

Mechanistic Support for Combined MET and AR Blockade in Castration-Resistant Prostate Cancer^{1,2}

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Abstract

A recent phase III trial of the MET kinase inhibitor cabozantinib in men with castration-resistant prostate cancer (CRPC) failed to meet its primary survival end point; however, most men with CRPC have intact androgen receptor (AR) signaling. As previous work supports negative regulation of MET by AR signaling, we hypothesized that intact AR signaling may have limited the efficacy of cabozantinib in some of these patients. To assess the role of AR signaling on MET inhibition, we first performed an *in silico* analysis of human CRPC tissue samples stratified by AR signaling status (+ or -), which identified *MET* expression as markedly increased in AR⁻ samples. *In vitro*, AR signaling inhibition in AR⁺ CRPC models increased *MET* expression and resulted in susceptibility to ligand (HGF) activation. Likewise, MET inhibition was only effective in blocking cancer phenotypes in cells with *MET* overexpression. Using multiple AR⁺ CRPC *in vitro* and *in vivo* models, we showed that combined cabozantinib and enzalutamide (AR antagonist) treatment was more efficacious than either inhibitor alone. These data provide a compelling rationale to combine AR and MET inhibition in CRPC and may explain the negative results of the phase III cabozantinib study in CRPC. Similarly, the expression of MET in AR⁻ disease, whether due to AR inhibition or loss of AR signaling, suggests potential utility for MET inhibition in select patients with AR therapy resistance and in AR⁻ prostate cancer.

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Introduction

Prostate cancer remains the second leading cause of cancer-related death in men in the United States [1]. Although advanced prostate cancer usually responds to therapies that suppress androgen-axis signaling, resistance inevitably develops, leading to the emergence of castration-resistant prostate cancer (CRPC). Importantly, the clinical efficacy of novel therapies targeting androgen receptor (AR) signaling, such as abiraterone and enzalutamide, has confirmed that most CRPC remains AR signaling intact (AR⁺) [2–4]. Resistance to these therapies inevitably develops, and approaches to improve the response time and address the key pathways of resistance are very much needed.

Despite promising early phase clinical trial results [5], a recently presented phase III trial evaluating the dual MET/VEGF inhibitor cabozantinib in CRPC did not meet its primary survival end point [6]. Although the AR signaling status was unknown in trial participants, the majority of men with CRPC retain active AR signaling [7,8]. Although MET has been reported to be overexpressed in CRPC, multiple studies have shown that AR signaling markedly downregulates MET expression [9–17]. Hence, we hypothesized that AR signaling status may mediate response to MET inhibition in prostate cancer. Herein we show in CRPC tissues and cell line models that *MET* expression is tightly linked to AR signaling status, with elevated MET expression and activity observed nearly exclusively in AR⁻ prostate cancer. Importantly, AR inhibition by enzalutamide results in MET overexpression and renders these functionally AR⁻ cells susceptible to HGF stimulation. In multiple *in vitro* and *in vivo* models, we credential MET as a target in AR⁺ CRPC when combined with antiandrogen therapy, as well as in AR⁻ disease models.

Materials and Methods

Cell Culture

All cell lines were purchased from ATCC, except LNCaP-AR which was a generous gift from Charles Sawyers's laboratory. PC3, DU145, LNCaP, and LNCaP-AR were maintained in RPMI1640, and VCaP in DMEM-GlutaMax; all were supplemented with 10% FBS (Invitrogen) in 5% CO₂ cell culture incubator.

Drugs

Cabozantinib and enzalutamide were purchased from SelleckChem. HGF was purchased from Invitrogen.

Invasion and Migration Assay

A total of 2 to 10 × 10⁴ cells were seeded in the upper chamber with 200 μl of serum-free medium and then incubate for 24 to 48 hours. For the invasion assays, 20 μg of growth factor reduced Matrigel was coated into the inner chamber. The crystal violet staining method used was described precisely [18]. Fluorescent-based invasion was performed with Calcium AM green (Invitrogen), and viable invaded cells were quantified by Tecan scanner for fluorescent intensity. Representative images were obtained with a fluorescent microscope.

Cell Viability and Proliferation Assay

Cell proliferation was measured by either CellTiterGlo or IncuCyte. Approximately 1000 to 30,000 cells were seeded in 96-well plates. Following drug treatment, viable cells were measured by CellTiterGlo every other day. IncuCyte was used to measure confluence rate.

Antibodies and Western Blot

For Western blot analysis, 30 μg of protein was separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (GE Healthcare). The membrane was incubated for 1 hour in blocking buffer (Tris-buffered saline, 0.1% Tween, 5% nonfat dry milk) followed by incubation overnight at 4°C with the primary antibody. After washing with Tris-buffered saline and 0.1% Tween, the membrane was incubated with HRP-conjugated secondary antibody, and signals were visualized using an enhanced chemiluminescence system as per the manufacturer's protocol (GE Healthcare). Antibodies used in Western blot were MET (D1C2, CST), AR (PG-21, Millipore), PSA (Dako), ERG (Abcam), GAPDH (CST), Actin (CST), pERK (CST), and p-Met (D26, CST).

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen), and cDNA was synthesized from 1 μg of total RNA using high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR was performed in duplicate or triplicate using standard SYBR green reagents and protocols on 7900 Real-Time PCR system (Applied Biosystems). The target mRNA expression was quantified using the ΔΔCt method and normalized to GAPDH expression.

RNA Interference

For knockdown experiments, cells were seeded in 6-well plates and transfected with 100 nM functionally verified FlexiTube siRNA (Qiagen) targeting MET or AllStars Negative Control siRNA (catalogue no. SI02655450) using RNAiMAX (Life Technologies) according to the manufacturer's instructions. siRNA sequences for MET knockdown were as follows: 1) AAGCCAATTTATCAG GAGGTG (catalogue no. SI00300860) and 2) ACCGAGGGAAT CATCATGAAA (catalogue no. SI00604814). Quantitative PCR and Western blot assays were performed after 24 or 48 hours to assess knockdown efficiency. Cell migration and Matrigel invasion assays were performed as described earlier.

In Silico Analysis of MET Expression and AR Signaling in CRPC

We queried the expression of *AR* and core AR signaling modules ($n = 7$ AR⁺ genes and $n = 3$ AR⁻ genes including *MET*) [19] from the Grasso Prostate [8] and Taylor Prostate [20] studies in the Oncomine database [21]. The same genes were similarly queried from microarray-based gene expression profiling of 11 commonly used prostate cancer cell lines, and from RNAseq data in the Robinson et al. CRPC profiling study [22] (downloaded from cBioPortal [23]).

Xenografts

To investigate combined inhibition of the AR and MET signaling axis, we used 30 mg/kg of cabozantinib, a dose shown previously to inhibit p-Met by >90% [24] and inhibit MET-dependent xenograft growth in a malignant peripheral nerve sheath model [25]. Dose of 10 mg/kg of enzalutamide was chosen to inhibit AR signaling, as this dose has previously been shown to block xenograft growth in LNCaP-AR tumors [26]. VCaP or LNCaP-AR (generously provided by Dr. Charles Sawyers) [27] subcutaneous xenografts were established in the bilateral flanks of male CB17 nu/nu mice. After 3 weeks, mice were treated by oral gavage with vehicle, enzalutamide (10 mg/kg), and/or cabozantinib (30 mg/kg) daily (5×/week). When the enzalutamide-only-treated group reached approximately half the final tumor volume (estimated at 400 mm³), this group was randomized: 14 xenografts were continued on enzalutamide

and 11 xenografts were switched to only 30 mg/kg of cabozantinib. Growth inhibition (as freedom from tumor volume tripling) was assessed using the log-rank test.

Results

To investigate the relationship of *MET* and AR signaling across CRPC, we queried the expression of *AR* and core AR signaling

modules ($n = 7$ AR⁺ genes and $n = 3$ AR⁻ genes including *MET*) which we recently demonstrated can stratify AR signaling status in prostate cancer through quantitative RT-PCR profiling of routine tissue specimens [19]. As shown in Figure 1A, expression of these genes in our previous expression profiling study of CRPC specimens obtained at rapid autopsy (Grasso et al., $n = 35$) [8] demonstrates a subset of samples with markedly reduced *AR* and AR⁺ module

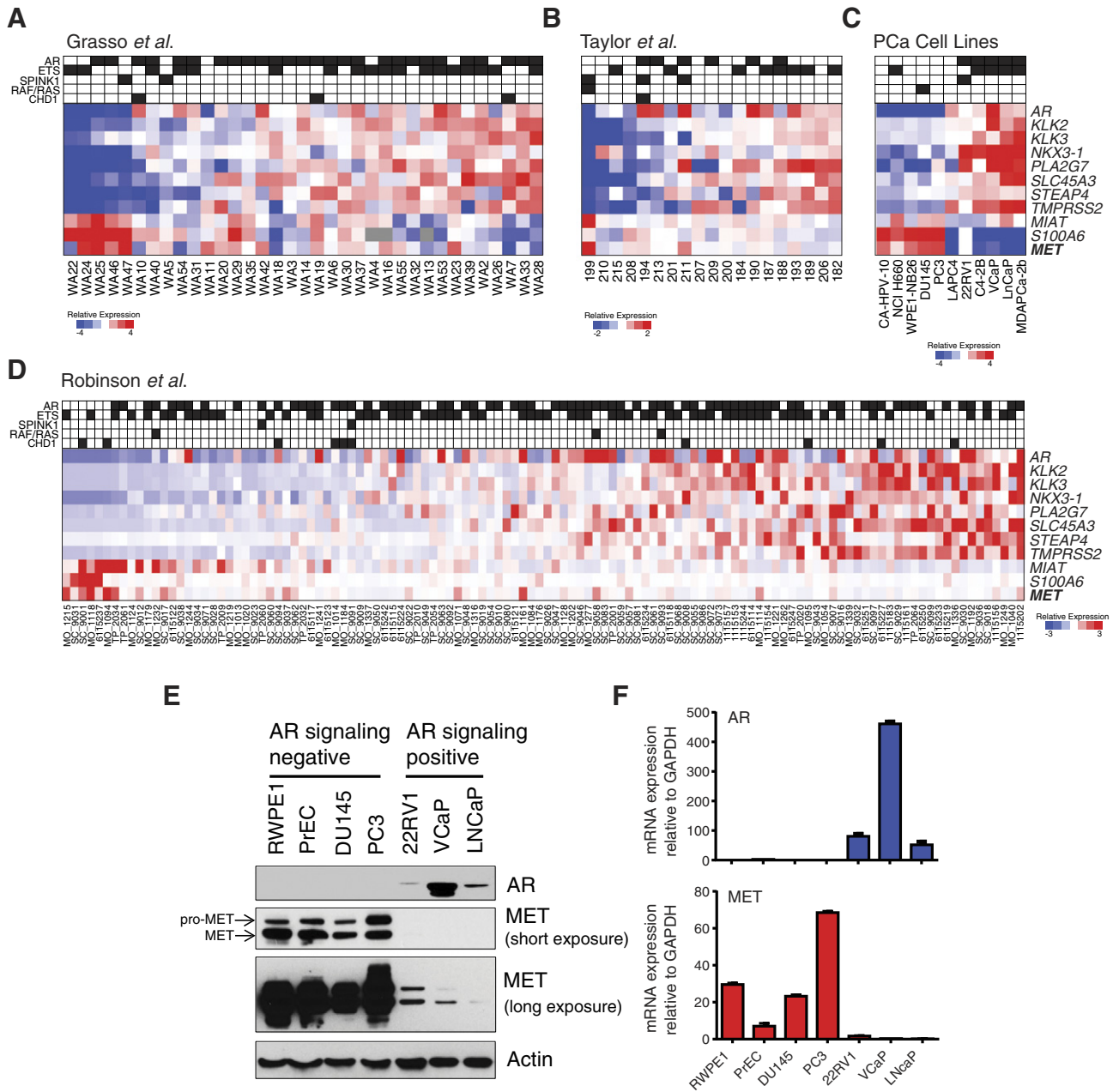


Figure 1. Overexpression of *MET* in AR⁻ CRPC. (A-D) *AR* and a core AR signaling module ($n = 7$ AR⁺ genes and $n = 3$ AR⁻ genes including *MET*) expressions were queried from multiple CRPC tissue and cell line profiling studies. *AR* and the AR signaling module expression in (A) CRPC tissue specimens from the Grasso et al. and (B) Taylor et al. prostate cancer profiling studies (accessed in Oncomine). Molecular subtyping (black indicates present) is indicated for all specimens, including AR amplification/mutation, ETS gene fusions, SPINK1 outlier expression, RAF/RAS family fusion or mutation, and *CHD1* deletion or mutation. Expression is shown according to the color scale, with red and blue indicating relative over- and underexpression. Samples are sorted by average AR⁺ module (including *AR*) expression. (C and D) As in A and B, except gene expression was queried from (C) a compendium of prostate cancer cell lines profiled by DNA microarrays and (D) the Robinson et al. RNAseq profiling study of CRPC tissues (accessed in cBioPortal). (E) Western blot analysis of MET protein expression in various prostate cell lines with different AR status, and (F) corresponding mRNA expression levels of AR and MET.

expression and corresponding increased AR⁻ module expression, including the three CRPC samples with the highest *MET* expression (WA24, WA47, and WA25). We confirmed similar findings in 17 antiandrogen-treated metastatic prostate cancer samples from the expression profiling study of Taylor et al. [20] and 11 commonly used prostate cancer cell lines. In the Taylor et al. study, although several samples had decreased expression of the AR⁺ module, a single specimen with the highest *MET* expression (sample 199) also had markedly reduced *AR* expression and AR⁻ module overexpression (Figure 1B). Likewise, *MET* expression was only observed in prostate cancer cell lines with low AR expression, low AR⁺ module expression, and elevated AR⁻ module expression (Figure 1C). Lastly, we assessed the relationship of *MET* and AR signaling in a large cohort of men with CRPC undergoing biopsy and comprehensive exome and transcriptome sequencing for precision medicine (SU2C International Dream Team, Robinson et al. [22]), which again demonstrated marked *MET* overexpression nearly exclusively in samples with low *AR* expression, low AR⁺ module expression, and high AR⁻ module expression (Figure 1D). Taken together, our integrative transcriptional analysis across human CRPC tissue and cell line profiling studies support the inverse correlation of *MET* and AR, with correlation coefficients of -0.27 to -0.81 (Supplementary Table 1). We confirmed these *in silico* findings through assessing *MET* and *AR* transcript and protein expression in a panel of prostate cell lines with differing AR status. The results confirmed that *MET* and AR expressions are inversely related at both protein and mRNA levels (Figure 1, E and F).

To directly assess the relationship of AR signaling and *MET* expression, we assessed the impact of hormone deprivation on *MET* expression in AR⁺ VCaP, LNCaP, and LNCaP-AR cells. As shown in Figure 2A and 2B, *MET* expression is increased after exposure to the AR antagonist enzalutamide and when cells are cultured in charcoal-stripped medium (to remove AR hormonal ligands). Similarly, stimulation with the synthetic androgen dihydrotestosterone (DHT) decreases *MET* expression under charcoal-stripped conditions in an enzalutamide-sensitive manner. Changes in AR pathway activity under these conditions were confirmed by quantitative RT-PCR analysis of canonical AR target genes (Supplementary Figure S1A). In addition, the inverse association of *MET* expression with androgen signaling activity was observed to be time dependent. As shown in Figure 2C, *MET* protein expression decreases as AR signaling becomes activated, with PSA and ERG serving as indicators of AR signaling. This was further supported by measuring the *MET* protein half-life in the setting of androgen deprivation. When protein synthesis was blocked by cycloheximide, *MET* protein is degraded along with ERG (Supplementary Figure S1B). Yet, AR protein does not bind to the *MET* promoter region as detected by AR ChIP-seq analysis performed after DHT stimulation and/or enzalutamide treatment (Supplementary Figure S2) [18]. Overexpression of *MET* in AR⁺ LNCaP cells does not affect endogenous AR protein level (Supplementary Figure S1C). It suggests that the regulation of AR and *MET* is one-directional in AR-positive CRPC, with suppression of *MET* by active AR signaling through possible posttranslational modification. Taken together,

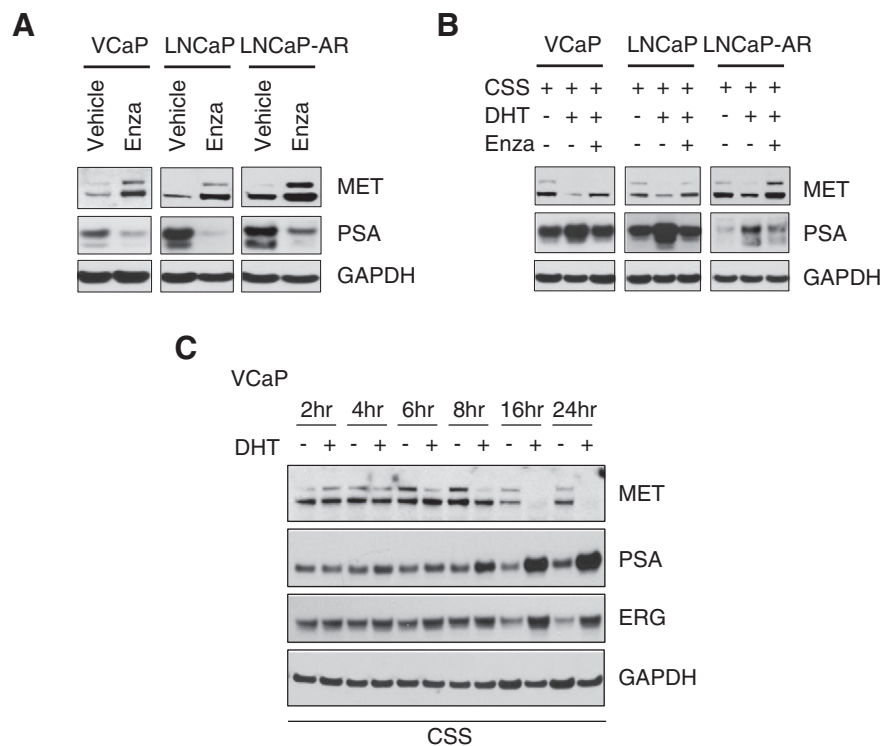


Figure 2. Blockade of androgen signaling increases *MET* expression *in vitro*. (A) AR-positive cells (VCaP, LNCaP, and LNCaP-AR) were treated with 10 μ M enzalutamide (Enza) followed by Western blot to measure *MET* and PSA protein levels. (B) Indicated cells were treated with charcoal-stripped serum (CSS) for 48 hours before stimulation with the synthetic androgen DHT (10 nM) and enzalutamide treatments for another 24 hours. Expression of indicated proteins was assessed by Western blot. (C) Western blot analysis of VCaP cells in the presence or absence of DHT (10 nM) for indicated time points after CSS for 48 hours. Protein levels of *MET*, ERG, and PSA were assessed.

these *in vitro* experiments support active AR signaling mediated repression of MET expression.

Given the overexpression of MET in AR⁻ prostate cancer models and the potential of MET as a target in AR inhibited AR⁺ CRPC models (therapeutic AR⁻), we sought to credential MET as a potential therapeutic target in AR⁻ prostate cancer. As MET has been reported to promote invasion upon ligand (HGF) binding-induced phosphorylation [28], Figure 3A shows that, in the presence of HGF, siRNA-mediated MET knockdown in PC3 and DU145 cells (AR⁻/high MET expression) significantly reduced invasion and migration. Likewise, in the presence of HGF, levels of both p-MET and pERK were substantially reduced after MET knockdown in both PC3 and DU145 cells (Figure 3B). Additionally, HGF-mediated invasion and migration in both DU145 and PC3 cells were sensitive to cabozantinib (Figure 3C). Taken together, these experiments support MET as a mediator of invasion in AR⁻ prostate cancer models and suggest potential utility of therapeutic targeting in advanced prostate cancer.

Given our results suggesting that MET is not expressed in AR⁺ CRPC, we first sought to determine if MET expression results in a cabozantinib-sensitive cancer phenotype in AR⁺ CRPC models. As shown in Figure 4A, MET overexpression promoted invasion in AR⁺

LNCaP cells (in the presence of androgen), which was sensitive to cabozantinib. We then confirmed that p-MET (Y1234/1235) is increased upon HGF stimulation in MET-transfected LNCaP cells cultured in the presence of androgen, and exposure to cabozantinib reverses this effect (Figure 4B). Importantly, cabozantinib had no effect on VCaP or LNCaP cell invasion under normal culture conditions (androgen present) (Figure 4C). However, when AR signaling in LNCaP cells was inhibited through the use of charcoal-stripped medium, HGF significantly increased invasion in a cabozantinib-sensitive manner (Figure 4D). Together, these results demonstrate that while cabozantinib has no significant effect on invasion in AR⁺ CRPC models when AR signaling is active (and MET is not expressed), blocking AR signaling primes AR⁺ cells for MET activation and cabozantinib sensitivity.

To more directly assess the potential of AR inhibition inducing therapeutic vulnerability in MET in AR⁺ CRPC models, we investigated combination treatment with the potent AR inhibitor enzalutamide and cabozantinib. Measuring viable cells by CellTiterGlo, we found that combined enzalutamide and cabozantinib drug treatment more significantly blocked VCaP and LNCaP cell proliferation than either agent alone (Figure 5A). Next, IncuCyte

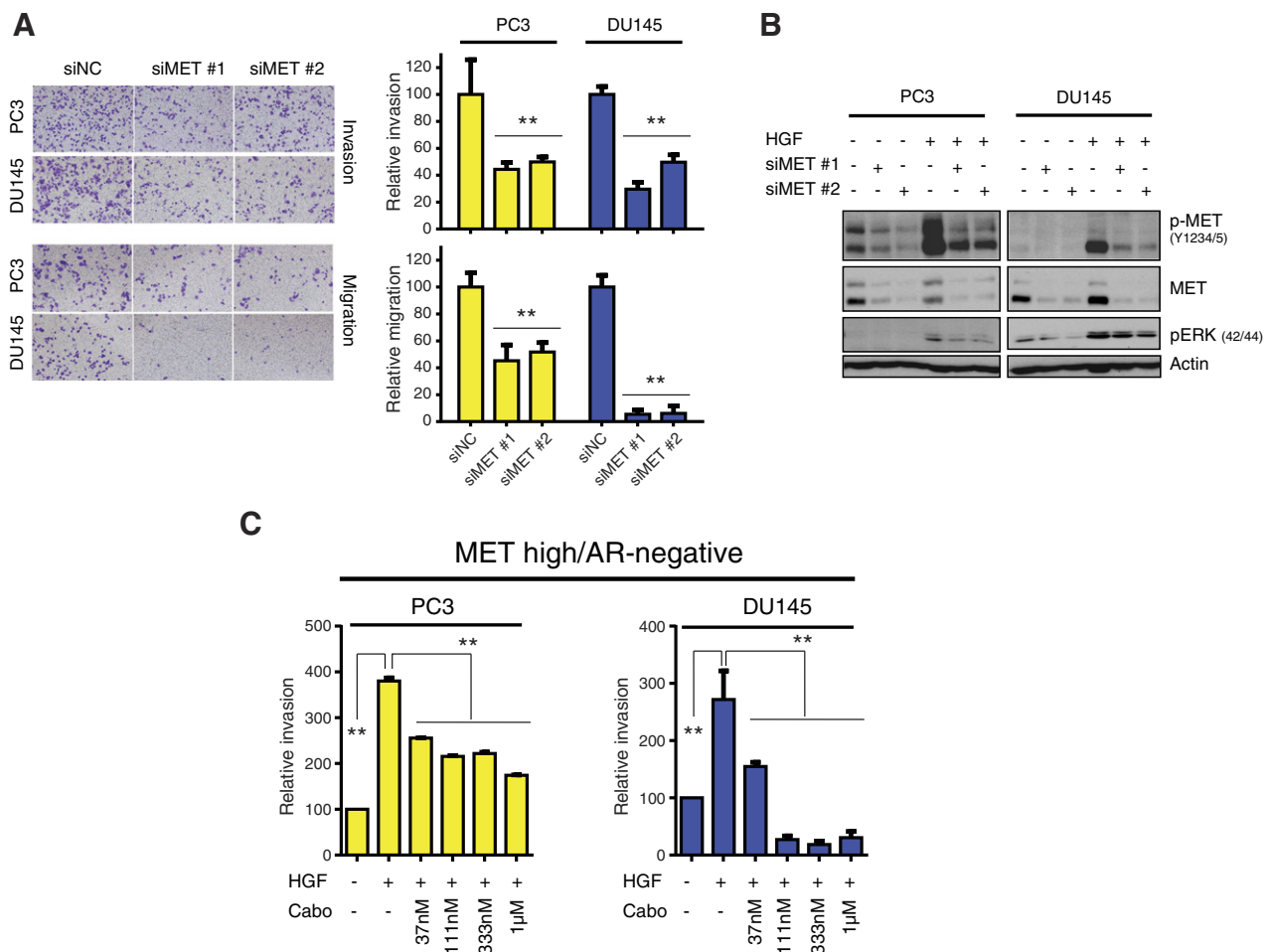


Figure 3. MET/HGF axis promotes invasion in prostate cancer *in vitro*. (A) Knockdown of MET in DU145 and PC3 (AR⁻) prostate cancer cells. Invasion or migration assays were done in the presence of MET ligand HGF for 24 hours. Representative pictures of crystal violet staining are shown on the left, and quantification is shown on the right. (B) Western blot analysis of (A) in the conditions of with or without HGF; indicated protein targets were assessed. (C) Invasion assay was performed in the presence of HGF and/or various treatment doses of cabozantinib (Cabo) in MET high/AR-negative prostate cancer cells for 24 hours.

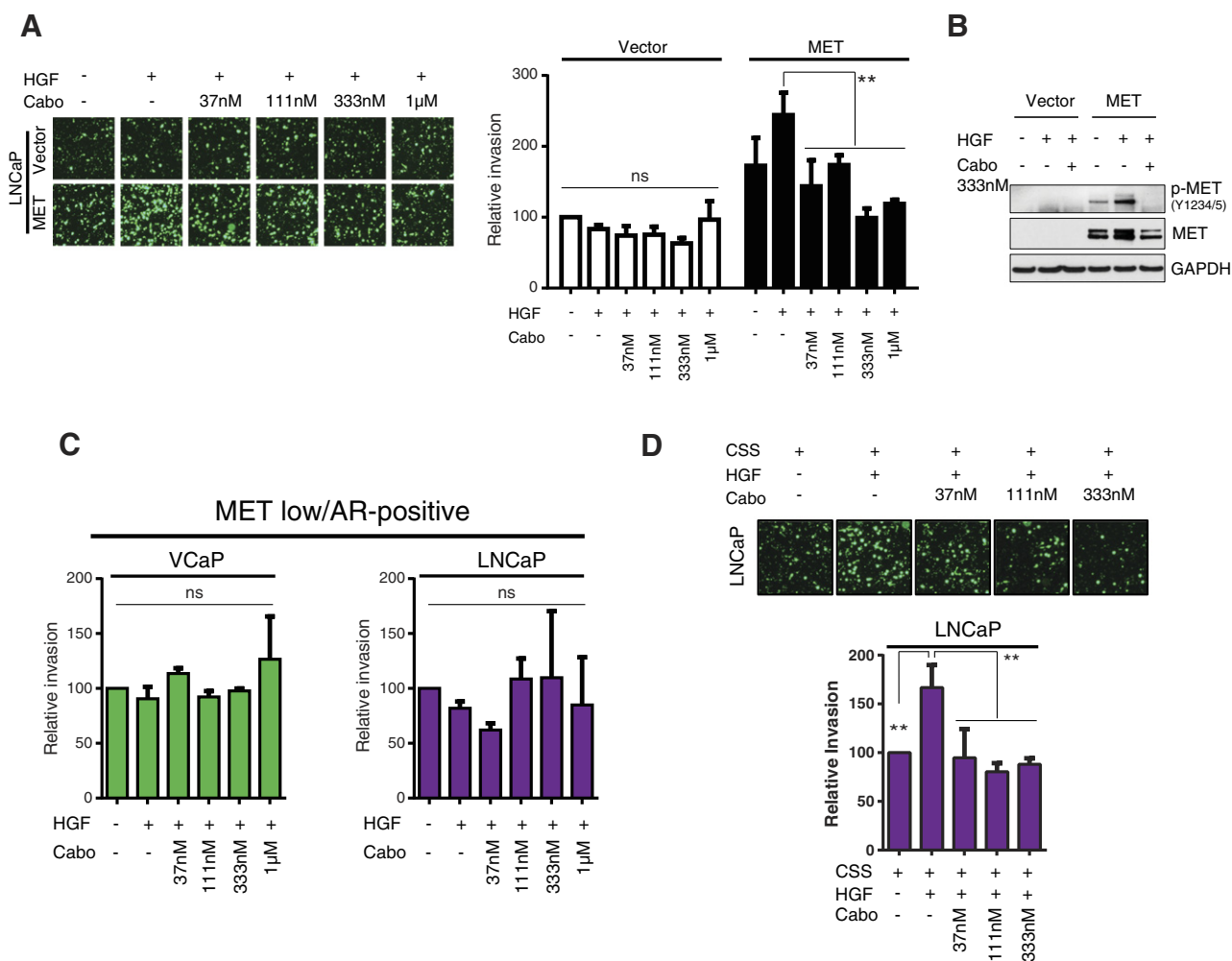


Figure 4. Elevated MET sensitizes AR⁺ prostate cancer to Cabo. (A) LNCaP (AR⁺) prostate cancer cells were stably transfected with either empty vector or MET, and invasion ability was assessed in the presence of HGF and/or various treatment doses of Cabo as indicated. (Left panel) Representative pictures of indicated treatment results by fluorescent staining. (Right panel) The quantification of invasion relative to vector control. (B) Selected treatment outcomes were measured by Western blot for phospho-MET (Y1234/1235) and total MET protein levels. (C) Invasion assay was performed in the presence of HGF and/or various treatment doses of Cabo in MET low/AR-positive prostate cancer cells for 48 hours. (D) LNCaP (AR⁺) prostate cancer cells were treated with CSS for 48 hours before invasion assay under indicated conditions for another 48 hours.

was used to measure the confluence rate for LNCaP cells upon single or combination treatment. Figure 5B shows that, whereas low-dose cabozantinib as a monotherapy has no effect on confluence, the effect of enzalutamide is significantly increased by combination with low-dose cabozantinib ($P < .01$).

To confirm these *in vitro* results, we first extended these observations to mouse xenograft experiments using the VCaP model. As shown in Figure 5C and Supplementary Figure S3A, although VCaP xenografts were minimally sensitive to enzalutamide and responded better to cabozantinib alone, tumors were significantly more responsive to combination of enzalutamide plus cabozantinib compared with either monotherapy ($P < .01$ vs. enzalutamide, $P < .05$ vs. cabozantinib, log-rank test) (Figure 5C and Supplementary Figure S3A). Importantly, sequential treatment was slightly better than enzalutamide alone, but the difference was not statistically significant. Next, we similarly assessed the effects of combined cabozantinib and enzalutamide in LNCaP-AR mouse xenografts. As shown in Figure 5D and Supplementary Figure S3B, combined cabozantinib and enzalutamide treatment was again more efficacious

than either monotherapy ($P < .01$). Taken together with our *in silico* and *in vitro* studies, these results support combined blockade of AR and MET in AR⁺ CRPC and may partially explain why cabozantinib treatment failed its primary end point in a CRPC population where the majority of men likely have active AR signaling.

Discussion

We have identified the HGF/MET axis as a potentially important driver of resistance to potent androgen suppression and provide a rationale for dual targeting of androgen and HGF/MET signaling in mCRPC. Although little is known about the compensatory pathways that facilitate resistance to next-generation AR-targeted therapies [8,29], we hypothesized that resistance may involve MET overexpression that occurs in response to decreased AR signaling. Although a recent study reported frequent MET amplification/gain in CRPC [30], in our analysis of the Grasso, Taylor, and Robinson data sets, we observed no correlation between *MET* copy number and gene expression. Additionally, although broad low-level gains of chromosome 7q (containing *MET*) were common in CRPC, high-level *MET*

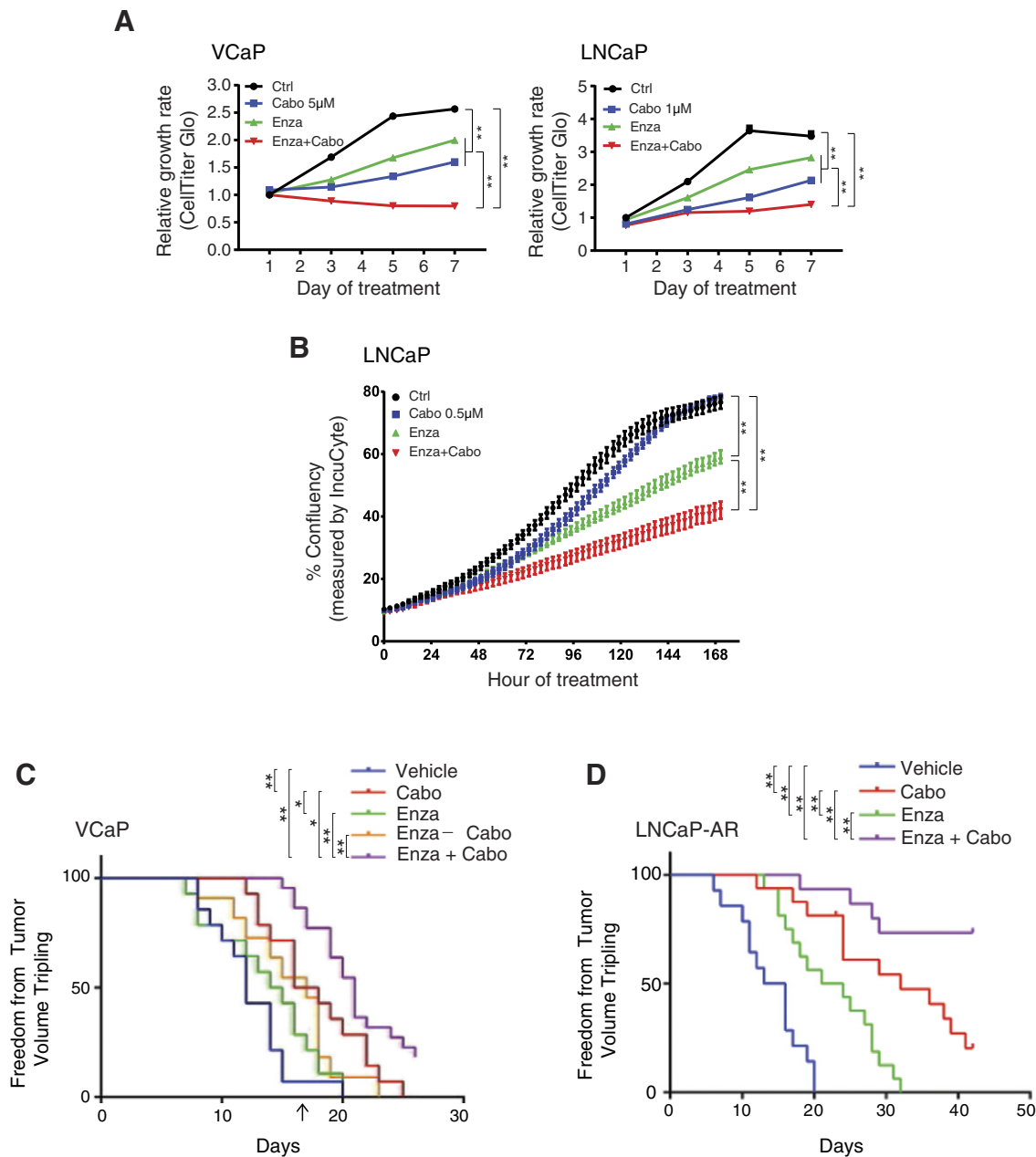


Figure 5. Cabozantinib, in combination with enzalutamide, inhibits growth of AR-positive cells *in vitro* and *in vivo*. (A) VCaP and LNCaP (AR⁺) prostate cancer cells were treated with either Cabo or enzalutamide (Enza) individually or in combination in the presence of HGF. Viable cells were measured by CellTiterGlo, and percentage was plotted against day 0. (B) LNCaP prostate cancer cells were treated with indicated treatments in the presence of HGF. Confluence rate was measured by IncuCyte. (C) VCaP subcutaneous xenografts were established and treated with 10 mg/kg of Enza (oral gavage) and/or 30 mg/kg of Cabo (oral gavage) 5 days per week. When the Enza-only-treated group reached approximately half the final tumor volume (estimated at 400 mm³), this group was randomized; 14 were continued on Enza, and 11 xenografts were switched to only 30 mg/kg of Cabo. This time point is indicated by an arrow on the plot. Tumor volumes were measured by caliper, and time to tumor volume tripling is shown on the freedom plots. Log-rank tests are reported for each treatment group. * and ** indicate $P < .05$ and $P < .01$, respectively. (D) As in C, except using LNCaP-AR tumors and without Enza to Cabo switching.

amplifications were exceedingly rare and did not result in MET overexpression, as would be expected for a driving oncogene (Supplementary Figure S4). Likewise, in our recent targeted profiling study of 116 cases including both aggressive untreated prostate cancers and CRPC, only a single AR⁻ NePC showed low-level focal *MET* amplification [31]. Hence, AR signaling may be the predominant mechanism regulating *MET* expression in CRPC.

The *in vitro* and *in vivo* data further establish the strong inverse relationship between MET expression and AR activity. From a

functional standpoint, the potential relevance was confirmed both through MET knockdown in AR⁻/MET⁺ cell lines and through overexpression of MET in AR⁺/MET⁻ cells. Cabozantinib also reversed the effects of MET overexpression in LNCaP cells. Wanjala et al. also showed that overexpression of MET in AR⁺ LAPC4 cells (which show low MET expression in the presence of active AR signaling) activated ERK and AKT signaling, and drove *in vitro* and *in vivo* growth in a manner that was sensitive to crizotinib (a multikinase inhibitor that potently inhibits MET) [30]. Thus,

therapeutic strategies that inhibit the MET pathway are likely to work only in those specific settings where MET is elevated, which generally correspond to substantially AR-repressed disease states.

The data shown here demonstrate that even in AR⁺ CRPC, where MET overexpression is absent, antiandrogen therapy can increase MET expression, potentially inducing therapeutic vulnerability. In multiple AR⁺ CRPC models, combined treatment with antiandrogen and anti-MET therapy showed at least additive tumor inhibition, which we hypothesize is due to concurrent targeting of AR signaling and inhibition of compensatory MET activity. Our results and prior work supporting the inverse relationship of AR signaling and MET expression suggest that optimal timing of treatment and combination therapy using cabozantinib (or other MET inhibitors) in prostate cancer may be crucial to efficacy [9–14,16,17,32]. Supporting this hypothesis, phospho-MET was recently shown to be increased in bone marrow metastases from men with CRPC showing primary abiraterone resistance [33].

These findings have important clinical implications, as there are currently conflicting data regarding the efficacy of MET inhibition in advanced prostate cancer. In a multicenter phase II randomized discontinuation trial of cabozantinib in mCRPC, 171 men with CRPC received 100 mg of cabozantinib daily, and those with stable disease per RECIST (Response Evaluation Criteria In Solid Tumors) at 12 weeks were randomized to cabozantinib or placebo [5]. Random assignment was halted early based on the observed activity of cabozantinib, with 68% of evaluable patients demonstrating improvement on bone scan, including complete resolution in 12%. Median progression-free survival was 23.9 weeks with cabozantinib and 5.9 weeks with placebo ($P < .001$). However, the recently presented phase III COMET-1 study (cabozantinib 60 mg daily) did not meet the primary end point of improved overall survival [6]. There were, however, significant improvements in bone scan response and progression-free survival in the cabozantinib group. Critically, neither MET nor AR signaling status was assessed in samples from these patients.

Our results suggest two potential strategies for more effectively implementing MET inhibition in CRPC. First, a strategy employing a more precision-based approach, such as selecting patients based on MET overexpression, would likely yield greater efficacy. Second, concurrently administering cabozantinib with androgen signaling inhibitors may prevent this resistance pathway from driving further tumor progression and significantly improve therapeutic response. Importantly, this work provides a hypothesis for the failure of cabozantinib in the phase III trial, as most men with CRPC have intact AR signaling. Our work therefore supports trials of MET inhibition combined with potent AR-signaling blockade in AR-signaling-intact CRPC. A number of novel therapeutics are in development that more selectively inhibit the HGF/MET axis, and these warrant significant attention based on the results presented here.

In conclusion, our work elucidates the potential rationale and impact of targeting AR and one of its compensatory pathways in advanced prostate cancer. We demonstrated through *in silico* analysis that most patients with pre-second-generation antiandrogen CRPC (with or without prior chemotherapy) have intact AR signaling and thus low MET expression. *In vitro*, MET expression increased in response to potent AR signaling inhibition, and when expressed (whether through AR inhibition or forced overexpression), MET drove tumorigenic potential and sensitized them to cabozantinib. Importantly, both *in vitro* and *in vivo*, combined cabozantinib and enzalutamide treatment in multiple AR⁺ CRPC models was more

effective than either treatment alone. These results offer a potential explanation for the failure of cabozantinib in a pivotal CRPC trial and provide a mechanistic basis for co-targeting AR and MET in AR-signaling-intact CRPC.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2015.11.009>.

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