Preclinical Development

Modulation of Akt/mTOR Signaling Overcomes Sunitinib Resistance in Renal and Prostate Cancer Cells

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Abstract
Tyrosine kinase inhibitors exhibit impressive activity against advanced renal cell carcinoma. However, recent clinical studies have shown an equivocal response to sunitinib in patients with castration-resistant prostate cancer. The tumor suppressor PTEN acts as a gatekeeper of the phosphoinositide 3-kinase (PI3K)/Akt/mTOR cell-survival pathway. Our experiments showed that PTEN expression inversely correlates with sunitinib resistance in renal and prostate cancer cells. Restoration of PTEN expression markedly increases sensitivity of tumor cells to sunitinib both in vitro and in vivo. In addition, pharmacologic manipulation of PI3K/Akt/mTOR signaling with PI3K/mTOR inhibitor, GDC-0980, mTOR inhibitor, temsirolimus, or pan-Akt inhibitor, GSK690693, was able to overcome sunitinib resistance in cancer cells. Our findings underscore the importance of PTEN expression in relation to sunitinib resistance and imply a direct cytotoxic effect by sunitinib on tumor cells in addition to its antiangiogenic actions.

Introduction
Small molecule multitargeted receptor tyrosine kinase inhibitors (TKI) represent a clinically relevant strategy for patients with advanced tumors such as renal cell carcinoma (RCC) and gastrointestinal stromal tumors (1, 2). It has been reported that tumoricidal activity of sunitinib is mediated mainly through inhibition of the platelet-derived growth factor receptor, the VEGF receptor (VEGFR) 1 and 3, KIT, and fms-related tyrosine kinase 3 (FLT3; ref. 3). In phase III clinical trials with sunitinib, median survival for patients with metastatic RCC increased from 10 to 24 months (4). As such, sunitinib received approval by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic RCC in 2006. Due to its remarkable success with metastatic RCC, the drug is also under intense investigation as a treatment for several other cancers, including breast, colorectal, and non–small cell lung (3).

More recently, initial clinical experience with sunitinib in castration-resistant prostate cancer (CRPC) patients has been reported. In phase II trials, a heterogeneous response to sunitinib was noted in both docetaxel-resistant and docetaxel-naive patients, with a significant proportion of tumors showing at least a partial radiographic response. In the most recent study of subjects with docetaxel-resistant CRPC, 11.1% of patients showed 30% tumor reduction by Response Evaluation Criteria In Solid Tumors (RECIST) criteria. Another 44.4% of patients had quantifiable but less significant tumor response (5).

Drug resistance to anticancer therapies remains a clinical challenge. Tumors may acquire stability to the drug during treatment or show intrinsic resistance from the outset of therapy. One possible mechanism implicated in TKI resistance may be the constitutive activation of various intracellular protumorigenic pathways downstream of receptor signaling. Activation of the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway, chiefly due to redundant autocrine signaling rather than mutations, is clearly involved in tumor initiation and progression (6). The PI3K/Akt/mTOR axis is a central crossroad of many intracellular signaling pathways, exhibiting direct and indirect control over a multitude of cellular events, including cell-cycle progression, cellular proliferation/growth, autophagy, and angiogenesis (6).

The tumor suppressor PTEN acts as a gatekeeper of the PI3K/Akt/mTOR cell-survival signaling pathway. Under normal cellular conditions, PTEN is a negative regulator of tumorigenic activity; however, when lost or inactivated, the PI3K/Akt/mTOR pathway is enhanced. As such, loss of PTEN can be an indicator of aggressive behavior in tumors of various cell types (6). In this study, we found that the status of PTEN expression inversely correlates with sunitinib resistance in renal and prostate cancer cells. Restored expression of PTEN in PTEN-negative tumor cells markedly increased tumor sensitivity to sunitinib, both in vitro and in vivo. Furthermore, we were able to circumvent sunitinib resistance in PTEN-negative
tumor cells by manipulation of PI3K/Akt/mTOR signaling with pharmacologic agents such as GDC-0980, GSK690693, and temsirolimus. These results indicate that lack of PTEN expression may contribute to intrinsic sunitinib resistance, and the activity of the PI3K/Akt/mTOR pathway may be a marker of TKI response/resistance in genitourinary tumors. Furthermore, coadministration of sunitinib with PI3K/Akt/mTOR inhibitors may represent a rational treatment strategy for patients with resistant urologic tumors.

Materials and Methods

Cells and culture conditions
Androgen-dependent LNCaP prostate cancer cells, androgen-independent PC-3 and DU-145 prostate cancer cells, and renal carcinoma 786-O cells were obtained from American Type Culture Collection. The 769-P, HRC-51, HRC-78, and SK-45 human renal cell carcinoma cell lines were a kind gift of Dr. Joseph Testa (Fox Chase Cancer Center, Philadelphia, PA). The SK-26B cell line was obtained through the generosity of Dr. Finke (The Cleveland Clinic Foundation, Cleveland, OH). Initial stocks were cryopreserved, and at every 6-month interval a fresh aliquot of frozen cells was used for the experiments. No authentication was done by the authors. Cells were cultured in RPMI 1640 (Bio-Whittaker) supplemented with 10% FCS (Hyclone), gentamicin (50 mg/L), sodium pyruvate (1 mmol/L), and nonessential amino acids (0.1 mmol/L) under conditions indicated in the figure legends.

Antibodies and reagents
Antibodies to PTEN, Akt, phospho-Akt(S473), phospho-Akt(T308), phospho-4EBP1(T37/46), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from LC Laboratories. Sunitinib and temsirolimus were obtained from Cell Signaling Technology. Sunitinib and temsirolimus were obtained from LC Laboratories. GDC-0980 was obtained from ChemieTek. GSK690693 was obtained from Selleck.

Stable transfection with PTEN
To generate the PTEN expression vector, PTEN open reading frame was amplified with specific forward 5'-atcttgaggtgcaggctcactgagcatcaacag-3' and reverse 5'-atcttgagaattctctagattttggtcctgagatc-3' primers carrying HindIII and EcoRI restriction sites (underlined) using as a template pOTB7 vector (clone ID#3977787) containing full-length PTEN cDNA (Thermo Scientific) and then cloned into HindIII/EcoRI sites of pcDNA3.1-puro vector. The sequence of the pcDNA3.1-PTEN-puro construct was validated in the DNA Sequencing Facility of the Fox Chase Cancer Center. Cells were transfected with either the PTEN expression vector or the pcDNA3.1-puro control vector using the TransIT-Prostate transfection kit (Mirus Bio). Selection was done using puromycin (1 μg/mL).

Western blot analysis
Whole-cell lysates preparation and Western blot analysis were carried out as described previously (7).

Measurement of apoptosis
DNA fragmentation was detected using the APO-BRDUC Kit (The Phoenix Flow Systems, Inc.) according to manufacturer’s instructions.

Measurement of IL-6 and IL-8
Interleukin (IL)-6 and IL-8 levels in cell culture supernatants were measured using ELISA Kits (R&D Systems).

Assessment of in vivo tumor growth
For in vivo studies, 1 × 106 PC-3-PTEN or PC-3-puro-mycin cells were inoculated subcutaneously in the flank region of 6-week-old male C.B17/1cr-scid mice using a 27-gauge needle. All animal procedures were done in accordance with institutional guidelines on animal care and with appropriate institutional certification. Animals were fed an autoclaved AIN-93M diet (Harlan Teklad) and water ad libitum. Two weeks after the injection of tumor cells, animals were randomly assigned to the control or experimental groups (n = 5 mice per group). The mice were treated with either vegetable oil (control) or sunitinib (40 mg/kg in vegetable oil, 5 days per week, orally).

To examine the effect of coadministration of sunitinib and temsirolimus on tumor growth in vivo in PTEN-deficient cells, subcutaneous tumors were established using PTEN-negative PC-3 cells. Two weeks after tumor cell injection, animals were randomly assigned to the control or experimental groups (n = 7 mice per group). The mice were treated with (i) vegetable oil (control), (ii) sunitinib (40 mg/kg in vegetable oil, 5 days per week, orally), (iii) temsirolimus (10 mg/kg, once a week, i.v.), or with sunitinib and temsirolimus combined. Tumors were measured twice weekly and their volumes were calculated by the formula: \[\text{volume} = \frac{1}{2} \times (\text{length} \times \text{width}^2)\]. To examine sunitinib levels in plasma and tumor tissue, sunitinib (40 mg/kg in vegetable oil) was administered orally daily to C.B17/1cr-scid mice with PC-3 xenografts for 7 consecutive days. Animals were euthanized via CO2 asphyxiation 3 hours following last sunitinib administration. Levels of sunitinib in plasma and tumor samples were examined at Wolfe Laboratories.

Statistical analysis was done using a 2-sided Student t test. A P value of less than 0.05 was considered statistically significant.

Results

Lack of PTEN expression coincides with sunitinib resistance in renal and prostate cancer cells in vitro
To investigate the potential mechanisms underlying TKI resistance in vitro, we first analyzed the effect of sunitinib on the viability of several renal and prostate cancer cell lines. The findings presented in Fig. 1A show...
that sunitinib was capable of inducing a significant level of apoptosis in 7 of the 10 renal and prostate cancer cell lines tested with the notable exception of 786-O (renal) and PC-3 and LNCaP (prostate) cancer cell lines. These 3 cell lines were nearly completely resistant to sunitinib-mediated apoptosis. Because constitutive activation of the PI3K/Akt/mTOR pathway due to deletion or mutation of PTEN expression has been detected in a large percentage of the most common human cancers (6, 8), we analyzed the status of PTEN expression in these 10 cell lines. As shown in Fig. 1B, PTEN expression was undetectable in the sunitinib-resistant 786-O, PC-3, and LNCaP cells. All other cell lines that were sensitive to sunitinib-mediated apoptosis manifested PTEN positivity.

To validate our observation that PTEN expression modulates the sensitivity of malignant cells to sunitinib, we generated 786-O renal and PC-3 prostate cancer cell lines capable of expressing PTEN (Fig. 2A). Consistent with the role of PTEN as a negative regulator of PI3K/Akt/mTOR signaling pathway, the level of Akt phosphorylation was markedly reduced in PTEN-expressing cells compared with 786-O and PC-3 cells transfected with the control vector (Fig. 2A). With successful restoration of PTEN expression in previously PTEN-deficient 786-O and PC-3 cells, a pronounced sensitivity to sunitinib-mediated apoptosis was attained (Fig. 2B).

**Restoration of PTEN expression suppresses the production of protumorigenic cytokines, IL-6, and IL-8 by PC-3 and 786-O cells**

IL-6 and IL-8 production is upregulated following treatment with sunitinib in sunitinib-resistant hepatocellular carcinoma and renal cell carcinoma (9, 10). To investigate the potential impact of PI3K/Akt/mTOR signaling on the production of IL-6 and IL-8, we measured IL-6 and IL-8 levels in cell culture supernatants of PTEN-negative and PTEN-positive 786-O and PC-3 cells treated with sunitinib for 12 hours. As shown in Fig. 2C, treatment with sunitinib significantly increased expression levels of IL-6 in parental but not PTEN-positive cells. In PC-3 cells, this effect was most pronounced in the presence of TNF-α (unpublished observation). Interestingly, administration of sunitinib upregulated IL-8 expression solely in 786-O but not PC-3 cells. Nevertheless, restitution of PTEN expression completely blocked sunitinib-mediated increase of IL-8 production by 786-O cells. It is important to note that IL-6 and IL-8 levels were measured before the onset of cell death at a point when cell viability was greater than 95%.

**Sunitinib exhibits a significant antitumor effect in vivo on PTEN expressing PC-3 prostate cancer cells**

To extend our in vitro findings to the in vivo setting, xenograft tumors were established in severe combined immunodeficient mice using PC-3 cells that exhibited stable expression of PTEN. Control tumors were established using PTEN-negative PC-3 cells transfected with the control vector as described above. In addition, a xenograft model was created using 786-O and 786-O-PTEN tumors, however it was severely limited by the slow and unpredictable growth of both populations of cells to make its use practical. Animals were treated with vehicle alone or sunitinib (40 mg/kg) through oral gavage. Vehicle-treated animals uniformly showed a progressive increase in primary tumor growth. In contrast, tumor growth was consistently and markedly reduced in animals carrying PC-3-PTEN xenografts treated with sunitinib ($P < 0.001$; Fig. 3A). Treatment with sunitinib also caused an observable decrease in the growth of tumors established from control PC-3 cells (Fig. 3A); however, this decrease was not statistically significant ($P = 0.08$). At the termination of the study, tumors were resected and weighed. The weight of the tumors in mice injected with PC-3-PTEN tumors and treated with sunitinib was significantly lighter compared with mice injected with PC-3 tumors ($P = 0.002$). There was no significant difference in...
the tumor weights between mice injected with PC-3 cells treated with vehicle or sunitinib (P = 0.065) (Fig. 3B). It is important to underscore that whereas plasma levels of sunitinib were within a low micromolar range, intratumoral sunitinib concentrations were significantly higher (mean ± SEM: 5.4 ± 0.8 μmol/L vs. 66.7 ± 15.8 μmol/L, respectively; Table 1). Our data confirms recent findings by Gotink and colleagues showing that intratumor sunitinib concentrations in the murine Renca RCC model and tumor tissue specimens obtained from cancer patients were 10-fold higher than the corresponding plasma concentrations (11). Indeed, our in vivo findings confirmed the crucial role of PTEN status as a marker of sensitivity to sunitinib and provide further validation that sunitinib has a direct effect on tumor cells, which extends beyond the established angiogenic inhibitory actions.

Pharmacologic manipulation of PI3K/Akt/mTOR signaling enhances antitumor efficacy of sunitinib in vitro and in vivo

To explore a clinically relevant strategy to manipulate the PI3K/Akt/mTOR pathway in cells exhibiting sunitinib resistance, we preincubated parental PTEN-negative 786-O and PC-3 cells with pharmacologic inhibitors of PI3K/mTOR inhibitor, GSK690693, a pan-Akt inhibitor, and temsirolimus, an mTORC1 inhibitor (Fig. 4A). Temsirolimus is currently approved by the FDA for the treatment of advanced RCC (12). Phase I clinical trials for GDC-0980 and GSK690693 are currently recruiting participants (13, 14). The cytotoxic effect of sunitinib in combination with either GDC-0980, GSK690693, or temsirolimus was evaluated using the DNA fragmentation assay (Fig. 4B). As previously shown, treatment of PTEN-deficient renal or prostate cancer cells with sunitinib alone for 24 hours induced a negligible level of apoptosis. Similarly, treatment with sunitinib alone resulted in modest PI3K/mTOR pathway activity. Cells incubated with sunitinib, GDC-0980, GSK690693, or temsirolimus resulted in a synergistic induction of DNA fragmentation in these previously resistant cell lines (Fig. 4B).

Next, we examined the effect of various treatment modalities on the status of PI3K/Akt/mTOR pathway activity. Cells incubated with sunitinib, GDC-0980, GSK690693, or temsirolimus alone did not show any significant decrease in Akt phosphorylation. Of note, treatment with sunitinib alone resulted in modest...
downregulation of p-AktT308 levels in PC-3 cells; however, overall Akt activity was not affected, elicited by the fact that phosphorylation level of its direct target, Tuberin/TSC-2 (phospho-T1462), remained unchanged. Concomitant treatment with sunitinib and either GDC-0980, GSK690693, or temsirolimus resulted in complete inhibition of PI3K/Akt/mTOR signaling, as indicated by lack of phosphorylation of TSC2 and 4EBP1 targets (Fig. 4C). Pharmacologic inhibition of mTORC1 signaling with temsirolimus also blocked sunitinib-mediated upregulation of IL-6 and IL-8 expression in PC-3 and 786-O cells (Fig. 4D).

In light of our encouraging in vitro data, we next examined the antitumor effects of sunitinib in combination with temsirolimus using a mouse xenograft model bearing PTEN-negative PC-3 tumors. Three weeks after the initiation of therapy, single-agent treatment with temsirolimus or sunitinib showed a notable decrease in the growth of xenograft tumors (Fig. 4E). However, combination treatment with sunitinib and temsirolimus resulted in vastly more impressive inhibition of tumor growth among the experimental groups. These results confirmed the importance of PTEN as an important surrogate for tumor cell resistance to TKIs, but also provide preclinical data to suggest a readily available clinical strategy to circumvent resistance patterns in PTEN-deficient tumors.

Discussion

Treatment with multitargeted TKIs has dramatically altered the landscape in the treatment of patients with advanced tumors such as kidney and certain gastrointestinal tumors (1, 2). Despite encouraging results, clinical studies of certain solid organ malignancies, that is, CRPC, have shown a suboptimal response to the TKI, sunitinib (5, 15–17). Our present work with urologic malignancies features sunitinib resistance as a factor of PTEN expression, which serves the role of a gatekeeper of the PI3K/Akt/mTOR signaling pathway. Reestablishment of PTEN expression in PTEN-deficient cancer cells restores sensitivity to TKI therapy. Furthermore, suppression of PI3K/Akt/mTOR signaling with clinically relevant agents, such as temsirolimus, GDC-0980, and GSK690693, serves to reinstate sensitivity of PTEN-deficient cancer cells to TKI-mediated apoptosis.

Treatment with sunitinib completely abrogates Akt-dependent and Akt-independent survival signaling in PTEN-positive cells. Importantly, PTEN-negative cells exhibit constitutively active Akt signaling downstream of the cellular targets of sunitinib (Fig. 5). As such, additional pharmacologic modulation is required to suppress aberrant Akt activity. To elaborate further, we conclude that inhibition of Akt signaling alone is incapable of inducing apoptosis due to the existence of Akt-independent survival pathways. Thus, only concomitant administration of sunitinib and PI3K/Akt/mTOR inhibitors is capable of triggering apoptosis in PTEN-negative cancer cells.

The ability of multitargeted TKIs to inhibit angiogenesis, and thereby affect tumor progression, has been well established (3, 18). Recent studies make evident that the sole influence of sunitinib stems from its antiangiogenic effect, further emphasizing its lack of ability to exert a direct antitumor effect. These observations are based on the...
notion that pharmacologically relevant plasma concentrations of sunitinib are insufficient to inhibit RCC tumor cell proliferation (19). Alternatively, other investigations have elucidated that actions of sunitinib are not limited to inhibition of angiogenesis but, additionally, show clear evidence of tumor growth inhibition (20, 21) and antitumor immune response modulation (22). Our present findings are consistent with latter, providing compelling evidence of its multitargeted effects, that is, direct antitumor effect in conjunction with the previously established...
antiangiogenic activity. These observations are made evident by the fact that intratumor sunitinib levels are vastly higher than the corresponding plasma concentrations, as previously reported (23, 24), and confirmed by our own results (Table 1). In fact, sunitinib modulates a series of complex interactions between tumor and the surrounding tissues, thereby influencing multiple cellular processes, such as angiogenesis, cell growth, and cell survival (Fig. 5).

Our in vitro experiments make evident that PTEN expression inversely correlates with sunitinib resistance in renal and prostate cancer cells. Our in vitro findings were further validated in vivo: resistance to sunitinib was shown in PTEN-negative PC-3 xenograft tumors, although profound sensitivity to sunitinib was gained when xenograft tumors were established from PTEN-positive PC-3 cells. Furthermore, we successfully overcame sunitinib resistance in PTEN-negative xenograft tumors using temsirolimus, a pharmacologic inhibitor of mTORC1. Although our findings require clinical validation, PTEN tumor status could be translated into a biomarker to assist guide selection of therapeutic agents presently approved by the FDA for treatment of advanced urologic malignancies.

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

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