Dual Targeting of the Akt/mTOR Signaling Pathway Inhibits Castration-Resistant Prostate Cancer in a Genetically Engineered Mouse Model

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Abstract

Although the prognosis for clinically localized prostate cancer is now favorable, there are still no curative treatments for castration-resistant prostate cancer (CRPC) and, therefore, it remains fatal. In this study, we investigate a new therapeutic approach for treatment of CRPC, which involves dual targeting of a major signaling pathway that is frequently deregulated in the disease. We found that dual targeting of the Akt and mTOR signaling pathways with their respective inhibitors, MK-2206 and ridaforolimus (MK-8669), is highly effective for inhibiting CRPC in preclinical studies in vivo using a refined genetically engineered mouse model of the disease. The efficacy of the combination treatment contrasts with their limited efficacy as single agents, since delivery of MK-2206 or MK-8669 individually had a modest impact in vivo on the overall tumor phenotype. In human prostate cancer cell lines, although not in the mouse model, the synergistic actions of MK-2206 and ridaforolimus (MK-8669) are due in part to limiting the mTORC2 feedback activation of Akt. Moreover, the effects of these drugs are mediated by inhibition of cellular proliferation via the retinoblastoma (Rb) pathway. Our findings suggest that dual targeting of the Akt and mTOR signaling pathways using MK-2206 and ridaforolimus (MK-8669) may be effective for treatment of CRPC, particularly for patients with deregulated Rb pathway activity. Cancer Res; 72(17); 4483–93. ©2012 AACR.

Introduction

All aspects of prostate development and function require androgen signaling, which is mediated by the androgen receptor, a nuclear steroid receptor (1, 2). Moreover, prostate cancer arises under the influence of androgen receptor signaling, whereas removal of androgens via androgen deprivation therapy is the most common first-line treatment for recurrent prostate tumors. However, in most cases, androgen deprivation therapy ultimately results in the emergence of a highly aggressive form of the disease, which is now referred to as castration-resistant prostate cancer (CRPC) to reflect its continued dependence on androgen receptor in the absence of testicular androgens (3). Among available therapeutic approaches for treatment of CRPC, conventional chemotherapy has limited efficacy (4–6). Recently, several agents that target androgen receptor and/or androgen synthesis, namely MDV3100 and Abiraterone, have been introduced into the clinic and have shown promising results (7, 8), although they are also not curative.

An alternative approach is targeted therapy directed against signaling pathways that are active in CRPC, including the Akt/mTOR signaling axis. Indeed, the culmination of several lines of evidence, including analyses of tissue microarrays (9–11), oncogenomic analyses of human clinical data (12), and functional studies in mouse and human prostate cancer cells (9, 13, 14), have established that activation of Akt/mTOR signaling is strongly and causally associated with advanced prostate tumors and particularly CRPC. Nonetheless, despite the relevance of Akt/mTOR pathway deregulation for disease progression and the availability of suitable therapeutic agents, targeting these pathways using single agents has not been effective in clinical settings (15, 16), although there are notable exceptions (17). It is widely believed that this may be due in part to feedback activation of these pathways, which occurs in response to drug treatment (18–21).

Therefore, we have now investigated the consequences of dual targeting of Akt/mTOR signaling in CRPC. By analyses of preclinical studies in vivo using genetically-engineered mouse (GEM) models, we show that combinatorial treatment using MK-2206 to target Akt and ridaforolimus (MK-8669) to target mTOR is highly effective for inhibition of CRPC. Parallel studies of human prostate cancer cell lines in culture revealed that the mechanism of action is via inhibition of cellular proliferation mediated by the retinoblastoma (Rb) signaling pathway.
Considering the importance of Rb status for progression to CRPC (12, 22) as well as response to therapy (23), our findings suggest that dual inhibition of Akt/mTOR signaling with MK-2206 and MK-8669 may be a promising approach for treatment of patients with CRPC, particularly those with deregulated Rb signaling.

Materials and Methods

Generation and analysis of GEM models

All experiments using animals were conducted according to protocols approved by the Institutional Animal Care and Use Committee at Columbia University Medical Center (New York, NY). The Nkx3.1CreERT2/+ allele, which is null for Nkx3.1, expresses Cre-ERT2 under the control of the Nkx3.1 promoter (24). The conditional allele for Pten (Ptenfllox/fllox) having loxP sites flanking exon 5 (25) was obtained from the National Cancer Institute Mouse Models of Human Cancer Consortium. Mice were bred to generate the full spectrum of genotypic combinations. Primers for genotyping are provided in Supplementary Table S1.

For induction of Cre activity, tamoxifen (Sigma Cat #T5648; or corn oil alone) was delivered by i.p. injection (225 mg/kg) or oral gavage (100 mg/kg) for 4 consecutive days, to mice at 2 to 3 months of age. Mice were androgen ablated by surgical castration at 4 months after tamoxifen. For phenotypic analyses, mice were sacrificed, the prostatic lobes (anterior, dorsolateral, and ventral) were collected individually and the weights determined. Tissues were fixed in 10% formalin, cyropreserved in optimal cutting temperature (OCT) compound, or snap frozen in liquid nitrogen.

Immunohistochemical staining was done on 3 µm paraffin sections as described previously (9) using an Intellipath FLX from Biocare Medical and visualized using a Nikon Eclipse E800 microscope equipped with a Nikon DXM1200 digital camera. Quantitation of proliferating cells was done as described previously (26), using 5 independent sections from 5 independent mice (i.e., 25 sections). SA β-galactosidase staining was done on tissues embedded directly in OCT (without prior fixation) as in ref. 27. Western blot analyses were done using total protein extracts obtained by sonicating mouse prostate tissue or human prostate cell in 1× radioimmunoprecipitation assay buffer [0.1% SDS, 1.0% deoxycholate–sodium Salt, 1.0% Triton X-100, 0.15 M NaCl, 10 mmol/L Tris–HCl (pH 7.5), 1 mmol/L EDTA] containing fresh 1% protease inhibitor (#1697498, Sigma-Aldrich) and 1% phosphatase inhibitor (#P2850, Sigma-Aldrich). Details of all antibodies are provided in Supplementary Table S1.

Real-time PCR was done on total RNA using the Quantitech SYBR Green PCR kit (Qiagen). A pairwise fixed reallocation randomization test using the relative expression software tool (Qiagen) was used to test the significance of the expression ratios of transcripts. Primers are listed in Supplementary Table S1. Statistical analyses were conducted using a 2-tailed t test, χ² test, or Fisher’s exact test as appropriate. GraphPad Prism software (version 5.0) was used for all statistical analysis and to generate data plots.

Preclinical analyses of GEM

MK-2206 and MK-8669 were obtained from Merck. MK-2206 was dissolved in 30% captisol (Cydex) in sterile water to make a working stock of 20 mg/mL and delivered via oral gavage at 120 mg/kg. MK-8669 was dissolved in 100% ethanol to make a working stock of 25 mg/mL, and then diluted to 1.25 mg/mL in a solution of 5.2% Tween 80, 5.2% PEG400 in sterile water, and delivered via i.p. at 10 mg/kg. Docetaxel was purchased from LC Labs and delivered at 10 mg/kg in a vehicle solution of 23% Tween 80 in 1× PBS. The optimal dosage of each of the drug was determined by delivering varied amounts of drug over a period of 1 month to mice of the same strain and genotypic background used for the preclinical studies, and selecting the maximal tolerated dose. The starting point for these dosage studies was the information provided by the supplier (Merck). The MK-2206 and/or MK-8669 (or vehicle) were delivered 3 times a week (Monday, Wednesday, and Friday) and docetaxel was delivered twice a week (Tuesday and Friday) for a period of 2 months. For the short-term therapeutic response group, agents were delivered once a day for 5 consecutive days, and mice were dissected 6 hours after last treatment. Mice were weighed daily and observed for signs of distress following dosing; none of the treatments resulted in appreciable weight loss exceeding 10%.

Computational analyses of expression profiling data

Gene expression profiling analysis was done using total RNA isolated form phenotypically wild-type (intact) Nkx3.1flox/flox (n = 3), intact Nkx3.1flox/flox; Ptenfllox/fllox (n = 6), and castrated Nkx3.1flox/flox; Ptenfllox/fllox (n = 6). RNA was isolated using the MagMAX-96 total RNA isolation kit (Ambion), which was reverse transcribed and biotin labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion). The cRNA (1.5 mg) was hybridized on mouseWG-6 v2 BeadArrays (Illumina) using an iScan BeadArray scanner (Illumina). Data were loaded and normalized using IlluminaExpressionFileCreator version 2 and Illumina normalizer with collapse mode using the maximum of all the probe values for each gene and without background subtraction. The resulting datasets were preprocessed to remove probesets whose difference between maximum and minimum values were less than 100. Data were log transformed for GSEA analysis (GEO: GSE39054).

Gene signatures comparing the intact wild type (Nkx3.1flox/flox) to the intact and castrated Nkx3.1flox/flox; Ptenfllox/fllox were defined using the Welch t-test to identify genes ranked by their differential expression in the intact and castrated Nkx3.1flox/flox; Ptenfllox/fllox mouse prostate tumors versus the phenotypically wild-type (Nkx3.1flox/flox) mouse prostate, respectively. To identify pathways commonly deregulated in intact and castrated Nkx3.1flox/flox; Ptenfllox/fllox prostate tumors, enrichment of these differentially expressed gene signatures in human pathways was evaluated using gene set enrichment analyses (GSEA; ref. 28) using pathways collected in the c2 curated gene sets (http://www.broad-institute.org/gsea/msigdb/index.jsp) with 1,000 gene label permutations (gene sets). Significantly enriched gene sets, defined by nominal P < 0.05, were compared between intact Nkx3.1flox/flox; Ptenfllox/fllox versus Nkx3.1flox/flox and castrated intact Nkx3.1flox/flox; Ptenfllox/fllox versus Nkx3.1flox/flox gene signatures.
Enrichment analyses to identify relevant biological processes were done by interrogating the REACTOME (29), KEGG (30), and BioCarta (http://www.biocarta.com/gene/allpathways.asp) databases.

Cell culture analyses
PC3 (CRL-1435) and LNCaP (CRL-1740) cells were obtained from the American Type Culture Collection (ATCC) and were used within 6 months of their receipt; verification of the cell lines was done by ATCC. Cells were grown in a RPMI medium supplemented with 10% heat-inactivated fetal calf serum and 100 U/mL penicillin–streptomycin. Cells were treated with vehicle (dimethyl sulfoxide) or with MK-2206 (1 \( \times \) 100 U/mL penicillin–streptomycin). 12.5 mmol/L siRNA or control siRNA (Ambion) was introduced by transfection using Lipofectamine RNAiMAX (Invitrogen). All cell culture assays were done in at least 3 independent experiments. Comparison of differences among the groups was carried out by two-tailed Student t test.

Results
A refined mouse model of CRPC
Among the genes that are known to be causally associated with prostate tumorigenesis are: (i) the Nkx3.1 homeobox gene, which is specifically expressed in the prostate epithelium and its reduced expression associated with prostatic intraepithelial neoplasia (PIN; refs. 1, 31) and (ii) the Pten tumor suppressor, whose loss-of-function is associated with tumor promotion via activation of the phosphoinositide 3-kinase/Akt signaling pathway (1, 32, 33). Previously, we showed that germline loss-of-function of Pten and Nkx3.1 leads to CRPC following surgical castration (34, 35), and other groups have shown that conditional loss-of-function of Pten in prostate also leads to CRPC (36–39). Indeed, several lines of evidence now support a causal role for Pten loss-of-function in the transition to CRPC (13, 39, 40), and therefore highlight the importance of using GEM models having Pten inactivation to study CRPC in vivo. However, germline loss of Pten results in tumors in many sites other than prostate, whereas most of the conditional deletion models reported thus far have used a constitutively active Probasin-Cre transgene (PB-Cre), which leads to recombination (deletion) in the prostate before complete maturation (41).

In the current study, we produced a refined GEM model of Pten-induced castration resistance by deleting Pten specifically in the prostatic epithelium of adult mice using a tamoxifen-inducible (rather than constitutively active) Cre under the control of the Nkx3.1 promoter (24). This Nkx3.1\( ^{CreERT2} \) knock-in allele simultaneously inactivates 1 allele of Nkx3.1 (which is haploinsufficient in prostate cancer (1, 31)) while driving tamoxifen-dependent Cre-mediated recombination specifically in prostate epithelium, including in a relevant cell of origin of prostate cancer (24). This Nkx3.1\( ^{CreERT2} \) allele was crossed with a Pten conditional allele (25) to obtain the Nkx3.1\( ^{CreERT2}; \) Pten\( ^{fl/fl} \) mice used in these studies (hereafter denoted Nkx3.1\( ^{CreZf}; \) Pten\( ^{fl/fl} \)). Importantly, Pten recombination was induced via tamoxifen activation of Cre in adult mice at 2 months of age, which is subsequent to when the prostate and other male secondary sexual organs are fully mature. In control experiments, we have shown that this temporally limited induction with tamoxifen has little or no effect on the prostate phenotype of control or mutant mice (24).

The Nkx3.1\( ^{CreZf}; \) Pten\( ^{fl/fl} \) mice, but not the control mice (Nkx3.1\( ^{CreZf}; \)), developed PIN lesions as early as 6 to 7 months after tamoxifen induction, which by 9 to 12 months progressed to high-grade PIN (\( N = 8/8 \) mice) and by 16 months to extensive high-grade PIN with areas of invasion (\( N = 8/8 \) mice; Fig. 1A–E; Table 1). Pathological evaluation of these lesions using the classification of Park and colleagues (42) revealed that mouse tumors displayed extensive PIN III and PIN IV (i.e., high-grade PIN) with areas of squamous metaplasia, found in some GEM models of prostate cancer.

Nkx3.1\( ^{CreZf}; \) Pten\( ^{fl/fl} \) mice that have undergone depletion of androgens by surgical castration initially display tumor regression (\( N = 4/4 \) mice), which is evident by 2 weeks following castration (Fig. 1I–L). However, this initial regression was followed by the emergence of castration-resistant lesions that resembled high-grade PIN by 6 to 7 months after tamoxifen induction (\( N = 14/14 \) mice); these lesions progressed to high-grade PIN with adenocarcinoma by 12 months and to extensive, poorly differentiated adenocarcinoma by 16 months following tamoxifen induction (\( N = 14/14 \) mice; Fig. 1F–H; Table 1). Pathological evaluation of these lesions (42) revealed that these tumors displayed extensive PIN IV (\( N = 14/14 \) mice) and some with squamous metaplasia (\( N = 2/14 \) mice).

![Figure 1.](http://cancerres.aacrjournals.org)
by 12 months following tumor induction, whereas the older mice (i.e., 16 months) displayed extensive microinvasive adenocarcinoma, which was not evident in the noncastrated mice (N = 14/14 mice; Table 1). Therefore, the Nkx3.1<sup>CreERT2/+; Pten<sup>lox/lox</sup> mice are initially sensitive to castration as has been reported previously for Pten inactivation in other GEM models (34, 36) but ultimately develop castration-resistant tumors that are histologically more advanced than the intact mice.

Notably, although the castrated Nkx3.1<sup>CreERT2/+; Pten<sup>lox/lox</sup> mice had a more severe phenotype, immunohistochemical analyses revealed that the lesions arising in prostates from the intact and castrated mice were otherwise more similar than they were different (Fig. 2). In particular, the prostatic lesions in both the intact and castrated mice expressed robust levels of androgen receptor, which was mainly nuclear (n = 5; Fig. 2A and B). These prostatic lesions were primarily luminal, as evident from the robust expression of cytokeratin (CK) 8, a marker of luminal epithelium, although the prostatic lesions from the castrated mice displayed an approximately 5-fold increase in the basal epithelium, evident from expression of CK5, a basal cell marker (n = 5; Fig. 2C–F and Q). Furthermore, both the intact and castrated prostates were similarly highly proliferative (~12%–14%) as evident by Ki67 staining (n = 5; Fig. 2G and H; Table 1). Finally, the prostatic lesions in both the intact and castrated mice displayed strong activation of Akt and mTOR signaling, as evident by the immunostaining for p-Akt and p-S6, respectively, as well as by Western blot analyses (Fig. 2I–L and Q).

Nonetheless, lesions arising in prostates of castrated Nkx3.1<sup>CreERT2/+; Pten<sup>lox/lox</sup> mice were consistently more advanced than those of the age-matched intact Nkx3.1<sup>CreERT2/+; Pten<sup>lox/lox</sup> mice (Fig. 1; Table 1). Interestingly, this was accompanied by decreased expression of senescence-associated (SA)-β-galactosidase (SA-β-gal). In particular, although prostates from the intact Nkx3.1<sup>CreERT2/+; Pten<sup>lox/lox</sup> mice expressed high levels of SA-β-gal, as has been reported previously for other prostate cancer models having inactivation of Pten (27), SA-β-gal expression was barely detectable in prostates from the castrated Nkx3.1<sup>CreERT2/+; Pten<sup>lox/lox</sup> prostate (Fig. 2M–P). Notably, this striking difference in SA-β-gal expression was observed in mice at 7 months following tumor induction (3 months after castration; Fig. 2M and N), when both the intact and castrated mice displayed a similar histopathology (see Fig. 1, Table 1), indicating that this difference is not due to the more advanced nature of the castrated tumors. These findings raise the interesting possibility that castration resistance is associated with a bypass of the senescence phenotype, which may contribute to cancer progression.

Computational analyses using gene expression profiling (Supplementary Tables S3 and S4) supports our observations from these phenotypic analyses that the prostate tumors from

Table 1. Summary of prostate cancer phenotype

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype</th>
<th>N</th>
<th>Age</th>
<th>Pathologic description</th>
<th>% Ki67 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Nkx3.1&lt;sup&gt;CreERT2/+; Pten&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>6</td>
<td>8</td>
<td>Hyperplasia, low-grade PIN (similar to Nkx3.1 phenotype)</td>
<td>3</td>
</tr>
<tr>
<td>Intact</td>
<td>Nkx3.1&lt;sup&gt;CreERT2/+; Pten&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>10</td>
<td>6–9</td>
<td>PIN I, PIN II, and PIN III</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>9–12</td>
<td>Extensive PIN III and PIN IV, with some (2 out of 8 mice) having areas of squamous carcinoma</td>
<td>12%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>16+</td>
<td>Extensive PIN III and IV, with some (2 out of 8 mice) having areas of solitary or extensive squamous metaplasia; acute inflammation and submucosal proliferation of Pale cells.</td>
<td>ND</td>
</tr>
<tr>
<td>Castrated</td>
<td>Nkx3.1&lt;sup&gt;CreERT2/+; Pten&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>12</td>
<td>6–9</td>
<td>PIN I, PIN II, and PIN III</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>9–12</td>
<td>PIN II, PIN III, and PIN IV with extensive fibrosis and inflammation; some (3 out of 14 mice) having areas of solitary or extensive squamous metaplasia</td>
<td>14%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>16+</td>
<td>Microinvasive adenocarcinoma with areas of poorly differentiated adenocarcinoma; some mice (2 out of 14 mice) having areas of large fibrous nodules lined by a glandular epithelium with extensive squamous metaplasia</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE: Summary of the prostate cancer phenotype of the mouse models used in this study. The numbers of mice analyzed are indicated; the age refers to the time following tumor induction. The pathological description follows the pathological classification of Park and colleagues (42). The % Ki67 staining refers to the total number of Ki67-positive epithelial cells relative to the total number of epithelial cells.

Abbreviations: N, total number of mice analyzed; ND, not determined.
intact and castrated Nkx3.1^{Cre/+}; Pten^{fl/fl} mice are more similar than they are different. In particular, using GSEA (28) to identify relevant biological processes, we found that about 50% of biological pathways were similarly deregulated in prostates of both intact and castrated mice (Fig. 2R; Supplementary Tables S6 and S7). Among the pathways that were similarly deregulated between the intact and castrated Nkx3.1^{Cre/+}; Pten^{fl/fl} prostate tumors were the NF-κB signaling, which is an indicator of the relative levels of inflammation, and the p53 pathway, which has been associated with abrogating the senescence phenotype following Pten inactivation (ref. 27; Supplementary Tables S6 and S7). Therefore, both the phenotypic and molecular features of the intact and castrated Nkx3.1^{Cre/+}; Pten^{fl/fl} mouse prostate tumors support the fact that they are highly similar.

Interestingly, the notable exception to this overall similarity was deregulation of a senescence signature (P < 0.001), which was only observed in the intact prostate (Fig. 2S), consistent with our phenotypic observation (Fig. 2M–P). Taken together, these findings further support a critical role for Pten loss-of-function for castration resistance and suggest that the transition to castration resistance is associated with alleviation of the senescence phenotype, which may contribute to the more aggressive phenotype of castrate-resistant tumors.

Combination targeted therapy inhibits CRPC

Although in general, targeted therapy using single agents directed against Akt/mTOR signaling has had limited efficacy in clinical contexts (15, 16), their actions as combined agents has shown some promise, including in prostate cancer cells (43). Given the robust activation of Akt and mTOR signaling in these mouse tumors (see Fig. 2I–L), we considered that the castrated Nkx3.1^{Cre/+}; Pten^{fl/fl} mice are well suited to evaluate the consequences of combinatorial targeting these signaling pathways for inhibition of CRPC. To do so, we used a specific non-ATP-competitive allosteric Akt inhibitor, MK-2206, that is highly effective for inhibiting Akt function in various in vivo and cellular contexts (17, 44, 45). We combined this Akt inhibitor with an mTOR inhibitor, MK-8669 (ridaforolimus, formerly termed deforolimus), an analog of rapamycin specific for the mTORC1 complex (11). Both of these agents have been evaluated in clinical trials where they have shown to be effective and well tolerated (17, 46–48), and the combination is currently being tested in a Phase 1 trial (clinicaltrials.gov #NCT01295632). Notably, we verified that each of these agents inhibited their respective pathways in the mouse prostate in vivo when delivered individually or in combination (Fig. 3G–I), and were well tolerated for the duration of the treatment period when delivered at their effective doses.

Thus, we conducted preclinical studies in the intact or castrated Nkx3.1^{Cre/+}; Pten^{fl/fl} mice by administering MK-2206 and/or MK-8669 for 2 months, following which the mice were sacrificed for analysis (Fig. 3A; Supplementary Fig. S1 and Supplementary Table S3). Treatment with either MK-2206 or MK-8669 alone had a modest impact on the overall tumor phenotype considering multiple endpoints (see below), although MK-8669 significantly reduced proliferation (Table 2). In striking contrast, the combination of MK-2206 + MK-8669 was highly effective for inhibiting castration-resistant tumors (Fig. 3 and Table 2). This was evident by analyses of several independent endpoints, including: (i) a profound alteration in histologic phenotype (Fig. 3B–F); (ii) a 13-fold reduction in tumor weight (P = 0.004; Table 2); (iii) a significant (3-fold) reduction in cellular proliferation (P < 0.001; Fig. 3Q–U, Table 2); and (iv) attenuated expression of the relevant pathway markers, namely p-Akt and p-S6 (Fig. 3G–P and V). Notably, MK-2206 + MK-8669 had only a modest effect on the tumor phenotype in the intact Nkx3.1^{Cre/+}; Pten^{fl/fl} mice as evident by the more limited effect on tumor weight, histology, and other end points, although these agents were still more effective in combination than individually (Supplementary Fig. S1, Supplementary Table S3). Interestingly, the strong efficacy of MK-2206 + MK-8669 for inhibition of these castration-resistant tumors contrasted with the weak efficacy of conventional chemotherapy (docetaxel: Fig. 3F, K, P, and U), paralleling the limited efficacy of conventional chemotherapy for CRPC in humans (6). Taken together, these preclinical studies suggest that dual targeting Akt/mTOR signaling using MK-2206 + MK-8669 may be beneficial for treatment of CRPC.

Dual targeted therapy of Akt/mTOR signaling affects Rb signaling

We next sought to understand the mechanism of action of MK-2206 + MK-8669 in human prostate cancer cells in culture, using PC3 and LNCaP cells (49; Fig. 4). First, we confirmed that these drugs inhibit their respective signaling pathways by assessing phosphorylation at serine 473 on Akt and mTOR and 4E-BP1, which are specific for mTORC1, as a read-out of inhibition by MK-2206, and phosphorylation of S6 and other end points, although these agents were still more effective in combination than individually (Supplementary Fig. S1, Supplementary Table S3). Interestingly, the strong efficacy of MK-2206 + MK-8669 for inhibition of these castration-resistant tumors contrasted with the weak efficacy of conventional chemotherapy (docetaxel: Fig. 3F, K, P, and U), paralleling the limited efficacy of conventional chemotherapy for CRPC in humans (6). Taken together, these preclinical studies suggest that dual targeting Akt/mTOR signaling using MK-2206 + MK-8669 may be beneficial for treatment of CRPC.
Figure 2. Phenotypic analyses of the prostate phenotype from intact versus castrated Nkx3.1<sup>CE2/+; Pten<sup>f/f</sup> mice. A–P, marker analyses of anterior prostate. Mice were induced with tamoxifen to form tumors at 2 months of age, castrated or left intact 4 months later, and then analyzed 12 months following tumor induction (unless otherwise indicated). A and B, immunostaining for androgen receptor shows similar levels of nuclear androgen receptor in intact and castrated mice. C and D, immunostaining for cytokeratin 8 (CK8) shows the predominance of luminal cells in the prostate of both intact and castrated mice. E and F, immunostaining for cytokeratin 5 (CK5) shows enrichment of basal cells in the prostate of castrated mice. G and H, immunostaining for Ki67 shows high level of proliferative activity in the prostates of both intact and castrated mice. I–L, immunostaining for p-Akt<sup>Ser473</sup> and for p-S6<sup>S235/236</sup> indicates strong activation of Akt and mTOR signaling both in the intact and castrated mice. M–P, SA-β-gal staining shows robust expression in the intact but not castrated prostate both at 7 or 12 months following tumor induction. Q, Western blot analyses using total protein extracts from dorsal prostate of mice from the indicated genotypes, 12
LNCaP cells (data not shown). In particular, treatment with either MK-2206 or MK-8669 individually resulted in a 1.8- or 2.0-fold reduction in cellular proliferation, respectively, whereas combined treatment with MK-2206 and MK-8669 resulted in a 2.6-fold reduction ($P < 0.0001$). This was accompanied by an increase in the percentage of cells in G1, from 41% for the vehicle-treated cells to 53% or 55% in the cells treated with each agent individually, and 75% in the cells treated with both agents together ($P < 0.0001$; Fig. 4E). It is noteworthy that the combined action of MK-2206 and MK-8669 is additive in cell culture, in contrast to their synergistic effects in vivo (compare Figs. 3 and 4), as observed previously with another

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Figure 3. Dual targeting of Akt/mTOR signaling inhibits CRPC. A, design of preclinical studies to evaluate combination treatment with MK-2206 and MK-8669 for CRPC. Nkx3.1CreERT2/þ; Ptenf/f mice were induced with tamoxifen at 2 months of age and castrated 4 months later (at 6 months). Treatment (with MK-2206 and/or MK-8669 or docetaxel) was initiated 10 months after tamoxifen induction (at 12 months) and continued for 2 months following which mice were sacrificed for analyses. B–F, representative hematoxylin and eosin images showing the histology of the anterior prostate of mice treated with agents as indicated. G–P, immunostaining for p-Akt and p-S6 showing reduced immunostaining following treatment with their respective inhibitors, MK-2206 and MK-8669, as was also evident by Western blotting (see V). Q–U, analyses of cellular proliferation by immunostaining for Ki67; quantitation of the data is shown in Table 2. V, Western blot analyses of tissues from the short-term treated mice showing relative expression of Akt/mTOR pathway markers following drug treatment. Scale bars, 100 mm.

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R and S, analyses of differential gene expression analyses from intact versus castrated Nkx3.1CreERT2/þ; Ptenf/f mice (see Supplementary Tables S3 and S4). R, venn diagram summarizing the overlap in biological pathways, identified using GSEA (see Supplementary Tables S5 and S6), which are differentially expressed in intact or castrated Nkx3.1CreERT2/þ; Ptenf/f mouse prostate. S, GSEA analyses shows enrichment of a senescence signature in the intact Nkx3.1CreERT2/þ; Ptenf/f mouse prostate tumor. Shown on the x-axis is the rank order of mouse genes from the most upregulated (position 1) to the most downregulated (position 12,435) between the intact Nkx3.1CreERT2/þ; Ptenf/f versus the control Nkx3.1CreERT2/þ; Ptenf/f mice; the barcode indicates the position of genes from the indicated human biologic pathway. The y-axis corresponds to the running enrichment score (ES) generated by the cumulative tally of the pathway genes. The total height of the curve indicates the extent of enrichment, with the normalized enrichment score (NES) and $P$ values indicated. Scale bars, 100 mm.
combination targeted therapy (9, 14). This may reflect additional non–cell autonomous consequences of these agents in vivo, such as effects on angiogenesis or other processes (50) and/or the duration of the treatment period, which is 2 months in vivo but only a few days in vitro.

Finally, we considered the mechanisms by which MK-2206 + MK-8669 inhibited cellular proliferation using Western blot analyses to assess the status of potential effectors. We found that treatment with both MK-2206 + MK-8669, but not either agent individually, resulted in a significant reduction in Rb phosphorylation at 2 key serines [Ser(807/811) and Ser(780); Fig. 4C], which are targets of cyclin E/CDK2 and cyclin D/CDK4, respectively. Considering that a major target of the Rb protein is the S-phase–inducing transcription factor E2F1 (51), we evaluated the status of E2F target genes in the castrated Nkx3.1CE2+/−; Ptenff mice (Fig. 5). Using GSEA

Table 2. Summary of tumor weights and proliferation of prostate tumor from castrated Nkx3.1CE2+/−; Ptenff mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Weight, g</th>
<th>Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6</td>
<td>0.38 ± 0.01</td>
<td>11% ± 1.1</td>
</tr>
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<td>MK-2206</td>
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<td>15% ± 3.1; P = 0.20</td>
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<tr>
<td>MK-8669</td>
<td>6</td>
<td>0.34 ± 0.22; P = 0.90</td>
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</tr>
<tr>
<td>MK-2206 + MK-8669</td>
<td>7</td>
<td>0.03 ± 0.005; P = 0.004</td>
<td>4% ± 1.0; P &lt; 0.001</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>4</td>
<td>0.19 ± 0.05; P = 0.080</td>
<td>17% ± 1.6; P = 0.005</td>
</tr>
</tbody>
</table>

NOTE: Summary of the data for castrated Nkx3.1CE2+/−; Ptenff mice showing the average tumor weights and percentage of proliferating epithelial cells following the indicated treatment. Where indicated, P values compare the drug-treated to vehicle-treated groups. Abbreviation: N, the total number of mice analyzed.

Table 2. Summary of tumor weights and proliferation of prostate tumor from castrated Nkx3.1CE2+/−; Ptenff mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Weight, g</th>
<th>Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6</td>
<td>0.38 ± 0.01</td>
<td>11% ± 1.1</td>
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Figure 4. Analyses of MK-2206 and MK-8669 on human prostate cancer cells. A, Western blot analyses of protein extracts from PC3 or LNCaP cells, as indicated, showing the expression levels of the indicated phosphoproteins following treatment with agents, as indicated, for 24 hours. B, Western blot analyses of PC3 cells incubated with the control or Rictor siRNA for 24 hours, followed by treatment with the agents as indicated for an additional 24 hours. C, Western blot analyses of PC3 cells showing the expression levels of the indicated phosphoproteins following treatment with the agents, as indicated, for 24 hours. D, cell growth assay. PC3 cells were seeded at low density (200,000/35 mm dish) on day 0 and then treated on day 1 with vehicle or MK-2206 (1 μM) and/or MK-8669 (1 mmol/L). Cell numbers were assessed by counting with trypan blue. E, flow cytometry analyses of PC3 cells showing the percentage of cells in the G1 phase following treatment with the indicated drug for 24 hours; similar results were obtained following incubation for 48 hours.
pathway analyses, we observed a significant activation of the E2F signaling pathway in the castration-resistant tumors (Fig. 5A and B). Furthermore, real-time quantitative PCR validation of relevant target genes revealed that E2F target genes that were upregulated in the Nkx3.1CreERT2/+; Ptenff prostate tumors, relative to the control prostate (i.e., Nkx3.1CreERT2/+ mice; the barcode indicates the position of genes from the indicated human biological pathway. The y-axis corresponds to the running enrichment score generated by the cumulative tally of the pathway genes. The total height of the curve indicates the extent of enrichment, with the normalized enrichment score (NES) and P values indicated.

Discussion

In this study, we provide in vivo preclinical evidence from a refined GEM model of CRPC, complemented by analyses of human prostate cancer cells in culture, to show the efficacy of dual targeting of Akt/mTOR signaling for treatment of CRPC. An important feature of our refined GEM model is the virtual absence of senescence, which is a hallmark of Pten loss-driven prostate tumors (27). Thus, our findings support the idea that cellular senescence restrains Pten loss-driven prostate tumors (27), and further suggests that castration unleashes a more aggressive form of the disease by bypassing senescence.
Our preclinical findings in this GEM model and complementary studies using human prostate cancer cells, along with a previous cell culture study (43), suggest that the combined use of agents targeting distinct components of Akt/mTOR signaling may be advantageous, in part because they overcome a feedback loop that is believed to limit the efficacy of single agents. However, it is important to note that the observed consequences of these agents may differ when evaluated in cell culture versus in vivo and in human cells versus mouse models (52). In particular, although a feedback mechanism was readily evident in our analyses of the GEM mice in vivo (see Fig. 3), we did not observe this in our analyses of human prostate cancer cell lines in culture (see Fig. 4), we did not observe this in our analyses of the GEM mice in vivo (see Fig. 3). Other studies have shown that although rapamycin blocks S6 phosphorylation, but not AktS473 phosphorylation in various cell lines, prolonged exposure to rapamycin can also target mTORC2 (53). Therefore, additional factors in vivo may contribute to the enhanced efficacy of the dual inhibition Akt/mTOR signaling, in addition to limiting feedback inhibition. Furthermore, our findings indicate that dual inhibition of Akt/mTOR signaling with MK-2206 + MK-8669 is linked to inhibition of cellular proliferation via the RB signaling pathway, which is deregulated in a large subset of patients with CRPC (12, 22).

On the basis of our findings showing the enhanced efficacy of dual inhibition of Akt/mTOR signaling, we propose that the combination of MK-2206 + MK-8669 should be evaluated in clinical trials for patients with CRPC. Given the intractable nature of this disease, which has resisted many treatment modalities thus far, we further suggest that MK-2206 + MK-8669 should be considered as part of a broader treatment regimen that includes agents directed toward alternative mechanisms relevant for the disease. In this regard, the androgen receptor agonist, MDV3100, has shown tremendous promise in the clinic (8), has improved efficacy in combination with a PI3K inhibitor in castration-resistant GEM mice (36). Therefore, it will be of interest to evaluate MK-2206 + MK-8669 combined with agents that target androgen signaling, such as MDV3100 as well as Abiraterone. In addition, our intriguing finding showing the inverse correlation of castration resistance and cellular senescence suggests the possibility of combining MK-2206 + MK-8669 with a prosenescence therapy, as proposed by Pandolfi and colleagues (54). Such combinations can be readily evaluated in preclinical studies using these GEM mice, which can provide an initial assessment of the efficacy of the various combinations, as well as mechanistic insights into drug actions. In summary, we propose that dual targeting of Akt/mTOR signaling using MK-2206 + MK-8669 may contribute to a treatment regime for CRPC.

Disclosure of Potential Conflicts of Interest

C. Abate-Shen has a commercial research grant from Merck. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: N. Floch, C.W. Kinkade, T. Kobayashi, M. Shen, C. Abate-Shen

Development of methodology: N. Floch, C.W. Kinkade, A. Califano, C. Abate-Shen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Floch, C.W. Kinkade, A. Aytes, M. Shen

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Floch, C.W. Kinkade, T. Kobayashi, A. Aytes, C. Lelebire, A. Mitrofanova, B.D. Cardillo, A. Califano, C. Abate-Shen

Writing, review, and/or revision of the manuscript: N. Floch, C.W. Kinkade, T. Kobayashi, A. Califano, M. Shen, C. Abate-Shen

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Floch, C. Abate-Shen

Study supervision: N. Floch, C. Abate-Shen

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References

Targeting Akt/mTOR in Castration-Resistant Prostate Cancer

Dual Targeting of the Akt/mTOR Signaling Pathway Inhibits Castration-Resistant Prostate Cancer in a Genetically Engineered Mouse Model

Nicolas Floc'h, Carolyn Waugh Kinkade, Takashi Kobayashi, et al.


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