Pleiotropic Impact of DNA-PK in Cancer and Implications for Therapeutic Strategies

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Abstract

Purpose: DNA-dependent protein kinase catalytic subunit (DNA-PK) is a pleiotropic kinase involved in DNA repair and transcriptional regulation. DNA-PK is deregulated in selected cancer types and is strongly associated with poor outcome. The underlying mechanisms by which DNA-PK promotes aggressive tumor phenotypes are not well understood. Here, unbiased molecular investigation in clinically relevant tumor models reveals novel functions of DNA-PK in cancer.

Experimental Design: DNA-PK function was modulated using both genetic and pharmacologic methods in a series of in vitro models, in vivo xenografts, and patient-derived explants (PDE), and the impact on the downstream signaling and cellular cancer phenotypes was discerned. Data obtained were used to develop novel strategies for combinatorial targeting of DNA-PK and hormone signaling pathways.

Results: Key findings reveal that (i) DNA-PK regulates tumor cell proliferation; (ii) pharmacologic targeting of DNA-PK suppresses tumor growth both in vitro, in vivo, and ex vivo; (iii) DNA-PK transcriptionally regulates the known DNA-PK–mediated functions as well as novel cancer-related pathways that promote tumor growth; (iv) dual targeting of DNA-PK/TOR kinase (TORK) transcriptionally upregulates androgen signaling, which can be mitigated using the androgen receptor (AR) antagonist enzalutamide; (v) cotargeting AR and DNA-PK/TORK leads to the expansion of antitumor effects, uncovering the modulation of novel, highly relevant protumorigenic cancer pathways; and (viii) cotargeting DNA-PK/TORK and AR has cooperative growth inhibitory effects in vitro and in vivo.

Conclusions: These findings uncovered novel DNA-PK transcriptional regulatory functions and led to the development of a combinatorial therapeutic strategy for patients with advanced prostate cancer, currently being tested in the clinical setting.

Introduction

Multiple DNA damage repair (DDR) mechanisms have been selected for through evolution to preserve genomic integrity. DNA double-strand breaks (DSB) are the most deleterious and toxic forms of damage that, if left unrepaired, lead to cell-cycle arrest and cell death (1, 2). Two main pathways are employed to repair DSB: homologous recombination (HR), which utilizes a sister chromatid as a template resulting in high-fidelity DSB repair (3, 4); and nonhomologous end-joining (NHEJ), which does not require a sister chromatid template resulting in a more error-prone form of repair that can occur throughout the cell cycle (5, 6). Although both the processes aid in maintaining genomic integrity in normal cells, cancer cells utilize these processes, including upregulation of key DDR proteins, to acquire more aggressive phenotypes, and develop resistance to DNA-damaging agents (7). Therefore, targeting the DNA repair machinery and/or its components that are deregulated in cancer has the potential to be employed as anticancer therapeutic strategies.

Among many DDR proteins deregulated in cancer, DNA-dependent protein kinase catalytic subunit (DNA-PKcs, referred to as DNA-PK herein), a key DNA repair protein involved in NHEJ, is known to play a protumorigenic role in many cancers including...
DNA-dependent protein kinase catalytic subunit (DNA-PK) is a driver of aggressive disease and has been nominated as a therapeutic target in multiple cancer types. Targeting DNA-PK is an attractive therapeutic strategy that can lead to significant anticancer effects. However, further understanding of DNA-PK functions, especially transcriptional regulation, is essential for the development of effective treatments. This study demonstrates that DNA-PK transcriptionally modulates gene networks beyond its known function in DNA repair, hormone signaling, and metastatic pathways. Data herein identified novel DNA-PK–mediated functions including the regulation of epithelial–mesenchymal transition, immune response, and metabolic processes. Moreover, the unbiased transcriptomic data in this study informed the investigation of a combinatorial strategy targeting DNA-PK/TOR kinase (TORK), and androgen receptor (AR), which is currently being evaluated in the clinical setting in castration-resistant prostate cancer (CRPC). The data presented in this study have led to bench-to-bed discoveries that have the potential to affect the management and treatment of CRPC in the clinical setting.

Materials and Methods

Proliferation assay

Cell lines. Cells lines, C4-2, 22Rv1, LNCaP, VCaP, and LN95 were authenticated by ATCC and checked for Mycoplasma upon thawing and at termination of maintenance after <20 passages.

Inhibitors. Cell lines LNCaP, VCaP, C4-2, 22Rv1, and LN95 were plated in 96-well plates at 1,500, 1,500, 500, 1,000, and 1,000 cells/well concentration, respectively. All cell lines were grown in full serum culture media, with the exception of LN95, which are normally cultured in charcoal-stripped serum. Cells were treated the next day with 0–25 μmol/L combinations of NU7441, CC-115, and CC-223 for 6 days and compared with vehicle control, DMso. IC25, IC50, and IC75 (when possible) were determined for CC-115 in each cell line. Combination treatments with enzalutamide and CC-115 + enzalutamide were conducted by treating the cells with CC-115 IC25, IC50, and IC75 dose and titrating the concentration of enzalutamide (0–25 μmol/L). After 6 days, Quant-it PicoGreen dsDNA Assay Kit (Abcam) was used according to protocol and data were recorded using a BioTek Synergy HT plate reader. Results were analyzed and graphed using GraphPad Prism7 to generate dose-response curves for the single-agent treatments. The combination treatment data were analyzed using CompuSyn Software analysis, to determine the anticancer effects. However, further understanding of DNA-PK functions, especially transcriptional regulation, is essential for the development of effective treatments. This study demonstrates that DNA-PK transcriptionally modulates gene networks beyond its known function in DNA repair, hormone signaling, and metastatic pathways. Data herein identified novel DNA-PK–mediated functions including the regulation of epithelial–mesenchymal transition, immune response, and metabolic processes. Moreover, the unbiased transcriptomic data in this study informed the investigation of a combinatorial strategy targeting DNA-PK/TOR kinase (TORK), and androgen receptor (AR), which is currently being evaluated in the clinical setting in castration-resistant prostate cancer (CRPC). The data presented in this study have led to bench-to-bed discoveries that have the potential to affect the management and treatment of CRPC in the clinical setting.
The combination index of CC-115 and enzalutamide using nonconstant ratio parameters.

siRNA. The CRPC cell lines, C4-2 and 22Rv1, cells were seeded at a 1 x 10^3 density on poly-l-lysine–coated plates in culture media for 24 hours. Cells were then transfected for 8 hours in serum-free media conditions with either control or PRKDC siRNA pools (Thermo Scientific Scientific) according to manufacturer's protocol as previously described (12). Cells were then maintained in complete media for 96 hours post transfection and processed for either RNA, protein, or growth assays.

Bromodeoxyuridine incorporation assay

Bromodeoxyuridine (BrdU) labeling and detection were performed as previously described (12, 14). Samples were acquired using a GUAVA easyCyte flow cytometer and analyzed using InCyte software for BrdU incorporation.

Western blotting

Cells were treated as specified and cell lysates were generated as previously described (12). AR (N-20, directed against amino acids 1-20), DNA-PK (Thermo Fisher Scientific, #MS-423-P), vinculin (Sigma-Aldrich, #V9264-200IL), VAV3 (EMD Millipore, #07-464), Prex1 (EMD Millipore, #MABC178), lamin B (Santa Cruz Biotechnology, #6217), pAKT (Cell Signaling Technology, 9271S), AKT (Cell Signaling Technology, 9272S), pS6 (Cell Signaling Technology, 2211S), S6 (Cell Signaling Technology, 2217S), PARP/cleaved PARP (Cell Signaling Technology, 9542S) antibodies were used for immunoblotting.

Gene expression

Cells were treated as specified above. RNA was isolated using TRIzol (Life Technologies) and quantitative PCR was conducted using primers as shown in Supplementary Table S1.

Mouse gene expression analysis was performed by extracting total RNA from tumor tissues using TRIzol Reagent (Sigma). RT-PCR was performed using One-Step RT-PCR Kit, SuperScript One-Step RT-PCR Systems (Life Technologies), following manufacturer’s instructions. Probes for human FRBP5 (Hs00188025) were purchased from Life Technologies.

PDE

PDE experiments were conducted as described previously (12, 27, 28). Tissues samples were treated as specified. IHC staining for Ki67 was performed by Thomas Jefferson Pathology Core Facilities and scored by a board-certified pathologist who reported percent Ki67 positivity after counting all cancer cells in the slide provided. PDEs are Institutional Review Board exempt due to deidentification of specimens. The Thomas Jefferson University Institutional Review Board has reviewed this procurement protocol and determined this research to be in compliance with federal regulations governing research on deidentified specimens and/or clinical data [45 CFR 46.102(f)].

Mouse models

Animals. Male 6- to 8-weeks-old CB17 SCID mice were obtained from Charles River Laboratories. All animal studies were performed under the protocols approved by Institutional Animal Care and Use Committees.

Formulation. Suspensions of CC-115 and enzalutamide were prepared in aqueous 0.5% carboxymethyl cellulose and 0.25% Tween-80. The formulations were homogenized using a Teflon pestle and mortar (Potter–Elvehjem tissue grinder). For multiday studies, the compound was freshly formulated every third day. Between doses, the formulated compound was stored under constant stirring using magnetic stirrer at 4°C in the dark. The treatment’s article and vehicle were administered by oral gavage.

Development of CRPC tumor model. LNCaP tumor cells (6 x 10^6/mouse) were injected subcutaneously into the hind flanks of male CB17 SCID mice. When the tumor volumes reached approximately 200 mm^3, the mice were surgically castrated, and the tumor growth was closely monitored. Relapsed tumors that reached 300 to 500 mm^3 in volume were used as donor tissue for transplanting to the next cohort of castrated mice. For transplantation, tumors from several donor mice were pooled, minced, and mixed with Matrigel (BD Biosciences) before implanting into the hind flank of the recipient mice. Approximately 25 to 50 mg of tumor tissue per mouse was transplanted for each mouse. Several cycles (2-5) of tumor transplantation were performed until enhanced tumorigenicity (80%-90% tumor take rate) was observed.

Efficacy studies with CRPC model. For efficacy studies, tumor fragments from passage 6 were used for inoculations. Twenty days after tumor fragment inoculation, mice bearing HR-LNCaP tumors of 200-400 mm^3 were randomly assigned to receive oral doses of vehicle, CC-115, enzalutamide, or combination of CC-115 and enzalutamide once a day for the duration of the study. Tumor volumes were determined before the initiation of treatment and were considered as the starting volume. Tumors were measured twice a week for the duration of the study. The long and short axes of each tumor were measured using a digital caliper in millimeters. The tumor volumes were calculated using the formula: width^2 x length/2 and expressed in cubic millimeters (mm^3).

In vivo target validation

pS6RP in tumor samples was determined using Mesoscale Discovery Kit [pS6RP: MA6000 p-S6RP (Ser 235/236)], Whole Cell Lysate Kit (MSD, #K110DFD-2), and expressed as the mean Mesoscale Counts ± SEM per manufacturer’s instructions.

pAKT (S473) and the total AKT in tumor samples were measured simultaneously using the Mesoscale Discovery Kit [pAKT (S473)]/Total AKT: MS6000 Phospho(S473)/Total AKT Whole Cell Lysate (MSD, #K1110D2-0) multiplex assay. The amount of phosphorylated AKT was calculated per manufacturer’s instructions and reported as a percentage of total AKT. The results were expressed as the mean percentage phosphorylated AKT ± SEM for each group.

pDNA-PK (S2056) and total DNA-PK in tumor samples were measured simultaneously using IHC. Five- to 10-µm thick cryostat sections were used. Shortly, frozen sections were fixed in 4% paraformaldehyde for 10 minutes at room temperature, washed in PBS, blocked, and permeabilized with 5% normal goat serum and 0.3% Triton X-100. Sections were then incubated with a cocktail of primary antibodies (1 µg/mL of anti-mouse anti-human DNA-PKcs mAb; Thermo Fisher Scientific, #MS-369-P0) and anti–pDNA-PK [rabbit anti-human pDNA-PK (S2056) polyclonal antibody (Abcam, ab18192)] for 2 hours followed by incubation with a cocktail of secondary antibodies (60 minutes). The sections were washed, counterstained with Hoechst dye (0.4 µg/mL), and mounted with antifade reagent.
were visualized with a Nikon E800 microscope and data were quantitated using Metamorph software. Using a 20× objective, five different fields from each section and four tumors from each treatment or control group were used for quantitation. The data are expressed as the percentage threshold area of pDNA-PK staining over the threshold area of DNA-PKs (total DNA-PK staining). The data from each individual animal was used to calculate the mean ± SEM for each group.

**Statistical analysis**

*In vitro* data are presented as mean ± SD, xenograft and *in vivo* biomarker data are presented as mean ± SEM. Statistical analyses, including *P* values, performed using GraphPad Prism7.

**RNA sequencing**

**RNA sequencing.** C4-2 cells were plated in hormone-proficient conditions at 50,000 cells per plate overnight, followed by 1-μmol/L single-agent drug treatments (NU7441, CC-115, CC-223, and enzalutamide) in biological triplicate. 22Rv1 cells were plated in hormone-proficient conditions at 100,000 cells per plate overnight, followed by 1-μmol/L treatment with CC-115 and vehicle control for 24 hours, with experiments collected in triplicate. RNA was extracted and purified using TRIzol and RNAeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA sequencing (RNA-seq) libraries were subsequently constructed using the TruSeq Stranded Total RNA Library Prep Gold Kit (protocol # 15031048 Rev E) and sequenced on Illumina’s NextSeq 500 sequencer at the Sidney Kimmel Cancer Sequencing core facility using paired-end 75 bp reads. For combination treatments all drugs were used at 1 μmol/L concentration.

**RNA-seq analyses.** RNA-seq was aligned against the hg19 human genome using STAR v2.5.2a (29). Differential gene expression was generated using DESeq2 v1.12.4 (30). Gene set enrichment analysis (GSEA) was performed using gene sets from the Molecular Signature Database (MSigDB) to identify the pathways modulated by DNA-PK (Fig. 1C, right); significantly over-represented pathways (FDR < 0.25) are shown using a circos plot where significantly enriched and pronounced pathways are represented by blue and green ribbons, respectively (FDR < 0.25). Consistent with previous reports, GSEA revealed the deenrichment in pathways known to be modulated by DNA-PK, including androgen response, DNA repair, cell cycle/proliferation, and protumorigenic processes, thus recapitulating the highly selective nature of DNA-PK’s transcriptional regulatory functions (8, 14). In addition, this study of DNA-PK inhibition revealed novel pathways affected by DNA-PK including TGF signaling, KRAS signaling, TNFα signaling via NF-κB, oxidative phosphorylation, and unfolded protein response (Fig. 1C, right). These clinically relevant pathways are known to play important roles in tumor progression, thus highlighting the importance of delineating the roles of DNA-PK with respect to transcriptional regulation in cancer to reveal novel mechanisms of action. Together, these data demonstrate that the pro-proliferative functions of DNA-PK in cancer cells are associated with distinct protumorigenic transcriptional regulatory events.

**Targets DNA-PK using a therapeutically active compound inhibits tumor cell proliferation and regulates known DNA-PK transcriptional processes**

Although NU7441 is a highly specific DNA-PK inhibitor, this agent is not suited for clinical use. However, the dual DNA-PK and TORK inhibitor (CC-115; Supplementary Fig. S1C) has been recently developed and is in multiple clinical trials. Assessment of the dual kinase targeting in hormone-sensitive prostate cancer (HSPC) and CRPC models revealed that targeting DNA-PK using both CC-115 and NU7441 reduced cell viability in a dose-dependent manner, with CC-115 inducing apoptosis (Fig. 2A, Supplementary Fig. S1D). Similarly, FACS analysis demonstrated that targeting of DNA-PK with CC-115 significantly reduced cells in active S-phase compared with vehicle control and NU7441-treated cells (Supplementary Fig. S1B and S1E). These data suggest that targeting DNA-PK using CC-115 more potently inhibits tumor cell proliferation compared with NU7441 in both HSPC and CRPC models. Considering the important role of DNA-PK in transcriptional regulation, it was imperative to identify the transcriptomic alterations caused by this therapeutically active agent.

To investigate the impact of targeting DNA-PK using the dual kinase inhibitor on the transcriptome, RNA-seq analysis was performed in CRPC cells treated with CC-115. Principal component analysis (PCA) and sample clustering provided a high level of confidence in the effects observed for each treatment as...
demonstrated by tight sample clustering between their respective treatments (Supplementary Fig. S1C). As CC-115 is a dual kinase inhibitor targeting both DNA-PK and TORK, enhanced effects on the transcriptome were anticipated as compared with DNA-PK–exclusive targeting agents. Indeed, CC-115 treatment resulted in the alteration of transcriptional networks, with 4,896 upregulated and 5,878 downregulated transcripts in C4-2, and 3,555 upregulated and 4,358 downregulated transcripts in 22Rv1 (Fig. 2B; Supplementary Fig. S2A; \( P_{\text{adj}} < 0.05 \)). Through the analyses of both transcript profiles and GSEA, pathways modulated by DNA-PK/TORK targeting via CC-115 were highly conserved in C4-2 and 22Rv1, with 3149 and 2187 transcript changes in common but down- and upregulated by CC-115, respectively. Furthermore, consistently modulated targets of CC-115 in both models systems were validated in multiple CRPC models (Supplementary Fig. S2C).
Moreover, the results of two GSEAs in C4-2 (CC-115 and NU7441 treatments) were overlaid to uncover high-confidence DNA-PK-exclusive pathways (Fig. 2C, left). The combined transcriptionally altered pathways are illustrated using a circos plot where significantly enriched and deenriched pathways exclusive to NU7441 (light and dark blue ribbons, respectively), versus pathways exclusive to CC-115 (orange and red ribbons respectively; FDR < 0.25) are shown. As expected, the dual kinase inhibitor CC-115 altered the TORK-regulated pathways of cancer relevance, including fatty acid metabolism, glycosylation, and apoptosis pathways (34, 35). By contrast, a robust core set of pathways commonly regulated by both the single DNA-PK inhibitor (NU7441) and the dual DNA-PK/TORK inhibitor (CC-115) were identified (purple and green ribbons). The number of commonly deenriched pathways (purple) exceeded the number of commonly enriched pathways (green) by 14 and two respectively, suggesting that DNA-PK primarily upregulates the transcriptional processes that play important roles in tumor progression. Among these processes were known DNA-PK effectors, such as DNA-repair and cell cycle/proliferation, but also novel pathways where the role of DNA-PK transcriptional regulation is less understood, including oxidative phosphorylation, epithelial–mesenchymal transition (EMT), TNFα signaling via NF-kB, IL6/Jak/Stat3 signaling, and TGFβ signaling (Fig. 2C, left). In summary, these data show that targeting DNA-PK utilizing a clinical grade inhibitor, CC-115, potently inhibits the proliferation of CRPC cells and impacts DNA-PK-regulated transcriptional events enriched for cancer-relevant pathways.

Cooperative effects of cotargeting AR and DNA-PK pathways

Among the common regulated pathways, GSEA identified androgen signaling, a known driver of prostate cancer at all stages of disease (36). As DNA-PK is a known modulator of AR signaling (14), it was not surprising that treatment with a DNA-PK-specific inhibitor (NU7441) resulted in deenrichment of androgen signaling (Fig. 3A, left; FDR < 0.25). In contrast, androgen signaling was enriched when targeting DNA-PK/TORK axis using CC-115 (Fig. 3A, middle; FDR < 0.25). Although androgen-signaling downregulation was expected upon DNA-PK suppression, it has been previously described that AR signaling upregulation can occur upon TORK inhibition in prostate cancer (37, 38). Upregulation of androgen signaling was associated with elevated AR protein levels in C4-2 (Fig. 3A, right; Supplementary Fig. S2D) and was confirmed via RT-PCR demonstrating the induction of AR target gene expression upon CC-115 treatment (Fig. 3B, Supplementary Fig. S2E).

Similar upregulation of AR target gene transcripts was also seen using a specific TORK inhibitor (CC-223, also in clinical trials NCT02031419, NCT01177397, and NCT01545947), which further indicates that the inhibition of TORK is likely causing AR upregulation (Supplementary Fig. S3A; ref. 39). In summary, these data suggest that TORK suppression leads to upregulation of AR signaling in CRPC.

Because AR targeting agents are used as first-line therapy for metastatic disease (40, 41), an FDA-approved AR antagonist, enzalutamide, was used in combination with NU7441 and CC-115. As expected, the combination with enzalutamide further downregulated the AR target gene transcript levels when used with NU7441 due to a negative feedback loop between DNA-PK and AR, which has been described previously (14). The combination of enzalutamide with CC-115 mitigated the upregulation of AR target genes induced by the inhibition of TORK in CRPC models (Fig. 3B, Supplementary Figs. S2E and S3A). In summary, the combination treatments of DNA-PK inhibition with enzalutamide reduce the upregulation of AR signaling in CRPC.

To further understand the impact of DNA-PK suppression in combination with AR targeting on the transcriptome, RNA-seq was performed on C4-2 (Supplementary Fig. S4A) and validated in two other models for specific targets (Supplementary Fig. S4B). Enzalutamide alone resulted in 638 upregulated and 791 downregulated transcripts. As expected, a larger number of transcripts were altered in enzalutamide + NU7441 (2,284 upregulated, 3,644 downregulated) and enzalutamide + CC-115 (3,632 upregulated, 334 downregulated) than enzalutamide alone (Fig. 3C, top; \( \frac{FDR}{0.05} < 0.05 \)). Up- and downregulated genes by 1.5-fold change for single-agent exposure (enzalutamide alone, NU7441 alone, and CC-115 alone) were compared with their respective combination exposure (enzalutamide + NU7441 and enzalutamide + CC-115) as represented by Venn diagrams (Fig. 3C, middle). An expansion of transcriptomic alterations was observed for both NU7441 and CC-115 in combination with enzalutamide. GSEA pathway analysis was performed to identify up- and downregulated pathways, represented as heatmaps, based on the gained genes exclusive to each combination treatment (Fig. 3C, bottom; FDR < 0.25). Transcriptional downregulated events associated with NU7441 + enzalutamide combination were enriched for 18 pathways, whereas upregulated gene expression events were enriched for only two pathways. The downregulated gene set exhibited enrichment of known DNA-PK and AR-modulated processes such as androgen response, cell cycle, and hypoxia, as expected. Similarly, CC-115 + enzalutamide down- and upregulated gene sets were enriched for known AR, TORK, and DNA-PK known processes. However, novel pathways including Wnt β-catenin signaling, Hedgehog signaling, inflammatory response, and immune response signaling were gained upon both combinatorial treatments, which were not previously seen in the single-agent targeting approach with either kinase inhibitor or enzalutamide (Supplementary Fig. S4B). In summary, targeting DNA-PK and AR signaling in concert leads to an expanded transcriptomic profile that modulates novel protumorigenic signaling pathways when compared with each treatment alone.

To uncover DNA-PK-specific effects in the context of AR inhibition with enzalutamide, RNA-seq was performed using the TORK-specific inhibitor, CC-223, in combination with enzalutamide to mitigate the effects of targeting AR and TORK, thus uncovering putative DNA-PK-specific effects (Supplementary Fig. S3B–SD). Utilizing the genes modulated by 1.5-fold change for CC-115 + enzalutamide and CC-223 + enzalutamide combinations, GSEA analysis was performed (Supplementary Fig. S3D). Although there was significant overlap between the two conditions signifying genes/pathways modulated by TORK and AR inhibition, CC-115 + enzalutamide and CC-223 + enzalutamide had distinct effects, which can be attributed to DNA-PK and TORK, respectively. Processes that can be attributed to DNA-PK (CC-115 exclusive) consisted of pathways involved in metabolism, inflammatory response, and protumorigenic signaling similar to the DNA-PK pathways previously uncovered in Fig. 3C. In summary, the upregulation of AR signaling upon DNA-PK/TORK dual targeting with CC-115 can be mitigated using enzalutamide and this combination strategy led to an expansion of transcriptomic alterations that are distinct from...
Combining DNA-PK/TORK/AR Targeting in Prostate Cancer

Figure 2.
Targeting DNA-PK with CC-115 potently inhibits tumor cell proliferation and regulates known DNA-PK transcriptional processes. A, Dose–response proliferation assays using NU7441 (DNA-PK inhibitor) and CC-115 (dual DNA-PK/TORK inhibitor) in HSPC and CRPC cell lines using Pico Green assay at 6 days following treatment compared with vehicle control. B, RNA-seq schematic of C4-2 cell line treated with 1 μmol/L of CC-115 in triplicate for 24 hours before RNA was harvested. MA plots were generated for CC-115 treatment compared with control showing gene expression modulation with the number of transcripts upregulated (top) and downregulated (bottom). C, GSEA using Hallmark gene set from MSigDB analysis was used to identify enriched and deenriched pathways for NU7441- and CC-115–treated cells compared with control using FDR < 0.25. Each pathway is depicted by a ribbon in the circos plot (blue, pathways deenriched exclusively by NU7441; dark blue, exclusive enrichment by NU7441; red, enrichment by CC-115; purple, commonly deenriched; and green, commonly enriched). Commonly enriched and deenriched pathways regulated by DNA-PK are represented using heatmaps to the right.
Deenriched (DNA-PK NU7441)  
FDR q-Value = 0.244  
NES = -1.19

Enriched (DNA-PK/TORKi CC-115)  
FDR q-Value = 0.196  
NES = 1.39

A

B

C

GSEA (Hallmark Geneset)
those observed with single-agent targeting. Furthermore, novel putative DNA-PK–exclusive transcriptional regulatory events were identified, further implicating DNA-PK as a transcriptional regulator of cancer-relevant pathways.

Feasibility of cotargeting DNA-PK/TORK and AR axis in vivo

To investigate whether the combination of DNA-PK–targeting agents with enzalutamide demonstrate superior efficacy in comparison with CC-115 and enzalutamide alone, combination index analyses were performed after treatments with CC-115 at IC_{50}, IC_{50}, or IC_{50} concentrations combined with varied doses of enzalutamide in both HSPC and CRPC models. Combination of CC-115 (IC_{50}, IC_{50}, or IC_{50}) when combined with enzalutamide (all doses) showed synergism in both HSPC and CRPC models (Fig. 4A). To extend these observations into a preclinical model of CRPC, the combination of CC-115 + enzalutamide was tested in a xenograft model generated as shown in Fig. 4B on the left. Following tumor engraftment, mice were treated with vehicle, CC-115 alone (2 mg/kg), enzalutamide alone (low and high dose, 5 mg/kg and 10 mg/kg, respectively), and the combination of CC-115 + enzalutamide at low and high enzalutamide doses (2 mg/kg CC-115 + 5 mg/kg enzalutamide and 2 mg/kg CC-115 + 10 mg/kg enzalutamide). In addition, mRNA expression of the AR target gene, FKBP5, was interrogated to validate the efficacy of targeting the AR axis in this model. As expected, FKBP5 mRNA, as well as TORK and DNA-PK activity (Supplementary Fig. S5A and S5B), were elevated upon treatment with CC-115 alone, however, the combination of CC-115 + enzalutamide (low and high dose), thus corroborating the in vitro finding as shown in Fig. 3B (Fig. 4B, right). Moreover, CC-115 and CC-115 + enzalutamide were shown to reduce DNA-PK activity and TORK target expression in an in vivo HSPC model study treated with CC-115 alone, enzalutamide, and CC-115 + enzalutamide (unpublished data). In sum, these data recapitulate androgen signaling upregulation upon DNA-PK/TORK inhibition observed in vitro and mitigation of this effect with the enzalutamide + CC-115 combination in vivo.

Tumor doubling time was increased upon treatment with enzalutamide alone and CC-115 + enzalutamide (low and high dose) led to significantly increased tumor doubling time compared with CC-115 alone, demonstrating that the combination treatment is more effective than single-agent treatment in vivo (Fig. 4C). Similarly, combination treatments led to higher survival (using a tumor volume of 1,500 mm^3 as an endpoint) compared with either single treatment alone, with CC-115 + enzalutamide low and high dose having a 75% and 87.5% survival rate, respectively (Fig. 4D). Tumor volume was monitored throughout the duration of the study (39 days total, 19 days post initial treatment). At the end of the study, CC-115 alone showed marginal activity leading to approximately 22% decrease in tumor volume, enzalutamide alone showed dose-dependent activity (35% and 48% tumor volume decrease for 5 mg/kg and 10 mg/kg, respectively), and the combination treatments of CC-115 + low- and high-dose enzalutamide showed additive effects with a 61% and 62% decrease in tumor volume with the combination at high enzalutamide dose demonstrated a 29% decrease of tumor volume as compared with enzalutamide (10 mg/kg) and 64% decrease when compared with vehicle (Fig. 4E). Moreover, similar studies performed in another CRPC model, LNCaP-AR, recapitulate the results presented herein where the combination treatment leads to greater tumor growth inhibition compared with enzalutamide or CC-115 alone (unpublished data). In summary, these data demonstrate that targeting the DNA-PK/TORK and AR with CC-115 in combination with enzalutamide results in decreased tumor proliferation both in vitro and in vivo when compared with control or either agent treatment alone.

Dual DNA-PK/TORK inhibition and cotargeting AR elicits cooperative antitumor effects in human prostate cancer explants

Because targeting of DNA-PK-TOR-AR axis showed synergistic and additive antiproliferative effects in vitro and in vivo respectively, the impact of this combination treatment was studied in a PDE model using human tumor samples from high-volume disease (Supplementary Table S2) that were obtained after radical prostatectomy as described previously (28) and summarized in Fig. 5A. Importantly, these tissues retain histarchitecture, AR expression, proliferation rate, and the microenvironment features of the original tumor (27, 28, 42). Upon resection, the tumor samples were subdivided and treated with vehicle control, CC-115 (0.1, 0.5, and 1 μmol/L) alone, enzalutamide (1 μmol/L) alone, and combination of CC-115 (0.1, 0.5, and 1 μmol/L) + enzalutamide (1 μmol/L). The levels of Ki67, an indicator of tumor cell proliferation, were measured using IHC. Seventy-five percent of PDEs (6/8) responded to enzalutamide treatment (lower Ki67 positivity) as compared with control. CC-115 at high dose (1 μmol/L) showed strong antiproliferative effects when used as a single agent. The antiproliferative effects were enhanced upon combining CC-115 with enzalutamide especially with 0.1 and 0.5 μmol/L CC-115 (PDEs #1, 3, 7, 8). When the concentration of CC-115 was increased to 1 μmol/L, robust antiproliferative effects were observed but no further benefit was seen with the addition of enzalutamide (1 μmol/L) (PDEs #2, 4, 5, 6; Fig. 5C). These data demonstrate that targeting DNA-PK/TORK alone and...
Dylgjeri et al.

Figure 4.
Cotargeting DNA-PK/ TORK and AR axis has additive growth inhibitory effects in vivo. A, Combination index determination in prostate cancer cell lines when using CC-115 at IC25, IC50, and IC75 in combination with varied concentrations of enzalutamide. Experiments were performed in biological triplicates. B, Schematic of CRPC mouse model developed by Celgene. Upon establishment of CRPC tumors, the AR axis was interrogated by measuring FKBP5 mRNA expression levels in tumors after treatments via qPCR (right). Data represented as mean ± SEM of biological triplicates. Student t test statistical analyses were used where * P < 0.05 compared with control (red) or as otherwise indicated by the brackets. C, Average tumor doubling time was calculated for each treatment cohort. D, The percentage of tumors reaching 1,500 mm³ was calculated for each cohort. E, Tumor growth was monitored for 19 days after single-agent or combination drug treatment. Relative tumor volume is shown for each treatment normalized to tumor volume at the start of treatment. Mouse data are presented as mean ± SEM, and one-way ANOVA was used for statistical analysis where * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001 compared with control. Enza, enzalutamide.
in combination with enzalutamide has antiproliferative effects in primary prostate human tumors, and provide the impetus for clinical evaluation of DNA-PK-targeting agents in combination with enzalutamide.

Based, in part, on the findings presented here, a first-in-man phase IB/II clinical trial (NC102833883) is being conducted in prostate cancer, with endpoints assessing the safety and pharmacokinetics of escalating doses of CC-115 in combination with enzalutamide (160 mg twice a day) to establish the MTD. These data will be used in a phase II trial to assess endpoints of safety, biochemical recurrence by looking at PSA levels, and progression-free survival (Fig. 5D). Combined the studies presented herein have further defined the transcriptional-regulatory roles of DNA-PK, and have identified the processes that are affected by combinatorial targeting strategies of DNA-PKi/TORi and enzalutamide (Fig. 5E). Furthermore, the combination of DNA-PK/TOR and enzalutamide has demonstrated to have superior anticancer effects to single-agent therapy using a clinical grade inhibitor, in concert with a standard-of-care AR antagonist, has cooperative antitumor effects in multiple human malignancies. In aggressive prostate cancer, with endogenous DNA damage. This study provides strong rationale for using DNA-PK/TOR-targeting agents and enzalutamide in the management of CRPC.

**Discussion**

DNA-PK is a multifunctional kinase that is deregulated in multiple human malignancies. In aggressive prostate cancer, DNA-PK is strongly associated with poor outcome (12). Despite being studied for its role in DNA repair and transcriptional regulation, much remains to be uncovered about the mechanisms by which DNA-PK promotes cancer phenotypes. The study presented here identifies DNA-PK as a transcriptional regulator of multiple known and novel cancer-relevant pathways in the absence of exogenous DNA damage in prostate cancer. Furthermore, data herein demonstrate that DNA-PK targeting using a clinical grade inhibitor, in concert with a standard-of-care AR antagonist, has cooperative antitumor effects in prostate cancer. Key findings reveal that: (i) DNA-PK regulates tumor cell proliferation; (ii) pharmacologic targeting of DNA-PK suppresses tumor growth both in vitro, in vivo, and ex vivo; (iii) DNA-PK transcriptionally regulates known DNA-PK-mediated functions as well as novel cancer-related pathways that promote tumor growth; (iv) dual targeting of DNA-PK/TORK transcriptionally upregulates androgen signaling, which can be mitigated using the AR antagonist, enzalutamide; (v) cotargeting AR and DNA-PK/TORK leads to the expansion of antitumor effects, uncovering modulation of novel, highly relevant protumorigenic cancer pathways; and (vi) cotargeting DNA-PK/TORK and AR has cooperative growth-inhibitory effects in vitro and in vivo. In sum, this study uncovered multiple novel cancer-relevant processes that are transcriptionally modulated by DNA-PK in models of advanced disease, and demonstrated that DNA-PK/TORK can be effectively targeted in combination with enzalutamide to prevent tumor growth in prostate cancer in the absence of exogenous DNA damage. This study provides strong rationale for using this three-pronged attack by targeting DNA-PK, TORK, and AR axes in the management of lethal CRPC.

DNA-PK has been previously shown to modulate cancer phenotypes through DNA repair via NHEJ and transcriptional regulatory mechanisms (8, 12, 14, 43–45). Consistent with these previous findings, unbiased profiling of transcriptional networks sensitive to DNA-PK inhibition further supports the concept that DNA-PK positively regulates gene networks involved in DNA repair and hormone signaling as observed in Figs. 1C and 2C. Previously published data have also linked DNA-PK to play a prometastatic role in cancer (12, 21). DNA-PK promotes metastasis, in part, through transcriptional modulation of prometastatic gene networks in the Rac-Rho pathway in prostate cancer (12). Another study in melanoma has shown that DNA-PK modifies the tumor microenvironment by modulating the secretion of promigratory molecules and promotes metastasis (21). Data herein identified EMT and TNF via NF-κB pathways as also responsive to DNA-PK function. These findings are impactful as EMT is an important step toward development of metastases, and TNF plays a critical role in tumor microenvironment and EMT plasticity. Previous studies have described DNA-PK and hormone signaling as promotes the protein Snail and drive metastasis (33); however, it is of high importance to understand how DNA-PK modulates EMT/TNF gene networks transcriptionally. Understanding the mechanisms by which DNA-PK transcriptionally modulates prometastatic signaling would allow for the development of new therapeutics to target these processes with the goal of suppressing metastasis.

In addition to regulation of metastatic pathways, the data presented here uncovered a novel DNA-PK mediated influence on immune and inflammation response pathways including: IFNα response, IFNβ response, inflammatory response, and TNF pathway. These data complement previous findings that identified DNA-PK as a modulator of V(DJ) recombination, activator of the innate immune response, and subsequent inflammatory response in the presence of foreign DNA and pathogens (46–49). Uncovering the crucial impact of DNA-PK on both the innate and adaptive immune response is the focus of ongoing investigations. Moreover, understanding how the innate and immune responses are modulated in patients treated with DNA-PK-directed therapeutics will likely give insight into DNA-PK-dependent immune-related mechanisms of response and/or resistance. A recent study identified DNA-PK inhibition as a therapeutic approach that modulates immunity in melanoma and may increase the efficacy of immunotherapies (50), thus it would be of interest to study immunotherapy and DNA-PK inhibitors in prostate cancer tumors that overexpress DNA-PK and assess their efficacy as novel therapeutic strategies. In addition to transcriptional regulation of immune response pathways, data herein suggest that DNA-PK modulates cancer cell metabolism including fatty acid metabolism, cholesterol homeostasis, and oxidative phosphorylation (Figs. 1C and 2C; Supplementary Fig. S3D). Although these findings support previously published data describing the role of DNA-PK in lipogenesis and mitochondrial biogenesis and function (51–53), this study is the first to describe DNA-PK directly affecting the transcriptional regulation of metabolic gene networks rather than modulating metabolic processes through phosphorylation of cofactors or protein–protein interactions. These findings support the rationale to assess the critical impact of DNA-PK in tumor-associated metabolic and immunomodulating processes.

On the basis of the protumorigenic role of DNA-PK in cancer and the data linking it to metastatic potential, DNA-PK has been nominated as a therapeutic target. Recently developed DNA-PK inhibitors have entered the clinical trial space in combination...
Figure 5.
Dual DNA-PK/TORK inhibition and cotargeting AR elicits cooperative antitumor effects in human prostate cancer. 

A, Schematic of human PDE model generation from human prostatectomy samples that are treated with single-agent CC-115 (0.1, 0.5, and 1 µmol/L DNA-PK/TOR inhibitor) and enzalutamide (1 µmol/L) and combination treatments (0.1, 0.5, and 1 µmol/L CC-115 + 1 µmol/L enzalutamide) for 6 days. 

B, Representative Ki67 IHC tissue staining after each treatment is shown at 40x magnification. Enza, enzalutamide. 

C, Each PDE sample (each patient is represented by a different color) was scored by Digital Imaging Analysis (Aperio System) and confirmed by a pathologist. Data is represented as Ki67 mean ± SD of each cohort of patient tissues treated with the same treatment. 

D, Schematic of the CC-115 and enzalutamide clinical trial currently conducted in patients with CRPC. BID, twice a day; Enza, enzalutamide; QD, every day; RP2D, recommended phase II dose. 

E, Model summarizing findings in this article.
Combined DNA-PK/TORK/AR Targeting in Prostate Cancer

with irradiation in multiple liquid and solid tumors. In the study presented here, two DNA-PK inhibitors were used: a specific laboratory-grade DNA-PK inhibitor (NU7441) and a clinical-grade dual DNA-PK/TORK inhibitor (CC-115). The advantage of using the dual DNA-PK inhibitor is multi-fold: (i) CC-115 inhibits not only DNA-PK but also TORK, which is commonly deregulated in cancer and is involved in cancer metabolism, tumor microenvironment, proliferation, and metastasis; (ii) TORK targeting is already under investigation in multiple cancers including prostate, and already FDA approved in renal cell carcinoma (54, 55), however, patients with CRPC have had negligible results, suggesting that targeting DNA-PK in combination with TORK may lead to better results in CRPC (38, 56); (iii) CC-115 can potently inhibit proliferation as a single agent in models of HSPC and CRPC to a greater magnitude than either DNA-PK or TORK inhibition alone; and (iv) CC-115 is under current clinical investigation. One of the challenges that was anticipated and observed in this study with the use of two TORK-targeting agents was the upregulation of the androgen response due to a feedback regulatory mechanism between TOR and AR (37, 38). Utilization of TORK inhibitors as single agents has demonstrated minimal efficacy in clinical trials in CRPC, which is mainly attributed to the feedback response with AR (57, 58). Combinations of TORK-targeting agents with a standard-of-care androgen antagonist, enzalutamide, are being investigated in clinical trials in CRPC (NCT02125084 and NCT02407054). The data herein show that DNA-PK/TORK/ enzalutamide combination performed better than enzalutamide alone and DNA-PK/TORKi alone in inhibiting proliferation in prostate cancer models in vitro, in vivo and ex vivo. Thus, cotargeting of DNA-PK, TORK, and AR would be a multifactorial treatment strategy that impinges upon multiple promorgenic pathways, with the potential to improve therapeutic response. Despite the likely benefits of the DNA-PK/TORK/AR combination treatment, there are potential concerns that can be hypothesized, such as increased toxicity, drug-drug interaction effects, and development of resistance mechanisms. On the basis of the findings presented here, a first-in-man DNA-PK-targeted therapeutic clinical trial was opened to study the combination treatment of the dual inhibitor CC-115 with enzalutamide in men with CRPC (NCT02833883). Clinical assessment of CC-115/ enzalutamide combination will shed more light on whether the toxicities are manageable and whether they outweigh the benefits.

In summary, DNA-PK is known to be overexpressed, hyperactivated, and a driver of aggressive phenotypes in advanced prostate cancer (12), however the underpinning mechanisms are not well understood. Data presented herein defined the global transcriptomic functions of DNA-PK in the absence of exogenous DNA damage, and uncovered novel processes that are modulated by DNA-PK including transcriptional regulation of EMT, immune response, and metabolism. Moreover, a combination therapy targeting multiple critical nodes involved in prostate cancer was identified, and was fast-tracked into a clinical study. CRPC remains universally lethal disease; however cotargeting DNA-PK, TORK, and AR could provide an effective antitumor therapeutic strategy for the management of CRPC.

Disclosure of Potential Conflicts of Interest

H.K. Raymon has ownership interests (including patents) in Celgene. B.E. Leiby is a consultant/advisory board member for Bayer Healthcare. L.G. Gomella is a consultant/advisory board member for Janssen, Astellas, Bayer, Merck, and Celgene. F.Y. Feng is a consultant/advisory board member for Janssen, Sanofi, Astellas, Bayer, dendreon, Ferring, Celgene, EMD Serono, RefleXion, Clovis, and blue earth diagnostics. E.H. Filvaroff has ownership interests (including patents) in Celgene. K. Hege has ownership interests (including patents) in Celgene. D. Rathkopf is a consultant/advisory board member for janssen. K.E. Knudsen reports receiving commercial research support from Celgene. No potential conflicts of interest were disclosed by the other authors.

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