**Prostate Cancer Cell Response to Paclitaxel Is Affected by Abnormally Expressed Securin PTTG1**

Carolina Castilla1, M. Luz Flores1, Rafael Medina2, Begoña Pérez-Valderrama3, Francisco Romero4, María Tortolero4, Miguel A. Japón1,5, and Carmen Sáez1,5

**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 down-regulation prevents mitotic entry and, subsequently, inhibits mitosis-associated, paclitaxel-induced apoptosis. In contrast, 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 enhances 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 nonresponsive 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 bioriented in the mitotic spindle. When this occurs, APC/CDephosphorylation-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 cofactor 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 G1 state, from which they either die, arrest in G1, or initiate a new round of the cell cycle (3). Slippage occurs when, despite continuous SAC signaling (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 Forkhead box O (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 G2-M (9). Subcutaneous

1Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocio, CSIC, Universidad de Sevilla, Seville, Spain. 2Department of Urology, Hospital Universitario Virgen del Rocio, Seville, Spain. 3Department of Oncology, Hospital Universitario Virgen del Rocio, Seville, Spain. 4Department of Microbiology, Faculty of Biology, Universidad de Sevilla, Seville, Spain. 5Department of Pathology, Hospital Universitario Virgen del Rocio, Seville, Spain.

**Note:** Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

C. Castilla and M. Luz Flores contributed equally to this article.

**Corresponding Authors:** Carmen Sáez, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocio, Avenida Manuel Siurot s/n, Seville, Spain 41013. Phone: 34955013028; Fax: 34955013029; E-mail: csaez@csic.es; and Miguel A. Japón. E-mail: mjapon@cica.es

doi: 10.1158/1535-7163.MCT-13-0405

©2014 American Association for Cancer Research.
injection of PTTG1-transfected cells into nude mice has been shown to induce tumors formation. Furthermore, PTTG1 overexpression has been reported in many human tumors (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 transcription-activating activity, inducing the upregulation of several other genes (19). As a cell-cycle regulator, PTTG1 is bound to separate and inhibits its proteolytic activity during most parts of the cell cycle (20). In metaphase, replicates 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\(C_{\text{dc20}}\). PTTG1 destruction releases separase inhibition, which in turn mediates degradation of the cohesin complex and sister chromatids separation proceeds (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 \(\mu\)mol/L paclitaxel for 3 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% FBS, 100 U/mL penicillin, 10 \(\mu\)g/mL streptomycin, 10 mmol/L HEPES buffer, and 1 mmol/L glutamine in a 37°C humidified incubator under 5% CO2. Cells were harvested by trypsinization. The stock solution of paclitaxel (Calbiochem) was prepared at 10 mmol/L in dimethyl sulfoxide (DMSO; Sigma) and stored frozen. In all the experiments, DMSO was added to untreated cells.

Small interfering RNA

siRNA transfections were carried out using the Dharmafect 2 reagent (Thermo Fisher Dharmacon) 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 nmol/L. Cells were subjected to different treatments 24 hours after silencing.

Plasmid transfections

Cells were transiently transfected with a plasmid carrying a nondegradable 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; ref. 22). Transfections were carried out by using the FuGENE reagent (Promega), according to the manufacturer’s instructions. Cells were subjected to different treatments 24 hours after transfection.

Antibodies

Rabbit polyclonal anti-Bax and anti-Bak were available from BD Biosciences. 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; mouse monoclonal anti–\(\beta\)-actin was from Sigma. Rabbit polyclonal anti–cleaved caspase-9 (Asp315) was from Cell Signaling. Rabbit anti-BubR1 antibody was from Bethyl Laboratories. 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–\(\beta\)-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 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 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10% glycerol, and 1% NP40]. Equal amounts of total protein, as determined by using BCA protein assay (Pierce), were separated by SDS-PAGE on 4% to 20% gradient polyacrylamide gels (Invitrogen). Gels were electroblotted onto nitrocellulose membranes (GE Healthcare). Membranes were stained with Ponceau S to ensure that protein amounts were comparable. For immunodetection, blots were blocked in 1% blocking reagent (Roche) in 0.05% Tween 20-PBS and incubated with primary antibody overnight at 4°C. 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 hour. Enhanced chemiluminescent system was applied according to the manufacturer’s protocol (GE Healthcare). The experiments were performed at least three times.

Apoptosis assays

Cells (1 \(\times\) 10\(^5\)) were washed in cold PBS and suspended in 100-\(\mu\)L Annexin V binding buffer (R&D Systems) containing 5 \(\mu\)g/mL propidium iodide and 0.5 \(\mu\)g/mL Annexin V–FITC, incubated for 15 minutes at room temperature in the dark, and diluted in 400 \(\mu\)L Annexin V binding buffer. Fluorescence was measured in a FACScan flow cytometer (BD Biosciences) within 1 hour. 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) was used to assess the cell cycle. The experiments were performed at least three times.

**FISH**

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

**Statistical analysis**

Data comparing differences between two conditions were statistically analyzed, when indicated, using the paired Student t test. Differences were considered significant when P < 0.05. Calculations were performed using Prism 4.0 (GraphPad).

**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 μmol/L paclitaxel during 24 and 48 hours, 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 hours of drug treatment, there is a clear apoptosis induction in LNCaP cells, whereas it is hardly detectable in PC3 cells. Apoptosis increases in both cell lines after 48 hours, but it continues to be higher in LNCaP than in PC3 cells (Fig. 1). Then, we examined several cell-cycle–related proteins to assess if differences in apoptosis induction were linked to changes in the cell cycle. The behavior of these proteins was similar in both cell lines after paclitaxel treatment during 24 hours, but it was markedly different at 48 hours. Cyclin B1, PTTG1, and phosphorylated histone H3 (used as a surrogate for mitosis) increased in both cell lines 24 hours 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. Forty-eight hours 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-hour paclitaxel-treated PC3 cells, Bcl-xL was predominantly phosphorylated, as observed by the decrease of the unphosphorylated 30-kDa band. However, 48 hours after treatment, there was a mixture of unphosphorylated and phosphorylated Bcl-xL. In LNCaP cells, Bcl-xL...
decreased after 48 hours of paclitaxel treatment. The expression level of Mcl-1 antiapoptotic protein diminishes in both cell lines and at both time points after paclitaxel treatment, but in PC3 cells, Mcl-1 level remains high after 24 hours of paclitaxel treatment, as compared with the great decrease observed at 48 hours. In contrast, Mcl-1 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 cell 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 siRNA and cells were subsequently treated with 2.5 μmol/L paclitaxel during 24 and 48 hours. 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 hours (Fig. 2A). These results were confirmed by flow cytometry experiments with cells labeled with Annexin V–FITC and propidium iodide. As observed in Fig. 2B, the percentage of early apoptotic cells is more elevated in 48-hour–treated siRNA control cells than in treated cells. As compared with 24 hours, paclitaxel treatment during 48 hours induces a 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 hours revealed a strong decrease of mitosis when LNCaP cells lack PTTG1 (Fig. 2A). Bax and Bak levels remain unchanged, but the decrease in Mcl-1 levels observed in paclitaxel-treated siRNA control cells at both time points disappears in paclitaxel-treated PTTG1-silenced cells. Similarly, the decrease in Bcl-xL levels observed in 48-hour–treated siRNA control cells also disappears 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 toward 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 hours. In contrast, 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 no 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-hour 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 nondegradable PTTG1 mutant and treated with 2.5 μmol/L paclitaxel during 48 hours. 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 the 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-1, 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 hours of paclitaxel treatment, whereas BubR1 levels remained similar to DMSO-treated siRNA control and PTTG1-silenced cells. As compared with 24 hours, paclitaxel treatment during 48 hours 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-1 levels diminished after paclitaxel treatment in all conditions, but more markedly at 48 hours. Bcl-xL remained higher in 24-hour 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
Figure 2. PTTG1 silencing impairs apoptosis upon paclitaxel treatment in LNCaP cells. Cells were silenced with PTTG1 siRNA or with a nontargeting control siRNA and treated with 2.5 μmol/L paclitaxel during 24 and 48 hours. 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–labeled 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 the Student t test, comparing paclitaxel-treated control and PTTG1-silenced cells, was not significant.
undistinguishable by this technique, FISH was performed. Representative photographs are shown in Supplementary Fig. S1C. Forty-eight-hour 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 μmol/L paclitaxel during 48 hours. 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; that 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 slippage 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 proapoptotic effect on PC3 cells during mitosis

As observed in previous results, paclitaxel treatment of PTTG1-silenced or PTTG1-transfected PC3 cells does not increase apoptosis despite increasing mitotic cells, so we reasoned that apoptosis can only be triggered after slippage or that alternative blockades might 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; that is, after 24 hours of paclitaxel treatment. PC3 cells silenced for Mcl-1 and treated with paclitaxel during 48 hours 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 hours showed increased PARP cleavage and caspase-9 activation. These apoptotic events were associated with increased mitosis, as indicated by phosphorylated histone H3 and cyclin B1 levels when compared with paclitaxel-treated siRNA control, siRNA PTTG1, or siRNA Mcl-1 cells (Fig. 6A). Annexin V binding experiment also confirmed a higher percentage of
Figure 4. PTTG1 silencing impairs apoptosis upon paclitaxel treatment in PC3 cells. Cells were silenced with PTTG1 siRNA or with a nontargeting control siRNA and treated with 2.5 μmol/L paclitaxel during 24 and 48 hours. 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–labeled 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, mean ± SEM (n = 3). *, P < 0.05 from the Student t test.
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 with increased mitosis and elevated Mcl-1 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-1 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-1 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 the cell cycle and ploidy in this context. Cycle profiles were similar in both cell lines (Fig. 7C); however, FISH results showed 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 cancer cell response to chemotherapy. Thus, elucidating the mechanisms underlying bad or incomplete responses will help to schedule more effective chemotherapy regimens. Paclitaxel and its semisynthetic analogue, docetaxel, are currently being used to induce regression in hormone-refractory prostate cancers after androgen deprivation therapy failure (24), that 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 hours 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 hours 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 hours of 2.5 μmol/L paclitaxel treatment; similarly treated LNCaP cells were also in mitosis but initiating apoptosis. After 48 hours of drug treatment, most PC3 cells have exited from mitosis by slippage, with a concomitant increase in apoptosis. Apoptosis in LNCaP cells was 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; ref. 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). On the 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 indicated by 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, that is, the inhibition of slippage, was associated with diminished apoptosis. The effect of PTTG1 silencing in LNCaP cells was quite

Figure 7. Paclitaxel-resistant PC3 cells (PC3 PTXR) show increased mitosis and elevated Mcl-1 levels associated with diminished apoptosis. PC3 and PC3 PTXR cells were treated with 2.5 μmol/L paclitaxel during 48 hours. 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–labeled 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, mean ± SEM (n = 3). ** P < 0.01 from the Student t test.
different. Paclitaxel-treated, PTTG1-silenced LNCaP cells lose their ability to reach mitosis and, therefore, to die by apoptosis. In contrast, 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 hours, 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 because 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 period 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 hours, when the majority of cells are in mitosis, we silenced PTTG1 and Mcl-1, and paclitaxel was added during 48 hours, 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 apoptosis 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.

Disclosure of Potential Conflicts of interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Development of methodology: M.I. Flores, B. Pérez-Valderrama
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Castilla, M.I. Flores, R. Medina
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Castilla, M.I. Flores, F. Romero
Writing, review, and/or revision of the manuscript: C. Castilla, R. Medina, F. Romero, M. Tortolero, M.A. Japón, C. Sáez
Study supervision: R. Medina, M.A. Japón, C. Sáez

Acknowledgments
pEF/Securin KAA-DM plasmid was kindly provided by M. Brandeis (Department of Genetics, Silberman Institute of Life Sciences). The authors thank M. Mora-Santos, S. Giraldez, and J. Herrero-Ruiz for their help.

Grant Support
This work was supported by grants from the Instituto de Salud Carlos III (FIS PI10/0226, PI13/0228, ISCIII-RETIC RD12/0036/0064; to C. Sáez) and Ministerio de Economía, Spain (SAF2011-30003-C02; to M.A. Japón and F. Romero), Consejería de Salud (AI-2012-SA001; to M.A. Japón), and Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía (P10-CTS-6243, to C. Sáez; P08-CVI-03603, to M. Tortolero). C. Castilla was supported by a predoctoral grant from the Spanish Ministerio de Educación (F.P.I.: BES200612419) cofunded by Fondo Social Europeo, and M.I. Flores by a predoctoral grant from the Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía (CTS-6243). C. Sáez is recipient of a contract from Programa Nicolas Monardes, Consejería de Salud, Junta de Andalucía.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked as paid advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 20, 2013; revised June 25, 2014; accepted August 5, 2014; published OnlineFirst August 13, 2014.

References

Mol Cancer Ther; 13(10) October 2014
Molecular Cancer Therapeutics

2382
Downloaded from mct.aacrjournals.org on June 14, 2021. © 2014 American Association for Cancer Research.
Molecular Cancer Therapeutics

Prostate Cancer Cell Response to Paclitaxel Is Affected by Abnormally Expressed Securin PTTG1

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


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0405

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/08/16/1535-7163.MCT-13-0405.DC1

Cited articles
This article cites 36 articles, 9 of which you can access for free at:
http://mct.aacrjournals.org/content/13/10/2372.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/13/10/2372.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/13/10/2372.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.