Cabozantinib Inhibits Abiraterone’s Upregulation of IGFIR Phosphorylation and Enhances Its Anti-Prostate Cancer Activity
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Abstract

Purpose: Abiraterone improves the overall survival of men with metastatic castration-resistant prostate cancer. However, de novo or adaptive resistance to abiraterone limits its activity. Rational combinations of drugs with different mechanisms of action that overcome resistance mechanisms may improve the efficacy of therapy. To that end, we studied the molecular and phenotypic effects of the combination of cabozantinib plus abiraterone.

Experimental Design: Three prostate cancer cell lines were used to interrogate the in vitro molecular and antiproliferative effects of the single agents and combination of cabozantinib and abiraterone. The in vivo impact of the combination was assessed using the LAPC4-CR xenograft mouse model.

Results: In vitro proliferation studies demonstrated single-agent doses between 2 μmol/L and 10 μmol/L for abiraterone and cabozantinib inhibit prostate cancer cell proliferation in a dose-dependent manner, and the anticancer activity of abiraterone is enhanced when combined with cabozantinib. In vivo LAPC4-CR xenograft mouse studies also showed that cabozantinib can improve the antitumor activity of abiraterone. Cabozantinib, a multiple receptor tyrosine kinase inhibitor, enhances the ability of abiraterone to inhibit AR activity in a cell-line-dependent manner. In addition, our cell line studies demonstrate abiraterone-stimulated insulin-like growth factor 1 receptor (IGFIR) phosphorylation with downstream activation of MEK1/2 and ERK1/2, and that this potential adaptive resistance mechanism was inhibited by cabozantinib.

Conclusions: Cabozantinib can enhance the efficacy of abiraterone by blocking multiple compensatory survival mechanisms, including IGFIR activation, and supports the assessment of the combination in a clinical trial.

Material and Methods

Introduction
Prostate cancer is the most common type of nonskin malignancy and the second leading cause of cancer death in adult males in the United States (1). Androgens and the androgen receptor (AR) have long been recognized to play crucial roles in prostate cancer development and progression (2–8). Prostate cancers depend on androgens for growth and androgen deprivation therapy (ADT), which suppresses testicular androgen production and diminishes AR activation, is the primary treatment for advanced disease, and is the most effective and widely used systemic therapy for patients with hormone-sensitive prostate cancer (9, 10). Although ADT is initially effective, most tumors will eventually become resistant and progress to castration-resistant prostate cancer (CRPC), which is usually lethal (11–14). It is well recognized that AR activation by residual androgens in CRPC patients still drives cancer growth in some patients (15). Abiraterone is an inhibitor of the cytochrome P450 17α-hydroxylase/17, 20 lyase (CYP17A1), a key enzyme in the synthesis of extragonadal and testicular androgens (16). As such, it decreases androgen production and AR signaling in CRPC and is FDA approved for the treatment of metastatic CRPC (mCRPC; ref. 17).

Cabozantinib is a multi receptor tyrosine kinase inhibitor including blocking cMet and VEGFR2 activation, and has been approved for the treatment of progressive metastatic medullary thyroid cancer based on inhibiting RET (18). In this study, we examined the impact of cabozantinib on the antitumor activity of abiraterone in prostate cancer. Our data show that cabozantinib significantly enhances abiraterone inhibition of prostate cancer cell growth and also blocks abiraterone’s compensatory activation of the IGFIR survival pathway.

Materials and Methods

Human cell lines
PC3 and VCaP cell lines were purchased from the ATCC. LAPC4 cell line was a gift from Dr. Robert Reiter, University of California, Los Angeles (Los Angeles, CA). LACP4-CR cell line was obtained from Dr. Steven Balk laboratory, Beth Israel Deaconess Medical Center. LACP4 cells were maintained in RPMI-1640 and...
supplemented with 10% FBS. LAPC4-CR cells were cultured in phenol-red-free RPMI-1640 with 10% charcoal-stripped FBS. For PC3 and VCaP cells, DMEM with 10% FBS was used. All culture mediums included 100 IU of penicillin and streptomycin (100 μg/mL). All cells were maintained at 37°C, 5% CO2, and 100% relative humidity, and regularly screened for mycoplasma using a Venor GeM Mycoplasma Detection Kit (Sigma). For the DFT induction study, LAPC4 and VCaP cells were cultured in phenol-red-free RPMI-1640 and DMEM medium, respectively, supplied with 10% charcoal-stripped FBS for 2 days before adding 5 nmol/L DHT.

### Cell proliferation assay
Cell proliferation was determined by using the WST-1 assay (Roche). Briefly, cells were cultured in 96-well plates with a confluence of approximately 20% for overnight to allow adherence and were treated with drugs in the defined concentration. Culture medium were changed every 2 days supplied with the fresh drugs. Cell proliferation assay was carried out at different days after treatment and read with a spectrophotometer at a wavelength of 450 nm following the manufacturer's instruction. Each experiment was performed in triplicate.

### Western blot analysis and quantitative RT-PCR
Protein concentration in the whole-cell lysis was measured by protein BCA assay (Bio-Rad) and equal amount of whole protein was loaded for Western blot analysis to detect protein expression level. Antibodies used for Western blot analyses are as follows: AR (1:2,000; Santa Cruz Biotechnology), PSA (1:5,000; Meridian Life Science), IGFR (1:500; Santa Cruz Biotechnology), p-IGFR (1:1,000; Cell Signaling Technology), MER1/2 (1:1,000; Cell Signaling Technology), p-MEK1/2 (1:1,000; Cell Signaling Technology), ERK1/2 (1:1,000; Cell Signaling Technology), p-ERK1/2 (1:1,000; Cell Signaling Technology), Akt (1:1,000; Cell Signaling Technology), p-Akt (1:1,000; Cell Signaling Technology), and β-actin (1:5,000; Sigma).

Of note, 100 ng total RNA extracted from prostate cancer cells was applied to cDNA synthesis and RT-PCR analysis. The following primers were purchased from Origene and used to measure the targeted gene level:
- AR, forward 5'-ATGGTGAAGGACAGTGCCCCTATC-3', reverse 5'-ATGGCTCCTGGCAGTCTCCCAA-3',
- PSA, forward 5'-CCGAACCTTCACCTGAGAGG-3', reverse 5'-GACCTATCCCTGAGACACAC-3',
- KLK2, forward 5'-GGCTCAGGACAGGTTAAGA-3', reverse 5'-CCGTAATGACCCCTGTTGTG-3',
- FKBPs, forward 5'-GCGAAGGAGGAGACCAGACAT-3', reverse 5'-TAGGCCTCCCTGGCTTCAAA-3',
- pLZF, forward 5'-GAGGCTCTGATTGAAGGGCTG-3', reverse 5'-AGCCGAAACTATCCAGGAAAC-3',
- GAPDH, forward 5'-CAGCTGCAAGATAGTACGCA-3', reverse 5'-GTCCTCTGGCTGCGACGTGAT-3'.

All RT-PCR experiments were performed in triplicate.

### Human phospho-receptor tyrosine kinase array assay
Cells were cultured for 2 days until approximately 80% confluent and then treated with 5 μmol/L cabozantinib for 6 hours. Cell lysis was extracted from 1 × 10^6 cells and quantification of sample protein concentrations was determined using protein BCA assay. Of note, 200 μg total cell lysis was used as the starting materials for the kinase array assay following the manufacturer’s instruction (R&D Systems). For the validation experiment, LAPC4 cells were cultured in regular medium for 2 days to allow adherence and then changed to serum-free culture medium for 24 hours followed by 5 ng/mL of IGF treatment for different time. The affected proteins were detected by Western blot analysis. To examine the specific effect of cabozantinib on the IGF-induced p-IGFR level, LAPC4 cells were cultured in serum-free culture medium for 18 hours and treated with 0.5 or 5 μmol/L of cabozantinib for 6 hours. Cells were then treated by 5 ng/mL of IGF for 1 hour before collecting the cell lysis for Western blot analysis.

### In vivo xenograft study
Human LAPC4-CR tumor fragment (30 mg) was injected subcutaneously in the right flank of 15 nude mice. Ten of the tumor-bearing mice were used to implant 70 mice for the efficacy study. The remaining 5 mice (1 with tumor, 4 without tumors) were used for a run-in toxicity study: the four mice without tumors were treated with 30 mg/kg cabozantinib oral daily plus abiraterone 0.5 mg oral daily for 14 consecutive days. The one tumor-bearing mouse was dosed with single-agent cabozantinib 30 mg/kg oral daily for 14 consecutive days.

Weights were monitored twice weekly. Abiraterone was dissolved in 5% benzyl alcohol and 95% safflower oil and cabozantinib was supplied in sterile water. The vehicle treatment was 5% benzyl alcohol plus 95% safflower oil. For the efficacy study, mice were divided into four treatment groups (n = 8 per group) when tumors reached approximately 80 to 250 mm^3 (average mean per group ~130 mm^3); vehicle, cabozantinib at 30 mg/kg oral daily, abiraterone 0.5 mg/day oral daily, and combination of cabozantinib with abiraterone at the same doses as single-agent arms. Mice were sacrificed when tumors reached 2 cm in any dimension. Each group received their assigned treatment until mice were sacrificed due to exceeding size criteria and the mean size per group could not be established. For the combination group, abiraterone was dosed first with cabozantinib dosed at least one hour later. Both drugs were made fresh daily and used within one hour of being made. Because all tumors did not reach an appropriate size at once, mice were entered onto study for 3 consecutive weeks when tumors fell within the acceptable size range, with mice randomly assigned to each treatment arm to ensure similar mean tumor volume at day 1. Tumor and body weight data were collected twice weekly for 2 months after start of treatment.

### IHC and spectral imaging
A total of 24 formalin-fixed, paraffin-embedded prostate cancer tissue specimens were utilized to construct the TMA. Each sample was represented by four TMA cores. IHC staining of p-cMet and p-
IGFIR was performed on 4 μm sections of the TMA, using the Bond Refine Detection System following the manufacturer's protocols on the Leica Bond III automated immunostainer. The sections were automatically deparaffinized; antigen retrieval was done with EDTA buffer (pH 9.0) and processed for 20 minutes. The slides were incubated with the antibody against p-cMet (Tyr1234/1235, D26, Cell Signaling Technology) at a dilution of 1:50, and p-IGFIR (Tyr1161, NB100-92555, NOVIS) at a dilution of 1:1,000 for 60 minutes, respectively. The sections were then treated according to the streptavidin-biotin-peroxidase complex method (Bond Polymer Refine Detection, Leica Microsystems) with diaminobenzidine (DAB) as a chromogen and counterstained with hematoxylin. The lung cell line H1993 was used as a positive control for p-cMet, and the breast cell line MCF-7 for p-IGFIR, respectively. Omission of the primary antibody was utilized as a negative control.

The TMA was scanned on a PerkinElmer Vectra 2 imaging work station. Appropriate filter cubes and ×20 objective exposure times were set for each chromogen. DAB and hematoxylin single stained slides were imaged separately with the established exposure time for generating the spectral library. A bright field spectral scan was run at ×4 magnification with the correct number of TMA rows and columns, as identified from the TMA map.

We ran an algorithm-learning tool utilizing the InForm Analysis software package to train for classifying the tumor, necrosis and stroma, and subsequently completed cell segmentation. Nuclei finding was enabled by detecting circular objects in the hematoxylin channel. A 2-pixel radii around the nuclei was defined as the cytoplasm. The algorithm was then applied to all the images contained within the TMA. The reported mean for a given cell was the average intensity of all the normalized total pixel values in each nucleus, cytoplasm, and membranous.

Statistical analysis

Statistical analysis for all in vitro studies was performed with Prism 6.0 software (GraphPad Software Inc.). All values in figures are presented as mean ± SD. Statistical significance was calculated on the basis of the Student t test (two-tailed) or log-rank test (Kaplan–Meier survival analysis), and the level of significance was set at P < 0.05. For the mouse study, statistical analysis was carried out using SPSS V.13.0. The data were presented as mean ± SD of independent results. The significance of difference was assessed by ANOVA. Two-tailed nonparametric independent samples t tests were used for evaluating statistical significance of differences between groups. The Spearman rank correlation was used to investigate the correlation of the protein expression between p-cMet and p-IGFIR. Two-sided P values less than 0.05 were considered statistically significant.

Results

Cabozantinib enhances antitumor activities of abiraterone in prostate cancer both in vitro and in vivo

Given the need to improve upon the efficacy of abiraterone since it can prolong survival but does not cure mCRPC, we assessed whether cabozantinib could improve its efficacy. Cabozantinib both inhibits prostate cancer cells’ growth and blocks cancer-associated bone turnover (19) and is rational to combine with abiraterone. To explore whether cabozantinib also enhances abiraterone’s direct anticancer activity, we examined cell proliferation of three prostate cancer cell lines after abiraterone alone, cabozantinib alone, and in combination treatments. The cells studied were androgen dependent (LAPC4 and VCaP) and androgen independent (LAPC4-CR, Fig. 1A). As expected, abiraterone effectively inhibits the in vitro cell proliferation of androgen-dependent cell lines, VCaP and LAPC4, but also inhibits the androgen-independent LAPC4-CR cells in a dose-dependent manner with doses between 5 μmol/L and 10 μmol/L abrogating cell proliferation. Cabozantinib, like abiraterone, also decreases cell proliferation in all three cell lines in a dose-dependent manner. We then examined the effect of cabozantinib on cell proliferation combined with abiraterone at either relatively high or low concentrations. Our data indicated that the cabozantinib significantly enhances the inhibitory effect of abiraterone in vitro for most conditions studied (Fig. 1A and Supplementary Fig. S1A).

Furthermore, using the castration and abiraterone-resistant CRPC cell line LAPC4-CR xenograft mouse model, we demonstrated that abiraterone had minimal single-agent activity (see discussion), cabozantinib also had some single-agent activity but the most durable suppression of xenograft growth was seen when abiraterone and cabozantinib were combined (Fig. 1B). Mice treated with the combination of cabozantinib and abiraterone also had smaller mean tumor volume than cabozantinib-treated mice throughout the whole study. The difference was significant when comparing the combination treatment with that of abiraterone monotherapy (P < 0.05 after day 10). All treatments did not affect the weight of each treatment group (Supplementary Fig. S1B).

Cabozantinib’s impact on the activity of abiraterone on the AR axis

Given abiraterone’s ability to inhibit androgen synthesis and decrease AR activity, we assessed cabozantinib’s impact on abiraterone’s ability to alter AR activity. We found that AR and PSA protein levels in the androgen-dependent cell lines, LAPC4 and VCaP, were further decreased when treated by the combination of high doses of cabozantinib with abiraterone, especially the AR level in VCaP and PSA level in LAPC4 (Fig. 2A). We also examined the expression levels of AR and several AR-regulated genes affected by treatments with cabozantinib and abiraterone at the mRNA level and noted cell line-specific changes (Fig. 2B). Overall, cabozantinib does not abrogate abiraterone-mediated gene changes of the AR pathway and in some cases, augments those changes. For example, mRNA level of PSA in VCaP is decreased by both cabozantinib and abiraterone as single treatments and is further decreased by the combination. To further characterize the effect of cabozantinib and abiraterone on the androgen–AR axis, we performed a DHT (dihydrotestosterone) induction experiment after cabozantinib or abiraterone treatment (Fig. 2C). We found that DHT rescues cabozantinib and abiraterone suppression of AR-regulated genes. In short, cabozantinib does not antagonize abiraterone’s activity on the AR axis and in many instances, it enhances the suppression or activation activity of abiraterone in a cell line–dependent manner.

Cabozantinib inhibits abiraterone-induced phosphorylation of IGFIR and its downstream targets in prostate cancer

Cabozantinib was designed as an inhibitor of receptor tyrosine kinases, including cMet, VEGFR2, RET, and Axl (20). We investigated which tyrosine kinases were directly impacted by cabozantinib in prostate cancer cell lines using Human Phospho-
Cabozantinib Enhances Anticancer Activity of Abiraterone

Receptor Tyrosine Kinase Array Kit (Fig. 3A and Supplementary Fig. S2). Interestingly, cMet (HGF-R) was only detected as a cabozantinib target in PC3 cells. Phosphorylation of VEGFR2 was not detected in any of the four prostate cancer cell lines (but maybe detectable in tumor-associated angiogenesis in microenvironment). In addition, phosphorylation of Axl in PC3 and EGF-R in LAPC4 and LAPC4-CR was detectable and in all cases, the phosphorylation was decreased by cabozantinib. EGF-R was also detectable in PC3 and VCaP; however, the phosphorylation level of EGF-R was unchanged in PC3 or increased in VCaP after cabozantinib treatment, suggesting that the impact of cabozantinib on EGF-R phosphorylation is context dependent. More importantly, we found that the phosphorylation of members of the insulin family receptors, including insulin-R and IGFIR, was detectable and decreased by cabozantinib in three prostate cancer cell lines (LAPC4, VCaP, and PC3).

To validate the observation that cabozantinib can inhibit the phosphorylation level of IGFIR, we examined the activity of cabozantinib in the LAPC4 cell line. (Fig. 3A and Supplementary Fig. S2). After treated by IGF at different time-points, the total level of IGFIR in cells was unchanged and the phosphorylation level of IGFIR (p-IGFIR at sites Tyr1135/1136) was induced at 5 minutes of IGF treatment. The Western blot analysis clearly showed that cabozantinib inhibits the level of p-IGFIR in the LAPC4-CR xenograft mouse. Tumor volume was measured twice weekly after start of treatment when tumors reached approximately 80 to 250 mm³ (average mean per group ~150 mm³). Mice were sacrificed when tumors reached 2 cm in any dimension. Viruses conditions for four groups (n = 8 per group) are as follows: vehicle (5% benzyl alcohol + 95% safflower oil); cabozantinib at 30 mg/kg, abiraterone at 0.5 mg/d, and combination of cabozantinib with abiraterone. Student t test was used to analyze the data through day 17, the last day on which all the animals in each group remained on study.

Figure 1.
Cabozantinib enhances the inhibitory activities of abiraterone for prostate cancer growth in prostate cancer cell lines and in xenograft mouse model. A, cell growth rates were plotted by measuring the relative cell numbers compared with that of day 0 for LAPC4, LAPC4-CR, and VCaP. All cells were treated by different concentrations of cabozantinib (cabo or C) or abiraterone (Abi or A) or combinations. Each point represents the average of three replicates with SD. B, cabozantinib or abiraterone treatment for the tumor volume in LAPC4-CR xenograft mice. Tumor volume was measured twice weekly after start of treatment when tumors reached approximately 80 to 250 mm³ (average mean per group ~150 mm³). Mice were sacrificed when tumors reached 2 cm in any dimension. The treatment conditions for four groups (n = 8 per group) are as follows: vehicle (5% benzyl alcohol + 95% safflower oil); cabozantinib at 30 mg/kg, abiraterone at 0.5 mg/d, and combination of cabozantinib with abiraterone. Student t test was used to analyze the data through day 17, the last day on which all the animals in each group remained on study.

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downstream targets of IGFIR, such as Akt, MEK1/2, and ERK1/2. Compared with the nontreatment control, we found that cabozantinib only significantly decreased the level of p-IGFIR and p-Akt, whereas the low levels of p-MEK1/2 and p-ERK1/2 were not substantially effected by cabozantinib treatment (Fig. 3D). Interestingly, we observed that abiraterone increases the level of p-IGFIR as well as the levels of p-MEK1/2 and p-ERK1/2 but decreases the level of p-Akt. In other words, abiraterone treatment in LAPC4 cell line may result in a compensatory activation of a survival pathway, the IGFIR signaling pathway with MEK1/2 and ERK1/2 activation. This suggests that this may be one of the mechanisms for abiraterone resistance in prostate cancer patients. Most interestingly, cabozantinib not only abolished p-IGFIR when combined with abiraterone, but also decreased the induction of higher levels of p-MEK1/2 and p-ERK1/2 with abiraterone treatment.

Figure 2.
Cabozantinib (C) does not block abiraterone (A) and in most situations augments abiraterone effect of the androgen-AR axis. A, AR and PSA expression levels were measured by Western blot analysis in LAPC4 and VCaP cells after treatment by different concentrations of cabozantinib and abiraterone for 48 hours. β-actin was included as a loading control. B, RT-PCR analysis of AR- and standard AR-regulated genes in LAPC4, LAPC4-CR, and VCaP cells after either single or combined treatments of cabozantinib and abiraterone for 48 hours. C, RT-PCR analysis of the effect of cabozantinib or abiraterone treatment on the DHT induction of standard AR-regulated genes in LAPC4 and VCaP cells. Cells were cultured in androgen-depleted culture medium (gFBS) with or without drug for 48 hours, then 5 nmol/L final concentration of DHT was added in culture medium for an additional 24 hours before total RNA extraction. Gene expression level at the condition of nontreatment (NT) was treated as 1. Each point represents the average of three replicates with SD.
Cabo\textsuperscript{z}antinib evidently inhibits the phosphorylation level of IGFIR enhanced by abiraterone in prostate cancer cells. A, the Human Phospho-Receptor Tyrosine Kinase Array detects tyrosine-phosphorylated receptors in LAPC4 cell lysis. LAPC4 cells were either untreated (NT, only DMSO) or treated by 5 \textmu m/L abiraterone (A), 5 \textmu m/L cabozantinib for 6 hours. Of note, 200 \textmu g of lysis was run on each array following the manufacturer's protocol, and data shown are from a 2-minute exposure to film. B, LAPC4 cells were cultured in serum-free culture medium for 24 hours and then treated by 5 ng/mL of IGF for the indicated time. Total IGFIR and phospho-level (Ty1135/1136) of IGFIR (P-IGFIR) in cell lysis were detected by Western blot analysis. C, LAPC4 cells were cultured in serum-free culture medium for 18 hours, followed by 0.5 or 5 \textmu m/L of cabozantinib treatment for 6 hours. Cells were then treated by 5 ng/mL of IGF for 1 hour before collecting the cell lysis. \textbeta -Actin was included as a loading control. D, total and phospho-proteins of IGFIR, MEK1/2, ERK1/2, and AKT were measured by Western blot analysis in LAPC4 cells either untreated (NT) or treated by 2.5 \textmu m/L abiraterone (A), 5 \textmu m/L cabozantinib (C), or 2.5 \textmu m/L abiraterone plus 5 \textmu m/L cabozantinib (A+C) for 6 hours.

Assessment of residual-resistant xenograft tumors after prolonged \textit{in vivo} treatment

Having noted abiraterone’s compensatory upregulation of p-IGFIR, we then assessed the expression of the residual-resistant tumors in the \textit{in vivo} LAPC4-CR xenograft model using IHC. IHC for p-cMet and p-IGFIR showed membrane, cytoplasmic, and nuclear staining in tumor cells (Fig. 4A). The expression level of total p-cMet and p-IGFIR were both increased in residual resistant tumors after treatment with abiraterone alone, cabozantinib alone, and the combination of abiraterone with cabozantinib, compared with the untreated control group (Supplementary Fig. S3A and S3B). Other key findings are cells viable after abiraterone alone had a modest increase in membrane, cytoplasmic, and total p-cMet but no change in p-IGFIR in any one specific cellular location. In contrast, the viable cells after cabozantinib as a single agent or in combination with abiraterone had an increase in p-cMet and p-IGFIR in all cellular locations compared with control and abiraterone as a single agent (Fig 4B and Supplementary Fig. S3C). These data are opposite to those seen after short-term \textit{in vitro} exposure, and it is proposed that the dynamics of the resistance mechanisms after long-term \textit{in vivo} setting evolve and is discussed in more detail in the discussion. In addition, we found a significant positive correlation between protein levels of p-cMet and p-IGFIR in all four treatment groups (Supplementary Fig. S3D).

Cabo\textsuperscript{z}antinib has the advantage of inhibiting prostate cancer cell proliferation combined with abiraterone by simultaneously targeting multiple receptor tyrosine kinases

Given the observation that cabozantinib enhanced the antitumor activity of abiraterone in all three prostate cancer cell lines and in the xenograft LAPC4-CR tumor and showing it inhibits the level of p-IGFIR in this study as well as the p-cMet and p-VEGFR2 in a cell line–dependent manner (20), we compared it with other specific tyrosine kinase inhibitors, including crizotinib (cMet inhibitor), sunitinib (VEGFR2 inhibitor), and NVP-AEW541 (IGFIR inhibitor). When treated by 2.5 \textmu m/L abiraterone combined with each inhibitor at the concentration of IC\textsubscript{50} (Fig. 5A and Supplementary Fig. S4), cell proliferation of LAPC4 was inhibited under each condition (Fig. 5B). However, cabozantinib exhibited the greatest effect. Similar results were obtained for the other two cell lines (data not shown). These data suggest that the spectrum of tyrosine kinases inhibited by cabozantinib, including IGFIR, cMet, and VEGFR2, could underlie the more pronounced ability of cabozantinib to enhance abiraterone. To further investigate the activity of simultaneously inhibition of IGFIR, cMet, and VEGFR2 in prostate cancer, we examined the cell proliferation affected by NVP-AEW541 combined with either single inhibitor of crizotinib or sunitinib or both (Fig. 5C). We choose IC\textsubscript{50} not IC\textsubscript{50} in this analysis to reduce the toxicity because the cells died very quickly if treated by combined drugs at IC\textsubscript{50}. IGFIR inhibitor combined with that of a cMet inhibition has a greater inhibition for the cell proliferation of LAPC4, LAPC4-CR, and VCap than that of the combination with VEGFR2 inhibition. IGFIR inhibitor combined with VEGFR2 inhibitor had no additional effect in LAPC4-CR. Notably, the greatest effect was always achieved when cells were treated with all three inhibitors. It suggests that different types of prostate cancer cells possess distinct responses for the combination of various inhibitors and cabozantinib has the advantage of inhibiting prostate cancer cell proliferation by simultaneously targeting cMet, VEGFR2, and IGFIR receptor tyrosine kinases (and potentially others) to enhance the antitumor activity of abiraterone.

\textbf{Discussion}

In this study, we present data to support the use of cabozantinib with abiraterone for treatment of prostate cancer. This paper also describes abiraterone’s compensatory upregulation of p-IGFIR and activation of the downstream survival pathway (p-MEK and p-ERK) and cabozantinib’s ability to inhibit phosphorylation of IGFIR as well as abiraterone-induced phosphorylation of MEK and ERK. In short, the work suggests the cabozantinib blocked one of the abiraterone’s short term compensatory adaptive resistance mechanisms.
The *in vivo* LAPC4-CR is a CRPC model that develops rapid resistance to abiraterone. As can be seen, cabozantinib has single-agent activity and more activity is seen when cabozantinib is combined with abiraterone. It is notable that the LAPC4-CR in *in vivo* is a completely androgen-independent prostate cancer cell line and had undergone two passages in mice in our experiments. Therefore, it is probable that the cellular processes may have already been changed to produce an abiraterone-resistant model. Moreover, it is clearly a robust model of resistance to hormonal therapies and supports the notion that abiraterone and cabozantinib is a regimen worthy of assessment in clinical trials, especially in view of its ability to inhibit VEGF and cMet tyrosine kinase receptors. In this paper, we screened the spectrum of the tyrosine kinases to its ability to inhibit VEGF and cMet tyrosine kinase receptors. In this paper, we screened the spectrum of the tyrosine kinases.

The IHC data of the residual tumors from the xenograft after long-term *in vivo* exposure provide some clues for the drivers of resistance. Notably, there was some upregulation of p-cMet in the abiraterone alone treated cells implicating this as a possible driver of resistance in this setting, which may have been blocked by cabozantinib and resulted in the enhanced activity with the combination in the cells that responded and were growth-suppressed for the long term. The lack of increase in p-IGFIR in the abiraterone alone treated cells in *vivo* (contrary to the *in vitro* data) may be due to it being a short-term self-limited event that supports the cells until other mechanisms take over. Notably, although cabozantinib is well characterized to be an inhibitor of p-cMet and our data indicate that it inhibits p-IGFIR *in vitro*, the residual tumors after long term have upregulation of both p-cMet and p-IGFIR. This implies part of the resistance mechanisms to cabozantinib and the residual viable cells have cellular alterations that bypass cabozantinib tyrosine kinase inhibitor effects and allows the cells to survive by upregulation of the pathways under the control of these receptors.

Figure 4.
Assessment of residual-resistant xenograft tumors after prolonged *in vivo* treatment. A, represents images (×400) of the expression of phospho-Met and phospho-IGFIR in the four prostate cancer treatment groups. A–D show IHC staining of p-cMet, and E–H for p-IGFIR. Tumors with a high level of p-cMet (C and D) or p-IGFIR (G and H) are represented after treatment with cabozantinib (Cabo) or a combination of abiraterone (Abi) with Cabo (Comb), compared with the untreated control group (A and E). High-power images (×400) are presented on the top right of each condition. B, *P* values for multivariate analysis of p-cMet and p-IGFIR expression in different treated LAPC4-CR-derived prostate cancer tissue groups. Two-sided *P* values were calculated by independent samples t test. *P* values < 0.05 are shown in bold.
cancer cells acquire metastatic capability (33–35). However, some studies showed no difference between IGFIR levels in normal prostate tissue and prostate cancer and even showed decreased levels of IGFIR in prostate cancer at the metastatic stage (36–39).

We assume that the active form of IGFIR, p-IGFIR, plays a more important role than that of total IGFIR itself, which might be the reason for the conflicting results for IGFIR levels in prostate cancer. Induction of phosphorylation of IGFIR by the conditioned medium of PrSC-inoculated immunodeficient mice dramatically increases the growth of LNCaP and DU-145, and an IGFIR inhibitor (crizotinib) decreases growth of LNCaP and DU-145.

**Figure 5.**
Cabozaantinib is a better candidate to enhance the antitumor activity of abiraterone. A, IC50 and IC20 of cMet inhibitor (crizotinib), VEGFR2 inhibitor (sunitinib), IGFIR inhibitor (NVP-AEW541), and cabozaantinib for growth inhibition of various prostate cancer cells (LAPC4, LAPC4-CR, and VCaP) after treatment for 4 days. B, the effect of different inhibitors (IC50) combined with 2.5 μmol/L abiraterone on the growth inhibition of LAPC4 cells. Relative cell numbers were measured at different times, and each point represents the average of three replicates with SD. C, responses are different for various prostate cancer cells with different combinations of inhibitors among IGFIR, cMet, and VEGFR2. Ii (IGFIR inhibitor, NVP-AEW541); Mi (cMet inhibitor, crizotinib); Vi (VEGFR2 inhibitor, sunitinib). The number following the two letters in the legend is the concentration used in the treatment, which is the IC20. D, study model indicates that cabozaantinib inhibits the enhanced phosphorylation level of IGFIR induced by the treatment of abiraterone and improves the efficacy of abiraterone treatment for CRPC patients.
inhibitor abolished the PrSC conditioned media-induced cell growth (40). This suggests that IGFIR function should be evaluated by assessing p-IGFIR levels. In addition, our p-IGFIR xenograft mouse tissue immunostaining data are consistent with previous studies by Aleksic and colleagues (26) that cell surface IGFIR can translocate to the nucleus of human prostate cancer cells and result in nuclear IGFIR phosphorylation. This suggests that nuclear p-IGFIR might enhance the transcriptional regulation in prostate cancer. Therefore, significant inhibition of p-IGFIR level by cabozantinib could be an important antitumor character for cabozantinib in prostate cancer, either alone or in combination with abiraterone.

Improving the antitumor activity of abiraterone could greatly benefit CRPC patients. Having compared cabozantinib with other multi receptor tyrosine kinase inhibitors with combinations cMet and/or VEGFR2 and/or IGFIR inhibitory attributes, cabozantinib had the most robust profile. This is presumably due to the spectrum of tyrosine kinases, including IGFIR, inhibited by cabozantinib and resultant blocking of relevant targets in the varied cellular and genomic backgrounds in the prostate cancer cell lines tested. In addition, most patients taking abiraterone also receive glucocorticoids, which could further suppress androgen synthesis (41). However, glucocorticoids could induce immunosuppression to facilitate cancer progression in patients (42). Recent studies have shown that cabozantinib can render tumor cells more sensitive to immune-mediated killing and generate a more permissive immune environment (43, 44), which suggests that cabozantinib can benefit prostate cancer patients when taking abiraterone with glucocorticoids through modulation of the immune system.

In summary, we have demonstrated that cabozantinib has single-agent activity and can enhance the antitumor activity of abiraterone in prostate cancer and that part of the underlying mechanism is by inhibition of p-IGFIR induced by abiraterone. Although a study has shown that cabozantinib did not improve overall survival as a single agent (17), it does have activity by delaying progression and causing regression of CRPC in some patients. As such, there is still the potential to improve prostate cancer care by enhancing abiraterone efficacy, and a phase I trial is ongoing that has shown the combination is viable for further testing (45).

Disclosure of Potential Conflicts of Interest

P.W. Kantoff has ownership interest (including patents) in Exelixis. C.J. Sweeney reports receiving other commercial research support from Exelixis and is a consultant/advisory board member for Janssen. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wang, Y. Huang, M. Bowden, G.-S.M. Lee, P.W. Kantoff, C.J. Sweeney
Writing, review, and/or revision of the manuscript: X. Wang, Y. Huang, M. Bowden, G.-S.M. Lee, P.W. Kantoff, C.J. Sweeney
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Cabozantinib Enhances Anticancer Activity of Abiraterone

Cabozantinib Inhibits Abiraterone's Upregulation of IGFIR Phosphorylation and Enhances Its Anti-Prostate Cancer Activity

Xiaodong Wang, Ying Huang, Amanda Christie, et al.


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