INTRODUCTION

Androgen-deprivation therapy is the backbone treatment for advanced prostate cancer and suppresses androgen receptor (AR) signaling in cancer cells by reducing androgen production and inhibiting androgen action.1 Recently, novel AR pathway inhibitors such as abiraterone, apalutamide, and enzalutamide in combination with androgen-deprivation therapy have been proven to prolong survival for patients with metastatic hormone-naïve prostate cancer.2 In addition to apalutamide and enzalutamide, another second-generation...
antiandrogen darolutamide has been shown to improve metastasis-free survival and overall survival in nonmetastatic castration-resistant prostate cancer. Notably, darolutamide was different in molecular structure from apalutamide and enzalutamide, leading to a different pharmaceutical outcome. Although the treatment-mediated manipulations on AR signaling initially show excellent anticancer effects, most tumors eventually recur and become fatal. Several molecular mechanisms underlying the cellular resistance to AR-signaling targeting therapies have been reported, including AR amplification and overexpression, AR mutations, AR co-regulators, AR activation by intracellular signal transduction pathways, and AR variants.

Y-box binding protein-1 (YB-1) functions as a transcription factor in the nucleus as well as an RNA-binding factor in the cytoplasm that is involved in RNA splicing and modulates the expression of its target genes. Ribosomal S6 kinase (RSK) phosphorylates and activates YB-1, and then plays a key role in the pathogenesis of various types of cancer, including prostate cancer. We previously showed that YB-1 was overexpressed in highly malignant prostate cancer and upregulated after androgen ablation; furthermore, YB-1 promoted castration resistance via AR overexpression and AR variant expression. Notably, YB-1 inhibition by gene knockdown and the RSK inhibitor SL0101 augmented cellular sensitivity to castration and enzalutamide. These studies indicated the potential of inhibitors that target RSK/YB-1 signaling to overcome the resistance of prostate cancer to AR-targeting therapies.

RSK contains two nonidentical functional kinase domains: the N-terminal kinase domain (NTKD) and the C-terminal kinase domain (CTKD). The CTKD functions to regulate RSK activation, whereas the NTKD is responsible for substrate phosphorylation. SL0101, which was isolated from *Forsteronia refracta*, is an extremely specific allosteric inhibitor for the NTKD. However, the RSK inhibitor SL0101 shows poor RSK specificity and poor pharmacokinetics, which are critical obstacles for its clinical application. The challenge has been to develop an inhibitor with favorable pharmacological and pharmacokinetic properties required for oral delivery. PMD-026, an oral first-in-class small molecule kinase inhibitor, is the first RSK inhibitor to be tested in a clinical trial for patients with breast cancer. Therefore, in this study, we investigated the effect of PMD-026 on YB-1/AR signaling and its potential antitumor activities in prostate cancer in vitro and in vivo.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human prostate cancer 22Rv1 (RPMI1640), PC-3 (MEM), DU145 (MEM), and LNCaP (RPMI1640) cells were obtained from the American Type Culture Collection (ATCC) and cultured in media (Life Technologies) with 10% fetal bovine serum. LNCaP cells propagated about 10–40 times were used. Cells were maintained in a 5% CO₂ atmosphere at 37 °C.

2.2 | Reagents

Enzalutamide and darolutamide were purchased from Selleck Chemicals. The RSK inhibitor PMD-026 was kindly provided by Phoenix Molecular Designs.

2.3 | Quantitative real-time PCR

Quantitative reverse transcription PCR was performed as described previously. Total RNA was prepared from cultured cells using the RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized from 1.0 μg of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) in accordance with the manufacturer’s protocol. Quantitative reverse transcription PCR was performed using TaqMan Gene Expression Assays for YB-1 (Hs00898625_g1), full-length AR (Hs00171172_m1), AR V7 (order-made; probe: FAM-TCTGGAGAGAAAATT-MGBNFQ, forward primer: 5’-TGTGCTTCGGAAATGTTATGA-3’, reverse primer: 5’-TCATTTGAGATGCTTGCAATTG-3’), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs02758991_g1; Life Technologies) and TaqMan Gene Expression Master Mix (Life Technologies) on a CFX Connect Real-Time System (Bio-Rad). The transcript levels of YB-1 and full-length AR, and AR V7 were normalized using GAPDH transcript levels. All values represent the results of three independent experiments.

2.4 | Western blot analysis

Western blot analysis was performed as described previously. Protein concentrations were quantified using a Protein Assay Kit (Bio-Rad). Whole-cell extracts (30 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride microporous membranes (GE Healthcare Bio-Science) using a semi-dry blotter. The blotted membranes were sequentially incubated with the appropriate primary antibodies for 1 h and peroxidase-conjugated secondary antibodies for 40 min at room temperature. Antibody against phosphorylated YB-1 (Ser102) (p-YB-1; #2900) was purchased from Cell Signaling Technology. Anti-YB-1 (2397-1), anti-RSK1 (2004-1), and phosphorylated p90RSK1 (Ser380) (p-RSK1; 1468-1) antibodies were purchased from Epitomics. Antibodies against AR (N-20, sc-816) and β-actin (A3854) were obtained from Santa Cruz Biotechnology and Sigma, respectively. The bound antibodies were visualized using an ECL kit (GE Healthcare Bio-Sciences Corporation), and images were obtained using an image analyzer (Ez-Capture MG, ATTO).

2.5 | Cytotoxicity analysis

Cytotoxicity analysis was performed as described previously. Briefly, 22Rv1 cells (2.5 × 10⁴) were seeded in 96-well plates. The following day, cells were exposed to various concentrations of enzalutamide/darolutamide with or without PMD-026 for various times. The surviving cells
were stained using the alamarBlue assay (TREK Diagnostic System) at 37 °C for 90 min. The absorbance of each well was measured using the ARVO™ MX plate reader (Perkin Elmer, Inc.).

2.6 | Apoptosis assay

Freshly prepared tumors were embedded in Tissue-Tec OCT compound (Sakura Finetek) and frozen at -80 °C. Cryosections (10 mm) were fixed with 4% (w/v) paraformaldehyde for 10 min at 37 °C. After blocking with 10% horse serum (Sigma), samples were stained with anti-cleaved caspase-3 (#9661, Cell Signaling) and anti-cleaved poly(ADP-ribose) polymerase (PARP) (#6544, Cell Signaling) antibody, followed by Alexa 488-conjugated goat anti-rabbit antibody (Invitrogen). Images were obtained with a laser-scanning confocal microscope (LSM700 META, Carl Zeiss).

2.7 | Cell cycle assay

Treated 22Rv1 cells were washed in PBS, resuspended in PBS, and stained with Cell Cycle Assay Solution Deep Red (Dojindo Molecular Technologies, Inc.) at 37 °C for 15 min. The cell cycle profiles were obtained using a FACSVerse at 640 nm and the data were analyzed using FlowJo software (Becton Dickinson).

2.8 | Mouse castration model

Male athymic nude mice (6 to 8 weeks old) were obtained from Japan SLIC Inc. We inoculated 22Rv1 cells (3 × 10^6) with 0.1 ml of Matrigel (BD Biosciences) in the flanks of mice. The following day, mice were randomly divided into four groups for treatment with enzalutamide and/or PMD-026: enzalutamide treatment group, PMD-026 treatment group, enzalutamide + PMD-026 group, and untreated controls. Each experimental group consisted of five mice. Enzalutamide was dissolved and given at 10 mg/kg/100 ml using an oral zonde needle once a day. PMD-026 was dissolved and given at 100 mg/kg/100 ml using an oral zoned needle twice a day at 4 weeks after cell inoculation. Tumor growth was monitored and tumor measurements were performed every 7 days using calipers. Tumor volume was calculated using the formula (V = A × B^2/2), where A and B represent the longest and shortest dimensions, respectively. When the tumor volume reached over 100 mm^3, castration was conducted.

![Figure 1](https://via.placeholder.com/150)
under anesthesia. Animal care was in accordance with institutional guidelines, following approval from the institutional Animal Ethical Board.

The coefficient of drug interaction was defined as the ratio between the percentage of tumor weight for a drug combination and the product of the percentage of tumor weight for the individual drugs. Coefficient values of <1 were considered to be synergistic, those of >1 antagonistic, and those of ~1 additive. 21

3 | RESULTS

3.1 | PMD-026 suppressed YB-1/AR signaling in 22Rv1 cells

First, we examined the effect of the novel RSK inhibitor PMD-026 on YB-1/AR signaling in 22Rv1 cells, which express AR variants even in a condition of normal AR activity. In 22Rv1 cells treated with 5 μM of PMD-026, AR V7 mRNA was decreased at 24 and 48 h, but no changes were observed in full length AR mRNA expression (Figure 1A). In addition, 5 μM of PMD-026 completely inhibited phosphorylation of YB-1 and decreased expression of the AR V7 variant, but not full-length AR, at 24 and 48 h (Figure 1B). We also examined the effect of various concentrations of PMD-026 on YB-1/AR signaling in 22Rv1 cells. PMD-026 decreased YB-1 phosphorylation as well as AR V7 mRNA and AR variant expressions at 2–5 μM in a dose-dependent manner although 5 μM of PMD-026 failed to show statistical significance on AR V7 mRNA expression (Figure 1C and D).

3.2 | PMD-026 in combination with second-generation anti-androgens suppressed cell proliferation and induced apoptosis in 22Rv1 cells

We next examined the effect of PMD-026 in 22Rv1 cell viability. As shown in Figure 2A, PMD-026 inhibited 22Rv1 cell viability in a dose-dependent manner. Meanwhile, the cytotoxicity of PMD-026 in AR-negative PC-3 and DU145 cells as well as LNCaP cells expressing full-length AR only was less prominent compared to 22Rv1 cells (Figure S1). In addition, the combination of PMD-026 with enzalutamide (Figure 2B) and darolutamide (Figure 2C) reduced the cell viability of 22Rv1 cells to a greater extent than any of the single treatments alone.

We next explored the mechanism of the antiproliferative effect induced by PMD-026 with anti-androgens. As shown in Figure 3A,B, PMD-026 treatment in combination with enzalutamide and darolutamide induced cleavage of PARP to a greater extent than the single treatments, indicating induction of cellular apoptosis. Cell cycle analysis also showed increased sub-G1 and G2/M fractions in cells...
FIGURE 3  Anticancer mechanism of PMD-026 in combination with anti-androgens in 22Rv1 cells. (A) and (B) 22Rv1 cells were treated with or without 5 μM of PMD-026 and 2 μM of enzalutamide (A) or darolutamide (B) for 72 h. Whole-cell extracts were subjected to western blot analyses. (C) 22Rv1 cells were treated with or without 5 μM of PMD-026 for 72 h and 2 μM of enzalutamide for 48 h. The cells were analyzed by flow cytometry. The percentage of cells in each cell cycle phase is shown. Boxes, mean; bars, ± standard deviation; P values were calculated by two-sided Student’s t-test. *P < 0.05, **P < 0.01
FIGURE 4    Legend on next page
treated with enzalutamide and PMD-026 to a greater extent than the single treatments, suggesting that the combination treatment with enzalutamide and PMD-026 induced G2/M arrest followed by cellular apoptosis (Figure 3C).

3.3 | Combination treatment with enzalutamide and PMD-026 suppressed 22Rv1 tumor growth in a mouse xenograft model

Finally, we examined the antitumor effect of PMD-026 combined with enzalutamide in a mouse xenograft model using 22Rv1 cells. The results showed that PMD-026 or enzalutamide alone suppressed tumor growth. Furthermore, PMD-026 in combination with enzalutamide exhibited the most antitumor effect in the mouse model, compared with PMD-026 or enzalutamide alone (Figure 4A). Coefficient values at day 21 and day 28 were 0.52 and 0.49, respectively. Those values were considered to be a synergistic effect of PMD-026 and enzalutamide. Mouse body weight remained unchanged during the 4 weeks of treatment in all treatment arms, suggesting no adverse effect of therapeutics, even in combination (Figure 4B).

To investigate the mechanism of the antitumor effect of PMD-026 combined with enzalutamide, we examined apoptosis in tumor sections from the treatment groups. In tumors from mice treated with enzalutamide or PMD-026 alone, cleaved caspase-3 and cleaved PARP were not detected (Figure 4C). However, the combination treatment of enzalutamide and PMD-026 induced prominent cleavage of caspase-3 and PARP.

4 | DISCUSSION

Castration resistance is a major issue in the clinical management of patients with advanced prostate cancer. This study investigated the antitumor effect of PMD-026 in castration-resistant prostate cancer 22Rv1 cells. We confirmed that PMD-026 treatment showed an excellent inhibitory effect on YB-1 phosphorylation in 22Rv1 cells. In breast cancer and melanoma cells, PMD-026 was shown to specifically target RSK and not to affect kinases upstream in the mitogen-activated protein kinase pathway, resulting in prominent inhibition of YB-1 phosphorylation. This finding suggested that PMD-026 inhibits YB-1 activity through RSK/YB-1 signaling across different cancer cell lines.

The excellent antitumor effects of PMD-026 as a single agent were reported in breast cancer and melanoma. The combination use of PMD-026 with standard systemic anticancer treatment was previously examined, and excellent synergistic anticancer effects were reported. PMD-026 in combination with paclitaxel showed anticancer effects in breast cancer in vitro and in vivo models. Jahan et al. reported that PMD-026 synergizes with the anti-estrogen agent fulvestrant in the hormone-receptor positive breast cancer cell line MCF-7 in vitro. In addition, Ramos et al. reported that PMD-026 resensitized resistant melanoma cells to the B-Raf inhibitor vemurafenib. Consistent with an important role of YB-1 in tumor growth and progression, this study showed the antitumor effect by PMD-026 in vitro and in vivo castration-resistant prostate cancer models. Although PMD-026 showed an anticancer effect in various prostate cancer cells, including AR-negative PC-3 and DU145 cells as well as LNCaP cells expressing full-length AR only, anticancer effects on 22Rv1 cells expressing full-length AR and AR variants were most prominent. A higher anticancer effect in 22Rv1 cells by RSK inhibitor PMD-026 may be explained by suppression of AR variants, which is critical molecules for survival and proliferation in 22Rv1 cells.

As a mechanism of anticancer effect of PMD-026, our study demonstrated for the first time that PMD-026 in combination with enzalutamide induced G2/M arrest and cellular apoptosis in prostate cancer cells. Consistently, previous study showed that cellular apoptosis was induced by PMD-026 in breast cancer cells. Thus, our results indicate an excellent antitumor effect of PMD-026 through G2/M arrest and cellular apoptosis in prostate cancer, especially in castration-resistant prostate cancer, in combination with second-generation antiandrogens, including enzalutamide and darolutamide.

Recently, promising clinical data for PMD-026 were reported in 15 metastatic breast cancer patients. PMD-026 showed excellent pharmacokinetics and was generally well tolerated, and tumor regression was reported in some patients. Notably, it was shown that PMD-026 achieves similar serum concentration in phase I/Ib clinical trials using the experimental conditions employed in this study. Thus, successful clinical application of PMD-026 in prostate cancer patients seems to be promising, although biomarker-driven selection of patients would be important.

In conclusion, this study demonstrated the excellent antitumor effects of the novel RSK inhibitor PMD-026 and a combination effect with the anti-androgen enzalutamide in castration-resistant prostate cancer. These findings warrant clinical trials of PMD-026 in prostate cancer patients.
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CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

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