Molecular Target Characterization and Antimyeloma Activity of the Novel, Insulin-like Growth Factor 1 Receptor Inhibitor, GTx-134

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Abstract

**Purpose:** Therapeutic strategies that target insulin-like growth factor 1 receptor (IGF-1R) hold promise in a wide variety of cancers including multiple myeloma (MM). In this study, we describe GTx-134, a novel small-molecule inhibitor of IGF-1R and insulin receptor (IR) and characterized its antitumor activity in preclinical models of MM.

**Experimental Design:** The activity of GTx-134 as a single agent and in combination was tested in MM cell lines and primary patient samples. Downstream effector proteins and correlation with apoptosis was evaluated. Cytotoxicity in bone marrow stroma coculture experiments was assessed. Finally, the in vivo efficacy was evaluated in a human myeloma xenograft model.

**Results:** GTx-134 inhibited the growth of 10 of 14 myeloma cell lines (<5 μmol/L) and induced apoptosis. Sensitivity to GTx-134 correlated with IGF-1R signal inhibition. Expression of MDR-1 and CD45 were associated with resistance to GTx-134. Coculture with insulin-growth factor-1 (IGF-1) or adherence to bone marrow stroma conferred modest resistance, but did not overcome GTx-134–induced cytotoxicity. GTx-134 showed in vitro synergies when combined with dexamethasone or lenalidomide. Further, GTx-134 enhanced the activity of PD173074, a fibroblast growth factor receptor 3 (FGFR3) inhibitor, against t(4;14) myeloma cells. Therapeutic efficacy of GTx-134 was shown against primary cells and xenograft tumors. Although dysregulation of glucose homeostasis was observed in GTx-134–treated mice, impairment of glucose tolerance was modest.

**Conclusions:** These studies support the potential therapeutic efficacy of GTx-134 in MM. Further, they provide a rationale for clinical application in combination with established antimyeloma treatments and novel targeted therapies. Clin Cancer Res; 17(14); 4693-704. ©2011 AACR.

Introduction

Multiple myeloma (MM) is a disease of malignant post-germinal center B lymphocytes which home to and accumulate in the bone marrow (BM), where they receive proliferative, survival, and migratory signals from the BM microenvironment. While the advent of autologous stem cell transplantation and several novel therapeutics including; the proteosome inhibitor, bortezomib and the immunomodulatory drugs, thalidomide and lenalidomide have improved patient outcomes, MM remains for the most part incurable (1). More recently, insights into the biology, genetics, and molecular pathology of MM have provided a platform upon which novel therapeutic strategies are being developed to target myeloma cells and/or the BM microenvironment more specifically and effectively. One such target is the insulin-like growth factor 1 receptor (IGF-1R).

IGF-1R is a receptor tyrosine kinase (RTK) widely expressed in normal tissues where it functions in growth regulation (2, 3). The primary ligands are IGF-1 and IGF-2 and, to a much lesser extent, insulin (4). Receptor activation has been shown to stimulate proliferation, survival, transformation, migration, and angiogenesis in various cell types and contexts (5, 6). Increased expression of IGF-1, IGF-2 and/or their corresponding receptor, IGF-1R, has been shown in a broad range of tumors and increased levels of circulating IGF-1 are associated with colon, prostate, breast, lung, and bladder cancers (5, 7). Given its pleiotropic role in these tumor types, several approaches have been taken to inhibit IGF-1R signaling, including RNA antisense (8, 9), anti-IGF-1R–blocking antibodies (10), dominant-negative IGF-1R (11), and small-molecule inhibitors (12–16).
Translational Relevance
Insulin-like growth factor 1 receptor (IGF-1R) is implicated in cancer through its pleiotropic effects on proliferation, survival, transformation, migration, and angiogenesis. Indeed, IGF-1R expression has been identified as a poor prognostic marker in multiple myeloma (MM) and is postulated to be a therapeutic target. This article is the first to characterize GTx-134, as a novel dual inhibitor of IGF-1R/insulin receptor with potent antmyeloma activity. Further we identified mechanisms of resistance to GTx-134 and showed synergism of GTx-134 with standard antimyeloma treatments and other targeted agents in high-risk myeloma subtypes. These results provide the scientific rationale for treatment strategies that should be explored to most effectively introduced GTx-134 into the clinic for the treatment of MM.

Many studies have also shown that IGF-1R stimuliates the proliferation and survival of MM cells as well as their migration, adhesion, and invasion (17–20). Despite the clinical and genetic heterogeneity of MM, IGF-1R is widely expressed in primary patient samples and almost universally expressed in human myeloma cell lines (HMCL; refs. 21–23). In addition, stromal cells are known to secrete IGF-1 (24). IL-6, another major MM growth factor, has further been shown to act, in part, via recruitment of IGF-1R (25, 26). Furthermore, IGF-1R expression is associated with high-risk clinical subtypes that have been shown to have a particularly poor prognosis (27, 28, 29), in particular t(4;14) and t(14;16) translocation groups (23), and abnormal IGF-1 expression has been linked to progression from monoclonal gammopathy of undetermined significance (MGUS) to MM (30).

As a result, inhibition of IGF-1R represents an attractive therapeutic target for MM. While preliminary preclinical studies have yielded encouraging results (15, 22), a better understanding of the mechanisms of anti-MM activity will allow clinicians to better apply IGF-1R inhibitors to patient care. In the present study, we establish the antimyeloma activity of GTx-134 and provide insight into its mechanism of action, features determining sensitivity or resistance, and combination strategies required to maximize patient benefit.

Materials and Methods

Chemical compounds and biologic reagents
GTx-134 stock solution was obtained from Chemtech, Inc. Myeloma cell lines were purchased from the DSMZ and ATCC cell banks.

Cell lines and tissue culture
All HMCLs were maintained in Iscove’s modified Dulbecco medium (IMDM) supplemented with 10% FCS (HyClone). Bone marrow stromal cells (BMSC) were derived from MM patients and prepared as previously described (31). For viability assays, BMSCs were plated on 96-well plates and once confluent, irradiated with 20 Gy. BM aspirates were obtained by consent under a protocol approved by the University Health Network Research Ethics Board (Toronto, ON).

Viability assays
Cell viability was assessed by MTT dye absorbance according to the manufacturer’s instructions (Boehringer Mannheim). Cells were seeded in 96-well plates at a density of 20,000 cells per well (HMCL). The cells were incubated with or without IGF-1 (50 ng/mL) or IL-6 (10 ng/mL) where indicated and increasing concentrations of GTx-134. For each concentration of the compound of interest, 10-μL aliquots of drug or DMSO diluted in culture medium were added. For drug combination studies, the cells were incubated with the indicated concentration of melphalan, bortezomib, dexamethasone, or PD173074 and GTx-134. For each dose combination, the combination index (CI) was calculated on the basis of following equation: CI = D1/(Dx1) + D2/(Dx2) where D1 and D2 are the doses of drug 1 and drug 2, respectively, that have the same effect when used in combination, and (Dx1) and (Dx2) are the doses of drug 1 and drug 2, respectively, that have the same effect when used alone. Finally, to evaluate the effect of GTx-134 on growth of MM cells adherent to BMSCs, 20,000 cells were cultured on BMSC-coated 96-well plates in the presence or absence of GTx-134. Plates were incubated at 37°C, 5% CO2 for 72 hours. Each experimental condition was carried out in triplicate.

Western blot and immunoblotting
MM cell lines were lysed in lysis buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, 10% Glycerol, 1 mmol/L M EDTA, and 1.5 mmol/L MgCl) supplemented with 1 mmol/L PMSF, 2 mmol/L Na3VO4 and protease inhibitor cocktail (Roche Applied Science) for 30 minutes on ice, and clarified by centrifugation at 12,000 g for 15 minutes. Samples were analyzed by 10% SDS-PAGE and immunoblotted with the specified antibody. Protein bands were visualized using secondary antibodies coupled to horseradish peroxidase and the enhanced chemiluminescence (ECL) kit from Pierce according to the manufacturer’s instructions.

Apoptosis analysis
Apoptosis of drug-treated myeloma cell lines was measured using the Annexin V-Fluos Staining Kit (Boehringer Mannheim). In addition, MNCs from patient-derived BMs were plated at a cell density of 5 × 10^5 cells/mL in IMDM
with 15% FCS in the presence of diluted DMSO, 0.6 or 1.8 μmol/L, GTx-134. Cells were harvested after 72 hours and stained with anti-CD138-PE (PharMingen) and FITC-conjugated Annexin V (Boehringer Mannheim) as previously described (31). Samples were analyzed by flow cytometry on a FACSCaliber flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences).

Intracellular phospho-protein staining

Determination of Akt phosphorylation by flow cytometry has been described previously. BM mononuclear cells (MNCs) were incubated with DMSO (2 hours), GTx-134 (2 hours) or LY294002 (0.5 hours) and then stimulated with 50 ng/ml IGF-1 at 37°C for 12 minutes where indicated. The cells were immediately fixed with 2% formaldehyde, permeabilized by adding ice-cold methanol (to a final concentration of 90%), whereas vortexing and incubated on ice for 30 minutes. The cells were washed with PBS plus 4% FCS, stained with anti-phospho-Akt for 15 minutes and then labeled with fluorescein isothiocyanate (FITC)-conjugated goat antirabbit and phycoerythrin (PE)-labeled anti-CD138. Flow cytometry was conducted on a FACSCaliber flow cytometer and analyzed using Cellquest software.

Xenograft mouse model

To further validate drug activity in vivo, the xenograft mouse model was prepared as previously described (31). Briefly, 6- to 8-week-old female NOD/SCID mice (Jackson Laboratory) were irradiated with 250 rads Cs-137 (cesium), 1 day before tumor cell administration. The vehicle or drug (20 mg/kg, twice daily for a total of 40 doses in the most sensitive cell lines (Fig. 1C and D). By comparing the drug concentrations required to achieve a 50% antiproliferative effect (IC50) GTx-134 showed IC50's of less than 5 μmol/L, whereas MM1.S, UTMC2, and H929 were most sensitive to GTx-134 inhibiting IGF-1R and downstream Akt activity in the HMCL, MM1.S (Fig. 1B). These cells express IGF-1R and upon exposure to IGF-1 markedly increase the phosphorylation of both IGF-1R and downstream Akt. IGF-1 induced phosphorylation of IGF-1R and Akt in these cells was inhibited in a dose-dependent manner by GTx-134. As IGF-1R and the IR bear strong homology, we further examined whether GTx-134 can also inhibit signaling downstream of the IR. To this end, MM1.S cells were similarly pretreated with vehicle control, 1 or 5 μmol/L GTx-134 for 4 hours, then stimulated with either IGF-1 or insulin. Examination of the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) showed that GTx-134 equally inhibited signaling downstream of both IGF-1R and IR (Supplementary Fig. S1).

In cellular proliferation assays, GTx-134 inhibited the growth of genetically diverse HMCLs at low micromolar doses in the most sensitive cell lines (Fig. 1C and D). By comparing the drug concentrations required to achieve a 50% antiproliferative effect (IC50) GTx-134 showed IC50's of less than 5 μmol/L, whereas MM1.S, UTMC2, and H929 were most sensitive to GTx-134 inhibiting IGF-1R and downstream Akt activity in the HMCL, MM1.S (Fig. 1B). These cells express IGF-1R and upon exposure to IGF-1 markedly increase the phosphorylation of both IGF-1R and downstream Akt. IGF-1 induced phosphorylation of IGF-1R and Akt in these cells was inhibited in a dose-dependent manner by GTx-134. As IGF-1R and the IR bear strong homology, we further examined whether GTx-134 can also inhibit signaling downstream of the IR. To this end, MM1.S cells were similarly pretreated with vehicle control, 1 or 5 μmol/L GTx-134 for 4 hours, then stimulated with either IGF-1 or insulin. Examination of the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) showed that GTx-134 equally inhibited signaling downstream of both IGF-1R and IR (Supplementary Fig. S1).

To analyze serum insulin levels, mouse serum was collected at 120 minutes post glucose challenge. Serum insulin was measured with the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem) as per manufacturers’ instruction.

Results

GTx-134 inhibits IGF-1R signaling and growth of HMCLs

GTx-134 is a novel small-molecule inhibitor, identified by screening of a kinase-focused small-molecule library and subsequently shown in biochemical assays to inhibit class II RTKs including IGF-1R and insulin receptor (IR) with IC50’s of 97 nmol/L and 187 nmol/L, respectively. The in vitro kinase inhibition profile of GTx-134 suggests it is selective for IGF-1R and closely related RTKs in a broad survey of the kinome (Supplementary Table S1). We first confirmed the ability of GTx-134 to inhibit cellular IGF-1R activity in the HMCL, MM1.S (Fig. 1B). These cells express IGF-1R and upon exposure to IGF-1 markedly increase the phosphorylation of both IGF-1R and downstream Akt. IGF-1 induced phosphorylation of IGF-1R and Akt in these cells was inhibited in a dose-dependent manner by GTx-134. As IGF-1R and the IR bear strong homology, we further examined whether GTx-134 can also inhibit signaling downstream of the IR. To this end, MM1.S cells were similarly pretreated with vehicle control, 1 or 5 μmol/L GTx-134 for 4 hours, then stimulated with either IGF-1 or insulin. Examination of the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) showed that GTx-134 equally inhibited signaling downstream of both IGF-1R and IR (Supplementary Fig. S1).

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GTx-134–induced apoptosis is accompanied by inhibition of IGF-1R and Akt phosphorylation

We next determined whether GTx-134 induces apoptosis of sensitive HMCLs such as MM1.S, H929, and UTMC2 and included SKM2 and RPMI8226 as resistant controls. These HMCLs were treated with 0.6 or 1.8 μmol/L GTx-134 for 72 hours and apoptosis was assayed by annexin V staining and flow cytometry (Fig. 2A). A marked increase in apoptotic cells (range 33%–67%) was observed in all 3 sensitive lines in response to 1.8 μmol/L GTx-134. In contrast, minimal apoptosis was induced (3%–10%) in the more resistant lines.
We next determined whether the effect of GTx-134 on inhibition of IGF-IR and Akt signaling (within a cell line) correlated with cytotoxicity. Sensitive cell lines, MM1.S, H929, and UTMC2 and the more resistant lines, SKMM2 and MY5, were preincubated with 1, 5, or 12.5 μmol/L GTx-134 for 4 hours and then stimulated with IGF-1 prior to harvesting for immunoblotting (Fig. 2B). All HMCLs showed an increase in phosphorylation of IGF-1R and Akt upon IGF-1 stimulation.

Pretreatment with GTx-134 induced dose-dependent inhibition of IGF-1–mediated phosphorylation that was concordant with its cytotoxic activity. That is, IGF-1R and Akt phosphorylation were almost completely inhibited at doses of 5 μmol/L or less in the sensitive cell lines, whereas higher doses of GTx-134 were required to effectively inhibit phosphorylation in SKMM2 and MY5 cells. Together, the data are consistent with the targeted activity of GTx-134, in that measurable...
inhibition of IGF-1R and Akt activity are required for induction of apoptosis.

We postulated that the higher doses necessary to produce target inhibition in My5 and SKMM2 may be a consequence of efflux pump-mediated resistance. By gene expression profiling SKMM2 cells express high levels of the multidrug-resistant gene, MDR-1. To test our hypothesis, SKMM2 were treated with GTx-134 together with cyclosporin A (CsA) or verapamil, broad-spectrum inhibitors of multidrug transporters (32). Addition of CsA or verapamil reduced the IC_{50} of SKMM2 cells from 30 μmol/L to less than 2.5 μmol/L (Supplementary Fig. S2), suggesting that GTx-134 is a P-glycoprotein (Pgp) substrate.

On the other hand, My5 cells do not express MDR-1 and were not sensitized by coculture with CsA or verapamil implicating other mechanisms of resistance in these cells. It has been proposed that CD45 negatively regulates IGF-1–dependent activation of PI3k and that expression of CD45 phosphatase renders myeloma cells resistant to inhibition of IGF-1 signaling (33, 34). Flow cytometry analysis of pan-CD45 expression among the panel of 14 HMCLs identified only My5 and U266 cells as expressing CD45 (Supplementary Fig. S3), both of which were among the 5 most GTx-134–resistant cell lines. To follow-up on this observation, U266 cells that harbor a subset of CD45−cells were sorted into CD45^+ and CD45^−populations,
and sensitivity to GTx-134 was evaluated by MTT assay. U266-CD45+, U266-CD45−, and the U266 pool population all showed similar dose–response curves suggesting that response to IGF-1R inhibitors is independent of CD45 status in these cells.

Coculture with IGF-1 or stroma confers modest protection from the cytotoxic effects of GTx-134

To further characterize the importance of IGF-1 in mediating sensitivity to GTx-134, MM1.S and H929 cells were treated with increasing concentrations of drug in either the absence or presence of IL-6 (Fig. 3B). By MTT assay, when stimulated with IGF-1, HMCLs are provided modest protection against the antiproliferative activity of GTx-134 with a shift in the IC50 values from 1.0 to 2.7 μmol/L and 0.6 to 1.3 μmol/L for MM1.S and H929, respectively. By comparison, MM1.S cells were not protected against GTx-134 by stimulation with IL-6 (Fig. 3A). Further, both HMCLs were protected when cultured in the presence of stromal cells (Fig. 3C), a condition which has been shown to enhance IGF-1 production in coculture supernatants (22).

GTx-134 potentiates the cytotoxic activity of therapeutics used in the treatment of MM

As novel anticancer agents are generally applied in combination with existing therapeutics, we examined whether GTx-134 could potentiate the action of drugs currently used to treat MM. To this end, MM1.S cells were incubated with increasing concentrations of GTx-134 and either melphalan, bortezomib, or dexamethasone. Cytotoxicity was assessed by MTT activity and synergy determined by combination indices (CI; Fig. 4). In all cases, GTx-134 potentiated the effects of the antimyeloma agents, and synergized (CI < 1) with dexamethasone and lenalidomide.

We have previously shown that IGF-1R and/or IR is modulated along with fibroblast growth factor receptor 3 (FGFR3) in the t(4;14)-positive myeloma cell line,
KMS11 (35). Using an unbiased proteomics approach, we identified the activation loop tryptic peptides from IGF-1R and/or IR (these are identical) as phosphorylated with FGF stimulation and inhibited by treatment with the selective FGFR inhibitor, PD173074 providing evidence for crosstalk with FGFR3 in KMS11 cells. In light of these findings, we assessed the effect of combining GTx-134 with FGFR3 inhibition in KMS11 cells. GTx-134 synergized with PD173074 suggesting that the therapeutic strategy of combining IGF-1R/IR inhibitors with FGFR3-targeted therapies that are already in the clinic for t(4;14) myeloma should be explored.

GTx-134 induces apoptosis of primary patient MM cells

To further assess the potential of GTx-134 as a novel antimyeloma agent, primary cells derived from patient BMs were treated with vehicle control, 0.6, or 1.8 μmol/L GTx-134 for 72 hours. Cells were then labeled with anti-CD138 to identify myeloma cells and annexin V to estimate apoptotic cells by multiparametric flow cytometry (Fig. 5A). A significant decrease in the viable myeloma cell population (annexin V negative; CD138 positive) was shown in 3 of 12 patient samples treated with 0.6 μmol/L GTx-134 and in 9 of 12 patient samples treated with 1.8 μmol/L. While there was some toxicity detected in the non-MM cell population treated at highest dose (Fig. 5B), this was proportionally much less than the effect observed in the CD138+ cell population. Of the 9 samples sensitive to GTx-134 in vitro, 6 were derived from patients that had shown clinical resistance to several lines of treatment including alkylating agents and high-dose dexamethasone (all), bortezomib (3 cases), and immunomodulatory drug–based regimens (4 cases; Supplementary Tables S2–4).

To further extend our findings from cell culture showing that GTx-134 exerts its anticancer activity via triggering the inhibition of Akt signaling, mononuclear cells derived from myeloma patients where treated for 2 hours with vehicle, 1 or 5 μmol/L GTx-134, and then stimulated for 12 minutes with IGF-1. A FITC-labeled anti-phospho-Akt antibody was used to measure the phosphorylation status of Akt in the CD138 cell population (Fig. 5C). By flow cytometry analysis, a dose-dependent decrease in the Akt activation in response to IGF-1 was observed showing target inhibition by GTx-134 in primary myeloma cells.

GTx-134 effectively reduces tumor burden in a HMCL xenografts model

Xenograft experiments were next conducted to assess the potential in vivo efficacy of GTx-134. NOD/SCID mice were inoculated subcutaneously with 2.5 × 10^7 MM1.S cells and
a matrigel basement membrane matrix. After approximately 1 week, when tumors were palpable, the animals were divided into 3 groups: vehicle controls, a group with a low tumor burden to receive GTx-134 immediately, and a group to receive GTx-134 2 weeks later and thus with a high tumor load. The vehicle or drug (20 mg/kg twice per day) were administered by i.p. injections and measurements were taken twice weekly to estimate tumor volume (Fig. 6A). When compared with vehicle controls, a significant ($P < 0.05$) antitumor effect was shown in both GTx-134 treatment groups. In mice with low tumor volumes at the initiation of treatment, almost complete inhibition of tumor progression was observed.

Glucose tolerance tests were conducted to assess the effect of GTx-134 on glucose homeostasis. Serum glucose measurements from multiple time points were obtained in mice treated for 24 days with 40 mg/kg/day of drug or solvent control (Fig. 6B). Under basal conditions, animals receiving GTx-134 exhibited lower blood glucose levels but, when the mice were administered glucose, the drug-treated group showed a greater peak blood glucose concentration. Glucose levels in both groups returned to their respective basal levels within 1 hours of glucose administration suggesting similar kinetics of glucose homeostasis. Serum insulin was also measured 2 hours after glucose administration (data not shown). Although serum levels were higher in the GTx-134–treated vs. placebo-treated animals (0.36 ± 0.18 ng/mL vs. 0.33 ± 0.10 ng/mL, respectively) this did not meet statistical significance (Fig. 6B).

Discussion

The IGF-1R signaling pathway is deregulated in a variety of human cancers (7) and plays an important role in tumor
GTx-134 is a Novel Antimyeloma Agent

Figure 6. GTx-134 inhibits growth of MM xenograft tumors. NOD/SCID mice were inoculated subcutaneously with MM1.S cells. After approximately 1 week, when tumors were palpable, the animals were divided into 3 groups: vehicle control (n = 7), a low tumor burden group to receive GTx-134 immediately (G1, n = 8), and a high tumor burden group to receive GTx-134 2 weeks later (G2, n = 5). Vehicle or drug (20 mg/kg twice daily) was administered by i.p. injection. A, measurements were taken twice weekly to estimate tumor volume. B, glucose tolerance tests were conducted by taking glucose measurements from the blood of mice treated with 40 mg/kg/day (○; n = 5) of GTx-134 or a solvent control (■; n = 5) at the indicated times. GTx-134 or vehicle was administered 4 hours prior to glucose administration.

progression, proliferation, metastasis, protection from apoptosis, and transformation (5, 7). As a result, several strategies to inhibit it with antibodies, antisense, siRNAs, IGF binding proteins, dominant-negative receptors, and small-molecule inhibitors have been explored to date (36, 37). In fact, some clinical efficacy has also been reported. When combined with paclitaxel and carboplatin, IGF-1R antibodies resulted in improved response rates in lung cancer patients (38). In addition, a dose-finding phase I trial of a fully humanized monoclonal antibody in solid tumor patients also showed some evidence of antitumor activity in the absence of severe toxicities (39). Several other trials of either monoclonal antibodies or small-molecule inhibitors of IGF-1R are currently recruiting or are under development.

IGF-1R has been shown to be a promising target in anti-myeloma therapy and several preclinical studies have reported encouraging results for a variety of IGF-1R inhibitors (15, 22, 40). For example, the small-molecule inhibitor cyclolignan picropodophyllin (PPP) induces apoptosis, inhibits proliferation, angiogenesis, and osteolysis, and dramatically increases survival in mice (40–42). Another small-molecule inhibitor, NVP-AEW541, has been found to enhance the in vitro activities of dexamethasone and bortezomib (43). 2 agents currently used for the treatment of MM, as well as the mTOR inhibitor, RAD001 (44). Similarly, treatment with the anti-IGF-1R antibody AVE1642 was most effective in combination with bortezomib (45).

Results from the present study confirm GTx-134 as a dual inhibitor of IGF-1R and IR with decreased autophosphorylation of its target receptor, and inhibition of signaling through the PI3k/Akt pathway. Despite the ubiquitous expression of IGF-1R, we observed a heterogeneous response with some HMCLs being very sensitive (IC50 values in the low micromolar range) and others being more resistant (IC50 values higher than 5 μmol/L). While basal activation of IGF-1R and Akt varied widely between cell lines, there was no association with sensitivity to GTx-134. On the other hand, the dose-dependent inhibition of IGF-1–mediated phosphorylation of IGF-1R and Akt correlated strongly with sensitivity and cytotoxic activity. In at least 1 cell line, the higher doses required to inhibit IGF-1R and Akt phosphorylation are a result of drug efflux, as CsA, and verapamil, broad spectrum inhibitors of multidrug transporters sensitized the MDR-1 expressing cell line to GTx-134. We also confirmed the report by Descamps and colleagues suggesting that anti-IGF-1R therapy may not be effective against CD45 expressing myeloma cells (46). An explanation is that the phosphatase activity of CD45 downregulates the kinase activity of IGF-1R making CD45 insensitive to IGF-1. Consistent with this observation, both CD45+ myeloma cell lines in our panel of 14 were most resistant to GTx-134. However, we found that U266 cells were relatively insensitive regardless of CD45 expression that is, both CD45+ and CD45− U266 cells were resistant to GTx-134. This result is contradictory to a previous report showing reversal of IGF-1R inhibitor resistance in CD45 positive cells with CD45-silencing (46). Our data suggests that additional intrinsic mechanisms of resistance exist in U266 cells that may not have been acquired in the cell line used for CD45-silencing experiment. Taken together, these results offer insights into potential mechanisms of resistance and provide evidence that molecular differences between HMCLs, independent of simply constitutive IGF-1R activation, play an important role in determining sensitivity to GTx-134.

As MM exists predominantly in the BM microenvironment, it is subject to an array of growth factor and cytokine stimulation. These cytokines and factors, such as IGF-1 and IL-6, are secreted by BM stromal cells, endothelial cells, and osteoclasts to promote MM cell growth, survival, migration and chemoresistance in the BM milieu (47). We therefore evaluated the effect of GTx-134 on cells cultured in the presence of extracellular factors (Fig. 3). HMCLs cultured with IGF-1 and on BM stroma were provided modest protection against GTx-134 with a shift in the dose-
response curves to the right. Although there are numerous mechanisms by which direct adhesion to BMSCs may induce resistance, coculture of MM cells with BMSC has been shown to significantly enhance production of IGF-1. Thus, one possible explanation for the observed resistance may be potentiation and/or prolongation of IGF-1 signal in the presence of excess ligand, necessitating higher doses of GTx-134 to achieve target inhibition. Further, comparison between GTx-134 and other published IGF-1R inhibitor studies in BM microenvironment models reveals qualitative differences. For example, IGF-1 and/or BM stroma coculture do not confer protection against the antmyeloma effects of IGF-1R inhibitors, NVP-ADW742 (22), and PPP (40) as is observed with GTx-134 and also reported for NVP-AEW541 (43). Potential explanations for this disparity includes differences in selectivity and potencies of these small molecules for multiple kinases and the molecular heterogeneity of the different cell lines used in each of these studies. Nevertheless, the significant antitumor activity shown in the xenograft animals suggests that biologically relevant levels of GTx-134 can be achieved in vivo with a favorable therapeutic window.

It has previously been reported that MM cells can be sensitized to anticancer agents with IGF-1R inhibition (22, 40) suggesting that agents targeting this pathway may improve the clinical efficacy of drugs employed in the treatment of this disease. To inform as to which treatment strategy should be considered in the clinic we examined the combinatorial effect of GTx-134 with dexamethasone, melphalan, bortezomib, or lenalidomide. GTx-134 enhanced the cytotoxic activity of all these agents. In particular, however, the combination with dexamethasone or lenalidomide proved to be synergistic and thus looks particularly attractive for the clinic. Our results are further supported by previous reports showing that PPP potentiated the effects of dexamethasone (40) and NVP-AEW541 synergized with both dexamethasone and lenalidomide (43).

Serum levels of IGF-1 have been linked to adverse outcome in myeloma. The data on the prognostic significance of IGF-1R expression have been divergent, however, a recent report by Sprynski and colleagues identified IGF-1R expression as a poor prognostic marker in 2 independent gene expression data sets (27). Interestingly, this finding could be largely explained by the strong association of IGF-1R expression with the presence of the adverse cytogenetic marker, t(4;14). The t(4;14; p16.3;q32) translocation, that occurs in approximately 15% of MM tumors results in the dysregulated expression of 2 putative oncoproteins, MMSET and FGFR3 (48). Using an unbiased mass spectrometry–based phospho-proteomics approach, we previously found evidence for crosstalk between IGF-1R and FGFR3, in a t(4;14) positive cell line. In the present study, we established that GTx-134 greatly improved the efficacy of a selective FGFR3 inhibitor in these cells suggesting that this combination maybe a useful treatment strategy for myeloma patients with the worst clinical outcomes.

Xenograft experiments to determine the in vivo efficacy and safety of this novel IGF-1R inhibitor were also carried out. Treatment of NOD/SCID mice inoculated subcutaneously with MM1.S cells resulted in substantial inhibition of tumor growth even in well-established tumors (Fig. 6A). According to the first reported clinical studies, IGF-1R-targeted therapies have been well tolerated with hyperglycemia described as one of the most common toxicities (38, 39). Although glucose levels after the i.p. glucose load were increased consistent with these clinical findings, it was surprising to observe that in the basal state (fasting, postabsorptive) glucose levels were lower in the GTx-134–treated mice. This was present in the absence of hyperinsulinemia which suggests a lack of insulin resistance, at least at the time of blood sampling, 6 hours after drug administration. Basal glucose levels are determined primarily by hepatic glucose production which is regulated both directly and indirectly by insulin as well as by counter-regulatory hormones. Thus, the pathophysiological mechanism responsible for the lower fasting glucose in the inhibitor-treated mice remains to be determined. Nevertheless, together, with the lack of significant weight loss as a marker of general toxicity the data suggest that doses of GTx-134 producing exposure that is efficacious are also safe and tolerable.

In summary, GTx-134 is a potent small-molecule inhibitor of IGF-1R with demonstrated antmyeloma efficacy in HMCLs, primary patient samples, and MM xenografts. Added potential includes its synergy with dexamethasone, lenalidomide, and targeted therapies for high-risk MM. Further study of GTx-134 is warranted to facilitate its use in patient care.

Disclosure of Potential Conflicts of Interest

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