Elevation of Receptor Tyrosine Kinases by Small Molecule AKT Inhibitors in Prostate Cancer Is Mediated by Pim-1

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Abstract
The PI3K/AKT pathway is hyperactivated in prostate cancer but its effective therapeutic targeting has proven difficult. In particular, the antitumor activity of AKT inhibitors is attenuated by upregulation of receptor tyrosine kinases (RTK) through an uncharacterized feedback mechanism. In this report, we show that RNA interference-mediated silencing or pharmacologic inhibition of Pim-1 activity curtails AKT inhibitor-induced upregulation of RTKs in prostate cancer cells. Although Pim kinases have been implicated in cap-dependent translational control, we find that in the context of AKT inhibition, the expression of RTKs is controlled by Pim-1 in a cap-independent manner by controlling internal ribosome entry. Combination of Pim and AKT inhibitors resulted in synergistic inhibition of prostate tumor growth \textit{in vitro} and \textit{in vivo}. Together, our results show that Pim-1 mediates resistance to AKT inhibition and suggest its targeting to improve the efficacy of AKT inhibitors in anticancer therapy, \textit{Cancer Res}; 73(11); 3402–11. ©2013 AACR.

Introduction
The PI3K/AKT pathway is commonly activated in human cancer and controls cellular processes that contribute to the initiation and maintenance of cancer (1). It is activated in 40% of primary and 70% of metastatic prostate cancers secondary to mutations or deletions in PTEN (1–3). Activation of the pathway can be associated with mutations in the phosphoinositide 3-kinase (PI3K) catalytic subunit P110\textalpha and regulatory subunit (1), mutations in each of the 3 AKT isoforms (1, 4), and activation of receptor tyrosine kinases (RTK) by mutation (e.g., EGF receptor; EGFR) or gene amplification (e.g., HER2), which can result in activation of downstream PI3K/AKT (1, 5). Multiple small-molecule inhibitors have been developed to target PI3K/mTOR or AKT (6), but the efficacy of these drugs is compromised by the stimulation of compensatory signaling pathways that have the potential to enhance tumor growth (7–9). There is accumulating evidence that inhibition of the PI3K/AKT pathway can lead to adaptive resistance due to upregulation and activation of RTKs (7–9). The mechanism underlying the AKT inhibition-induced upregulation of some of these RTKs, including HER3, INSR, and insulin-like growth factor-1 receptor (IGF-1R), has been shown to, in part, involve FOXO transcription factors (7); however, these transcription factors do not seem to be involved in the AKT inhibition-induced upregulation of other RTKs, including MET, HER2, and RET (7).

The PI3K/AKT pathway is hyperactivated in prostate cancer but its effective therapeutic targeting has proven difficult. In particular, the antitumor activity of AKT inhibitors is attenuated by upregulation of receptor tyrosine kinases (RTK) through an uncharacterized feedback mechanism. In this report, we show that RNA interference-mediated silencing or pharmacologic inhibition of Pim-1 activity curtails AKT inhibitor-induced upregulation of RTKs in prostate cancer cells. Although Pim kinases have been implicated in cap-dependent translational control, we find that in the context of AKT inhibition, the expression of RTKs is controlled by Pim-1 in a cap-independent manner by controlling internal ribosome entry. Combination of Pim and AKT inhibitors resulted in synergistic inhibition of prostate tumor growth \textit{in vitro} and \textit{in vivo}. Together, our results show that Pim-1 mediates resistance to AKT inhibition and suggest its targeting to improve the efficacy of AKT inhibitors in anticancer therapy, \textit{Cancer Res}; 73(11); 3402–11. ©2013 AACR.

Materials and Methods

Reagents and antibodies
GSK690693 was provided by GlaxoSmithKline for \textit{in vitro} and \textit{in vivo} studies. MK2206, PP242, AZD8055, and BEZ235 were purchased from Selleck Biochemicals. Antibodies are listed in the Supplementary Data.

Plasmids
The S'-untranslated region (UTR) of human \textit{Met} (15) was amplified by PCR using genomic DNA extracted from PC3-LN4
cells as template with the following 2 primers: 5'-ATAC-TAGCTGAGCCGGGCGGTTGCTGA-3' and 5'-AACCAT-GCCCTCACCTCCAGGTGCTGGCGCA-3'. The PCR product was sequenced and cloned into the EcoRI and NcoI sites of the plasmid of pRF to create pR-MET-F.

**Immunoblotting**

Cells were harvested in lysis buffer A consisting of 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% NP-40, and 5 mmol/L EDTA. Protein concentrations were determined by DC Protein Assay (Bio-Rad).

**Cell culture and transfections**

Cell lines were grown in RPMI (PC3-LN4, DU145, 22RV1, VCAP, and BT474) or Dulbecco’s Modified Eagle Medium [HeLa, mouse embryo fibroblasts (MEF)] in 5% CO2. DU145, 22RV1, VCAP, BT474, and HeLa cells were supplied by American Type Culture Collection and passaged in our laboratory for less than 6 months after receipt. PC3-LN4 cells were described before (16). The MEFs, which were triple knockout (TKO) for all Pim genes, were previously described (17). Cells were transfected with Lipofectamine 2000 reagent according to manufacturer’s instructions.

**Real-time PCR analyses**

SYBR Green reactions were done using a BioRad q5 quantitative real-time PCR (qRT-PCR) system. For data analysis, raw counts were normalized to the housekeeping gene averaged for the same timepoint and condition (ΔCt). Counts are reported as fold change relative to the untreated control (2^−ΔΔCt). All primers were designed and synthesized by Integrated DNA Technologies. Primers are listed in the Supplementary Data.

**Luciferase assays**

Firefly luciferase and Renilla luciferase activities were measured in a luminometer (Model TD 20/20; Turner Designs) using the reagents provided with the Dual Luciferase Reporter kit (Promega).

**Soft-agar colony formation assays**

The soft-agar assay was conducted on 6-well plates in duplicate. For each well, 5,000 cells were mixed in growth medium containing 0.7% agarose and GSK690693 or SMI-4a. Cells were then layered over 1% agarose in regular medium. Medium containing GSK690693 or SMI-4a was added to each well every 4 days. The assays were terminated after 21 days, and colonies were stained with crystal violet and counted under a microscope.

**Cell proliferation measurement**

Cells were plated in 96-well plates at 3,000 cells per well in 100 μL of 10% FBS-containing medium. After 24-hour incubation, the medium was replaced with 0.2% FBS medium with GSK690693, SMI-4a, or dimethyl sulfoxide (DMSO) for 72 hours. Cell viability was measured using a MTT assay. The absorbance was read at 590 nm with a reference filter of 620 nm.

**In vitro transcription and RNA transfection**

The mRNAs were purified with MEGA Clear Kit (Ambion), quantified spectrophotometrically, and their qualities were verified on a denaturing agarose gel. RNA transfection was conducted with TransIT-mRNA Transfection Kit (Mirus) according to the manufacturer’s suggestion. An aliquot of 1 μg of capped mRNAs and 2 μL of TransIT-mRNA reagent together with 1 μL of mRNA boost reagent was used to transfect 80% confluent cells grown in 12-well plates. At 16 hours after transfection, cells were harvested and lysed for luciferase assay.

**Animal experiments**

Four- to six-week-old nu/nu nude male mice were obtained from Charles River Laboratories and maintained in pressurized ventilated caging. All studies were conducted in compliance with Institutional guidelines under an Institutional Animal Care and Use Committee-approved protocol (MUSC#3081). For efficacy studies, mice with well-established tumors were selected and randomized 14 days after implantation (size > 150 mm³); PC3-LN4 xenograft tumors were established in nude mice by subcutaneously injecting 5 × 10² cells suspended in PBS into the right flank. Mice were treated with vehicle, GSK690693, or SMI-4a, or GSK690693 + SMI-4a at the indicated doses. GSK690693 was dissolved in 30% propylene glycol, 5% Tween-80, 65% of 5% dextrose in water (pH4.5), and administered intraperitoneally daily, whereas SMI-4a was dissolved in the same solvent and administered by oral gavage twice daily. Tumor dimensions were measured with a caliper and tumor volumes were calculated [tumor volume (mm³) = (length × width²)/2].

**Statistical analysis**

The results of quantitative studies are reported as mean ± SD or mean ± SEM (for animal experiments). Differences were analyzed by Student t test. P < 0.05 was regarded as significant.

**Results**

**AKT inhibition induces Pim-1 expression in prostate cancer cells**

Treatment of the prostate cancer PC3-LN4 cells with the pan-AKT inhibitor GSK690693 markedly increased the levels of Pim-1 protein in a time- and concentration-dependent fashion (Fig. 1A and B) but had a minimal effect on the expression of Pim-3 protein and reduced the levels of Pim-2 (Fig. 1C). Similar results were obtained using another AKT inhibitor, MK2206, and a PI3K/mTOR dual inhibitor, BEZ235 (Fig. 1C). The induction of Pim-1 was also observed with GSK690693 treatment of human prostate cancer cell lines DU145, 22RV1, and VCAP (Supplementary Fig. S1A). The effect of GSK690693 on Pim-1 was not secondary to an off-target effect as knockdown in PC3-LN4 cells of all 3 AKTs with siRNAs increased the levels of Pim-1 protein (Fig. 1D). Treatment of PC3-LN4 cells with GSK690693 or MK2206 resulted in elevations in the level of Pim-1 mRNA, but not Pim-2 or Pim-3 (Fig. 1E). Similarly, treatment of PC3-LN4 cells with siRNAs directed at AKT1, AKT2, and AKT3 also resulted in the elevation of Pim-1 mRNA.
To further determine whether GSK690693 regulates the transcription of the \textit{Pim-1} gene, a 3.0 kb promoter fragment of the \textit{Pim-1} promoter was cloned upstream of a luciferase reporter. Addition of GSK690693 increased the activity of this promoter in PC3-LN4 cells (Fig. 1G).

**Upregulation of \textit{Pim-1} is required for AKT inhibitor-associated induction of RTKs**

Consistent with previous reports of upregulation of these RTKs in response to AKT inhibition (7), treatment of PC3-LN4 cells with GSK690693 increased the protein levels of multiple RTKs, including MET, EPHA2, RON, EGFR, HER2, HER3, INSR, and IGF-IR (Supplementary Fig. S1B). In addition, we observed increased extracellular signal-regulated kinase (ERK) phosphorylation resulting from treatment with GSK690693 (Supplementary Fig. S1B). This is in keeping with previous finding that PI3K inhibition leads to mitogen-activated protein kinase pathway activation (18–19).

GSK690693 also completely blocked the phosphorylation of 2 well-known AKT substrates, GSK3\(\beta\) and PRAS40, showing the effectiveness of this compound (Supplementary Fig. S1C) and caused the paradoxical hyperphosphorylation of AKT at its 2 regulatory sites (Thr308 and Ser473; Supplementary Fig. S1C), a common property of ATP-competitive AKT inhibitors (20).

To determine whether \textit{Pim-1} plays an important regulatory role in the ability of AKT inhibitors to modulate RTKs, we first determined the effects of \textit{Pim}-directed siRNAs and small-molecule inhibitors. The use of siRNA directed at \textit{Pim-1} showed that a forced reduction in \textit{Pim-1} levels markedly reduced the ability of GSK690693 to elevate the protein levels of multiple RTKs, including MET and EPHA2, HER3, HER2, INSR, and IGF-IR (Fig. 2A). The addition of SMI-4a, a small-molecule \textit{Pim} kinase inhibitor (21), reduced GSK690693-induced upregulation of RTK protein levels in PC3-LN4 (Fig. 2B), DU145, 22RV1, and VCAP cells (Supplementary Fig. S2).

The results of phospho-RTK antibody array (reverse-phase protein array; RPPA) analysis revealed that treatment of PC3-LN4 cells with GSK690693 increased the tyrosine phosphorylation of a number of RTKs tested in the assay, that is, MET, EPHA2, HER3, HER2, INSR, and IGF-IR (Supplementary Fig. S3). The lack of complete correlation in these assays may arise from the differing specificity of the antibodies used in the RPPA analysis. This change in RTK phosphorylation is consistent with the AKT inhibitor-induced increases in the protein levels of the...
RTKs; however, it cannot be ruled out that GSK690693 stimulates RTK phosphorylation through an alternative mechanism (7). Treatment with SMI-4a blocked the GSK690693-induced RTK phosphorylation (Supplementary Fig. S3), showing that the inhibition of Pim reverses the activity of this AKT inhibitor. To further evaluate the role of Pim-1 in regulating AKT inhibitor-induced upregulation of RTKs, MEFs were treated with GSK690693. In wild-type cells, but not in the Pim kinase-decient (TKO) cells, GSK690693 treatment of the cells increased the levels of the RTKs tested, that is, MET, HER3, IGF-IR, and EPHA2 protein, as well as the phosphorylation of ERK (Fig. 2C). We treated PC3-LN4 (Fig. 2D) and VCAP (Supplementary Fig. S4) cells with 3 different Pim kinase inhibitors, SMI-4a (4a, 10 μmol/L), SMI-16a (16a, 10 μmol/L), or K00135 (K, 5 μmol/L) for 24 hours (D); PC3-LN4 cells were transfected with a nontargeting control siRNA, siRNA against Pim-1, an empty vector, or a Pim-1–expressing plasmid for 72 hours (E).

AKT inhibition increases cap-independent translation

AKT protein kinase activity controls protein synthesis by regulating the multistep process of mRNA translation at multiple stages from ribosome biogenesis to translation initiation and elongation (23). Although GSK690693 treatment of prostate cancer cells did not modify phosphorylation of 4E-BP1, this compound increased phosphorylation of eIF2α and eliminated phosphorylation of ribosomal protein S6 (Fig. 3A). To further define the role of cap-dependent translation in the mechanism of action of this agent, GSK690693 was combined with 2 potent inhibitors of mTOR complex (mTORC)-1/mTORC2 and thus cap-dependent translation, PP242 and AZD8055 (24–25). These inhibitors in combination with...
GSK690693 resulted in reduced phosphorylation of 4E-BP1 and increased eIF2α phosphorylation compared with GSK690693 alone (Fig. 3A), suggesting inhibition of 5'-cap-dependent translation. We measured the binding of eIF4G and 4E-BP1 to the 5' mRNA cap by using m7GTP-sepharose. The structure of these beads mimics the 5' mRNA cap and precipitates cap-interacting proteins. In agreement with the effect on phosphorylation of 4E-BP1, PP242, or AZD8055 in combination with GSK690693 strongly reduced eIF4G and increased 4E-BP1 binding to m7GTP-sepharose, whereas GSK690693 alone did not have a significant effect (Supplementary Fig. S5A). However, the treatment of prostate cancer cells with these mTORC1 inhibitors did not reduce the GSK690693-induced elevation of MET, EPHA2, HER3, and IGF-IR (Fig. 3A). A recent study (26) using Torin 1, an ATP-competitive mTOR inhibitor, showed that Torin 1-resistant mRNAs are enriched for RTKs such as MET, IGF-IR, and INSR, indicating that the translation initiation of these mRNAs do not depend on mTOR activity (27). We found that treatment of PC3-LN4 cells with PP242 or AZD8055 indeed did not inhibit the expression of MET, EPHA2, HER3, IGF1R, or INSR (Supplementary Fig. S5B). In addition, the expression of Bcl-2 whose translation under cellular stress has been shown to be controlled by a cap-independent mechanism was not suppressed by treatment with mTOR inhibitors, whereas proteins known to be sensitive to mTOR inhibition, YB-1, HSP90, RPS7 (26, 29), were reduced (Supplementary Fig. S5B). Reduced eIF4G and increased 4EBP1 binding to m'GTP-sepharose, and increased eIF2α phosphorylation (Supplementary Fig. S5C) confirmed that cap-dependent translation was efficiently inhibited. Together these data suggest that upregulation of RTKs is not controlled by cap-dependent mechanisms.

Under conditions of decreased cap-dependent translation, the internal ribosome entry site (IRES)-mediated translation can play a larger role in regulating protein synthesis (30). Recently, it has been shown that inhibition of PI3K/mTOR leads to increased IRES-mediated translation (8). Inhibition of AKT by GSK690693 resulted in increased IRES activity measured by ratio of firefly to Renilla luciferase activities in constructs containing either cellular [hypoxia-inducible factor-1α (HIF1α), Myc, and VEGF; ref. 31] or viral [cricket paralysis virus (CrPV) and hepatitis C virus (HCV); ref. 32] IRES sequences (Fig. 3B). In agreement with these findings, GSK690693 induced expression of Bcl-2, Myc, VEGF, and HIF1α, all of which can be translated in a cap-independent manner under cellular stress (28, 31, 33–34), further suggesting the possibility that cap-independent translation is upregulated (Fig. 3C).

**Pim-1 regulates RTK expression through cap-independent translation**

Expression of human Pim-1 in PC3-LN4 cells did not affect the levels of RTK mRNAs (Supplementary Fig. S6A and B) or the half-life of the RTKs (Supplementary Fig. S6C), suggesting that Pim-1 may control the levels of these proteins through a translational mechanism. Plus, GSK690693 increased cap-
independent translation (Fig. 3). Taken together, we speculated that the upregulation of the RTKs induced by AKT inhibitors could be controlled, at least in part, by a cap-independent mechanism. We first determined whether the MET 5′-UTR contains an IRES that could be stimulated by either GSK690693 or Pim-1. The MET 5′-UTR is relatively long (408 nt) and is guanine-cytosine (GC)-rich (15), which are common properties of IRES-containing 5′-UTRs. The 5′-UTR of MET was cloned and inserted in front of firefly luciferase in the dicistronic vector pRF (35). The presence of the MET 5′-UTR sequence increased the expression of downstream firefly luciferase relative to Renilla by 38-fold compared with the vector control (Fig. 4A), suggesting that it could function as an IRES. In comparison, the IRESs of encephalomyocarditis virus (EMCV), HIF1α, and VEGF produced 18-, 9-, and 13-fold increases, respectively. In PC3-LN4 cells transfected with the pRF vector containing the MET IRES, overexpression of Pim-1 or treatment of GSK690693 resulted in an increase in ratio of firefly/Renilla luciferase activities as compared with control treatment (Fig. 4A). Knockdown of Pim-1 suppressed GSK690693-induced MET IRES activities (Supplementary Fig. S7). Collectively, these results indicated that Pim-1 can potentially regulate translation of MET in a cap-independent fashion.

To determine whether the MET 5′-UTR is sufficient to drive translation by acting as an IRES and to rule out the possibility of a cryptic promoter in the 5′-UTR of MET, we in vitro transcribed the pRF vector containing the MET IRES yielding a capped dicistronic mRNA, and then transfected this mRNA directly into PC3-LN4 cells. Insertion of the MET or VEGF 5′-UTR resulted in a 7- or 5-fold increase in the firefly/Renilla ratio, respectively. In comparison, when the pRF vector containing the viral EMCV IRES was transcribed and transduced into these cells, the firefly/Renilla ratio increased by 114-fold (Fig. 4B). Thus, in comparison with a viral IRES, both the MET and VEGF sequences have relatively weak IRES activities. Besides MET, other RTKs including IGF-IR have been reported to have IRES elements in their 5′-UTRs (36). As shown in Fig. 4C, the IRES activity of the 5′-UTR of IGF-IR was increased on treatment of the cells with GSK690693 or Pim-1 overexpression and, conversely, was decreased on knockdown of endogenous Pim-1 protein levels. Furthermore, knockdown of Pim-1 suppressed GSK690693-induced IGF-IR IRES activities (Supplementary Fig. S7). It is possible that this mechanism is important for the control of other RTKs because in general these genes have long 5′-UTRs. In addition, knockdown of Pim-1 in PC3-LN4 cells led to a reduction of IRES activities of viral, CrPV...
and HCV, and cellular, HIF1α and Myc, IRESs (Fig. 4D). These data suggest that Pim-1 could be a more general regulator of IRES-mediated translation. This concept is further supported by our finding that the upregulation of proteins whose translation can be controlled by an IRES-mediated mechanism under cellular stress, Bcl-2, Myc, VEGF, and HIF1α, is stimulated by GSK690693 and requires Pim-1 expression (Fig. 4E).

Ribosomal stress abrogates AKT inhibition-induced upregulation of RTK expression

Pim-1 has been shown to physically interact with ribosomal protein S19 and to cosediment with ribosomes (37–38). Knockdown of ribosomal protein S19 or S6 abolished upregulation of MET, EPHA2, HER3, and IGF-IR induced by GSK690693 without affecting Pim-1 induction (Fig. 5A). Consistent with findings from other laboratories (38–40), reduced protein expression of ribosomal protein S6 was seen when S19 was decreased by siRNA and vice versa (Fig. 5A). To test the effect of ribosomal stress on RTK upregulation independent of ribosomal protein knockdowns, low concentrations of actinomycin D (ActD) were used to inhibit RNA polymerase I, and thus induce ribosomal stress (41–42). Similar to S19 and S6 knockdowns, ActD treatment blocked upregulation of MET, EPHA2, HER3, and IGF-IR induced by GSK690693 (Fig. 5B). ActD treatment also inhibited upregulation of MET, EPHA2, and HER3 resulting from direct Pim-1 overexpression in PC3-LN4 cells (Fig. 5C). Ribosomal stress did not seem to affect global translation as the expression of Src and ERK1/2 proteins was not altered (Fig. 5A and B). These data suggest that Pim-1 may work through intact ribosomes to control RTK expression.

Combination treatment with an AKT and a Pim inhibitor synergistically blocks prostate tumor growth in vitro and in vivo

As a preliminary test of whether combined inhibition of AKT and Pim kinases might provide synergistic antitumor efficacy, we tested the effects of the inhibitors on the proliferation of PC3-LN4 cells in vitro. Treatment of PC3-LN4 cells with the Pim inhibitor SMI-4a in combination with the AKT inhibitor GSK690693 resulted in a synergistic enhancement of the inhibition of proliferation as shown by combination index of less than 0.5 (Fig. 6A; data not shown), and a markedly greater reduction in both the numbers and the size of colonies seen in a soft-agar colony formation assay (Fig. 6B). GSK690693 and SMI-4a blocked the proliferation of DU145 in a similar fashion (Supplementary Fig. S8).

To test the activity of these agents in vivo, PC3-LN4 cells were injected into mice and treated with GSK690693 alone, SMI-4a alone, or both drugs in combination on a daily basis for 21 days starting at 15 days after tumor implantation. When used alone, treatment of these drugs caused a modest inhibition of tumor growth, whereas the combined treatment resulted in a markedly greater inhibition of tumor growth (Fig. 6C). As shown in Fig. 6D, immunoblot analysis of lysates of tumors harvested at the termination of the experiment on day 36 had upregulated the levels of MET, EPHA2, and HER3 protein in mice treated with GSK690693 as compared with the tumors from mice treated with vehicle (Fig. 6D). Interestingly, the levels of Pim-1 were increased in the combined therapy, and could suggest an in vivo interaction between these agents cannot be ruled out. This upregulation of the RTKs was significantly reduced in the tumors from mice treated with a combination of GSK690693 and SMI-4a (Fig. 6D).

Figure 5. Ribosomal stress abrogates RTK upregulation induced by GSK690693. A, PC3-LN4 cells were treated for 48 hours with siRNAs against Pim-1, ribosomal protein S19, S6 as well as a nontargeting control siRNA (2 left lanes) followed by adding GSK690693 (5 μmol/L) for an additional 24 hours. B, PC3-LN4 cells were treated with increasing dose of ActD with and without 5 μmol/L GSK690693 for 24 hours. C, PC3-LN4 cells were transfected with a Pim-1–expressing plasmid or a control vector. ActD (5 nmol/L) was added 24 hours after transfection for an additional 16 hours. Whole-cell lysates were subjected to immunoblot analyses with the indicated antibodies.

Figure 6. Combination treatment with an AKT and Pim inhibitor synergistically blocks prostate tumor growth in vitro and in vivo. A, PC3-LN4 cells were treated with increasing doses of each inhibitor alone or in combination with the indicated IC50 for 72 hours. Control cells were treated with DMSO vehicle alone. B, PC3-LN4 cells were treated with increasing doses of ActD with and without 5 μmol/L GSK690693 for 24 hours. C, PC3-LN4 cells were transfected with a Pim-1–expressing plasmid or a control vector. ActD (5 nmol/L) was added 24 hours after transfection for an additional 16 hours. Whole-cell lysates were subjected to immunoblot analyses with the indicated antibodies.
Discussion

The results of these experiments provide insights into the mechanisms underlying the compensatory interplay between AKT and Pim-1 in the regulation of prostate cancer cell behavior influenced by the expression of RTKs. They suggest a model in which reduction in AKT activity is associated with an increase in the levels of Pim-1 protein kinase that occurs through a transcriptional mechanism. This increase in Pim-1 kinase is associated, in turn, with promotion of the expression of RTKs through a cap-independent mechanism. Downregulation of Pim-1 blocks the feedback elevation in RTKs associated with inhibition of AKT (Fig. 6E). Likewise inhibitors of Pim synergize with small-molecule AKT inhibitors to block the growth of prostate cancer cells.

The control of Pim-1 protein levels is complex and has been shown to involve the ubiquitin proteasome pathway and translational mechanisms (43). In the current study, we show that inhibition of AKT can increase the levels of Pim-1 through a transcriptional mechanism; however, it is possible that additional alternative mechanisms could also play a role in increasing Pim-1 protein levels. The induction of Pim-1 by AKT inhibition coincides with suppression of total protein synthesis (Supplementary Fig. S9) and is not inhibited by further treatment with mTORC inhibitors (Fig. 3A), suggesting that Pim-1 protein levels could also be regulated in a cap-independent manner. The Pim-1 5'UTR may contain an IRES that could also be regulated by specific cellular growth conditions (44), although the existence of this IRES is controversial (45).

It has been shown previously that inhibition of AKT regulates the transcription of RTKs by modulating the activity of Foxo transcription factors (7); however, in the same study no change was seen in the level of HER2, RET, or MET mRNAs.
suggesting that the levels of specific RTKs might be controlled by other mechanisms. Cap-dependent translation plays a role in both PI3K/AKT and Pim-2 enhancement of the synthesis of specific proteins (46). It should be noted, however, that molecules that blocked mTORC1 activity could not inhibit the Pim-2 protein kinase and an agent that blocked eIF4A function, which is known to take part in IRES mediated translation, was required. In addition, small-molecule mTORC inhibitors can decrease the translation of many mRNAs, for example, 5'-terminal oligopyrimidine tracts mRNAs, while increasing the level of translation of RTKs (26), again suggesting that these RTK mRNA may be translated in a cap-independent fashion. Moreover, further inhibition of cap-dependent translation with the mTORC1/2 inhibitors, PP242 and AZD8055, had no effect on the ability of GSK960693 or Pim-1 to induce RTKs (Fig. 3A), suggesting that in the experimental conditions used in these studies, the mechanism by which this agent controls RTK levels is not cap dependent.

Our results are consistent with the hypothesis put forward by Muranen and colleagues that inhibition of PI3K/mTOR could lead to enhanced cap-independent translation (8). Cloning of the Met 5'-UTR into a dicistronic luciferase vector showed that it can function as an IRES element, although weakly in comparison with viral sequences, and its activity is enhanced by GSK960693 and Pim-1 overexpression (Fig. 4A and B). Further supporting evidence of the ability of GSK960693 and Pim-1 to regulate the activity of the IRES is the observation that the IGF-IR IRES (47) is stimulated by these agents and that Pim-1 knockdown decreases the activity of this element (Fig. 4C). Our data further suggest that Pim-1 may be essential for full IRES activity of additional viral and cellular IRES elements, including HCV, CrPV, HIF1α, and Myc (Fig. 4D), suggesting a general role of Pim in the control of cap-independent translation.

It has been suggested previously that because they are both survival kinases, AKT and Pim protein kinases could be important pharmacologic targets to inhibit tumor growth (12). Our experiments show a high degree of synergism between small-molecule inhibitors of AKT and Pim in their ability to kill prostate cancer cells both in tissue culture and in a xenograft model (Fig. 6). Analysis of the tumors from treated animals showed that AKT inhibitor treatment elevates RTKs in the tumor cells grown in vivo and that simultaneous treatment with a Pim inhibitor downregulates this effect (Fig. 6D). Because both these kinase pathways are highly activated in human prostate cancer, dual inhibitor treatment of these tumors could be a particularly attractive chemotherapeutic strategy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed by the authors.

Authors’ Contributions
Conception and design: B. Cen, A.S. Kraft
Development of methodology: B. Cen, W. Wang
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Cen, W. Wang, A.S. Kraft
 Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Cen, S. Mahajan, A.S. Kraft
 Writing, review, and/or revision of the manuscript: B. Cen, A.S. Kraft
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