Therapeutic Destruction of Insulin Receptor Substrates for Cancer Treatment

Hadas Reuveni1,2*, Efrat Flashner-Abramson2, Lilach Steiner1,2, Kirill Makedonski1,2, Renduo Song3, Alexei Shir1, Meenhard Herlyn4, Menashe Bar-Eli3, and Alexander Levitzki2*

Abstract

Insulin receptor substrates 1 and 2 (IRS1/2) mediate mitogenic and antiapoptotic signaling from insulin-like growth factor 1 receptor (IGF-IR), insulin receptor (IR), and other oncoproteins. IRS1 plays a central role in cancer cell proliferation, its expression is increased in many human malignancies, and its upregulation mediates resistance to anticancer drugs. IRS2 is associated with cancer cell motility and metastasis. Currently, there are no anticancer agents that target IRS1/2. We present new IGF-IR/IRS-targeted agents (NT compounds) that promote inhibitory Ser-phosphorylation and degradation of IRS1 and IRS2. Elimination of IRS1/2 results in long-term inhibition of IRS1/2-mediated signaling. The therapeutic significance of this inhibition in cancer cells was shown while unraveling a novel mechanism of resistance to B-RAFV600E/K inhibitors. We found that IRS1 is upregulated in PLX4032-resistant melanoma cells and in cell lines derived from patients whose tumors developed PLX4032 resistance. In both settings, NT compounds led to the elimination of IRS proteins and evoked cell death. Treatment with NT compounds in vivo significantly inhibited the growth of PLX4032-resistant tumors and displayed potent antitumor effects in ovarian and prostate cancers. Our findings offer preclinical proof-of-concept for IRS1/2 inhibitors as cancer therapeutics including PLX4032-resistant melanoma. By the elimination of IRS proteins, such agents should prevent acquisition of resistance to mutated-B-RAF inhibitors and possibly restore drug sensitivity in resistant tumors. Cancer Res; 73(14); 4383–94. ©2013 AACR.

Introduction

The insulin-like growth factor 1 receptor (IGF-IR) signaling pathway is pivotal in many human malignancies (1–5). Upregulation of IGF-IR signaling in cancer cells results from its overexpression or from upregulation of its ligands, IGF-I and IGF-II (6–8). IGF-IR signaling is crucial for the establishment and maintenance of transformation as well as for anchorage-independent growth (9). Moreover, IGF-IR–mediated signaling significantly contributes to the emergence of resistance to chemotherapy (10), to radiation (11), and to targeted therapies (12–17). These pro-oncogenic activities of IGF-IR are highly dependent on its proximal downstream effectors, IRS1 and IRS2. IRS proteins, once phosphorylated on tyrosine residues by IGF-IR, transmit mitogenic, antiapoptotic, and antidiiferentiation signals to the cell, mainly through the phosphoinositide 3-kinase (PI3K)–PKB module (18). IRS1/2 also mediate the termination of IGF-IR signaling. Ser-phosphorylation of IRS1/2 by various cellular kinases blocks their interaction with the receptor and targets them for degradation by the proteasome (19). This negative feedback loop is the major cellular pathway that shuts off IGF-IR signaling.

The role of IRS proteins in human malignancies has been established: overexpression of IRS1/2 causes cell transformation (20, 21) and IRS1 is constitutively activated in many human tumors, including tumors that display no aberrant activation of IGF-IR (22). Downregulation of IRS1 (by antisense or siRNA procedures) reverses the transformed phenotype (23). While IRS1 is critical for tumor growth, IRS2 is essential for tumor metastasis (2, 18, 24–26). Importantly, IRS proteins integrate signals from multiple kinases other than IGF-IR, such as insulin receptor (IR), IR/IGF-IR hybrids, EGF receptor (EGFR), and Src, all of which are involved in transformation (18, 27–30). Furthermore, IRS1 was found to be a mediator of resistance to EGFR and mTOR inhibitors (16, 17).

The prominent role of IRS proteins in cancer initiation, progression, and metastasis, as well as in acquired drug
resistance, establishes them as potential targets for novel anticancer drugs.

Here, we present and characterize a unique family of small molecules that lead to Ser-phosphorylation and destruction of IRS1 and IRS2. The elimination of IRS1/2 results in long-term inhibition of IGF-IR signaling and powerful inhibition of tumor cell growth.

Materials and Methods

Reagents and antibodies

For details, see Supplementary Methods

Cell lines

A375 (human melanoma), HCT16 (colon cancer), HCT15 (colon cancer), SK-ES.1 (Ewing sarcoma), NCI-H460 (lung cancer) were cultured in RPMI with 10% fetal calf serum (FCS). HepG2 (hepatocarcinoma) were cultured in Dulbecco's Modified Eagle Medium (DMEM) and F12 (1:1) containing 10% FCS. DU145 (prostate cancer) were cultured in RPMI containing 5% FCS and 5 mg/L insulin. All cell lines were obtained from the American Type Culture Collection. YUMAC, YURIF, YUSIK (all human melanoma, kindly provided by Prof. Ruth Halaban, Yale University, New Haven, CT) were cultured in OptiMEM containing 5% FCS. M571, M2068, M560n (all human melanoma), normal melanocytes, and normal fibroblasts (kindly provided by Dr. Michal Lotem, Hadassah Hospital, Jerusalem, Israel) were maintained in RPMI, DMEM and F12 (1:3:1) containing 10% FCS. A375SM (metastatic A375 cells; ref. 31) were maintained in MEM containing 10% FCS. 451-Lu (human melanoma) and 451-Lu-Br (PLX4032-resistant melanoma; ref. 32) were maintained in RPMI containing 5% FCS (media for resistant lines contained 1 μmol/L PLX4032). All media were supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin and all cells were grown at 37°C/5% CO₂.

Cell proliferation

Cells were grown in complete medium and treated with inhibitors one day following seeding. Seventy-two hours later, the surviving cells were quantified by methylene blue staining or by WST-1 staining for nonadherent cells (Roche).

Anchorage-independent growth assay

Cells were plated in 50 μL growth medium containing 0.3% agar on top of a layer of 100 μL of growth medium containing 1% agar. Fifty microliters of growth medium containing inhibitors was added on top. A week later, representative images were taken using a microscope eyepiece camera (ANMO Electronics Corporation) and colonies were stained with 0.5% MTT for 4 hours. The dye was extracted with dissolving buffer (5 g SDS/8.75 mL DDW/12.5 mL DMF/0.5 mL acetic acid/0.07 mL HCl). Following overnight incubation at 37°C and absorbance was read at 570 nm.

Migration assay

Cells were treated with 3 μmol/L NT157 for 18 hours and wounded at t = 0 using a pipette tip. The medium was replaced with medium lacking inhibitor, and images were taken at the indicated times using a microscope eyepiece camera (ANMO Electronics Corporation).

Subcellular fractionation

Assays were conducted according to the protocol described by Abcam: http://www.abcam.com/index.html?pageconfig=resource&rid=11473. Cytoplasmic fractions were concentrated using Amicon ultra-4 filters (Millipore).

Immunoblots

Cells were treated as indicated and lysed with boiling sample buffer (10% glycerol, 50 mmol/L Tris-HCl, pH 6.8, 3% SDS, and 5% 2-mercaptoethanol). Western blot analysis was conducted as described previously (33).

Coimmunoprecipitation

Serum-starved cells were treated as specified and lysed on ice. Coimmunoprecipitation was conducted using 0.5 to 1 mg total protein, 2 μg antibody, and 50 μL protein A–agarose beads at 4°C. Bound proteins were eluted with boiling sample buffer, and analyzed by Western blot analysis. Detailed methods can be found in the Supplementary Data.

Quantitative PCR

Serum-starved cells were treated with NT157 and then RNA was extracted using TRIzol reagent (Invitrogen) and subjected to reverse transcription using M-MLV RT (Invitrogen). Quantitative PCR amplification was conducted using SYBR Green in a 7900HT Fast Real-Time PCR system (ABI). The relative quantities of gene transcripts were normalized to HuPO (Human acidic ribosomal protein) transcripts. The following primers were used: HuPO: forward 5'-GCTTCCTGGAGGG-TGTC-3', reverse 5'-GGATCTGTGTTATACCCGTTG-IRS1: forward 5'-CTTCTGTACAGGTTCCATCC-3', reverse 5'-CTTCG-ACGATGCTGTTTC-3'; IRS2: forward 5'-ACAAATGTGCTA- CACCGAG-3', reverse 5'-CTGCTTTCTGAGAGAC-3'.

In vivo tumor growth and survival models

Athymic Nude-nu mice were used for the in vivo studies described in Fig. 6A–C. To study tumor growth, A375 cells were injected subcutaneously into nude mice. Once tumor size reached approximately 75 mm³, mice were treated daily for 12 days by intravenous or intraperitoneal administration of vehicle or NT157 (Fig. 6A and B). For the survival melanoma model (Fig. 6C), A375 cells were injected intravenously into female nude mice (9 mice/group). Administration of vehicle or NT157 (100 mg/kg, 10 mL/kg) intravenously 3 times a week for 2 weeks (arrows) was initiated 10 days later. Animals that developed tumor signs were sacrificed by an overdose of anesthetic. The internal organs of the sacrificed animals were then excised to verify tumor development. To study the growth of 451-Lu-Br tumors (Fig. 7E), cells were injected subcutaneously into female nude mice (10 mice/group). Administrations of vehicle, PLX4032 (12.5 mg/kg) orally, NT157 (70 mg/kg) intraperitoneally, or PLX4032 and NT157 were initiated 10 days later. Tumor volume was measured 3 times a week and animals were sacrificed on day 26.

For further details see Supplementary Methods.
In vivo model of lung metastasis of A375SM cells
A375SM cells were injected intravenously (7–9 mice/group) into female athymic BALB/c nude mice, 8–10 weeks. Administration of NT157 (70 mg/kg) intravenously 3 times a week was initiated 3 days later. Temozolomide (100 mg/kg) was administered intraperitoneally twice a week. A control group was given vehicle intravenously. The mice were sacrificed after 4 weeks; the lungs were removed and fixed in Bouin’s solution for 24 hours. Surface tumor nodules were counted using a dissecting microscope. For further details, see Supplementary Data.

Immunohistochemistry
A total of $5.0 \times 10^5$ A375SM cells were injected subcutaneously into the right flank of female athymic BALB/c nude mice (10 mice/group). Ten days later, NT157 was administered intravenously or intraperitoneally (70 mg/kg) 3 times a week for 4 weeks. The mice were sacrificed 48 hours after the last treatment, and tumors were processed for immunohistochemical and (terminal deoxynucleotidyl transferase–mediated dUTP end labeling) TUNEL assay. To detect the levels of pS-IRS1/2 and pY(1131)IGF-IR, antibodies were used at 1:50 dilution. Assays were conducted as previously described (34).

TUNEL assay
The TUNEL assay was conducted using a commercial kit (Promega) according to the manufacturer’s protocol, as previously described (34).

Results
The NT compounds constitute a novel family of anticancer agents that target IRS1/2 to degradation
During our quest for IGF-IR kinase inhibitors, we discovered a unique subfamily of IGF-IR signaling inhibitors. These compounds, represented by NT52, NT75, NT157, and NT205 (Fig. 1A; ref. 35) were developed by rational design based on a structure–activity relationship study of our earlier compounds (Supplementary Table S1 and Supplementary Fig. S1A). In a cell-free assay, the NT compounds exhibited ATP noncompetitive as well as substrate noncompetitive inhibition of the full-length IGF-IR (Supplementary Fig. S1B) while they did not inhibit the isolated kinase domain of the receptor (data not shown).

Figure 1. NT inhibitors lead to the dissociation of IRS1/2 from IGF-IR and induce Ser-phosphorylation of IRS1/2. A, chemical structures of the NT compounds. The active NT compounds all possess an OH at the 5' position of the aminobenzyl moiety. The control compound C133 possesses a Br substitution at this position. B, NT compounds, but not C133, induce IRS1/2 Ser-phosphorylation and strongly inhibit IGF-I–induced PKB activation. Serum-starved A375 cells were treated with NT inhibitors for 5 hours, then stimulated with IGF-I for 5 minutes, and lysed. C, NT-induced IRS1/2 Ser-phosphorylation is IGF-I–independent. Serum-starved A375 cells were treated with NT inhibitors for 5 hours and lysed without IGF-I stimulation. D, NT157 induces the translocation of IRS1/2 to the cytosol. Serum-starved A375 cells were treated with NT157 as indicated. Cells were stimulated with IGF-I for 5 minutes, lysed, and fractionated by differential centrifugation.
shown). These data show that the NT compounds are mixed-competitive inhibitors (36) and namely inhibit IGF-IR in an allosteric manner. The new compounds were simultaneously tested for their ability to inhibit IGF-I-induced signaling in cells (Fig. 1B) and for their antiproliferative activity against various cancer cell types (Supplementary Table S2). Remarkably, the NT compounds induced extensive Ser-phosphorylation of IRS proteins (Fig. 1B and C), in correlation with their ability to inhibit cancer cell growth (see below).

The inhibitory Ser-phosphorylation of IRS1/2 led to the suppression of signaling to PKB in A375 human melanoma cells (Fig. 1B). We further investigated this phenomenon using NT157 as the lead compound. NT157 induced the dissociation of IRS1/2 from the receptor within 30 minutes of treatment before the Ser-phosphorylation of IRS was evident (Fig. 1D and Fig. 2A). This dissociation was probably due to a conformational change in IGF-IR induced by the allosteric mode of binding of NT157. The Ser-phosphorylation of IRS1/2 is known...
to preclude their rebinding to the receptor and transduction of signaling downstream (19).

IRS1/2 Ser-phosphorylation increased with time (Fig. 2A), and within 20 hours of NT treatment, IRS1/2 proteins disappeared (Fig. 2B). The autophosphorylation of IGF-IR on tyrosine 1131 remained intact (Fig. 2A), and only at higher concentrations of the inhibitors was pTyr-IGF1R directly inhibited (Fig. 1B). These findings highlight the unique mode of action of the NT compounds in intact cells: The primary mechanism of the NT-induced inhibition of IGF-IR signaling is a result of the inhibitory Ser-phosphorylation and the subsequent elimination of IRS1/2. In the absence of the key mediators in the pathway, signaling downstream is impeded regardless of IGF-IR activity. The details of this exceptional mechanism will be further discussed below.

In fact, the targeting of IRS1/2 for Ser-phosphorylation and proteasome-dependent degradation is the major cellular mechanism for shutting off IGF-IR signaling (19, 37). Indeed, when NT compounds were combined with the proteasome inhibitor, MG132, Ser-phosphorylated IRS1/2 accumulated (Fig. 2C). Furthermore, NT157 did not decrease the mRNA levels of IRS1 or IRS2 (Fig. 2D). Treatment with NT compounds eventually led to apoptosis, as shown by the appearance of cleaved PARP (Fig. 2B). The NT-induced Ser-phosphorylation of IRS1/2 committed them to degradation. The treatment of A375 cells with NT157 for a period of 4 hours was sufficient to induce massive Ser-phosphorylation of IRS1/2 and subsequent IRS1/2 degradation, effects that were sustained for at least 48 hours after the removal of NT157 from the medium (Fig. 2E). Cellular proliferation was strikingly inhibited after 72 hours of NT treatment. The IC50 values of A375 cell proliferation were unchanged when cells were treated only once with medium containing NT157 or NT75, without daily refreshment of the medium (Supplementary Fig. S2A and S2B), or when the NT compounds were washed out 24 hours after treatment (Supplementary Fig. S2C and S2D). These data show that a short treatment with the NT compounds triggers an irreversible cellular cascade that culminates in cell death.

The elimination of IRS1/2 is a key feature of the NT compounds. The depletion of IRS1/2 from cancer cells should lead to the inhibition of all signals transmitted through IRS proteins. Indeed, the effects of the NT compounds on IRS1/2 were IGF-I–independent (Fig. 1C, Fig. 2 and Supplementary Fig. S1C). Because IRS1/2 can also mediate IR signaling, we tested whether IR activity is affected by NT157. NT157 inhibited signaling to PKB following stimulation by IGF-II and insulin, as well as by IGF-I (Supplementary Fig. S3A; 27, 30). Immunoprecipitation of IR from NT157-treated A375 cells confirmed that its autophosphorylation, like that of IGF-IR, was not inhibited (Supplementary Fig. S3B and S3C). We conclude that NT compounds bring about the degradation of IRS1/2, irrespective of the upstream signal, leading to the inhibition of all signal transduction pathways that converge on IRS1/2.

The Ser-phosphorylation and degradation of IRS1/2 that were induced by NT compounds were observed in various human cancer cell lines (Fig. 2F). Thus, the unique mode of inhibition of IGF-IR signaling by NT compounds is widespread. The NT compounds displayed potent antiproliferative activity on many cancer cell lines (Fig. 3A and Supplementary Table S2), including lines that are resistant to anticancer agents.
NT compounds also showed efficacy in inhibiting anchorage-independent colony formation and cell migration of human melanoma A375 cells (Fig. 3B and C). Notably, NT157 displayed little to no effect on the survival of normal melanocytes and fibroblasts (Fig. 3D).

The NT compounds constitute a highly defined family of chemical entities (Supplementary Table S1 and Fig. 1A). C133 is a structural analog of the NT family in which the 5'-OH was substituted with a Br atom (Fig. 1A). This minor modification abolished the effects on IRS1/2 (Fig. 1B). We found that the anti-IRS1/2 activity of the NT compounds highly correlated with their anticancer activities and indeed C133 was only a weak inhibitor of cancer cell proliferation and of the growth of A375 cells in soft agar (Supplementary Table S2; Fig. 3A and B).

The ERK<sup>MAPK</sup> pathway in A375 melanoma cells mediates NT157-induced Ser-phosphorylation of IRS1/2

We studied the mechanism of NT action in A375 melanoma cells, with NT157 as the lead compound. We found that concomitant to the detachment of IRS1/2 from the receptor NT157 induced an increased interaction of IGF-IR with the adaptor protein Shc (Fig. 4A). Shc and IRS proteins bind the

![Figure 4](image-url)

**Figure 4.** NT157 shifts IGF-IR complexation from IRS1/2 to Shc and leads to ERK<sup>MAPK</sup>-dependent Ser-phosphorylation of IRS1/2 in A375 melanoma cells. A, NT157 promotes recruitment of Shc to IGF-IR. Serum-starved A375 cells were treated with NT157, stimulated with IGF-I, and lysed. IGF-IR was immunoprecipitated (IP) and Shc levels in the immunoprecipitated samples were detected. B, time-dependent activation of the ERK-MAPK pathway. Serum-starved A375 cells were treated with NT157 for the indicated times, stimulated with IGF-I for 5 minutes, and lysed. The kinase activity of ERK was assessed by analysis of the phosphorylation of its direct substrate, RSK. C, the ERK-MAPK pathway mediates the NT157-induced Ser-phosphorylation of IRS1/2. Serum-starved A375 cells were treated with an inhibitor of mutated B-RAF (PLX4720) or of MEK1/2 (PD184352) for 30 minutes, and then NT157 was added for 4 hours. Cells were then stimulated with IGF-I for 5 minutes and lysed. D, the NT157-induced C-RAF activation is Ras dependent. Serum-starved A375 cells were treated with an inhibitor of Ras (FTS) for 10 minutes and then NT157 was added for 4 hours. Cells were stimulated with IGF-I for 5 minutes and lysed. E, the ERK-MAPK pathway mediates the antiproliferative effect of NT157. A375 cells were treated with PLX4720 and NT157 for 72 hours and then survival was quantified by methylene blue staining.
same tyrosine-phosphorylated Tyr950 of IGF-IR (38), and thus the detachment of IRS1/2 may allow enhanced access of IGF-IR to Shc. Following the recruitment of Shc to IGF-IR, but before the Ser-phosphorylation of IRS1/2, NT157 treatment induced the activation of the ERKMAPK pathway in A375 cells (Fig. 4B). In melanoma cells that harbor the mutant B-RAF^{V600E/K}, such as A375, the ERKMAPK pathway is constitutively active and therefore plays the key role in IRS1/2 Ser-phosphorylation. The treatment of A375 cells with the MEK1/2 inhibitor, PD184352, or the B-RAF^{V600E} inhibitor, PLX4720, resulted in nullification of the NT157-induced Ser-phosphorylation of IRS1 and in alleviation of the NT157-induced inhibition of IGF-1–dependent activation of PKB (Fig. 4C), whereas inhibition of mTOR or PI3K exhibited no effect (Supplementary Fig. S4B), confirming that the NT-induced Ser-phosphorylation of IRS1/2 is dependent on the ERKMAPK pathway in these cells. It has been previously shown that ERK1/2 is capable of phosphorylating IRS1 on certain Ser-residues, such as Ser636/639 (19). To study the mode by which NT157 induced activation of ERK1/2 beyond their basal state in A375 cells, which harbor a constitutively active B-RAF^{V600E}, we checked the phosphorylation status of C-RAF, which can participate in the amplification of the signal elicted by IGF-IR through Shc (39). C-RAF was strongly activated following NT157 treatment (Fig. 4B) in a Ras-dependent manner (Fig. 4D). The events described above occurred regardless of the kinase activity of IGF-IR, namely in the absence of IGF-1 stimulation (Supplementary Fig. S4), or in the presence of a specific IGF-IR kinase inhibitor (Supplementary Fig. S4F). Importantly, the combined treatment of A375 cells with NT157 and PLX4720 abrogated the antiproliferative effects of NT157, confirming that these effects of NT157 are mediated by IRS1/2 phosphorylation and degradation (Fig. 4E).

We propose a model (Fig. 5) whereby the binding of NT157 to an allosteric site on IGF-IR leads to the detachment of IRS1/2 from the receptor, followed by the recruitment of Shc to the receptor. This leads to the activation of the ERKMAPK pathway, which in turn leads to Ser-phosphorylation of IRS1/2. This inhibitory Ser-phosphorylation precludes re-binding of IRS1/2 to IGF-IR, blocks IGF-1 signaling, and targets IRS1/2 to degradation. The downstream activation of PKB is inhibited and cell death is induced.

Melanoma tumor growth and metastasis is efficiently inhibited by NT157

We determined the effect of the NT inhibitors on tumor growth and metastasis in mice. Daily treatment with NT157 resulted in more than 80% growth inhibition of established human A375 melanoma tumors (Fig. 6A). The antitumor effect of NT157 was dose-dependent (Fig. 6B). In models of metastasis, NT157-treated mice survived longer than vehicle-treated controls (Fig. 6C). Moreover, NT157 strongly inhibited the development of lung metastases of melanoma cells (Fig. 6D). NT157 was at least as effective as temozolomide, which is often used in the treatment of metastatic melanoma (Fig. 6D). The effect of NT157 on melanoma metastasis correlated with the inhibitory action of NT157 on melanoma cell migration and colony formation (Fig. 3B and C). Immunohistochemical analysis of the A375SM tumors confirmed our biochemical findings reported above (Fig. 1B): Treatment of mice with NT157 induced increased IRS1 Ser-phosphorylation and apoptosis of tumor cells (Fig. 6E). These observations were detected 48 hours following the last treatment, validating the long-lasting inhibitory effect of NT157 also in vivo. At the doses applied, inhibition of IGF-IR autophosphorylation was also detected.

Increased expression of IRS1 has been reported in both ovarian and androgen-refractory prostate cancers (26, 40). The efficacy of NT compounds on tumor growth and mouse survival was shown using A2780 ovarian cancer and PC3 androgen-refractory prostate cancer xenografts (Supplementary Fig. S5).

Resistance to B-RAF^{V600E/K} inhibitor drugs can be mediated by IRS1/2 upregulation

A new drug, PLX4032/Vemurafenib (41), targeted against melanomas carrying the common B-RAF^{V600E/K} mutation, shows remarkable efficacy against metastatic melanoma, but resistance to PLX4032 emerges and tumors eventually progress in almost all patients (42). We examined the potential involvement of IRS proteins in the acquired resistance of mutated B-RAF melanomas to PLX4032. To this end, we used 2 melanoma cell lines that have developed resistance to B-RAF^{V600E/K} inhibition in vitro: 451-Lu-BR and Mel1617-BR. These cell lines were cloned from the parental metastatic melanoma lines 451-Lu and Mel1617, respectively, following continuous exposure to a B-RAF^{V600E/K} inhibitor (32).

IRS1/2 upregulation is induced by short-term treatment with B-RAF^{V600E/K} inhibitor and reversed by NT157.

In melanoma cells harboring the constitutively active B-RAF^{V600E/K}, the ERKMAPK pathway plays the most important role in the basal Ser-phosphorylation of IRS1/2. Indeed, treatment of the parental 451-Lu melanoma cells with PLX4032 led to inhibition of pERK and to inhibition of the basal Ser-phosphorylation of IRS2 (as indicated by the electrophoretic downshift of IRS2, Fig. 7A). Concomitantly, an increase in IRS2 levels was observed (Fig. 7A). Treatment of 451-Lu cells with NT157 resulted in Ser-phosphorylation and degradation of IRS1, and accordingly to inhibition of IGF-1-induced PKB activation (Fig. 7B). Combined treatment with both inhibitors resulted in inhibition of both ERK and PKB pathways (Fig. 7B). Correspondingly, a synergistic cytotoxic effect was observed when the cells were treated with both inhibitors (Supplementary Fig. S6A). As the ERK pathway is crucial for the NT157-induced Ser-phosphorylation of IRS1/2, a sequential treatment in which NT157 is added to the medium a few hours ahead of PLX4032 was crucial to gain a synergic effect and avoid an antagonistic effect of the treatments.

As reported by Villanueva and colleagues, the levels of pERK were unperturbed by PLX4032 in PLX4032-resistant 451-Lu-BR cells. PLX4032 did not affect IRS1/2 (Fig. 7A).

In the parental Mel1617 cells, similar to 451-Lu cells, treatment with PLX4032 resulted in upregulation of IRS1 and IRS2, and the IGF-1–induced activation of PKB was concomitantly increased (Supplementary Fig. S6B). The levels of pY-IGF-IR were unperturbed by PLX4032, suggesting that the increase in pT-PKB levels is a results of the upregulation of IRS1/2.
Enhanced levels of IRS1 in PLX4032-resistant cells. Next, we tested the relative levels of IRS1 in the PLX4032-resistant cell lines: 451-Lu-BR and Mel1617-BR. Remarkably, we found a significant increase in the levels of IRS proteins in both cell lines, compared with the levels of IRS proteins in the corresponding parental melanoma lines, which are sensitive to mutant B-RAF inhibition (Fig. 7A and Supplementary Fig. S6C). The dramatic increase in IRS1 level in 451-Lu-BR cells far exceeded the upregulation of IGF-IR already reported by Villanueva and colleagues (Fig. 7A; ref. 32). Accordingly, the IGF-I–induced activation of PKB was enhanced in the PLX4032-resistant 451-Lu-BR cells, as compared with the parental sensitive metastatic melanoma cells, 451-Lu (Fig. 7A). In Mel1617-BR, the levels of both IRS1 and IRS2 were highly upregulated as compared with the parental Mel1617 cells (Supplementary Fig. S6C). The fact that a similar upregulation of IRS levels was found in both PLX4032-resistant cell lines tested suggests that this may be a common mechanism of acquired resistance to B-RAF<sup>V600E/K</sup> inhibition. To further investigate the broad relevance of this mechanism, we expanded our research and analyzed a panel of 6 patient-derived human melanoma cell lines (Fig. 7C and D): YUMAC, YURIF, and YUSIK are human melanoma patient-derived cell lines that contain the mutated B-RAF (43). These patients were not treated with a mutated B-RAF inhibitor. M2068, M560n, and M571 are human melanoma cell lines containing the mutated B-RAF, derived from patients that had been treated with PLX4032 and developed resistance to the drug. Our biochemical analysis validated that in M2068, M560n, and M571, ERK activation was resistant to PLX4032 treatment, whereas in YUMAC, YURIF, and YUSIK, ERK activation was highly sensitive to PLX4032 treatment (Fig. 7C). Interestingly, we found significantly higher levels of IRS1 in the PLX4032-resistant patient-derived cells as compared with the PLX4032-sensitive cells (Fig. 7D). To summarize, we detected high levels of IRS1 in melanoma cells that acquired resistance to mutated B-RAF inhibitors both in culture and in patients.
NT157 induced downregulation of IRS1/2 in PLX4032-resistant cells and inhibited PLX4032-resistant tumor growth. In PLX4032-resistant 451-Lu-BR cells, NT157 induced striking Ser-phosphorylation of IRS1/2, inhibited signaling to PKB, and led to cell death (Fig. 7B and Supplementary Fig. S6A; Similar data for Mel1617-BR cells is shown in Supplementary Fig. S6D and S6E). Correspondingly, NT157 inhibited the growth of 451-Lu-BR tumors in nude mice (Fig. 7E). Interestingly, enhanced 451-Lu-BR tumor growth was observed when mice were treated with PLX4032, possibly due to ‘drug addiction’ (44) and upregulation of the IGF-IR/IRS1 pathway (Fig. 7E). Combined treatment with NT157 and PLX4032 suppressed this accelerated tumor growth (Fig. 7E). Notably, all the cell lines tested, which had acquired resistance to PLX4032 either in culture or in patients, showed sensitivity to NT157 in a cytotoxicity assay (not shown).

To summarize, we found increased levels of IRS1/2 in melanoma cells treated with PLX4032 and in melanoma cells that had acquired resistance to B-RAFV600E/K inhibition. The downregulation of IRS1/2 by NT157 led to cell death and tumor inhibition of PLX4032-resistant cells.

Discussion
We have discovered an exceptional family of IGF-IR-IRS1/2 inhibitors. Unlike existing IGF-IR signaling inhibitors, the NT inhibitors represent a new concept in targeted therapy, whereby the inhibitor shuts off signaling by taking advantage of a negative feedback loop normally used by cells. This is brought about by the Ser-phosphorylation and the degradation of IRS1/2, resulting in long-lasting inhibition of signaling to PKB, and leading to cancer cell death. The anticancer activity of NT compounds on both IRS1 and IRS2 is highly effective, because IRS1 promotes tumor growth and IRS2 promotes metastasis. Furthermore, inhibition of both IRS proteins precludes the compensation of one for the other.

Importantly, we showed that a short exposure to NT compounds sufficed to induce Ser-phosphorylation of IRS1/2, directing IRS1/2 to degradation, even after the removal of the inhibitors. Moreover, the NT157-induced Ser-phosphorylation of IRS1/2 in A375 tumor cells in mice persisted for at least 48 hours following the administration of the drug. The finding that a short exposure to the NT compounds suffices to gain a long-lasting antitumoral effect has clinical ramifications. It
allows treatment at relatively low frequencies, which should lead to reduced side effects.

IR is highly homologous to IGF-IR in structure and function. It has been shown that selective inhibition of IGF-IR, for example, by using monoclonal antibodies, can result in compensatory activation of IR by IGF-II, leading to drug resistance (45). Because IRS1 and IRS2 mediate signaling from both IGF-IR and IR, the NT compounds lead to the disruption of signaling downstream of both receptors induced by IGF-I, IGF-II, or insulin and reduce the probability of drug resistance.

Approximately 50% of melanomas are driven by B-RAFV600E/K, and resistance to B-RAFV600E/K inhibition can be mediated by different pathways (46, 47). Our results reveal that upregulation of IRS1 and IRS2 proteins is a novel mechanism that leads to acquired resistance to B-RAFV600E/K inhibition. Similar mechanisms have been reported for acquired resistance to EGFR and mTOR inhibitors (16, 17). In all of these cases, the inhibitors reduce the levels of Ser-phosphorylation of IRS1/2, and consequently stabilize IRS1/2 and increase prosurvival signaling via PKB.

Figure 7. IRS1 is upregulated in PLX4032-resistant melanoma. A, IRS1 levels and downstream signaling are increased in PLX4032-resistant human melanoma 451-Lu-BR cells as compared with PLX4032-sensitive human melanoma 451-Lu cells. Serum-starved 451-Lu cells (resistant) and 451-Lu cells (parental) were treated with 1 µmol/L PLX4032 for 18 hours, stimulated with IGF-I for 5 minutes, and lysed. B, NT157 induces Ser-phosphorylation and degradation of IRS1 in 451-Lu and 451-Lu-BR cells in the presence or absence of PLX4032. Serum-starved cells were treated with 5 µmol/L NT157, and 3 hours later, 1 µmol/L PLX4032 was added for an additional hour. The cells were stimulated with IGF-I for 5 minutes and lysed. C, responsiveness of the ERK–MAPK pathway to PLX4032 in patient-derived melanoma cells. B-RAFV600E/K-carrying cells derived from 6 patients with melanoma were serum-starved, treated with 1 µmol/L PLX4032 for 24 hours, and lysed. D, cells derived from PLX4032-resistant melanoma patients show increased levels of IRS1 compared with cells derived from patients with melanoma not treated with PLX4032. Patient-derived cells were grown with complete growth medium or under starvation conditions and lysed. IRS1 levels were analyzed and STAT3 levels served as loading controls. E, NT157 inhibits the growth of PLX4032-resistant tumors in the presence or in the absence of PLX4032. 451-Lu-BR cells were injected subcutaneously into nude mice (10 mice/group), and administration of vehicle, PLX4032 (12.5 mg/kg; 5 times per week; orally), NT157 (70 mg/kg; 3 times per week; i.p.), or PLX4032 + NT157 was initiated 10 days later.
It has been previously shown that in some cases of melanoma IGF-IR is upregulated, leading to enhanced signaling through IRS proteins (32, 48). The upregulation of IGF-IR and/or IRS proteins can be effectively reversed by treatment with NT compounds, leading to tumor cell death. The present study suggests that combining NT compounds with B-RAF<sup>V600E/K</sup> inhibitors should be effective for the treatment of patients with metastatic melanoma harboring the mutated B-RAF, and that NT compounds should be effective for patients showing resistance to B-RAF<sup>V600E/K</sup> inhibitors (49). Furthermore, NT compounds may resensitize resistant tumors to B-RAF<sup>V600E/K</sup> inhibitors. Our results highlight IRS proteins as critical mediators of tumor progression, metastasis, and drug resistance and further establish them as important drug targets in cancer.

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

Authors' Contributions
Conception and design: H. Reuveni, E. Flashner-Abramson, L. Steiner, K. Makedonski, M. Bar-Eli, A. Levitzki
Development of methodology: H. Reuveni, E. Flashner-Abramson, K. Makedonski, R. Song, A. Shir, A. Levitzki
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Reuveni, L. Steiner, R. Song, M. Herlyn
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Reuveni, E. Flashner-Abramson, L. Steiner, A. Shir, A. Levitzki
Writing, review, and/or revision of the manuscript: H. Reuveni, E. Flashner-Abramson, M. Herlyn, A. Levitzki

References


Therapeutic Destruction of Insulin Receptor Substrates for Cancer Treatment

Hadas Reuveni, Efrat Flashner-Abramson, Lilach Steiner, et al.


Updated version Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-3385

Supplementary Material Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2013/05/09/0008-5472.CAN-12-3385.DC1

Cited articles This article cites 48 articles, 26 of which you can access for free at: http://cancerres.aacrjournals.org/content/73/14/4383.full.html#ref-list-1

Citing articles This article has been cited by 9 HighWire-hosted articles. Access the articles at: /content/73/14/4383.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.