**Abstract**

MEDI-573 is a human antibody that neutralizes insulin-like growth factor (IGF) I and IGFII. IGFs are overexpressed in multiple types of cancer; their overexpression is a potential mechanism for resistance to IGF receptor (IGFIR)-targeting therapy. Effects of IGF on cell proliferation, differentiation, and survival are mediated through its binding to and activation of IGFIR or insulin receptor A (IR-A). In this study, we measured the mRNA levels of IGF, IGFI, and IGFIR in human pediatric sarcoma xenografts, and protein levels in sarcoma cell lines. MEDI-573 potently inhibited in vitro proliferation of sarcoma cell lines, with Ewing sarcoma cell lines being the most sensitive. In addition, MEDI-573 inhibited IGFI- and IGFII-induced sarcoma cell proliferation in vitro. The effect of MEDI-573 on IGF signaling was also examined. Treatment with MEDI-573 markedly reduced levels of pIGFIR, pIR-A, and pAKT and significantly blocked IGFI- and IGFII-induced activation of the IGFIR and AKT pathways. MEDI-573 inhibited the growth of sarcoma xenografts in vivo and inhibition correlated with neutralization of IGFII and IGFI. Combination of MEDI-573 with either rapamycin or AZD2014, another mTOR inhibitor (mTORi), significantly enhanced the antitumor activity of MEDI-573, and this response correlated with modulation of AKT and mTOR signaling. In summary, sarcoma cells respond to autocrine or paracrine growth stimulation by IGFI and IGFII, and inhibition of IGFI and IGFII by MEDI-573 results in significant slowing of tumor growth rate in sarcoma models, particularly in Ewing sarcoma. These data provide evidence for the potential benefits of MEDI-573 and mTORi combinations in patients with Ewing sarcoma. Mol Cancer Ther; 13(11); 2662–73. ©2014 AACR.

**Introduction**

Insulin-like growth factors (IGFI and IGFII) are involved in regulating cell proliferation, survival, differentiation, and transformation. Both IGFI and IGFII are expressed ubiquitously and act as endocrine, paracrine, and autocrine growth factors (1–3). They activate multiple intracellular signaling cascades including the insulin receptor substrate (IRS) protein family, AKT, and MAPK pathways by binding to the insulin-like growth factor 1 receptor (IGFIR) and insulin receptor A isoform (IR-A; refs. 4–6).

High levels of circulating IGFI and IGFII are associated with an increased risk for the development of several cancers (7), including breast, prostate, pancreatic, colorectal, non–small cell lung cancer, hepatocellular carcinoma, and sarcomas. It has been shown that downregulation of IGFIR expression or blocking its signaling leads to inhibition of tumor growth, both in vitro and in vivo (8, 9), and overexpression of IR-A and IGFII may lead to resistance to IGFIR-directed therapies (10, 11). In addition, inhibition of IGF signaling increases the susceptibility of tumor cells to chemotherapeutic agents in vivo (2, 4, 8, 12). Therefore, inhibition of both IR-A and IGFIR signaling may enhance therapeutic efficacy against IGF-driven cancers (9).

Sarcomas arise from transformed cells of mesenchymal origin, and include bone sarcoma and soft tissue sarcoma. Soft tissue sarcomas are the fifth most common solid cancers in the population under 20 years of age, with rhabdomyosarcoma being the most common. Bone sarcomas are the third most prevalent cancer in adolescence, with osteosarcoma and Ewing sarcoma being the most frequent (13). Current treatment options for sarcomas include surgery, chemotherapy, and radiation. Unfortunately, the overall survival rate has not improved significantly over the last 20 years. The growth and survival of sarcoma cells is influenced by the IGF signaling pathways (14, 15). Expression of IGFIR, IGFI, or IGFII is high in most Ewing sarcomas,
osteoaromas, and rhabdomyosarcomas (16). Ewing sarcomas secrete more IGFI whereas rhabdomyosarcomas secrete more IIIF (14, 17). IGFI is highly expressed in osteosarcoma and stimulates tumor cell growth (18). Genetic alterations in the IGFI pathway are prevalent in several sarcomas. Loss of imprinting at the IGFI locus is commonly detected in embryonal rhabdomyosarcomas (19). PAX3-FKHR fusion protein resulted from chromosomal translocation activates IGFIIR transcription in alveolar rhabdomyosarcomas (20).

Given the role of the IGFI signaling pathway in cancers in general and sarcoma in particularly, targeted therapeutic approaches that inhibit the IGFIIR receptor, including mAbs, have been tested in a number of sarcomas. Several anti-IGFIIR mAbs with in vitro and in vivo antitumor activities in sarcoma models have been developed (21–25). These mAbs inhibit IGFIIR and IGFI signaling through IGFIIR and heterodimeric IGFIIR/IR-A, but do not inhibit IGFI signaling through IR-A. As a result of their inability to target the IR-A signaling pathway, the efficacy of anti-IGFIIR mAbs may be limited.

MedImmune is developing MEDI-573 as a potential anticancer therapy for patients with solid tumors (26). MEDI-573 is a human IgG2 lambda mAb. This antibody selectively binds to human IGFI and IGFIIR, and inhibits their ability to trigger IGFIIR and IR-A signaling without affecting insulin activation of IR. In vitro, MEDI-573 inhibits human tumor cell proliferation. In vivo, treatment of tumor-bearing mice with MEDI-573 significantly inhibits the tumor growth of engineered mouse embryonic fibroblast (MEF) cells that overexpress human IGFIIR and IGFI or IIIF (26). Therefore, treatment with MEDI-573 represents a novel therapeutic approach to modulating both the IGFIIR and IR-A signaling pathways.

The mTOR plays an important role in regulating cell growth, proliferation, and survival (27). Activation of mTOR leads to phosphorylation of the downstream signaling molecules ribosomal protein S6 kinase β-1 (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). mTOR signaling has been an attractive therapeutic target for cancer treatment (28); mTOR complex 1 (mTORC1) inhibitors temsirolimus and everolimus have been approved for treating metastatic renal cell carcinoma and pancreatic neuroendocrine tumors, respectively. The importance of the mTOR pathway in the growth and survival of specific sarcomas has recently been recognized (29). In a number of sarcomas, the mTOR and IGFIIR pathways are active, and are functionally relevant pathways for tumor cell survival. However, inhibition of mTORC1 with either rapamycin or its derivatives induces AKT activation by releasing the negative feedback between S6K and IRS/PDK and subsequently reactivating IGFIIR signaling (30). This IGFIIR reactivation, in turn, provides a possible mechanism of resistance to mTORC1 inhibitors and suggests a potential benefit of combining these drugs with agents targeting the IGFI pathway, and several such combinations are under evaluation in early-phase clinical trials. Although rapamycin and its derivatives only inhibit mTORC1, the drug AZD2014 is a potent inhibitor of both mTORC1 and mTORC2 (31). AZD2014 also has a greater potency in suppressing protein synthesis and broad antiproliferative activity against multiple tumor types. AZD2014 is currently in phase I clinical testing.

Here, we describe studies examining the role of IGFI ligands in sarcomas and the activity of MEDI-573 in these tumor types. We profiled the levels of IGFI ligands and receptors in primary sarcoma xenografts and cell lines, and MEDI-573 was found to inhibit several sarcomas, and this inhibition was enhanced when combined with rapamycin or AZD2014, and these activities were associated with suppression of known IGFI receptor and mTOR signaling mechanisms.

Materials and Methods

Cells and reagents

Sarcoma cell lines RD-ES, SK-ES, SJCRH30, RD, SJSA-1, MG-63, SAOS-2, and HS729 were purchased from American Type Culture Collection. TC-71 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Upon delivery, cells were expanded and low passage vials were stored in liquid nitrogen. Studies were carried out within 8 weeks after resuscitation. Cell line authentication was conducted by short tandem repeat-based DNA fingerprinting and multiplex PCR. IMPACT tests were also performed on all cell lines.

CellTiter-Glo (CTG) reagents were obtained from Promega. MSD kits for phosphor-IGFIIR (pIGFIIR), phospho-IR (pIR), and phospho-AKT (pAKT) were purchased from Promega. MSD kits for total IGFI and IGFIIR were purchased from R&D Systems. ELISA kits for total IGFI and IGFII were purchased from Insight Genomics. ELISA detecting free IGFI and IGFIIR were developed in house. Human IGFI and IGFII were obtained from R&D Systems. Antibodies for detecting pAKT, phospho-4EBP1 (p4EBP1), phospho-S6K (pS6K), and GAPDH are from Cell Signaling Technology.

mRNA samples from 20 primary sarcoma xenografts were provided by Dr. Peter Houghton at Nationwide Children’s Hospital (Columbus, OH). The primary xenografts came from pediatric patients, age from 6 months to 25 years. The samples included 5 Ewing sarcomas, 7 osteosarcomas, and 8 rhabdomyosarcomas.

RT-PCR assays for measuring IGFI, IGFIIR, IR-A, and IR-B mRNA levels

Total RNA was purified using the ZR RNA MicroPrep Kit (Zymo Research) following the manufacturer’s instructions.

Single-stranded cDNA was generated from total RNA using the SuperScript III First-Strand Synthesis SuperMix. Samples of cDNA were preamplified using TaqMan Pre-Amp Master Mix Kit (Life Technologies), according to the manufacturer’s instructions.
RT-PCR assays were performed according to standard procedures. Briefly, sample mix (5 μL) containing 2.5 μL of 2× Universal Master Mix, 0.25 μL of sample loading buffer, and 2.25 μL of preamplified cDNA were loaded into each sample inlet of the dynamic array (Fluidigm). Of note, 5 μL of 10× gene expression assay mix was loaded into each detector inlet. Before loading the samples and assay reagents into the inlets, the chip was primed in the IFC Controller (Fluidigm) and the array was placed on the IFC Controller for loading and mixing. The array was loaded on the BioMark RT-PCR System (Fluidigm) for thermal cycling (95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, and finally 60°C for 1 minute). The number of replicates and the composition of the samples varied depending on the particular experiment but were never less than triplicate determinations. Average cycle threshold (Ct) values were used to quantify the designed probes. The average Ct values of all available reference gene assays within a sample were utilized for calculation of ΔCt.

IGFI, IGFII, IGFR, IR-A, and IR-B were tested. TaqMan mRNA expression assays of IR-A and IR-B were previously described in detail by Huang and colleagues. (31). Other TaqMan gene expression assays were purchased from Applied Biosystems.

In vitro cell proliferation assays

Sarcoma cell lines were cultured overnight in RPMI or DMEM medium with 10% FBS. Medium containing 0.1% charcoal-stripped FBS (Life Technologies) was added the following day and incubated overnight. The next day, various amounts of MEDI-573 were added to the cells and incubated for 3 days. At the end of 3-day incubation, cell proliferation was quantified using the CTG reagent.

To assess the effect of MEDI-573 on IGF-induced proliferation, MEDI-573 or isotype control antibody was added to the cells and incubated for 30 minutes at 37°C. IGFII (75 ng/mL) or IGFII (50 ng/mL) was then added to the appropriate wells and incubated for 3 days. Cell proliferation was then quantified using the CTG reagent.

Assays for pIGFIR, pIR, and pAKT

The sarcoma lines were cultured overnight in 10% FBS growth medium. Medium containing 0.1% charcoal-stripped FBS was added the following day and incubated overnight. The next day, cells were treated with either IGFII (75 ng/mL) or IGFII (50 ng/mL) or IGFII/IGFII premixed with MEDI-573 for 5 minutes. After 15 minutes incubation, media were removed; cells were washed and lysed with 1.0% Triton X lysis buffer with protease and phosphatase inhibitors. Approximately 8 to 20 μg of total protein was loaded on MSD 96-well MULTI-SPOT plates and the level of total and phosphorylated IGFIIR and IR protein was determined using the Insulin Signaling Panel (total protein and phosphoprotein) Whole Cell Lysate kits (MSD) according to the manufacturer’s protocol. The level of total and phosphorylated AKT was determined using the Phospho (Ser473)/Total AKT Assay Whole Cell Lysate kit according to manufacturer’s standard protocol.

Xenograft studies in mice

All procedures using mice were approved by the MedImmune Institutional Animal Care and Use Committee according to established guidelines. For in vivo efficacy studies, 5 × 10^6 sarcoma cells in 50% Matrigel were inoculated subcutaneously into female athymic nude mice (Harlon Laboratories). When tumors reached approximately 150 to 200 mm^3, mice were randomly assigned into groups (10 mice per group). MEDI-573 was administered intraperitoneally (i.p.) twice per week at indicated doses. AZD2014 was given via oral gavage daily, whereas rapamycin was administered intraperitoneally every 3 days. Tumor volumes were measured twice weekly with calipers. Tumor growth inhibition was calculated on the last day of study relative to the initial and final mean tumor volume of the control group.

For in vivo mechanism of action studies, when tumors reached approximately 400 mm^3, a single dose of MEDI-573 (60 mg/kg) was administered. Tumor and plasma samples were collected 4 hours after dosing to assess the effect of MEDI-573 on autocrine IGF signaling. In another set of mice, 6 hours after administration of MEDI-573, human IGFII or IGFII was injected by tail-vein. Tumor and plasma samples were collected 15 minutes after IGF injection to assess the effect of MEDI-573 on IGFII- and IGFII-induced signaling.

Results

IGFI, IGFII, and IGFR levels and IR-A:IR-B ratio in primary sarcoma xenografts and sarcoma cell lines

mRNA levels of IGFII, IGFII, IGFR, and the IR-A:IR-B ratio in 20 primary sarcoma (Ewing sarcoma, osteosarcoma, and rhabdomyosarcoma) xenografts were determined by qRT-PCR (Fig. 1A). The mRNA levels of IGFII were significantly higher in Ewing sarcomas than in osteosarcomas (P = 0.029) and rhabdomyosarcomas (P = 0.0024). The mRNA levels of IGFII were significantly higher in rhabdomyosarcomas than in Ewing sarcomas (P = 0.0005) and osteosarcomas (P = 0.0066). All three types of sarcomas expressed equivalent mRNA levels of IGFR. The majority of sarcoma xenografts assayed had a high IR-A:IR-B ratio, with rhabdomyosarcomas being the highest. The IR-A:IR-B ratios were much higher than those reported previously in breast tumors (32).

We also screened sarcoma cell lines for IGFII, IR-A, and IGFII protein levels by ELISA (Fig. 1B). Consistent with the mRNA results from primary xenograft samples, Ewing sarcoma cell lines secreted the highest IGFII protein levels. The levels were almost equivalent to that in P12 cells, a MEF line engineered to overexpress human IGFII and IGFR. Although high levels of IGFII mRNA were detected in primary sarcoma xenografts (Fig. 1A) and cell lines (data not shown), only a few rhabdomyosarcoma cell lines and several osteosarcoma cell lines secreted IGFII protein, and then at only low levels. None of the Ewing...
Most sarcoma cell lines expressed detectable amounts of IGFII. The growth of three Ewing sarcoma cell lines (RD-ES, TC-71, and SK-ES-1) and one rhabdomyosarcoma cell line (SJCRH30) was inhibited by MEDI-573 in the absence of exogenous IGFI or IGFII. This indicated that growth of these cell lines was driven by endogenous IGFI or IGFII (Fig. 2A). A maximum of approximately 30% growth inhibition was also observed in RD and SJSA-1 cell lines (Fig. 2A). However, treatment with MEDI-573 did not have an effect on Hs729, KHOS, MG-63, SAOS2 sarcoma cell line proliferation (Supplementary Table S1).

The antiproliferative activity of MEDI-573 was also tested in a number of sarcoma cell lines that were stimulated with exogenous IGFs. Addition of IGFI or IGFII increased cell proliferation by approximately 2-fold in Ewing sarcoma cell lines (RD-ES, TC-71, and SK-ES-1) and osteosarcoma cell lines (MG-63, SAOS2; Fig. 2B). Treatment with MEDI-573 potently inhibited IGFI- and IGFII-stimulated proliferation of these cell lines. By comparison, treatment with MEDI-573 inhibited IGFI-stimulated proliferation (IC_{50} ranged from 0.29 to 5.4 μg/mL) more than IGFII-stimulated proliferation (IC_{50} ranged from 3.1 to 33.4 μg/mL; Supplementary Table S1). This correlates with the higher binding affinity of MEDI-573 for IGFII (K_d = 2 pmol/L) compared with IGFI (K_d = 294 pmol/L). Some cell lines, such as KHOS and RD, did not respond to IGFI or IGFII stimulation (Supplementary Table S1). Treatment with MEDI-573 did not have a significant effect in modulating the proliferation of KHOS and RD cell lines with or without exogenous IGFI stimulation; this indicated that IGFI signaling does not drive growth or survival in these nonresponsive cell lines.

To evaluate the mechanism for the antiproliferative effect of MEDI-573, the activation of caspase-3/-7 in RD-ES, SJSA-1, and KHOS cell lines was determined following treatment with MEDI-573 at increasing concentrations for 48 hours. In RD-ES and SJSA-1 cell lines, treatment with MEDI-573 induced activation of caspase-3/-7 in a dose-dependent manner compared with a negative, isotope-matched control antibody. In contrast, activation of caspase-3/-7 was not detected in KHOS cell lines (data not shown). The flow cytometry results for cell-cycle analysis indicated that MEDI-573 treatment increased the percentage of cells in G0–G1. The MEDI-573–treated RD-ES cells showed a typical apoptosis pattern of DNA content that reflected G0–G1, S, and G2–M phases of the cell cycle, together with a sub-G0–G1 phase (corresponding to apoptotic cells).

MEDI-573 inhibits tumor growth in sarcoma xenograft models

Treatment of mice bearing RD-ES (Ewing sarcoma) xenografts with MEDI-573 twice per week at dose levels of 10, 30, or 60 mg/kg resulted in tumor growth inhibition of 25%, 44%, and 52%, respectively (Fig. 3A). Treatment with MEDI-573 also resulted in tumor growth inhibition
Figure 2. MEDI-573 inhibits proliferation of sarcoma cell lines. A, no exogenous IGFs added. B, cells were stimulated with 75 ng/mL of IGF I or 50 ng/mL of IGF II and increasing concentrations of MEDI-573 (red), or an IgG2 isotype control antibody (black). After 3 days of incubation, cell proliferation was assessed using Cell Titer Glo assay and expressed as the percentage of those that were stimulated with IGFs only.
in the SJSA-1 xenograft model of osteosarcoma. Treatment with MEDI-573 did not affect the growth of KHOS cells in vivo (Fig. 3A), which was consistent with the in vitro results. Treatment with MEDI-573 was well tolerated in mice as no body weight loss was observed.

Free IGF ligand levels were measured in xenograft tumors harvested from untreated mice and mice treated with MEDI-573. In RD-ES xenograft tumors, MEDI-573 at a dose level of 10 mg/kg had no effect on free IGFI levels, but almost completely suppressed free IGFI levels at dose levels of 30 and 60 mg/kg (Fig. 3B). The levels of free IGFI were too low to be detected in RD-ES xenograft model. In contrast, SJSA-1 xenograft tumors had detectable levels of free IGFI, but not free IGFI. The free IGFI in SJSA-1 xenograft tumors was almost completely eliminated by MEDI-573 even at the lowest dose of 10 mg/kg due to the higher binding affinity of MEDI-573 for IGFI compared to IGFI. Although dose-dependent inhibition of IGFI levels in the KHOS/NP xenograft model was observed, free IGFI was still detected even at the highest dose level of 60 mg/kg MEDI-573. This level was higher than the baseline IGFI levels in SJSA-1 xenograft tumors (Fig. 3B). MEDI-573 treatment also inhibited tumor growth in TC-71 xenograft model, and complete suppression of free IGFI was observed in this model (data not shown). Although proliferation of SK-ES-1 and SJCRH30 cell lines was inhibited by treatment with MEDI-573 in vitro in the absence of exogenous IGFs (Fig. 2A), treatment with MEDI-573 did not affect the in vivo growth of these two models. This could attribute to the different levels of IGF ligands produced in vitro and in vivo. The levels of IGF secreted by SK-ES-1 and SJCRH30 cells in vitro were comparable with RD-ES cells (Fig. 1B). However, the baseline levels of IGFI or IGFI were very high in SK-ES-1 and SJCRH30 xenograft tumors. There was no complete knockdown of IGFs even at the highest dose of MEDI-573 (data not shown).

Inhibition of IGF signaling by MEDI-573

MEDI-573 inhibited autophosphorylation of IGFI, IR, and AKT in RD-ES, SK-ES-1, TC-71, and SJSA-1 but not in KHOS cell lines (Fig. 4A); this finding correlated with the antiproliferative effects of MEDI-573. When exogenous IGFI or IGFI was added to cells, phosphorylation of IGFI and IR was observed in all cell lines examined (Fig. 4B). Pretreatment with MEDI-573 inhibited IGFI or IGFI-induced activation of IGFI and IR. IGFI and IGFI also stimulated phosphorylation of AKT in RD-ES, SK-ES-1,
TC-71, and SJSA-1 cell lines; MEDI-573 blocked this effect. However, in KHOS cell line, activation of AKT was not observed although receptor phosphorylation was observed with IGFI and IGFII stimulation (Fig. 4B).

The effects of MEDI-573 on IGF signaling in vivo using sarcoma xenografts were also examined. To be consistent with in vitro experiments, in vivo pharmacodynamic studies were performed in two ways. First, effects of MEDI-573 on signaling that was induced by IGF ligands secreted by tumors in an autocrine manner were evaluated. A single dose of MEDI-573 was administered to mice bearing approximately 400 mm³ RD-ES, SJSA-1, or KHOS tumors. Autophosphorylation of AKT, 4EBP1, and S6K was examined by Western blot analysis. Both 4EBP1 and S6K are markers of mTOR activation. Treatment with MEDI-573 inhibited phosphorylation of AKT, 4EBP1 and S6K in RD-ES tumors (Fig. 5A) and SJSA-1 tumors (data not shown). In contrast, there was no change in the phosphorylation of any of these three proteins upon treatment with MEDI-573 in KHOS tumors (data not shown). Second, because adult mice do not produce IGFI and MEDI-573 has low binding affinity for mouse IGFI, we injected human IGFI or IGFII into mice in an attempt to model the effect of these ligands on signaling when delivered by endocrine or paracrine secretion. Fifteen minutes after IGFI or IGFII injection, high levels of IGFI or IGFII were detected in RD-ES tumor lysates and plasma. Pretreatment with MEDI-573 6 hours before ligand injection decreased the human IGFI level by approximately 50% in both tumor lysates and plasma, whereas the human IGFII level was reduced by more than 80% (Fig. 5B). Phosphorylation of AKT and S6K was increased upon IGFI or IGFII injection compared with mice that did not receive exogenous ligand (Fig. 5C). Pretreatment with MEDI-573 led to a dramatic reduction in IGF-induced pAKT and pS6K levels, particularly in the IGFII-injected mice. Administration of IGFI or IGFII did not change the baseline level of p4EBP-1. However, treatment with MEDI-573 caused a reduction of p4EBP-1 even below the baseline level.
MEDI-573 combined with mTOR inhibitors suppresses sarcoma cell proliferation in vitro and blocks signaling

The effects of MEDI-573 in combination with rapamycin or AZD2014, a dual inhibitor of mTORC1 and mTORC2, on cell proliferation were evaluated in a cytotoxicity assay. In RD-ES cells, treatment with MEDI-573 alone and rapamycin alone led to a 56% and 34% decrease in cell number compared with untreated control, respectively.

The combination treatment with MEDI-573 and rapamycin resulted in an 80% reduction in cell number ($P < 0.01$). AZD2014 as a single agent reduced cell number by 55% compared with untreated control, and the combination treatment with MEDI-573 and AZD2014 led to an 85% reduction in cell number ($P < 0.01$; Fig. 6A). MEDI-573 alone and in combination with either mTOR inhibitor (mTORi) had no effect on cell proliferation in KHOS cell line (Supplementary Table S1 and data not shown).

Figure 5. Effect of MEDI-573 on IGF signaling in vivo. A, Western blot analysis of pAKT, p4EBP1, and pS6K from three RD-ES xenografts in nude mice administered with single dose of MEDI-573 (60 mg/kg). GAPDH was used as loading control. B, analysis of free IGF1 and IGFII levels in tumor lysates and plasma. Mice bearing RD-ES xenografts were administered MEDI-573 at a dose level of 60 mg/kg or left untreated before recombinant IGF1 or IGFII was injected into mice. C, tumors from figure 5B were collected and analyzed for levels of pAKT, p4EBP1, and pS6K by Western blot analysis.
To examine the effect of MEDI-573, mTORi, and the combination of both on IGF signaling, RD-ES, SJSA-1, and KHOS cell lines were treated with these agents for 4 hours (Fig. 6B). MEDI-573 (30 µg/mL) alone inhibited phosphorylation of S6K and AKT in RD-ES and SJSA-1 cells, but not in KHOS cells. MEDI-573 had no effect on phosphorylation of 4EBP-1 in any cell line tested. Combination of MEDI-573 with rapamycin or AZD2014 resulted in complete inhibition of phosphorylation of S6K in RD-ES and SJSA-1 cell lines. Combination of MEDI-573 and AZD2014 resulted in a greater reduction of phosphorylation of 4EBP-1 compared with combination of MEDI-573 and rapamycin in the two responsive cell lines RD-ES and SJSA-1. Consistent with previous findings, treatment with rapamycin induced phosphorylation of AKT in all three cell lines tested. In the presence of MEDI-573, rapamycin-
induced AKT phosphorylation was significantly inhibited to baseline levels in RD-ES and SJSA-1 cells, but not in KHOS cells. As anticipated, AZD2014 did not induce AKT phosphorylation (Fig. 6B). These results indicate that the inhibition of tumor cell proliferation in vitro by MEDI-573, in combination with mTORi, correlates with their increased inhibition of the cognate signaling pathways. There was no detectable change in phosphorylation of ERK with MEDI-573 single-agent treatment or in combination with mTORi (data not shown).

**MEDI-573 combined with mTORi inhibits sarcoma tumor growth in vivo**

Treatment with MEDI-573 or AZD2014 as single agents resulted in a 52% and 51% tumor growth inhibition, respectively, in the RD-ES xenograft model. Combination treatment with these two agents led to further tumor growth inhibition (96%), which was statistically greater than either agent alone (\(P < 0.001\); Fig. 6C, left). Similarly, a combination effect was observed in SJSA-1 xenograft model (Supplementary Fig. S1). No combination effect was observed in the KHOS xenograft model (data not shown). The combination treatment was well tolerated as no body weight loss was observed.

A combination of MEDI-573 with rapamycin was also tested in the RD-ES xenograft model. Although this combination treatment had an enhanced antitumor activity (79% tumor growth inhibition) compared with either agent alone (59% for MEDI-573, and 44% for rapamycin), the effect was weaker than that seen with combining MEDI-573 with AZD2014 (Fig. 6C, right). The combination with rapamycin was also well tolerated as no body weight loss was observed.

Discussion

Insulin and IGF signaling have been extensively studied over the last several decades. Considerable preclinical evidence points toward the IGF pathway as an important regulator of tumor biology, justifying targeting this pathway for developing cancer therapies. Several IGFIR inhibitors have been tested in clinical trials for various cancers. Although early clinical results showed some evidence of response, larger randomized trials have not shown a clear clinical benefit to patients. Several companies have subsequently discontinued their anti-IGFIR programs. However, targeting IGFII and IGFII offers a different approach that may avoid adaptive responses such as upregulation of insulin receptor and circulating IGFII or IGFII levels that arise from targeting receptor directly (10, 33). MEDI-573 targets IGFII and IGFII and thereby inhibits IGFII-mediated signal transduction through both IGFIR and IR-A. Preclinical studies conducted with human cancer cells suggest that MEDI-573 has the potential to achieve broad antitumor efficacy owing to its ability to inhibit both IGFIR and IR-A pathways. Furthermore, MEDI-573 has the potential to achieve this without perturbing glucose homeostasis, which is an adverse effect observed with drugs that target IGFIR directly. Currently, MEDI-573 is being evaluated in a phase II clinical trial for metastatic breast cancer.

In this study, we have shown that MEDI-573 effectively inhibited the growth of several sarcoma cell lines. Dose-dependent growth inhibition was most obvious in three Ewing sarcoma cell lines (RD-ES, SK-ES-1, TC-71) examined with or without addition of IGFII and IGFII. All three cell lines secrete high levels of IGFII in vitro. Following implantation of RD-ES cells into athymic nude mice, there was a detectable level of IGFII, but not IGFII, in the xenografts. Because mice do not express IGFII, this result demonstrates that the growth of the RD-ES xenograft model is driven by IGFII only. MEDI-573 significantly inhibited the growth of RD-ES tumors in vivo by completely neutralizing the IGFII. This in vivo activity of MEDI-573 is a consequence of inhibiting autocrine signaling initiated by human IGFII produced by the tumor, as MEDI-573 has low cross-reactivity to mouse IGFII, which can also activate human IGFIR at comparable levels as human IGFII. We show that RD-ES tumors responded to exogenously added IGFII and IGFII, and that MEDI-573 efficiently blocked this response (Fig. 5). Therefore, paracrine or endocrine production of IGFs may also play a role in stimulating the growth of RD-ES tumors. MEDI-573 may have a profound antitumor activity in human by inhibiting IGFs that function in autocrine, paracrine, and endocrine manner. In contrast, the osteosarcoma cell line SJSA-1 secretes both IGFII and IGFII in vitro, but only IGFII in vivo. SJSA-1 cells did not respond to exogenously added IGFs. Therefore, the growth of SJSA-1 tumors was mainly driven by autocrine production of IGFII. MEDI-573 completely neutralized IGFII and inhibited the growth of SJSA-1 tumors.

The functional activity of MEDI-573 on the growth of sarcoma cells correlated well with its effect on IGF signaling. MEDI-573 inhibited both IGFII- and IGFII-stimulated phosphorylation of IGFIR, IR, and the downstream signaling protein AKT in the responsive sarcoma cell lines. Furthermore, MEDI-573 inhibited autocrine phosphorylation of these signaling molecules. In the nonresponsive KHOS model, MEDI-573 did not inhibit activation of IGF signaling stimulated by autocrine IGFs. Although exogenous IGFII and IGFII induced phosphorylation of IGFIR and IR in KHOS cells, there was no activation of AKT, and therefore no effect on growth of KHOS cells. Thus, neither autocrine nor paracrine/endocrine production of IGFs is a major driver in KHOS cell tumorigenesis.

One interesting finding from our study was that MEDI-573 was more active in inhibiting IGFII-stimulated than IGFII-stimulated cell proliferation (Fig. 2B and Supplementary Table S1). This is likely due to the higher binding affinity of MEDI-573 for IGFII than IGFII. IGFII activates IR-A with greater affinity than IGFII. It has been shown that the IGFII/IR-A pathway plays a predominant role in certain types of cancer, such as colon and breast cancers (34, 35). Our data suggested that MEDI-573 would be
effective in treating those types of cancer. Moreover, because overexpression of IGFI and IR-A is responsible for resistance to IGFIR–targeting therapies (36, 37), MEDI-573 treatment may be able to overcome resistance to anti-IGFIR agents.

One possible reason for the disappointing results with IGFIR targeting agents in clinical studies could be the biologic complexity as compensatory pathways are turned on when IGF signaling pathways are disrupted. For example, treatment with cixutumumab (an anti-IGFIR antibody) has been found to activate AKT and mTOR, induced expression of EGFR, Akt1, and survivin proteins, and activated the EGFR pathway (38). Thus, an optimal combination strategy to use with IGF pathway inhibition needs to be defined. The combination of IGFIR inhibitors with mTORC1 inhibitors such as rapamycin has been validated in vitro and in vivo as a potential therapeutic strategy to treat patients with sarcoma. The results of early clinical trials revealed that combining IGFIR and mTORi has some clinical benefits in Ewing sarcoma (39). Our data showed that blocking rapamycin-induced AKT activation by MEDI-573 was associated with enhanced efficacy in RD-ES and SJSA-1 sarcoma models. This supports the idea of combining inhibition of IGF and PI3K/AKT/mTOR signaling pathways. In addition, we showed that combining MEDI-573 with AZD2014, which inhibits both mTORC1 and mTORC2, provided best inhibition of pAKT compared with MEDI-573 in combination with rapamycin. MEDI-573 in combination with AZD2014 also led to a greater knockdown of p4EBP-1 and pS6K in RD-ES and SJSA-1 models. Whether combining IGF1, IGFII, and IGFIR inhibition would provide improved clinical benefits compared with combining IGFIR and mTORC1 inhibition needs to be further tested in the clinic. In addition to PI3K/AKT inhibitors, other rational combinations could include EGFR/HER2 or HSP90 inhibitors, which have been implicated as resistance mechanisms to IGFIR inhibition in sarcoma cell lines (40, 41).

Despite the promising clinical response reported in phase I trials of IGFIR targeting antibodies in patients with Ewing sarcoma, phase II trials suggested approximately 10% objective response, indicating not all patients benefit from IGFIR inhibition. Therefore, there is a need to identify patient selection strategies. In clinical trials of IGFIR-targeting agents, predictive biomarkers were not used as none had been defined when trials were initiated. Recently, a phase III trial testing ganitumab (anti-IGFIR antibody) was terminated because of lack of efficacy in patients with metastatic pancreatic cancer. However, subset analysis from phase II trial data showed patients with high circulating levels of IGFI, IGFII, or IGFBP-3, or low levels of IGFBP-2, had improved median overall survival (42). Baseline circulating factors related to the IGF signaling axis may predict a clinical response to ganitumab in patients with metastatic pancreatic adenocarcinoma. However, this hypothesis needs to be further validated.

In summary, we have shown that IGF1 and IGFII play important roles in the growth of different types of sarcomas. MEDI-573 efficiently neutralizes IGF1 and IGFII and inhibits tumor growth in sarcoma mouse xenograft models. In the RD-ES Ewing sarcoma model, the in vivo efficacy of MEDI-573 was significantly enhanced when used in combination with mTORi. In addition, our data also demonstrate the effect of MEDI-573 used as a single agent or in combination with mTORi on downstream signaling. MEDI-573 was most active in Ewing sarcoma models. These results support the consideration of clinical evaluations of MEDI-573 alone or in combination with mTORi for treatment of Ewing sarcoma.

Disclosure of Potential Conflicts of Interest

J. Huang received a commercial research grant from Medimmune. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: H. Zhong, C. Chen, J. Huang, Y. Yao, R.E. Hollingsworth
Development of methodology: H. Zhong, C. Chen, J. Huang, Y. Yao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Fazenbaker, S. Breen, C. Chen, Y. Yao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Zhong, C. Fazenbaker, S. Breen, C. Chen, J. Huang, C. Morehouse, Y. Yao
Writing, review, and/or revision of the manuscript: H. Zhong, C. Fazenbaker, C. Chen, Y. Yao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Zhong, C. Chen, Y. Yao
Study supervision: H. Zhong, R.E. Hollingsworth

Acknowledgments

The authors thank Dr. Peter Houghton for generously providing us with the primary sarcoma xenografts and Dr. Dirk Mendel for insightful comments and critical review of the article.

Grant Support

This work was supported by Medimmune.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 20, 2014; revised August 24, 2014; accepted August 24, 2014; published OnlineFirst September 5, 2014.

References


Correction: MEDI-573, Alone or in Combination with Mammalian Target of Rapamycin Inhibitors, Targets the Insulin-like Growth Factor Pathway in Sarcomas

In this article (Mol Cancer Ther 2014;13:2662–73), which appeared in the September 2014 issue of Molecular Cancer Therapeutics (1), the acknowledgments section was incomplete. The corrected acknowledgments section is below. The authors regret the error.

The authors thank Dr. Peter Houghton for generously providing the primary sarcoma xenografts; Dr. Dirk Mendel for insightful comments and critical review of the article; Drs. Sylvie Guichard, Teresa Klinowska, and Stephen Green from AstraZeneca’s AZD2014 team for providing AZD2014 and helpful discussion; and Karen Mitz from MEDI-573 Product Development Team for reviewing the article.

Reference

Published online March 10, 2015.
doi: 10.1158/1535-7163.MCT-15-0014
©2015 American Association for Cancer Research.
Molecular Cancer Therapeutics

MEDI-573, Alone or in Combination with Mammalian Target of Rapamycin Inhibitors, Targets the Insulin-like Growth Factor Pathway in Sarcomas

Haihong Zhong, Christine Fazenbaker, Shannon Breen, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0144

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/09/06/1535-7163.MCT-14-0144.DC1

Cited articles
This article cites 40 articles, 19 of which you can access for free at:
http://mct.aacrjournals.org/content/13/11/2662.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/13/11/2662.full#related-urls

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

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

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/13/11/2662.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.