Pharmacodynamic and antineoplastic activity of BI 836845, a fully human IGF ligand neutralizing antibody, and mechanistic rationale for combination with rapamycin

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Abbreviations
DMEM, Dulbecco’s modified Eagle’s medium; EC₅₀, half-maximal effective concentration; EGF, epidermal growth factor; GAPDH, glycerinaldehyd-3-phosphate-dehydrogenase; GH, growth hormone; HRP, horse radish peroxidase; IGF, Insulin-like growth factor; IGF-1R, Insulin-like growth factor 1 receptor; IGFBP, IGF binding protein; InsR, insulin receptor; i.p., intraperitoneal; IR-A, insulin receptor variant A; Kᵣ, dissociation constant; mAb, monoclonal antibody; MMRM, Mixed Model for Repeated Measurements; mTORC1, mammalian target of rapamycin complex 1; n.d., non-detectable; NEAA, non-essential amino acids; NSCLC, non-small cell lung cancer; pAKT, phosphorylated AKT; PMSF, phenylmethylsulfonylfluorid; RT, room temperature; SCLC, small cell lung cancer; TGI, tumor growth inhibition; TMB, tetramethylbenzidine
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ABSTRACT:

Insulin-like growth factor (IGF) signaling is thought to play a role in the development and progression of multiple cancer types. To date, therapeutic strategies aimed at disrupting IGF signaling have largely focused on antibodies that target the IGF-1 receptor. Here, we describe the pharmacologic profile of BI 836845, a fully human monoclonal antibody that utilizes an alternative approach to IGF signaling inhibition by selectively neutralizing the bioactivity of IGF ligands. Biochemical analyses of BI 836845 demonstrated high affinity to human IGF-1 and IGF-2, resulting in effective inhibition of IGF-induced activation of both IGF-1R and IR-A in vitro. Cross-reactivity to rodent IGFs has enabled rigorous assessment of the pharmacologic activity of BI 836845 in preclinical models. Pharmacodynamic studies in rats showed potent reduction of serum IGF bioactivity in the absence of metabolic adverse effects, leading to growth inhibition as evidenced by reduced body weight gain and tail length. Moreover, BI 836845 reduced the proliferation of human cell lines derived from different cancer types and enhanced the antitumor efficacy of rapamycin by blocking a rapamycin-induced increase in upstream signaling in vitro as well as in human tumor xenograft models in nude mice. Our data suggest that BI 836845 represents a potentially more effective and tolerable approach to the inhibition of IGF signaling compared with agents that target the IGF-1 receptor directly, with potential for rational combinations with other targeted agents in clinical studies.
Introduction

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are structurally related polypeptides that promote cell growth and survival (1). In normal physiology, IGFs have key roles in the control of cellular proliferation and survival and of organism growth (2). Their concentrations in blood are physiologically regulated and reflect hepatic production, but IGFs are also expressed locally in many tissues in a paracrine or autocrine manner (3). Tissue IGF bioactivity varies not only with circulating ligand concentration and with local ligand production, but also with the concentrations of the various serum IGF-binding proteins (4;5).

IGF-1 signals by binding to the IGF-1 receptor (IGF-1R) or to hybrids of IGF-1R and insulin receptors (InsR) (1;6). IGF-2 initiates signaling via these same receptor species, but can also activate the A isoform of the InsR (IR-A) (7;8). This is of particular interest from an oncologic perspective as IR-A is preferentially expressed in fetal and cancer cells (9;10). In contrast to members of the epidermal growth factor (EGF) family, which are frequently amplified and/or mutationally activated in neoplasia, resulting in ligand-independent receptor activation, IGF-1R/InsR signaling always requires active ligands.

More than a decade of preclinical research has provided a rationale for targeting IGF signaling in the treatment of cancer (1;11). Oncogenic transformation of mouse embryonic fibroblasts, for instance, has been described to be dependent on the expression of IGF-1R (12). Expression of IGF-2 by transformed cells has been observed (13) and this can lead to activation of IGF-1R in an autocrine manner. In many preclinical cancer models, tumor growth can be stimulated by IGF-1 or IGF-2, and tumors grow substantially slower in mice carrying a mutation that reduces IGF ligand levels (4). Moreover, a separate line of indirect evidence for the relevance of IGF signaling for neoplasia comes from the observation that there is considerable interindividual variation in circulating IGF-1 levels, and that risk and prognosis of certain cancers is related to circulating IGF-1 concentration (2;14;15). Finally, IGF signaling may also play a role
as a resistance mechanism which limits the effectiveness of cytotoxic or targeted anti-cancer agents, including rapalogs (16-21).

This preclinical evidence has motivated the development of several IGF-1 receptor-targeted mAbs (22-24). However, efficacy of anti-IGF-1R antibodies has been disappointing in clinical trials to date (25-30). One possible explanation for these results is that in clinical use, drugs studied to date do not adequately inhibit IGF signaling, especially in the context of the need to block autocrine loops in IGF-dependent cancers (31-33), particularly those driven by IGF-2, which can also signal via IR-A (34). For the alternative approach of targeting all receptor species in the insulin/IGF tyrosine kinase receptor family using small molecule inhibitors, the dosing of these agents must be well balanced to avoid metabolic toxicity and it remains to be demonstrated that clinical use at tolerable doses leads to adequate receptor blockade in neoplastic tissue.

A third therapeutic strategy is to target the IGF ligands rather than the receptors, aiming to abrogate signaling via IGF-1R and IR-A as well as their hybrid receptors, without impact on circulating insulin and glucose levels and their metabolic functions (30). Two agents, BI 836845 and MEDI-573 (35), with this mode of action have been developed. Here, we describe the pharmacological activity of BI 836845, a fully human monoclonal antibody that neutralizes the activities of both IGF-1 and IGF-2. In contrast to MEDI-573, BI 836845 cross-reacts with mouse and rat IGF-1 and IGF-2, allowing a comprehensive in vivo characterization of the pharmacodynamic properties of the antibody in preclinical rodent models.
Materials and Methods

Reagents and Cell Lines

BI 836845 was isolated by selection of specific Fab fragment clones from the human combinatorial antibody phage display library (HuCAL Gold) (36) that bind human IGF-1 with low nanomolar affinity, in three panning cycles as previously described (37) and subsequently subjected to in vitro affinity maturation (38). IGF-1R antibodies used were αIR-3 (Calbiochem, No. GR11L) and one which was generated based on sequence ID 2.13.2 of the patent WO 02/053596 A2 (termed IGF-1R mAb). Other reagents included rapamycin (LC laboratories, Woburn, MA, USA), and purified IGF-1 (CM001) and IGF-2 (FM001; GroPep Bioreagents Pty Ltd, Thebarton, Australia).

Recombinant mouse cell lines expressing human IGF-1R and IR-A, respectively, were kindly provided by R. Vigneri (University of Catania, Italy). GEO cell line (originally established by M. Brattain (Roswell Park Cancer Institute, Buffalo, NY, Baylar College of Medicine, Houston, TX ) was obtained from S. Pepe (Università degli Studi di Napoli, Italy). RD-ES (ATCC #HTB-166), SK-ES-1 (ATCC, #HTB-86), and all other cell lines used were purchased from ATCC, DSMZ, JCRB, or the Memorial Sloan-Kettering Cancer Center. Cell line authentication was conducted in-house or by the provider using short-tandem-repeat PCR. Upon delivery, cell lines were expanded and low-passage vials stored in liquid nitrogen. Experiments were carried out within 12 weeks after resuscitation.

Biacore analysis

Binding affinity (K_D) was determined using a Biacore™ 3000 high-resolution surface plasmon resonance system in HBS-EP buffer + 1 mg/mL CM-Dextran and 0.25 mg/mL bovine serum albumin as running buffer at a flow rate of 20 µL/min. The sensor chip was coated with approximately 1000 RU of an unspecific reference antibody in flow cell 1 and approximately
1000 RU of a rabbit-anti-human Fc-gamma-specific antibody in flow cell 2 using reagents from an amine coupling kit. The test antibody was captured by running a 1 µg/mL solution over the sensor surfaces for 3 min. IGF antigens were diluted to 500, 250, 125, 62.5 and 31.3 nM and measured in random order using 5 min for association and dissociation. Data evaluation was performed using the BIAevaluation software, version 4.1. Biacore 3000 instrument; HBS-EP buffer, amine coupling kit and BIAevaluation software were provided by Biacore/GE Healthcare, Freiburg, Germany.

**Cell-Based IGF-1R and IR-A Phosphorylation Assays**

Mouse embryonic fibroblast cell lines derived from IGF-1R-deficient mice and engineered to overexpress human IGF-1R or human IR-A were used to measure IGF bioactivity, defined here as the ability of a sample (serum, plasma, cell culture media) to stimulate receptor phosphorylation as determined by ELISA. The cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 0.075% sodium bicarbonate, non-essential amino acids (NEAA; Gibco), and 0.3 µg/mL puromycin at 37°C and 5% CO₂. The ELISA was performed following standard procedures. Briefly, 10,000 cells/well were seeded on 96-well plates followed by a 24-hour incubation. Cells were starved in medium with 0.5% FBS overnight before a dilution range of BI 836845 or IGF-1R antibodies was added to the cells, followed by either IGF-1 (20 ng/mL), IGF-2 (100 ng/mL) or a human serum pool (20%). Cells were incubated for 30 min at 37°C and 5% CO₂ and subsequently fixed in 4% formaldehyde/PBS (20 min, room temperature (RT)), quenched (1.2 wt% hydrogen peroxide in wash buffer, 30 mins, RT) and blocked (5% BSA in wash buffer, 60 min, RT with agitation). Subsequently, cells were incubated with primary antibody (phospho-IGF-1R β (tyr1135/1136)/insulin receptor β (tyr1150/1151) antibody; Cell Signaling #3024, 1:1,000 in blocking buffer, incubation overnight at 4°C with agitation) and secondary antibody (anti-rabbit IgG goat immunoglobulins conjugated with horseradish peroxidase (HRP); Dako #P0448, 1:500
in blocking buffer for 60 min at RT with agitation) followed by substrate solution (tetramethylbenzidine (TMB; Bender MedSystems #BMS406.1000), for 5-20 min with agitation). Finally, the reaction was stopped by adding 1 M phosphoric acid and the absorbance was read using a photometer (OD 450 nm, OD 650 nm as reference).

**Rat Studies**

Six to eight week-old male rats (Crl:WI[Han]) were treated intravenously once weekly for 13 weeks with 20, 60, or 200 mg/kg of BI 836845 and compared to vehicle-treated animals. The treatment phase was followed by a 12-week recovery phase. Plasma samples were taken at time points prior to, during, and after treatment. Rat body weight was measured twice weekly throughout the study and tail length at the end of the treatment period.

**Xenograft Studies**

Tumors (< 100 mm³) were established from cultured GEO or RD-ES cells (5 x 10⁶ in matrigel) by subcutaneous injection into the right flanks of female BomTac:NMRI-Foxn1nu mice. Animals were randomized (n=7/group) and treated with vehicle (0.5% Natrosol) or rapamycin (5 mg/kg) intraperitoneally once a day for five consecutive days per week, twice weekly with BI 836845 (100 mg/kg), or a combination of both agents. Tumor volumes were determined three times a week using a digital caliper. Body weights of the mice were measured daily as an indicator of tolerability of the compounds. Four hours after the last treatment (day 20), serum was sampled and RD-ES tumors were explanted, lysed in Bio-Plex Cell Lysis Kit (BioRad #171-304012) containing 2 mM PMSF, and analyzed for AKT phosphorylation by Western blotting (n=4/treatment group). Rapamycin treatment of non tumor-bearing mice for one week and subsequent serum sampling was performed similarly.
Additional Materials and Methods

2D cell viability assays, Western blot and qRT-PCR analyses (primers/probes listed in Supplementary Table 2), and determination of total rat serum IGF-1 levels and ex vivo IGF-1 bioactivity are described in the Supplementary Materials and Methods.

Statistics

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software Inc.). Control (pre-treatment or vehicle-treated animals) and treatment groups were compared in one-way analyses of variance (ANOVA) followed by a Tukey’s post test if not indicated otherwise. (p) values of less than 0.05 (*), 0.01 (**) and 0.001 (***), were considered to be statistically significant.
Results

**Biochemical Characterization of BI 836845**

BI 836845 is a fully human monoclonal antibody selected from a naïve phage display library and affinity optimized for IGF-1 binding. As shown in Supplementary Table 1, surface plasmon resonance analysis demonstrated that in addition to the high affinity for human IGF-1 (0.07 nM) BI 836845 also shows high affinity for human IGF-2 (0.8 nM). BI 836845 was also shown to strongly cross-react with mouse and rat IGF-1 and IGF-2 (Supplementary Table 1). No binding to human insulin was detected at up to 100-fold higher antibody concentrations (data not shown).

In order to measure IGF bioactivity (IGF-1R or IR-A kinase activating activity) in serum, plasma, or cell culture media, we developed a cell-based assay utilizing mouse fibroblasts engineered to express human IGF-1R or IR-A, in which receptor phosphorylation can be quantified by ELISA. This assay was initially used to determine the potency and effectiveness of BI 836845 in neutralizing recombinant IGF-1 and IGF-2. As shown in Figure 1A and 1B, BI 836845 inhibited both IGF-1 (20 ng/mL) and IGF-2 (100 ng/mL) induced IGF-1R phosphorylation with an IC₅₀ of 90 ng/mL (0.6 nM) and 1120 ng/mL (7.5 nM), respectively. In the same assay, two IGF-1R mAbs were less effective with respect to IGF-1-induced activation and displayed only weak effects on IGF-2-induced activation with more than 30% of IGF-2-induced IGF-1R phosphorylation remaining even at an antibody concentration of 100 nM (Figure 1B). Figure 1C demonstrates that BI 836845 also potently inhibited IGF-2 (100 ng/mL)-induced IR-A activation with an IC₅₀ of 815 ng/mL (5.4 nM), unlike IGF-1R-targeted antibodies which had no effect. When cell culture medium containing 20% human serum was used to stimulate receptor phosphorylation, this could be completely neutralized by BI 836845 with an IC₅₀ of 246 ng/mL (1.6 nM). By contrast, an IGF-1R-targeted antibody showed only partial inhibition of IGF bioactivity (Figure 1D).
Pharmacodynamic Effects of BI 836845 in Vivo

The cross-reactivity of BI 836845 to mouse and rat IGF-1 and IGF-2 has allowed a comprehensive preclinical characterization of the pharmacodynamic properties of the antibody in these species. Pharmacodynamic effects of BI 836845 were studied in 6 - 8 week-old rats treated once weekly for 13 weeks with 20, 60, or 200 mg/kg of the antibody (last treatment on day 85). Treatment with BI 836845 at all doses levels was well tolerated and resulted in a clear reduction in plasma IGF bioactivity as determined ex vivo by the bioassay described above (Figure 2A). These effects of BI 836845 on plasma IGF bioactivity were seen despite a corresponding increase in total IGF-1 plasma levels. Figure 2B shows that treatment with BI 836845 resulted in elevated total IGF-1 from day 3 onwards, with up to 25-fold increases seen for the groups treated with 60 and 200 mg/kg. Following cessation of treatment, a dose-dependent return of both plasma IGF bioactivity (Figure 2A) and total IGF-1 levels (Figure 2B) toward control levels was seen by day 169. A dose-dependent effect of BI 836845 treatment was observed on rat body weight gain (Figure 2C). Compared with controls, the 60 and 200 mg/kg doses showed a similar reduction in body weight gain, which was less pronounced for the 20 mg/kg dose level (Figure 2C). To confirm that the effect of BI 836845 on body weight was due to a reduction in body growth, tail length was monitored. Figure 2D shows that there was a dose-dependent reduction in tail length compared to vehicle controls on day 85 of the study. In addition to these findings, we noted that essentially all organ weights were reduced. Moreover, we observed a reduction in secondary spongiosa of bones, where IGF-1 is known to play an important role (39) (data not shown).
Antineoplastic Activity of BI 836845 in Cancer Models

BI 836845 was assayed for \textit{in vitro} growth inhibitory activity in cancer-derived cell lines grown in 10\% FBS. The proliferation of several cell lines derived from different cancer types, including non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), Ewing’s sarcoma, and multiple myeloma, was found to be potently inhibited by BI 836845 (low nM EC\textsubscript{50} values; Table 1). Approximately one third of the responsive cell lines showed elevated levels of IGF-1 and/or IGF-2 mRNA expression compared to median expression of 625 cell lines (examples are shown in Figure 3A), indicating that BI 836845 is capable of attenuating \textit{in vitro} proliferation of cell lines showing autocrine ligand production. The colorectal carcinoma cell line GEO showed a particularly high level of IGF-2 expression (50-fold over median, Figure 3B), which was associated with constitutive IGF pathway activation in tumors \textit{in vivo} (Figure 3C). In addition, we assessed mRNA expression of InsR and its variants by qRT-PCR and identified expression of both InsR splice variants in GEO cells (Supplementary Figure 1A). In contrast, the Ewing’s sarcoma cell line RD-ES exclusively expressed the IR-A variant (Supplementary Figure 1C), in line with previously published findings (40). In both cell lines, no insulin mRNA expression was detectable (Supplementary Figure 1A and 1C) and InsR mRNA levels were significantly lower than those of IGF-1R (Supplementary Figure 1B and 1D). This difference was also reflected at the protein level in RD-ES cells \textit{in vitro} and \textit{in vivo} (Supplementary Figure 1E). The GEO cell line was established as a subcutaneous xenograft model in immunodeficient (nude) mice to determine if BI 836845 could inhibit the \textit{in vivo} growth of a tumor with high IGF-2 expression. Figure 3D shows that treatment with 100 mg/kg of BI 836845 twice weekly resulted in 54\% tumor growth inhibition (TGI) after 27 days of treatment.
**BI 836845 Enhances the Antineoplastic Efficacy of Rapamycin**

Previous studies have shown improved efficacy when combining IGF-1R mAbs with rapamycin (41;42). We were therefore interested in assessing whether the combination of an IGF ligand-neutralizing antibody with rapamycin would be an effective antineoplastic strategy. We therefore tested the combined effect of BI 836845 and rapamycin on the viability and signaling of two BI 836845-sensitive Ewing’s sarcoma-derived cell lines, SK-ES-1 and RD-ES. Cell viability studies *in vitro* demonstrated that the combination of BI 836845 and rapamycin was more potent and effective than either single agent at inhibiting proliferation of both cell lines (Figure 4A and 4C; p<0.001 at concentrations ≥ 12.33 and ≥ 0.1 nM, respectively). To investigate the molecular mechanism behind the improved efficacy seen when both agents were combined, we assessed AKT phosphorylation levels (serine 473; pAKT) as a biomarker reflecting the consequences of rapamycin-induced relaxation of an inhibitory feedback loop (43). Treatment of SK-ES-1 (Figure 4B) and RD-ES cells (Figure 4D) with 100 nM rapamycin resulted in increased pAKT compared with untreated cells. When BI 836845 was combined with rapamycin phosphorylation of AKT was inhibited, indicating that the feedback increase in pAKT in the presence of rapamycin is dependent on IGF ligand-driven signaling (Figures 4B and 4D). The inhibitory effects on cell proliferation of the combination versus each single agent were tested in 60 cancer cell lines (Supplementary Figure 2A). In 13 out of 17 cell lines derived from different cancer types, the effects on proliferation were consistent with the effects on AKT phosphorylation (Supplementary Figure 2B).

An RD-ES xenograft model was used to determine if the additive effects of BI 836845 and rapamycin seen *in vitro* could be recapitulated *in vivo*. The combination of BI 836845 (twice weekly, 100 mg/kg i.p.) and rapamycin (5 x weekly, 5 mg/kg i.p.) was more effective (TGI 93%) at inhibiting tumor growth than either single agent (rapamycin TGI 75%, BI 836845 TGI 53%) after 20 days of treatment (Figure 5A). Single agent treatments were significantly different from control treatment from day 6 onward (p < 0.05 for rapamycin and p < 0.01 for BI 836845 on day...
6), while combination treatment differed significantly from controls from day 3 onward (p < 0.05 on day 3). Differences between single agent treatments and combination treatment were statistically significant from day 8 onward (p < 0.05 on day 8). Further analyses of pAKT levels in the RD-ES tumors showed that rapamycin increased pAKT levels and that this increase could be inhibited by BI 836845 (Figure 5B). IGF bioactivity in the serum of RD-ES tumor-bearing mice was increased upon rapamycin treatment compared to control mice (Figure 5C). BI 836845 treatment alone, and in combination with rapamycin, completely inhibited IGF bioactivity in plasma (Figure 5C). To investigate the contribution of RD-ES tumors to the elevated levels of plasma IGF bioactivity, we treated non-tumor-bearing mice similarly and found that rapamycin induced a significant increase in plasma IGF bioactivity comparable to that seen in the RD-ES tumor-bearing mice (Figure 5D).
Discussion

BI 836845, a fully human monoclonal antibody, blocks the activation of IGF-1R and IR-A by binding to and neutralizing both IGF ligands. The preclinical data presented in this study contributed to the rationale for a clinical trial program, and phase I studies are now in progress.

The mechanism of action of BI 836845 is distinct from that of IGF-1R-targeted antibodies which to date have proved disappointing in clinical studies (30). By binding to and neutralizing the IGF ligands, BI 836845 blocks activation not only of the IGF-1R, but unlike anti-IGF-1R antibodies also reduces IGF-2-stimulated activation of IR-A. This may be an important advantage, as the expression of this receptor variant is often increased in tumor cells (7;8;10;44) and may play a role in autocrine IGF-2-mediated growth stimulation (34;40). In addition, some IGF-1R antibodies have been shown to be weak inhibitors of IGF-2-driven IGF-1R signaling, which is thought to be due to differences in the binding sites of IGF-1 and IGF-2 on the IGF-1 receptor (45).

The rodent models presented here are informative not only with regards to in vivo efficacy but also to tolerability of BI 836845, as this antibody blocks the bioactivity of both human and rodent IGF ligands. Long term administration of BI 836845 to rats was well tolerated at doses sufficient to reduce somatic growth. Growth inhibition of rats was associated with a substantial decrease of serum IGF bioactivity, a relevant pharmacodynamic endpoint. This was achieved in the presence of significantly elevated total IGF-1 levels in the plasma following BI 836845 treatment. A plausible explanation for the increase in total serum IGF-1 relates to a compensatory increase in growth hormone (GH) secretion, resulting in increased hepatic IGF-1 expression (1). While we did not observe a significant increase in GH levels in BI 836845-treated rats, this does not exclude this mechanism, as the pulsatile nature of GH secretion limits the utility of single GH measurements (data not shown). A similar phenomenon has been observed with the anti-VEGF ligand targeting approach of bevacizumab, where following treatment serum VEGF levels have been found to be increased, presumably as a result of an increase in VEGF...
synthesis and/or a decrease in VEGF clearance caused by complex formation between VEGF and the antibody (46).

In human cancers, autocrine expression of IGF-1 or IGF-2 is not a rare event (2). It is unlikely that this represents a random deregulation of gene expression. Rather, a malignant clone that expresses both a growth factor and its receptor will likely have a proliferative advantage that will be selected for. Thus, autocrine expression of IGF may indicate a cancer that is responsive to and/or dependent on IGF-1R activation (1). However, this does not necessarily imply that such tumors will be responsive to any particular IGF targeting strategy. Interruption of an autocrine loop in vivo requires a high drug concentration in the tumor, and it is conceivable that pharmacokinetic or tolerability issues may limit efficacy of IGF-targeting therapies even for the subset of cancers that depend on IGF stimulation. Therefore, it was of interest to observe that BI 836845 was capable of significantly inhibiting not only in vitro proliferation of IGF-secreting cell lines but also the in vivo growth of a colorectal cancer model that expresses high levels of IGF-2. Despite the important pharmacodynamic information that has been generated from the profiling of BI 836845 in rats and mice, the translational limitation of using rodent models for studying IGF physiology is that they express far less IGF-2 than humans (47), and this needs to be taken into consideration when interpreting such studies in rodents.

Besides being potential drivers of tumor growth in certain subsets of cancer, IGFs may contribute to resistance mechanisms limiting the efficacy of other anti-cancer agents. Previous studies have shown a potential for IGF targeting in rational combinations, such as IGF-1R and IGF ligand antibodies (48), or combinations of IGF-1R mAbs with rapamycin to more efficiently block angiogenesis (41;42). We studied the combination of BI 836845 with rapamycin in view of these prior preclinical and clinical data indicating that the efficacy of rapalogs may be limited because inhibition of mTORC1 results in AKT activation due to IGF-1R-dependent interference with negative feedback loops constraining signaling downstream of the IGF-1R (21;43;49;50). Our in vitro and in vivo findings provide evidence that the activity of rapamycin can be improved
by co-administration of BI 836845 by abolishing AKT activation, suggesting a rational combination that deserves clinical evaluation. Apart from the known cell-autonomous actions (43;49), we observed a novel homeostatic response to mTORC1 inhibition at the whole organism level, characterized by increases in serum IGF-1R activating activity, a phenomenon observed in nude mice in the presence or absence of xenograft tumors. This response was clearly attenuated by BI 836845, suggesting that rapamycin-induced AKT activation is dependent on IGF ligand activity (mechanistic model depicted in Supplementary Figure 3). Interestingly, a similar effect was seen in a study using vascular smooth muscle cells (51). However, the precise definition of the mechanism by which rapamycin increases serum IGF bioactivity requires additional investigation and is an active area of research. Similarly, the evaluation of angiogenesis and proliferation markers, such as CD34 and Ki67, will be important since enhanced anti-angiogenic effects may also play a role in explaining the improved efficacy of the combination (48).

Despite the recent clinical setbacks with agents targeting the IGF-1R (30), the data presented here support the differentiated therapeutic concept of IGF-1/2 ligand neutralization which warrants clinical investigation. Preclinical antiproliferative activity was observed with BI 836845 both in vitro and in vivo, and long term exposure at levels sufficient to abolish IGF serum bioactivity and strongly attenuate somatic growth was not associated with significant toxicity in rats. The results presented also provide clues that may be relevant to the design of phase II clinical trials. Presuming that dosing of BI 836845 will not be limited by toxicity, a crucial issue will be defining a phase II dose. Our preclinical studies have identified measurable pharmacodynamic endpoints such as serum IGF bioactivity that will be useful for defining the PK/PD relationship in humans. With regards to rational rather than arbitrary drug combinations, our findings indicate that co-administration of BI 836845 with rapalogs deserves consideration, and with respect to potential selection criteria for tumors that may be particularly responsive, autocrine expression of IGF-1 or IGF-2 may define a subpopulation of interest (52).
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References


### Table 1: Activity of BI 836845 in cancer-derived cell lines

The effect of BI 836845 on viability of cancer-derived cell lines was tested in 2D assays. EC$_{50}$ values of selected cell lines inhibited by BI 836845 at a low nM concentration are shown.

<table>
<thead>
<tr>
<th>Cancer Indication</th>
<th>Cell Line</th>
<th>EC$_{50}$ µg/mL (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple myeloma</td>
<td>NCI-H929</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>LP-1</td>
<td>0.06 (0.4)</td>
</tr>
<tr>
<td>Ewing’s sarcoma</td>
<td>TC-71</td>
<td>0.7 (5)</td>
</tr>
<tr>
<td>Ewing’s sarcoma</td>
<td>SK-ES-1</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Ewing’s sarcoma</td>
<td>RD-ES</td>
<td>1.8 (12)</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>TC-177</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>NCI-H128</td>
<td>6.8 (45)</td>
</tr>
<tr>
<td>Non-small cell lung cancer</td>
<td>NCI-H2122</td>
<td>15 (100)</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1

Effect of BI 836845 or receptor-targeted mAbs on recombinant IGF or human serum-induced receptor phosphorylation

In mouse cells engineered to express human IGF-1R, BI 836845 (up to 15 µg/mL (100 nM)) effectively inhibited IGF-1R phosphorylation induced by (A) IGF-1 (20 ng/mL) and (B) IGF-2 (100 ng/mL), with an IC_{50} of 90 ng/mL (0.6 nM) and 1120 ng/mL (7.5 nM), respectively. Two IGF-1R-targeted monoclonal antibodies were much less effective at inhibiting IGF-2 activity. (C) In IR-A-expressing cells, BI 836845 blocked IGF-2 (100 ng/mL)-induced IR-A phosphorylation with an IC_{50} of 815 ng/mL (5.4 nM), whereas IGF-1R-targeted antibodies showed no effect. (D) BI 836845 potently neutralized serum IGF bioactivity in 20% pooled human serum (IC_{50} of 1.6 nM), while an IGF-1R monoclonal antibody showed only partial inhibition. Results presented are representative of three independent experiments.

Figure 2

Comprehensive preclinical characterization of the pharmacodynamic properties of BI 836845 in growing rats

Intravenous treatment of growing male rats with 20, 60 or 200 mg/kg of BI 836845 (n ≥ 24/group) once weekly for 13 weeks followed by a 12-week recovery period showed (A) a clear reduction in plasma IGF bioactivity at all dose levels relative to untreated controls with significant inhibition at 60 (p < 0.05) and 200 mg/kg (p < 0.01). At the end of the recovery phase (day 169), a dose-dependent increase toward baseline was observed. (B) BI 836845 treatment led to elevated total IGF-1 levels in the plasma which returned to baseline during the recovery period. The 200 and 60 mg/kg doses showed significant effects (p < 0.05) compared to control animals, the increase being greater than that seen with 20 mg/kg. Treatment with BI 836845 at all dose levels resulted
in a significant reduction of (C) body weight gain (p < 0.0001) and (D) tail length in growing male rats at day 85. Kruskal-Wallis test with Dunn’s posthoc test was performed for statistical evaluation of differences in tail lengths.

Figure 3
Activity of BI 836845 in cancer-derived cell lines in the presence of autocrine IGF ligands

(A) IGF-1, IGF-2 and IGF-1R expression data of selected sensitive cancer cell lines and (B) of colorectal GEO cells (Probe sets NM_000618_at, NM_000612_at and NM_000875_at of Exon chip data set of 625 cell lines). Median expression was calculated from all cell lines in data set. (C) 50-fold over median IGF-2 mRNA expression in the GEO cell line was associated with constitutive pathway activation in explanted GEO xenograft tumors as assessed by p-Tyr1135/1136-IGF-1R and p-Ser473-AKT Western blot analyses (n=2); the respective bands shown were cropped from the complete blots. (D) Intraperitoneal administration of 100 mg/kg of BI 836845 twice weekly for 27 days significantly inhibited the growth of GEO xenograft tumors by 54% (p < 0.01); two-tailed t-test was used for statistical evaluation.

Figure 4
Combination potential of BI 836845 with rapamycin

In SK-ES-1 cells, (A) treatment with the combination of BI 834845 and rapamycin for 5 days resulted in a significant reduction of cell viability and (B) treatment with 100 nM rapamycin for 24 hours increased AKT Ser473-phosphorylation, which was abolished by the addition of 100 nM BI 836845. (C) Cell viability and (D) pAKT levels of RD-ES cells were altered similarly. For cell viability assays (A + C), samples were measured in triplicates. Results of both cell lines are representative of two independent experiments each. Statistical analysis was performed using a Mixed Model for Repeated Measurements (MMRM) with SAS 9.2 (SAS Institute Inc., Cary, NC,
USA). AKT Ser473-phosphorylation (B + D) was normalized to GAPDH loading control. The results are representative for three independent experiments; respective bands shown were cropped from the complete blots.

Figure 5
Enhanced in vivo antitumor efficacy of BI 836845 in combination with rapamycin in the RD-ES xenograft model

(A) Treatment of mice bearing RD-ES tumors with BI 836845 (twice weekly, 100 mg/kg i.p.) or rapamycin (5 x weekly, 5 mg/kg i.p.) led to a tumor growth inhibition (TGI) of 53% and 75%, respectively, compared to control animals (0.5 % Natrosol, 10 mL/kg i.p. daily). Combining these agents improved efficacy (93% TGI). Median tumor volumes are shown in the plot. n = 7/treatment group. For statistical analysis, tumor volumes were log-transformed to stabilize the variance over the time course and analyzed utilizing MMRM. (B) Effect of BI 836845, rapamycin (p < 0.05), and the combination of BI 836845 and rapamycin on pAKT levels in explanted RD-ES tumors (n = 4/group) as determined by densitometric analysis of Western blot bands. Phosphorylated AKT values were normalized to total AKT values and are expressed as % pAKT/AKT. IGF bioactivity was increased by rapamycin in serum samples of (C) RD-ES tumor-bearing (n = 4/group; p < 0.01) and (D) non-tumor-bearing mice (n = 4/group; p < 0.005). (C) Treatment with BI 836845 showed a complete and potent neutralization of serum IGF bioactivity relative to control samples and also inhibited the rapamycin-induced increase of IGF bioactivity (reduction to baseline levels of the assay).
Figure 1

A

IGF-1R Phosphorylation (% Control)

log [antibody] (ng/mL)

○ BI 836845
△ αIR3 IGF-1R mAb
□ IGF-1R mAb
▼ Isotype Control

B

IGF-1R Phosphorylation (% Control)

log [antibody] (ng/mL)

○ BI 836845
△ αIR3 IGF-1R mAb
□ IGF-1R mAb
▼ Isotype Control

C

+ IGF-2 (100 ng/ml)

IR-A Phosphorylation (% Control)

log [antibody] (ng/mL)

○ BI 836845
△ αIR3 IGF-1R mAb
□ IGF-1R mAb
▼ Isotype Control

D

+ 20% pooled human serum

IGF-1R Phosphorylation (% Control)

log [antibody] (ng/mL)

○ BI 836845
△ IGF-1R mAb
□ IGF-1R mAb
▼ Isotype Control