Title: Efficacy of Ganitumab (AMG 479), Alone and in Combination With Rapamycin, in Ewing's and Osteogenic Sarcoma Models

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Abstract

Ewing’s and osteogenic sarcoma are two of the leading causes of cancer deaths in children and adolescents. Recent data suggest that sarcomas may depend on the type I insulin-like growth factor receptor (IGF1R) and/or the insulin receptor (INSR) to drive tumor growth, survival, as well as resistance to mammalian target of rapamycin complex 1 (mTORC1) inhibitors. We evaluated the therapeutic value of ganitumab (AMG 479), an anti-IGF1R, fully human monoclonal antibody, alone and in combination with rapamycin (mTORC1 inhibitor) in Ewing’s (SK-ES-1 and A673) and osteogenic (SJSA-1) sarcoma models. IGF1R was activated by IGF-1 but not by insulin in each sarcoma model. INSR was also activated by IGF-1 in the SJSA-1 and SK-ES-1 models, but not in the A673 model where insulin was the preferred INSR ligand. Ganitumab significantly inhibited the growth of SJSA-1 and SK-ES-1 xenografts; inhibition was associated with decreased IGF1R and Akt phosphorylation, reduced total IGF1R and BrdU detection, and increased caspase-3 expression. Ganitumab inhibited rapamycin-induced IGF1R, Akt, and GSK3β hyperphosphorylation in each sarcoma model. However, ganitumab in combination with rapamycin also resulted in a marked increase in INSR expression and activity in the SJSA-1 and A673 models. The in vivo efficacy of ganitumab in the two ganitumab-sensitive models (SJSA-1 and SK-ES-1) was significantly enhanced in combination with rapamycin. Our results support studying ganitumab in combination with mTORC1 inhibitors for the treatment of sarcomas and suggest that INSR signaling is an important mechanism of resistance to IGF1R blockade.
Introduction

Sarcomas are among the most common cancers of childhood and early adolescence (Ries et al., 2008). Osteogenic and Ewing’s sarcoma most frequently occur in adolescents during rapid bone growth and present clinical challenges due to their aggressiveness and metastatic tendency. As a result, treatment is aggressive, typically involving surgical resection preceded and followed by chemotherapy. Despite refinements in surgical techniques (Longhi et al., 2006; Iwamoto, 2007), overall treatment strategies for sarcomas have not substantially improved patient survival over the last decade (Hughes, 2009). An autocrine loop involving the type I insulin-like growth factor receptor (IGF1R) and both of its ligands, IGF-1 and IGF-2, has long been thought to be a key mechanism driving the proliferation and survival of sarcoma cells (Kim et al., 2009). Evidence supporting the importance of this axis includes: a) the association between the IGF-1-driven adolescent growth spurt and peak incidence of osteogenic sarcoma, b) the direct control of IGF1R pathway-related proteins by the EWS/FLI-1 fusion protein (oncogenic transcription factor that controls expression of IGF1R, IGF-1, and IGF binding proteins [BPs] (Arvand and Denny, 2001; Prieur et al., 2004; Mateo-Lozano et al., 2006)) in Ewing’s sarcomas, and c) overexpression of IGF-2 in rhabdomyosarcomas (Rikhof et al., 2009). IGF-1 activity is predominantly mediated through IGF1R (homodimers) and IGF1R / insulin receptor (INSR) hybrid receptors (heterodimers). However, IGF-2 action can also be mediated through the activity of type A INSR (INSR-A) (Belfiore et al., 2009). Expression of INSR-A on sarcoma tumor cells has been shown to be complementary to IGF1R activity and ensures successful activation of the phosphatidylinositol-3-kinase (PI3K) signaling pathway even in the presence of IGF1R inhibition (Avnet et al., 2009).

The mammalian target of rapamycin (mTOR) is a protein kinase that plays a pivotal role in the growth and proliferation of cells in response to changes in their environmental and...
nutritional status (Bjornsti and Houghton, 2004). mTOR is activated by a variety of growth factors and is up-regulated in many human cancers (Wan and Helman, 2007). Treatment of tumor cells with mTOR Complex 1 (mTORC1) inhibitors, such as rapamycin and everolimus, inhibits phosphorylation of p70S6K and 4E-BP1 resulting in inhibition of protein synthesis. mTORC1 inhibitors have been shown to inhibit the proliferation of sarcoma cells, the expression of EWS/FLI-1 fusion proteins, and the growth of sarcoma xenografts, supporting the investigation of rapamycin and its analogs as therapeutics for the treatment of sarcomas (Mateo-Lozano et al., 2003; Wan and Helman, 2007; Wang et al., 2008a).

The limited efficacy of approved mTORC1 inhibitors in cancer patients has motivated researchers to evaluate potential mechanisms of resistance (O’Reilly et al., 2006; Breuleux et al., 2009; Meric-Bernstam and Gonzalez-Angulo, 2009). Inhibition of mTORC1 with either rapamycin or rapamycin analogs has been shown to induce phosphorylation of Akt S473, highlighting a possible mechanism of resistance to mTORC1 inhibitors (O’Reilly et al., 2006). Interestingly, rapamycin-induced Akt activation seemed to be dependent on IGF1R / insulin receptor substrate-1 (IRS-1) / PI3K activity (Sun et al., 2005; Wan et al., 2007; Wang et al., 2008a), suggesting a potential benefit of combining rapamycin with IGF1R pathway inhibitors (Kurmasheva et al., 2006; Wang et al., 2008a). Fully human monoclonal antibodies that disrupt IGF1R signaling without cross reacting with the INSR are currently in clinical development (Manara et al., 2007; Pollak, 2008; Rodon et al., 2008). Combination of one of these monoclonal antibodies with rapamycin has been shown to inhibit rapamycin-induced Akt reactivation (O’Reilly et al., 2006; Cao et al., 2008). Early phase clinical trials of anti-IGF1R monoclonal antibodies in combination with mTORC1 inhibitors are currently testing this hypothesis in cancer patients.
Ganitumab is an investigational, fully human monoclonal antibody (IgG1) against the human IGF1R and is currently in phase 2 clinical development for multiple oncology indications, including sarcomas. Previously, we showed that ganitumab blocked the activation of IGF1R and hybrid receptors by both IGF-1 and IGF-2 and enhanced the effects of gemcitabine against human pancreatic carcinoma xenograft models (Beltran et al., 2009). The objective of the present study was to evaluate the efficacy of ganitumab against sarcoma xenograft models displaying distinct responses to IGF-1 and insulin and to understand the molecular mechanisms involved in driving the sensitivity and resistance to the combination of ganitumab plus rapamycin in these models.
Materials and Methods

Animals

Female athymic nude mice, 4 to 6 weeks old (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used in all experiments. The laboratory housing the animals met all Association for Assessment and Accreditation of Laboratory Animal Care specifications. Experimental procedures were performed in accordance with Institutional Animal Care and Use Committee and United States Department of Agriculture regulations.

Reagents and Cell Lines

Human sarcoma cell lines, SJSA-1 (osteogenic sarcoma), SK-ES-1 (Ewing's sarcoma), and A673 (Ewing's sarcoma), were purchased from the American Type Culture Collection in 2006 (Manassas, VA). Each cell line was authenticated through identification of EWS/FLI-1 type I and type II translocations and mutational status of b-raf. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovin serum (FBS) (SJSA-1 and A673), or McCoy's 5A with 15% FBS (SK-ES-1). Human anti-streptavidin IgG1 (hIgG1) was used as control antibody. Rapamycin was obtained from LC Laboratories (Woburn, MA). IGF-1 and insulin were obtained from Sigma-Aldrich (St. Louis, MO).

Determination of Cell-Surface Levels of IGF1R and INSR

SJSA-1, SK-ES-1, and A673 cells were harvested and incubated with 1µg phycoerythrin-conjugated antibodies specific for human IGF1R or INSR (BD Pharmingen, San Jose, CA) for 1.5h at 4°C. Receptor levels were determined using a quantitative flow cytometry assay relating the geometric mean cell fluorescence intensity to that in Quantum microbead standards (Bangs...
Laboratories, Fishers, IA). Fluorescence levels were determined in duplicate, and at least 2
determinations were made for each cell line.

**In Vitro Cell Growth Assays**

SJSA-1 cells were seeded in 96-well plates (2 x 10^3 per well) in DMEM with 10% FBS, 2mM
glutamine, and either 500nM ganitumab or hlgG1 plus 0 to 77nM rapamycin (3-fold serial
dilutions). A673 cells were seeded (1.7 x 10^4 per well) in DMEM with 10% FBS, 2mM
glutamine, and either 250nM ganitumab or hlgG1 plus 0 to 54nM rapamycin (2.5-fold serial
dilutions). SK-ES-1 cells were seeded (1 x 10^4 per well) in McCoy’s 5A medium with 15% FBS,
2mM glutamine, and either 500nM ganitumab or hlgG1 plus 0 to 54nM rapamycin (3-fold serial
dilutions). Confluence measurements were performed in duplicate for each well at 4-h intervals
over a period of 5 to 7 days using an Incucyte™ phase contrast optical imaging system (Essen
Instruments, Ann Arbor, MI). Prism (GraphPad Software, La Jolla, CA) was used to analyze
dose-response data.

**Analysis of IGF1R and INSR Signaling**

To determine IGF-1 and insulin EC50 and ganitumab half maximal inhibitory concentration (IC50),
sarcoma cells were plated into 96-well plates (3 to 4 x 10^4 per well) in FBS-containing growth
medium as described above. After an overnight incubation, the cells were washed once in
phosphate-buffered saline (PBS) and serum-deprived for 18 to 24h in DMEM or McCoy’s 5A.
Dilutions of either hlgG1 or ganitumab with fixed concentrations of either IGF-1 or insulin were
added to the cells in an equal volume of fresh serum-free media, and the cells were harvested
after 20 minutes.

The short-term effects of ganitumab plus rapamycin on the phosphorylation of IGF1R,
p70S6K, and Akt were determined in serum-starved sarcoma cells (6-well format) treated for 1h
with hIgG1 (0.5µM), ganitumab (0.5µM), rapamycin (27nM), or ganitumab and rapamycin, all in the presence of 10nM IGF-1.

The long-term effects of ganitumab plus rapamycin on cell proliferation and IGF1R pathway markers were determined in vitro using an Incucyte™ optical imaging system as described above. Cells were harvested for analysis after 52h (SJSA-1), 96h (SK-ES-1), or 76h (A673) of growth for analysis of IGF1R pathway markers.

In each of the experiments described above, levels of total (t) and/or phosphorylated (p) INSR, IGF1R, IRS-1, Akt, p70S6K, and GSK3β were measured using Meso Scale Discovery™ (MSD) multiplex assays (Meso Scale Discovery, Gaithersburg, MA).

**In Vivo Pharmacodynamic Studies**

Tumor cells (5 x 10^6 cells) were injected subcutaneously into the flank of female athymic nude mice in a 2:1 ratio of cells to Matrigel (BD Bioscience, San Jose, CA). When the average tumor size reached approximately 300 to 450mm³, mice were randomly assigned into four groups (n=3). Mice received either a single intraperitoneal dose of ganitumab (1mg) or hlgG1 (1mg) (total of 4 groups: 2 groups received ganitumab and 2 received hlgG1). After 6h, an ganitumab group and a hlgG1 group received human IGF-1 (15µg) by tail-vein injection while the other ganitumab and hlgG1 groups received PBS injections. Xenografts were collected 15 minutes after IGF-1 or PBS challenge and snap-frozen in liquid nitrogen. Samples were homogenized with a Polytron in 3 volumes of Tris-buffered saline (TBS) (20mM Tris-HCl pH 8.5, 0.15M NaCl) and diluted with 3 volumes of TBST (TBS, 2% Triton X-100). Cell lysates were cleared by centrifugation at 14,000rpm and protein levels (t and p) were analyzed using the MSD™ multiplex assays.
In a separate experiment, 12 mice bearing 300 to 450 mm\(^3\) tumors were assigned to four groups (n=3) and received single intraperitoneal doses of hlgG1 (300 \(\mu\)g), ganitumab (300 \(\mu\)g), rapamycin (0.5 mg/kg), or the combination of two of these agents. Six hours later, xenografts were excised, snap frozen in liquid nitrogen, and processed for MSD™ assays as described above.

**In Vivo Antitumor Efficacy Studies**

Female athymic nude mice were injected with 5 \(\times\) 10\(^6\) cells in a suspension containing a 2:1 ratio of cells to Matrigel (BD Bioscience, San Jose, CA). After 7 to 15 days, mice bearing tumors (approximately 200 mm\(^3\)) were randomly assigned into groups (n=10 for each) and administered ganitumab (30, 100, or 300 \(\mu\)g per dose) or hlgG1 (300 \(\mu\)g) as single agents or in combination with rapamycin (0.5 mg/kg) by intraperitoneal injection twice weekly for the duration of the experiment. Tumor volumes were measured twice per week with calipers. Tumor growth inhibition for each treatment group was calculated on the last day of each study and was expressed relative to the initial and final mean tumor volume of the control group. Body weights were monitored to assess tolerability of the treatments.

**Detection of BrdU and Caspase-3 in Ganitumab-Treated Xenografts and Cultured Cells**

Mice bearing SJSA-1 or A673 xenografts (approximately 300 mm\(^3\)) were administered ganitumab (1 mg) or hlgG1 (1 mg) by intraperitoneal injection. Xenografts were harvested after 3 or 24 h, fixed, and stained for cleaved caspase-3 or BrdU as described previously (Polverino et al., 2006). Briefly, cleaved caspase-3 was detected using a rabbit anti-caspase-3 antibody (Cell Signaling, Danvers, MA) followed by a peroxidase-labeled goat anti-rabbit IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) and diaminobenzidine. Incorporation of BrdU was detected with a rat anti-BrdU antibody (Accurate, Westbury, NY), a biotin-labeled
rabbit anti-rat IgG secondary antibody, and a Vectastain Elite ABC detection kit (Vector Laboratories, Burlingame, CA).

Cell lines were incubated in serum-free media in low-adherence culture plates with or without IGF-1 (200nM) and ganitumab (0.5μM) for 24h. Cells were then pulsed with BrdU labeling reagent (Invitrogen, Carlsbad, CA) and fixed in ice-cold, 90% methanol. For flow cytometry, cells were stained with anti-BrdU-Alexa647 antibody (0.2µg/µL, Invitrogen, Carlsbad, CA) and fluorescein isothiocyanate-labeled anti-caspase-3 antibody (1:5 dilution, Invitrogen, Carlsbad, CA), followed by treatment with PI/RNAse staining solution (BD Pharmingen, San Jose, CA).

**Statistical Analysis**

For *in vivo* efficacy studies, repeated-measures analysis of variance was used to compare the reduction in tumor volume in mice treated with the combination of ganitumab plus rapamycin versus those treated with either agent alone. The *in vivo* ganitumab dose-response experiments were analyzed with repeated-measures analysis of variance followed by a post hoc Scheffé test. Changes in phosphorylation markers in the *in vivo* or *in vitro* acute assays were compared using one-way analysis of variance.
Results

IGF1R and INSR Levels in Sarcoma Cells

The levels of IGF1R and INSR in each sarcoma cell line were determined by quantitative flow-cytometry. The levels of IGF1R were: SJSA-1, 9,000/cell; A673, 14,000/cell; and SK-ES-1, 18,000/cell. The levels of INSR were: SJSA-1, 350/cell; A673, 2,100/cell; and SK-ES-1, 13,000/cell. The relatively low expression of INSR in SJSA-1 cells compared with A673 and SK-ES-1 cells allowed us to distinguish the effect of ganitumab on signaling through IGF1R homodimers, INSR homodimers, and IGF1R/INSR heterodimers.

Sensitivity of Serum-Starved Sarcoma Cells to IGF-1 and Insulin

The sensitivity of sarcoma cells to IGF-1 and insulin stimulation was determined by measuring IGF1R, INSR, Akt, IRS-1, GSK3β, and p70S6K activation with MSD™ multiplex assays (Table 1). The IGF1R response to IGF-1 was similar for the three cell lines (EC$_{50}$ 0.45nM to 1.1nM). However, each cell line displayed a distinct INSR response. IGF-1 was the preferred INSR agonist in SJSA-1 cells, which have a low INSR/IGF1R ratio (0.04). SK-ES-1 cells, which express higher INSR/IGF1R ratios (0.72), exhibited similar INSR sensitivity to insulin and IGF-1 (EC$_{50}$ 3.0nM and 7.7nM), whereas in A673 cells, INSR was more responsive to insulin than to IGF-1 (EC$_{50}$, 2.2nM and 32nM).

Inhibition of IGF1R, INSR, and Akt Activity by Ganitumab

Ganitumab inhibited IGF-1-induced phosphorylation of IGF1R (IC$_{50}$=2.0nM in SJSA-1 cells; 1.6nM in A673 cells; and 3.7nM in SK-ES-1 cells) and INSR (albeit at higher concentrations, IC$_{50}$>50nM) in all three cell lines (Fig. 1A,B). In SJSA-1 cells, the inhibitory effect of ganitumab on IGF-1-induced Akt phosphorylation was similar to its effect on IGF1R.
phosphorylation (IGF1R IC$_{50}$, 2.0nM; Akt IC$_{50}$, 4.7nM) but was about 30-fold less than for IGF1R phosphorylation in A673 cells (IGF1R IC$_{50}$, 1.6nM; Akt IC$_{50}$, 50nM) and SK-ES-1 cells (IGF1R IC$_{50}$, 3.7nM; Akt IC$_{50}$, 102nM) (Fig. 1C).

**Inhibition of IGF-1 Signaling and Tumor Growth by Ganitumab**

The *in vivo* effects of ganitumab on IGF-1 signaling were first studied in sarcoma xenografts using a pharmacodynamic assay. Fifteen minutes after IGF-1 treatment, phosphorylation of IGF1R, INSR, and Akt was increased 4- to 17-fold relative to mice that did not receive IGF-1 (Fig. 2A). Net levels of pIGF1R, pINSR, and pAkt were plotted to allow comparison of absolute levels of pINSR between cell lines (Fig. 2A). No changes in the effect of ganitumab were observed when levels of phosphorylated protein were normalized to levels of total protein (Supp. Fig. 1). Pretreatment with intraperitoneal ganitumab (1mg) for 6h led to a significant reduction (73% to 97%) in IGF-1-induced IGF1R phosphorylation in each model relative to mice pretreated with hlgG1. Basal pIGF1R was significantly inhibited in the SJSA-1 (83%) and SK-ES-1 (64%) models. Ganitumab treatment also significantly inhibited IGF-1-stimulated pAkt (SJSA-1, 41%; SK-ES-1, 36%; A673, 53%) and basal pAkt (SJSA-1, 80%; SK-ES-1, 32%). As expected, IGF-1-induced INSR phosphorylation was not inhibited by ganitumab in either of the two INSR expressing cell lines (SK-ES-1 and A673). Ganitumab did inhibit IGF-1-induced INSR activation in the SJSA-1 cell line as well as basal INSR in the SK-ES-1 line (Fig. 2A).

Treatment of mice bearing SJSA-1 xenografts with ganitumab twice per week resulted in significant tumor-growth inhibition of 75% at 30µg, 74% at 100µg, and 84% at 300µg (Fig. 2B). SK-ES-1 xenografts were slightly less sensitive to inhibition by ganitumab with significant tumor-growth inhibition of 47% at 30µg, 61% at 100µg, and 74% at 300µg. Reduced sensitivity to
ganitumab was observed in the insulin-responsive A673 tumor model: 300µg ganitumab resulted in 35% tumor-growth inhibition that did not reach statistical significance (p=0.053) (Fig. 2B). Ganitumab treatment did not result in loss of body weight (data not shown).

Tumors harvested from the animals on the last day of the aforementioned study were assessed for t and pIGF1R (Fig. 2C). Ganitumab significantly reduced the level of tIGF1R in each model, with the largest reduction (approximately 50%) observed in SJSA-1 tumors. Ganitumab inhibited IGF1R phosphorylation in SJSA-1 and SK-ES-1 tumors in a dose-dependent manner, but only modest, non-significant inhibition was observed in A673 tumors.

Effect of Ganitumab on Caspase-3 Activation and BrdU Incorporation

To assess the biological consequences of IGF1R and Akt inhibition by ganitumab, cleavage of caspase-3 and incorporation of BrdU were examined in SJSA-1 xenografts (most sensitive to inhibition by ganitumab) and A673 xenografts (most resistant to inhibition by ganitumab). Ganitumab treatment (1mg) of SJSA-1 xenografts led to increased expression of cleaved caspase-3 after 3h and reduced BrdU incorporation after 24h (Fig. 3A). In A673 xenografts, ganitumab did not alter the expression of cleaved caspase-3 but reduced BrdU-labeling after 24h (Fig. 3B). Flow cytometry analysis showed that SJSA-1 cells incubated in serum-free media with IGF-1 had lower levels of cleaved caspase-3 and higher incorporation of BrdU than cells maintained in serum-free medium alone; the effects of IGF-1 were either partially or fully reversed by simultaneous exposure of the cells to ganitumab (data not shown).

Ganitumab and Rapamycin Inhibition of Cell Growth In Vitro

Growth curves (% cell confluency) were generated to evaluate the concentration-dependent effect of rapamycin in combination with excess ganitumab or hlgG1. Ganitumab treatment alone consistently increased the cell doubling time (SJSA-1, 1.4 fold; SKES-1, A673...
1.1 fold data not shown). The time at which each cell line treated with hlgG1 reached about 85% confluency was selected to illustrate the effect of ganitumab and rapamycin on cell growth (Fig. 4). In the presence of hlgG1, the rapamycin IC$_{50}$ (95% confidence intervals) estimates were 1.5 nM (0.2-13.7) for SJSA-1, 1.3 nM (0.8-2.1) for SK-ES-1, and 5.0 nM (2.7-9.2) for A673. In the presence of ganitumab, the rapamycin IC$_{50}$ estimates were 0.14 nM (0.01-2.8) for SJSA-1, 0.6 nM (0.2-1.4) for SK-ES-1, and 1.7 nM (0.8-3.5) for A673. Thus, ganitumab treatment of SK-ES-1 and A673 cells reduced the rapamycin IC$_{50}$ by 2-3 fold and appeared to potentiate rapamycin inhibition of SJSA-1 cells. The scatter in SJSA-1 data reflects the tendency of this cell line to migrate and undergo changes in cell shape (Fig. 4).

**Effects of Short-Term Ganitumab Treatment on Rapamycin-Induced Activation of IGF1R, Akt, and p70S6K**

The short-term treatment of established xenografts and of IGF-1-stimulated, serum-starved cells was performed to further differentiate the immediate effects of ganitumab, rapamycin, and ganitumab plus rapamycin on IGF1R signaling from the adaptive pathway responses that occur after long-term treatment. We consider this is an important distinction as interpretation of long-term treatment experiments can be complicated by changes in cell proliferation, cell metabolism, and cell death. Ganitumab significantly inhibited *in vitro* phosphorylation of IGF1R and Akt in all three cell lines. Inhibition of pIGF1R was greater than 80% in all three cell lines. Inhibition of pAkt was highest in the SJSA-1 and A673 cell lines (>80%) while suboptimal inhibition (<50%) was observed in the SK-ES-1 cell line (Fig 5A). Ganitumab also inhibited phosphorylation of p70S6K (~50%) in all three sarcoma cell lines *in vitro* (Fig. 5A). Rapamycin significantly inhibited pp70S6K (>80%) *in vitro* but induced pAkt (~2-fold) (Fig. 5A). In the presence of ganitumab, rapamycin-induced Akt activation was significantly and substantially inhibited to levels observed in untreated samples (Fig. 5A). Strong inhibition of
pIGF1R by ganitumab was observed in all three sarcoma tumor xenografts (Fig. 5B). This in vivo receptor blockade was associated with inhibition of pAkt with the most potent activity seen in low INSR expressing SJSA-1 tumors. In contrast to our in vitro observations, ganitumab did not consistently inhibit pp70S6K in tumors. Consistent with our in vitro observations, rapamycin significantly induced pAkt (~2-fold) in the SJSA-1 model and ganitumab completely blocked this effect. Similar effects were observed in the SK-ES-1 and A673 xenografts but significance was not achieved. Rapamycin also significantly inhibited pp70S6K (>50%) in the SJSA-1 and SK-ES-1 models (Fig. 5B).

**Effects of Long-Term Ganitumab Treatment on Rapamycin-Induced Activation of IGF1R Pathway Proteins**

The effect of prolonged drug exposure on IGF1R pathway signaling was determined in an experiment performed in parallel with the growth analysis shown in Figure 4. The overall pattern of marker phosphorylation observed with ganitumab treatment alone (Supp. Fig. 2) suggests that IGF-1 and IGF-2 are responsible for the majority of the PI3K/Akt pathway activity stimulated by FBS (pAkt inhibition of 55% to 85%). In SJSA-1 and A673 cells, rapamycin reduced the amount of tp70S6K and p:t p70S6K per cell in a concentration-dependent manner (Fig. 6A,B). In the presence of ganitumab, higher concentrations of rapamycin were required to reduce the levels of tp70S6K, and a slight enhancement in the reduction of pp70S6K was also observed (Fig. 6A,B).

The p:t ratios of Akt and GSK3β (corrected for differences in cell confluency) were increased in rapamycin-treated cells, and this effect was inhibited by ganitumab (Fig. 6C,D). A separate plot of phosphorylated and total signals (corrected for cell confluency) suggested that the feedback response was predominantly the result of increased pAkt and decreased tGSK3β
(data not shown). To illustrate feedback at the receptor level, we plotted pIGF1R and pINSR corrected only for differences in cell confluency since rapamycin exhibited strong positive effects on total protein levels (Fig. 6E,F). Long-term rapamycin treatment increased both pIGF1R and pINSR levels in all three sarcoma cell lines. Ganitumab treatment inhibited the pIGF1R induction, but the pINSR increase was inhibited only in SK-ES-1 cells (Fig. 6F).

We determined if levels of tIGF1R, tINSR, tIRS-1, tAkt, and tGSK3β correlated to levels of tp70S6K after treatment with ganitumab and rapamycin (Table 2). The levels of tp70S6K and tGSK3β decreased in parallel (slope approximately equal to 1) in response to rapamycin treatment in the presence and absence of ganitumab. A similar correlation was obtained for tAkt although the slope was reduced by ganitumab treatment. There was a less consistent association between the other pathway proteins and tp70S6K. For example, in SJSA-1 cells, tIGF1R and tINSR remained relatively constant as tp70S6K levels decreased.

**Effect of Ganitumab on the Efficacy of Rapamycin Against SJSA-1 and SK-ES-1 Tumor Xenografts**

Twice-weekly treatment with intraperitoneal ganitumab or rapamycin as single agents led to 32% to 45% tumor-growth inhibition in mice bearing SJSA-1, SK-ES-1, or A673 xenografts (Fig. 7A). The combination of ganitumab and rapamycin led to additive tumor-growth inhibition (72%) in the SJSA-1 model, which was significantly better than either agent alone ($p<0.001$). The drug combination also led to increased tumor-growth inhibition in the SK-ES-1 model ($p=0.02$ versus ganitumab and $p=0.005$ versus rapamycin). No significant combination effect was observed in the A673 model. The combination of ganitumab and rapamycin did not result in significant body weight loss (data not shown).
Tumors were harvested from the animals on the last day of the study and were assayed for pIGF1R, pINSR, and pAkt (Fig. 7B). Consistent with findings from the in vivo monotherapy studies (Fig. 2C), xenografts exposed for approximately 3 weeks to a suboptimal (30µg/dose SJSA-1) or optimal (300µg/dose SK-ES-1 and A673) dose of ganitumab had significantly reduced levels of net pIGF1R (Fig. 7B). Normalized (p:t) analysis of IGF1R (Supp. Fig. 3) resulted in the appearance of less-than-optimal pIGF1R inhibition by ganitumab because of the receptor-downregulating effects of ganitumab following repeated dosing. Rapamycin seemed to induce pAkt in all xenografts, but statistical significance versus control was only achieved in the A673 tumors ($p=0.076$ for SK-ES-1, and $p=0.099$ for SJSA-1). In contrast to our previous observations (pharmacodynamic assay, Fig. 5A,B), ganitumab did not inhibit rapamycin-induced pAkt in these SJSA-1 and A673 efficacy experiments. This lack of activity correlated with enhanced activation of INSR in the same models ($p<0.05$ versus hlgG1). In contrast, the combination treatment resulted in reduced pINSR and pAkt in SK-ES-1 xenografts (Fig. 7B).
Discussion

Emerging phase 2 clinical data are providing increasing support for targeting IGF1R for the treatment of human cancers (Rodon et al., 2008). A number of anti-IGF1R antibodies have demonstrated antitumor activity in patients with Ewing's sarcoma (Pollak, 2008; Tolcher et al., 2009), and there is a need to understand the mechanisms underlying this activity (Scotlandi and Picci, 2008). We have shown that ganitumab inhibited IGF-1 and IGF-2 signaling through IGF1R homodimers and IGF1R/INSR hybrids (not INSR homodimers) in models of pancreatic cancer via ligand blockade and receptor downregulation (Beltran et al., 2009). Here we investigated the efficacy of ganitumab alone and in combination with rapamycin in three sarcoma models that exhibit distinct signaling responses to IGF-1 and insulin resulting in distinct dependency and cross-talk of IGF1R and INSR signaling.

Ganitumab significantly inhibited ligand-induced phosphorylation of IGF1R in each model. Inhibition of IGF1R phosphorylation was associated with significantly reduced tumor growth in SJSA-1 and SK-ES-1 xenografts; however, A673 xenografts were relatively resistant to ganitumab treatment. SJSA-1 xenografts, which expressed the lowest level of INSR, exhibited the greatest sensitivity to ganitumab. SK-ES-1 xenografts expressed INSR but demonstrated a preference for IGF-1 over insulin and remained sensitive to ganitumab albeit at a 10-fold higher dose. This decreased sensitivity may be driven by the requirement to achieve adequate coverage of IGF1R/INSR hybrids. These effects of ganitumab are consistent with the results reported previously for pancreatic cancer models (Beltran et al., 2009). However, in contrast to the pancreatic cancer models, in which ganitumab treatment resulted in either anti-proliferative or pro-apoptotic activities, ganitumab inhibition of SJSA-1 xenografts involved both potent anti-proliferative and pro-apoptotic activities. This dual mechanism of action may explain
the high sensitivity of SJSA-1 xenografts to ganitumab. These data suggest that low tINSR expression and incorporation of INSR into IGF1R/INSR hybrid receptors may be significant factors determining the efficacy of ganitumab as monotherapy.

The relative resistance of A673 xenografts to ganitumab was unexpected given that the expression of IGF1R and INSR is within the range observed in other sensitive cells. Our attempts to try to model this resistance in vitro were not successful: no more than a 2-fold difference in ganitumab IC_{50} for IGF1R was observed, and ganitumab treatment led to similar percent growth inhibition among the three cell lines. The resistance of A673 xenografts to IGF1R blockade in vivo appeared to be associated with a sub-optimal reduction in the level of IGF1R expression and appeared to have emerged during repeated dosing of ganitumab (IGF1R was strongly inhibited by a single ganitumab treatment in established A673 tumors). One possible explanation for the differential in vitro and in vivo sensitivity of A673 cells is exposure to circulating hormones (e.g. insulin) or stromal factors within the tumor environment. In this regard, we observed that A673 cells displayed hypersensitivity to insulin, whereas the preferred ligand in SJSA-1 and SK-ES-1 cells appeared to be IGF-1. Resistance of INSR to ganitumab treatment was also observed in long-term cell culture and xenograft experiments. These observations suggest that INSR homodimers in A673 cells may be disproportionately active relative to other cell lines that are more sensitive to ganitumab. Alternatively, hybrid receptors in A673 cells may be preferentially activated by insulin rather than IGF-1 via unknown mechanisms.

It is well established that inhibition of mTORC1 by rapamycin results in reduced phosphorylation of p70S6K, a direct target of the mTOR kinase. This effect was readily detected in serum-starved cultures in the present study. However, it is important to note that in growing cells (serum-fed) the inhibition of p70S6K activity by rapamycin was due to a reduction
in the levels of t and pp70S6K. As reported by others (O’Reilly et al., 2006), we found that rapamycin treatment generated a durable induction of Akt phosphorylation in cancer cell lines \textit{in vitro} and in established tumor xenografts \textit{in vivo}. The ability of ganitumab to inhibit induction of Akt phosphorylation strongly suggests that this effect depends on IGF1R activity and the PI3K pathway, a result that is consistent with other studies of rapamycin in combination with an anti-IGF1R antibody or PI3K inhibitors (Sun et al., 2005; Wang et al., 2008b). Rapamycin treatment also resulted in a striking increase in GSK3β phosphorylation resulting not from an increase in pGSK3β, but from a preferential reduction in non-phosphorylated protein. Ganitumab inhibited the effect of rapamycin on tGSK3β suggesting that Akt activity is essential for this effect.

Several normalization methods (p/t protein, correction for cell-confluency, division by tp70S6K) were explored to separate direct drug-related effects on IGF1R pathway markers from indirect drug-related changes on cell size and number. The changes in tGSK3β and tAkt in response to rapamycin were closely correlated with levels of tp70S6K. This observation supports the direct involvement of mTOR in regulation of the stability of these three pathway proteins. The association between tp70S6K and other IGF1R pathway proteins (IRS-1, INSR, IGF1R) was less consistent (SJSA-1 < A673 < SK-ES-1) although it improved with ganitumab treatment. The increases in tIGF1R, tINSR, and tIRS-1 relative to tp70S6K (and per unit cell confluence) with rapamycin exposure in SJSA-1 and A673 cells suggests that receptor levels and tIRS-1 can be strongly influenced by mTOR-independent mechanisms.

The modest cytostatic effects obtained \textit{in vitro} with sarcoma cells treated with ganitumab falls in the range previously reported for IGF1R inhibitors (Maloney et al., 2003; Mitsiades et al., 2004; Buck et al.). We believe that the rich nutritional composition of cell culture media may be compensating for the inhibition of metabolic pathways that results from PI3K/Akt pathway inhibition (Nawijn et al., 2011). The addition of excess ganitumab plus sub-saturating
concentrations of rapamycin to each sarcoma cell line resulted in growth inhibition that was generally additive. In vivo, this additive effect was observed in the ganitumab-sensitive SJSA-1 and SK-ES-1 models. It has been suggested that upregulation of Akt activity in response to mTORC1 inhibition is an acquired mechanism of rapamycin resistance in tumor models and cancer patients (Sun et al., 2005; O’Reilly et al., 2006; Wang et al., 2008b). This concept is not universally accepted, as recent preclinical studies have suggested that rapamycin sensitivity can occur in the presence of Akt feedback induction (Breuleux et al., 2009). The observation that ganitumab prevented the increase in pAkt in A673 tumors after a single dose but not after repeated dosing of rapamycin, suggests that regulation of other PI3K/Akt pathway inputs are important in determining resistance to this drug combination. This is highlighted by the increase in INSR activation observed after multiple dosing in A673 xenografts and the lack of enhanced efficacy in the combination group.

We believe that activation of the INSR is a plausible mechanism for the intrinsic resistance of A673 cells to ganitumab and for the acquired resistance of SJSA-1 cells to the combination of ganitumab plus rapamycin. In vivo, ganitumab plus rapamycin resulted in significant increases in pINSR in SJSA-1 and A673 xenograft models, and phosphorylation of INSR was associated with phosphorylation of Akt. Analysis of small “adapted” SJSA-1 tumors present in the combination group at the end of the study, suggests that surviving tumor cells are able to adjust to the combination treatment by activating INSR homodimers, which are resistant to ganitumab activity. A similar INSR effect was observed with A673 tumors, even though they are inherently less sensitive to ganitumab. Interestingly, this INSR-mediated resistance was not observed in the SK-ES-1 model, in agreement with the in vitro data observed in Figure 6F.

Aside from INSR-mediated resistance, it is likely that other factors contribute to the resistance of A673 cells to ganitumab. One important factor is mutant b-raf (Oliner et al., 1992),
which has been shown to detach MAPK pathway activation from growth factor receptor activation at the plasma membrane resulting in constitutive Erk signaling. This results in a strong proliferative signal that might be insensitive to IGF1R blockade. In addition, it is also possible that response to ganitumab is governed by the expression of other important IGF1R axis proteins. For example, SK-ES-1 cells secrete IGF-1 and IGFBP-3 into conditioned media and SJSA-1 cells produce IGF-2 (unpublished data), which is known to bind to and activate INSR. The expression of IGFBPs, which can modify local IGF-1 and IGF-2 activity, is known to vary widely among these cell lines. Moreover, the three cell lines carry distinct genetic alterations: SJSA-1 cells are amplified for Mdm2; and A673 and SK-ES-1 cells possess EWS-FLI-1 translocations. (Davies et al., 2002; Smith et al., 2006).

It has been suggested that inhibition of either PI3K or IGF1R may be a useful strategy to enhance the clinical efficacy of mTORC1 inhibitors like rapamycin (Sun et al., 2005; O'Reilly et al., 2006; Wang et al., 2008b). This concept is supported by our data, which showed that blockade of rapamycin-induced Akt activation by ganitumab was associated with increased efficacy in 2 of the 3 tumor models. These results strongly suggest that maintenance of PI3K/Akt activity though IGF1R stimulation may be essential for the pathway alterations induced by inhibition of mTORC1. Our results also point to the potential role of INSR as a mechanism of resistance and highlights the importance of an effective biomarker strategy to distinguish tumor phenotypes (SJSA-1 and SK-ES-1) that can benefit from this combination. The effects we observed on GSK3β suggest that more attention should be devoted to this marker in clinical studies of IGF1R and mTORC1 inhibitors. Clinical trials are required to determine whether addition of ganitumab can translate into therapeutic benefit in malignancies influenced by IGF1R activity, and in those for which the activity of mTORC1 inhibitors has been limited by Akt-mediated resistance.
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Authorship Contributions

Wrote or reviewed the manuscript and approved the final draft: Beltran, Chung, Moody, Mitchell, Cajulis, Vonderfecht, Kendall, Radinsky, Calzone

Designed the experiments: Calzone, Beltran, Kendall, Radinsky

Conducted experiments: Chung, Moody, Mitchell, Cajulis

Data analysis: Beltran, Calzone, Vonderfecht, Moody, Cajulis, Chung, Mitchell
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Footnotes

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Figure Legends

Figure 1. Inhibition of IGF-1-stimulated signals in osteogenic and Ewing’s sarcoma cell lines. Sarcoma cells were treated with increasing concentrations of ganitumab added simultaneously with 2nM IGF-1 for 20 minutes. Ganitumab inhibition of IGF1R (A), INSR (B), and Akt (C) phosphorylation was determined using MSD™ multiplex assays. Cells were grown and serum-starved in a 6-well format prior to each drug treatment. Phosphorylated and total protein levels from 40µg of protein extract were analyzed. The percent activity remaining was normalized to ‘no antibody’ treatment after subtraction of background. Lowest concentration indicated = no drug treatment.

Figure 2. Inhibitory effects of ganitumab on IGF1R signaling and tumor growth in sarcoma xenografts. A, Effect of ganitumab on basal and IGF-1-induced levels of pIGF1R, pINSR, and pAkt. Mice bearing SJSA-1, SK-ES-1, or A673 xenografts (300 to 450mm³) were randomly assigned into treatment groups and injected intraperitoneally with 1mg ganitumab or 1mg hIgG1 (n=6 for each treatment group). After 6h, the treatment groups were divided in half (n=3) and received either intravenous IGF-1 (15μg) or PBS. After 15 minutes, tumors were collected and analyzed for levels of pIGF1R and pINSR (left axis) and pAkt (right axis). Data are shown as mean phosphorylated protein signal ± S.E.M. *p<0.05 versus control hIgG1 groups in the presence or absence of IGF-1. RLU, relative light units. B, In vivo efficacy of ganitumab as monotherapy. Mice bearing SJSA-1, SK-ES-1, or A673 xenografts (~200mm³) were randomly assigned into treatment groups (n=10) and treated intraperitoneally (starting the day of first measurement) twice per week with hIgG1 (300µg/dose) or ganitumab (30, 100, or 300µg/dose) for the duration of the experiment. Tumor volumes were measured twice per week. Data are shown as mean tumor volume ± S.E.M. The significance of differences in tumor-growth
inhibition between ganitumab treatment versus hIgG1 treatment was: *p<0.0001, †p=0.0011, ‡p=0.0172. C, Status of tIGF1R and pIGF1R after long-term ganitumab treatment. Six hours after the last dose (as described in Fig. 2B), xenografts were excised and processed for MSD™ detection of tIGF1R and pIGF1R. Data are shown as mean ± S.E.M. *p<0.05 versus control hIgG1 groups (n=3).

Figure 3. Effects of ganitumab on cell proliferation and apoptosis in vivo. Mice bearing SJSA-1 (A) or A673 (B) xenografts (~300mm³) were treated by intraperitoneal injection with ganitumab or hIgG1 (1mg). Xenografts were harvested, fixed and processed for immunohistochemical detection of caspase-3 (after 3 hours) or BrdU (after 24 hours). Images were viewed with a Nikon Eclipse 90i microscope (Nikon Instruments, Melville, NY) and representative images were captured with a Nikon DXM1200F digital camera, 20X objective, and Digital Sight ACT-1 software.

Figure 4. The effect of ganitumab and rapamycin on cell growth in vitro. Cell lines were grown in 10-15% FBS-containing culture media (96-well format) containing a fixed concentration of ganitumab or hIgG1 (250nM for A673 cells; 500nM for SJSA-1 and SK-ES-1 cells) and 3-fold serial dilutions of rapamycin. We estimated that the concentration of IGF-1 in the assay media was 0.6-0.9 nM. Cell confluency was measured every 4 h. The times at which each cell line treated with hIgG1 reached about 85% confluency (52 h for SJSA-1, 96 h for SK-ES-1, and 76 h for A673) were selected to illustrate the effect of ganitumab and rapamycin on cell growth. The percent confluency at these time points in the presence or absence of ganitumab was plotted against rapamycin concentration and changes in IC₅₀ calculated using Prism. Data are shown as the mean ± SD.
Figure 5. Acute effects of ganitumab plus rapamycin on IGF1R activation and signaling. Levels of pIGF1R, pAkt, pp70S6K were determined using MSD™ multiplex assays. The pIGF1R and p70S6K results are plotted against the left axis; those for pAkt are plotted against the right axis. *p<0.05 between hlgG1 versus rapamycin or ganitumab alone and **p<0.05 between rapamycin alone versus rapamycin plus ganitumab. A, In vitro study. The status of pIGF1R and pp70S6K (left axis) and pAkt (right axis) was determined in serum-starved sarcoma cells treated for 1h with hlgG1 (0.5µM), ganitumab (0.5µM), rapamycin (27nM), or ganitumab plus rapamycin, all in the presence of 10nM IGF-1. Data are shown as the mean ± SD. B, In vivo study. Pathway markers were analyzed in tumor-bearing mice (n=3) treated for 6h with a single intraperitoneal dose of hlgG1 (300µg), ganitumab (300µg), rapamycin (0.5mg/kg), or rapamycin plus ganitumab. Data are shown as the mean ± S.E.M.. RLU, relative light units.

Figure 6. Analysis of IGF1R pathway markers in growing cells after long-term treatment with ganitumab and rapamycin. In parallel with the experiment shown in Figure 4, sarcoma cell lines were seeded in serum-containing media in 96-well microtitre plates and treated with ganitumab and rapamycin. IGF1R and Akt MSD™ multiplex assays were performed in triplicate at the incubation times indicated in Figure 4B. The signals for each treatment were normalized to cells treated with hlgG1. A,B, The relative change (Δ) in tp70S6K and pp70S6K (corrected for differences in cell confluency) plotted against log rapamycin concentration in the presence of ganitumab or hlgG1. C,D, The relative change (Δ) in Akt and GSK3β (p:t) (corrected for differences in cell confluency) plotted against log rapamycin concentration. E,F, The relative change (Δ) in pIGF1R and pINSR (corrected for differences in cell confluency) plotted against log rapamycin concentration. Relative marker changes without normalization are shown in Supp. Fig. 2.
Figure 7. In vivo efficacy and pharmacodynamics of ganitumab in combination with rapamycin. Mice bearing ~200mm³ SJSA-1, SK-ES-1, or A673 xenografts were randomly assigned into treatment groups (n=10) and treated intraperitoneally (starting the day of first measurement) twice per week with hIgG1 (300µg/dose), ganitumab (30µg/dose for SJSA-1 and 300µg/dose for SK-ES-1 and A673), hlgG1 plus rapamycin (0.5mg/kg), or ganitumab plus rapamycin for the duration of the experiment. A, In vivo efficacy. Data are shown as mean tumor volume ± S.E.M. The significance of differences between growth inhibition due to the combination versus single agent alone was: *p<0.001 versus ganitumab or rapamycin alone; **p=0.02 versus ganitumab alone; and **p=0.005 versus rapamycin alone. B, Pharmacodynamic marker analysis. Six hours after the last dose of treatment as described above, xenografts were excised and processed for MSD™ detection of pIGF1R and pINSR (left axis), and pAkt (right axis). Data are shown as the mean phosphorylated level + S.E.M. *p<0.05 between mice treated with hlgG1 versus rapamycin, ganitumab, or the combination treatment. RLU, relative light units.
Table 1. Cell Line Sensitivity to IGF-1 and Insulin

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IGF1R Pathway Marker</th>
<th>IGF-1 (Fold Induction)</th>
<th>Insulin (Fold Induction)</th>
<th>IGF-1 EC$_{50}$ (range) nM</th>
<th>Insulin EC$_{50}$ (range) nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJSA-1</td>
<td>pIGF1R</td>
<td>6.5</td>
<td>ND</td>
<td>0.45 (0.11 to 1.8)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>pINSR</td>
<td>6.3</td>
<td>3.0</td>
<td>5.1 (2.7 to 9.5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>pAkt-1</td>
<td>17.6</td>
<td>6.5</td>
<td>0.42 (0.23 to 0.80)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>pIRS-1</td>
<td>7.0</td>
<td>2.3</td>
<td>1.7 (0.7 to 3.6)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>pGSK3β</td>
<td>2.2</td>
<td>2.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>pp70S6K</td>
<td>2.7</td>
<td>2.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A673</td>
<td>pIGF1R</td>
<td>13.5</td>
<td>3.4</td>
<td>1.1 (0.9 to 1.5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>pINSR</td>
<td>25.3</td>
<td>52.9</td>
<td>32 (20 to 51)</td>
<td>2.2 (1.8 to 2.6)</td>
</tr>
<tr>
<td></td>
<td>pAkt-1</td>
<td>99.2</td>
<td>76.5</td>
<td>2.5 (1.8 to 3.6)</td>
<td>6.4 (5.9 to 6.9)</td>
</tr>
<tr>
<td></td>
<td>pIRS-1</td>
<td>18.6</td>
<td>22.9</td>
<td>2.3 (1.7 to 3.0)</td>
<td>3.1 (2.2 to 4.4)</td>
</tr>
<tr>
<td></td>
<td>pGSK3β</td>
<td>5.5</td>
<td>4.0</td>
<td>0.9 (0.4 to 2.1)</td>
<td>0.77 (0.5 to 1.1)</td>
</tr>
<tr>
<td></td>
<td>pp70S6K</td>
<td>4.8</td>
<td>3.4</td>
<td>1.4 (0.6 to 3.5)</td>
<td>1.0 (0.4 to 2.2)</td>
</tr>
<tr>
<td>SK-ES-1</td>
<td>pIGF1R</td>
<td>13.0</td>
<td>2.6</td>
<td>0.6 (0.5 to 0.7)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>pINSR</td>
<td>37.7</td>
<td>81.4</td>
<td>7.7 (5.3 to 11)</td>
<td>3.0 (2.6 to 3.4)</td>
</tr>
<tr>
<td></td>
<td>pAkt-1</td>
<td>31.0</td>
<td>27.5</td>
<td>0.24 (0.1 to 0.5)</td>
<td>0.7 (0.5 to 0.9)</td>
</tr>
<tr>
<td></td>
<td>pIRS-1</td>
<td>8.8</td>
<td>16.1</td>
<td>1.5 (1.1 to 2.1)</td>
<td>1.4 (1.0 to 2.0)</td>
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<tr>
<td></td>
<td>pGSK3β</td>
<td>3.4</td>
<td>3.4</td>
<td>0.07 (0.02 to 0.3)</td>
<td>0.1 (0.02 to 0.2)</td>
</tr>
<tr>
<td></td>
<td>pp70S6K</td>
<td>5.8</td>
<td>4.6</td>
<td>0.06 (0.01 to 0.6)</td>
<td>0.14 (0.07 to 0.3)</td>
</tr>
</tbody>
</table>
The maximum fold induction of IGF-1- or insulin-induced phosphorylation after 20-minute stimulation with 200 nM IGF-1 or insulin was calculated by dividing the stimulation MSD assay value by the non-stimulation MSD assay value for each phosphorylated marker.

EC$_{50}$ = the concentration (nM) of IGF-1 or insulin required to achieve 50% maximal phosphorylation, calculated using a sigmoidal dose-response equation (GraphPad Software, La Jolla). The range in parenthesis is the 95% confidence interval for the curve fit.

An EC$_{50}$ was not determined (ND) when the fold stimulation was less than 3-fold.
Table 2. Correlation of Total IGF1R Pathway Markers Relative to tp70S6K After Ganitumab and Rapamycin Treatment

<table>
<thead>
<tr>
<th>Marker</th>
<th>SJSA-1</th>
<th>SK-ES-1</th>
<th>A673</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker</td>
<td>hlgG1</td>
<td>Ganitumab</td>
<td>hlgG1</td>
</tr>
<tr>
<td>IGF1R</td>
<td>0.1 (0.11)</td>
<td>0.3 (0.8)</td>
<td>0.6 (0.9)</td>
</tr>
<tr>
<td>INSR</td>
<td>-0.2 (0.7)</td>
<td>0.1 (0.3)</td>
<td>0.8 (0.9)</td>
</tr>
<tr>
<td>IRS-1</td>
<td>ND</td>
<td>ND</td>
<td>1.1 (0.8)</td>
</tr>
<tr>
<td>Akt</td>
<td>0.7 (0.9)</td>
<td>0.5 (0.9)</td>
<td>0.9 (1.0)</td>
</tr>
<tr>
<td>GSK3β</td>
<td>1.0 (1.0)</td>
<td>0.9 (1.0)</td>
<td>1.0 (1.0)</td>
</tr>
</tbody>
</table>

The relative change of IGF1R, INSR, IRS-1, Akt, and GSK3β was plotted against tp70S6K, and the slope of the line fit through the data was determined using GraphPad Prism software by linear regression. The R² value for each regression analysis is given in parenthesis. The total protein levels were taken from the experiment shown in Figure 6 without normalization for cell confluence. ND indicates that IRS-1 level was too low to generate significant data. Background for all other markers was generally insignificant (<1% total signal).
Figure 1

A. IGF1R

B. INSR

C. Akt

Percentage of Control vs. log [Ganitumab] (nM)

- SJSA-1
- SK-ES-1
- A673
Figure 2

A

SJSA-1

SK-ES-1

A673

B

SJSA-1

SK-ES-1

A673

C

SJSA-1

SK-ES-1

A673
Figure 4

SJSA-1, 52 h

SK-ES-1, 96 h

A673, 76 h

% Confluence vs. Log [Rapamycin] (nM)

O hlgG1

■ Ganitumab
Figure 5

A

SJSA-1 *In Vitro*

SK-ES-1 *In Vitro*

A673 *In Vitro*

B

SJSA-1 *In Vivo*

SK-ES-1 *In Vivo*

A673 *In Vivo*
Figure 6

A

$\Delta \text{tp70S6K}$

B

$\Delta \text{p:tp70S6K}$

C

$\Delta \text{p:t Akt}$

D

$\Delta \text{p:GSK3}\beta$

E

$\Delta \text{p:IGF1R}$

F

$\Delta \text{p:NSR}$

Log [Rapamycin] (nM)

Log [Rapamycin] (nM)

Log [Rapamycin] (nM)

Log [Rapamycin] (nM)

Log [Rapamycin] (nM)

Log [Rapamycin] (nM)

hlgG1 Treated

- SJSA-1
- A673
- SK-ES-1

Ganitumab Treated

- SJSA-1
- A673
- SK-ES-1
**Title:** Efficacy of Ganitumab (AMG 479), Alone and in Combination With Rapamycin, in Ewing’s and Osteogenic Sarcoma Models

**Authors:** Pedro J. Beltran, Young-Ah Chung, Gordon Moody, Petia Mitchell, Elaina Cajulis, Steven Vonderfecht, Richard Kendall, Robert Radinsky, Frank J. Calzone

Beltran Supplementary Figure 1

**Supplementary Figure 1.** Effect of ganitumab on basal and IGF-1-induced levels of activated IGF1R, INSR, and Akt in sarcoma xenografts. The phosphorylation (p) signals for IGF1R, INSR, and Akt obtained in the experiment described in Figure 2A were divided by total (t) protein signals determined for each sample in parallel to eliminate effects attributed to cell-line or drug-induced changes in total marker level. Data are shown as mean phosphorylated protein level divided by total protein ± S.E.M. *p<0.05 versus control hIgG1 groups in the presence or absence of IGF-1. RLU, relative light units.
Supplementary Figure 2. IGF1R pathway marker profiles in SJSA-1 (A), SK-ES-1 (B), and A673 (C) cells treated with ganitumab and rapamycin. Sarcoma cells were plated in triplicate in a 96-well format in growth media (10% FBS DMEM, SJSA-1 and A673; 15% FBS McCoy’s 5A, SK-ES-1) with freshly thawed serum at an initial confluence of 25% SJSA-1, 8% SK-ES-1, and 20% A673. Pathway markers were assayed using MSD™ IGF1R and Akt multiplex assays. The drug incubation times are indicated in Figure 4B. To represent the marker changes on a standardized scale, the total and phosphorylated signals were divided by the average of the hlgG1 control.
Supplementary Figure 3. Pharmacodynamic marker analysis in sarcoma xenografts treated with ganitumab and rapamycin. The phosphorylation signals for IGF1R, INSR, and Akt obtained in the efficacy study described in Figure 7B were divided by total signals determined for each sample in parallel to eliminate effects attributed to cell-line or drug-induced changes in total marker level. The less-than-optimal inhibition of p:t IGF1R in this analysis reflects the significant downregulation of tIGF1R induced by ganitumab following repeated dosing in vivo. RLU, relative light units.