Prostate cancer cell response to paclitaxel is affected by abnormally expressed securin PTTG1

Running title: PTTG1 and response to paclitaxel treatment

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Abstract

PTTG1 protein, the human securin, has a central role in sister chromatid separation during mitosis and its altered expression has been reported in many tumor types. Paclitaxel is a widely used chemotherapeutic drug, whose mechanism of action is related to its ability to arrest cells in mitosis and the subsequent induction of the intrinsic apoptotic pathway. By using two prostate cancer cell lines with different responses to paclitaxel treatment, we have identified two situations in which PTTG1 influences cell fate differentially. In slippage-prone PC3 cells, both PTTG1 downregulation and overexpression induce an increase in mitotic cells that is associated with diminished apoptosis after paclitaxel treatment. In LNCaP cells, however, PTTG1 downregulation prevents mitotic entry and, subsequently, inhibits mitosis-associated, paclitaxel-induced apoptosis. By contrary, PTTG1 overexpression induces an increase in mitotic cells and apoptosis after paclitaxel treatment. We have also identified a role for Mcl-1 protein in preventing apoptosis during mitosis in PC3 cells, as simultaneous PTTG1 and Mcl-1 silencing enhance mitosis-associated apoptosis after paclitaxel treatment. The finding that a more efficient mitotic arrest alone in PC3 cells is not enough to increase apoptosis, was also confirmed with the observation that a selected paclitaxel-resistant PC3 cell line showed an apoptosis-resistant phenotype associated with increased mitosis upon paclitaxel treatment. These findings could contribute to identify putative responsive and non-responsive cells and help us to approach incomplete responses to paclitaxel in the clinical setting.
Introduction

The spindle assembly checkpoint (SAC) is responsible to delaying chromosome segregation until all duplicated sister chromatids are bound and bi-oriented in the mitotic spindle. When this occurs, APC/C\(^{Cdc20}\)-mediated degradation of securin (PTTG1 in humans) and cyclin B1 takes place, and cells enter anaphase (1). Paclitaxel is a chemotherapeutic drug of the taxane group widely used in the treatment of different cancers. It interacts with β-tubulin and triggers cell cycle arrest in mitosis, blocking normal mitotic spindle assembly and cell division. The interruption of mitotic spindle formation activates the SAC. Several components of the SAC have been identified (Cdc20, Mad1, Mad2, BubR1/Mad3, Bub1, Bub3, etc), but the main target of SAC is Cdc20, a co-factor of the ubiquitin ligase APC/C. The SAC negatively regulates the ability of Cdc20 to activate the APC/C-mediated polyubiquitylation of PTTG1 and cyclin B1, thereby preventing their destruction by the 26S proteasome and generating an anaphase inhibitory signal (1, 2). Following paclitaxel-induced mitotic arrest, cells die in mitosis or exit mitosis by slippage into a tetraploid G\(_1\) state, from which they either die, arrest in G\(_1\) or initiate a new round of cell cycle (3). Slippage occurs when, despite continuous SAC signalling (as induced by paclitaxel treatment), PTTG1 and cyclin B1 are degraded and cells exit mitosis without cytokinesis (4). When cell death occurs, it takes place mainly via the intrinsic pathway of apoptosis. Among the apoptotic events initiated by paclitaxel there have been included several modifications of Bcl-2 family proteins such as Bcl-2 and Bcl-xL phosphorylation, Bax activation (2, 5) or Bim induction by FOXO transcription factors (6, 7).

PTTG1, the product of pituitary tumor-transforming gene 1, is involved in sister chromatid separation during mitosis (8). Its expression level varies within the cell cycle, reaching the maximum in G\(_2\)/M (9). Subcutaneous injection of PTTG1-transfected cells
into nude mice has been shown to induce tumors formation. Furthermore, it has been reported PTTG1 overexpression in different tumor types (10-18). PTTG1 is involved in several cellular processes including DNA damage repair, apoptosis, and angiogenesis. It also interacts with a number of factors both in vivo and in vitro. PTTG1 also possesses transactivating activity, inducing the upregulation of several other genes (19). Regarding PTTG1 function as a cell cycle regulator, during most of cell cycle PTTG1 is bound to a protease called separase and thus inhibits its proteolytic activity (20). In metaphase, replicated sister chromatids are held together by the cohesin complex, an essential process to prevent premature separation of chromatids (21). During metaphase to anaphase transition, once chromosomes are properly oriented, PTTG1 is degraded via APC/C-Cdc20. PTTG1 destruction releases separase inhibition, which in turn mediates degradation of the cohesin complex and sister chromatids separation proceed (10). PTTG1 function in apoptosis is currently under debate. In fact, it has been reported that PTTG1 overexpression promotes and inhibits apoptosis (10). Thus, we sought to investigate the influence of PTTG1 protein on cell cycle and apoptosis induction in two prostate cancer cell lines that respond differentially to paclitaxel.

**Materials and methods**

**Cell culture**

Human prostate cancer PC3 and LNCaP cell lines were obtained from the Interlab Cell Line Collection (Genoa, Italy). No further authentication was made by the authors. All the experiments were done using vials from the first ten passages after receipt. Paclitaxel-resistant PC3 cell line (PC3 PTXR) was generated in our laboratory. Parental PC3 cells were treated with 1 μM paclitaxel for three days; then, the drug was withdrawn from the medium and surviving cells were allowed to grow. These cells were
again subjected to the same treatment and the resulting paclitaxel-resistant cells were used for the assays. Cell lines were routinely grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer and 1 mM glutamine in a 37°C, humidified incubator under 5% CO₂. Cells were harvested by trypsinization. The stock solution of paclitaxel (Calbiochem, San Diego, CA) was prepared at 10 mM in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) and stored frozen. In all the experiments, DMSO was added to untreated cells.

**Small interfering RNA**

Small interfering RNA (siRNA) transfections were carried out using the Dharmafect 2 reagent (Thermo Fisher Dharmacon, Lafayette, CO) according to the manufacturer’s instructions. The PTTG1 and Mcl-1-specific siRNAs and the control negative siRNA were validated pools from Thermo Fisher Dharmacon (On-Target plus Smart pools L-004308, L-004501, and D-001810). All siRNAs were used at 100 nM. Cells were subjected to different treatments 24 h after silencing.

**Plasmid transfections**

Cells were transiently transfected with a plasmid carrying a non degradable PTTG1, mutated in both KEN and D boxes, pEF/Securin KAA-DM, that was provided by M. Brandeis (Department of Genetics, Silberman Institute of Life Sciences) (22). Transfections were carried out by using the FuGENE reagent (Promega, Madison, WI), according to the manufacturer’s instructions. Cells were subjected to different treatments 24 h after transfection.
Antibodies

Rabbit polyclonal anti-Bax and anti-Bak were available from BD Biosciences (San Jose, CA). Mouse monoclonal anti-Bcl-xL, rabbit polyclonal anti-Mcl-1, anti-cyclin B1 and anti-phospho-histone H3 (Ser10) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-β-actin from Sigma. Rabbit polyclonal anti-cleaved caspase 9 (Asp 315) was from Cell Signaling (Danvers, MA). Rabbit anti-BubR1 antibody was from Bethyl Laboratories (Montgomery, TX). Rabbit polyclonal anti-PTTG1 was previously described (23). Dilutions used in Western blots were anti-Bax (1:1,000); anti-Bak (1:3,000); anti-Bcl-xL (1:100); anti-Mcl-1 (1:1,000); anti-cyclin B1 (1:1,000); anti-phospho-histone H3 (1:500); anti-β-actin (1:16,000); anti-cleaved caspase 9 (1:500); anti-BubR1 (1:3,000) and anti-PTTG1 (1:1,000). Caspase-mediated cleavage of poly-(ADP-ribose) polymerase (PARP) was assessed by Western blot using a mouse monoclonal anti-human PARP (BD Biosciences) at 1:250 dilution.

Western blotting

Cells were lysed in Nonidet P-40 (NP40) lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol and 1% NP40). Equal amounts of total protein, as determined by using BCA protein assay (Pierce, Rockford, IL), were separated by SDS-PAGE on 4–20% gradient polyacrylamide gels (Invitrogen, Carlsbad, CA). Gels were electroblotted onto nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Membranes were stained with Ponceau S to ensure that protein amounts were comparable. For immunodetection, blots were blocked in 1% blocking reagent (Roche, Mannheim, Germany) in 0.05% Tween 20-PBS for 1 h and incubated with primary antibody overnight at 4°C diluted in blocking buffer. Blots were then washed in 0.05% Tween 20-PBS and incubated with either goat anti-mouse (1:20,000; GE Healthcare) or
goat anti-rabbit (1:20,000; GE Healthcare) peroxidase-labeled antibodies in blocking buffer for 1 h. Enhanced chemoluminescent system was applied according to the manufacturer’s protocol (GE Healthcare). The experiments were performed at least three times.

**Apoptosis assays**

$1\times10^5$ cells were washed in cold PBS and suspended in 100 μl Annexin V binding buffer (R&D Systems, Minneapolis, MN) containing 5 μg/ml propidium iodide and 0.5 μg/ml annexin V-fluorescein isothiocyanate (FITC), incubated for 15 min at room temperature in the dark, and diluted in 400 μl Annexin V binding buffer. Fluorescence was measured in a FACScan flow cytometer (BD Biosciences) within 1 h. The collected events were gated on the forward and side scatter plots to exclude cellular debris. CellQuest Pro software (BD Biosciences) was used to analyze the data.

**Flow cytometric analysis of cell cycle**

Cells were trypsinized and fixed in 70% ethanol. Propidium iodide staining of nuclei was performed with a CycleTest Plus DNA reagent kit (BD Biosciences), and the DNA content was measured with a FACScan flow cytometer. Data were acquired with CellQuest Pro software. ModFit LT2 software (Verity Software, Topsham, ME) was used to assess cell cycle. The experiments were performed at least three times.

**Fluorescence in situ hybridization (FISH)**

Cultured cells were imprinted onto silanized slides, fixed in ice-cold methanol/glacial acetic acid (3:1) for 10 min and air-dried. Then, slides were immersed in a 2×SSC (saline-sodium citrate)/0.3% NP40 solution at 37°C during 30 min,
dehydrated in increasing graded ethanols and air-dried. Cellular DNA and centromeric probes for chromosomes 8 (Spectrum red) and 17 (Spectrum aqua) from Vysis (Downers Grove, IL) were co-denatured at 72°C for 5 min and hybridized at 37°C overnight in a humid chamber. After hybridization, slides were washed in 2×SSC /0.3% NP40 solution at 72°C for 5 min, counterstained with 4',6-diamidino-2-phenylindole (DAPI)/antifade (Vysis) and visualized under fluorescence microscopy equipped with appropriate filter sets and a digital camera (Leica, Wetzlar, Germany). The experiments were performed at least three times.

**Statistical analysis**

Data comparing differences between two conditions were statistically analyzed, when indicated, using paired Student’s t test. Differences were considered significant when P < 0.05. Calculations were performed using Prism 4.0 (GraphPad, San Diego, CA).

**Results**

*Paclitaxel-treated LNCaP cells undergo apoptosis during mitosis but less sensitive PC3 cells mostly die after slippage*

First, we examined the sensitivity of PC3 and LNCaP prostate cancer cells to paclitaxel-induced apoptosis. Cells were treated with 2.5 μM paclitaxel during 24 and 48 h, and apoptosis was assessed by the appearance of the 85 kDa-cleaved fragment of PARP protein and by caspase 9 activation. Both cleaved PARP and active caspase 9 were clearly more evident in paclitaxel-treated LNCaP than in paclitaxel-treated PC3 cells at both time points. A delay can be observed between both cell lines; after 24 h of
drug treatment, there is a clear apoptosis induction in LNCaP cells, whereas it is hardly detectable in PC3 cells. Apoptosis increases in both cells lines after 48 h, but it continues to be higher in LNCaP than in PC3 cells (Fig. 1). Then, we examined several cell cycle-related proteins in order to assess if differences in apoptosis induction were linked to changes in cell cycle. The behavior of these proteins was similar in both cell lines after paclitaxel treatment during 24 h, but it was markedly different at 48 h. Cyclin B1, PTTG1 and phosphorylated histone H3 (used as a surrogate for mitosis) increased in both cell lines 24 h after drug treatment. BubR1 protein, indicative of SAC activation, also increased in LNCaP cells and remains similar in PC3 cells at the same time point. 48 h after paclitaxel treatment, all of these proteins decreased in PC3 cells and remain similar (or even higher in the case of phospho-histone H3) in LNCaP cells. It has been described that PTTG1 becomes phosphorylated in mitosis (9) and, interestingly, paclitaxel treatment induces PTTG1 phosphorylation in both cell lines, observed as a PTTG1 band shift, but the protein decreases in paclitaxel-treated PC3 cells and accumulates in paclitaxel-treated LNCaP cells (Fig. 1). These data suggest that PC3 cells were reaching mitosis and subsequently undergoing slippage, whereas LNCaP cells were also reaching mitosis but remained fully blocked in this phase.

Several proteins belonging to the Bcl-2 family were also examined. As previously described (2), antiapoptotic protein Bcl-xL becomes phosphorylated in both paclitaxel-treated cell lines, more markedly in PC3 cells. In the case of 24 h paclitaxel-treated PC3 cells, Bcl-xL was predominantly phosphorylated, as observed by the decreased of the unphosphorylated 30 kDa band. However, 48 h after treatment, there was a mixture of unphosphorylated and phosphorylated Bcl-xL. In LNCaP cells, Bcl-xL decreased after 48 h of paclitaxel treatment. The expression level of Mcl-1L antiapoptotic protein diminishes in both cell lines and at both time points after paclitaxel
treatment, but in PC3 cells, Mcl-1L level remains high after 24 h of paclitaxel treatment, as compared with the great decrease observed at 48 h. By contrary, Mcl-1L expression after paclitaxel treatment was low and similar at both time points in LNCaP cells. Proapoptotic Bax and Bak remain practically unchanged upon paclitaxel treatment in both cell lines (Fig. 1).

PTTG1 gene silencing impairs LNCaP cells progression through cell cycle and their sensitivity to paclitaxel-induced apoptosis

To test the role of PTTG1 in the response to paclitaxel of LNCaP cells, endogenous PTTG1 was silenced with a small interfering RNA (siRNA) and cells were subsequently treated with 2.5 μM paclitaxel during 24 and 48 h. First, we examined apoptosis induction in this context. PTTG1-silenced LNCaP cells showed clearly diminished PARP cleavage and caspase 9 activation after paclitaxel treatment as compared with siRNA control cells at both 24 and 48 h (Fig. 2A). These results were confirmed by flow cytometry experiments with cells labelled with annexin V-FITC and propidium iodide. As observed in Fig. 2B, the percentage of early apoptotic cells is more elevated in 48h-treated siRNA control cells than in treated cells silenced for PTTG1 (36.4% vs. 20.8%).

The decrease in phosphorylated histone H3, BubR1 and cyclin B1 levels in paclitaxel-treated PTTG1-silenced LNCaP cells in relation to paclitaxel-treated siRNA control cells during 24 and 48 h revealed a strong decrease of mitosis when LNCaP cells lack PTTG1 (Fig. 2A). Bax and Bak levels remain unchanged, but the decrease in Mcl-1L levels observed in paclitaxel-treated siRNA control cells at both time points disappear in paclitaxel-treated PTTG1-silenced cells. Similarly, the decrease in Bcl-xL levels observed in 48 h paclitaxel-treated siRNA control cells also disappear upon
PTTG1 silencing (Fig. 2A). Next, cell cycle analysis was performed to ascertain whether the diminished mitosis in paclitaxel-treated PTTG1-silenced cells was due to the appearance of a slippage phenomenon or to impaired transition towards G2/M. DMSO-treated LNCaP cells silenced for PTTG1 display an increase in G1 and a decrease in S and G2/M phases respectively as compared with DMSO-treated siRNA control cells. The change in cell cycle distribution was still deeper when cells were treated with paclitaxel. siRNA control cells accumulate in S and G2/M following paclitaxel exposure, although the effect is more marked at 48 h. By contrary, the proportion of paclitaxel-treated PTTG1-silenced cells that accumulate in S and G2/M is dramatically lower at both times (Fig. 2C). Finally, to check that there were not any ploidy changes provided that cells were either in mitosis or not reaching mitosis, a FISH was carried out. As observed in Fig. 2D the percentage of cells with higher ploidy than the normal was low and similar in both 48 h paclitaxel-treated siRNA control and siRNA PTTG1 cells, supporting western blot and cell cycle data. Representative FISH images are shown in Supplementary Fig. S1A.

**PTTG1 overexpression increases paclitaxel-induced mitotic arrest of LNCaP cells with a concomitant increase in apoptosis**

To further investigate the role of PTTG1 in the response to paclitaxel, LNCaP cells were transiently transfected with a non-degradable PTTG1 mutant and treated with 2.5 μM paclitaxel during 48 h. PARP cleavage and caspase 9 activation were higher in paclitaxel-treated PTTG1-transfected cells as compared with treated cells transfected with empty vector (Fig. 3A). The annexin V binding experiment also confirmed these results (Fig. 3B). In relation to the effect on cell cycle, phosphorylated histone H3, BubR1 and cyclin B1 were increased in PTTG1-transfected LNCaP cells after paclitaxel
treatment, indicating a more efficient mitotic arrest. Bcl-xL, Mcl-1L, Bax and Bak remain similar in both PTTG1 and empty vector transfected cells (Fig. 3A). FISH results also confirmed that cells were not slipping out the mitotic arrest imposed by paclitaxel (Fig. 3C and Supplementary Fig. S1B).

**PTTG1 gene silencing diminishes paclitaxel-induced apoptosis by inhibiting mitotic slippage in PC3 cells**

The same experimental approach used above was extended to PC3 cells. As observed by the 85 kDa band of PARP protein, caspase 9 activation (Fig. 4A) and annexin V binding experiment (Fig. 4B), PTTG1 silencing diminishes the apoptotic sensitivity of PC3 cells to paclitaxel treatment. Both siRNA control and PTTG1-silenced PC3 cells showed increased levels of phosphorylated histone H3 and cyclin B1 after 24 h of paclitaxel treatment, whereas BubR1 levels remained similar to DMSO-treated siRNA control and PTTG1-silenced cells. As compared with 24 h, paclitaxel treatment during 48 h induces a decrease in phosphorylated histone H3, BubR1 and cyclin B1 levels in siRNA control cells that were much less obvious in PTTG1-silenced cells (Fig. 4A). Mcl-1L levels diminished after paclitaxel treatment in all conditions, but more markedly at 48 h. Bcl-xL remained higher in 24 h paclitaxel-treated cells silenced for PTTG1 in relation to siRNA control cells treated in the same way. We did not detect any appreciable changes in Bax and Bak proteins (Fig. 4A). Cell cycle was also examined to further elucidate the effect of PTTG1 silencing on this cell line. DMSO-treated PTTG1-silenced cells showed an increase in G1 phase and a decrease in S phase, as compared with DMSO-treated siRNA control cells (Fig. 4C). Cell cycle profiles were essentially similar in paclitaxel-treated siRNA control and PTTG1-silenced cells, although the percentage of paclitaxel-treated PTTG1-silenced cells in G1 phase was
slightly higher than in paclitaxel-treated siRNA control cells (Fig. 4C). As both paclitaxel-treated siRNA control and PTTG1-silenced cells peak in a DNA content corresponding to G2/M phases and, provided that mitotic and slipped cells are indistinguishable by this technique, FISH was performed. Representative photographs are shown in Supplementary Fig. S1C. 48 h paclitaxel-treated siRNA control cells show 71.6% of cells with higher ploidy than the normal, whereas paclitaxel-treated PTTG1-silenced cells show a reduction of 26.3% in such percentage (Fig. 4D). Altogether, these data point to an inhibition of slippage upon PTTG1 silencing.

**PTTG1 overexpression increases paclitaxel-induced mitotic arrest of PC3 cells but diminishes the apoptotic effect of the drug**

PC3 cells were also transiently transfected with the PTTG1 mutant and treated with 2.5 μM paclitaxel during 48 h. Paclitaxel-treated PTTG1-transfected PC3 cells were more blocked in mitosis than treated cells transfected with empty vector, as observed by increased levels of phosphorylated histone H3, BubR1 and cyclin B1, but, as in the case of PTTG1 silencing, this event was associated with diminished PARP cleavage, caspase 9 activation and with a reduced percentage of apoptotic cells; this is, with a minor induction of apoptosis (Fig. 5A and B). Bcl-xL, Bax and Bak levels remain essentially unchanged, whereas Mcl-1L levels were higher in paclitaxel-treated PTTG1-transfected PC3 cells than in empty vector transfected cells treated in the same way. To test if there were changes in the proportion of mitotic and slipped cells under these conditions, a FISH was performed. PC3 cells transfected with empty vector and treated with paclitaxel showed 64.1% of cells with higher ploidy than the normal, whereas paclitaxel-treated PTTG1-transfected cells showed a decrease of 27.6% in that
percentage, indicating an inhibition of slippage in the last condition (Fig. 5C and Supplementary Fig. S1D).

Simultaneous PTTG1 and Mcl-1 downregulation has a pro-apoptotic effect on PC3 cells during mitosis

As results obtained in PC3 cells indicate that the increase in mitotic cells achieved with paclitaxel in both PTTG1-silenced and transfected cells not only does not increase apoptosis but diminishes it, we reasoned that apoptosis only can be triggered post-slippage or that other blockades might exist that prevent apoptosis during mitosis. To test this hypothesis, we focused on Mcl-1 because of its elevated level during the moment of maximum mitosis in this cell line; this is, after 24 h of paclitaxel treatment. PC3 cells silenced for Mcl-1 and treated with paclitaxel during 48 h, showed an increase in phosphorylated histone H3 and cyclin B1 proteins in relation to paclitaxel-treated siRNA control cells. Strikingly, the increases in phosphorylated histone H3 and cyclin B1 proteins were also slightly higher than in paclitaxel-treated siRNA PTTG1 cells, but PARP and caspase 9 cleavages were clearly more elevated in drug-treated siRNA Mcl-1 cells (Fig. 6A). Cells simultaneously silenced for both PTTG1 and Mcl-1 and treated with paclitaxel during 48 h showed an increase in PARP cleavage and caspase 9 activation, associated to increased mitosis, as indicate phosphorylated histone H3 and cyclin B1 levels, comparing with treated-siRNA control, siRNA PTTG1 and siRNA Mcl-1 cells (Fig. 6A). Annexin V binding experiment also confirmed a higher percentage of apoptosis in treated cells simultaneously silenced for PTTG1 and Mcl-1 (Fig. 6B). These data indicate that apoptosis resistance induced with PTTG1 silencing may be bypassed; surpassing even the apoptotic induction obtained in siRNA control cells, by simultaneously silencing Mcl-1.
Paclitaxel-resistant PC3 cells show diminished apoptosis associated to increased mitosis and elevated Mcl-1L levels after paclitaxel treatment

Finally, we examined apoptosis, cell cycle and Bcl-2 family proteins in a PC3 cell line resistant to paclitaxel-induced apoptosis (PC3 PTXR) developed in our laboratory. As observed in Fig. 7A and B, cleavage of PARP, caspase 9 activation and the percentage of apoptotic cells, were clearly diminished in PC3 PTXR cells after paclitaxel treatment, as compared with parental PC3 cells. This reduced apoptosis was associated with higher levels of phosphorylated histone H3, BubR1, cyclin B1 and PTTG1 in the resistant cell line, illustrating again an association between inhibition of slippage and the decrease of apoptosis. In relation to Bcl-2 family proteins, Bak and Bcl-xL levels remain unchanged but we observe a decrease in Bax protein expression level in the paclitaxel-resistant cell line upon drug treatment. Interestingly, the decrease in Mcl-1L level observed in the parental cell line after paclitaxel treatment is clearly less marked in the resistant cell line under the same condition (Fig. 7A), supporting the role of Mcl-1L protein in the inhibition of apoptosis during mitosis. As western blot data suggested a stronger paclitaxel-induced mitotic arrest in the resistant cells as compared with the parental ones, we studied cell cycle and ploidy in this context. Cycle profiles were similar in both cell lines (Fig. 7C), however, FISH results showed a 66.7% of paclitaxel-treated PC3 cells with higher ploidy than the normal, whereas in paclitaxel-treated PC3 PTXR cells this percentage was 54.3%, corroborating the diminished slippage in PC3 PTXR cells upon paclitaxel treatment (Fig. 7D and Supplementary Fig. S1E).
Discussion

Defects in cell cycle and apoptosis machineries influence cell responses to chemotherapeutic drugs, thus elucidating the mechanisms underlying bad or incomplete responses could help to improve therapy regimens. Paclitaxel and its semi-synthetic analogue, docetaxel, are currently being used to induce regression in hormone-refractory prostate cancers after androgen deprivation therapy failure (24), this is, first line treatment for hormone-refractory prostate cancers is mainly taxane-based chemotherapy (24, 25). As it has been reported (26), PC3 and LNCaP cells show a different apoptotic response after paclitaxel treatment. It has also been described that PC3 cells treated with 50 ng/ml of paclitaxel during 24 h and then withdrawn, exit from mitosis without cytokinesis, leading to the appearance of polyploid cells. In this context, cell death was a slow and delayed process (a sub-G₁ population appeared after 72 h of treatment), occurring after DNA endoreduplication (27). Also, PC3 cells have been shown to exit from mitosis after a prolonged mitotic arrest induced by 150 nmol/L paclitaxel, and remained in an abnormal G₁-like state for extended period of time (28).

Within our experimental conditions, we have observed that most PC3 cells had reached mitosis but were not in apoptosis after 24 h of 2.5 μM paclitaxel treatment; similarly treated LNCaP cells were also in mitosis but initiating apoptosis. After 48 h of drug treatment, most PC3 cells have exited from mitosis by slippage, with a concomitant increase in apoptosis. Apoptosis in LNCaP cells were also increased at that time, surpassing the apoptotic induction observed in PC3 cells, but this was associated with an increase in mitotic cells. Apoptosis induction by paclitaxel is related to its ability to arrest cells in mitosis (28); our data also suggest that LNCaP, the cell line capable of maintaining a more efficient mitotic arrest, is more sensitive to the apoptotic effect of paclitaxel than slippage-prone PC3 cell line.
There are several reports addressing PTTG1 functions in apoptosis. Concretely, PTTG1 overexpression may cause both p53-dependent and p53-independent apoptosis in MCF7 breast cancer cells (p53 wild type) and MG63 osteosarcoma cells (p53 deficient) (29). It also has been published that PTTG1 induces p53 promoter activation through c-myc and that PTTG1 overexpression stimulates Bax expression, a known downstream target of p53 (30). By contrary, it has been described that PTTG1 interacts with p53 in vitro and in vivo, and that such interaction blocks the specific binding of p53 to DNA, inhibiting its transcriptional activity. In this context, Bax promoter activity decreases due to PTTG1-p53 interaction, leading to diminished apoptosis (31). It also has been observed in hepatoma cell lines that PTTG1 overexpression attenuates p53 induced apoptosis (32). Specifically in prostate cancer, it has been observed that LNCaP cells stably transfected with a PTTG1 construct showed higher S and G2/M percentages than vector-only transfectants. Reciprocally, LNCaP cells stably transfected with an antisense PTTG1 construct showed increased G1 phase cells, as compared with control cells (33), a finding that was in accordance with siRNA PTTG1 effects reported in JEG-3 cells (34).

In the context of apoptosis induction by an antimitotic drug such as paclitaxel, we have identified two situations in which PTTG1 influences cell fate differentially. PTTG1 silencing in PC3 cells does not affect their ability to reach mitosis in response to paclitaxel treatment, moreover, the maintenance of the mitotic state is strengthened; similarly, paclitaxel-treated, PTTG1 overexpressing PC3 cells also showed an increased maintenance of mitosis. Both PTTG1 overexpression and silencing lead to increased SAC activation after paclitaxel treatment, as indicate elevated BubR1 levels; this could explain the increase in mitotic cells achieved after paclitaxel treatment. The most striking and paradoxical effect observed in both situations is that the increase in mitosis,
this is, the inhibition of slippage, was associated with diminished apoptosis. The effect of PTTG1 silencing in LNCaP cells was quite different. Paclitaxel-treated, PTTG1-silenced LNCaP cells lose their ability to reach mitosis and, therefore, to die by apoptosis. By contrary, paclitaxel-treated, PTTG1 overexpressing LNCaP cells, reach mitosis more efficiently and showed increased apoptosis. These data are in agreement with the classic behavior of paclitaxel sensitive cells: LNCaP cells reach mitosis and die by apoptosis during mitosis. When mitotic arrest is potentiated, as occurs in the case of PTTG1 overexpression, apoptosis induction is also enhanced. As explained above, several data indicate that the induction of a mitotic arrest in PC3 cells is not sufficient to trigger apoptosis. First, PC3 cells reach mitosis quickly after paclitaxel treatment; however, after 24 h, apoptosis is barely detectable. In second place, both PTTG1 overexpression and downregulation raise the proportion of mitotic cells by slippage inhibition after paclitaxel treatment, but none of these two situations is linked to increased apoptosis. Thus, the crosstalk between mitotic and apoptotic machineries seems to be uncoupled since mitotic arrest, regardless of its duration, does not facilitate apoptosis. In fact, within this context, apoptosis only takes place after PC3 cells have undergone slippage. We reasoned that apoptosis only could be triggered post-slippage or that other blockades might exist that prevented apoptosis during mitosis. To test this hypothesis, we focused on antiapoptotic Mcl-1 protein because of its elevated level during the moment of maximum mitosis in this cell line. The relevance of Mcl-1 during mitotic arrest and the subsequent apoptosis has been highlighted in several studies (35, 36). These studies suggest that Mcl-1 stabilization during a mitotic arrest induces apoptosis resistance. As we have observed high levels of Mcl-1L in PC3 cells treated with paclitaxel during 24 h, when the majority of cells are in mitosis, we silenced PTTG1 and Mcl-1 and paclitaxel was added during 48 h, to test the effect of Mcl-1
downregulation alone or in the context of a more efficient mitotic arrest induced by PTTG1 silencing. In both situations, PC3 cells showed increased mitosis linked to increased apoptosis, more markedly in the case of simultaneously silenced cells.

Finally, the results obtained with PC3 PTXR cells also support our previous results. Concretely, the effect of paclitaxel treatment on these cells resembles the situation achieved upon PTTG1 overexpression, as they also showed increased mitosis and diminished apoptosis after drug treatment. However, in the case of paclitaxel-resistant cells, diminished slippage does not explain completely the strong reduction in apoptosis; the decrease of Bax protein and the inability to decrease Mcl-1L levels upon treatment could also account for the resistant phenotype, illustrating again the implication of Mcl-1L protein in the development of apoptosis resistance to paclitaxel.

In summary, within our model, the highest apoptotic induction after paclitaxel treatment is achieved when cells arrest in mitosis efficiently, PTTG1 protein levels are high and Mcl-1L protein is rapidly downregulated during mitosis. In the case of slippage-prone cells, the increase in mitotic arrest that occurs upon PTTG1 silencing does not facilitate apoptosis, unless Mcl-1 downregulation takes place at the same time. These different possibilities could help to understand the molecular basis underlying bad responses to paclitaxel therapy and emphasize the need for seeking new therapeutic regimens targeting apoptosis-resistant forms of prostate cancer.

**Author’s Contributions**

C. Castilla and M. L. Flores designed research, acquired and analyzed data, and wrote the manuscript. R. Medina and B. Pérez-Valderrama interpreted data and reviewed the manuscript. M. Tortolero and F. Romero designed research and reviewed
the manuscript. M. A. Japón and C. Sáez conceived and designed research, supervised the study, and revised the manuscript.

Acknowledgments

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References


22. Zur A, Brandeis M. Securin degradation is mediated by fzy and fzr, and is required for complete chromatid separation but not for cytokinesis. EMBO J 2001;20:792-801.


Figure legends

Figure 1
Differential effect of paclitaxel on apoptosis induction and cell cycle in PC3 and LNCaP cells. Cells were treated with 2.5 μM paclitaxel during 24 and 48 h. Intact and cleaved PARP, active caspase 9 (cleaved CASP-9), PTTG1, cyclin B1, phospho-histone H3 (p-His-H3), BubR1, Bcl-xL, Mcl-1, Bax and Bak proteins were detected by western blot. β-actin is shown as loading control.

Figure 2
PTTG1 silencing impairs apoptosis upon paclitaxel treatment in LNCaP cells. Cells were silenced with PTTG1 siRNA or with a non-targeting control siRNA and treated with 2.5 μM paclitaxel during 24 and 48 h. A, Western blot analysis of the indicated proteins is shown. β-actin was used to ensure equal loading. B, Apoptosis detection by flow cytometry of annexin V and propidium iodide labelled cells. C, Cell cycle analysis of propidium iodide-stained cells by flow cytometry. Percentage of cells in each phase of the cell cycle was computed with ModFit software. D, Ploidy analysis by FISH. The number of signals per cell was determined for chromosomes 8 and 17 in at least 100 cells. Histogram represents the percentage of cells with normal or higher ploidy for each condition. P from Student’s t test, comparing paclitaxel-treated control and PTTG1 silenced cells, was not significant.

Figure 3
Paclitaxel-induced mitotic arrest and apoptosis are increased in PTTG1-overexpressing LNCaP cells. Cells were transiently transfected with a plasmid carrying a non degradable PTTG1 mutant (pEF/Securin KAA-DM) or with empty vector and treated
with 2.5 μM paclitaxel during 48 h. A, Western blot analysis of the indicated proteins is shown, using β-actin as loading control. B, Apoptosis detection by flow cytometry of annexin V and propidium iodide labelled cells. C, FISH analysis of chromosomes 8 and 17 in at least 100 cells. Histogram represents the percentage of cells with normal and higher ploidy for each condition. Data are presented as mean ± S.E.M. (n = 3). P from Student’s t test, comparing paclitaxel-treated empty vector and PTTG1 transfected cells, was not significant.

Figure 4

PTTG1 silencing impairs apoptosis upon paclitaxel treatment in PC3 cells. Cells were silenced with PTTG1 siRNA or with a non-targeting control siRNA and treated with 2.5 μM paclitaxel during 24 and 48 h. A, Western blot analysis of the indicated proteins is shown. β-actin was used to ensure equal loading. B, Apoptosis detection by flow cytometry of annexin V and propidium iodide labelled cells. C, Cell cycle analysis of propidium iodide-stained cells by flow cytometry. Percentage of cells in each phase of the cell cycle was computed with ModFit software. D, Ploidy analysis by FISH. The number of signals per cell was determined for chromosomes 8 and 17 in at least 100 cells. Histogram represents the percentage of cells with normal or higher ploidy for each condition. Data are presented as mean ± S.E.M. (n = 3). * P < 0.05 from Student’s t test.

Figure 5

Paclitaxel-induced mitotic arrest is increased in PTTG1-overexpressing PC3 cells and is associated with decreased apoptosis. Cells were transiently transfected with a plasmid carrying a non degradable PTTG1 mutant (pEF/Securin KAA-DM) or with empty vector and treated with 2.5 μM paclitaxel during 48 h. A, Western blot analysis of the indicated
proteins is shown, using β-actin as loading control. B, Apoptosis detection by flow cytometry of annexin V and propidium iodide labelled cells. C, FISH analysis of chromosomes 8 and 17 in at least 100 cells. Histogram represents the percentage of cells with normal and higher ploidy for each condition. Data are presented as mean ± S.E.M. (n = 3). Data are presented as mean ± S.E.M. (n = 3). ** P < 0.01 from Student’s t test.

Figure 6
Simultaneous PTTG1 and Mcl-1 silencing improve paclitaxel-induced apoptosis in PC3 cells during mitosis. PC3 cells were silenced with non-targeting control, PTTG1, Mcl-1 and both PTTG1 and Mcl-1 siRNAs. To ensure an equal quantity of siRNAs in all conditions, non-targeting control siRNA was added at 100 nM in individual non-targeting control, PTTG1 and Mcl-1 siRNAs. Cells were then treated with 2.5 μM paclitaxel during 48 h. A, Intact and cleaved PARP, active caspase 9, PTTG1, cyclin B1, phospho-histone H3, BubR1, Bcl-xL, Mcl-1, Bax and Bak proteins were detected by western blot. β-actin is shown as loading control. B, Apoptosis detection by flow cytometry of annexin V and propidium iodide labelled cells.

Figure 7
Paclitaxel-resistant PC3 cells (PC3 PTXR) show increased mitosis and elevated Mcl-1 levels associated to diminished apoptosis. PC3 and PC3 PTXR cells were treated with 2.5 μM paclitaxel during 48 h. A, Expression levels of the indicated proteins were assessed by western blot using β-actin as loading control. B, Apoptosis detection by flow cytometry of annexin V and propidium iodide labelled cells. C, Cell cycle analysis by flow cytometry. Percentage of cells in different phases of the cell cycle is shown. D, Ploidy analysis by FISH. The number of signals per cell was determined for
chromosomes 8 and 17 in at least 100 cells. Histogram represents the percentage of cells with normal and higher ploidy. Data are presented as mean ± S.E.M. (n = 3). ** P < 0.01 from Student’s t test.
Figure 1

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Figure 3

(a) Western blot analysis of PARP, cleaved CASP-9, PTTG1, cyclin B1, p-His H3, BubR1, Bcl-xL, Mcl-1L, Bax, Bak, and β-actin in empty vector and hSec KAA-DM treated with paclitaxel. 
(b) Flow cytometry analysis of Propidium iodide staining in DMSO and paclitaxel treated cells with siRNA control and siRNA PTGG1.
(c) Annexin V-FITC staining of LNCaP cells in DMSO and paclitaxel treated cells with empty vector and hSec KAA-DM. 

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Figure 6

(a) Western blot analysis showing the effects of siRNA on PARP, cleaved CASP-9, PTTG1, Mcl-1, cyclin B1, p-His H3, and β-actin under paclitaxel conditions.

(b) Flow cytometry analysis showing the percentage of cells in different stages of apoptosis under siRNA and paclitaxel conditions.
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Prostate cancer cell response to paclitaxel is affected by abnormally expressed securin PTTG1

Carolina Castilla, M. Luz Flores, Rafael Medina, et al.

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