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Pim-1 knockdown potentiates paclitaxel-induced apoptosis in human hormone-refractory prostate cancers through inhibition of NHEJ DNA repair

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Abstract

The knockdown of Pim-1 or inhibition of Pim-1 activity significantly increased c-H2A.X expression. The effect was correlated to apoptosis and was attributed to the inhibition of nonhomologous DNA-end-joining (NHEJ) repair activity supported by the following observations: (1) inhibition of ATM and DNA-PKcs activities, (2) down-regulation of Ku expression and nuclear localization and (3) decrease of DNA end-binding of both Ku70 and Ku80. The data suggest that Pim-1 plays a crucial role in the regulation of NHEJ repair. In the absence of Pim-1, the ability of DNA repair significantly decreases when exposed to paclitaxel, leading to severe DNA damage and apoptosis.

Keywords

Pim-1; Paclitaxel; H2A.X phosphorylation; NHEJ DNA repair; Prostate cancers

1. Introduction

Pim-1, a serine/threonine kinase, has been implicated in numerous biological functions, including cell migration and invasion, cell survival, proliferation and differentiation [1–3]. The crystal structure of Pim-1 reveals that it is a constitutively active kinase [4]. Pim-1 is predominantly present in the cytoplasm and the nucleus, and has been identified to induce cell cycle progression and to inhibit cell death. Several cell cycle regulators have been reported to be phosphorylated by Pim-1, including CDC25A, CDC25C, p27^{Kip1} and p21^{Cip1/WAF1} [5–7]. Although Pim-1 is expressed in normal cells, a wide variety of human cancer cell lines have been identified to display a relatively high level of Pim-1 expression, such as lymphoid and myeloid cells, prostate cancer cells, colorectal carcinoma and gastric cancer cells [3,8]. Moreover, expression of Pim-1 is regulated by several growth factors,

cytokines and cellular stresses, such as hypoxia and cancer chemotherapeutic drugs [3,9–12].

Pim-1 is over expressed in a number of cancer cells and is involved in numerous cellular signaling pathways that regulate cell cycle progression and cell survival. Several studies have analyzed the prognostic impact of the proto-oncogene Pim-1 in certain types of solid tumors. The evidence shows that up-regulation of Pim-1 might be a tumor marker for gastric cancer, prostate cancer and pancreatic cancer [13–15]. Accordingly, Pim-1 has been elucidated to serve as a target for cancer chemotherapeutic intervention. Recently, several ATP competitive small-molecule inhibitors of Pim-1 have been developed. Some of these inhibitors are initially known as the inhibitors on blocking other kinases – staurosporine (a broad-spectrum kinase inhibitor), LY333531 (a protein-kinase C inhibitor), LY294002 (a phosphatidylinositol 3-kinase inhibitor) and quercetagenin (a flavonoid) for example [12,16–18]. Pim-1 inhibitors display effective inhibitory activity of cell growth against several human cancer cell types, including leukemia and several solid tumors [12,19]. The Pim-1-mediated mechanisms leading to its effects on cell survival, cell cycle progression and apoptosis are complicated. Pim-1 can phosphorylate Bad, a BH3-containing Bcl-2 family member protein, leading to this pro-apoptotic protein to be sequestered by 14-3-3 proteins and blocking the apoptotic effect [20]. Besides, the increase of anti-apoptotic proteins, Bcl-2 and Bcl-xL induced by Pim-1 has been suggested to be attributed to the cell survival [20,21]. Recently, Pim-1 has been implicated in the regulation of mammalian target of rapamycin (mTOR) translational pathway through phosphorylation of 4E-BP1 protein [22] and PRAS40 (proline-rich Akt substrate 40 kDa) [23]. Moreover, Pim-1 may also regulate cell cycle progression through modification of p21, p27 and Cdc25A and by phosphorylating Cdc25C phosphatase [5–7,16].

A wide variety of intracellular survival pathways can be induced in cancer cells to cope with the imposed stresses caused by cancer chemotherapeutic drugs. Over expression of Pim-1 kinase is one of the examples of survival pathways. Pim-1 has been indicated to regulate cell survival after cytokine withdrawal [21,24] and exposure to cancer chemotherapeutic agents [25]. Because Pim-1 confers drug resistance [3,9,12], the associated induction of Pim-1 in cells when treated with anticancer drugs [9,12] becomes a major obstacle to be solved. Tubulin-binding drugs, such as docetaxel, have been widely used for the treatment of prostate cancers. Since Pim-1 kinase has been implicated in the progression of prostate cancers and to serve as a prognostic marker, the Pim-1-involved mechanism study in prostate cancers exposed to tubulin-binding drugs is particularly important. We present data in this study demonstrating that Pim-1 is highly involved in cellular DNA repair system. Knockdown of Pim-1 in human hormone-refractory prostate cancer cells largely facilitates apoptosis induced by tubulin-binding drug through impairment of DNA repair system. The data may provide insight into anticancer approach targeting Pim-1 and tubulin in an impact in DNA repair mechanism.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). The following antibodies were used: anti-mouse and anti-rabbit IgGs, Bcl-2, Bcl-xL, Bax, Bid, Bad, PARP-1, nucleolin, cyclin B1, DNA-PK catalytic subunit (DNA-PKcs), phospho-DNA-PKcs^{Thr2609} and replication protein A 32 kDa subunit (RPA32) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), α -tubulin, Ku70, Ku80, phospho-H2A.X^{Ser139}, ATM, phospho-ATM^{Ser1981}, Pim-1, phospho-STAT3^{Ser727} (signal transducer and activator of transcription 3), GAPGH, phospho-Bad^{Ser112} and actin (Cell Signaling, Beverly, MA) and MPM-2 (Upstate Biotechnology, Lake Placid, NY). Paclitaxel, vincristine, evodiamine, colchicine, quercetin, propidium iodide (PI), phenylmethylsulfonylfluoride (PMSF), leupeptin, dithiothreitol and rhodamine 123 were obtained from Sigma–Aldrich (St. Louis, MO). Pim-1 siRNA was obtained from (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

2.2. Cell culture

PC-3 cell line was from American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI1640 medium with 10% FBS (v/v) and penicillin (100 units/ml)/streptomycin (100 lg/ml). Cultures were maintained in a humidified incubator at 37 °C in 5% CO₂/95% air.

2.3. Flow cytometry cell cycle analysis

After the treatment, the cells were harvested by trypsinization, fixed with 70% (v/v) alcohol at 4 °C for 30 min and washed with PBS. After centrifugation, cells were incubated in phosphate–citric acid buffer (0.2 M NaHPO₄, 0.1 M citric acid, pH 7.8) for 30 min at room temperature. The cells were centrifuged and resuspended with 0.5 ml PI solution containing Triton X-100 (0.1% v/v), RNase (100 lg/ml) and PI (80 lg/ml). DNA content was analyzed with the FACSscan and CellQuest software (Becton Dickinson, Mountain View, CA).

2.4. DNA fragmentation assay

The DNA fragmentation was determined using the Cell Death Detection ELISA-plus kit (Roche, Mannheim, Germany). The assay was based on the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death. After the treatment with the indicated agents, the cells were lysed and centrifuged, and the supernatant was used for the detection of nucleosomal DNA according to the manufacturer's protocol.

2.5. Western blotting

After the treatment, the cells were washed twice with ice-cold PBS and reaction was terminated by the addition of 100 μ l ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 10 lg/ml aprotinin, 10 lg/ml leupeptin, and 1% Triton X-100). The amount of proteins (40 lg) were separated by electrophoresis in a 10% or 15% polyacrylamide gel and transferred to a PVDF membrane. After an overnight incubation at 4

LC in PBS/5% nonfat milk, the membrane was washed with PBS/0.1% Tween 20 for 1 h and immuno-reacted with the indicated antibody for 2 h at room temperature. After four washings with PBS/0.1% Tween 20, the anti-mouse or anti-rabbit IgG (dilute 1:2000) was applied to the membranes for 1 h at room temperature. The membranes were washed with PBS/0.1% Tween 20 for 1 h and the detection of signal was performed with an enhanced chemiluminescence detection kit (Amersham Biosciences). The data were quantified using the computerized image analysis system ImageQuant (Amersham Biosciences).

2.6. Nuclear extraction

Nuclear extracts were prepared by sequential cell lysis and nuclear lysis. Cells were suspended in 30 l buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol and 0.2 mM PMSF. The cells were subjected to vigorous vortex for 20 s, ice-cold treatment for 10 min, and centrifuged at 2000 rpm for 2 min. The pelleted nuclei were resuspended in buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM PMSF. After 20 min on ice, the lysates were centrifuged at 15,000 rpm for 2 min. The supernatants containing the solubilized nuclear proteins were used for Western blotting.

2.7. Immunofluorescence examination

After the treatment, the cells were fixed with 100% methanol at -20 °C for 5 min and incubated in 1% bovine serum albumin (BSA) containing 0.1% Triton X-100 at 37 °C for 30 min. The cells were washed twice with PBS for 5 min and incubated with primary antibodies at 37 °C for 1 h. The cells were washed twice with PBS and the tetramethyl rhodamine isothiocyanate or fluorescein isothiocyanate conjugated secondary antibody was used. The nuclei were recognized by the staining with DAPI (1 mg/ml). The labeled targets in cells were detected by a confocal laser microscopic system (Leica TCS SP2).

2.8. Detection of tubulin polymerization by cellular fractionation

After the treatment, the cells were harvested by trypsinization and collected by centrifugation. The cells were lysed with 0.1 ml of hypotonic buffer (1 mM MgCl₂, 2 mM EGTA, 0.5% NP-40, 2 mM PMSF, 200 U/ml aprotinin, 100 lg/ml soybean trypsin inhibitor, 5.0 mM ϵ -amino caproic acid, 1 mM bezamidine and 20 mM Tris-HCl, pH 6.8). The cytosolic and cytoskeletal fraction of cell lysate were separated by centrifugation at 16,000 g for 15 min. The supernatant contained cytosolic tubulin. The pellet representing the particulate fraction of polymerized tubulin was resuspended in 0.1 ml hypotonic buffer. Tubulin contents in both fractions were detected by Western blotting.

2.9. Small interfering RNA (siRNA) transfection

The Pim-1 siRNA was obtained from Invitrogen (Invitrogen Stealth™ select 3 RNAi Set). The 3 RNAi Set against Pim-1 were 5'-UAU ACA CUC GGG UCC CAU CGA AGU C-3', 5'-AGA ACA UCU UGC AUC CAU GGA UGG U-3', and 5'-UUC UUC AGC AGG ACC ACU UCC AUG G-3'. The negative control sequence was 5'-GCC TAC CGT CAG GCT ATC GCG TAT C-3'. For transfection, PC-3 cells were seeded into 60-mm tissue culture dishes with 30% confluence and grown for 24 h to 50–60% confluence. Each

dish was washed with serum-free Opti-MEM (Life Technologies, Grand Island, NY), and 2 ml of the same medium was added. Aliquots containing siRNA in serum-free Opti-MEM were transfected into cells using Lipofectamine 2000 following the manufacturer's instructions. After incubation for 6 h at 37 °C, cells were washed with medium and incubated in 10% FBS-containing RPMI-1640 medium for 48 h. Then, the cells were treated with or without paclitaxel for the indicated time and the subsequent experiments were performed.

2.10. DNA end-binding activity of Ku proteins

Assessment of DNA end-binding activity of Ku proteins were carried out by using a Ku70/Ku80 DNA Repair kit (Active Motif). Briefly, equivalent amounts of nuclear proteins (4 µg) were loaded into an oligonucleotide coated 96-well plate. Then, Ku proteins contained in nuclear extract specifically bound to this oligonucleotide. Ku proteins antibody provided by this kit detected DNA binding-Ku70 or Ku80. Addition of a secondary HRP-conjugated antibody provided a colorimetric readout quantified by spectrophotometry.

2.11. Pim-1 kinase assay

The assay was performed according to the supplier's protocol (Abnova Pim-1 Kinase Assay/Inhibitor Screening Kit, Heidelberg, Germany). Plates were pre-coated with a substrate corresponding to recombinant p21^{waf1} containing threonine residues that could be efficiently phosphorylated by Pim-1. The detector antibody specifically detected only the phosphorylated form of Thr¹⁴⁵ residue on p21^{waf1}.

2.12. Data analysis

The compound was dissolved in DMSO. The final concentration of DMSO exposed to cells was 0.1%. Data are presented as the mean ± SE of three independent experiments. Statistical analysis of the data was performed with one-way analysis of variance (ANOVA) followed by Bonferroni t-test and p-values less than 0.05 were considered significant.

3. Results

3.1. Induction of Pim-1 up-regulation by tubulin-binding agents

Tubulin-binding agents are widely used in cancer chemotherapy. The activation of STAT3 has been indicated to be associated to the cellular stress caused by tubulin-binding agents in prostate cancer cells [12]. Moreover, STAT3 can bind directly to the Pim-1 promoter and, therefore, is an important mediator for Pim-1 expression [9]. Several tubulin-binding agents were used in this study to examine the effect on STAT3 activity and Pim-1 expression in human hormone-refractory prostate cancer PC-3 cells. The data demonstrated that all of these agents resulted in the activation of STAT3 and a dramatic increase of Pim-1 expression (Fig. 1A). Because Pim-1 is implicated in cell survival and progression of prostate carcinomas, the knockdown of Pim-1 was performed to study its role on intrinsic drug resistance of tubulin-binding agents. As a result, apoptotic cell death not only was significantly increased in Pim-1 knockdown cells but also was potentiated by the exposure to these agents (Fig. 1B and C). Furthermore, the mitotic arrest and the increased expression of cyclin B1 and MPM2, phosphorylation of mitosis-specific proteins, induced by tubulin-

binding agents were significantly inhibited in the cells with Pim-1 knockdown (Fig. 1B and D).

3.2. Effect of Pim-1 on microtubule formation

Confocal microscopic examination showed that Pim-1 was primarily located at the cytosol of PC-3 cells. After the exposure to paclitaxel, the microtubule was profoundly stabilized showing strong fluorescence intensity. Notably, Pim-1 localization was associated with the microtubule. Cells treated with the other anti-tubulin agents, vincristine and evodiamine, exhibited a more diffuse cytoplasmic tubulin staining pattern, and colchicine-treated cells displayed almost a complete loss of tubulin staining, all of most probably indicating on tubulin de-polymerization induced by these agents (Fig. 2A). Furthermore, the tubulin polymerization assay in PC-3 cells showed that paclitaxel induced a dramatic increase of tubulin polymerization detected in particulate fraction; whereas, the other anti-tubulin agents inhibited the tubulin polymerization. The knockdown of Pim-1 did not modify the effect of these agents on tubulin polymerizing activity (Fig. 2B), indicating that the induction and potentiation of apoptosis in Pim-1 knock-down cells (Fig. 1B) were not relevant to tubulin dynamics when exposed to these agents.

3.3. Effect of paclitaxel on Bcl-2 family member proteins and DNA damage response

The Bcl-2 family of proteins plays a central role on the regulation of mitochondria-mediated apoptosis. Tubulin-binding agents are well identified to induce the phosphorylation and degradation of anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL. The data showed that paclitaxel induced a time-dependent phosphorylation of Bcl-2, Bcl-xL and Bad (Fig. 3A). The knockdown of Pim-1 did not change the effect on Bcl-2 and Bcl-xL phosphorylation. The Bad phosphorylation, however, was as expected to be completely abolished in Pim-1 knockdown cells since Bad was a direct substrate for Pim-1 [26] (Fig. 3A). H2A.X is required for DNA fragmentation and is phosphorylated at Ser¹³⁹ when exposed to apoptotic stimuli [27,28]. Paclitaxel induced a dramatic increase of H2A.X phosphorylation after a 24-h treatment (Fig. 3B). The data were time correlated with the occurrence of cell apoptosis (supplementary Fig. 1). Moreover, Pim-1 knockdown also significantly accelerated paclitaxel-induced DNA damage response by comet assay (supplementary Fig. 2). Of note, the apoptosis-associated H2A.X phosphorylation was significantly facilitated in Pim-1 knockdown cells (Fig. 3B). The data reveal that in the presence of paclitaxel, Pim-1 knockdown may trigger a mechanism that leads to an early onset of DNA damage response.

3.4. Effect of Pim-1 knockdown on DNA repair system

ATM plays a central role in DNA damage checkpoint. It is activated in response to DNA damage, particularly in the presence of double-strand breaks (DSBs). Ku proteins and DNA-PK also play crucial roles in DNA repair, participating in the nonhomologous DNA-end-joining (NHEJ) repair for repairing DSBs [29]. Paclitaxel induced a time-dependent increase of the phosphorylation of ATM, DNA-PK and RPA32 (serving as a DNA-PK substrate), indicating the formation of DNA breaks and processing of DNA repair as a result to paclitaxel action (Fig. 4). However, the activity of these DNA repair kinases was

significantly inhibited in Pim-1 knock-down cells. Furthermore, Pim-1 knockdown induced a modest but statistically significant decrease of Ku80 expression (Fig. 4).

3.5. Effect of Pim-1 knockdown on nuclear localization and DNA binding of Ku proteins

Ku proteins work in nuclei for functional DNA repair. However, several studies have reported cytoplasmic localization of Ku proteins [30]. To determine the nuclear localization of Ku proteins, the nuclei were separated from PC-3 cells after the Pim-1 knock-down or not. As a consequence, the nuclear Ku80 was significantly decreased in Pim-1 knockdown cells (Fig. 5A). Besides, the assessment of DNA end-binding activity of Ku proteins was also carried out. The data demonstrated that the Pim-1 knockdown dramatically decreased the DNA end-binding of both Ku70 and Ku80 (Fig. 5B). The data were highly correlated with the Pim-1 knock-down-induced reduced activity of the DNA repair kinases described above (Fig. 4).

3.6. Effect of Pim-1 inhibitors on DNA repair system

To examine if the inhibition of Pim-1 kinase activity by Pim-1 inhibitors exhibits similar effect to Pim-1 knockdown, the pharmacological inhibitor quercetin was used. The quercetin-mediated inhibitory activity of Pim-1 was determined using recombinant Pim-1 kinase and an in vitro kinase assay. The half maximal inhibitory concentration (IC_{50}) was 0.17 ± 0.2 μ M ($n = 4$, supplementary Fig. 3). However, a high concentration of 30 μ M was used in the PC-3 cell model because the high lipophilicity made the quercetin hard to get to the target [31]. The data showed that in the presence of quercetin, paclitaxel-induced H2A.X phosphorylation and PARP-1 cleavage were facilitated, which was associated with the inhibition of ATM phosphorylation as well as RPA32 phosphorylation (Fig. 6). The data confirm the impact of Pim-1 knockdown on DNA repair activity.

4. Discussion

Pim-1 has been implicated in the development and progression of hematopoietic malignancies and also in prostate carcinomas by promoting cell survival/proliferation and antagonizing cell apoptosis. In cancers, STAT3 activity is critical for the transcriptional and translational regulation of Pim-1. However, STAT3 by itself is susceptible to a wide variety of cellular stresses, including the insult by anticancer drugs. Accordingly, the increased STAT3 and Pim-1 kinase activity may contribute to the intrinsic resistance of cancer chemotherapeutic drugs. The data presented in this study showed that tubulin-binding agents induced an increase of STAT3 activity and Pim-1 expression in prostate cancer PC-3 cells. Zemskova and the colleagues [12] have also reported that docetaxel (100 nM) can activate STAT3 and Pim-1 kinase in human prostate epithelial cell line RWPE-2 and prostate cancer cell line DU145. On the contrary, paclitaxel of high concentrations (1.75–7 μ M) has been reported to inhibit STAT3 activity in breast cancer cell lines [32]. Although it needs further investigation to clarify the discrepancy between these studies, the data raise the resistance issue in patients when tubulin-binding drugs are used for the treatment of prostate cancers.

The knockdown of Pim-1 significantly potentiated the apoptotic cell death caused by tubulin-binding agents. Because the increased expression of Pim-1 by these agents in Pim-1

wild-type cells was tightly associated with tubulin/microtubule, one of the possibilities for the potentiated apoptosis was to act on the tubulin/micro-tubule dynamics. However, neither the paclitaxel-induced microtubule stabilization nor the tubulin de-polymerization caused by anti-tubulin agents was modified in Pim-1 knockdown cells. Thus, the possibility that Pim-1 affects tubulin/microtubule dynamics can be excluded as a cause for Pim-1 knockdown-potentiated apoptosis.

Mitochondria-dependent signaling pathway contributes a major part of cell apoptosis induced by tubulin-binding agents. The Bcl-2 family members play a central role in controlling the mitochondria integrity. A large body of evidence suggests that the phosphorylation of Bcl-2 and Bcl-xL caused by tubulin-binding agents disrupts the mitochondria integrity, leading to the release of cytochrome c and activation of caspase cascades [25]. The data in the present study showed that Pim-1 knockdown did not change the phosphorylation state of Bcl-2 and Bcl-xL, indicating that Bcl-2 and Bcl-xL were not downstream mediators of Pim-1 kinase. In contrast, the phosphorylation of the pro-apoptotic protein Bad was completely abolished in Pim-1 knockdown cells. Pim-1 has been identified to directly phosphorylate Bad at serine 112, a critical site for its inactivation [26]. Bad phosphorylation is, therefore, suggested to be one of the mechanisms that explain Pim-1-mediated pro-survival activity. Nevertheless, the Bad involved mechanism could not fully explain the Pim-1 knockdown facilitation of paclitaxel-mediated DNA damage and apoptosis (please see below).

The phosphorylation of histone variant H2A.X at serine 139, also named γ H2A.X, has been extensively used as a marker for DSBs, the most lethal form of DNA damage. DSBs can be induced by a lot of events, such as ultraviolet and ionizing radiation, anti-cancer drugs, free radicals and some other apoptotic stimuli [27]. Normally, cells initiate DNA repair mechanism when DNA DSBs occur. Similar effect is also present in paclitaxel-induced cellular DNA damage by which the DNA repair mechanism follows [33]. NHEJ and homologous recombination are two predominant repair pathways. In response to DSBs, γ H2A.X is rapidly formed by several kinases, including ATM, ATR and DNA-PKcs [27]. Interestingly, the present data showed that the activation of ATM and DNA-PKcs (at a 4-h and 8-h treatment, Fig. 4) occurred much earlier than the formation of γ H2A.X (at a 24-h treatment, Fig. 3B) in cells responsive to paclitaxel. In other words, paclitaxel induced a γ H2A.X-independent mechanism of DNA damage response during a 4-h to 12-h treatment. It has been suggested that γ H2A.X is dispensable for the initial recognition of DNA breaks [34]. The mild defects in DNA damage signaling and DNA repair also have been detected in H2A.X-deficient cells and animals [27,35]. Our data also indicated that γ H2A.X-independent while ATM- and DNA-PKcs activated event might provide a mechanism promoting cell survival because paclitaxel did not cause apoptosis during the time of the event. The evidence that ATM and DNA-PKcs promote cell survival has also been reported by Guo and the colleagues [36]. However, Pim-1 knockdown largely facilitated paclitaxel-induced γ H2A.X formation and apoptosis, revealing that in the absence of Pim-1 paclitaxel could trigger a rapid DNA damage, leading to apoptotic cell death. The effect was attributed to the inhibition of NHEJ repair activity supported by the following observations: (1) the significant inhibition of ATM and DNA-PKcs activities, (2) the significant down-regulation of Ku protein expression and its nuclear localization and (3) the decrease of the DNA end-

binding of both Ku70 and Ku80. The Pim-1 knockdown-mediated inhibition of DNA repair and potentiation of cell apoptosis are particularly critical in prostate cancers because it has been extensively identified that Pim-1 plays a crucial role in survival of prostate cancers [12]. Pim-1 also serves as a resistance factor in prostate cancers exposed to tubulin-binding drugs [12,37]. To substantiate the role played by Pim-1, the pharmacological Pim-1 inhibitor quercetin was used to mimic the knockdown of Pim-1. As expected, quercetin inhibited ATM activation, facilitating the formation of cH2A.X and cell apoptosis. There is evidence showing that prostate cancers are over-expressed with ATM [38]. It indicates that ATM may play a central role in DNA repair of prostate cancers. The report correlates with the present study that both Pim-1 knockdown and quercetin-mediated Pim-1 inhibition block ATM-involved DNA repair system, leading to the sensitization of apoptosis caused by paclitaxel.

In conclusion, the data suggest that Pim-1 plays a crucial role in the regulation of DNA repair in human prostate cancers. Under cellular stress, Pim-1 may promote cell survival through ATM-, DNA-PKcs- and Ku protein-involved DNA repair pathway. In the absence of Pim-1, the capability of DNA repair significantly decreases in PC-3 cells in response to paclitaxel, leading to severe DNA damage and apoptosis. The data provide evidence supporting that Pim-1 may serve as a potential target for the treatment of hormone-refractory prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Mutual regulation of tubulin-binding agents and Pim-1. (A) PC-3 cells were treated with or without the indicated agent for 8 h. The cells were harvested and lysed for the detection of protein expression by Western blot analysis. The expression was quantified using the computerized image analysis system ImageQuant. (B and C) PC-3 cells were transfected with or without Pim-1 siRNA. The cells were treated with the indicated agent for 18 or 6 h, respectively. The cell cycle progression (B) and expressions of MPM2 and cyclin B1 (D) were detected using flow cytometric analysis of propidium iodide staining and Western blot, respectively. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the respective control. (C) The cells were treated with or without the indicated agent for 8 h. The cells were harvested and lysed for the detection of DNA fragmentation using the Cell Death Detection ELISaplus system.

Fig. 2.

Effect of Pim-1 on cellular microtubule assembly and disassembly. (A) PC-3 cells were treated with the indicated agent for 9 h. The cells were fixed and the protein expression was detected using confocal microscopic examination. Areas of co-localization between Pim-1 and tubulin in the merged images are the yellow demonstration. DAPI was used for nuclear counterstains with blue fluorescence. Scale bar, 20 μ m and (B) both parent and Pim-1 knockdown cells were treated with the indicated agent for 18 h. The cells were harvested and the particulate fractions were obtained for the detection of microtubule assembly by Western blot analysis. The tubulin expression was quantified using the computerized image analysis system ImageQuant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Effect of Pim-1 knockdown on the expression of γ -H2A.X and Bcl-2 family protein members in PC-3 cells. Both parent and Pim-1 knockdown cells were treated without or with paclitaxel (0.1 μ M) for the indicated times. The cells were harvested for the detection of protein expression by Western blot analysis. The protein expression was quantified using the computerized image analysis system ImageQuant. *** $p < 0.001$ compared with the control.

Fig. 4.

Effect of Pim-1 knockdown on several protein expressions in PC-3 cells. Both parent and Pim-1 knockdown cells were treated without or with paclitaxel (0.1 μM) for the indicated times. The cells were harvested for the detection of protein expression by Western blot analysis. The protein expression was quantified using the computerized image analysis system ImageQuant. * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.001$ compared with the respective control siRNA.

Fig. 5.

Effect of Pim-1 knockdown on nuclear localization and DNA binding of Ku proteins in PC-3 cells. Both parent and Pim-1 knockdown cells were harvested. The nuclei were separated and collected for the detection of protein expression by Western blot analysis (A) or for the assessment of DNA end-binding activity of Ku proteins described in Section 2.

*** $p < 0.001$ compared with the respective control.

Fig. 6.

Effect of quercetin and paclitaxel on several protein expressions. (A) PC-3 cells were treated with or without paclitaxel (0.1 μ M) in the absence or presence of quercetin (30 μ M) for the indicated times. The cells were harvested for the detection of protein expression by Western blot analysis and (B) the protein expression was quantified using the computerized image analysis system ImageQuant. * $p < 0.05$ compared with quercetin-free control.