We previously showed that the 44-kDa serine/threonine kinase Pim-1 (Pim-1L) can protect prostate cancer cells from apoptosis induced by chemotherapeutic drugs (Xie, Y., Xu, K., Dai, B., Guo, Z., Jiang, T., Chen, H., and Qiu, Y. (2006) Oncogene 25, 70–78). To further explore the mechanisms of Pim-1L-mediated resistance to chemotherapeutic drugs in prostate cancer cells, we employed a yeast two-hybrid screening to identify cellular proteins that were associated with Pim-1L, and we found the ABC transporter BCRP/ABCG2 as one of the potential interacting partners of Pim-1L. We also showed that the expression level of Pim-1L and BCRP was up-regulated in mitoxantrone and docetaxel-resistant prostate cancer cells. Pim-1L was co-localized with BCRP on the plasma membrane and induced phosphorylation of BCRP at threonine 362. Knocking-down Pim-1L expression in the drug-resistant prostate cancer cell lines, Pim-1L was essential for its functionality. This is further corroborated by our finding that the plasma membrane localization and drug-resistant activity of BCRP were compromised by T362A mutation. Our data suggest that Pim-1L may protect prostate cancer cells from apoptosis, at least in part, through regulation of transmembrane drug efflux pump. These findings may provide a potential therapeutic approach by disrupting Pim-1 signaling to reverse BCRP-mediated multidrug resistance.

The proto-oncogene pim-1 encodes two serine/threonine kinases with molecular masses of 33 and 44 kDa by utilizing two alternative translation sites (1, 2). These two Pim-1 proteins exhibit comparable in vitro kinase activity. However, the 44-kDa Pim-1 appears to be more stable (1). Studies have shown that the 33-kDa Pim-1 is monomeric in vivo, whereas the 44-kDa Pim-1 is found in a complex (1), suggesting the latter may interact with more protein partners. Our current knowledge on Pim-1 kinases is largely derived from study on the 33-kDa isoform. The 33-kDa Pim-1 has been implicated in the regulation of cell cycle and transcription by phosphorylating a number of substrates such as cdc25A, HP1, and p100 (3–6). Moreover, it has been shown that Pim-1 may play a role in the regulation of the survival signaling by phosphorylating BAD (7). Pim-1 is thought to play an integral role in the development of a number of human cancers, such as hematolymphoid malignancies (8, 9). A number of studies demonstrated that Pim-1 is up-regulated in both human prostate cancer as well as animal models and may play an important role in prostate cancer development and progression (10–12). Pim-1 has emerged as a potential diagnostic marker in prostate cancer (10). Recently, we have shown that the 44-kDa isoform Pim-1L may play a more prominent role in anti-apoptosis signaling in response to chemotherapeutic drugs in prostate cancer cells (2). The localization of the 44-kDa Pim-1L is primarily on the plasma membrane, and it contains an N-terminal proline-rich motif and interacts directly with tyrosine kinase Etk through an interaction between the PXXP motif and the SH3 domain of Etk. Such interaction competes with tumor suppressor p53 for binding to Etk and activates Etk kinase activity (13).

Advanced metastatic prostate cancer treated by hormone manipulation or orchietomy frequently leads to the development of progressive hormone-refractory prostate cancer and highly chemoresistant tumors. Several biochemical mechanisms of drug resistance have been identified in prostate cancer cell lines, including alterations of glutathione metabolism, altered topoisomerase activity, and up-regulation of the transmembrane drug efflux pumps (14), and in particular ATP binding cassette (ABC)3 transporter family members such as multidrug resistance protein-1 (MDR1/Pgp/ABCB1) and multidrug resistance-associated protein-1 (MRP1/ABCC1). Recently it

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3 The abbreviations used are: ABC, ATP binding cassette; GST, glutathione S-transferase; HA, hemagglutinin; MX, mitoxantrone; DTX, docetaxel; PR, proline-rich; TPT, toptecan.
has been reported that MRP1 but not MDR1 overexpression contributes to acquired drug resistance in two prostate cancer cell lines derived from PC3 and DU145 (14). It has been shown that the presence of a half ABC transporter, breast cancer resistance protein BCRP/ABCG2, isolates the putative prostate stem cells from the prostate tissue microenvironment through constitutive efflux of androgen and protects the putative tumor stem cells from androgen deprivation, hypoxia, or adjuvant chemotherapy, and provides the nidus for recurrent prostate cancer (15). In addition to androgen, a large set of BCRP substrates has been identified including chemotherapeutic agents, fluorescent dyes, as well as chemical toxicants (16). BCRP overexpression has been detected in a variety of mitoxantrone- or other chemotherapeutic agents selected cell lines (17, 18). The molecular mechanism of BCRP in drug efflux has been well studied in breast cancer cells. Several immunohistochemical studies using monoclonal and polyclonal antibodies have confirmed that BCRP is mainly localized to the plasma membrane of mammalian cells. Recent studies suggest that BCRP may function as a homodimer (19) or homotetramer (20). However, whether BCRP is involved in the multidrug resistance in prostate cancer remains elusive.

To further explore the mechanisms of Pim-1L-mediated resistance to chemotherapeutic drugs in prostate cancer cells, we employed the yeast two-hybrid screening to identify cellular proteins that are associated with 44-kDa Pim-1L, and we found BCRP as one of the potential interacting partners of Pim-1L. We also showed that expression levels of Pim-1L and BCRP are up-regulated in mitoxantrone- and docetaxel-resistant prostate cancer cell lines. Pim-1L can directly interact with and phosphorylate BCRP, which promotes BCRP dimerization and ultimately its plasma membrane localization. Our data suggest that Pim-1L may protect prostate cancer cells from apoptosis, at least in part, through interacting with and phosphorylating BCRP. These findings may provide a potential therapeutic approach by disrupting Pim-1 signaling to reverse BCRP-mediated multidrug resistance.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening**—The full-length 44-kDa human Pim-1 cDNA was amplified by PCR using kinase inactive mutant plasmid described previously (2) as template with high fidelity polymerase (Invitrogen) and primers 5'-CGGAATTCTAGCCTCCTGCACCAGGCGGCG-3' and 5'-CGGAATTCTATTGGCCTGGCCGCCGCGAC-3'. The products were digested with EcoRI and inserted into the pGBK7T vector (Clontech). The expression library was the human HeLa cell cDNA library (Clontech). Plasmids were introduced into yeast strain Y187, and interacting proteins were double selected for growth on His/Leu/Trp-deficient plates and strain Y187, and interacting proteins were double selected for growth on His/Leu/Trp-deficient plates and his/mia biotech) with the forward primer 5'-CGGAATTCCCTACCCAACCTCCAGGATG-3' and their respective to generate the N-terminal tagged HA-BCRP and Myc-BCRP construct. All human Pim-1 constructs contain the N-terminal FLAG-tag and described previously (2). To generate the BCRP T362A or T362D mutant, the threonine residue at position 362 was mutated to alanine or aspartic acid via oligonucleotide-directed mutagenesis with the sense primers 5'-GAAGAAGATCGACGTCTTCAAGG-3' or 5'-GAAGAAGATCGACGTCTTCAAGG-3', respectively and their complementary antisense primers by using the QuickChange Mutagenesis kit (Stratagene). GST-Pim-1L and GST-proline-rich region of Pim-1L (GST-PR) constructs were generated by PCR cloning into pGEX-6P vector (Amersham Biosciences Pharmacia biotech) with the forward primer 5'-CGGAATTCTAGCCTCCTGCCCCGCCGGCG-3' with the reverse primers 5'-CGGAATTCTATTGGGCGCCGCCGAC-3' and 5'-CGGAATTCTACCCACACTCCAGGATG-3', respectively. GST-Pim-1L construct is described previously (21).

**Cell Culture and Transfection**—CWR-R1 cells were kindly provided by Dr. C. W. Gregory (22). All other cell lines used in this study were purchased from American Tissue Culture Collections (Manassas, VA). The 293T cells were maintained in

![FIGURE 1. Interaction between Pim-1L and BCRP.](image-url)
Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. CWR-R1 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Transfections were performed by using FuGENE 6 (Roche Applied Science), Lipofectamine 2000 (Invitrogen), or the calcium phosphate precipitation method (Biological Mictions Inc.) according to the manufacturer’s instructions.

**GST Pull-down Assay**—GST fusion proteins were expressed and purified as described previously (13, 23). Briefly, the GST fusion proteins were pulled down by glutathione beads at 4 °C for 1 h and then washed three times with the lysis buffer (21, 24). The immobilized GST fusion proteins were incubated with the lysates of 293T cells transfected with the HA-tagged BCRP for 1 h at 4 °C. The beads were washed with the lysis buffer four times and then the protein complexes were loaded in SDS-PAGE, followed by immunoblotting with anti-HA antibody.

**Immunoprecipitation, Compartimental Protein Extraction, Western Blot, and in Vitro Kinase Assay**—The transfected cells were lysed in the buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na3VO4, 1 mg/ml aprotinin, 1 mg/ml leupentin, and 1 mM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation, and antibodies were added to lysates and incubated for 1–3 h at 4 °C. The immunocomplexes were collected by using protein A or protein G-Sepharose beads, and then the beads were washed extensively for three times at 4 °C with the lysis buffer. Cell fractionation was carried out using compartmental protein extraction kit (BioChain, Inc.). Immunoblotting was performed as described previously (24). Briefly, blots were incubated with primary antibodies, 1:1000 dilution of anti-Myc tag, 1:500 dilution of anti-phosphothreonine, 1:2000 dilution of anti-FLAG tag, 1:2000 dilution of anti-HA tag, 1:100 dilution of anti-Pim-1, 1:500 dilution of anti-BCRP (BXP21) at room temperature for 1 h and followed by the detection with horseradish peroxidase-conjugated secondary antibody. The polyclonal phospho-specific antibody for BCRP Tyr-362 (anti-pBCRP) was developed by immunizing the rabbits with a peptide containing phosphothreonine 362 of BCRP, and the terminal bleeds were affinity-purified using Sepharose-
immobilized peptide by a commercial carrier (Bethyl). The polyclonal anti-Pim-1L antibody is described previously (2), and the monoclonal anti-Pim-1 (catalog no. sc13513) was purchased from Santa Cruz Biotechnology Inc. The Pim-1L in vitro kinase assays were carried out as described previously (2, 21). Briefly, the purified kinase active GST-Pim-1L or kinase-inactive Pim-1LKM protein was mixed with immunoprecipitated HA-BCRP and incubated at room temperature in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, and 200 μM ATP) for 30 min. The reaction was terminated by adding the equal volume of 2× SDS samples buffer. The phosphorylation of BCRP was detected by Western blot with anti-pBCRP Tyr-362 antibody.

Immunofluorescence Staining and Microscopy—LNCaP cells were seeded on coverslips coated with poly-D-lysine and trans-
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Among 16 positive clones identified in our yeast two-hybrid screening using the kinase inactive Pim-1L mutant as bait, two independent clones harbored in-frame DNA sequence coding for BCRP. This interaction appears to occur in mammalian cells because BCRP was co-immunoprecipitated with Pim-1L but not Pim-1S, which lacked the N-terminal proline-rich region (Fig. 1A), suggesting that BCRP preferentially binds to Pim-1L. The GST-pull-down experiments showed that BCRP was associated with GST-Pim-1L but not with GST, GST-Pim-1S, or GST-PR (Fig. 1B), suggesting that only Pim-1L is able to directly interact with BCRP, and this interaction requires both the N-terminal proline-rich region and the kinase domain of Pim-1L. We also observed the co-localization of BCRP and Pim-1L on the plasma membrane (Fig. 1C). Furthermore, the interaction between endogenous BCRP and Pim-1L in a prostate cancer cell line CWR-R1 was confirmed by the co-immunoprecipitation experiments shown in Fig. 1D.

To determine whether the interaction of Pim-1L and BCRP is involved in drug response, we treated CWR-R1 cells with levetircept, the siRNAs specific for Pim-1, BCRP, or together and then tested their response to chemotherapeutic drugs. As shown in Fig. 2A, knocking down Pim-1 or BCRP sensitizes CWR-R1 cells to MX and DTX compared with the vector controls. These data suggest that both Pim-1L and BCRP are required for the survival of CWR-R1 cells in response to these drugs. In addition, we established two drug-resistant cell lines R1/MX and R1/DTX from an AR-positive hormone refractory prostate cancer cell line CWR-R1 by culturing them in media containing MX or DTX, two Food and Drug Administration (FDA) approved drugs for treatment of advanced hormone refractory prostate cancer. As shown in Fig. 2B, both R1/DTX and R1/MX cells demonstrated increased resistance to multiple drugs including MX, DTX, and TPT compared with the parental cells. The expression level of BCRP and Pim-1L was also increased in these drug-resistant cell lines compared with the parental line. Meanwhile, we did not detect an appreciable increase of MDR1 in these resistant lines (Fig. 2C), suggesting that BCRP may play a major role in the drug resistance in these cell lines.

To further examine if BCRP and Pim-1L act together to protect cells, we infected LNCaP cells with lentiviruses encoding the HA-tagged BCRP and/or the FLAG-tagged Pim-1L and then examined their effects on drug response. LNCaP cells expressed very little BCRP and were very sensitive to MX or DTX. However, overex-

RESULTS

FIGURE 3. Pim-1L induces BCRP phosphorylation at threonine 362. A, alignments of phosphorylation consensus sequences from identified Pim-1 kinase substrates, B, endogenous BCRP in prostate cancer cells is threonine phosphorylated. Lysates from CWR-R1 parental and R1/MX, R1/DTX cells were immunoprecipitated with anti-BCRP or IgG control followed by immunoblotting with anti-phosphothreonine (pThr) or anti-BCRP. C, Pim-1L phosphorylates BCRP. 293T cells were transfected with the plasmids as indicated. BCRP phosphorylation was examined as described in the legend to Fig. 1A. D, Pim-1L phosphorylates BCRP at Thr-362. 293T cells were transfected with the plasmids as indicated. At 24 h post-transfection, the cells were serum-starved overnight. The cell lysates were subjected to immunoprecipitation with anti-HA antibody and followed by immunoblotting with the anti-phosphothreonine 362 (pThr362). E, down-regulation of Pim-1L decreases endogenous BCRP phosphorylation at Thr-362. CWR-R1/DTX cells were treated with the lentivirus encoding the siRNA for Pim-1 or the vector control. The endogenous BCRP threonine phosphorylation was determined by immunoprecipitation with anti-BCRP followed by immunoblotting with the anti-phosphothreonine (pThr-362). F, Pim-1L phosphorylates BCRP at T362 in vitro. In vitro kinase assays were carried out as described under “Experimental Procedures”. The phosphorylation of BCRP was detected by immunoblotting with anti-pBCRP362 antibody. The amount of GST-Pim-1L and BCRP in the reactions was monitored by immunoblotting with anti-Pim-1 and anti-BCRP, respectively. G, resensitization of drug-resistant cells by down-regulation of BCRP or Pim-1L. R1/MX, R1/DTX cells were treated with the lentivirus encoding the siRNA for Pim-1L or BCRP, or the vector or the scrambled siRNA control. At 48 h post-infection, the cells were maintained in MX or DTX containing medium for 72 h. Cell growth was determined by WST-1, *p < 0.01 compared with the vector control. Meanwhile, the cell colonies were visualized by Coomassie Blue staining.
expression of either BCRP or Pim-1L alone in LNCaP cells could increase survival to some degree as previously reported, and co-expression of Pim-1L and BCRP led to a dramatic increase in cell viability (Fig. 2D). These data suggest that an additive effect of BCRP and Pim-1L in anti-apoptosis signaling against chemotherapeutic drugs and the drug-resistant activity of BCRP could be enhanced by the elevated Pim-1L activity in these cells.

As shown in Fig. 3A, several identified Pim-1 substrates contain the consensus sequence of the preferred substrates of Pim-1: (Lys/Arg)_3-X-(Ser/Thr)-X (where X stands for any residue). Interestingly, the threonine 362 (Thr-362) residue of BCRP is embedded in the similar sequence context, suggesting that BCRP could serve as a substrate for Pim-1L. This is supported by the observation that the immunoprecipitated BCRP from R1/MX and R1/DTX cells could be recognized by a phosphothreonine-specific antibody (Fig. 3B). Co-expression of BCRP and Pim-1L in 293T cells induced threonine phosphorylation of BCRP, and the substitution of Thr-362 with alanine (T362A) or the phosphorylation-mimicking aspartate (T362D) completely abolished threonine phosphorylation of BCRP, suggesting that Thr-362 may indeed be phosphorylated by Pim-1L in vivo (Fig. 3C). To further characterize phosphorylation of BCRP Thr-362, we developed a polyclonal antibody that specifically recognized phosphorylated Thr-362 of BCRP (anti-pT362). As shown in Fig. 3D, this antibody recognized the wild-type BCRP phosphorylated by Pim-1L but not the unphosphorylatable T362A mutant. In addition, the anti-pT362 antibody detected the phosphorylated endogenous BCRP in R1/DTX and R1/MX cells and this phosphorylation was reduced when Pim-1L was knocked down by the siRNA (Fig. 3E). Furthermore, our in vitro kinase assays demonstrated that purified GST-Pim-1L, but not the kinase-inactive mutant Pim-1LKM, could directly phosphorylate BCRP in vitro (Fig. 3F). These data suggest that BCRP is threonine phosphorylated and Pim-1L may be responsible, at least in part, for phosphorylating BCRP in these cells. The requirement of both BCRP and Pim-1L for their drug resistance was further supported by the fact that the down-regulation of either BCRP or Pim-1L by the specific siRNAs resensitized these cells to drugs (Fig. 3G).

To test whether phosphorylation of Thr-362 of BCRP is important for BCRP-mediated drug resistance, we infected LNCaP cells with the lentivirus encoding the HA-tagged BCRP or its mutant and then examined whether LNCaP cells were protected by the overexpression of BCRP or its mutant from apoptosis induced by chemotherapeutic drugs MX, TPT, and DTX. As shown in Fig. 4A, the wild-type BCRP-mediated drug resistance, we infected LNCaP cells with the lentivirus encoding the HA-tagged BCRP or the T362A mutant and then examined whether LNCaP cells were protected by the overexpression of BCRP or its mutant from apoptosis induced by chemotherapeutic drugs MX, TPT, and DTX. As shown in Fig. 4A, the wild-type BCRP significantly increased the survival of LNCaP cells in response to these drugs, but the T362A mutant failed to do so, suggesting that the integrity of Thr-362 is essential for the functionality of BCRP. Co-expression of BCRP and Pim-1L in LNCaP cells exerted an additive effect on protecting LNCaP cells from apoptosis, which was significantly diminished when Thr-362 was mutated into alanine. In addition, the BCRP drug-resistant activity was significantly reduced when endogenous Pim-1L expression was knocked down by the siRNA, whereas the phosphorylation-mimicking mutant T362D remained active independent of Pim-1L (Fig. 4B). These results suggest that the phosphorylation of
BCRP Thr-362 induced by Pim-1L may be required for its optimal efflux activity.

Based on the topological structure of BCRP, Thr-362 localizes in the linker region between the ATP binding pocket and the transmembrane domain. To test whether Thr-362 phosphorylation plays a role for BCRP membrane localization, we infected LNCaP cells by the lentivirus encoding the wild-type BCRP or the T362A mutant. Immunofluorescence microscopy revealed that the wild-type BCRP and the phosphorylation-mimicking mutant T362D were predominantly localized on the plasma membrane, whereas the T362A mutant was mainly localized in cytoplasmic compartment (Fig. 5A). The retention of BCRP in the cytoplasmic compartment caused by the T362A mutation was further confirmed by fractionation experiments (Fig. 5B). It has been reported that the dimerization/oligomerization of BCRP on the plasma membrane plays a critical role for its drug efflux activity (19). We therefore examined the effects of T362A mutation on BCRP dimerization. We co-transfected the wild-type HA-tagged BCRP with the Myc-tagged BCRP or its T362A mutant into 293T cells. The status of BCRP dimerization/oligomerization was examined by the co-immunoprecipitation assays. As shown in Fig. 5C, the wild-type Myc-BCRP was efficiently associated with the wild-type HA-BCRP but such interaction was disrupted by the T362A mutation. This is further corroborated by our observation that the formation of endogenous BCRP multimers in R1/MX cells was compromised when Pim-1L expression was knocked down by the specific siRNA (Fig. 5D). Taken together, these data suggest that phosphorylation of BCRP Thr-362 induced by Pim-1L may modulate the dimerization/oligomerization of BCRP and its plasma membrane presentation.

**DISCUSSION**

Previously we have shown that the 44 kDa Pim-1 kinase (Pim-1L) predominantly localizes on plasma membrane and plays a more prominent role than the 33 kDa isofrom (Pim-1S) in anti-apoptosis signaling in response to chemotherapeutic drugs in prostate cancer cells. To identify the potential Pim-1L-specific substrates, we carried out the yeast two-hybrid screening. Interestingly, none of the preys identified in our survey was previously reported as Pim-1S-associated proteins by using the similar screening approach. This suggests that Pim-1L and Pim-1S may interact with a different pool of protein partners and therefore play distinct roles in cell signaling. Further study on the Pim-1L interacting and signaling network would reveal the mechanisms by which Pim-1L exerts its biological activity. In this study, we provided the evidence that BCRP/ABCG2 is a preferential substrate of Pim-1L and is an important mediator for Pim-1L-promoted drug resistance in prostate cancer cells. It appears to require both the N-terminal proline-rich domain and the kinase domain for Pim-1L to interact with BCRP, suggesting that more than one interacting site may be present between BCRP and Pim-1L. We also showed that phosphorylation of BCRP Thr-362 by Pim-1L modulates the dimerization/oligomerization of BCRP and its plasma membrane localization. Previous *in vitro* studies showed that BCRP may form...
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homodimers through disulfide bond mediated by cysteine 603; however, such covalent intermolecular link does not appear to be prerequisite for BCRP to exert its transport activity (25, 26). It remains to be investigated how phosphorylation of Thr-362 affects the intermolecular interaction between BCRP dimers or multimers. It is possible that phosphorylation of this site would induce a conformational change of BCRP and promote the nearby highly conserved Cys-374 to form intermolecular disulfide bond. Additional experiments have to be done to test whether it is indeed the case. We previously showed that Pim-1L can interact with tyrosine kinase Etk on the plasma membrane, and Etk is known to be involved in the regulation of vesicle trafficking and promotes plasma membrane presentation of EGFR (13, 24). It is possible that BCRP could be regulated by both kinases in a dynamic fashion. Future investigations are needed to elucidate how these proteins work together to confer drug resistance.

A previous study showed that overexpression of BCRP in breast cancer cells does not confer resistance to paclitaxel (9). However, in this study, we showed the evidence that BCRP is involved in resistance to docetaxel, a taxol derivative, in prostate cancer cells, suggesting that BCRP may be able to transport a broader range of substrates than we previously thought depending on cellular context. It is not yet clear whether phosphorylation of BCRP induced by Pim-1L may alter the substrate spectrum of the transporter. BCRP has been shown to be highly expressed in a subset of prostate cancer stem/progenitor cells. It would be interesting to examine whether the drug resistant lines we established in this study may possess the property of cancer stem/progenitor cells. To further investigate whether BCRP may play a role in docetaxel resistance in human prostate cancer, we conducted a survey on tissue samples from patients who were treated with docetaxel for at least six months using our anti-pThr-362 antibody. Our preliminary analysis revealed a significant increase of BCRP Thr-362 phosphorylation in the docetaxel-resistant samples compared with the untreated control groups, suggesting that BCRP activity may contribute to docetaxel resistance in prostate cancer. It remains to be tested whether the anti-pThr362 antibody could be used to predict patient outcome in response to chemotherapy. Given that down-regulation of BCRP and Pim-1L expression sensitizes prostate cancer cells to chemotherapeutic drugs, it is conceivable that combination treatment with Pim-1 or BCRP inhibitors would significantly enhance the efficacy of conventional chemotherapy for prostate cancer and prevent the development of drug resistance.

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