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 insulin-like growth factor type 1 (IGF-1) receptor (IGF1R) and/or the insulin receptor (INSR) to drive tumor growth, survival, and resistance to mammalian target of rapamycin complex 1 (mTORC1) inhibitors. We evaluated the therapeutic value of ganitumab (AMG 479; C47H10028N1728O2020S42), an anti-IGF-1R, 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 IGFI-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 bro-modeoxyuridine detection, and increased caspase-3 expression. Ganitumab inhibited rapamycin-induced IGF1R, Akt, and glycogen synthase kinase-3β 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 (Heare et al., 2009). Osteogenic and Ewing’s sarcoma most frequently occur in adolescents during rapid bone growth and present clinical challenges because of 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 insulin-like growth factor type 1 (IGF-1) 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: 1) the association between the IGF-1-driven adolescent growth spurt and peak incidence of osteogenic sarcoma, 2) the direct control of IGF1R pathway-related proteins by the EWS/FLI-1 fusion protein [oncogenic transcription factor that controls expres-

ABBREVIATIONS: IGF, insulin-like growth factor; IGF1R, IGF type 1 receptor; pIGF1R, phosphorylated IGF1R; tIGF1R, total IGF1R; pAkt, phosphorylated Akt; tAkt, total Akt; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; IRS-1, insulin receptor substrate 1; pIRS-1, phosphorylated IRS-1; tIRS-1, total IRS-1; INSR, insulin receptor; pINSR, phosphorylated INSR; tINSR, total INSR; p70S6K, phosphorylated p70S6K; p70S6K, total p70S6K; GSK3β, glycogen synthase kinase-3β; pGSK3β, phosphorylated GSK3β; tGSK3β, total GSK3β; pAkt, phosphorylated Akt; pPI3K, phosphatidylinositol-3-kinase; MSD, Meso Scale Discovery; BrdU, bromodeoxyuridine; PBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle medium; PBS, phosphate-buffered saline; IC50, half-maximal inhibitory concentration; TBS, Tris-buffered saline; RLU, relative light units; HgG1, human IgG1; AMG 479, C47H10028N1728O2020S42; ANOVA, analysis of variance.
sion of IGF1R, IGF-1, and IGF binding proteins (Arvand and Denny, 2001; Prieur et al., 2004; Mateo-Lozano et al., 2006) in Ewing’s sarcomas, and 3) overexpression of IGF-2 in rhabdomyosarcomas (Rikho et al., 2009). IGF-1 activity is mediated predominantly 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).

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-binding protein 1, 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). It is noteworthy that rapamycin-induced Akt activation seemed to depend 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 sequence of ganitumab can be found at: http://www.who.int/medicines/publications/druginformation/INN_PLJ103.pdf.

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, 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 (Manassas, VA) in 2006. 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 bovine serum (FBS) (SJSA-1 and A673), or McCoy’s 5A with 15% FBS (SK-ES-1). Human antistreptavidin 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 1 µg of phycoerythrin-conjugated antibodies specific for human IGF1R or INSR (BD Pharmingen, San Jose, CA) for 1.5 h 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 two determinations were made for each cell line.

In Vitro Cell Growth Assays. SJSA-1 cells were seeded in 96-well plates (2 × 10³ per well) in DMEM with 10% FBS, 2 mM glutamine, and either 500 nM ganitumab or hIgG1 plus 0 to 77 nM rapamycin (3-fold serial dilutions). A673 cells were seeded (1.7 × 10⁴ per well) in DMEM with 10% FBS, 2 mM glutamine, and either 250 nM ganitumab or hIgG1 plus 0 to 54 nM rapamycin (2.5-fold serial dilutions). SK-ES-1 cells were seeded (1 × 10⁴ per well) in McCoy’s 5A medium with 15% FBS, 2 mM glutamine, and either 500 nM ganitumab or hIgG1 plus 0 to 54 nM rapamycin (3-fold serial dilutions). Confluence measurements were performed in duplicate for each well at 4-h intervals over 5 to 7 days using an Incucyte phase contrast optical imaging system (Essen Instruments, Ann Arbor, MI). Prism (GraphPad Software Inc., San Diego, 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 (3–4 × 10⁴ 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 24 h in DMEM or McCoy’s 5A. Dilutions of either hIgG1 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 min.

The short-term effects of ganitumab plus rapamycin on the phosphorylation of IGF1R, p70S6K, and Akt were determined in serum-starved sarcoma cells (six-well format) treated for 1 h with hIgG1 (0.5 µM), ganitumab (0.5 µM), rapamycin (27 nM), or ganitumab and rapamycin, all in the presence of 10 nM 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 52 h (SJSA-1), 96 h (SK-ES-1), or 72 h (A673) for growth of analysis for IGFR1 pathway markers.

In each of the experiments described above, levels of total and/or phosphorylated INSR, IGFR1, 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 × 10⁶ 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 450 mm³, mice were randomly assigned into four groups (n = 3, each group). Mice received either a single intraperitoneal dose of gani-tumab (1 mg) or hlgG1 (1 mg) (total of four groups; two groups received ganitumab and two received hlgG1). After 6 h, a ganitumab group and a hlgG1 group received human IGF-1 (15 μg) by tail-vein injection, whereas the other ganitumab and hlgG1 groups received PBS injections. Xenografts were collected 15 min 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) (20 mM Tris-HCl, pH 8.5, 0.15 M NaCl) and diluted with 3 volumes of TBS/2% Triton X-100. Cell lysates were cleared by centrifugation at 14,000 rpm, and protein levels (total and phosphorylated) were analyzed using MSD multiplex assays.

In a separate experiment, 12 mice bearing 300- to 450-mm³ tumors were assigned to four groups (n = 3, each group) and received single intraperitoneal doses of hlgG1 (300 μg), ganitumab (300 μg), rapamycin (0.5 mg/kg), or a 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 × 10⁶ cells in a suspension containing a 2:1 ratio of cells to Matrigel (BD Bioscience). After 7 to 15 days, mice bearing tumors (approximately 200 mm³) were randomly assigned into groups (n = 10, each group) and administered ganitumab (30, 100, or 300 μg per dose) or hlgG1 (300 μg) as single agents or in combination with rapamycin (0.5 mg/kg) by intraperitoneal injection twice per week 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 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 mm³) 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). In brief, cleaved caspase-3 was detected using a rabbit anticaspase-3 antibody (Cell Signaling Technology, Danvers, MA) followed by a peroxidase-labeled goat anti-rabbit IgG antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) and diaminobenzidine. Incorporation of BrdU was detected with a rat anti-BrdU antibody (Accurate Chemical & Scientific, 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 (200 ng/ml) and ganitumab (0.5 μM) for 24 h. 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/Alexa Fluor 647 antibody (0.2 μg/μl, Invitrogen) and fluorescein isothiocyanate-labeled anticaspase-3 antibody (1:5 dilution, Invitrogen), followed by treatment with propidium iodide/RNase staining solution (BD Pharmingen).

Statistical Analysis. For in vivo efficacy studies, repeated-measures analysis of variance (ANOVA) 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 ANOVA 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 ANOVA.

Results

IGFR1 and INSR Levels in Sarcoma Cells. The levels of IGFR1 and INSR in each sarcoma cell line were determined by quantitative flow cytometry. The levels of IGFR1 were: SJSA-1, 9000/cell; A673, 14,000/cell; and SK-ES-1, 18,000/cell. The levels of INSR were: SJSA-1, 350/cell; A673, 2100/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 IGFR1 homodimers, INSR homodimers, and IGFR1/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 IGFR1, INSR, Akt, IRS-1, GSK3β, and p70S6K in xenografts and cultured cells. The IGFR1 response to IGF-1 was similar for the three cell lines (EC50 = 0.45–1.1 nM). However, each cell line displayed a distinct INSR response. IGF-1 was the preferred INSR agonist in SJSA-1 cells, which had a low INSR/IGF1R ratio (0.04). SK-ES-1 cells, which expressed higher INSR/IGF1R ratios (0.72), exhibited similar INSR sensitivity to insulin and IGF-1 (EC50 = 3.0 and 7.7 nM, respectively), whereas in A673 cells INSR was more responsive to insulin than to IGF-1 (EC50 = 2.2 and 32 nM, respectively).

Inhibition of IGFR1, INSR, and Akt Activity by Ganitumab. Ganitumab inhibited IGF-1-induced phosphorylation of IGFR1 (IC50 = 2.0 nM in SJSA-1 cells; 1.6 nM in A673 cells; and 3.7 nM in SK-ES-1 cells) and INSR (albeit at higher concentrations, IC50 > 50 nM) in all three cell lines (Fig. 1, A and B). In SJSA-1 cells, the inhibitory effect of ganitumab on IGF-1-induced Akt phosphorylation was similar to its effect on IGF1 phosphorylation (IGFR1 IC50 = 2.0 nM; Akt IC50 = 4.7 nM) but was approximately 30-fold less than for IGFR1 in A673 cells (IGFR1 IC50 = 1.6 nM; Akt IC50 = 50 nM) and SK-ES-1 cells (IGFR1 IC50 = 3.7 nM; Akt IC50 = 102 nM) (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 IGFR1, INSR, and Akt was increased 4- to 17-fold relative to mice that did not receive IGF-1 (Fig. 2A). 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 (Supplemental Fig. 1). Pretreatment with intraperitoneal ganitumab (1 mg) for 6 h led to a significant reduction (73 to 97%) in IGF-1-induced IGFR1 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.
Table 1

Cell line sensitivity to IGF-1 and insulin

The maximum fold induction of IGF-1 or insulin-induced phosphorylation after 20-min stimulation with 200 nM IGF-1 or insulin was calculated by dividing the stimulation MSD assay value by the nonstimulation 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 Inc.). The range in parentheses is the 95% confidence interval for the curve fit.

<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)</th>
<th>Insulin $EC_{50}$ (Range)</th>
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<tbody>
<tr>
<td>SJSA-1</td>
<td>plIGF1R</td>
<td>6.5</td>
<td>N.D.</td>
<td>0.45 (0.11–1.8)</td>
<td>N.D.</td>
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<tr>
<td></td>
<td>plINSR</td>
<td>6.3</td>
<td>3.0</td>
<td>5.1 (2.7–9.5)</td>
<td>N.D.</td>
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<tr>
<td></td>
<td>pAkt-1</td>
<td>17.6</td>
<td>6.5</td>
<td>0.42 (0.23–0.80)</td>
<td>N.D.</td>
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<tr>
<td></td>
<td>pIRS-1</td>
<td>7.0</td>
<td>2.3</td>
<td>1.7 (0.7–3.6)</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>pGSK3β</td>
<td>2.2</td>
<td>2.1</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>pp70S6K</td>
<td>2.7</td>
<td>2.0</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>A673</td>
<td>plIGF1R</td>
<td>13.5</td>
<td>3.4</td>
<td>1.1 (0.9–1.5)</td>
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<tr>
<td></td>
<td>plINSR</td>
<td>25.3</td>
<td>52.9</td>
<td>32 (20–51)</td>
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<tr>
<td></td>
<td>pAkt-1</td>
<td>99.2</td>
<td>76.5</td>
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<td>6.4 (5.9–6.9)</td>
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<td>pIRS-1</td>
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<td>pGSK3β</td>
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<td>0.9 (0.4–2.1)</td>
<td>0.77 (0.5–1.1)</td>
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<td></td>
<td>pp70S6K</td>
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<td>3.4</td>
<td>1.4 (0.6–3.5)</td>
<td>1.0 (0.4–2.2)</td>
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<tr>
<td>SK-ES-1</td>
<td>plIGF1R</td>
<td>13.0</td>
<td>2.6</td>
<td>0.6 (0.5–0.7)</td>
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<tr>
<td></td>
<td>plINSR</td>
<td>37.7</td>
<td>81.4</td>
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<td>0.06 (0.01–0.6)</td>
<td>0.14 (0.07–0.3)</td>
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</table>

N.D., an $EC_{50}$ was not determined when the fold stimulation was less than 3-fold.

Fig. 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 2 nM IGF-1 for 20 min. 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 six-well format before each drug treatment. Phosphorylated total protein levels from 40 μg of protein extract were analyzed. The percentage of activity remaining was normalized to “no antibody” treatment after subtraction of background. Lowest concentration indicated equals no drug treatment.

(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 30 μg, 74% at 100 μg, and 84% at 300 μg (Fig. 2B). SK-ES-1 xenografts were slightly less sensitive to inhibition by ganitumab, with significant tumor growth inhibition of 47% at 30 μg, 61% at 100 μg, and 74% at 300 μg. Reduced sensitivity to ganitumab was observed in the insulin-responsive A673 tumor model: 300 μg of 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 tIGF1R and plIGF1R (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, nonsignificant 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 (1 mg) of SJSA-1 xenografts led to increased expression of cleaved caspase-3 after 3 h and reduced BrdU incorporation after 24 h (Fig. 3A). In A673 xenografts, ganitumab did not alter the expression of cleaved caspase-3 but reduced BrdU labeling after 24 h (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 (percentage of cell confluence) 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; SK-ES-1 and A673, 1.1-fold; data not shown). The time at which each cell line treated with hlgG1 reached approximately 85% confluence 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- to 3-fold and seemed 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 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 to be an important distinction because 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 more than 80% in all three cell lines. Inhibition of pAkt was highest in the SJSA-1 and A673 cell lines (>80%), whereas 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 cells. 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 statistical 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 Fig. 4. The overall pattern of marker phosphorylation observed with ganitumab treatment alone (Supplemental 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 55–85%). In SJSA-1 and A673 cells, rapamycin reduced the amount of tp70S6K and p:tp70S6K per cell in a concentration-dependent manner (Fig. 6, A and 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. 6, A and B).

The p:t ratios of Akt and GSK3β (corrected for differences in cell confluence) were increased in rapamycin-treated cells, and this effect was inhibited by ganitumab (Fig. 6, C and D). A separate plot of phosphorylated and total signals (corrected for cell confluence) 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 confluence because rapamycin exhibited strong positive effects on total protein levels (Fig. 6, E and 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 whether 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...
Acute effects of ganitumab plus rapamycin on IGF1R activation and signaling. Levels of pIGF1R, pAkt, and 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.

Fig. 5. Acute effects of ganitumab plus rapamycin on IGF1R activation and signaling. Levels of pIGF1R, pAkt, and 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 hIgG1 versus rapamycin or ganitumab alone; ††, 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 1 h with hIgG1 (0.5 μM), ganitumab (0.5 μM), rapamycin (27 nM), or ganitumab plus rapamycin, all in the presence of 10 nM IGF-1. Data are shown as the mean ± S.D.

Fig. 6. Analysis of IGF1R pathway markers in growing cells after long-term treatment with ganitumab and rapamycin. In parallel with the experiment shown in Fig. 4, sarcoma cell lines were seeded in serum-containing media in 96-well microtiter plates and treated with ganitumab and rapamycin. IGF1R and Akt MSD multiplex assays were performed in triplicate at the incubation times indicated in Fig. 4B. The signals for each treatment were normalized to cells treated with hlgG1. A and B, the relative change (Δ) in tp70S6K and pp70S6K (corrected for differences in cell confluence) plotted against log rapamycin concentration in the presence of ganitumab or hlgG1. C and D, the relative change (Δ) in Akt and GSK3β (pt) (corrected for differences in cell confluence) plotted against log rapamycin concentration. E and F, the relative change (Δ) in pIGF1R and pINSR (corrected for differences in cell confluence) plotted against log rapamycin concentration. Relative marker changes without normalization are shown in Supplemental Fig. 2.
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 (30 μg/dose SJSA-1) or optimal (300 μg/dose SK-ES-1 and A673) dose of ganitumab had significantly reduced levels of net pIGF1R (Fig. 7B). Normalized (p:t) analysis of IGF1R (Supplemental Fig. 3) resulted in the appearance of less than optimal pIGF1R inhibition by ganitumab because of the receptor down-regulating effects of ganitumab after repeated dosing. Rapamycin seemed to induce pAkt in all xenografts, but statistical significance versus control was achieved only in the A673 tumors (p = 0.076 for SK-ES-1 and p = 0.099 for SJSA-1). In contrast to our

![Fig. 7. In vivo efficacy and pharmacodynamics of ganitumab in combination with rapamycin. Mice bearing ~200-mm³ 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 hlgG1 (300 μg/dose), ganitumab (30 μg/dose for SJSA-1 and 300 μg/dose for SK-ES-1 and A673), hlgG1 plus rapamycin (0.5 mg/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 caused by the combination versus single agent alone was as follows: *, p < 0.001 versus ganitumab or rapamycin alone; **, p = 0.02 versus ganitumab alone or p = 0.005 versus rapamycin alone. B, pharmacodynamic marker analysis. Six hours after the last dose of treatment as described in A, 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 a combination.](https://www.jpet.org/content/147/1/651/F1)

<table>
<thead>
<tr>
<th>Marker</th>
<th>SJSA-1</th>
<th>SK-ES-1</th>
<th>A673</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>hlgG1</td>
<td>Ganitumab</td>
<td>hlgG1</td>
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<tr>
<td>IGF1R</td>
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<td>0.8 (0.9)</td>
</tr>
<tr>
<td>IRS-1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.1 (0.8)</td>
</tr>
<tr>
<td>Akt</td>
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<td>0.5 (0.9)</td>
<td>0.9 (1.0)</td>
</tr>
<tr>
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<td>0.9 (1.0)</td>
<td>1.0 (1.0)</td>
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N.D., IRS-1 level was too low to generate significant data; background for all other markers was generally insignificant (<1% total signal).
previous observations (pharmacodynamic assay; Fig. 5), 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 hIgG1). 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 down-regulation (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 dependence 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 antiproliferative or proapoptotic activities, ganitumab inhibition of SJSA-1 xenografts involved both potent antiproliferative and proapoptotic 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 percentage growth inhibition among the three cell lines. The resistance of A673 xenografts to IGF1R blockade in vivo seemed to be associated with a suboptimal reduction in the level of IGF1R expression and seemed 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 seemed 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 mTOR kinase activity. 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 caused by a reduction in the levels of total p70S6K 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 in vivo and in established tumor xenografts 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 nonphosphorylated 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 confluence, 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 the 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 of 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 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., 2010). 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 subsaturating 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 up-regulation 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, because 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 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. It is noteworthy that this INSR-mediated resistance was not observed in the SK-ES-1 model, in agreement with the in vitro data observed in Fig. 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 mitogen-activated protein kinase pathway activation from growth factor receptor activation at the plasma membrane, resulting in constitutive extracellular signal-regulated kinase signaling. This results in a strong proliferative signal that might be insensitive to IGF1R blockade. In addition, it is 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 IGF binding protein 3 into conditioned media, and SJSA-1 cells produce IGFI-2 (P. J. Beltran and F. J. Calzone, unpublished data), which is known to bind to and activate INSR. The expression of IGFI binding proteins, which can modify local IGF-1 and IGFI-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 such as 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 two of the three tumor models. These results strongly suggest that maintenance of PI3K/Akt activity through 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 highlight 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

Participated in research design: Beltran, Kendall, Radinsky, and Calzone.

Conducted experiments: Chung, Moody, Mitchell, and Cajulis.

Performed data analysis: Beltran, Chung, Moody, Mitchell, Cajulis, Vonderfecht, and Calzone.

Wrote or contributed to the writing of the manuscript: Beltran, Chung, Moody, Mitchell, Cajulis, Vonderfecht, Kendall, Radinsky, and Calzone.

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