Quantification of Binding of IGF-1 to BI 836845, a Candidate Therapeutic Antibody Against IGF-1 and IGF-2, and Effects of This Antibody on IGF-1:IGFBP-3 Complexes in Vitro and in Male C57BL/6 Mice

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IGF-1 and IGF-2 are potent mitogens acting through the IGF-1 receptor (IGF-1R). The importance of the IGF system in neoplasia has been demonstrated in several models, and IGF-1 signaling has become a target for drug development. The drug candidate BI 836845 is a fully human IgG1 ligand-neutralizing antibody that cross-reacts with IGF-1 and IGF-2. It has been shown to reduce both IGF-1R phosphorylation and cellular proliferation in preclinical studies. In rodent studies, administration of BI 836845 leads to large increases in total IGF-1 concentration in serum, despite reduced serum IGF-1 activity as measured by a kinase activation assay. Despite the fact that anti-IGF-ligand antibodies have entered clinical trials, their effect on IGF-binding proteins has not been described. In this report, we developed a novel technique to measure ligand-BI 836845 binding, and we apply it to a mouse model in various contexts. We show that although large increases in total serum IGF-1 levels are observed, the vast majority of ligand is present as a complex with BI 836845, and total serum IGF-binding protein-3 levels are decreased. Finally, we show that BI 836845 treatment induces an increase in GH levels, a finding consistent with attempted compensation at the level of the pituitary. Our results reveal complexities in the physiologic sequelae of BI 836845 administration that have implications for determination of optimal dosing regimens and for development of pharmacodynamic endpoints for clinical trials.

IGF-1 and IGF-2 are peptide hormones similar in structure to insulin that regulate a variety of cellular activities, including metabolism, proliferation, and growth. The IGF-1R binds both IGFs and initiates a signaling cascade that results in the activation of the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin) pathway (1–5). The IGF-2 receptor, which binds to IGF-2, does not possess intrinsic tyrosine kinase activity and is therefore considered as a sink that decreases IGF-2 bioactivity (6).

IGF actions are tightly modulated by a family of proteins called IGF-binding proteins (IGFBPs), of which IGFBP-1–6 have been characterized. The liver is the main source of serum IGFs and IGFBPs, but their expression is ubiquitous. IGFBP-3 is the most abundant IGFBP in the circulation. It forms a ternary complex with IGFs and a third protein termed acid-labile subunit (7, 8). IGF-binding proteins are known to modulate actions of IGFs both in vitro and in vivo (7).

Activation of the IGF-1R is generally accepted to play an important role in progression of neoplasia through increases in cell growth and proliferation and inhibition of apoptosis (9). Therefore, the IGF axis represents a potential target for cancer therapy (10–12). Early candidate drugs that have been clinically evaluated for the treatment of neoplasia are IGF-1R-specific antibodies and IGF-1R tyrosine kinase inhibitors (13). Despite encouraging early clinical data, definitive phase III clinical studies have been disappointing (14). A newer targeting strategy based on

Abbreviations: ALS, acid-labile subunit; FGF-21, fibroblast growth factor 21; GHR, GH receptor; IGFBP, IGF-binding protein; IGF-1R, IGF-1 receptor; qRT-PCR, quantitative RT-PCR; STAT, signal transducer and activator of transcription.

ligand-specific antibodies (15, 16) has recently entered clinical trials. A precedent for the antiligand approach is provided by an approved therapeutic antibody directed against vascular endothelial growth factor (17).

The drug candidate BI 836845 (Boehringer Ingelheim) (15) is a monoclonal, fully human dual specific IgG1 antibody with high affinity for both human and rodent IGF-1 and IGF-2. It was shown in preclinical studies to inhibit phosphorylation of the IGF-1R in vitro and to lead to decreased proliferation of a panel of cancer cell lines. In rodents, animal weight gain was reduced in a dose-dependent manner after a 13-week period of weekly iv administration of BI 836845. Additionally, experimental tumor volumes were consistently lower in mice treated with BI 836845 as compared with vehicle. Interestingly, BI 836845 treatment also leads to substantial increases of total IGF-1 serum levels in both normal and immunocompromised rodents (15). This effect may be the result of a host-compensatory mechanism by which GH levels rise as a consequence of the relaxation of the inhibitory IGF-1 feedback loop to the pituitary. This effect is common with IGF-1R-specific antibodies and was recently reviewed (13). On the other hand, it is also plausible that the half-life of IGF-1 is extended by binding to BI 836845, and increased serum levels may represent a decrease in IGF-1 degradation/elimination. Of course, these mechanisms are not mutually exclusive.

In the present study, we further characterize the therapeutic antibody BI 836845 by examining its effects in a mouse model. We develop a novel technique to measure IGF-1:BI 836845 complexes in serum, and we show that BI 836845 has the ability to sequester the vast majority of IGF-1, suggesting that presence of additional IGF-1 binding sites in serum are the major cause for increases in total IGF-1 levels.

Materials and Methods

Materials

BI 836845 was kindly provided by Dr. Paul Adam (Boehringer Ingelheim). Recombinant mouse IGF-1 and IGFBP-3 were purchased from R&D Systems. Control nonspecific human IgG1 isotype antibody was purchased from Cell Sciences, and C57 BL/6 mouse pooled serum was purchased from GeneTex.

Animals

C57 BL/6 male mice 6–8 weeks of age were purchased from Charles River Laboratories and were randomized into 4 groups for the first 2 experiments. In the first study, 3 mice received 10 mg/kg BI 836845, 3 mice received 30 mg/kg BI 836845, 3 mice received 100 mg/kg BI 836845, and 4 mice received vehicle. The treatments were performed ip alternatively every third or fourth day, for 4 doses and animals were humanely destroyed 4 days after the last dose. A sample of plasma was obtained before death, and total mouse serum was collected by cardiac puncture and further used in column-processing experiments. In the second study, 12 male mice were treated ip with 100 mg/kg BI 836845, and 3 mice were humanely destroyed after 2 hours, 3 mice were killed after 6 hours, 3 mice were killed after 24 hours, and 3 mice were killed after 48 hours. Four control mice received vehicle and 3 mice were humanely destroyed after 48 hours. A plasma sample was obtained for all mice alive at each time point (including mice that were to be killed at that time point), and total serum was collected by cardiac puncture and used in subsequent column-processing experiments. In the third study, 6 mice were randomized into 2 groups of 3 mice each and received either 100 mg/kg BI 836845 or vehicle daily for 4 days. Plasma samples were collected at times 0 and 24-, 48-, and 96-hour time points prior to treatment administration. Plasma samples were collected from the saphenous vein for all experiments.

Protein A agarose columns

Small 0.2-mL Nab Protein A-agarose plus columns and Protein A binding buffer were purchased from Thermo Fischer. Because the columns are prepacked with more resin than is optimal, they were emptied and the resin was stored separately. For each condition, a column was packed with 140 μL of resin, centrifuged at 5000 rpm for 1 minute to remove storage solution and then washed 3 times with Protein A binding buffer. After the last centrifugation, 150 μL of serum were added to the resin and mixed with thorough up and down movements for 10 minutes. The columns were then centrifuged, and the first fraction was collected and termed flow-through. A volume of 400 μL of elution buffer (100 mM glycine at pH 2.2) was applied to the resin for dissociating antibody-bead complexes. After a brief mix, the columns were centrifuged, and the second fraction termed "elution 1" was collected. The process was repeated for elution fractions 2 and 3 (see Supplemental Figure 1 published on the Endocrine Society's Journals Online web site at http://endo.endojournals.org).

ELISAs and variants

IGF-1 and IGFBP-3 immunoassay kits, purchased from R&D Systems, and a human IgG1 immunoassay kit, purchased from Abcam, were used according to manufacturers specifications except for the following experiments.

For the IGF-1 assay, the high temperature pretreatments were achieved by heating a sample of the first required serum dilution and using this heated sample to generate the second dilution.

For immunoprecipitation, undiluted serum samples were incubated in microwell plates precoated with IGFBP-3-specific antibody and incubated for 2 hours. Then, wells were washed and the immunoprecipitated material was dissociated with a solution containing 100 mM glycine at pH 2.3. The samples were neutralized and used in IGF-1 or IGFBP-3 immunoassays.

RNA isolation and quantitative RT-PCR (qRT-PCR)

Mouse liver samples (10 mg) were homogenized, and total RNA was extracted using a tissue RNA extraction kit (QIAGEN) according to manufacturer's specifications. RNA samples were monitored for degradation on agarose gel. Then, a total of 5 μg of RNA were reverse transcribed using random primers and a recombinant version of the Moloney Murine Leukemia Virus

reverse transcriptase (Life Technologies). To eliminate the RNA template, RNase H was added to the reaction for 20 minutes. Then, total cDNA was purified from the reaction mixture by silica column treatment (QIAGEN), and total cDNA was quantified using absorbance spectrometry. A total of 500 ng of cDNA were used downstream in qRT-PCR experiments. Taqman probes for each gene assayed, enzyme mix, and 96-well plates were purchased from Life Sciences, and qRT-PCR was performed on a Light Cycler. Results were either used raw (normalized to quantified total cDNA) or normalized to β-actin mRNA levels.

Immunoprecipitation and Western blotting

For immunoprecipitation, liver samples were homogenized in radioimmune precipitation assay buffer supplemented with protease and phosphatase inhibitors (Roche). Then, 500 μg of total protein was incubated for 1 hour with 40 μL protein A/G conjugated beads (Santa Cruz Biotechnology) for preclearance at 4°C. Beads were removed by centrifugation, and the lysates were further incubated with 5 μg GH receptor (GHR)-specific antibody (Santa Cruz Biotechnology) for 1 hour at 4°C. Afterward, 70 μL of protein A/G-conjugated agarose beads were added to each tube and incubated overnight on a rotating device at 4°C. The following day, the tubes were washed once in 1 mL of radioimmune precipitation assay buffer supplemented with protease and phosphatase inhibitors (centrifugation at 9000 rpm for 1 minute) and final resuspension was done in SDS-PAGE 6× concentrated loading buffer. Samples were boiled for 15 minutes and loaded on a SDS-PAGE gel. Following transfer, membranes were immunoblotted with antibodies specific to phosphotyrosine (Santa Cruz Biotechnology) or GHR. In Western blot experiments, liver homogenates were assayed with specific primary antibodies for IGF-1 (R&D Systems), IGFBP-3 (Santa Cruz Biotechnology), acid-labile subunit (ALS) (R&D Systems), phosphotyrosine 694 signal transducer and activator of transcription (STAT)5a/ phosphotyrosine 699 STAT 5b (Cell Signaling Technology) and STAT 5 a/b (Cell Signaling Technology).

Results

Use of a protein A column to remove BI 836845 from mouse serum in vitro

In order to study the distribution of IGF-1 between complexes with the therapeutic IgG1 class antibody BI 836845 and serum IGFBPs, we conducted an immunodepletion experiment using protein A agarose conjugates and C57 BL/6 mouse serum to which different amounts of BI 836845 or a nonspecific control human IgG1 were added. The immunodepletion method is illustrated in Supplemental Figure 1 and requires running serum through a column containing protein A-agarose, collecting the flow through, and then eluting 3 fractions under suitable conditions.

BI 836845 monoclonal antibody was added to mouse serum samples at final concentrations ranging from 0–300 μg/mL. Samples were then incubated 16 hours at 37°C and then subjected to the protein A column treatment. Each condition yielded 4 fractions labeled flow through, elution 1, elution 2, and elution 3. A sample of nonspecific human IgG1 was used as control.

Total human IgG1 concentration was measured in each fraction as well as in the total sample prior to column loading. Figure 1A shows that IgG1 levels measured in the mouse serum after the 16-hour incubation were comparable to the initial concentration added, indicating that both BI 836845 and control antibody degradation was minimal and that the measurement technique was appropriate. More importantly, Figure 1B indicates that the immunodepletion method was suitable for the effective removal of BI 836845 from serum because less than 5% of the added antibody was found in the flow through. Also shown is the amount of IgG1 present in the elution fractions, with most BI 836845 eluting in the first fraction and a much smaller amount eluting in the second fraction. The third elution values are not shown because they were consistently 0 across all conditions. The recovery rate was calculated as the sum of the 3 fractions (Flow through + Elution 1 + Elution 2) divided by the amount measured prior to column loading and yielded values between 83% and 92% for all conditions.

Measuring IGF-1 bound to BI 836845 in mouse serum in vitro

We then measured IGF-1 in serum samples to which BI 836845 was added and observed that the therapeutic antibody, as may have been expected a priori, interfered with the IGF-1 detection system. To overcome this, we used evidence, both from the literature (18) and pilot experiments, that IGF-1 is heat stable. On the other hand, IgGs are relatively heat labile and, at least for rat IgG1, the denaturing temperature was reported to be 60°C for the F_{ab} domain and 71°C for the F_c domain (19). Therefore, we used heat as a sample pretreatment technique in order to dissociate the antibody-ligand complexes with the expectation that most IGF-1 would survive this manipulation. We determined that sample preincubation at 80.6°C for 15 minutes was sufficient to eliminate the BI 836845-induced interference while maintaining almost all of the IGF-1 immunoreactivity (Supplemental Figure 2).

At this stage, we concluded that we could measure IGF-1 reliably in the presence of BI 836845 using this pretreatment prior to the ELISA. Figure 2A shows the amount of IGF-1 present in the flow-through fraction of serum passed through the protein A column as a function of BI 836845 concentration, measured with and without the 80.6°C preincubation. As expected, the amount of IGF-1 retained in the column increases as initial concentrations of BI 836845 added to the serum are raised. Fur-

thermore, the preincubation treatment has no effect on the measurement of IGF-1 in the flow-through, which is consistent with the low abundance of BI 836845 in these fractions. Figure 2B indicates that the vast majority of bound IGF-1 elutes in the first elution fraction, which is also the fraction containing the vast majority of eluted BI 836845 (Figure 1B). The third elution fraction is not shown because it was consistently 0 across all conditions. The recovery rate of IGF-1, measured again as the sum of 3 fractions (Flow through + Elution 1+ Elution2) divided by the amount prior to column loading, varied between 77% and 97%, with an average of 84%.

The data shown in Figure 2 suggest a loss of IGF-1 after column filtration as evidenced by the fact that about 75%–80% of initial IGF-1 is present in the flow-through of samples containing no BI 836845 or 300 μg/mL of control human IgG1. This is not the outcome of nonspecific binding because prior blocking of the columns with either BSA or Tween 20 had no effect on this measurement (data not shown). Rather, this observation is due to the amount of fluid inherently retained in the column (measured to be around 50 μL), which acts as a diluent, particularly in the initial step of the technique.

Effect of BI 836845 on IGF-1/IGFBP-3 binding

A further consideration was the impact of BI 836845 on IGFBP-3 distribution between the flow-through and elution fractions. We observed that IGFBP-3 is not retained by the protein A agarose resin for any of the BI 836845 concentrations tested (Supplemental Figure 3). This finding suggests that ternary complexes of IGF-1:IGFBP-3:BI 836845 are not formed and that IGF-1 shifts gradually from IGFBP-3 to BI 836845 as the concentration of the therapeutic antibody is increased. In order to confirm this hypothesis, we isolated total IGFBP-3 from BI 836845-treated mouse serum as described in Figure 3A and then measured total associated IGF-1. Figure 3A shows that as the concentration of BI 836845 increases, less IGF-1 is bound to IGFBP-3, indicating that IGFBP-3 exists in free form. Interestingly, the addition of 5 μg/mL antibody shows almost no effect on IGFBP-3-associated IGF-1 levels despite the fact that more than half of total IGF-1 is

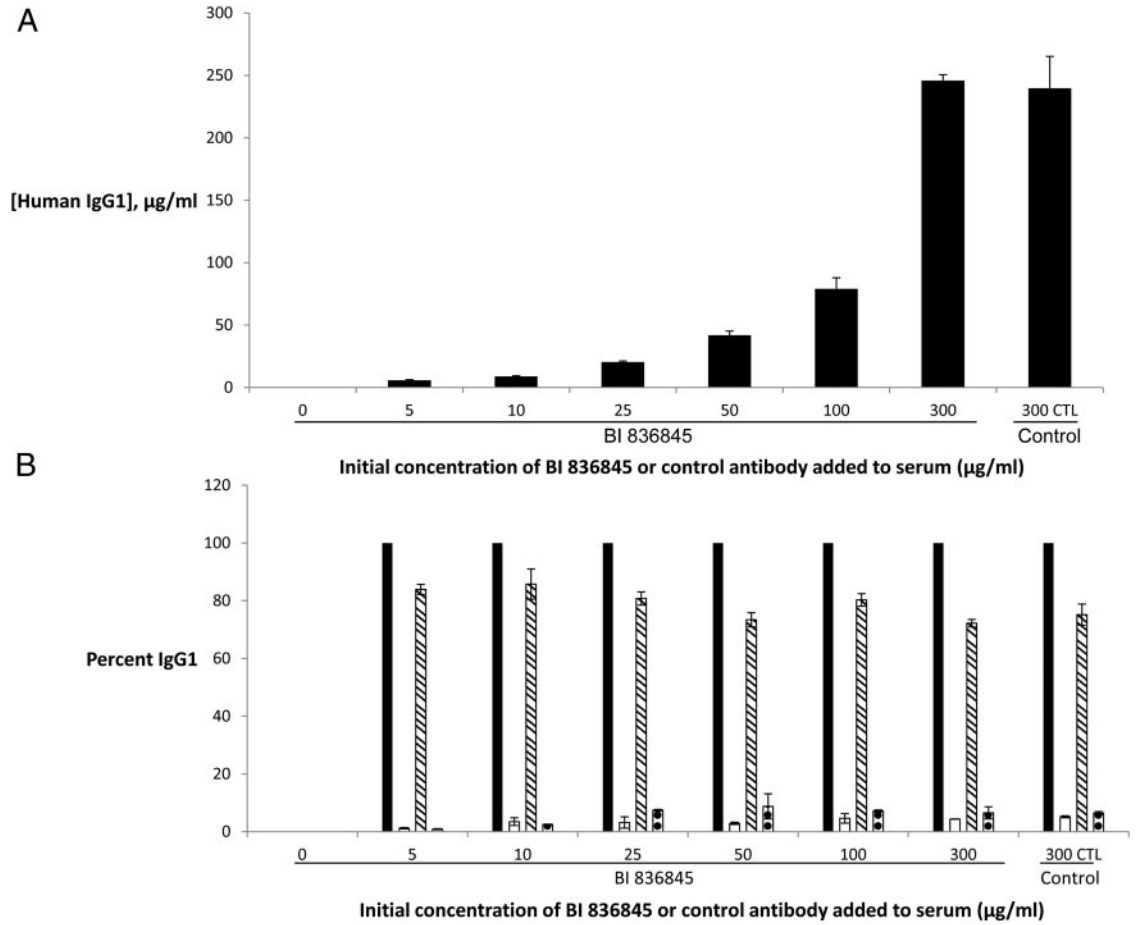


Figure 1. Concentration of human IgG1 in column fractions as measured by ELISA A, Human IgG1 concentration measured by ELISA in mouse serum incubated for 16 hours at 37°C with respect to initial antibody concentrations added. B, The percentage of IgG1 measured by ELISA in flow-through (white), first-elution (striped), and second-elution (dotted) fractions with respect to total fraction (black). Error bars indicate SD of measurements from at least 2 independent column-processing experiments. CTL, control.

bound to BI 836845 in this sample (Figure 2B). Because the affinity of IGFBP-3 for IGF-1 is among the highest in the IGFBP family (20), we speculate that, in the presence of BI 836845, IGF-1 may dissociate in a first step primarily from IGFBPs other than IGFBP-3.

Figure 3B is a summary illustration and depicts the reciprocity between IGF-1:IGFBP vs IGF-1:BI 836845 complexes in vitro. The amount of BI 836845 required for equal distribution of ligand between IGFBPs and the therapeutic antibody complexes is less than 33 nM, which corresponds to the amount of IGFBP-3 in serum. This indicates that, at least in serum ex vivo, the affinity of BI 836845 for IGF-1 is comparable to that of IGFBP-3.

Supplemental Figure 4 confirms the validity of the IGF-1 and IGFBP-3 measurements by Western blot. The serum concentration of IGF-2 was not assayed. In contrast to humans, serum IGF-2 levels drop in mice after birth, and very low levels are observed throughout adulthood (21). Therefore, given the low abundance of this ligand in the mouse serum used herein, we deemed its importance to be minor in the context of the current experiments.

Effect of BI 836845 on IGF-1 and IGFBP-3 in vivo

In order to investigate the effect of BI 836845 on IGF-1 levels in vivo, we treated groups of C57 BL/6 mice with various doses of the therapeutic antibody for 2 weeks, following which animals were humanely destroyed 4 days after the last injection. Figure 4A shows that the concentration of BI 836845 achieved in serum is dose dependent. Figure 4B illustrates that total levels of serum IGF-1 increase substantially, as previously shown (15), and Figure 4C illustrates that the concentration of IGF-1 not complexed to BI 836845, as measured in the flow-through fraction after protein A column purification, is similar to levels of total IGF-1 in control mice. Additionally, serum GH levels are not significantly different among groups (Figure 4D). From this experiment, we concluded that although total levels of IGF-1 rise, the vast majority is complexed to the therapeutic antibody. However, levels of potentially bioactive IGF-1 (as estimated by the concentration of IGF-1 not complexed to BI 836845), total levels of GH, as well as total levels of IGFBP-3 (data not shown) were identical to concentrations observed in control

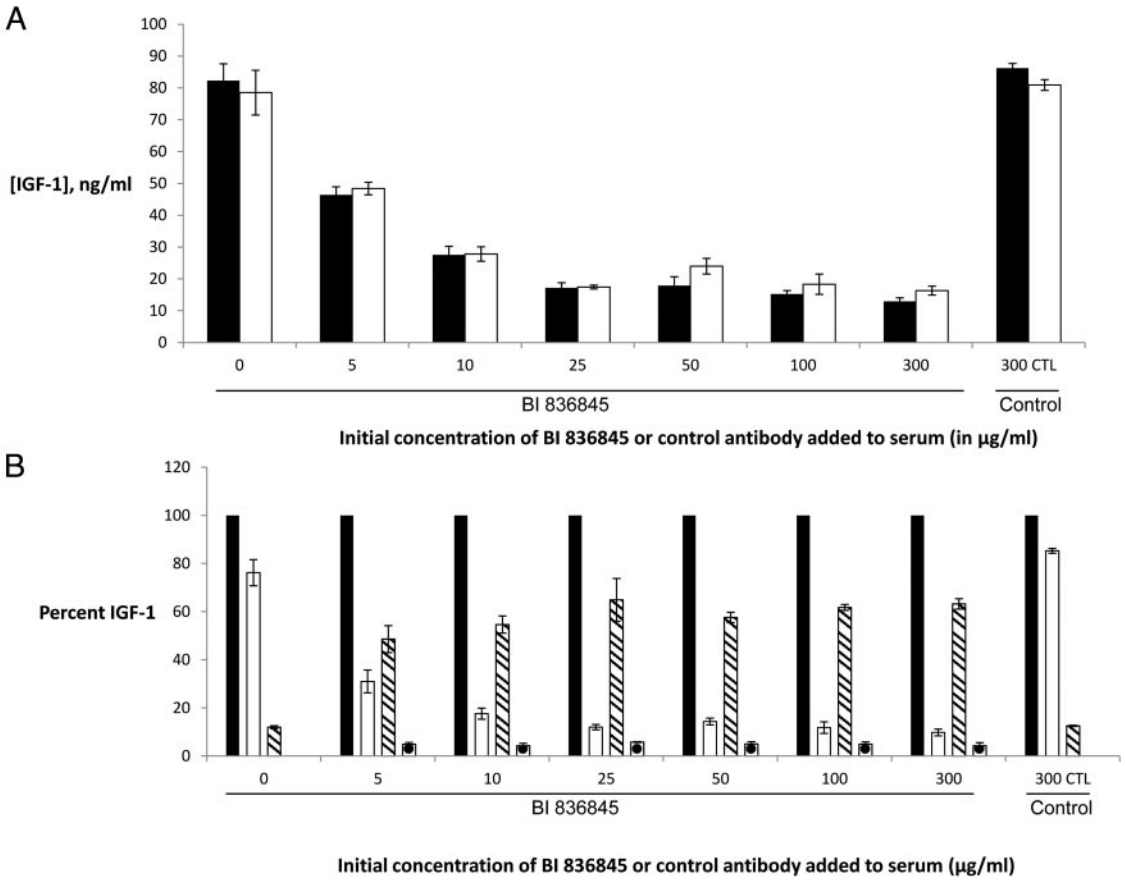


Figure 2. Concentration of mouse IGF-1 in column fractions as measured by ELISA A, Mouse IGF-1 concentration in the flow-through fraction detected by ELISA with no pretreatment (black) or following an 80.6°C pretreatment (white) according to initial BI 836845 or control antibody concentration. B, The percentage of IGF-1 measured by ELISA (with 80.6°C pretreatment) in flow-through (white), first-elution (striped), and second-elution (dotted) fractions with respect to total fraction (black) after column processing. Error bars indicate SD of measurements from at least 2 independent column-processing experiments. CTL, control.

mouse serum. Therefore, these data provided evidence neither for an increase in free IGFBP-3 nor for a decrease in IGF-1 bioactivity following BI 836845 treatment, despite prior evidence for reduced IGF-1 bioactivity in terms of end points such as somatic growth (15). Antibody separation and recovery rates for both IGF-1 and BI 836845 were similar to in vitro studies (data not shown).

We next examined the effect of 100 mg/kg BI 836845 treatment short term. C57 BL/6 mice were treated with BI 836845 and then humanely destroyed at several time points over a 2-day period. Figure 5A shows that serum levels of antibody greater than 200 μg/mL are achieved after treatment but that these levels are maintained for less than 24 hours. Importantly, these antibody levels (1400 nM) are in vast molar excess of IGF-1 (80 nM). Figure 5B indicates that a 10-fold increase in total IGF-1 concentration occurs over 48 hours. Interestingly, Figure 5C shows that levels of IGF-1 uncomplexed to BI 836845 are lower than total IGF-1 levels in control mice for a period of at least 48 hours, which is consistent with the decrease in IGF-1 bioactivity previously reported (15). The amount of bioactive IGF-1 is lowest 6 hours after administration and gradually increases with time approaching control levels after 48 hours. Figure 5D illustrates a timeline of serum levels of both BI 836845 and IGF-1 in molar terms. At earlier time points, there is a vast excess of binding sites over ligand, presumably leading to an initial decrease in bioavailable IGF-1, which is then gradually attenuated as ligand and binding sites become equimolar. Again, antibody separation and recovery rates for both IGF-1 and BI 836845 were similar to in vitro studies (data not shown).

We then examined the effect of BI 836845 administration on IGFBP-3 and ALS plasma levels. Figure 6A shows a decline in total IGFBP-3 levels that is of similar magnitude to the decrease observed in bioavailable IGF-1 (Figure 5C). Therefore, this set of data does not suggest that BI 836845 leads to an increase in free IGFBP-3 in vivo, in contrast to our findings in vitro (Figure 3A). Figure 6B illustrates that a decline in serum ALS is apparent at the 6-hour time point.

An important additional endpoint was the measurement of GH levels, particularly in the context of the decline in potentially bioactive IGF-1 (Figure 5C). Figure 7A shows that GH levels are increased after BI 836845 treatment, with significant changes observed 6 hours after treatment and maintained up to 24 hours after treatment.

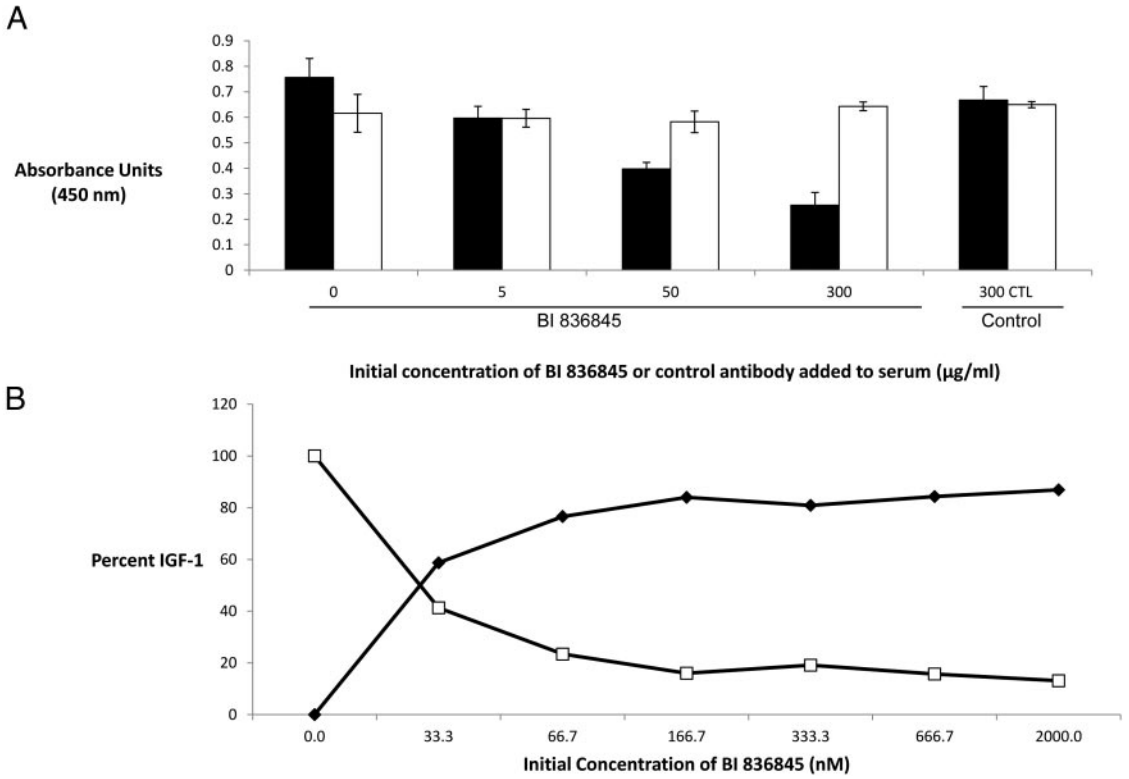


Figure 3. Mouse serum levels of free IGFBP-3 A, Samples of undiluted serum containing different concentrations of BI 836845 or 300 μg/mL control IgG1 were incubated in microplate wells coated with IGFBP-3-specific antibody. Molecular species bound to the anti-IGFBP-3-coated wells were eluted in an acidic condition (100 mM glycine, pH 2.3), neutralized, and amounts of IGF-1 (black) or IGFBP-3 (white) were quantified by ELISA. Error bars represent SD of measurements from at least 2 independent experiments. B, Schematic representation of results from Figure 2 illustrating the reciprocity of IGF-1-IGFBP (white squares) or IGF-1:BI 836845 (black squares) complexes. As the concentration of BI 836845 increases, the proportion of IGF-1 bound to IGFBP-3 decreases. The concentration of IGFBP-3 in mouse serum is approximately 20 nM. CTL, control.

The variance of experimental measurements was high, which is not surprising given the pulsatile secretion of this hormone. However, in an attempt to decrease group variation in GH, we performed an additional experiment in which mice were given 100 mg/kg dose of BI 836845 daily and starved 3 hours prior to blood collection. Figure 7B illustrates that starvation leads to a decrease in global GH levels, which is consistent with previous reports for mice (22). Additionally, BI 836845-treated animals display significantly higher GH levels compared with animals receiving vehicle, which is consistent with inhibition of the IGF-1-mediated feed-back loop to the pituitary.

We then measured liver transcripts of IGF-1, IGFBP-3, and ALS in order to understand the role of liver secretion on serum levels of these analytes. Figure 7, C, D, and E, illustrate the mRNA levels of IGF-1, IGFBP-3, and ALS, as measured by real-time PCR, in liver samples of mice humanely destroyed at the relevant time points. Figure 7C indicates no change in liver IGF-1 mRNA abundance after BI 836845 administration, a finding that is surprising given that serum GH levels are more than 10-fold higher in these animals than in control animals. Additionally, the data revealed no statistically significant difference in IGFBP-3 mRNA levels (Figure 7D). The liver mRNA abundance of ALS was significantly lower at the 2-hour time point (Figure 7E), which provides evidence that decreased liver transcription may be responsible for the observed decline in plasma levels at 6 hours after BI 836845 administration (Figure 6B).

Finally, we measured the levels of fibroblast growth factor 21 (FGF-21) following BI 836845 treatment in an attempt to understand why the induced increase in GH levels (Figure 7, A and B) did not lead, as would have been expected, to an increase in IGF-1 liver mRNA levels (Figure 7C). Although FGF-21 was recently shown to be a mediator of GH resistance in mice during starvation (23), we were unable to establish such a role in our current experiments. Figure 8A illustrates that levels of FGF-21 are variable and are not statistically different following injection of BI 836845 or vehicle. Then, we investigated the changes in the liver GHR activation levels in animals treated with BI 836845. We observed an increase of both GHR tyrosine phosphorylation (Figure 8C) and downstream mediators STAT 5 a/b phosphorylation in livers of animals following BI 836845 administration (Figure 8B).

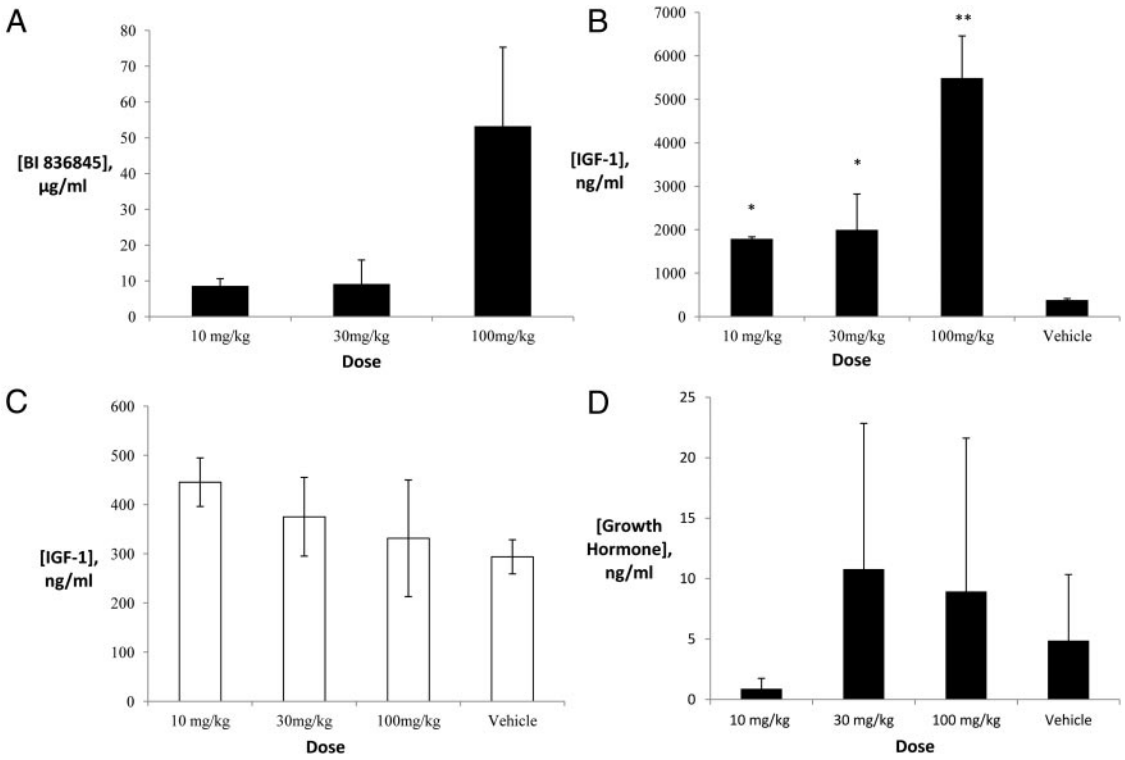
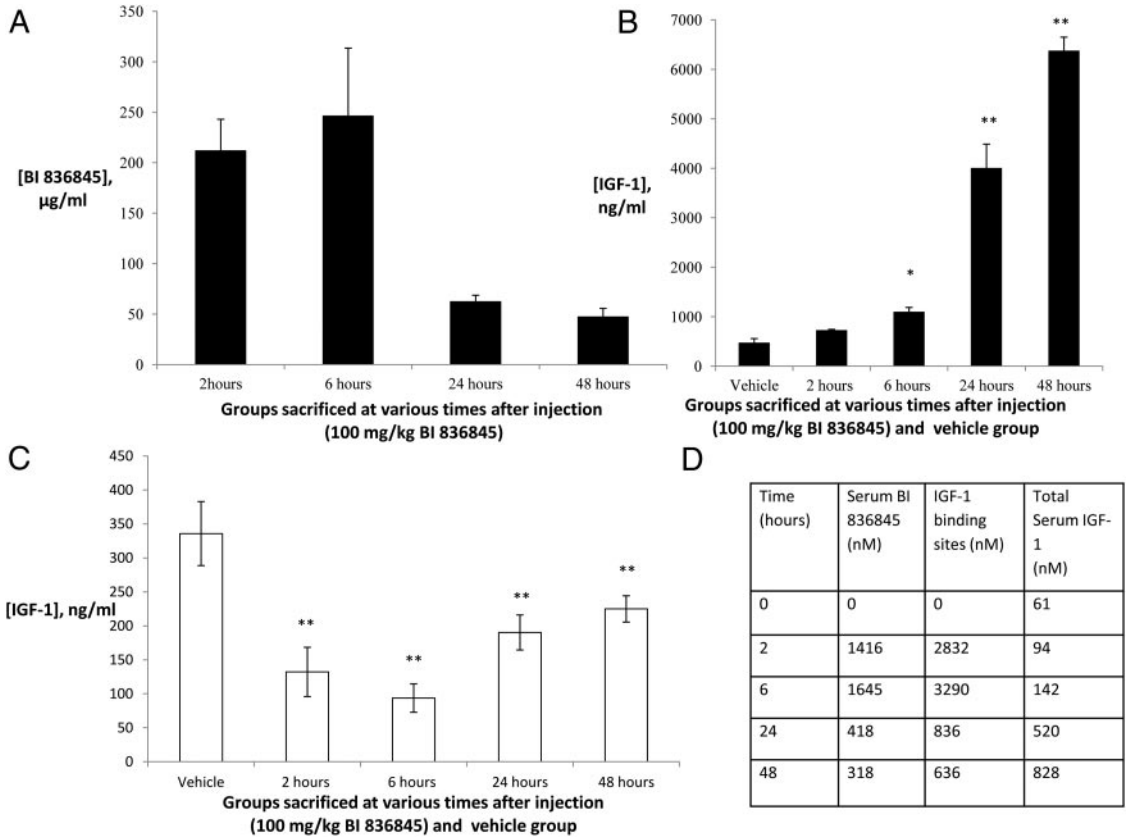


Figure 4. Mouse serum levels of BI 836845, total IGF-1, and potentially bioactive IGF-1 (ie, non-BI 836845-complexed IGF-1) A, BI 836845 concentration assayed by ELISA in serum from C57 BL/6 mice treated with indicated doses of BI 836845 for 2 weeks, administered twice weekly, and humanely destroyed 4 days after the last injection. B, IGF-1 concentration in the same sera as in panel A. C, IGF-1 concentration in the flow-through fraction after Protein-A agarose column separation of sera used for panel A. D, GH concentration in the same sera as in panel A. In all panels error bars represent SD of measurements from n = 3 mice except for vehicle value, where n = 4. For panel B, global *P* value of ANOVA is < 0.0001; *, $P < .05$; **, $P < .01$ using Dunnett's multiple-comparison test.

Discussion

The human IgG1 antibody BI 836845 is a novel candidate therapeutic agent for the treatment of cancers that are growth stimulated by IGFs, which may originate from endocrine, paracrine, or autocrine sources. It represents a relatively novel strategy for targeting the IGF-1 axis by a ligand-binding approach rather than an antireceptor approach, such as figitumumab (24) or small receptor tyrosine kinases inhibitors, such as BMS-554417 (25). In vitro, it has been shown that BI 836845 has a strong affinity for both human and rodent IGFs and that it inhibits IGF-1R phosphorylation in multiple cell line models (15). In rodent studies, BI 836845 significantly inhibits growth of xenografted tumors but also leads to large increases in total serum IGF-1 concentrations (15). The mechanisms responsible for this increase in total IGF levels and the impacts of BI 836845 on IGF physiology as a whole are poorly described. In the light of this increase in ligand levels, the main goal of the present manuscript was to quantify IGF-1:BI 836845 complexes in serum. We recognized that although the therapeutic goal of BI 836845 was to reduce IGF-1R activation, this drug candidate may also influence IGFBP physiology.

We developed a novel technique requiring minimal serum dilution to measure IGF-1:BI 836845 complexes and applied it in a mouse model. We show that, in vitro, the addition of BI 836845 shifts the binding of IGF-1 from IGFBPs to the therapeutic antibody. Although IGF ligands complexed to IGFBPs can be released to "free" or "bioactive" ligands by physiological processes such as IGFBP proteolysis, the ligand bound to BI 836845 is likely unavailable for receptor binding. Additionally, because IGFBP-3 has among the highest affinity for ligands, our results suggest that other IGFBPs are also uncomplexed to ligand in the presence of BI 836845 ex vivo. We observed that 5 μg/mL BI 836845, although capable of binding approximately 50% of IGF-1, did not induce a change in levels of IGF-1 bound to IGFBP-3. This would imply that the amount of IGF-1 carried by IGFBP-3 is less than the 70%–90% that is generally assumed. However, we do not feel that this interpretation is definite because our experimental conditions likely resulted in a disturbance of the



Time (hours)	Serum BI 836845 (nM)	IGF-1 binding sites (nM)	Total Serum IGF-1 (nM)
0	0	0	61
2	1416	2832	94
6	1645	3290	142
24	418	836	520
48	318	636	828

Figure 5. Mouse serum levels of BI 836845, total IGF-1, and potentially bioactive IGF-1 A, BI 836845 concentration assayed by ELISA in total serum from C57 BL/6 mice treated once with 100 mg/kg BI 836845 and humanely destroyed at various time points over 48 hours. B, IGF-1 concentration in the same sera as in panel A. C, IGF-1 concentration in the flow-through fraction after Protein-A agarose column separation of sera used for panel A. D, Data from panels A, B, and C represented in molar form. Binding sites are obtained by a doubling of BI 836845 concentrations because 2 binding sites per antibody are present. In all panels error bars represent SD of measurements from n = 3 mice. Global *P* values of ANOVAs are $< .0001$; *, $P < .05$; **, $P < .01$ using Dunnett's multiple-comparison test.

competition between the various IGFBPs for ligands. In particular, for our ex vivo assays, we have used a commercially available pooled C57/BL6 mouse serum, which could have possibly originated from animals of different age, feeding status, etc. Therefore, it is likely that equilibria between IGF-1 and the various IGFBPs in such a pool are different from those achieved in serum from a single individual.

We also present evidence that the affinity of the therapeutic antibody in serum ex vivo for IGF-1 is comparable to that of IGFBP-3, which is in agreement with previous SPR studies conducted in simple buffer solutions (15). Because BI 836845 and another anti-IGF antibody (16) are currently in clinical trials, an extension of the technique described in this report may be applicable in the quantification of IGF-1:BI 836845 complexes directly in human serum. Of course, the use of human IgG1 concentration to indicate BI 836845 levels is not applicable in human serum samples. Although many general methods exist (26), the accurate measurement of therapeutic antibody levels in human samples often requires custom tailored solutions, and this is not unique to BI 836845. However, the serum fractionation technique using protein A is potentially applicable in clinical samples and could be useful in measuring the amount of IGF-1 bound/unbound to BI 836845.

In this report, we also confirm that administration of BI 836845 in vivo leads to large increases in total IGF-1 levels, and we show that more than 90% of ligand is bound to the therapeutic antibody. The amount of IGF-1 unbound to BI 836845, and therefore potentially bioactive, decreases below control levels after treatment. However, once most available binding sites of the antibody are occupied, bioactive IGF-1 levels revert to normal IGF-1 concentrations seen in untreated animals. Our results are therefore consistent with the described inhibitory effects of BI 836845 on endpoints such as body weight gain or tail length of rats (15). However, our data suggest that particular care must be exercised as to the dosing regimen. In the case of C57 BL/6 mice, we showed that administration of BI 836845 every 3 or 4 days (as was done for rats in Ref. 15) is not optimal because bioavailable IGF-1 levels are below control levels for 48 hours but revert to untreated

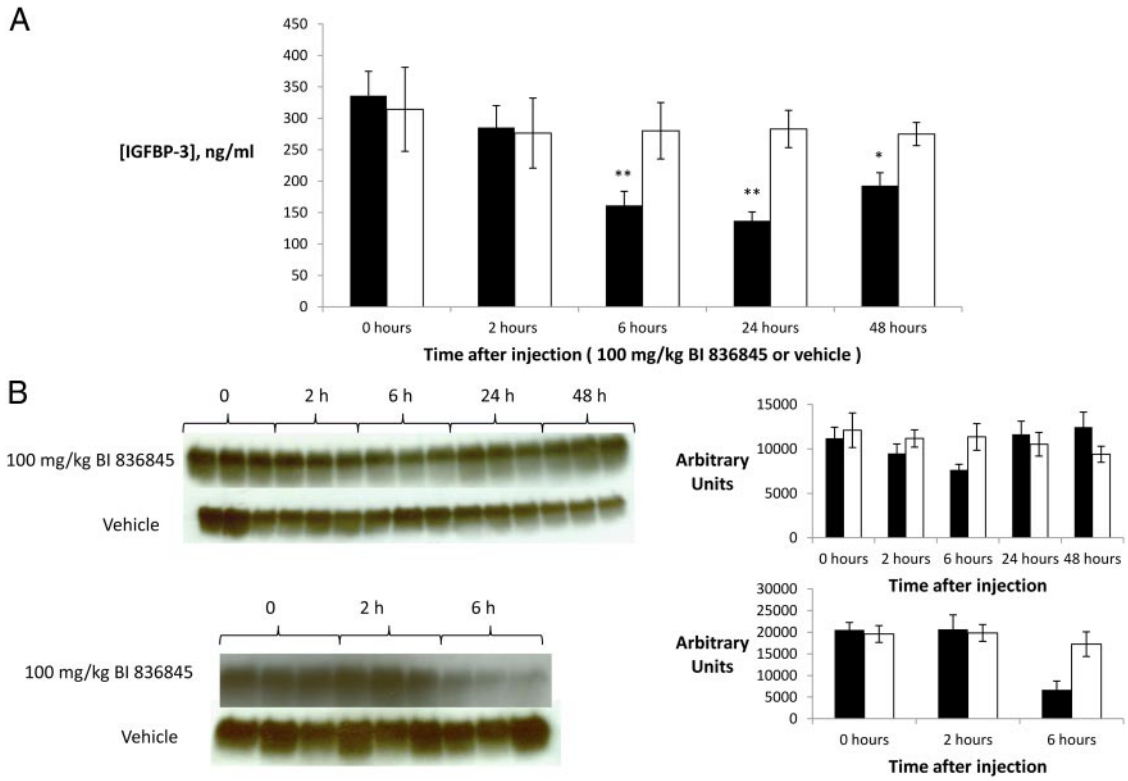


Figure 6. Mouse plasma levels of IGFBP-3 and ALS A, IGFBP-3 concentration assayed by ELISA in plasma from C57 BL/6 mice treated once with 100 mg/kg BI 836845 (black) or vehicle (white) over 48 hours. In treated set, error bars represent SD of measurements from n = 13 mice for the 0 time point, n = 12 for the 2-hour time point, n = 9 for the 6-hour time point, n = 6 for the 24-hour time point, and n = 3 for the 48 hours time point. In vehicle set, n = 4 at all time points. Global *P* value is less than 0.01 for repeated-measures ANOVA; *, $P < .05$; **, $P < .01$ with respect to control at specific time points using a Bonferroni correction for multiple testing. B, ALS concentration in plasma samples from a single group of 3 mice measured by Western blot sequentially at 0, 2, 6, 24, and 48 hours after BI 836845 treatment and in a second group of 3 mice measured sequentially at 0, 2, and 6 hours. Below each blot are identical measurements and time points from a group of 3 mice having received vehicle treatment. Densitometry analysis of the Western blots is presented as a summary and error bars indicate SD of the 3 values at each time point. The experiment is representative of at least 3 independent blots.

levels by 4 days. Similar measurements in human sera may be useful in developing optimal dosing schedules for clinical trials. These could be defined as treatment regimens that lead to levels of ligands not complexed to BI 836845 that are substantially lower than total ligand levels before treatment for all time points. It is also anticipated that the half-life of BI 836845 will be considerably longer in humans than in the immunologically competent mice used in our models, given that it is a fully human antibody. We recognize that prolonged treatment with BI 836845 may lead to physiologic conditions characterized by elevated GH levels, the consequence of which may be species specific, and which will have to be monitored in clinical trials.

We show that levels of GH rise after BI 836845 treatment in vivo, a finding that is consistent with a compensatory mechanism due to loss of the inhibitory effect of bioactive IGF-1 on pituitary GH secretion. This implies that the drug candidate does indeed reduce IGF-1 bioactivity, consistent with the results reported by the kinase activation assay (15). A similar outcome has previously been observed following administration of IGF-1R-specific antibodies (13). However, despite evidence of increased liver GHR activation, we were unable to show the anticipated increase in liver levels of IGF-1, IGFBP-3 or ALS mRNA, all of which have been shown to be positively regulated by GH (13, 27, 28). On the contrary, we observed that serum protein levels of both IGFBP-3 and ALS decrease at some time points following BI 836845 treatment. Therefore, given that the vast majority of serum IGF-1 is bound to the therapeutic antibody and that liver IGF-1 mRNA abundance is not increased, our data suggest that the presence of excess ligand-binding sites in serum is the major factor responsible for the observed rise in total IGF-1 levels, although other contributing factors such as IGF-1 posttranslational modulation cannot be ruled out. The mechanism by which BI 836845-treated animals become, at least with respect to IGF-1 gene expression, GH insensitive remains to be elucidated. For example, it may be that the monoclonal antibody leads to

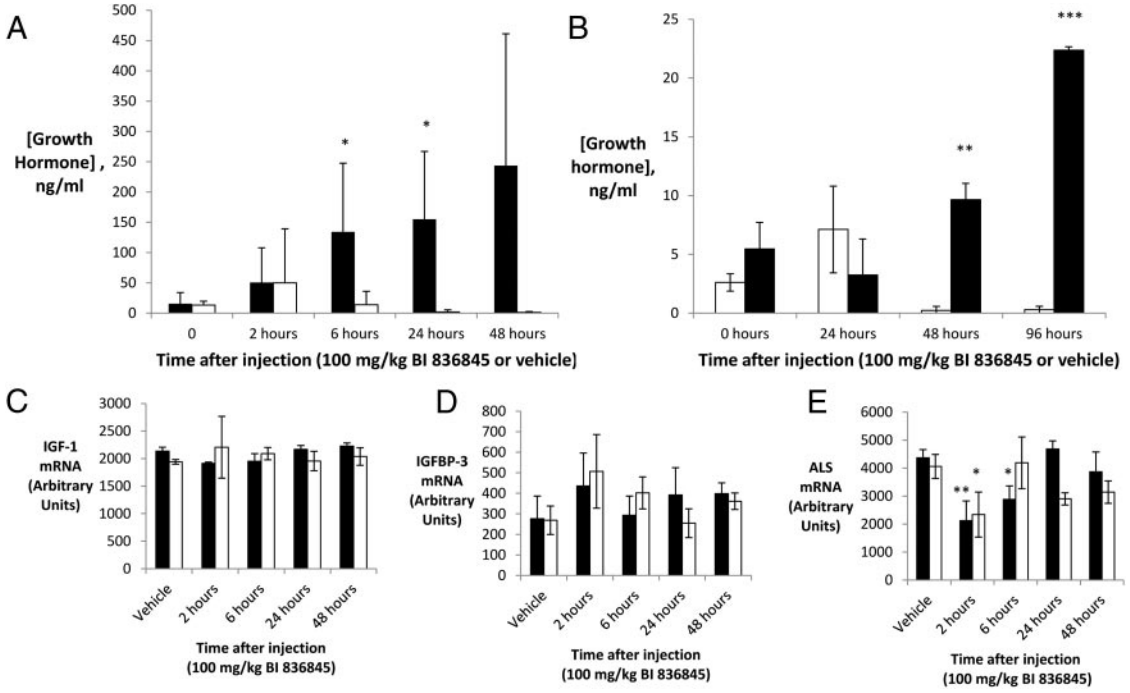


Figure 7. Mouse plasma levels of GH and mouse liver mRNA levels of IGF-1, IGFBP-3, and ALS A, GH concentration assayed by ELISA in plasma from C57 BL/6 mice treated once with 100 mg/kg BI 836845 (black) or vehicle (white) over 48 hours. In treated set, error bars represent SD of measurements from n = 13 mice for the 0 time point, n = 12 for the 2-hour time point, n = 9 for the 6-hour time point, n = 6 for the 24-hour time point, and n = 3 for the 48-hour time point. In vehicle set, n = 4 at all time points. Global *P* value is less than .05 for repeated-measures ANOVA; *, $P < .05$ with respect to control at specific time points using a Bonferroni correction for multiple testing. B, GH concentration assayed at various time points by ELISA in plasma from C57 BL/6 mice treated daily with 100 mg/kg BI 836845 (black) or vehicle (white). Error bars represent SD of measurements from n = 3 mice for all time points. Global *P* value is $< .0001$ for repeated-measures ANOVA; **, $P < .01$; ***, $P < .001$ and with respect to time 0 using a Bonferroni correction. C, IGF-1 mRNA levels were quantified in livers of mice humanely destroyed at different time points following a single injection of 100 mg/kg BI 836845 with respect to total cDNA (white) or with respect to β-actin as a reference gene (white). The term "vehicle" refers to mice having received vehicle treatment and humanely destroyed at the end of the experiment, ie, 48 hours later. D and E, IGFBP-3 and ALS mRNA levels quantified exactly as in panel C. Panels C, D, and E represent a typical experiment that was repeated at least 3 times, and error bars represent SDs of mRNA levels from n = 3 mice per group. *, $P < .05$; **, $P < .01$ using Dunnett's multiple-comparison test.

reduced caloric intake of animals and to a GH insensitivity that is not mediated by FGF-21.

Hepatic secretion rates of IGF-1 are incompletely described, but one study in rats estimates that liver secretion of IGF-1, per hour, is approximately 40% of the total amount of IGF-1 in serum (29). Under the assumption that similar rates of hepatic secretion apply to mice, our observed increases in total IGF-1 (~10 fold over 48 hours) are achievable under baseline hepatic IGF-1 secretion rates.

Interestingly, we observed that the therapeutic antibody leads to increases in IGFBP-3 uncomplexed to ligand in mouse serum ex vivo. However, animal experiments did not indicate that a similar increase in free IGFBP-3 levels occurs in serum in vivo because total levels of IGFBP-3 vary in the same direction and magnitude as levels of bioactive IGF-1. One possible explanation for this discrepancy is an increased serum clearance of free IGFBP-3 following BI 836845 treatment. It was shown that ternary complexes of IGFBP-3:IGF-1:ALS are confined to the circulation as opposed to binary ligand complexes that readily cross the endothelial barrier due to smaller size (30). Therefore, it is possible that newly generated free IGFBP-3 is able to diffuse out of the circulation and into tissues. This idea is attractive from a therapeutic point of view because it has been shown that IGFBP-3 can act independently of IGFs to inhibit cell growth and to increase apoptosis in a variety of models (31–33).

A second possibility to account for the decrease in serum IGFBP-3 levels is a decreased liver output. In rodents, liver IGFBP-3 was reported to be positively regulated by IGF-1 (27, 34), which, in terms of bioavailable levels, decreases after BI 836845 administration. Although we were unable to show a statistically significant change in hepatic IGFBP-3 gene expression following BI 836845 administration, the high variation among animals and the relatively small size of treatment groups do not allow us to rule out a possible decrease in expression. The implications and mechanism of the observed decrease in ALS plasma levels and liver mRNA are under investigation.

Another possibility is an increase in IGFBP-3 proteolysis that can occur directly in serum, or at even higher rates in tissues (35). This becomes particularly important if the rate of IGFBP-3 diffusion into tissues increases significantly.

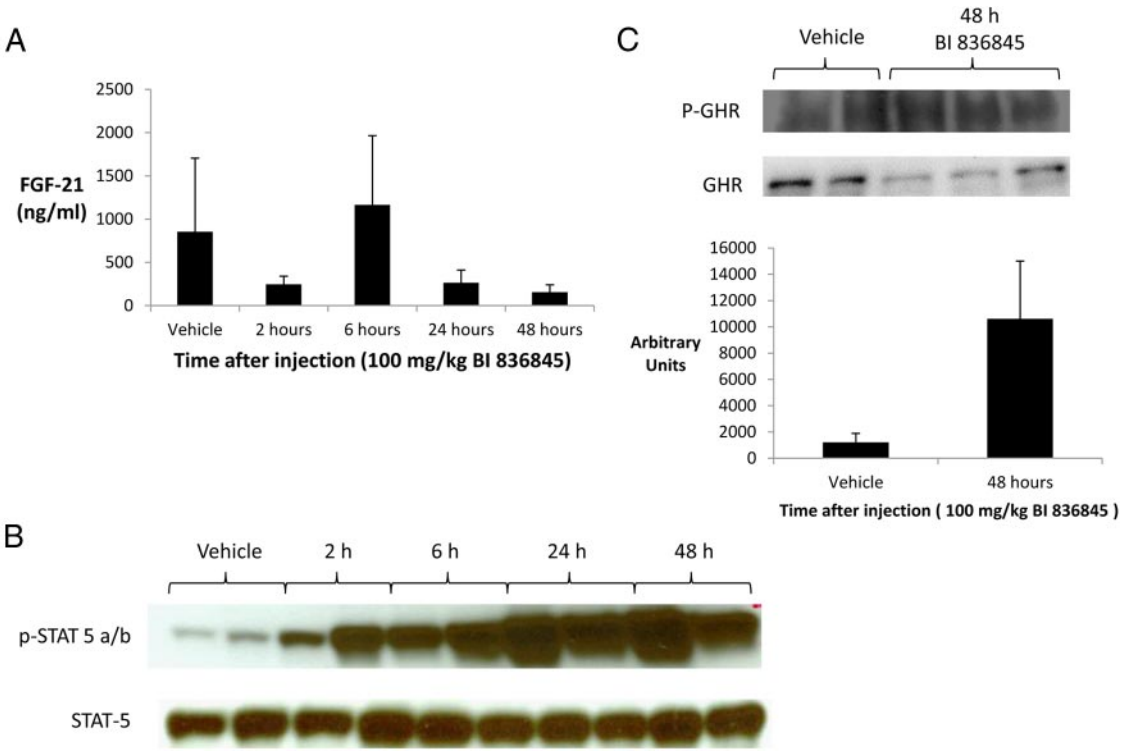


Figure 8. Mouse serum levels of FGF-21 and mouse liver GHR signaling A, FGF-21 concentration assayed by ELISA in total serum from C57 BL/6 mice treated once with 100 mg/kg BI 836845 and humanely destroyed at various time points over 48 hours. Error bars represent SD of measurements from n = 3 mice for all time points. B, STAT 5 a (Tyr 694)/b (Tyr 699) phosphorylation in liver samples from 2 mice at each time point over 48 hours after treatment with 100 mg/kg BI 836845 or vehicle. C, GHR total tyrosine phosphorylation in livers of 2 mice treated with vehicle and 3 mice 48 hours after treatment with 100 mg/kg BI 836845. Densitometry is presented as a summary, phosphorylated GHR (P-GHR) is normalized to total immunoprecipitated GHR, and error bars represent SD of measurements n = 2 and n = 3. The experiment is representative of at least 3 independent blots. p-STAT, phosphorylated STAT.

Finally, we have observed that, at least in C57 BL/6 mice, BI 836845 serum clearance is high and more than 75% of initially achieved serum levels are eliminated within 24 hours of treatment. This finding may be explained by an immune response due to the nonmurine nature of the antibody, or it may be a species peculiarity. The mg/kg dose of BI 836845 required to achieve a specified serum concentration in humans is likely lower than that in murine models. The half-life of BI 836845 in humans should be an important consideration because antibody serum levels may be expected, as was the case in mice, to determine the extent of increase in total ligand levels, the amount of bioavailable ligands, and the effectiveness of a specific dosing regimen.

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