Synthetic Lethality in PTEN-Mutant Prostate Cancer Is Induced by Combinatorial PI3K/Akt and BCL-XL Inhibition
Wenying Ren, Raghav Joshi, and Paul Mathew

Abstract

The bone-conserved metastatic phenotype of prostate cancer is a prototype of nonrandom metastatic behavior. Adhesion of prostate cancer cells to fibronectin via the integrin α5 (ITGA5) has been proposed as a candidate bone marrow niche localization mechanism. We hypothesized that the mechanisms whereby ITGA5 regulates the adhesion-mediated survival of prostate cancer cells will define novel therapeutic approaches. ITGA5 shRNA reduced expression of BCL-2 family members and induced apoptosis in PC-3 cells. In these PTEN-mutant cells, pharmacologic inhibition of the PI3K signaling pathway in combination with ITGA5 knockdown enhanced apoptosis. Chemical parsing studies with BH3 mimetics indicated that PI3K/Akt inhibition in combination with BCL-XL-specific inhibition induces synergistic apoptosis specifically in PTEN-mutant prostate cancer cells, whereas single-agent PI3K/Akt inhibitors did not. Given the importance of PTEN loss in the progression of prostate and other cancers, synthetic lethality induced by combinatorial PI3K/Akt and BCL-XL inhibition represents a valuable therapeutic strategy.

Introduction

The metastatic phenotype of prostate cancer is an exemplar of the nonrandom nature of metastases. Long into its natural history, the illness is dominated by and often confined to progressive dissemination of tumor cells within the bone marrow microenvironment. This outlier biological behavior suggests a narrow range of molecular themes that constrain the metastatic phenotype of the disease. Typically, the bone metastatic disease is distributed to areas of active hematopoiesis inferring the likely concordance of the hematopoietic niche and the bone metastatic niche. Bone-targeted therapy with bone-homing radioisotopes have altered the natural history of metastatic castration-resistant disease (1), offering impetus to the idea that a deeper understanding of the specific survival advantages that prostate cancer cells leverage in the niche could provide a more elegant and effective tailored strategy.

Multiple lines of evidence have suggested that mesenchymal stromal cells and/or their derivative osteoblasts are architects of the hematopoietic niche (2, 3). Experimental evidence has suggested that PC-3 and C4-2B prostate cancer cells demonstrated to compete for the hematopoietic niche (4). Adhesion, migration, and invasion of prostate cancer cells to fibronectin and its fragments via the integrin α5 (ITGA5) has been proposed as a candidate bone marrow localization mechanism controlled by bone-derived mesenchymal stromal cells (5, 6). Integrin-mediated cellular adhesion to extracellular matrix components is a crucial regulator of tumor cell survival (7). We hypothesized that the ITGA5 could specifically mediate survival signals in prostate cancer cells demonstrated to compete for the hematopoietic niche in experimental models and that insights from these observations could lead to novel therapeutic strategies in the disease.

Materials and Methods

Reagents

The inhibitors (target) BKM120 (buparlisib, pan-PI3K), picitlisib (pan-PI3K), ipatasertib (pan-Akt), navitoclax (BCL-XL),...
EMD Millipore (Chemicon), and caspase-8, PTEN, HSP90, Akt, and phospho-Akt antibodies were from Cell Signaling Technology. GAPDH antibody was from Thermo Fisher Scientific-Invitrogen.

Plasmid construction

Two human \( I T G A 5 \) shRNAs were generated using the following primers: \( \text{shIT} \text{GTA} \text{5} \ #1, 5'-\text{GCTACCCCTCCACAGATACGTCGGAGAGGTAGCCCTTTTTG-3'} \) (forward) and 5'-\( \text{AATTCAAAAAGGCTACCTCTCCACAGATAACTCGGAA} \) (reverse); \( \text{shIT} \text{GTA} \text{5} \ #2, 5'-\text{GCAGAGACAGAAGATCCTCAACGCGAGATCGTCCTTCCTTTG-3'} \) (forward) and 5'-\( \text{AATTCAAAAAGGCTACCTCTCCACAGATAACTTCGTAT-} \text{CTGAGAGGTAGCCCTTTTTG-3'} \) (reverse). Single-strand DNA oligos were annealed and cloned into pKSU6 expression vector between MscI and EcoRI sites. Luciferase and scramble shRNA expression vectors were gifts from Dr. Keyong Du (Tufts Medical Center, Boston, MA).

Cell culture and transient transfection

Human prostate cancer cell lines PC-3, LNCaP, and DU145 were obtained from ATCC. C4-2B was obtained from MD Anderson Cancer Center. Characterized Cell Line Core Facility. PC-3 and DU145 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin. LNCaP and C4-2B cells were cultured in RPMI supplemented with 10% (v/v) FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin. Cells were maintained in a humidified 37°C incubator with 5% \( \text{CO}_2 \). The transient transfection was carried out with Lipofectamine 2000 (Thermo Fisher Scientific-Invitrogen) according to the manufacturer's instructions.

Annexin V apoptosis assay

PC-3 cells were transiently transfected with shLuciferase or \( \text{shIT} \text{GTA} \text{5} \) expression vector, and cell apoptosis was assayed using a FITC-labeled Annexin V (Annexin V-FITC) Apoptosis Detection Kit (BD Biosciences). Cells were harvested 36 hours posttransfection, washed twice with cold PBS, and then resuspended in 1× binding buffer, followed by staining with Annexin V-FITC and propidium iodide (PI) at room temperature in the dark for 15 minutes. Immediately after staining, the percentage of apoptotic cells was quantified by flow cytometry (Beckman Coulter CyAn ADP Analyzer) according to the manufacturer's instructions. Cells that were Annexin V negative and PI positive were considered viable cells, whereas cells positive for Annexin V only were considered early apoptotic, and cells positive for PI only were considered necrotic. However, the Annexin V-FITC and PI double positively stained cells were deemed nonviable, late apoptotic, and necrotic.

Cell viability assay

The effect of \( \text{IT} \text{GTA} \text{5} \) silencing or drug treatment on cell viability was monitored by cell viability assay using alamarBlue (Thermo Fisher Scientific) and Vita-Blue (Selleckchem-Biotool) cell viability reagents. Briefly, the cells (2,000–5,000 depending on cell lines and treatments) were seeded in a 96-well microtiter plate (100 \( \mu \)L per well) with replications under the designated treatments as described in the figure legends. After incubation with differential drugs at the indicated concentrations and time points, cell viability was measured with GloMax-Multi Microplate Reader (Promega) quantitatively by recording the relative fluorescence units using the optical filter (Ex = 530–570 nm; Em = 590–620 nm). Percent cell viability (%) was calculated and shown as a ratio of absorbance in treated cells to absorbance in control cells (vehicle) after subtracting the average absorbance of background fluorescence.

Statistical analysis

Means ± SD were calculated, and statistically significant differences among groups were determined by one-way ANOVA analysis followed by post hoc comparisons, or by two-tailed unpaired Student t test between two groups as appropriate, with minimal significance at \( P < 0.05 \).

Results

\( \text{IT} \text{GTA} \text{5} \) knockdown induces apoptosis in PC-3 cells

To explore the prosurvival role of \( \text{IT} \text{GTA} \text{5} \), we inactivated \( \text{IT} \text{GTA} \text{5} \) in the PC-3 null androgen receptor–negative prostate cancer cell line PC-3 using shRNA. As hypothesized, an apoptotic response and reduction in cell viability concurrent with reduction in \( \text{IT} \text{GTA} \text{5} \) was observed (Fig. 1A–C).

BCL-2 family proteins are downregulated and apoptosis is enhanced when PI3K inhibition is combined with \( \text{IT} \text{GTA} \text{5} \) knockdown in PTEN-deficient cells

We next hypothesized that in these PTEN-deficient cells, the PI3K/Akt signaling pathway would collaborate with \( \text{IT} \text{GTA} \text{5} \) in regulating prosurvival signals. To test this further, we assessed a combination of PI3K inhibition with the pan-PI3K inhibitor buparlisib and genetic inactivation of \( \text{IT} \text{GTA} \text{5} \) compared with either strategy alone. Combined PI3K inhibitor therapy and knockdown of \( \text{IT} \text{GTA} \text{5} \) each was associated with downregulation of BCL-2, BCL-X\(_L\), and MCL-1 proteins, but an enhanced apoptotic response was seen in combination compared with either strategy alone (Fig. 1D).

Synergistic apoptosis is obtained when PI3K or Akt inhibition is combined with pharmacologic inhibition of BCL-2/BCL-X\(_L\) in PTEN-mutant prostate cancer cells

We hypothesized that potent and specific pharmacologic inhibitors of the BCL-2 family downstream of \( \text{IT} \text{GTA} \text{5} \) could further enhance the apoptotic response when combined with PI3K pathway inhibition in PTEN-deficient prostate cancer cells. Pharmacologic inhibition of the BCL-2/BCL-X\(_L\) proteins with the BH3 mimetic ABT263 (navitoclax) in combination with PI3K inhibition (buparlisib) demonstrated synergistic induction of an apoptotic response compared with either single agent as assessed by cleaved caspase-3 and cleaved PARP expression (Fig. 2A). No evidence of apoptosis was detectable with dose titration of the PI3K inhibitor despite adequate suppression of pAkt, and only a weak induction of apoptosis with navitoclax was observed in higher doses alone. Interestingly, an increase in \( \text{IT} \text{GTA} \text{5} \) expression was noted with dose titration of navitoclax, suggestive of a feedback loop mechanism that is activated by BCL-2/BCL-X\(_L\) inhibition in these cells. In contrast, \( \text{IT} \text{GTA} \text{5} \) expression decreased with titrated doses of and inhibition of pAkt, suggesting a direct regulation of \( \text{IT} \text{GTA} \text{5} \) by the PI3K pathway (Fig. 2A). In a second PTEN-mutant
prostate cancer line, LNCaP, we found that synergistic apoptosis was also induced with PI3K or a pan-Akt inhibitor (sunitinib) in combination with navitoclax. The induction of the apoptotic response as assessed by cleaved PARP expression was sustained over 72 hours with buparlisib and navitoclax (Fig. 2B). Once again single-agent PI3K or Akt inhibitors were not capable of inducing apoptosis in this PTEN-deficient background despite pharmacodynamic evidence of Akt inhibition with both agents. A similar pattern of apoptotic induction was confirmed in a third PTEN-deficient LNCaP derivative line C4-2B, with PI3K/Akt and BCL-2/BCL-X_L inhibitor combination therapy. In contrast, no evidence of apoptotic induction was seen in the PTEN wild-type DU145 cells with titrated doses of PI3K inhibitors alone or in combination with either navitoclax or the BCL-2–specific inhibitor ABT199 (venetoclax; Supplementary Fig. S1). Absence of PTEN expression and constitutive pAkt expression was confirmed in all three PTEN-mutant prostate cancer lines by Western blot analysis (data not shown).

Chemical parsing indicates that BCL-X_L inhibition is essential for induction of synthetic lethality with PI3K/Akt inhibitors in PTEN-deficient prostate cancer cells

Given these observations, we sought to identify whether BCL-2 or BCL-X_L inhibition was critical to the induction of synthetic lethality in PTEN-mutant cells using chemical parsing with specific BH3 mimetics as described previously (8). Using highly specific and potent BCL-2 and BCL-X_L inhibitors, respectively, we found that single-agent BCL-X_L inhibition induced apoptosis in PC-3 cells, whereas single-agent BCL-2 inhibition did not (Fig. 3A). Synergistic apoptosis was demonstrated when PI3K or Akt inhibition was combined with either BCL-X_L inhibition alone or BCL-2/BCL-X_L inhibition but not with BCL-2 inhibition alone. These parsing studies in PC-3 (Fig. 3A) and C4-2B cells (Fig. 3B) confirmed the essential role of BCL-X_L inhibition in regulating the apoptotic threshold in these PTEN-mutant prostate cancer cells. Interestingly, the extrinsic pathway of apoptosis is also activated with the potent BCL-X_L inhibitor A-1331852 as evidenced by cleaved caspase-8 (Fig. 3A and B).

Downregulation of MCL-1 expression with PI3K or Akt inhibition was observed in PC-3 cells, suggesting that combined MCL-1 and BCL-X_L inhibition may contribute to the synergistic apoptotic response with the combination. However, when the specific MCL-1 inhibitor A-1210477 was combined with A-1331852, no enhancement in apoptotic response was observed (Fig. 3C), suggesting that MCL-1 does not contribute significantly to BCL-X_L in the regulation of the apoptotic threshold in these PTEN-deficient cells.

Our earlier observations indicated that ITGA5 expression increased with BCL-2/BCL-X_L inhibition, consistent with a feedback-regulatory loop that further links ITGA5 with the BCL-2 family in these cells. This feedback loop is nevertheless countermanded by concomitant PI3K inhibition, which decreases ITGA5 expression (Fig. 2A). Expression analysis demonstrates that ITGA5 transcription and expression is decreased by PI3K inhibition and increased with combined BCL-2/BCL-X_L inhibition (Supplementary Fig. S2A). To address whether the ITGA5 feedback loop can generate resistance to therapy, ITGA5 knockdown combined with both PI3K inhibition and navitoclax enhanced apoptosis, suggesting that a feedback loop via ITGA5 may mediate resistance to combination therapy (Supplementary Fig. S2B). However, we do not have evidence that when PI3K inhibition is combined with specific and more potent single-agent BCL-X_L therapy, that is, with A-1331852, that this feedback loop can generate resistance to therapy.

Cell viability data appear to be highly consistent with the biochemical data, with specific inhibition observed in PTEN-deficient PC-3, LNCaP, and C4-2B cells with combined PI3K/Akt and BCL-X_L inhibition contrasted with PTEN wild-type DU145 cells (Fig. 3D; Supplementary Table S1), likely explained by a...
combination of antiproliferative effects and induction of apoptosis in the PTEN-deficient cells. The combination of Akt inhibitor and BCL-X\(_\text{i}\) inhibition appeared particularly potent in LNCaP cells with intermediate sensitivities noted in PC-3 and C4-2B cells (Fig. 3D; Supplementary Table S2).

**Discussion**

We determined whether ITGA5, implicated in fibronectin-mediated adhesion as a putative niche localization mechanism of prostate cancer cells in the bone marrow (5, 6, 9), regulates the survival of prostate cancer cells. The results implicate a functional role of ITGA5 in mediating prosurvival signals to BCL-X\(_\text{i}\), which collaborates in regulating the apoptotic threshold with the PI3K signaling pathway in PTEN-mutant prostate cancer cells. In contrast to effects observed with single-agent PI3K or Akt inhibition, synthetic lethality is obtained when PI3K or Akt inhibitors are combined specifically with BCL-X\(_\text{i}\) inhibitors in PTEN-mutant cancers, suggesting a novel therapeutic strategy for this major subset of the disease.

PTEN is one of the most commonly mutated and deleted tumor suppressor genes in human cancer (10). PTEN loss is one of the genetic hallmarks of disease progression in prostate cancer and functions as an oncogene in transgenic models of disease pathogenesis (11–13). Increasing frequency of PTEN loss is observed in progressively higher grades and stages of localized disease (14), and in metastatic castration-resistant prostate cancer, at least 40% of cancers will exhibit allelic loss of PTEN; and a smaller proportion will have point mutations and epigenetic silencing that also result in PTEN deficiency (15). Genomic alterations of PIK3CA, PIK3CB, and Akt1 are low-frequency events (1%–6%) by contrast. PTEN/PI3K/Akt pathway appears to be critical for the viability and maintenance of stem-like properties in prostate cancer cells (16) and the PTEN dose appears to fine tune the progression of the neoplastic phenotype (10). Yet, the results of PI3K, Akt, and mTOR pathway inhibitors (17) thus far have not reported significant single-agent activity in prostate cancer, raising questions as to the value of this therapeutic approach in the illness. Hitherto, combinatory therapy that has resulted in synthetic lethality in the context of the loss of the most common tumor suppressor gene in prostate cancer has not been identified.

Figure 2.

Synergistic induction of apoptosis with combinatorial BCL-2/BCL-X\(_\text{i}\) and pan-PI3K or pan-Akt inhibitors in PTEN-deficient PC-3 and LNCaP cells. A and B, Western blot demonstrating that buparlisib/ipatasertib and navitoclax synergistically induce apoptosis in PC-3 cells (A) in a dose-dependent fashion and LNCaP cells (B). Doses are in \(\mu\text{mol/L}\), and if not indicated 1 \(\mu\text{mol/L}\).
of ITGA5. Furthermore, we identified a feedback signaling loop that upregulates ITGA5 transcription and expression when BCL-2/BCL-XL are inhibited, providing further evidence of the connection of these pathways. Taken together, PTEN loss appears to be instrumental in the upregulation of ITGA5 expression and functions to leverage prosurvival signals via the BCL-XL protein.

PTEN-dependent regulation of BCL-XL independent of the PI3K/Akt pathway is plausible given that significant apoptosis is observed only with both PI3K/Akt and BCL-XL inhibitors in combination specifically in PTEN-mutant cells (Overview).

Validation of this novel synthetic lethality principle in in vivo models of PTEN-deficient prostate cancer and other neoplasms is

Figure 3.
Chemical parsing indicates that BCL-XL inhibition is essential for synthetic lethality with PI3K/Akt inhibitors in PTEN-mutant cells. A and B, Enhanced induction of synergistic apoptosis with A-1331852 over navitoclax and not venetoclax in PC-3 cells (A) and C4-2B cells (B) confirming the specificity for BCL-XL inhibition and potency of A-1331852. C, Although MCL-1 downregulation in A and B seems to be correlated with synergistic induction of apoptosis, combined A-1210477 and A-1331852 does not recapitulate this synergy. D, Cell viability assay showing differential impact of combined PI3K/Akt and BCL-XL in PTEN-WT DU145 versus PTEN-deficient PC-3 and LNCaP cells. All drugs were used at 1 μmol/L.
required to assist design of translation to the clinic. BCL-X\textsubscript{L} inhibitors have demonstrated thrombocytopenia as a dose-limiting toxicity, although low initial lead-in doses, intrapatient escalation, and intermittent schedules may permit biologically effective dose schedules to combine with Akt or PI3K inhibitors. Overlapping toxicity between BCL-X\textsubscript{L} and PI3K/Akt inhibitors is not otherwise anticipated. It is not yet established whether PI3K or Akt isoform-specific inhibition can phenocopy the synthetic lethality induced by the pan-PI3K/Akt inhibitors utilized in this study.

Although the Akt pathway regulates the apoptotic threshold in PTEN-mutant cells via phosphorylation and inactivation of the proapoptotic BAD protein (20), potent PI3K/Akt inhibition is insufficient to trigger an apoptotic response in PTEN-mutant prostate cancer cells without concomitant BCL-X\textsubscript{L} inhibition. In PTEN-mutant cells in which constitutive activation of the EGFR pathway is present, a modified strategy targeting that pathway may be required (20).

In summary, by tracing a putative mechanism of bone-homing behavior in PTEN-mutant prostate cancer cells (5, 6), we have decoded a collaborative mechanism of cell survival in PTEN-deficient prostate cancer cells linking the PI3K/Akt and BCL-X\textsubscript{L} pathways with translational implications. In vivo modeling experiments will be required to validate and define a feasible and effective biomarker-driven strategy in the clinic, in prostate cancer and potentially other PTEN-deficient neoplasms.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: W. Ren, R. Joshi, P. Mathew

Development of methodology: W. Ren, R. Joshi, P. Mathew

 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Ren, R. Joshi, P. Mathew

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Ren, R. Joshi, P. Mathew

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Joshi, P. Mathew

Study supervision: P. Mathew

**Grant Support**

These studies were enabled and supported by funding support from Will and Julie Peterson.

Received June 10, 2016; revised August 18, 2016; accepted August 25, 2016. Published OnlineFirst September 2, 2016.

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Molecular Cancer Research

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Mol Cancer Res 2016;14:1176-1181. Published OnlineFirst September 2, 2016.

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