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

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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 anti-myeloma activity. Further we identified mechanisms of resistance to GTx-134 and demonstrate synergism of GTx-134 with standard anti-myeloma 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.
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 co-culture experiments was assessed. Lastly, the in vivo efficacy was evaluated in a human myeloma xenograft model.

Results: GTx-134 inhibited the growth of 11 of 14 myeloma cell lines (< 5 μM) 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. Co-culture 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 demonstrated 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 anti-myeloma treatments and novel targeted therapies.
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 widely expressed in normal tissues where it functions in growth regulation (2, 3). The primary ligands are insulin-like growth factors 1 (IGF-1) and 2 (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).

Many studies have also shown that IGF-1R stimulates 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 (HMCLs) (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 anti-myeloma 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 ChemBridge Corporation (San Diego, CA). Human recombinant interleukin-6 (IL-6) and IGF-1 were obtained from R&D systems (Minneapolis, MN) and PeproTech (Rocky Hill, NJ), respectively. LY294002 (phosphatidylinositol 3-kinase kinase (PI3K) inhibitor), Akt rabbit monoclonal antibody (mAb), phospho-Akt rabbit mAb and phospho-IGF-1Rβ/IR rabbit mAb were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Myeloma cell lines were purchased from the DSMZ (Braunschwieg, Germany) and ATCC (Manassas, VA) cell banks.

Cell lines and tissue culture

All HMCLs were maintained in Iscove’s modified Dulbecco medium (IMDM) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT). Bone marrow stromal cells (BMSCs) 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 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium (MTT) dye absorbance according to the manufacturer’s instructions (Boehringer Mannheim, Mannheim, Germany). Cells were seeded in 96-well plates at a density of 20,000 cells per well (HMCLs). 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, 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 based on the following equation: CI = D1/(Dx)1 + D2/(Dx)2 where D1 and D2 are the doses of drug 1 and drug 2, respectively, that have x effect when used in combination, and (Dx)1 and (Dx)2 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 performed in triplicate.

*Western blot and Immunoblotting*

MM cell lines were lysed in lysis buffer (25 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% Glycerol, 1 mM M EDTA and 1.5 mM MgCl2) supplemented with 1mM PMSF, 2 mM Na3VO4 and protease inhibitor cocktail (Roche Applied Science, Benzberg, Germany) for 30 minutes on ice, and clarified by centrifugation at 12,000g for 15 minutes. Samples were analyzed by 10% sodium dodecyl–polyacrylamide gel electrophoresis (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 (Rockford, IL) 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, Indianapolis, IN). 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 μM GTx-134. Cells were harvested after 72 h and stained with anti-CD138-PE (PharMingen, San Diego, CA) and FITC-conjugated Annexin V (Boehringer Mannheim, Indianapolis, IN) as previously described (31). Samples were analyzed by flow cytometry on a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences).

*Intracellular phospho-protein staining*
Determination of Akt phosphorylation by flow cytometry has been described previously. BM MNCs were incubated with DMSO (2h), GTx-134 (2 h) or LY294002 (0.5 h) and then stimulated with 50 ng/m 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%) while vortexing and incubated on ice for 30 minutes. 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 anti-rabbit and phycoerythrin (PE) labeled anti-CD138. Flow cytometry was performed 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, Bar Harbor, ME) were irradiated with 250 rads γray in Gammacell 40 exactor (MDS Nordion International Inc., Kanata, Ontario, Canada), source Cs-137 (cesium), one day before tumor cell administration. The mice then were inoculated subcutaneously into the right flank with 2.5x10^7 MM1.S cells and 200 μL of a matrigel basement membrane matrix (Becton Dickinson, Bedford, MA). When tumors were palpable, the animals were randomized to three groups: group 1 (n=7) were given vehicle, group 2 (n=8) were given GTx-134 daily for four weeks, and group 3 (n=5) were observed and treatment initiated with GTx-134 once tumor volumes reached approximately 500 mm^3. The vehicle or drug (20 mg/kg, twice daily for a total of 40 mg/kg/day) was administered by i.p. Injection. Caliper measurements were performed twice weekly to estimate tumor volume, using the formula 1/2(length x width^2). All mice were scarified on day 36-post tumor inoculation. One-way analysis of variance was used to compare differences between vehicle and drug treated groups.

**Glucose Tolerance Test**

Glucose tolerance tests were performed two days before the mice were scarified in five mice from each of group 1 and 2. Briefly, mice that were fasted for 8 h were administered 20% glucose (*D*-dextrose in water) at 2 g/kg by i.p. injection 4 h after the morning dose of GTx-134 was given on day 24 of treatment. Blood glucose levels were measured at -240, -120, 0, 15, 30 60, 90 and 120 minutes after glucose challenge using blood glucose meter (Accu-Chek, Mannheim, Germany).
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, Downers Grove, IL) 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 demonstrated in biochemical assays to inhibit class II receptor tyrosine kinases (RTKs) including IGF-1R and insulin receptor (IR) with IC$_{50}$’s of 97 nM and 187 nM, 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 (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 pre-treated with vehicle control, 1 or 5 µM 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 (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 & D). By comparing the drug concentrations required to achieve a 50% antiproliferative effect (IC$_{50}$; Fig. 1B), 11 of 14 HMCLs tested showed IC$_{50}$’s of less than 5µM. KMS18, SKMM2, U266 and MY5 cells were most resistant (IC$_{50}$ > 5 µM), whereas MM1.S, UTMC2 and H929 were most sensitive to the cytotoxic effects of GTx-134 (IC$_{50}$ < 1.5 µM). Although we found ubiquitous expression of IGF-1R across the 14 cell lines, there was no correlation between basal activation of IGF-1R and its downstream target, Akt and susceptibility of HMCLs to GTx-134 (Fig. 1E).

*GTx-134-induced apoptosis is accompanied by inhibition of IGF-IR and Akt phosphorylation*
We next determined whether GTx-134 induces apoptosis of sensitive HMCLs such as MM1.S, H929 and UTMC2 and included SKMM2 and MY5 as resistant controls. These HMCLs were treated with 0.6 or 1.8 µM 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 three sensitive lines in response to 1.8 µM 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 pre-incubated with 1, 5, or 12.5 µM GTx-134 for 4 h and then stimulated with IGF-1 prior to harvesting for immunoblotting (Fig. 2B). All HMCLs demonstrated an increase in phosphorylation of IGF-1R and Akt upon IGF-1 stimulation. Pre-treatment 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 µM 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 maybe a consequence of efflux pump-mediated resistance. By gene expression profiling SKMM2 cells express high levels of the multidrug resistant gene, *MDR1*. To test our hypothesis, SKMM2 were treated with GTx-134 together with cyclosporin A (CsA) or verapamil, broad-spectrum inhibitors of multi-drug transporters (32). Addition of CsA or verapamil reduced the IC50 of SKMM2 cells from 30 µM to less then 2.5 µM (Fig. S2), suggesting that GTx-134 is a P-glycoprotein (Pgp) substrate.

On the other hand, My5 cells do not express *MDR1* and were not sensitized by co-culture with CsA or verapamil implicating other mechanisms of resistance in these cells. It has been 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 (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 demonstrated similar dose-response curves suggesting that response to IGF-1R inhibitors is independent of CD45 status in these cells.

**Co-culture 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 presence or absence of IGF-1 (Fig. 3B). By MTT assay, when stimulated with IGF-1, HMCLs are provided modest protection against the anti-proliferative activity of GTx-134 with a shift in the IC$_{50}$ values from 1.0 to 2.7 μM and 0.6 to 1.3 μM 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 co-culture 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 anti-myeloma 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 anti-myeloma agent, primary cells derived from patient BMs were treated with vehicle control, 0.6, or 1.8 µM GTx-134 for 72 h. 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 demonstrated in 3 of 12 patient samples treated with 0.6 µM GTx-134 and in 9 of 12 patient samples treated with 1.8 µM. 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) (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 h with vehicle, 1 or 5 µM 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 demonstrating 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 2.5×10⁷ MM1.S cells and a matrigel basement membrane matrix. After approximately 1 week, when tumors were palpable, the animals were divided into three groups: vehicle controls, a group with a low tumor burden to receive GTx-134 immediately, and a group to receive GTx-134 two 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 to vehicle controls, a significant (p<0.05) anti-tumor 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 1h 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 progression, proliferation, metastasis, protection from apoptosis, and transformation (5, 7). As a result, several strategies to inhibit it with antibodies, antisense, small interfering RNAs, 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 anti-tumor 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), two 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 receptors, and inhibition of signaling through the PI3k/Akt pathway. Despite the ubiquitous expression of IGF-1R we observed a heterogeneous responses with some HMCLs being very sensitive (IC₅₀ values in the low micromolar range) and others being more resistant (IC₅₀ values higher than 5 µM). 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 one 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 inhibitor of multi-drug transporters sensitized the MDRI expressing cell line to GTx-134. We also confirmed the report by Descamps et al. 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+ HMCLs 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 demonstrating 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 curve to the right. Although there are numerous mechanisms by which direct adhesion to BMSCs may induce resistance, co-culture 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-1R signaling in the presence of excess ligand, necessitating higher doses of GTx-134 to achieve target inhibition. Further, comparison between GTx-134 and other published IGF1-R inhibitor studies in BM microenvironment models reveals qualitative differences. For example, IGF-1 and/or BM stroma co-culture do not confer protection against the anti-myeloma 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 to 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 anti-tumor activity demonstrated 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 et al., identified IGF-1R expression as a poor prognostic marker in two 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 two putative oncogenes, MMSET and FGFR3 (48). Using an unbiased mass spectrometry-based phospho-proteomics approach, we previously found evidence for crosstalk between IGF-1R/IR 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 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 performed. 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 ip glucose load were increased consistent with these clinical findings, it was surprising to observe that in the basal state (fasting, post-absorptive) 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 anti-myeloma 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.
References

Figure Legends

Figure 1. GTx-134 inhibits IGF-1R signaling and viability of HMCLs. (A) MM1.S cells were serum starved overnight and cultured with the indicated concentrations of GTx-134 for 2 h. Cells were then stimulated with 50 ng/ml IGF-1 for 12 min and harvested for Western blot analysis. Blots were probed for IGF-1R, Akt, and phosphorylated IGF-1R/IR and Akt. GAPDH was probed as a loading control. (B-C) A panel of 14 HMCLs were treated with indicated concentrations of GTx-134 for 72 h and then subjected to MTT assay to determine cell viability. (B) Dose response curves were generated and (C) IC50 values were calculated. Values are the mean ± SD percentages from triplicate experiments. (D) Cell extracts from the 14 HMCLs were prepared and basal levels of IGF-1R, phosphorylated IGF-1R/IR, Akt, and phosphorylated Akt were assessed by immunoblotting. GAPDH was probed as a loading control.

Figure 2. Differential sensitivity to GTx-134 is associated with inhibition of signaling downstream of IGF-1R. (A) HMCLs that are most sensitive (H929, MM1.S, and UTMC2) or relatively resistant (SKMM2 and MY5) to GTx-134 were treated with vehicle control ( ), 0.6 ( ) or 1.8 µM ( ) of drug for 72 hours prior to being assayed for annexin V staining by flow cytometry, as a measure of apoptosis. Values are the mean ± SD percentages from four experiments and (*) indicates p<0.05. (B) These HMCLs were cultured with vehicle control, 1, 5, or 12.5 µM GTx-134 for 2 h or 10 µM LY294002 for 0.5 h and then stimulated with 50 ng/ml IGF-1 for 12 min prior to being harvested for Western blot analysis. Blots were probed for IGF-1R, phosphorylated IGF-1R/IR, Akt, and phosphorylated Akt and were compared to GAPDH as a loading control.

Figure 3. Co-culture with IGF-1 and BM stroma provides protection against GTx-134. MM1.S and H929 cells were treated with increasing concentrations of GTx-134 in either the presence ( ) or absence ( ) of (A) IL-6 (B) IGF-1 or (C) BMSCs for 72 h. The MTT assay was used as an indicator of cell viability. Values are the mean ± SD percentages from triplicate experiments.

Figure 4. GTx-134 enhances the activity of common anti-myeloma and targeted agents. MM1.S cells were incubated with increasing concentrations of GTx-134 (GTx) and/or either (A) melphalan (MEL), (B) bortezomib (BOR), (C) dexamethasone (Dex), (D) lenalidomide (Len) or (E) PD173074 (PD) for 48 h. Cytotoxicity was assessed by MTT activity and synergy determined by
calculating combination indices (* indicates CI=1; ** indicates CI<1.0). Values are the mean ± SD percentages from triplicate experiments.

**Figure 5. GTx-134 inhibits IGF-1R signaling and induces apoptosis of primary MM cells.** Primary cells derived from patient BM were treated with vehicle control ( □ ), 0.6 ( □ ), or 1.8 µM ( □ ) GTx-134 for 72 h. (A) Cells derived from 14 patient BM aspirates were stained with anti-CD138-PE and annexin V and examined by flow cytometry; the proportion of the CD138 positive, annexin V negative (viable MM cell) population is charted. (B) Representative dot plots are shown, demonstrating a dose-dependent decrease in the viable MM cell population. (C) and dose-dependent inhibition of IGF-1 induced Akt phosphorylation. Phosphorylated-Akt in response to IGF-1 in CD138 gated cells was measured by flow cytometry and compared to unstimulated control (solid). BM derived mononuclear cells were serum starved for 6 h, and treated with either vehicle control (green), 1 µM (red) or 5 µM (blue) GTx-134, or 10 µM (black) LY294002 for 30 minutes and then stimulated with 50 ng/ml IGF-1 for 12 minutes prior to being stained with a FITC-conjugated phosphorylated-Akt antibody and PE-conjugated CD138.

**Figure 6. GTx-134 inhibits of growth 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 three 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 two 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.


**Figure 1**

A. A molecule with a structural formula.

B. Western blot images showing the expression of various proteins:
- p-IGF-1R/IR
- IGF-1R
- p-Akt
- Akt
- GAPDH

C. Viability graph showing the effect of GTX-134 on different myeloma cell lines. The x-axis represents GTX-134 concentration (µM), and the y-axis represents viability as a percentage of untreated control.

D. IC50 values for GTX-134 in different myeloma cell lines. The graph shows the concentration (µM) on the x-axis and IC50 values on the y-axis.

E. Western blot images showing the expression of various proteins under different conditions.
- p-IGF-1R/IR
- IGF-1R
- p-Akt
- Akt
- GAPDH

Human Myeloma Cell Lines:
- MM1.S
- MM1.R
- U266
- SKM12PF
- RPMI8226
- UTMC2
- OPM2
- KMS11
- H929
- My5
- MM12PE
- MY7
- SKM12PE
- MY7
Figure 2

A

B

Human Myeloma Cell Lines

<table>
<thead>
<tr>
<th></th>
<th>MM1.S</th>
<th>UTMC2</th>
<th>H929</th>
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GTx-134
LY294002
IGF-1

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3

**A**

- **MM1.S**
  - MM1.S
  - MM1.S_IL-6

- **H929**
  - H929
  - H929_IL-6

**B**

- **MM1.S**
  - MM1.S
  - MM1.S_IGF-I

- **H929**
  - H929
  - H929_IGF-I

**C**

- **MM1.S**
  - MM1.S
  - Stroma_MM1.S
  - Stroma

- **H929**
  - H929
  - Stroma_H929
  - Stroma

GTx-134 (μM)
Figure 4

Graph A: Viability (Percent of Untreated) vs. GTx (μM) and Mel (μM)

Graph B: Viability (Percent of Untreated) vs. GTx (μM) and BOR (nM)

Graph C: Viability (Percent of Untreated) vs. GTx (μM) and Dex (nM)

Graph D: Viability (Percent of Untreated) vs. GTx (μM) and Len (μg/ml)

Graph E: Viability (Percent of Untreated) vs. GTx (μM) and PD (uM)
Figure 5

A

![Graph showing the percentage of viable myeloma cells among different patients treated with DMSO, 0.6 uM, and 1.8 uM.]

B

![Flow cytometry images for CD138-PE and Annexin V-FITC with percentage values for each treatment group.]

C

![Histogram showing the distribution of p-AKT-FITC levels.]

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Figure 6

A

![Graph A]

B

![Graph B]
Molecular Target Characterization and Anti-myeloma Activity of the Novel, Insulin-like Growth Factor 1 Receptor Inhibitor, GTx-134

Sheng-ben Liang, Xiu-Zhi Yang, Young Trieu, et al.

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