CCI-779 Inhibits Cell-Cycle G2–M Progression and Invasion of Castration-Resistant Prostate Cancer via Attenuation of UBE2C Transcription and mRNA Stability

Hongyan Wang1,4, Chunpeng Zhang2,4, Anna Rorick2,4, Dayong Wu2,4, Ming Chiu1, Jennifer Thomas-Ahner3,4, Zhong Chen2,4, Hongyan Chen2,4, Steven K. Clinton3,4, Kenneth K. Chan1,4, and Qianben Wang2,4

Abstract

The cell-cycle G2–M phase gene UBE2C is overexpressed in various solid tumors including castration-resistant prostate cancer (CRPC). Our recent studies found UBE2C to be a CRPC-specific androgen receptor (AR) target gene that is necessary for CRPC growth, providing a potential novel target for therapeutic intervention. In this study, we showed that the G1–S cell-cycle inhibitor-779 (CCI-779), an mTOR inhibitor, inhibited UBE2C mRNA and protein expression in AR-positive CRPC cell models ab1 and C4-2B. Treatment with CCI-779 significantly decreased abl cell proliferation in vitro and in vivo through inhibition of cell-cycle progression of both G2–M and G1–S phases. In addition, exposure of abl and C4-2B cells to CCI-779 also decreased UBE2C-dependent cell invasion. The molecular mechanisms for CCI-779 inhibition of UBE2C gene expression involved a decreased binding of AR coactivators SRC1, SRC3, p300, and MED1 to the UBE2C enhancers, leading to a reduction in RNA polymerase II loading to the UBE2C promoter, and attenuation of UBE2C mRNA stability. Our data suggest that, in addition to its ability to block cell-cycle G1 to S-phase transition, CCI-779 causes a cell-cycle G2–M accumulation and an inhibition of cell invasion through a novel UBE2C-dependent mechanism, which contributes to antitumor activities of CCI-779 in UBE2C overexpressed AR-positive CRPC. Cancer Res; 71(14): 4866–76. ©2011 AACR.

Introduction

The androgen receptor (AR), a ligand-dependent transcription factor, is expressed in both androgen-dependent prostate cancer (ADPC) and castration-resistant prostate cancer (CRPC; refs. 1, 2). One important function of AR in prostate cancer is to drive cell-cycle progression (3). Although it is well known that AR mainly regulates cell-cycle G1 to S-phase transition in ADPC through AR-dependent regulation of CCND1, p21, and p27 (3), recent integrated analysis of AR cistrome and gene expression data in prostate cancer found that AR selectively binds to the enhancers of G2–M phase genes in CRPC but not in ADPC, leading to higher G2–M phase gene expression and accelerated cell-cycle G2–M progression in CRPC versus ADPC (4, 5).

One of such AR-regulated G2–M phase genes in CRPC is UBE2C, a gene whose translation product is an anaphase-promoting complex/cyclosome (APC/C)-specific E2 ubiquitin-conjugating enzyme (6). Significantly, UBE2C mRNA and protein expression levels are overexpressed in CRPC cases (4, 7, 8). Consistent with the essential role of UBE2C in driving M-phase cell-cycle progression by inactivating the M phase checkpoint (9) or increasing the pool of active APC/C (10), silencing of UBE2C in CRPC cells arrests cell cycle in G2–M phase and decreases CRPC cell proliferation, suggesting that UBE2C is a potential therapeutic target in CRPC (4).

In this study, we screened several clinically active compounds for their ability to decrease UBE2C expression. CCI-779 (cell-cycle inhibitor-779; temsirolimus), an ester analogue of mTOR inhibitor rapamycin currently under clinical evaluation (11), emerged from screening to have significant efficacy and potency in inhibition of UBE2C protein and mRNA expression in AR-positive CRPC cell lines ab1 and C4-2B. Although previous studies found that mTOR inhibitors, including CCI-779, decrease the growth of cancer cell lines (e.g., AR-negative CRPC cell lines PC-3 and DU-145) via G1 to S-phase inhibition (12, 13), we show that CCI-779 inhibits the abl in vitro and in vivo growth by blocking both cell-cycle G2–M and G1–S transitions. Consistent with the newly identified role of UBE2C in promoting tumor invasion and metastasis.
(14–16), we found that CCI-779 treatment decreases UBE2C-dependent cell invasion of abl and C4-2B cells. Finally, we found that the combined effects on attenuating UBE2C transcription and mRNA stability of CCI-779 lead to decreased mRNA levels of UBE2C. Collectively, this study identifies CCI-779 as a UBE2C inhibitor in CRPC.

Materials and Methods

Reagents and cell culture

CCI-779 (temsirolimus) was purchased from LC Laboratories. LNCaP cells were obtained from the American Type Culture Collection (ATCC) and C4-2B cells were purchased from ViroMed Laboratories. LNCaP and C4-2B cells were cultured in RPMI-1640 media (Invitrogen) supplemented with 10% FBS and authenticated by the suppliers. abl cells, an androgen-independent derivative of the LNCaP cell line, were kindly provided by Zoran Culig (Innsbruck Medical University, Innsbruck, Austria) and authenticated by Culig Laboratory, using AR sequence analysis, cytogenetic analysis, and comparative genome hybridization analysis (17). The abl cells were maintained in RPMI-1640 media containing 10% charcoal-stripped FBS. All 3 cell lines were passaged in our laboratory for less than 6 months after resuscitation.

Western blot

Cells or tumor tissues were collected and lysed as previously described (18). The total lysate sample (50 μg per lane) was resolved by SDS-PAGE and immunoblotted with primary antibodies. Antibodies against various proteins were purchased from the following sources: anti-UBE2C (A650) from Boston Biochem; anti-AR (441), anti-GATA2 (H116), anti-SRC1 (M341), anti-p300 (C20), and anti-MED1 (M255) from Santa Cruz Biotechnology; anti-CCND1 (ab24249) and anti-FoxA1 (M341), anti-p300 (C20), and anti-MED1 (M255) from Santa Cruz Biotechnology; anti-CCND1 (ab24249) and anti-FoxA1 (ab23738) from Abcam; anti-calnexin from Stressgen, and anti-β-actin from Sigma-Aldrich. An anti-SRC3 antibody has been described previously (19).

Real-time reverse transcriptase PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). Real-time reverse transcriptase PCR (RT-PCR) was conducted on 100 ng of RNA by using MultiScribe Reverse Transcriptase (Applied Biosystems), according to the manufacturer's instructions. The following primers were used: UBE2C (5'-TGGTCTGCCCTGTATGATGT-3' and 5'-AAAAGCTGTGGGGTTTTCC-3'; ref. 20); CCND1 (5'-TCCCTCTCAAATGCAGAG-3' and 5'-GGGGATTG-GAAATGACCTT-3'); GAPDH (5'-TCCACCCATGGCAAAATTCC-3' and 5'-TCCACCTGTGGATTTTGG-3'; ref. 19), and actin (5'-AGGCACAGGGGTGAT-3' and 5'-GCCACATAGGAA-TCTCTGAC-3'; ref. 21).

RNA interference

ON-TARGETplus siRNAs targeting CCND1 and UBE2C (siCCND1 and siUBE2C) and ON-TARGETplus control siRNA (siControl) were purchased from Dharmacon. siRNAs were transfected using Lipofectamine 2000 (Invitrogen).

Synchronization and fluorescence-activated cell-sorting analysis

Cells were arrested in G2–M phase by using a thymidine-nocodazole block as previously described (22). Briefly, cells were first synchronized by arresting them at the G1–S border with 2 mmol/L thymidine for 24 hours, followed by a 4-hour release and then cells were arrested at M phase with 100 ng/mL nocodazole for 12 hours. CCI-779 (50 nmol/L) or vehicle control was added at the same time as nocodazole. The cells were released from the nocodazole block with 2 washes of fresh medium and allowed to progress to G1 phase. Cells were collected after the release (2 hours for abl cells, 1 hour for C4-2B cells, and 1.5 hours for LNCaP cells), stained with propidium iodide (PI; Sigma) and subjected to analysis by using a FACSCalibur cell flow cytometer (Becton Dickinson Biosciences). Fluorescence-activated cell-sorting (FACS) analysis was also done on unsynchronized cells after 13 hours of exposure to 50 nmol/L CCI-779.

Cell proliferation assay

Cell proliferation was measured by WST-1 (4-[3-(4-Iodo-phenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1-benzene disulfonate) assay according to the manufacturer's instruction (Roche). Briefly, this assay entails the addition of 10 μL WST-1 reagents per 100 μL cell cultures in a 96-well plate. These cultures were incubated for 30 minutes and the absorbance at 450 nm was determined by an ELISA Microplate Reader (Bio-Rad).

Xenograft model

Male, 6-week-old, Balb/c athymic nude mice were obtained from Charles River Laboratory and acclimated for 1 week in a pathogen-free enclosure before start of study. All experiments were conducted in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). abl cells (2 × 10^6 cells/flank) were suspended in 50% Matrigel (Becton Dickinson) and subcutaneously inoculated bilaterally into the flanks of mice, monitored daily, and tumor size was quantified with calipers twice a week (17, 23). When tumors had grown to 100 mm^3, treatments were initiated. Mice were randomly assigned into 2 cohorts with 10 mice per group. CCI-779 (treated group) or vehicle solution [5% Tween 80 (Sigma) and 5% polyethylene glycol 400 (Sigma); ref. 13; control group] was given intraperitoneally (i.p.) at the dose of 10 mg/kg for 4 consecutive days per week (24). The injection volume was 0.1 mL/10 g body weight. Tumor volume was calculated by using the standard formula: V = length × width^2 × 0.5. Body weight was also monitored biweekly. After 4 weeks, mice were euthanized and tumor tissues were weighed and subjected to Western blot analysis.

Transfection and invasion assay

Cells grown in 6-well plates were transfected with siUBE2C or siControl, or 2 μg of pCS2-myc-UBE2C (kindly provided by Michael Rape, University of California, Berkeley) or a control pCS2-myc vector (a gift from David Turner, University of Michigan), using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, 4 × 10^5 cells were seeded on the
Matrigel-coated filters (BD BioCoat BD Matrigel Invasion Chamber; Becton Dickinson) in the upper chamber, which was filled with media containing 0.1% FBS. The lower chamber was filled with media supplemented with 10% FBS. Both chambers were treated with 50 nmol/L CCI-779 or vehicle. The cells were allowed to invade for 48 hours. The cells on the underside of the filter were then fixed with 80% methanol, stained with 0.3% crystal violet, and counted using a light microscope. The invasion results were normalized by cell proliferation under the same treatment conditions.

Chromatin immunoprecipitation and ReChIP
Chromatin immunoprecipitation (ChIP) was done as previously described (19). The antibodies used were: anti-AR (N20), anti-GATA2 (H116), anti-SRC1 (M341), anti-SRC3, anti-p300 (C20), anti-MEDI (M255) from Santa Cruz Biotechnology; anti-FoxA1 (ab23738) from Abcam; and anti-RNA pol II (8WG16) from Covance. SRC3 ChIP was done with SRC3 antibodies as previously described (19). The ChIP-enriched DNA was then quantified by quantitative PCR (qPCR) using specific primers for the UBE2C enhancers 1 and 2 (4) and the UBE2C promoter, respectively. Negative controls used were sequences containing androgen-responsive elements (ARE) that do not have an actual binding to AR and FoxA1 (4). The primer sequences used in Chip assay were as follows: UBE2C enhancer 1 (5′-TGCCCTTGAAGTAAAGCTGAGTG-3′ and 5′-TCGTCTTCTCATAGTGCAAG-3′); UBE2C enhancer 2 (5′-CCCAAACTCTCTAGTGAGTG-3′ and 5′-CTCTTTCCTCTCCCTTACCC-3′); UBE2C promoter (5′-GGGGGAGATTCGATCTGGGAGACT-3′); control ARE region 1 (5′-CCACAATTCCAATGTTGCGCTCTT-3′ and 5′-GTGCTGTACCATGGATCTCAGGATG-3′), and control ARE region 2 (5′-GCTGATT-CATTACCTCCCCAGA-3′ and AGTTGGGACAGACGGGAAA-3′). ReChIP assays were carried out as previously described (25).

mRNA stability assay
abl cells were treated with 50 nmol/L CCI-779 or vehicle. Simultaneously, 5 μg/mL actinomycin D (Sigma) was used to block mRNA synthesis. Cells were collected at various time points (0, 6, 9, 12, 18, and 24 hours) after treatment and UBE2C mRNA level was then quantified by quantitative reverse transcriptase PCR (qRT-PCR). The t1/2 was calculated using the iterative curve-fitting software SigmaPlot (SPSS) by fitting 4-parameter exponential decay curves described by the formula y = a * e^(-x/b) + d. Estimations of c, designated as C, and corresponding standard errors, designated as SE(C), were used to calculate standard error (t1/2 = log(2)/SE(t1/2)). C4-2B cells (Fig. 1A and B). Interestingly, the inhibitory effect of CCI-779 on UBE2C protein and mRNA levels was also seen in an ADPC cell line LNCaP, although the effect was less effective than that observed in abl and C4-2B cells (Fig. 1A and B). Consistent with previous studies showing that the mTOR pathway is required for translation of mRNAs of critical G1 phase cell-cycle genes such as CCND1 (12), we found that treatment of abl, C4-2B, and LNCaP cells with CCI-779 significantly reduced CCND1 protein, but not mRNA, expression level (Fig. 1A and B). Thus, CCI-779 decreases protein expression levels of both UBE2C and CCND1, as well as UBE2C mRNA expression level in prostate cancer cells.

To further investigate whether CCI-779–mediated decrease in UBE2C mRNA expression was dependent on CCI-779–induced reduction in CCND1 protein expression, we examined the effect of CCI-779 on UBE2C mRNA level in CCND1-silenced and control-silenced abl cells. Silencing of CCND1 caused a complete cell-cycle G1 arrest, which was barely enhanced by CCI-779 treatment (Fig. 1C and D). Significantly, treatment of abl cells with CCI-779 decreased UBE2C mRNA level in abl cells already arrested in G1 phase (Fig. 1D). These results indicate that CCI-779 can directly decrease UBE2C mRNA expression in a CCND1 expression and G1 arrest independent manner. We obtained essentially similar results in C4-2B cells (Supplementary Fig. S1).

CCI-779 blocks both G2–M and G1–S cell-cycle progression and decreases cell proliferation in CRPC and ADPC cells
Because UBE2C plays an essential role in promoting G2–M phase cell-cycle progression in prostate cancer cells (4) and CCI-779 inhibited UBE2C expression (Fig. 1), we next examined the effect of CCI-779 on G2–M phase cell-cycle progression. abl, C4-2B, and LNCaP cells were synchronized to G2–M phase by using a thymidine–nocodazole block and then released for 1 to 2 hours. As shown in Figure 2A, whereas treatment of cells with CCI-779 had no effect on G2–M synchronization, CCI-779 treatment led to an increase in the G2–M phase and a decrease in the G1 phase after releasing from G2–M synchronization, suggesting that CCI-779 markedly delayed G2–M to G1 transition in all 3 cell lines. Consistent with the functional role of CCI-779 in decreasing CCND1 protein expression level (Fig. 1A) and a recent study showing that CCI-779 arrests prostate and breast cancer cells in G1 phase (24), CCI-779 blocked unsynchronized abl, C4-2B, and LNCaP cells in G1 phase (Fig. 2B). The inhibition of CCI-779 on G2–M and G1–S cell-cycle progression was correlated with a significantly decreased cell proliferation of abl, C4-2B, and LNCaP (but more notably abl and C4-2B cells; Fig. 2C).

Results
CCI-779 downregulates UBE2C protein and mRNA expression levels in CRPC and ADPC cells
Our initial compound screenings for identification of UBE2C inhibitors were carried out on abl cells. As a CRPC cell model, abl mimics the clinical properties of a significant proportion of CRPC cases. For example, recent studies reporting that AR upregulates cell-cycle genes (e.g., UBE2C, CDC20, and CDK1) in abl cells mimic the pattern of upregulated genes observed in human CRPC versus ADPC cases (4, 7, 8). CCI-779 emerged from screening because it potently decreased both protein and mRNA levels of UBE2C in abl cells (Fig. 1A and B). We further extended our study to another CRPC cell model C4-2B that overexpresses the AR (26). We confirmed that UBE2C protein and mRNA levels were significantly decreased in CCI-779–treated C4-2B cells (Fig. 1A and B). Interestingly, the inhibitory effect of CCI-779 on UBE2C protein and mRNA levels was also observed in an ADPC cell line LNCaP, although the effect was less effective than that observed in abl and C4-2B cells (Fig. 1A and B). Consistent with previous studies showing that the mTOR pathway is required for translation of mRNAs of critical G1 phase cell-cycle genes such as CCND1 (12), we found that treatment of abl, C4-2B, and LNCaP cells with CCI-779 significantly reduced CCND1 protein, but not mRNA, expression level (Fig. 1A and B). Thus, CCI-779 decreases protein expression levels of both UBE2C and CCND1, as well as UBE2C mRNA expression level in prostate cancer cells.

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To further delineate the role of UBE2C in CCI-779–mediated inhibition of CRPC cell proliferation, the effects of CCI-779 on cell proliferation of UBE2C-silenced or control-silenced abl and C4-2B cells were examined. The inhibitory effect of CCI-779 on abl cell proliferation was markedly decreased in UBE2C-silenced (28%) versus control-silenced (45%) abl cells (Fig. 2D), suggesting that UBE2C-silencing-mediated abl cell growth inhibition (Fig. 2D) significantly contributes to growth-inhibitory effect of CCI-779 on abl (Fig. 2C). In contrast, as UBE2C silencing only slightly decreased C4-2B cell proliferation (Fig. 2D), CCI-779–mediated inhibition of C4-2B cell proliferation (Fig. 2C) was presumed to be mostly due to CCI-779-induced decreased expression of CCND1 rather than UBE2C in C4-2B cells (Figs. 1A and 2D).

**CCI-779 inhibits in vivo growth of abl xenograft through downregulation of UBE2C and CCND1**

We further extended our studies to an *in vivo* xenograft model to validate the significance of our *in vitro* findings. Approximately 2 weeks after the inoculation of abl cells, mice were treated with CCI-779 (10 mg/kg, i.p.) for 4 consecutive days weekly for 4 weeks. Mice generally tolerated CCI-779 without showing any apparent toxicity throughout the experiment. No significant difference in body weight was observed between groups after the 4-week period (data not shown).

![Figure 1. Effects of CCI-779 on UBE2C and CCND1 expression in CRPC and ADPC cells.](image)
Remarkably, the tumor growth was inhibited by CCI-779 even after 1 week of treatment. By the end of the study, the tumor volume dramatically decreased from 234 ± 33 mm$^3$ in control group to 57 ± 4 mm$^3$ in CCI-779–treated group (Fig. 3A and B). In addition, there was no measurable tumor in 4 of 10 CCI-779–treated mice. Tumor weight measurement further supported our findings, as the average value was 78.6 ± 15.3 mg for control group as compared with 13.5 ± 2.5 mg for the CCI-779 group (Fig. 3C). More importantly, Western blot analysis confirmed that levels of both UBE2C and CCND1 protein were significantly decreased in tumor tissues following treatment with CCI-779 (Fig. 3D). These data suggest that CCI-779 significantly decreases CRPC cell in vivo growth through inhibition of UBE2C and CCND1. Similar effect of CCI-779 on CRPC cell growth and protein expression of CCND1 and UBE2C was observed in castrated mice (Supplementary Fig. S2).

**CCI-779 inhibits UBE2C-dependent CRPC cell invasion in vitro**

The role of UBE2C is not limited to promoting cell growth. Recent studies have found that UBE2C expression is positively correlated with metastasis in patients with various cancer types, including colorectal cancer (14), breast cancer (15), and soft tissue tumors (16). Consistent with these clinical observations, an in vitro study has shown that UBE2C downregulation...
and overexpression decreases and increases invasiveness of a human colon cancer cell line HT-29, respectively (14). To investigate whether UBE2C expression affects abl, C4-2B, and LNCaP cell invasion, we transfected a siRNA targeting UBE2C (siUBE2C), a control siRNA (siControl), UBE2C vector, or a control vector in all 3 cell lines (Fig. 4A) followed by Matrigel invasion assays. Control vector- or siControl-transfected abl and C4-2B cells were significantly more invasive than similarly transfected LNCaP cells (Fig. 4B and C; Supplementary Fig. S3). Interestingly, silencing of UBE2C significantly decreased, whereas overexpression of UBE2C significantly increased, invasiveness of abl and C4-2B but not LNCaP cells (Fig. 4B and C; Supplementary Fig. S3), suggesting that UBE2C is necessary for CRPC cell invasion but not sufficient for ADPC cell invasion. It is possible that additional invasion-related proteins are required for UBE2C to mediate ADPC cell invasion. We next examined the effect of CCI-779 on cell invasion. Exposure of control vector-transfected abl, C4-2B, and LNCaP cells to CCI-779 significantly reduced invasion of abl and C4-2B but not LNCaP cells (Fig. 4C; Supplementary Fig. S3). Importantly, UBE2C overexpression reversed most of this CCI-779–induced invasion inhibitory effect (Fig. 4C; Supplementary Fig. S3). These results suggest that CCI-779–induced prevention of CRPC cell invasion is mediated mostly by UBE2C.

Mechanisms for CCI-779 inhibition on UBE2C mRNA expression in CRPC cells

To investigate the underlying mechanisms for UBE2C mRNA inhibition by CCI-779 in CRPC cells, we first examined the effect of CCI-779 on recruitment of AR, its collaborating transcription factors FoxA1 and GATA2 (25), and its coactivators histone acetyltransferases [HAT]; SRC1, SRC3, and p300] and Mediator subunit (MED1; ref. 19) to the 2 UBE2C enhancers located −32.8 and +41.6 kilobases (kb) away from the transcription start site (TSS) of UBE2C gene in abl cells (4). abl cells were treated with CCI-779, and ChIP assays were conducted using antibodies against AR, FoxA1, GATA2, SRC1, SRC3, p300, MED1, and RNA polymerase II (pol II). Although exposure to CCI-779 did not affect AR binding at the 2 UBE2C enhancers, CCI-779 treatment decreased and increased FoxA1/GATA2 recruitment to the UBE2C enhancers 1 and 2, respectively (Fig. 5A–Fig. 5C). Significantly, CCI-779 treatment attenuated the recruitment of AR coactivators SRC1, SRC3, p300, and MED1 to both UBE2C enhancers but not the 2 negative control regions (Fig. 5D–Fig. 5G). Consistent with
the notion that HAT modifies chromatin structure to allow Mediator facilitating pol II recruitment to target gene promoters (27–29), exposure to CCI-779 significantly reduced the pol II level at the UBE2C promoter (Fig. 5H).

Because exposure to CCI-779 had no effect on protein expression levels of these coactivators (Fig. 5I), ReChIP assays were conducted to investigate whether CCI-779 treatment affected AR–coactivator interaction on chromatin. First-round ChIP was carried out with AR or MED1 antibodies, followed by second-round ChIP with p300 or AR antibodies. CCI-779 treatment significantly decreased interactions between AR and p300, and between AR and MED1 (Fig. 5J).

Figure 4. CCI-779 inhibits UBE2C-dependent CRPC cells invasion in vitro. A, left, UBE2C silencing decreases UBE2C protein expression. abl, C4-2B, and LNCaP cells were transfected with siControl or siUBE2C. Forty-eight hours posttransfection, Western blot analyses were carried out using the antibodies indicated. Right, UBE2C overexpression increases UBE2C protein expression. abl, C4-2B, and LNCaP cells were transiently transfected with pcDNA3.1 (myc) vector or pcDNA3.1 (myc)-UBE2C. Forty-eight hours later, cell lysates were analyzed by Western blotting, using the antibodies indicated. B, left, representative photomicrographs (100× magnification) show that UBE2C silencing inhibits invasiveness of abl and C4-2B cells. The invaded cells were stained and photographed. Right, quantification of the invaded cells for each cell line after siRNA transfection. The stained cells were manually counted from 5 randomly chosen 100× fields and normalized with cell proliferation (see Supplementary Fig. S3). ***. P < 0.001. C, left, representative photomicrographs (100× magnification) show that CCI-779 inhibits invasion of abl and C4-2B cells, and this effect is mostly reversed by UBE2C overexpression. Right, quantification of the invaded cells for each cell line in the absence or presence of CCI-779 (50 nmol/L). The stained cells were manually counted from 5 randomly chosen 100× fields and normalized with cell proliferation (see Supplementary Fig. S3). ***. P < 0.001 as compared with vehicle-treated groups; #, P < 0.001 as compared with CCI-779–treated group without UBE2C overexpression.
and K), indicating that CCI-779 treatment reduces coactivator binding through disruption of AR–coactivator interactions. Taken together, these data suggest that CCI-779 decreases AR–transcription–complex loading on UBE2C regulatory regions, which may account, at least in part, for the decreased UBE2C mRNA expression after CCI-779 treatment.

We further addressed whether CCI-779 affects UBE2C mRNA stability in CRPC cells. abl cells were incubated in the presence of either actinomycin D (to block de novo transcription)/CCI-779 or actinomycin D/vehicle for 6 to 24 hours. As shown in Figure 6, the UBE2C mRNA was destabilized by CCI-779 with a t_{1/2} = 8.52 ± 0.19 hours, as compared with the vehicle t_{1/2} = 10.97 ± 0.21 hours. These data suggest that both attenuated gene transcription and mRNA stability contribute to CCI-779 inhibition of UBE2C mRNA level in CRPC cells.

**Discussion**

The AR is often expressed and functional in most CRPC patients, and current clinical studies on CRPC focus on targeting AR itself by using AR antagonists (e.g., MDV-3100; ref. 30) or inhibitors of androgen synthesis (e.g., abiraterone acetate; ref. 32) and contributes to undesirable effects such as bone loss (33) and metabolic syndrome (34). An alternative approach for the inhibition of the cancer-promoting AR signaling pathway in CRPC is to target AR downstream target genes involved in CRPC growth. Given our recent findings showing that knocking down of CRPC-specific AR-target G2–M phase

Figure 5. CCI-779 inhibits the recruitment of AR coactivators and pol II to UBE2C regulatory regions in CRPC cells. abl cells were treated with 50 nmol/L CCI-779 or vehicle for 16 hours and subjected to ChIP analysis with antibodies against AR (A), FoxA1 (B), GATA2 (C), SRC1 (D), SRC3 (E), p300 (F), MED1 (G), or pol II (H), respectively. The DNA precipitates were then quantified by qPCR, using primers for the UBE2C promoter 

- ar
- foxa1
- gata2
- src1
- src3
- p300
- med1
- pol ii

and the UBE2C promoter [mean (n = 3) ± SE]. Negative controls (NC1 and NC2) were sequences containing AREs but without actual binding of AR and FoxA1. *, P < 0.05; **, P < 0.01 as compared with vehicle. I, protein levels of AR, FoxA1, GATA2, SRC1, SRC3, p300, and MED1 remained unchanged following treatment with 50 nmol/L CCI-779 for the indicated duration (0, 4, 8, 16, and 24 hours). J and K, ReChIP assays were conducted with antibodies against MED1 (J) and AR (K), for first ChIP, and AR (J) and p300 (K), for ReChIP (mean (n = 3) ± SE). *, P < 0.05; **, P < 0.001 as compared with vehicle.
genes (e.g., UBE2C, CDK1, and CDC20) significantly decreases CRPC cell growth (4), we propose that G2–M phase genes could serve as new targets for therapeutic intervention.

In this study, we identified the mTOR inhibitor CCI-779 as an inhibitor for UBE2C in CRPC cells. We showed that CCI-779 treatment significantly decreases UBE2C mRNA and protein expression in CRPC cells at its pharmacologically attainable concentrations in clinical trials (refs. 35, 36; Fig. 1). Although it is well known that inhibition of mTOR decreases protein expression levels of some genes by dephosphorylation of p70 ribosomal S6 kinase (S6K1) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1; refs. 12, 37), recent studies have found that mTOR inhibitors also decrease RNA levels of some genes (37, 38) through a variety of mechanisms. It has been shown that rapamycin inhibits mitochondrial gene transcription by disruption of protein–protein interactions between a transcription coactivator PGC-1α and a transcription factor yin-yang 1 (YY1), resulting in decreased recruitment of PGC-1α to the promoters of mitochondrial genes (39). Furthermore, rapamycin prevents sterol regulatory-element–binding protein 1 (SREBP1) target gene expression through inhibition of nuclear accumulation of SREBP1 (40). Additionally, rapamycin blocks PPAR-γ protein expression leading to decreased PPAR-γ target gene expression (41). With regard to the mechanisms for CCI-779 inhibition of UBE2C mRNA level, while CCI-779 does not affect AR binding and protein expression levels of AR and its coactivators, CCI-779 inhibits the recruitment of AR coactivators to the UBE2C enhancers through disruption of AR–coactivator interactions, leading to decreased pol II loading on the UBE2C promoter (Fig. 5). Interestingly, we also found that CCI-779 attenuates UBE2C mRNA stability (Fig. 6), suggesting that CCI-779-induced direct downregulation of UBE2C mRNA levels is caused by decreased UBE2C mRNA transcription and stability.

Although mTOR inhibitors have shown great potential as antitumor agents and CCI-779 has been approved by the U.S. Food and Drug Administration (FDA) as the first-line treatment in patients with advanced refractory renal cell cancer (RCC; ref. 11), results from clinical studies on mTOR inhibitors in CRPC have been somewhat disappointing. For example, it was reported that that therapeutic response was observed in only 17% to 25% CRPC patients treated with rapamycin alone (42, 43). One of the explanations for such clinical observations is that rapamycin and CCI-779 may activate AR target genes such as PSA and KLK4 in cultured CRPC cells and xenografts, leading to a decreased effect of mTOR inhibitors on inhibition of cell proliferation (42, 44). However, as the AR target genes examined in these studies (42, 44) are not directly relevant to cell growth and invasion, it is not very clear that the failure of mTOR inhibitors as monotherapy is caused by mTOR inhibitor–activated AR signaling. Interestingly, our studies found that CCI-779 significantly decreases the expression of a CRPC-specific AR target gene UBE2C in CRPC cell models abl and C4-2B (Fig. 1). The overexpressed UBE2C in abl and C4-2B cells, as compared with LNCaP cells (4), plays a critical role in cell proliferation and/or invasion (Figs. 2–4; ref. 4). CCI-779, acting partially through a UBE2C-dependent mechanism, significantly decreases abl cell growth in vitro and in vivo (Figs. 2 and 3). Importantly, we also found that CCCI-779–induced inhibition of abl and C4-2B cell invasion is mediated mostly by UBE2C (Fig. 4). Although the average level of UBE2C expression in CRPC patients is significantly higher than that in ADPC patients, UBE2C expression in CRPC cases is highly variable (4). Thus, it is possible that those CRPC patients with high UBE2C expression will have better therapeutic response for CCI-779 than those with low UBE2C expression. Further studies are needed to investigate whether UBE2C is able to serve as a biomarker for predicting CCI-779 therapy response in CRPC patients.

Figure 6. CCI-779 decreases mRNA stability of UBE2C in CRPC cells. A and B, degradation of UBE2C mRNA in abl cells in the absence or presence of CCI-779. C, CCI-779 shortens the half-life (t1/2) of UBE2C mRNA. abl cells were treated with 50 nmol/L CCI-779 or vehicle with transcription was blocked by actinomycin D. UBE2C mRNA level was quantified at the indicated time points after actinomycin D treatment. Data are presented as the percentage of the mRNA level measured at time 0 (without adding actinomycin D). **, P < 0.01 as compared with vehicle.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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CCI-779 Inhibits Cell-Cycle G2–M Progression and Invasion of Castration-Resistant Prostate Cancer via Attenuation of UBE2C Transcription and mRNA Stability

Hongyan Wang, Chunpeng Zhang, Anna Rorick, et al.

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