The Tyrphostin NT157 Suppresses Insulin Receptor Substrates and Augments Therapeutic Response of Prostate Cancer

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Abstract

Insulin-like growth factor (IGF) signaling is associated with castrate-resistant prostate cancer (CRPC) progression. Insulin receptor substrates 1 and 2 (IRS1/2) mediate mitogenic and antiapoptotic signaling from IGF1 receptor (IGFIR), insulin receptor, and other oncoproteins. This study demonstrates that IRS1/2 expression is increased in prostate cancer, and persists in CRPC. Furthermore, this study assesses the anticancer activity of NT157, a small molecule tyrphostin targeting IRS proteins, using androgen-responsive (LNCaP) and -independent (PC3) prostate cancer cells in vitro and in vivo. NT157 treatment resulted in dose-dependent inhibition of IGF1R activation, suppression of IRS protein expression, inhibition of IGF1-induced AKT activation, but increased ERK activation in NT157-treated cells in vitro. These effects were correlated with decreased proliferation and increasing apoptosis of LNCaP cells and increasing G2–M arrest in PC3 cells. NT157 also suppressed androgen-responsive growth, delayed CRPC progression of LNCaP xenografts, and suppressed PC3 tumor growth alone and in combination with docetaxel. This study reports the first preclinical proof-of-principle data that this novel small molecule tyrosine kinase inhibitor suppresses IRS1/2 expression, delays CRPC progression, and suppresses growth of CRPC tumors in vitro and in vivo. Demonstration that IRS expression can be increased in response to a variety of stressors that may lead to resistance or reduced effect of the therapies indicate that NT157-mediated IRS1/2 downregulation is a novel therapeutic approach for management of advanced prostate cancer. Mol Cancer Ther; 13(12); 2827–39. ©2014 AACR.
response (11, 12). Once tyrosine is phosphorylated, the IRS proteins function as adaptors to organize signaling complexes predominantly through activation of PI3K (13). IRS protein expression can be regulated by steroid, cytokine, and integrin signaling (14–16). There are five human IRS genes (reviewed in ref. 11). IRS1 and IRS2 are ubiquitously expressed and are the primary mediators of IGF-dependent mitogenesis and insulin-mediated regulation of glucose metabolism in most cell types (17). IRS1 plays a central role in cancer cell proliferation, its expression is increased in many human malignancies and its upregulation in human hepatocellular carcinoma mediates resistance to anticancer drugs (18). IRS2 is associated with cancer cell motility and metastasis (19). These findings make the IRS proteins potential targets of anticancer therapies. Indeed, we have recently reported that tyrphostins targeting IRS proteins possess antitumor effects (20).

Here, we report elevated IRS1/2 expression in prostate cancer and CRPC and characterize the preclinical anticancer activity of NT157, a small molecule tyrosine kinase inhibitor that downregulates IRS expression. We assess the potency of this tyrophostin drug candidate by characterizing its effects on IRS knockdown, suppressed proliferative signaling, induction of apoptosis, and cell-cycle arrest using LNCaP and PC3 prostate cancer models. We demonstrate the ability of NT157 to delay CRPC progression of LNCaP and to augment taxane sensitivity of PC3 cells in vitro and in vivo.

Materials and Methods

Prostate cancer cell lines

LNCaP cells were maintained in RPMI-1640 (Invitrogen) supplemented with 5% fetal bovine serum (FBS) and 2 mmol/L L-glutamine. PC3 cells were purchased from the American Type Culture Collection and maintained in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen Life Technologies, Inc.) containing 5% FBS. Cell lines were cultured in a humidified 5% CO2 atmosphere at 37 °C. LNCaP and PC3 prostate cancer models. We demonstrate the ability of NT157 to delay CRPC progression of LNCaP and to augment taxane sensitivity of PC3 cells in vitro and in vivo.

Small molecule tyrosine kinase inhibitor

The small molecule tyrosine kinase inhibitor targeting the IGF1R–IRS signaling pathway, NT157, was kindly provided by TyrNovo, Ltd. and used for in vitro and in vivo studies as previously described (20), wherein the chemical characterization, including molecular structure, is reported. Briefly, for in vitro studies, NT157 was dissolved in dimethyl sulfoxide (DMSO) as a 10 mmol/L stock solution and stored at 4 °C. For the in vivo studies, NT157 was dissolved in 20% 2-hydroxypropyl-β-cyclo-dextrin (2-HP-β-CD; Sigma) at 5 mg/mL and stored at −80°C.

Cell growth assays

Cell growth was assessed using crystal violet assay, as described previously (21). Cells were plated in 24-well plates and treated with NT157 as described. Crystal violet staining was carried out for time course, and 72 hours after treatment. The absorbance was determined with a microtiter plate reader (Becton Dickinson Labware) at 562 nm. Cell survival after NT157 treatment was calculated as the percentage of the absorbance in vehicle-treated cells.

Immunoblot analysis

After the indicated treatments, detached cells were pelleted from media and pooled with attached cells and lysed in RIPA buffer (50 mmol/L Tris, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 100 mmol/L NaCl, 1× Roche complete protease inhibitor cocktail). Samples containing equal amounts of whole-cell lysate protein (30 μg) were subjected to SDS–PAGE, transferred to nitrocellulose filters, blocked in PBS containing 5% nonfat milk powder for 1 hour, and incubated at 4°C overnight with primary antibody: anti-phospho-IGF1Rβ subunit (Y980) rabbit monoclonal antibody (mAb), anti-IGF1Rβ subunit rabbit polyclonal antibody (PAb), anti-phospho-akt (S473) rabbit PAb, anti-AKT rabbit PAb, anti-phospho-ERK1/2 (T202/Y204) rabbit PAb, anti-ERK1/2 rabbit PAb, anti-phospho-IRS1 (S636/639) rabbit PAb, anti-phospho-IRS1 (Thr1222) rabbit PAb, anti-IRS1 rabbit PAb, anti-phospho-IRS2 (Ser731) rabbit PAb (Abcam), anti-IRS2 rabbit PAb (Abcam), anti-cleaved PARP (N214) rabbit PAb, anti-cyclin D1 (M-20) rabbit PAb (Santa Cruz Biotechnology), anti-phospho-ERK (Ser807/811) rabbit PAb, anti-RB mouse mAb, anti-p21 mouse mAb (Santa Cruz Biotechnology), anti-p27 mouse mAb (Santa Cruz Biotechnology), anti-p53 rabbit PAb, anti-cdc2 rabbit PAb, anti-cdc25C rabbit mAb, and anti-vee1 rabbit. All antibodies were from Cell Signaling Technology unless otherwise indicated. Immunoblots were incubated horseradish peroxidase–conjugated anti-mouse or rabbit IgG antibody (Santa Cruz Biotechnology) and visualized with enhanced chemiluminescence immunoblotting analysis system (Amersham Life Science) using a DYZERSITY imaging system with GeneSnap Ver. 7.04 software (SynGene). Immunoblot images are representative of at least three independent experiments.

Flow cytometric analysis

Flow cytometric analysis of propidium iodide (PI)–stained nuclei was done, as described previously (22). In brief, cells were plated in 10-cm2 dishes and, on the day after, were treated as described above. The cells were trypsinized, washed twice, and incubated in PBS containing 0.12% Triton X-100, 0.12 mmol/L EDTA, and 100 mg/mL ribonuclease. PI (50 mg/mL) was then added to each sample for 20 minutes at 4°C. Cell-cycle
distribution was analyzed by flow cytometry (Beckman Coulter Epics Elite; Beckman, Inc.) based on G1–G0 and G2–M DNA content. Each assay was performed in triplicate.

Assessment of in vivo tumor growth

For xenograft studies, 2 × 10^6 LNCaP (suspended in 0.1 mL Matrigel; BD Biosciences) or PC3 cells (suspended in 0.1 mL serum-free DMEM) were inoculated s.c. in the flank of 6- to 8-week-old male athymic nude mice (Harlan Sprague–Dawley, Inc.) via a 27-gauge needle under isoflurane anesthesia. For LNCaP xenografts, when tumor volume exceeded 200 mm^3, mice were castrated and randomly selected for treatment with 50 mg/kg NT157 or vehicle (10% 2-HP-beta-CD + 0.67% NaCl/H_2O) injected i.p. three times per week. Each experimental group consisted of 12 mice. Tumor volume was measured twice weekly (length × width × depth × 0.5432). Data points were expressed as average tumor volume ± SEM. When PC3 tumors reached 100 mm^3, mice were randomly selected for treatment with 50 mg/kg NT157 or vehicle (10% 2-HP-beta-CD + 0.67% NaCl/H_2O) injected i.p. three times per week or treated with 10 mg/kg docetaxel injected i.p. three times a week for 1 week or combination of 50 mg/kg NT157 and 10 mg/kg docetaxel as indicated. Experimental groups consisted of 7 mice (vehicle), 9 mice (NT157), 10 mice (docetaxel), and 16 mice (combination). Tumor volume and body weight (BW) were measured once weekly. Data points were expressed as average tumor volume ± SEM. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

Immunohistochemistry

Immunohistochemistry (IHC) analysis of IRS1 and IRS2 in prostate cancer was performed on formalin-fixed, paraffin-embedded 4-mm sections of tissue microarrays composed of duplicate 5-mm cores from tumor samples from 92 patients distributed, as described in Supplementary Table S1. Immunohistochemical staining was conducted using IRS1- and 2-specific primary antibodies in the Ventana autostainer Discover XT (Ventana Medical System) with enzyme-labeled biotin streptavidin system and solvent resistant 3,3'-diaminobenzidine map kit. Antibody validation was performed by immunogenic peptide competition (Supplementary Fig. S1). All comparisons of staining intensities were made at ×200 magnification. Staining intensity was scored by a blinded pathologist (L. Fazli) using 0–3 scale.

Statistical analysis

Immunohistochemical staining comparisons were made by the ANOVA and Dunn multiple comparisons. In vitro data comparisons were assessed using the Student t test or ANOVA and Mann–Whitney posthoc test as indicated in respective legends. Tumor growth rates were compared using the Kruskal–Wallis test. Levels of statistical significance were set at P < 0.05.

Results

IRS1 and IRS2 expression are increased in prostate cancer, downregulated after hormone therapy, and increased with disease progression

Previous reports indicated a slight, but nonsignificant, increase in percentage of IRS1-positive cells (23), and significantly increased IRS2 expression (24) in prostate cancer tumors compared with benign epithelium; however, what influence ADT might have on IRS expression in prostate cancer has not been reported. We therefore, examined expression of IRS1 and IRS2 in the prostate cancer microenvironment of untreated, short-term (<6 months) neoadjuvant hormone therapy (NHT), long-term (6–12 months) NHT, and CRPC disease by IHC (Fig. 1). In this prostate cancer specimen series, IRS1 expression was elevated approximately 20% in therapy-naive prostate cancer and CRPC when compared with benign epithelium, but was indistinguishable from benign epithelium in NHT-treated specimens. IRS2 expression was upregulated 2-fold in therapy-naive prostate cancer and CRPC specimens, and while indistinguishable from naive specimens in short-term NHT specimens, was increased ~2-fold compared with benign epithelium in the long-term NHT cohort. These results indicate that IRS1 and IRS2 expression may be linked to increased AR activity in prostate cancer, downregulated as a consequence of androgen ablation, and reemerge during CRPC progression and suggest that IRS1 and IRS2 expression may be important for maintenance of malignant cell viability in androgen-responsive and CRPC states.

Dose-dependent and time-dependent suppression of IGF1R/PI3K signaling correlates with enhanced ERK activation, and IRS1/2 serine phosphorylation and degradation in NT157-treated LNCaP and PC3 cells

IGF signaling has previously been implicated in growth and survival signaling in prostate cancer models, including LNCaP and PC3 cells (7–10). The small molecule tyrphostin, NT157, is a novel potential therapeutic agent designed to disrupt IGF signaling that promotes degradation of IRS1 and IRS2 (20). To evaluate whether NT157 might have utility as an anticancer agent for prostate cancer, we first tested whether NT157 was capable of antagonizing IGF1R-mediated signaling in vitro as a precursor to assessing the physiologic impact of IGF1R expression on these prostate cancer models (Fig. 2). We observed that 24 hours of pretreatment with NT157 dose-dependently suppressed IGF1-stimulated activation site tyrosine phosphorylation of the IGF1R without significantly affecting IGF1Rβ2 subunit expression in both LNCaP and PC3 cells (Fig. 2A). In LNCaP cells, IGF1R Y980 phosphorylation was suppressed >60%...
at 2.5 μmol/L NT157 and >80% at 5 μmol/L NT157. In PC3 cells, IGF1R Y980 phosphorylation was suppressed approximately 40% at 2.5 μmol/L NT157, and >90% at 5 μmol/L. This effect generally correlated with decreased IGF1-stimulated Akt and GSK3β phosphorylation and, as previously noted (20), increased ERK phosphorylation and suppressed IRS2 expression in LNCaP cells and IRS1 and -2 expression in PC3 cells.

We next evaluated how NT157 pretreatment might affect IRS1/2 expression at 5 μmol/L NT157 from 0.5 to 8 hour-pretreatment (Fig. 2B and C). In both LNCaP and PC3 cells, prolonged NT157 treatment resulted in increased IRS5 serine phosphorylation and decreased steady-state IRS protein levels. In LNCaP, these effects were observed by 30 minutes, with increased IRS2 serine phosphorylation dramatically increasing by 2 hours of NT157 exposure and maximal decreased IRS2 levels by 8 hours. Similar kinetics was observed in PC3 cells but with maximal decreased IRS1 and IRS2 expression by 2 hours. These results indicate that NT157 can mediate suppression of IGF1R-mediated survival signaling through the established mechanism for negative feedback of IGF1R signaling: targeting IRS1/2 for serine phosphorylation and subsequent degradation (25, 26).

**NT157 treatment induces cell-cycle arrest and apoptosis in LNCaP and PC3 Cells**

To assess the effect of NT157 on cell viability, LNCaP cells cultured in 5% of FBS media or 5% of charcoal-stripped serum (CSS) media were treated with varying doses of NT157 for 3 days and population density was compared with that of DMSO-treated cells using crystal
violet assay (Fig. 3A). The density of LNCaP cells grown in FBS was decreased approximately 20% at 1 \( \mu \text{mol/L} \), approximately 70% at 2 \( \mu \text{mol/L} \), and >90% at 5 \( \mu \text{mol/L} \) (IC\(_{50}\), 1.4 \( \mu \text{mol/L} \)). Growth of LNCaP cells is suppressed >60% when cultured in CSS but still exhibited significant density at 2 \( \mu \text{mol/L} \) and maximal decreased density at 5 \( \mu \text{mol/L} \). The density of FBS-cultured PC3 cells was similarly decreased by NT157 treatment (~40% at 2 \( \mu \text{mol/L} \) and >70% at 5 \( \mu \text{mol/L} \); IC\(_{50}\), 2.5 \( \mu \text{mol/L} \)). These observations are in contrast with the dose-dependent effect of NT157 on viability of an immortalized, but benign, prostate epithelial cell line (RWPE1) and immortalized prostate fibroblasts (Supplementary Fig. S2). Although RWPE1 cells exhibit some cytotoxicity above 5 \( \mu \text{mol/L} \), viability was not inhibited by >50% at doses up to 10 \( \mu \text{mol/L} \), while the prostatic fibroblasts exhibit no more
than a 20% decrease in viability at 10 \mu mol/L. These results indicate that the tumor models exhibit an increased sensitivity to NT157, consistent with previous reports indicating a key role for IGF1R signaling for their viability (4–6).

Decreased density of FBS-cultured LNCaP and PC3 cells treated with NT157 was observed to be proportional to apoptotic induction as assessed by flow cytometry of fixed, PI-stained cells (Fig. 3B) and by increased levels of cleaved PARP (Fig. 3D). The fraction of cells undergoing apoptosis (sub \(G_0\)–\(G_1\) fraction or cleaved PARP) was significantly increased after treatment of LNCaP and PC3 cells with \(\geq 2.5 \mu\)mol/L NT157 for 2 days, except that no increase in cleaved PARP was detected in NT157-treated, dextran-coated, activated charcoal-stripped FBS (CSS)-cultured LNCaP cells below 10 \mu mol/L. Although this correlated with decreased numbers of LNCaP cells in \(G_0\)–\(G_1\) and \(G_2\)–\(M\) fractions under both FBS and CSS conditions, in PC3 cells there was a dose-dependent increase in the \(G_2\)–\(M\) fraction. When analyzing the \(G_0\)–\(G_1\) to \(S + G_2\)–\(M\) fractions of nonapoptotic cells, no obvious differences were observed in the NT157-treated, FBS or CSS LNCaP cultures; however, PC3 cells treated with \(\geq 2.5 \mu\)mol/L NT157 exhibited a significantly increased \(G_2\)–\(M\) fraction indicative of \(G_2\)–\(M\) arrest (Fig. 3C).

The above results suggest that suppression of IGF1R-mediated activation of IRS proteins promotes cell-cycle arrest and induction of apoptosis in these prostate cancer models. The impact of NT157 treatment on cell-cycle progression was analyzed by immunoblotting against a panel of mitotic and apoptotic regulatory factors (Fig. 3D). Induction of \(G_1\)–\(S\) arrest in NT157-treated, FBS-cultured LNCaP and PC3 cells is indicated by the dose-dependent increased p21 and p27 (LNCaP only) expression, decreased cyclin D1 and E2 expression, and decreased RB phosphorylation (P-RB). Decreased cyclin D3 and CDK4 expression was also observed (Supplementary Fig. S3); however, expression of the cyclin–linker-dependent kinase, CDK2, was low and no apparent changes in response to NT157 treatment were noted. In both cell types, the dose-dependent decrease in \(G_0\)–\(G_1\) populations (Fig. 3B) indicated that cells were undergoing apoptosis from the \(G_1\)–\(S\) checkpoint.

Induction of \(G_2\)–\(M\) arrest was indicated by elevated p53 expression in LNCaP cells and decreased cdc2/CDK1, cdc25c, and Wee1 expression in both cell lines when cultured in FBS. The relatively low expression of cyclin D1, cdc25c, Wee1, and lack of detection of cyclin E2 and P-RB as well as increased p27 and cleaved PARP only at the 10 \mu mol/L NT157 dose is indicative of senescence of CSS-cultured LNCaP cells. Cyclin B1 expression was not directly correlated to cdc2/CDK1 expression in NT157-treated LNCaP cells (Fig. 3 and Supplementary Fig. S3). NT157 treatment suppressed both cyclin B1 and cdc2/CDK1 levels in PC3 cells while cdc2/CDK1 expression persisted in CSS-cultured LNCaP cells even though cyclin B1 levels were suppressed by NT157 treatment. We consistently observed decreased cyclin B1 expression in FBS-cultured LNCaP cells treated with 2.5 \mu mol/L NT157, but not at higher doses. We speculate that this may be due to the rapid activation/stabilization of p53 and subsequent apoptotic induction indicated by PARP cleavage (Fig. 3D) and decreased \(G_2\)–\(M\) population (Fig. 3B). The accumulation of a \(G_2\)–\(M\) population, and proportionally lower apoptotic induction in PC3 cells would therefore be consistent with their p53-null status.

**NT157 enhances docetaxel-induced apoptosis in PC3 cells**

A recent report indicates that IRS1 enhances sensitivity to the chemotherapy (27). Given the above data indicating that NT157 treatment results in suppressed IRS expression, we evaluated the efficacy of NT157 in combination therapy with docetaxel on PC3 cells in vitro and determine whether this effect was additive or synergistic using the dose-dependent effects with constant ratio design according to the Chou and Talalay median effect principal (28). Docetaxel decreased PC3 viability at \(\geq 1 \mu\)mol/L, whereas NT157 decreased PC3 viability at \(\geq 1 \mu\)mol/L (Fig. 4A). At all concentrations tested, when combined, NT157 treatment significantly enhanced docetaxel cytotoxicity such that cell density was decreased 60% for cells treated with 1 \(\mu\)mol/L docetaxel and 1 \(\mu\)mol/L NT157; more than 2-fold greater than either agent at these doses alone (30% and 20%, respectively). Comparing the combination index (CI) value for each constant ratio combination and assessing the ED50 and ED75 CIs revealed that NT157 synergistically enhances the cytotoxic effect of docetaxel on PC3 cell growth (Fig. 4B). Moreover, this effect was found to be due to enhanced apoptotic induction as measured by PARP cleavage (Fig. 4C) and accumulation in the sub \(G_0\)–\(G_1\) fraction (Fig. 4D). This enhanced apoptotic induction be a consequence of apoptotic induction from cells arresting at both the \(G_1\)–\(S\) and \(G_2\)–\(M\).

**NT157 suppresses growth of LNCaP xenografts following castration**

We next evaluated the efficacy of NT157 as a combination therapy with castration on growth of LNCaP xenografts (Fig. 5). When tumor volume exceeded 200 mm3,
animals were castrated and randomly assigned to groups treated with either NT157 or vehicle. The mean tumor volume of the two groups was indistinguishable. Animals were treated for 6 weeks as described in the Materials and Methods. In castrated mice bearing LNCaP xenografts, NT157 significantly delayed tumor growth (Fig. 5A). Although tumors in vehicle-treated mice continued to grow following castration, average tumor volume decreased at 1 week following castration and NT157 treatment, remained static through 4 weeks of treatment, and did not exceed precastrate tumor volume at end of study. Average tumor growth rate in the NT157 treated cohort was decreased >35-fold (Supplementary Table S2 and Supplementary Fig. S4; vehicle growth rate = 129.5 mm$^3$/day versus NT157-treated growth rate = 3.5 mm$^3$/day). The tumor volume change waterfall plot analysis demonstrated that only one vehicle-treated animal exhibited a tumor growth change that was less than the greatest increase in tumor volume of the NT157-treated cohort and that half of the NT157-treated cohort tumor volumes decreased over the course of the study (Fig. 5B). No significant effect on animal body weight was observed in the vehicle- or NT157-treated cohorts during this treatment period (Supplementary Fig. S5). Immuno-histochemical analysis reveals decreased P-IGF1R, IRS2, and Ki67 detection and higher apoptotic rates, as shown by increased terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, in...
the NT157-treated group as compared with the vehicle-treated group (Fig. 5C). Decreased IRS2 expression in the NT157-treated group was corroborated by immunoblot and qPCR of selected tumors (Fig. 5D). These studies indicate that NT157 treatment affects IGF1R and IRS targets in xenografts and significantly delays castration-resistant progression of LNCaP androgen-responsive xenografts when combined with castration.

**NT157 potentiates docetaxel activity in PC3 xenografts**

The cooperative activity of NT157 and docetaxel on PC3 growth in vitro prompted an evaluation of the combined treatment on PC3 xenografts (Fig. 6). Once PC3 tumor volume exceeded 100 mm³, animals were randomly assigned for treatment with vehicle, NT157, docetaxel, or NT157 and docetaxel in combination as described in the

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Figure 5. NT157 treatment inhibits LNCaP xenograft growth and delays castration-resistant progression. A, LNCaP cells were inoculated s.c. and, when tumor volume exceeded 200 mm³, mice were castrated and randomly selected for treatment with 50 mg/kg of NT157 or vehicle (10% 2-HP-beta-CD + 0.67% NaCl in DDW) injected i.p. three times per week. Each data point represents the mean tumor volume in each group containing 12 mice ± SEM. B, the percentage change in volume for each tumor (12 tumors per treatment group) after 6 weeks is shown as a waterfall plot. C, tumors were collected 42 days following treatment initiation and P-IGF1R, IRS2, Ki67, and TUNEL were evaluated by immunohistochemical analysis (original magnification, ×200). D, total proteins were extracted from tumors and IRS2 was analyzed by immunoblotting. Total RNAs were extracted from the xenografts treated with castration, NT157 as indicated. IRS2 mRNA expression levels were determined by quantitative RT-PCR. Experimental groups are as indicated under each data column, representing relative levels of expression normalized to the mRNA level of GAPDH. *, P < 0.05, significant difference from vehicle-treated group (Student t test).
Figure 6. NT157 potentiates docetaxel activity in PC3 xenograft model. A, PC3 cells were inoculated s.c. and when tumors reached 100 mm³, mice were randomly selected for treatment with the same protocol as LNCaP. Each point represents the mean tumor volume in each group containing 7 mice in vehicle, 9 mice in NT157, 10 mice in docetaxel, and 16 mice in combination-treated as described in Materials and Methods (±SEM). B, the percentage change in volume for each tumor after 6 weeks is shown as a waterfall plot. C, tumors were collected after 42 days and P-IGF1R, IRS2, Ki67, and TUNEL were evaluated by immunohistochemical analysis (original magnification, ×200). D, total proteins were extracted from tumors, and IRS1 and IRS2 were analyzed by immunoblotting. Total RNAs were extracted from the xenografts treated with vehicle, NT157, docetaxel, and the combination of NT157 and docetaxel as indicated. IRS1 and IRS2 mRNA expression levels were determined by quantitative RT-PCR. Experimental groups are as indicated under each data column, representing relative levels of expression normalized to the mRNA level of GAPDH. *, P < 0.05, significant difference from vehicle-treated group; **, P < 0.05, significant difference from Docetaxel group (Dunn multiple comparisons test).

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Materials and Methods. Both NT157 and docetaxel significantly suppressed PC3 xenograft growth rate. Growth rate of tumors in the NT157 (106.4 mm$^3$/day) and docetaxel (87.7 mm$^3$/day) cohorts were suppressed approximately 4-fold relative to that of the vehicle cohort (387.2 mm$^3$/day) while the growth rate of the tumors in the combination cohort (12.5 mm$^3$/day) were suppressed >30-fold relative to the vehicle cohort and approximately 7-fold relative to the NT157 and docetaxel alone cohorts (Fig. 6A; Supplementary Table S3; Supplementary Fig. S6). The tumor volume change waterfall plot analysis demonstrated that while all NT157 and docetaxel tumors grew less than half as large as the average vehicle tumors, all increased in volume over the course of the study; however, in the combination cohort, half the tumors exhibited no growth or decreased in size over the course of the study (Fig. 6B). No significant effect on animal body weight was observed between NT157, docetaxel or combination, and vehicle-treated mice bearing PC3 xenografts during this treatment period (Supplementary Fig. S7). By immunohistochemical analysis, detection of P-IGF1R, IRS1, IRS2, and Ki67 were lower in the NT157 treatment cohort. In the docetaxel-treated group, while Ki67 index was decreased, P-IGF1R, IRS1, and IRS2, staining intensities were indistinguishable from that of the vehicle cohort. The combination therapy cohort staining for P-IGF1R, IRS1, and IRS2, staining intensities were indistinguishable from that of the vehicle cohort. The combination therapy cohort staining for P-IGF1R, IRS1, and IRS2, paralleled that of the NT157 treatment cohort, but Ki67 index was lower than in the NT157 and docetaxel alone cohorts and only in this cohort was there a substantial increase in TUNEL-positive cells in the xenograft samples (Fig. 5C). Decreased IRS1 and IRS2 expression in the NT157-treated groups was corroborated by immunoblot and qPCR of selected tumors (Fig. 5D). Intriguingly, docetaxel alone appeared to increase expression of IRS2 that was reversed when combined with NT157. These studies again indicate that NT157 treatment affects IGF1R and IRS targets of xenografts with NT157. These studies again indicate that NT157 suppresses IRS1/2 and augments prostate cancer therapeutic response.

Discussion

There have been few reports that attempt to link IRS1 and IRS2 expression to prostate cancer etiology. Early studies indicated that there is a trend toward increased IRS1 expression in malignant biopsies (29) and that IRS1 expression is slightly but nonsignificantly increased in prostate cancer when compared with benign prostate tissue (23). More recently, strong IRS2 expression has been reported in prostate cancer (24), and increased expression of IRS2 and insulin receptor is correlated with CRPC progression (30, 31). In this work, we confirm that IRS1 and IRS2 are elevated in prostate cancer, then go on to demonstrate that expression tends to be decreased in response to hormone therapy, and increased with disease progression. Although we observed that IRS1 and -2 expressions are elevated in cancer versus benign prostatic epithelium and remain expressed at elevated levels in CRPC, there is no further elevation in expression in advanced disease. Intriguingly, consistent with a previous report indicating that the IRS2/IRS1 transcript ratio is increased in benign versus prostate cancer (32), we observed that increased IRS2 expression is greater than that of IRS1 due primarily to the relatively low basal level of IRS2 in benign prostatic epithelium.

Although these findings are suggestive of a role for IRS1 and IRS2 in CRPC progression, mechanisms for how IRS proteins impact prostate cancer remains to be resolved. Although steroid pathways have been implicated in their expression, IRSes are ubiquitously expressed. Of the IRS family, only IRS4 was identified as an AR target gene by ChIP analysis (33); however, in the Androgen Responsive Gene Database (http://argdb.fudan.edu.cn/), IRS1, but not IRS2, is identified as possessing a nominal androgen-response element consensus in its upstream noncoding region. Furthermore, the AR-null PC3 cell line robustly expresses IRS1 and -2 as well, so their expression in prostate cancer would not appear to be dependent on AR activity. Although there is a trend in IRS2 (and indeed in IRS1) expression with regard to putative AR activity during progression to CRPC, we consider the most probable reason being attributable to the overall effect of androgens on prostatic epithelia and naïve prostate cancer quiescence response to hormone withdrawal (34, 35).

Several small molecule tyrosine kinase inhibitors and antibodies targeting IGF1R are in clinical development in prostate and many other cancers; however, until the recent identification of a novel investigational IRS1/2 small molecule inhibitor, NT157 (20), there have been no anticancer drugs targeting IRS proteins. Here, we demonstrate that NT157 decreases expression of IRS proteins and downregulates IGF1R-mediated AKT activation in the prostate cancer models, LNCaP and PC3 cell. Although androgen-independent PC3 cells express both IRS1 and -2 (29) and androgen-dependent LNCaP cells express IRS2, but not IRS1 (36), NT157 treatment similarly disrupted IRS-mediated signaling, inducing cell-cycle arrest and apoptosis of both cell lines. Although expression of cell-cycle regulatory factors is confounded by the induction of apoptosis during the course of these studies, we conclude that these results correlate NT157-mediated suppressed IRS expression with decreased IGF1-mediated PI3K signaling, cell-cycle arrest, and apoptotic activation in androgen-responsive and -independent prostate cancer cells indicative of apoptosis due to suppressed prosurvival signaling and induction of cell-cycle arrest. Furthermore, NT157 treatment delayed growth of LNCaP xenografts following castration and suppressed growth of PC3 xenografts cotreated with docetaxel. As we have previously described (37), p53 wild-type LNCaP cells readily respond to cell-cycle stress by inducing apoptosis from both G1 and G2–M stages, while p53-mutant PC3 cells accumulate in G2–M before apoptotic induction.
Consistent with the model proposed by Reuveni and colleagues (20), NT157 treatment also results in elevated ERK1/2 activation that may mediate phosphorylation and degradation of IRS proteins. Although activation of both the PI3K–AKT and Ras–ERK pathways have been implicated in controlling IGF1R-mediated growth and survival signaling (38), these results are consistent with our previous reports indicating that activation of PI3K/AKT, not ERK1/2, is primarily responsible for growth and survival responses to IGF1 (39–41). Therefore, the ability of NT157 to disrupt IGF1R-mediated signaling via IRS adaptor proteins, delay CRPC progression, and suppress growth of CRPC tumors is at least, in part, due to inhibition of prosurvival signaling of the PI3K–AKT pathway.

Furthermore, treatment of LNCaP cells with NT157 does not impact the ability of epidermal growth factor to activate PI3K-mediated signaling to drive pS473 on AKT or to drive pT5EY of ERK1 and -2 (Supplementary Fig. S8). We conclude that this supports our contention that NT157 selectivity targets IGF axis signaling regarding the selectivity of NT157 for the IGFIR versus other tyrosine kinases (20).

This ability of NT157 to promote apoptotic induction from G2–M arrest compliments the apoptotic induction of taxane-treated cells from G2–M arrest and provides a rationale for combining the two therapeutic modalities to improve response in patients with CRPC. A range of therapeutic approaches including reducing ligand availability by IGF1 antibodies and recombinant IGFBPs, reducing IGF1R expression by antisense and RNA interference, or inhibiting of IGF1R signaling by IGF1R antibodies and small molecule tyrosine kinase inhibitors are under investigation in CRPC (42). Although several of these, particularly the humanized IGFIR antibodies, are showing great promise, continued research to understand how the IGF axis functions to promote prostate cancer growth and progression and to identify new ways to target the IGF axis in prostate cancer should be pursued.

Targeting IRS expression is one such opportunity. Reuveni and colleagues (20) reported that increased IRS expression promotes vemurafenib resistance in several cases, while Buck and colleagues (43) reported a requirement for IRS proteins in resistance to tyrosine kinase receptor, and mTOR inhibitors. Similarly, we observe that IRS2 expression is elevated in prostate cancer, rebounds during CRPC progression, and in PC3 xenografts, increases in response to docetaxel treatment. These observations indicate that expression of IRS proteins is tightly regulated and can be increased in response to a variety of stressors that may lead to resistance or reduced effect of the therapies.

There is precedence for targeted agents to restore therapeutic sensitivity. Suppressing clusterin either through direct antisense targeting (44) or intriguingly through STAT1 interfering RNA treatment (45), has been demonstrated to restore taxane sensitivity in prostate cancer models. These responses appear to be due to the ability of clusterin to protect against cellular stress responses. IGF signaling, particularly through the PI3K axis, can also provide cytotoxic protection from a variety of stressors. The ability of NT157 to downregulate IRS1/2 expression in malignancies provides a novel therapeutic approach to enhance the sensitivity of cancers to other cytotoxic agents as established.

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

Authors’ Contributions
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Ibuki, M. Ghaffari, H. Azuma, M.E. Gleave
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The Tyrphostin NT157 Suppresses Insulin Receptor Substrates and Augments Therapeutic Response of Prostate Cancer

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