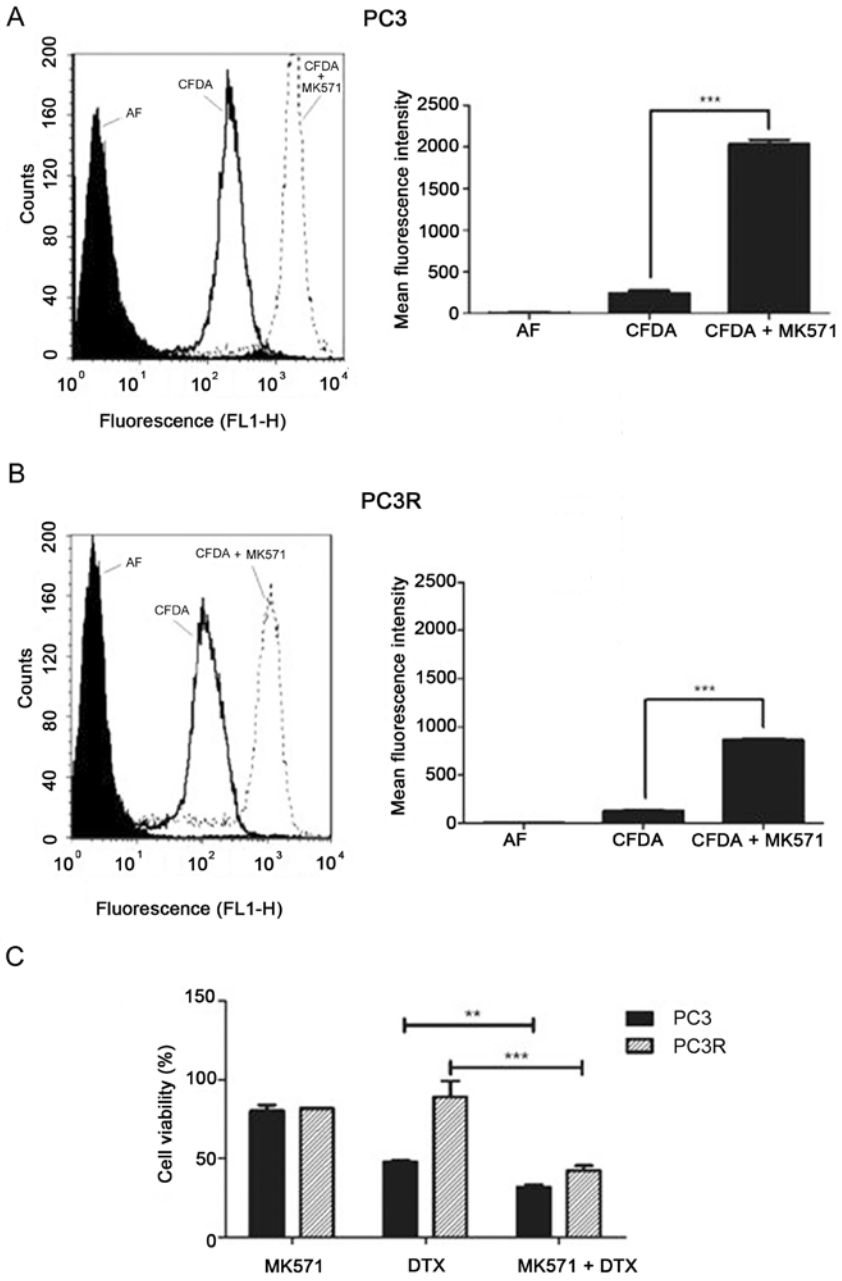

Figure 4. PC3 and PC3R cells display MRP1/ABCC1 activity. PC3 (A) and PC3R (B) cells were incubated for 30 min in presence of medium (AF), CFDA or CFDA plus MK571, and intracellular fluorescence was evaluated by flow cytometry as described in Materials and methods. (Left panels) Representative histograms. (Right panels) Graphs of the mean fluorescence intensity (MFI) ± SD of 3 experiments expressed as arbitrary units (a.u.). (C) Inhibition of MRP1/ABCC1 reverts docetaxel (DTX) resistance. PC3 and PC3R cells were plated ($2.5x10^3$ cells/well) and 24 h later treated with MRP1/ABCC1 inhibitor MK571, DTX or DTX plus MK571, and cellular viability was assessed by MTT assay, 72 h later. Results are expressed as mean ± SD of at least 3 experiments performed in triplicate. Asterisks indicate significance in relation to the control ($^{**}P<0.01$; $^{***}P<0.001$).

assessed by MTT assay 72 h later. PA appeared to be more active then DTX against PC as it acted on the two lineages in a similar way decreasing their viability up to 90% (Fig. 5).

In order to investigate whether the decrease in cell viability was due to apoptosis, we analyzed the DNA content of the sub-G0/G1 population in the cell cycle after 48 h of PA treatment. As shown in Fig. 6, PA increased the DNA fragmentation of both lineages in a dose-dependent manner indicating that death was mediated by apoptosis.

Pomolic acid has no effect on P-gp/ABCB1 but downmodulates MRP1/ABCC1 activity. Since inhibition of P-gp/ABCB1 or MRP1/ABCC1 increases the susceptibility of the resistant cell line to DTX, we analyzed whether the enhanced cytotoxic effect of PA would be due to an effect on these proteins. PC3 and PC3R cells were incubated with substrates of each protein in the presence or absence of inhibitors or 15 μg/ml PA and accumulation of the substrate was evaluated by cytometry. The results obtained demonstrated that PA-induced death in PC3R is independent of the increase in P-gp/ABCB1 since it does not interfere with the protein activity of the cell line (Fig. 7). Moreover, PA was able to modulate MRP1/ABCB1 activity in both parental and PC3R cell lines (Fig. 8), although less efficiently then the commercial inhibitor MK571.

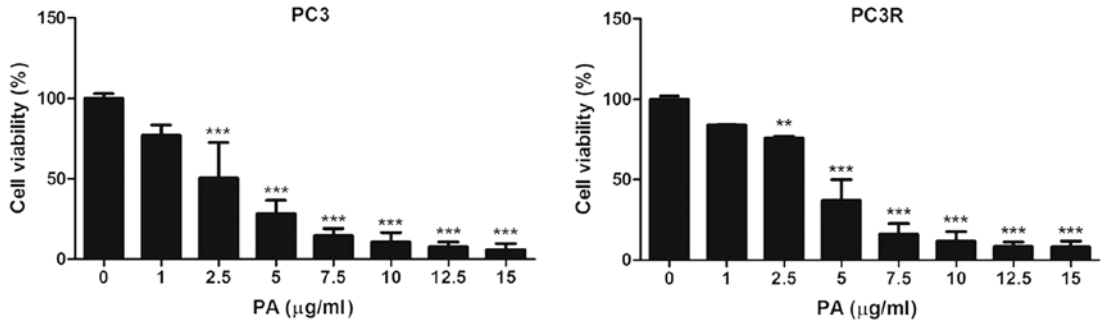

Figure 5. Sensitivity of the cell lines to pomolic acid (PA). PC3 and PC3R cells were plated ($2.5x10^3$ cells/well) and 24 h later treated with the indicated concentrations of PA. Cell viability was determined by MTT assay, 72 h later. Results are expressed as mean ± SD of at least 3 experiments performed in triplicate. Asterisks indicate significance in relation to the control ($^{**}P<0.01$; $^{***}P<0.001$).

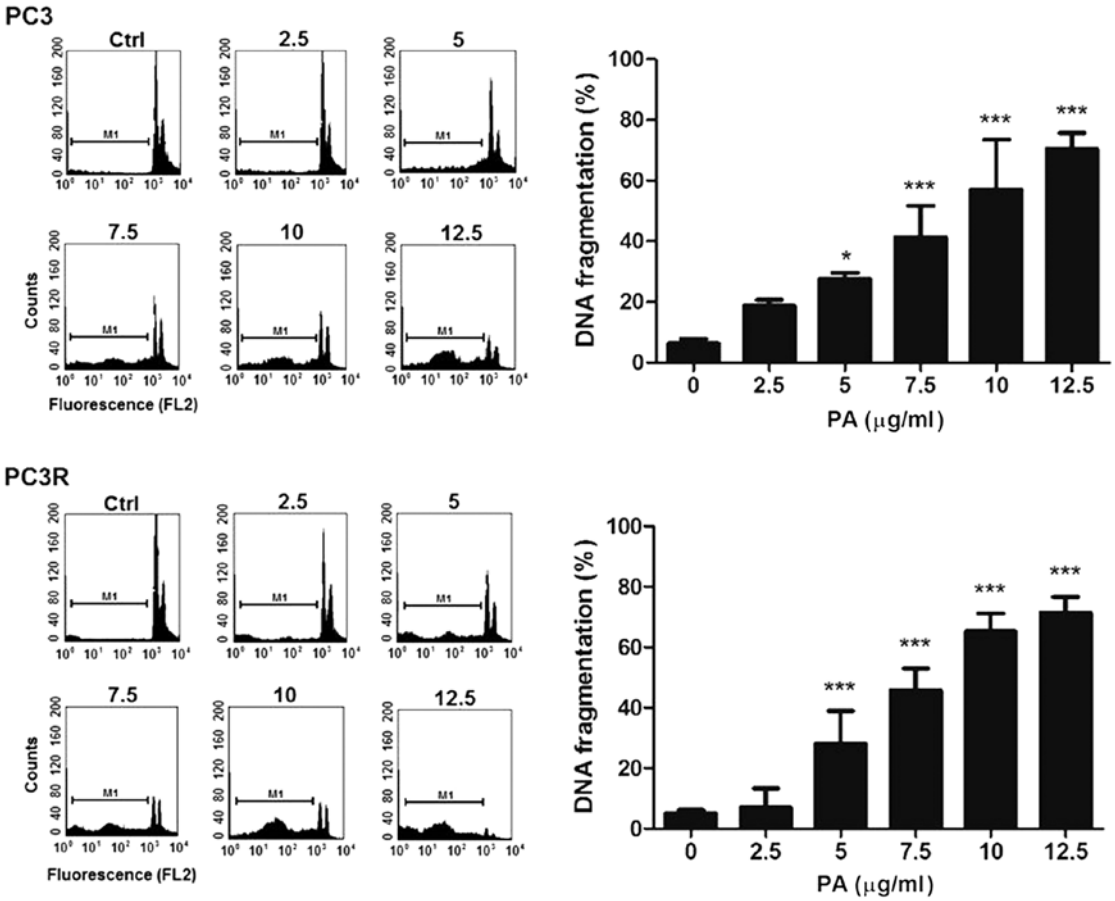

Figure 6. DNA content of the sub-G0/G1 population in the cell cycle after 48 h of PA treatment. PC3 and PC3R cells ($2x10^4$ cells/well) were plated for 24 h and then incubated for 48 h with the indicated concentrations of pomolic acid (PA). Subsequently, the cells were harvested and the hypodiploid population (considered apoptotic) was evaluated by flow cytometry (FL-2 channel) as described in Materials and methods. Results are expressed as mean ± SD of at least 3 experiments performed in duplicate. Asterisks indicate significance in relation to the control ($^{*}P<0.05$; $^{***}P<0.001$).

Pomolic acid partially reverts EMT changes induced by DTX treatment. Progression from the localized to invasive and metastatic tumors is partially dependent on a process called epithelial-mesenchymal-transition (EMT) in which the tumor epithelial cells loose expression of adhesion molecules such as E-cadherin and express proteins characteristic of mesenchymal cells such as vimentin (23). This process is also involved in the deregulation of androgenic signalization that occurs during the development of CRPC and confers resistance to apoptosis and stem cell like properties to tumoral cells (12).

Morphological analyses of the cells showed that DTX treatment changed the shape of the parental cells to a more elongated, fibroblastic appearance suggestive of EMT. Indeed, PC3R cells showed an increased amount of N-cadherin and vimentin ($P<0.001$), and a slight decreased amount of E-cadherin ($P<0.01$) compared with the PC3 parental cells suggesting an EMT phenotype in PC3R cells. Since EMT is a process involved in the progression of PC to CRPC and therefore, a possible target for the treatment of this tumor, to explore the mechanism of action of PA on PC, we evaluate the effects

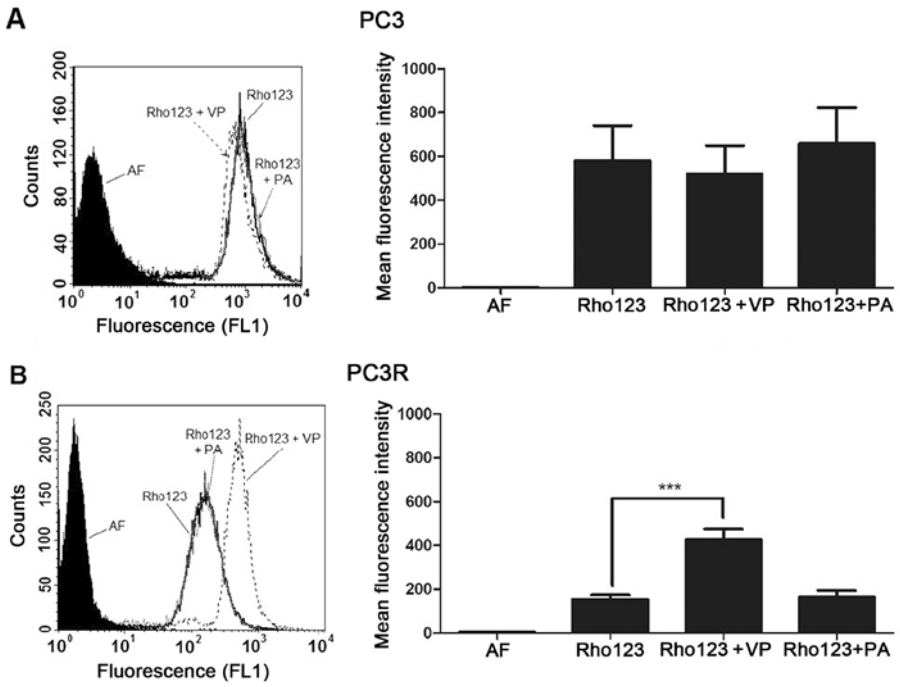

Figure 7. PA does not interfere with P-gp/ABCB1 activity in both parental and PC3R cell lines. PC3 (A) or PC3R (B) cells were incubated for 30 min in the presence of medium (AF), rhodamine 123 (Rho123), Rho123 plus verapamil (VP) or Rho123 plus 15 μg/ml pomolic acid (PA) and the intracellular fluorescence was measured by flow cytometry (FL-1 channel). (Left panel) Histograms representative of 3 independent experiments. (Right graphs) Mean fluorescence intensity (MFI) expressed as arbitrary units (a.u.). Asterisks indicate significance in relation to the control (***P<0.001).

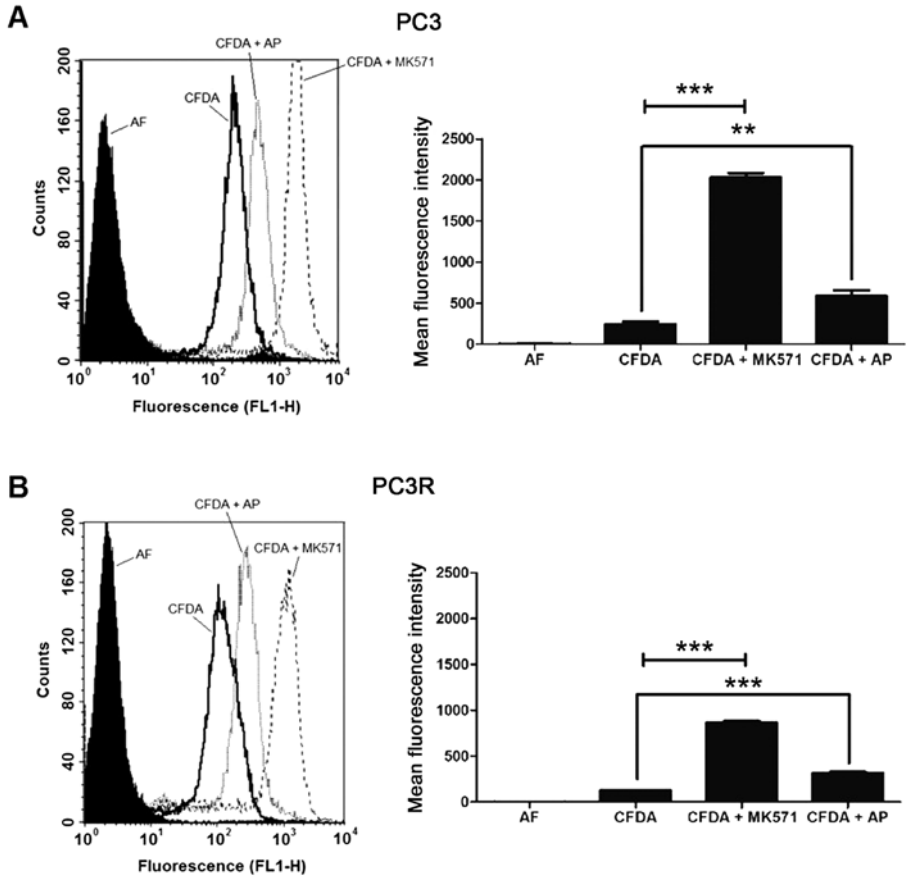

Figure 8. Pomolic acid (PA) modulates MRP1/ABCB1 activity in both parental and PC3R cell lines. Cells were incubated for 30 min in the presence of medium (AF), CFDA, CFDA plus MK571 or CFDA plus PA and the intracellular fluorescence was measured by flow cytometry (FL-1 channel). (Left panels) Histograms representative of 3 independent experiments. (Right graphs) Mean fluorescence intensity (MFI) expressed as arbitrary units (a.u.). Asterisks indicate significance in relation to the control (**P<0.01; ***P<0.001).

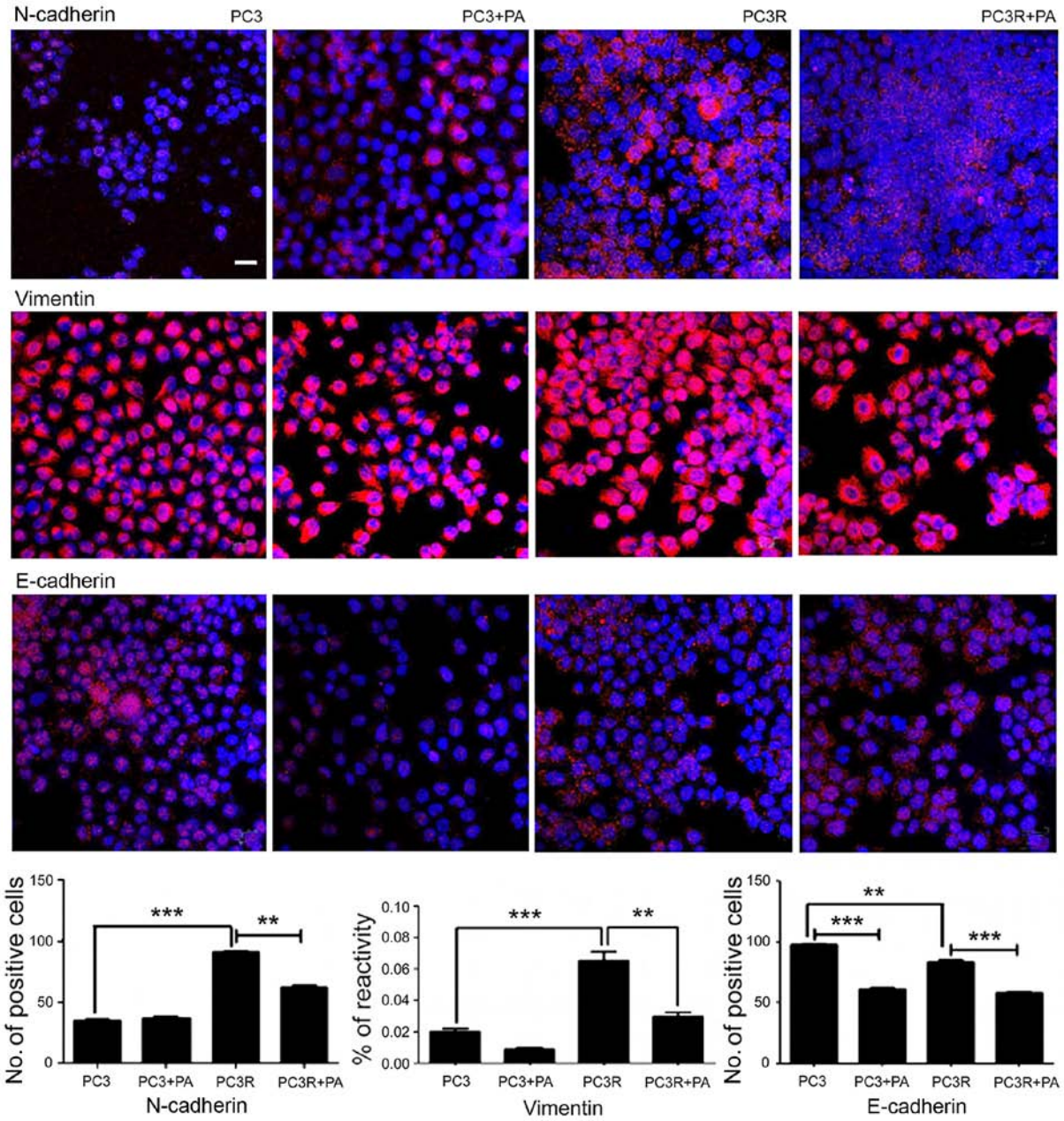

Figure 9. Fluorescence images of epithelial to mesenchymal transition (EMT) proteins (upper panels) and graphical representation of N-cadherin, vimentin, and E-cadherin (lower panels) in PC3 and PC3R cells treated or not with pomolic acid (PA). Cells were seeded on coverslips, treated with medium or 5 μg/ml PA for 48 h and processed as described in Materials and methods. At least 20 images of each coverslip were captured by epi-fluorescence microscopy and quantitative analysis was performed using an Image-Pro Plus 4.5 system. Calibration bar, 25 μm. Data represent mean ± SEM of the number of positive cells (E-cadherin and N-cadherin) or percentage of immune reactive cells calculated from DAPI (vimentin) in PC3 and PC3R cells treated compared with non-treated cells (**P<0.01, ***P<0.001), submitted to ANOVA followed by Tukey's post hoc test.

of this compound on various markers of EMT. The treatment of PC3R cells with 5 μg/ml PA for 48 h, decreased N-cadherin, vimentin, and E-cadherin levels in PC3R cells compared with non-treated PC3R cells (Fig. 9) indicating that the triterpene partially reverts the EMT phenotype observed in the resistant cell line.

Discussion

Prostate cancer (PC) is one of the leading causes of cancer-related death in the world in the male population (1). The mortality of PC is dependent on tumor recurrence and its progression to metastatic disease. To date, there is no effective treatment for metastatic disease which remains incurable (24).

Resistant PC strains are an important tool for understanding castration-resistant prostate cancer (CRPC) and for the search for alternatives to its treatment (22,25,26). The present study showed that the continuous treatment of PC3 with docetaxel (DTX) generated a resistant cell lineage (PC3R) as demonstrated by its lower sensitivity to the drug (Fig. 1). Thus, while 8 nM DTX was able to reduce the cell viability of the parental line (PC3) by 60%, ~100 nM of the drug was required to attain the same effect on PC3R cells. These data have been corroborated by the work of other researches describing PC3 cell lines resistant

to DTX (22,26) and to paclitaxel (25). The DTX-resistant lineage (PC3R) also exhibited cross-resistance to doxorubicin and vincristine, characterizing the MDR phenotype of this lineage.

Transporter proteins of the ABC family are one of the main mechanisms of MD, and differential expression of these pumps has been demonstrated in several tumor types including PC. In fact, an increase in P-gp/ABCB1 and MRP1/ABCC1 expression in PC samples in comparison with normal prostate tissue has already been observed (27).

Functional analysis of the major ABC transporters showed P-gp/ABCB1 activity in the resistant (PC3R) cell line but not in the parental cell line (PC3) (Fig. 3A and B). Expression of P-gp/ABCB1 in DTX-resistant PC lines (22) and correlation of this expression with DTX resistance has been previously reported (28,29). In the TaxR line, the deletion (knockout) of P-gp/ABCB1 expression by shRNA reversed the response to DTX (30), indicating that increased expression and/or activity of this protein could be responsible for the increased resistance to DTX noted in the PC3R cells. In fact, our results demonstrating that treatment with verapamil (VP), a specific inhibitor of P-gp/ABCB1, increased the sensitivity to DTX (Fig. 3C), corroborates this hypothesis.

Different form P-gp/ABCB1, MRP1/ABCC1 transporter activity was detected in both the resistant and parental PC3 cells (Fig. 4A and B). The presence of active MRP1/ABCC1 has also been described in PC cell lines (31) and increased expression of this protein was found in doxorubicin-resistant prostate lines (32). The presence of an active MRP1 in the studied lineages suggests a possible contribution of this transporter in the intrinsic resistance of PC. Moreover, treatment of PC3R with MK57, a specific inhibitor of MRP1/ABCC1 activity, only partially reversed the response to DTX (Fig. 4C) indicating that other mechanisms are also involved in the intrinsic resistance of these cells to DTX. Absence of BCRP/ABCG2 activity (data not shown), indicates that this resistance was not mediated by this transporter.

Several chemotherapeutic drugs have been used for the treatment of PC. However, in spite of several new therapies, they generally induce resistance, leading to treatment failure, disease progression and patient death (12,13). New approaches able to bypass the resistance mechanisms and to increase the survival of patients with CRPC are urgently needed in the clinic. With the objective of searching for new alternatives for CRPC, we evaluated the effect of the triterpene pomolic acid (PA) on the PC3 and PC3R cell lines. The antitumor activity of triterpenes in several cancer lineages, including PC, is well known (13,15,21,33,34). Similar to other triterpenes, PA shows potent antitumor activity, reducing the viability and inducing apoptosis of different tumor cell lines (17,35,36) including cancer cells presenting different MDR mechanisms (18). Our results (Fig. 5) showed that PA is quite effective, reducing the viability of the studied cell lines to levels below that obtained by treatment with DTX. The similarity of PA effects on PC3 and PC3R indicates that the triterpene is able to bypass not only the resistance mechanisms induced by DTX but also the intrinsic resistance mechanism present in the lineages. Indeed, PA did not interfere with P-gp/ABCB1 activity (Fig. 7B) present on PC3R cells, confirming previous findings in leukemic cell lines (17,18) that this triterpene is not a substrate for this transporter. Moreover, in agreement with previous observations of our group (36), PA negatively modulated the activity of MRP1/ABCC1 (Fig. 8A and B), suggesting that this effect contributes to PA cytotoxicity. However in addition to the activity of MDR proteins, resistance mechanisms present in CRPC may also be mediated by alterations of antiapoptotic proteins of the Bcl2 family (37) and innate activation of pathways involved in the EMT process, such as NF-κB and PI3K/AKT/mTOR among others (24). Literature data show that the cytotoxic effect of PA includes modulation of several of these pathways. Thus, PA is capable of killing Bcl-2 overexpressing cells (14), a protein whose inhibition restores the response to DTX (37). PA also inhibits the activation of NF-κB (38) a factor involved in PC progression (39), drug resistance (40) and activation and maintenance of the AR (41). Moreover, PA inhibits the activation of PI3K/AKT/mTOR (38) a pathway involved in disease progression and whose inhibition sensitizes PC cells to the apoptotic effect induced by chemotherapy (42).

Resistance of CRPC to DTX has also been related to endogenous cellular mechanisms or mechanisms dependent on the tumor microenvironment such as the EMT process. Previous studies have demonstrated that resistant prostate lines, including those resistant to docetaxel, exhibit an EMT phenotype (43,44). Analysis of the expression of EMT markers in the cell lines by fluorescence microscopy revealed that PC3R exhibited an increase in N-cadherin and vimentin expression and a slight decrease in E-cadherin when compared to PC3 indicating that PC3R underwent the EMT process (Fig. 9). PA partially reverted the increase in the expression of N-cadherin and vimentin induced by DTX treatment although its effect on E-cadherin was negligible. These results corroborate literature data showing that the triterpene ursolic acid blocks EMT by increasing E-cadherin expression and decreasing vimentin expression in different strains (45,46).

The results obtained in the present study demonstrated that PA may be a possible candidate for the development of novel antitumor agents for future PC chemotherapy. Indeed, in addition to being a powerful cytotoxic agent, this triterpene was found to interfere with important mechanisms of resistance in the treatment of PC such as that mediated by transporter proteins (P-gp/ABCB1 and MRP1/ABCC1) and its effects on EMT may be useful in preventing disease progression. Moreover, literature data have shown that PA also acts on several pathways involved in DTX resistance and the EMT process. Together, these data suggest a possible advantage of using PA as a co-adjuvant to prevent progression of PC and to the treatment of CRCP.

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Availability of data and materials

The datasets generated in the present study are available from the corresponding author on reasonable request.

Authors' contributions

CRG and CMT conceived and designed the study. CAM performed the experiments with the collaboration of GGR. CRG, CAM, GGR and CMT reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests. The authors alone are responsible for the content and writing of this article.

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