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In vitro and *in vivo* Characterization of MOD-4023, a Long-Acting Carboxy-Terminal Peptide (CTP)-Modified Human Growth Hormone

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

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Abstract

MOD-4023 is a novel long-acting version of human growth hormone (hGH), containing the carboxy-terminal peptide (CTP) of human chorionic gonadotropin (hCG). MOD-4023 is being developed as a treatment for adults and children with growth hormone deficiency (GHD), which would require fewer injections than currently available GH formulations and thus reduce patient discomfort and increase compliance. This study characterizes MOD-4023's binding affinities for the growth hormone receptor, as well as the pharmacokinetic and pharmacodynamics, toxicology and safety profiles of repeated dosing of MOD-4023 in Sprague-Dawley rats and Rhesus monkeys. Although MOD-4023 exhibited reduced *in vitro* potency and lower affinity to the GH receptor than recombinant hGH (rhGH), administration of MOD-4023 every 5 days in rats and monkeys resulted in exposure comparable to daily rhGH, and the serum half-life of MOD-4023 was significantly longer. Repeated administration of MOD-4023 led to elevated levels of insulin-like growth factor 1 (IGF-1), and twice-weekly injections of MOD-4023 resulted in larger increase in weight gain with fewer injections and a lower accumulative hGH dose. Thus, the increased half-life of MOD-4023 in comparison to hGH may increase the frequency of protein-receptor interactions and compensate for its decreased *in vitro* potency. MOD-4023 was found to be well-tolerated in rats and monkeys, with minimal adverse events, suggesting an acceptable safety profile. These results provide a basis for the continued clinical development of MOD-4023 as a novel treatment of GHD in children and adults.

1. Introduction

Growth hormone (GH) is a 191-amino-acid pituitary protein that stimulates the hepatic production and release of insulin-like growth factor-1 (IGF-1) into the systemic circulation. IGF-1 is instrumental in the promotion of linear growth in children and in the control of metabolism and body-mass composition in adults. Growth hormone deficiency (GHD) may occur as an isolated disorder or as part of multiple hormone deficiencies. Adult-onset GHD has been estimated to affect 1 per 100,000 people annually, and the incidence rate of childhood-onset GHD is approximately 2 per 100,000, although there are estimates as high as 1:4000. A deficiency of GH results in inadequate circulating IGF-1 levels and is manifested as abnormal linear growth in children, and as decreased lean body mass, increased fat mass, weakness, reductions in exercise capacity, muscle mass/strength, cardiac performance and bone density, as well as neuropsychological disturbances in adults [1,2]. The standard treatment for GHD in adults and children is replacement with recombinant human GH (rhGH). Current rhGH therapy requires daily subcutaneous (SC) injections that may decrease compliance, especially in children and adolescents [3]. In GHD adults, daily hGH administration may involve concomitant side effects, such as injection site reactions, edema, and arthralgia [1].

Several long-acting products have been studied in GHD patients, with the promise of requiring fewer injections and minimizing the adverse events involved with daily administration [4,5]. In order to increase the half-life of hGH, several approaches currently being explored involve the fusion of hGH to a moiety that lowers the clearance rate of the active drug, without requiring a large dose, and preferably providing an improved safety

profile. Conjugation of GH to albumin decreased renal clearance and increased half-life; however, this formulation did not exhibit an improved bioavailability in comparison to rhGH [6]. PEGylated GH demonstrated local injection site lipoatrophy in adults and children, thus raising a safety concern [7]; an additional study with PEGylated GH demonstrated an inadequate IGF-1 response [8]. Another approach involved the fusion of an unstructured amino acid sequence (XTEN) to hGH in order to reduce its renal clearance (VRS-317) [9]. This compound exhibited a longer elimination half-life and a prolonged IGF-1 response in a recent clinical trial in GHD adults [10]. Alternatively, Kim *et al.* generated a long-acting form of hGH by fusing it to the Fc domain of IgG. The safety profile of this chimeric protein, however, is yet to be established [11].

MOD-4023 (carboxyl-terminal peptide [CTP]-modified hGH) is a novel long-acting version of hGH, developed as a treatment for adults and children suffering from GHD. The CTP is derived from the naturally occurring 28 carboxy-terminal residues of human chorionic gonadotropin (hCG). This relatively conserved peptide (the sequence identity to its monkey and rat homologs is ~79% and ~74%, respectively) has been shown to provide hCG with the required longevity to maintain pregnancy [12,13]. Previous studies have demonstrated that fusion of CTP prolonged the circulatory half-life and increased the *in vivo* potency of FSH [14] and erythropoietin [15]. A long-acting GH was generated by fusing three CTP sequences to the coding sequence of hGH (CTP-hGH-CTP-CTP; MOD-4023). This chimera exhibited dramatically enhanced bioactivity and pharmacokinetics in hypophysectomized rats in comparison to commercial rhGH [16]. The present study characterizes the pharmacokinetic and pharmacodynamic profile of MOD-4023 *in vivo* and *in vitro* in Sprague-Dawley rats and Rhesus monkeys. In addition, toxicology and

safety assessments of long-term, repeated dosing of MOD-4023 are presented. Based on the results of this study, MOD-4023 has the potential to be injected once per week, while providing similar clinical efficacy to daily injections of rhGH.

2. Materials and Methods

2.1. Expression and Purification of MOD-4023

The cDNA of MOD-4023 is composed of three copies of cDNA encoding for CTP and a cDNA-encoding moiety for hGH. One copy of CTP cDNA was fused to the 5' end of the hGH and two copies of CTP cDNA were fused to the 3' end of hGH. MOD-4023 was manufactured by recombinant DNA technology using CD DG44 cells. Transfection was performed using FuGENE-6, and high-throughput cell line development was performed.

2.2. Binding Analysis

The binding affinity of MOD-4023 and rhGH to rat, monkey and human growth hormone receptors (raGHR, maGHR and hGHR, respectively) was compared using surface plasmon resonance analysis (Biacore 3000, GE Healthcare, UK). Recombinant raGHR-Fc, hGHR-Fc (R&D Systems, Minneapolis, MN), and maGHR-Fc chimeras (obtained from Prof. Angel Porgador, Ben-Gurion University, Israel) were immobilized on a CM5 sensor chip (GE Healthcare) in 10 mM acetate (pH=4.5) according to the manufacturer's protocol. This was followed by injection of MOD-4023 (Batch #4-09-X003B-S1,) or rhGH (Biotropin, Ferring Pharmaceuticals, Saint-Prex, Switzerland). The running buffer contained 10 mM Hepes (pH=7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20 (HBS-ET). The immobilization response was 600 resonance units (RU), equal to 0.6 ng protein. All experiments were performed at a flow rate of 50

µl/min. Each injection cycle was followed by surface regeneration using 100 mM NaOH. Data processing was performed using BIA Evaluation Software 4.1 with 1:1 Langmuir model. An empty flow cell was used as a control, and was subtracted from the responses obtained from the reaction surface. Average χ^2 was < 1.5.

2.3. BAFB2B2 Cell Proliferation Assay

The effect of MOD-4023 (Batch #RS0809) and rhGH (Biotropin) on the proliferation of murine BAFB2B2 cells (obtained from Prof. Michael J. Waters, Queensland University, Australia) was evaluated using the MTS method (CellTiter 96 Aqueous One, Promega, Madison, WI). EC_{50} was calculated using PRISM software (GraphPad Software, La Jolla, CA) and a best-fit dose-response stimulation curve. To evaluate the binding of hGHR to MOD-4023, an inhibition dose-response curve of the receptors was prepared using recombinant hGHR-Fc chimeras pre-incubated with MOD-4023 or rhGH. IC_{50} was calculated using PRISM software with a best-fit dose-response inhibition curve.

2.4. STAT5 Phosphorylation Assay by Western Blot

Western immunoblotting was used to evaluate the phosphorylation of STAT5 in hGHR-expressing HEK293 cells [17]. Following treatment with increasing doses of MOD-4023 or rhGH, samples were electrophoresed on 8% SDS polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membranes were incubated with antibodies against phosphorylated STAT5 (Cell Signaling Technology, Danvers, MA) or against total STAT5b (Santa Cruz Biotechnology, Dallas, TX). HRP-linked anti-mouse IgG secondary antibodies were obtained from Amersham (GE Healthcare). The proteins were detected using ECL chemiluminescence reagents (PerkinElmer, Waltham, MA)

according to the manufacturer's instructions. All treatments were performed in duplicates, two independent times.

2.5. Luciferase Activity Assay

The luciferase activity assay utilized the luciferase reporter construct, which carries eight GH response elements (GHREs) from the rat Spi2.1 gene in pGL2 (pGHRE-LUC) [17]. Briefly, hGHR-expressing HEK293 cells were seeded in 6-well plates and transfected with 1 μg of pGHRE-LUC. After treatment with rhGH or MOD-4023 at the indicated concentrations for 24 h, cell lysates were collected and analyzed for reporter activity using the luciferase assay system (Promega) according to the manufacturer's protocol. Luciferase activity was measured using a luminometer (BioTek Instruments, Winooski, VT). Transfection experiments were performed twice independently, and each experiment was performed in duplicates. Results were pooled and standard deviation (SD) scores were calculated.

2.6. Animals

All animal experiments were conducted at MPI Research (Mattawan, MI), except for the weight gain assay in hypophysectomized rats, which was performed in BTG (Kiryat Malachi, Israel). Both facilities complied with all applicable regulations governing the care and use of laboratory animals. A total of 106 male and 106 female experimentally naïve Sprague-Dawley (SD) CD (Crl:CD[SD]) rats (approximately 6 weeks of age) were obtained from Charles River Laboratories (Portage, MI) and housed in an environmentally controlled room. During the 10-day acclimation period, the animals were observed daily with respect to general health and any signs of disease. A total of 100 hypophysectomized (interaural method) SD male rats were obtained from Harlan

(Rehovot, Israel) and were 3-4 weeks of age at study initiation, with an average body weight of 85-90 grams. The animals were weighed upon arrival and their weight was monitored for three weeks during acclimation. Animals that had incomplete hypophysectomy (evidenced by weight gain of more than 5-10% during the acclimatization period) were eliminated from the experiment. All animals were given a detailed clinical examination prior to selection for study.

A total of 30 male and 30 female immature Rhesus monkeys (2-4 years old at the time of the study) were received from Covance Research Products (Princeton, NJ), with approximate weights of 2-5 kg, and housed in an environmentally controlled facility. Prior to assignment to study, all animals underwent a quarantine and acclimation period.

2.7. Pharmacokinetic and Pharmacodynamic Analyses in Rats

The toxicokinetic profile of MOD-4023 was evaluated in naïve SD rats (n = 11/sex/treated group, or 5/sex for the control group) who received subcutaneous injections of vehicle or 3.6, 36, or 180 mg/kg of MOD-4023 twice a week for four weeks. Blood samples were collected from cohorts of three animals/sex/dose group from the orbital sinus with carbon dioxide and oxygen inhalation. Samples were collected prior to dosing and at 1, 2, 4, 8, 24, 48 and 72 hours after dosing on Days 1 and 26 (after the last dose). Mean serum MOD-4023 concentrations were determined for each group at each time point. Parameters evaluated included cageside observations, clinical observations, body weight, food consumption, clinical pathology evaluations, anti-MOD-4023 antibodies, gross pathology, organ weights, and histopathology.

2.8. Efficacy Evaluation in Rats

The effect of MOD-4023 on weight gain was also evaluated in male hypohysectomized rats. MOD-4023 (0.48, 1.45 and 4.34 mg/kg, n=10/group) was injected subcutaneously every 4 days for 12 days. hGH (Biotropin, 0.1 mg/kg) was injected daily (n=10) for 14 days. Individual body weights were determined at randomization, prior to the first dosing, and every 2 days for 21 days. The IGF-1 response to MOD-4023 was evaluated in a repeated dose experiment in naïve SD rats. Four groups of rats (n = 10/sex/group) were administered MOD-4023 at doses of 3.6, 36, and 180 mg/kg by SC injection twice weekly. Additional 5 rats/sex were included in the control and high-dose groups and were maintained after treatment for a 2-week recovery period.

2.9. Pharmacokinetic and Pharmacodynamic Analyses in Monkeys

Four groups of Rhesus monkeys (n = 6/sex/group) were administered MOD-4023 by SC injection at doses of 0 (10 mM citrate, 147 mM NaCl), 1.5, 15, and 30 mg/kg/dose every five days for 26 weeks. Additional 6 monkeys/sex were administered daily with recombinant hGH as a comparator agent at 3.6 mg/kg/day. At the end of the treatment period, four animals/sex/group were sacrificed and the remaining two animals/sex/group were maintained for a 4-week recovery after treatment. The parameters evaluated included cage-side observations, detailed clinical observations, body weight, food consumption, ophthalmoscopy, electrocardiography, clinical pathology, anti-MOD-4023 antibodies, IGF-1, gross pathology, organ weights, and histopathology. Blood samples for determination of the serum concentrations of MOD-4023 and IGF-1 were collected pre-dose and at 1, 2, 4, 8, 24, 48, 72, 96, and 120 hours post-dose on Days 1, 91 and 181.

From the hGH group, samples were collected pre-dose and at 1, 2, 4, 8 and 24 hours post-dose on Days 1, 91, and 181.

2.10. Quantitation of MOD-4023 Serum Levels by ELISA

A quantitative sandwich ELISA was used to evaluate MOD-4023 levels in rat and monkey serum. Samples were incubated with anti-hGH immobilized on a microtiter tissue culture plate. The unbound material was removed by washing with buffer. Biotinylated rabbit anti-CTP was added to the plate, incubated and washed. This process was followed in the same manner using streptavidin-HRP. Superblue tetramethylbenzidine (TMB) was subsequently added to the wells and incubated. The colorimetric reaction was stopped using Stopping Solution when the A650 was between 0.6 and 0.8. The color of the samples was determined at 450 nm with a wavelength correction set at 650 nm using a microplate reader. The range of quantitation for this assay was 1400-75000 pg/ml.

2.11. Immunogenicity Analysis

In rats, blood samples (approximately 2-3 ml) were collected for determination of anti-MOD-4023 Ab level in the serum using bridging ELISA. Samples were collected pre-dose (from two unassigned animals/sex [baseline animals]), prior to the terminal necropsy, at euthanasia, and prior to the recovery necropsy. Samples were placed in plastic tubes containing no anticoagulant, allowed to clot, and stored on wet ice until centrifuged. The samples were divided into 200 µl aliquots and stored as needed on dry ice until transferred to frozen at -70°C. Only samples from the control and high-dose animals were analyzed for the presence of anti-MOD-4023 Abs. The samples were incubated with MOD-4023 immobilized on an ELISA plate and subsequently washed.

Bound antibodies were detected using biotinylated MOD-4023 and streptavidin-HRP, and visualized with TMB. Color development was stopped and A450 mas measured, with wavelength correction set to 650 nm.

Qualitative bridging ELISA was also used for the anti-MOD-4023 and anti-CTP antibody assays in monkey serum. Samples were collected at pre-dose, 120 h post Day 1 dosing, 120 h post-Day 91, on Day 181, and following a recovery period. Tissue culture plates were coated with MOD-4023 or CTP, incubated, and washed. Samples were then added to the plates, incubated and washed. The same process was followed first for biotinylated MOD-4023 and CTP, and then for streptavidin-HRP. Finally, TMB was added to the wells and incubated. The colorimetric reaction was stopped when the A650 was between 0.7 and 1. The color of the samples was determined as described above. For anti-MOD-4023, the acceptable OD range was 0.029-0.087 for rat serum and 0.038-0.115 for monkey serum. For anti-CTP, the acceptable range was 0.007-0.022 for rat serum and 0.007-0.02 for monkey serum.

2.12. Measurement of IGF-1 Levels

The Quantikine Mouse/Rat IGF-1 Immunoassay kit was used for rats and the Quantikine Human IGF-1 Immunoassay kit was used for monkeys (R&D Systems). The kit utilizes a quantitative sandwich ELISA technique. A monoclonal antibody specific for IGF-1 was pre-coated onto microplates, followed by the addition of standards and serum samples. Any IGF-1 present was bound by the immobilized antibody. After a wash step, an enzyme-linked polyclonal antibody specific for IGF-1 was added. Following a second wash to remove unbound antibody-enzyme reagent, a substrate solution was added to the wells. The color developed is in proportion to the amount of IGF-1 bound in the initial

step. The color development was stopped and A450 was measured. In rat and monkey serum, the lower and upper limits of quantitation were 0.39 and 3.78 ng/ml, respectively.

3. Results

3.1. Binding Affinity of MOD-4023 to the GH Receptor

The binding affinities of MOD-4023 and rhGH to rat, human and monkey GHR were compared using surface plasmon resonance analysis. Overall, when the two hGH derivatives are compared in terms of their affinities to rat, monkey or human GHR, the affinity of MOD-4023 to the receptors is ~5-10-fold lower than that of rhGH. The calculated KD values for MOD-4023-raGHR and MOD-4023-hGHR were 5.12 ± 0.50 and 6.38 ± 3.47 nM, respectively, indicating a comparable affinity of MOD-4023 to rat and human GH receptors. Recombinant hGH also showed similar affinities to rat and to human GHR, with rhGH-raGHR and rhGH-hGHR KD values of 0.54 ± 0.19 and 0.91 ± 0.79 nM, respectively. (Table 1).

3.2. Proliferation of BAFB2B2 Cells

BAFB2B2 are murine primary pro-B BAF-3 cells, stably transfected and highly expressing hGHR. The cells were incubated with escalating concentrations of MOD-4023 or rhGH, and the extent of proliferation was quantified using the MTS colorimetric assay. Based on this assay, the in vitro potency of MOD-4023 was 43-fold lower compared to the potency of rhGH. The ability of soluble hGHR or maGHR to inhibit MOD-4023-induced cell proliferation was about 18-20 times lower in comparison to rhGH-induced cell proliferation, further confirming the lower binding affinity of MOD-4023 to the GH receptor (Table 2).

3.3. In vitro GH-Induced STAT5b Phosphorylation and Luciferase Activity

MOD-4023 or rhGH treatment of HEK293 cells expressing hGHR led to activation of the STAT5b signaling pathway. The reproducible STAT5b phosphorylation responses, visualized as band intensity in western immunoblotting analysis, indicated that higher concentrations of MOD-4023 were necessary to induce STAT5b phosphorylation when compared to hGH (Figure 1A). The effects of phosphorylated STAT5b on gene regulation, evaluated by luciferase activity assays, showed luciferase activities correlating to increased STAT5b phosphorylation (Figure 1B). Furthermore, the results indicated that a higher concentration of MOD-4023 (425 ng/ml) was needed to achieve a response similar to 100 ng/ml of rhGH, suggesting a reduced sensitivity of human GHR for MOD-4023.

3.4. Pharmacokinetic Profile of MOD-4023 in SD Rats and Rhesus Monkeys

The pharmacokinetic parameters of MOD-4023 administered to SD rats twice a week for four weeks are presented in Figure 2 and Table 3. Systemic exposure was demonstrated in all MOD-4023-treated animals throughout the dosing period. T_{max} generally occurred at 8 hours on Day 1, and was more prolonged on Day 26 (8 or 24 hours). Exposure, as measured by C_{max} and AUC, increased in an approximately dose-proportional manner. With repeated dosing, exposure tended to increase and CL/F decreased in all groups. As pre-dose concentrations on Day 26 were very low, this trend cannot be due to MOD-4023 accumulation. There was a trend toward somewhat longer $T_{1/2}$ on Day 26 compared to Day 1. For most groups, T_{max} was later on Day 26 compared to Day 1 (24 hours vs. 8 hours).

To evaluate the pharmacokinetic profile of MOD-4023 in Rhesus monkeys, a repeated dose experiment was performed. The animals (n = 6/sex/group) received a subcutaneous injection of MOD-4023 at doses of 0, 1.5, 15, and 30 mg/kg/dose every five days for 26 weeks. An additional group of 12 animals received daily injections of 3.6 mg/kg hGH as a comparator agent (data not shown). The pharmacokinetic analyses for Day 1 and Day 181 are shown in Figure 3 and Table 4. Systemic exposure was demonstrated in all MOD-4023-treated animals. T_{max} generally occurred between 8 and 20 hours, and C_{max} tended to increase in a dose-proportional manner, with no differences between males and females. On Day 91 (data not shown), exposure to MOD-4023 was greater than on Day 1; no marked differences were noted between Day 91 and Day 181.

3.5. Primary Pharmacodynamics – Weight Gain Assay

The effect of MOD-4023 on weight gain, a primary activity of growth hormone, was evaluated in a comparative, repeat-dose experiment in hypophysectomized rats. MOD-4023 (0.48, 1.45 and 4.34 mg/kg, reflecting net hGH content of 0.35, 1.05 and 3.15 mg/kg, respectively) was injected 4 days apart for 12 days (n=10 per dose/group, 4 total doses) and weight gain was compared to animals receiving daily rhGH injections (0.1 mg/kg/day; n=10 per dose) for 14 days. As shown in Figure 4, MOD-4023 at a dose of 0.48 mg/kg (equivalent to actual GH content of 0.35 mg/kg) every 4 days produced a similar increase in weight gain to 0.1 mg/kg rhGH injected daily. A dose-response in weight gain was demonstrated following administration of MOD-4023 at doses of 1.45 and 4.34 mg/kg. To further examine the effect of MOD-4023 on body weight gain, naïve, intact SD rats were administered with 3.6, 36, or 180 mg/kg of MOD-4023 twice a week for four weeks (n = 10/sex/group; additional 5 male and 5 female rats were included in

the control and high-dose groups). Weight gain was measured twice a week during the study. Repeated doses of 36 and 180 mg/kg of MOD-4023 induced dose-proportional weight gain in both male and female rats. At 3.6 mg/kg/dose, an apparent weight gain effect was observed in females only (Figure 5).

3.6. Secondary Pharmacodynamics – IGF-1 Assessment

GH stimulates the hepatic production and release of IGF-1 into the systemic circulation. The mean percent changes of serum IGF-1 concentrations in the repeated dose study in intact SD rats are shown in Figure 6 (% change is shown for Day 1 relative to T = 0; baseline IGF-1 values are presented in Table 5). The results demonstrate an increase in IGF-1 levels relative to pre-dose on both Day 1 and Day 26 (data not shown), indicating that MOD-4023 was pharmacologically active during the study in all dosed groups. The apparent delay of several hours between GH administration and increase in IGF-1 serum levels has been demonstrated previously in rats, where an increase in IGF-1 mRNA was observed several hours after GH treatment and serum IGF-1 peaked after ~12 hours. [18] There appeared to be a sustained response to MOD-4023, as the Day 26 pre-dose IGF-1 concentrations in all groups dosed with 3.6 and 36 mg/kg of MOD-4023 were elevated above the Day 1 pre-dose concentrations (data not shown). As expected from the use of naïve animals, in which endogenous growth hormone is involved in the induction of normal levels of IGF-1, the response was not dose-proportional, as the IGF-1 response at 3.6 mg/kg was higher than observed at 36 mg/kg in both males and females. In females, the highest IGF-1 response was observed at the high dose of 180 mg/kg on both Day 1 and Day 26.

The IGF-1 response to MOD-4023 was also evaluated during the 26-week chronic repeat dose experiment in Rhesus monkeys. On Day 91 and Day 181, the serum IGF-1 concentrations from dosing through 120 hours (24 hours for rhGH-treated animals) were averaged to produce an estimate of the IGF-1 response. These responses were compared to the baseline IGF-1 concentration measured prior to the first dose on Day 1. As can be seen in Figure 7, MOD-4023 produced a dose-dependent increase in IGF-1. A considerable increase in IGF-1 response was observed from Day 0 to Day 90, after which the IGF-1 response appeared to stabilize, with only a slight increase on Day 180. As anticipated, the IGF-1 response to the low dose of MOD-4023 (1.5 mg/kg every five days) was greater than the response to vehicle injection but less than the response following treatment with the higher doses of MOD-4023 and rhGH. The middle and high doses of MOD-4023 (15 and 30 mg/kg every 5 days) produced IGF-1 responses similar to those affected by rhGH administered daily.

3.7. Toxicology Assessments

MOD-4023 was well-tolerated when given once to two times per week SC at doses up to 30 mg/kg/dose for 26 weeks in monkeys, or 180 mg/kg/dose for 4 weeks in rats. The NOAEL was determined to be the highest dose administered in all toxicological studies. No adverse effects were observed in MOD-4023-treated rats and monkeys in terms of clinical signs, body weight, ophthalmoscopy, electrocardiography, clinical pathology, and organ weight parameters. Aside from minor, reversible inflammatory responses at the site of injection and mammary gland changes considered to be a result of an exaggerated pharmacological response of GH as previously reported [19], no evidence of any MOD-

4023-related microscopic alterations were observed. Immunogenicity analyses in rats revealed a mild immunogenic response in both the control and the high-dose groups. However, this response did not affect either exposure to MOD-4023 or biological responsiveness (weight gain and IGF-1). In monkeys, although low antibody titers (directed at the hGH portion of MOD-4023) were detected by ELISA at the end of the study in approximately half of the animals administered with either rhGH or MOD-4023, there did not appear to be any effect on exposure or on IGF-1 response.

4. Discussion

MOD-4023 (CTP-modified hGH) is a novel long-acting recombinant human growth hormone analog for the treatment of children with growth failure due to inadequate endogenous growth hormone secretion, and of adults with GHD. Unlike other long-acting hGH products, fusion of the CTP to hGH extends the hormone's half-life without the use of polymers, encapsulation techniques, or nanoparticles. In contrast to other GH conjugates such as PEG or XTEN, CTP was shown to be directly involved in prolonging the half-life of the original protein from which it is derived (i.e. hCG) [12, 13] and is used for the generation of a long-acting FSH-CTP, which is being marketed as Elonva in Europe.

This study presents *in vitro* and *in vivo* characterization of the pharmacological activity of MOD-4023. In particular, comparisons were made to a currently marketed rhGH product (Biotropin). In all of these experiments, MOD-4023 demonstrated similar mechanism of action as daily hGH, albeit with reduced activity. The calculated equilibrium dissociation constant for rhGH-hGHR interaction correlates with the reported KD for the interaction between GH produced in *E. coli* and hGHR [20]. MOD-4023

exhibited lower affinity than rhGH to rat, monkey and human GHR, as well as a reduced *in vitro* potency in terms of the ability to induce proliferation of BAFB2B2 cells (mediated specifically by hGHR), reduced STAT5b phosphorylation, and a lower relative luciferase activity. The reduced potency of MOD-4023 might be attributed to the additional three copies of CTP, a highly glycosylated peptide, which might affect the ability of MOD-4023 to bind to the GHR. In comparison, hGH fused to XTEN (VRS-317) also exhibited relatively low affinity to hGHR [9].

Pharmacokinetic analysis of MOD-4023 in rats and monkeys indicated a dose-proportional exposure (in terms of C_{max} and AUC) comparable to rhGH, with no major differences between males and females. The presence of antibodies against the hGH component of MOD-4023 in rats and monkeys did not lead to significant neutralization, as they did not affect exposure or IGF-1 response. The serum half-life of MOD-4023 (4-6 hours in rats and 12-20 hours in monkeys) was considerably longer than that of native hGH (3.4 hours after SC injection in humans) [21]. The observation that C_{max} values of MOD-4023 were higher in monkeys than in rats is likely to be explained by the difference in serum half-life in the two species. Serum clearance and terminal half-life were fairly constant across the range of doses tested. This indicates that administration of MOD-4023 every 5 days was adequate to produce exposure throughout the dosing period.

The present study demonstrates that repeated administration of MOD-4023 resulted in elevated IGF-1 levels in rats and monkeys, an observation correlated with the pharmacological action of hGH. Twice-weekly injections of MOD-4023 in rats led to a larger increase in weight gain response as compared to rhGH with fewer injections and a lower total dose of hGH, consistent with its prolonged half-life. The prolonged half-life

of MOD-4023 compared to rhGH can possibly increase the frequency of protein-receptor interactions for MOD-4023. This, in turn, may compensate for the decreased *in vitro* potency of MOD-4023 resulting from its reduced binding affinity to the GHR.

MOD-4023 administered in monkeys at a dose of 30 mg/kg every 5 days resulted in an IGF-1 response similar to rhGH injected daily at an equivalent dose (in terms of GH content). These results suggest that therapeutic pharmacological GH activity could be attained in humans following less frequent injections of MOD-4023, i.e. weekly or every other week, avoiding the need for daily injections of hGH.

MOD-4023 was well-tolerated in rats and monkeys, with minimal adverse effects. The observed changes were anticipated, either based on the pharmacological activity of the drug, or related to local effects at the injection site [19]. Collectively, the results presented in these experiments indicate an acceptable safety profile for MOD-4023. The NOAEL in all of the toxicological studies were the highest administered doses, providing significant margins above the exposures obtained in GHD patients. Based on findings obtained in a Phase 2 study in adults (ClinicalTrials.gov identifier NCT01225666), exposure to MOD-4023 at the highest administered clinical dose was approximately 3800 times lower than exposure at the NOAEL dose in the long-term study in monkeys.

To conclude, GH activity is attained following less frequent injections of MOD-4023, as compared to daily injections of rhGH. These results support the ongoing clinical development program of MOD-4023 in adults and pediatric GHD populations.

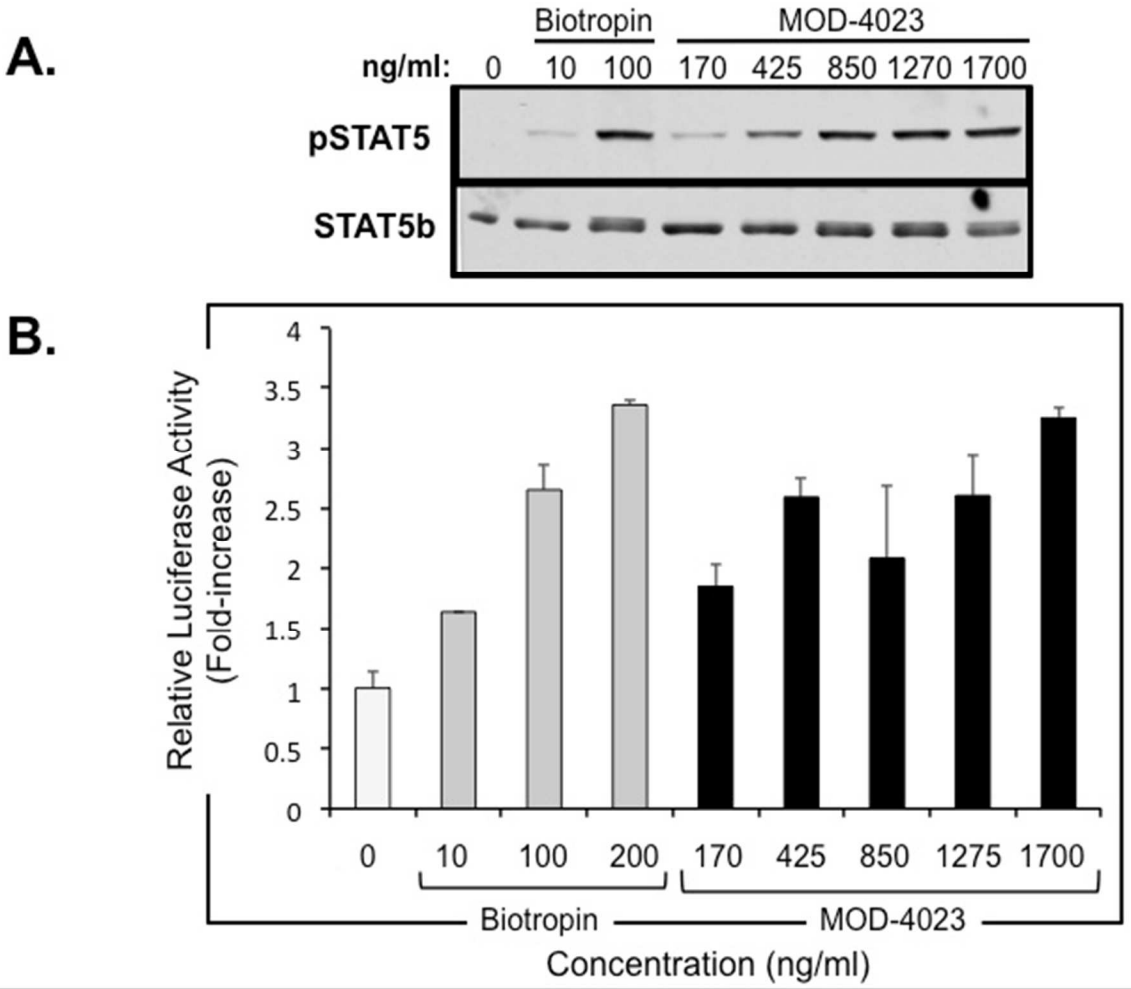
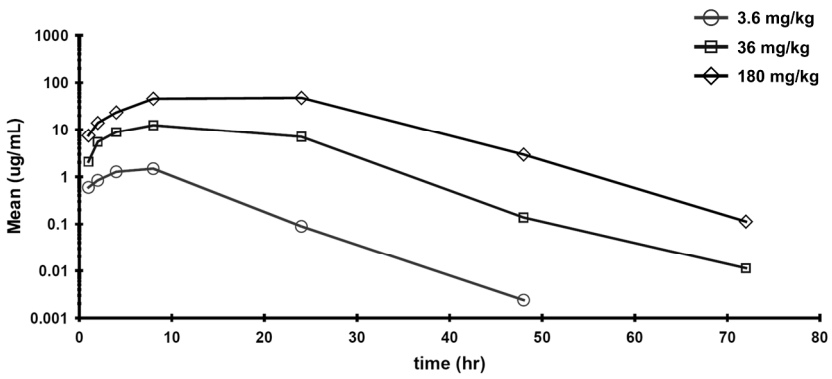
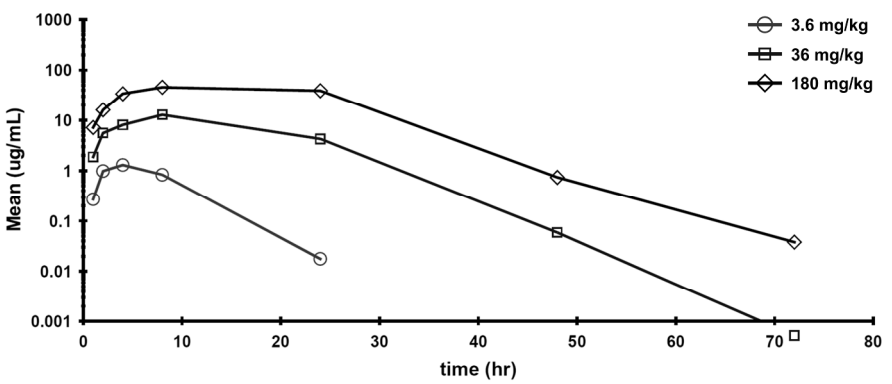

A.
Biotropin
MOD-4023
ng/ml: 0 10 100 170 425 850 1270 1700
pSTAT5
STAT5b
B.
Relative Luciferase Activity (Fold-increase)
4
3.5
3
2.5
2
1.5
1
0.5
0
0 10 100 200 170 425 850 1275 1700
Biotropin
MOD-4023
Concentration (ng/ml)

Figure 1. GH-induced STAT5b Phosphorylation and Luciferase Activity.

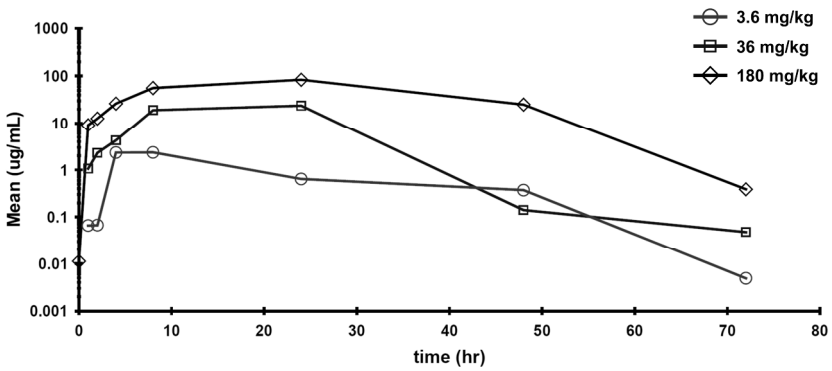
HEK293 cells expressing hGHR were treated (20 min) with concentrations of MOD-4023 and Biotropin as indicated. A. Representative western immunoblot of induced STAT5b phosphorylation. B. Relative luciferase activity of MOD-4023 (black bars) and rhGH (Biotropin; dark grey bars), compared to untreated conditions.


3.6 mg/kg
36 mg/kg
180 mg/kg
Mean (ug/mL)
time (hr)

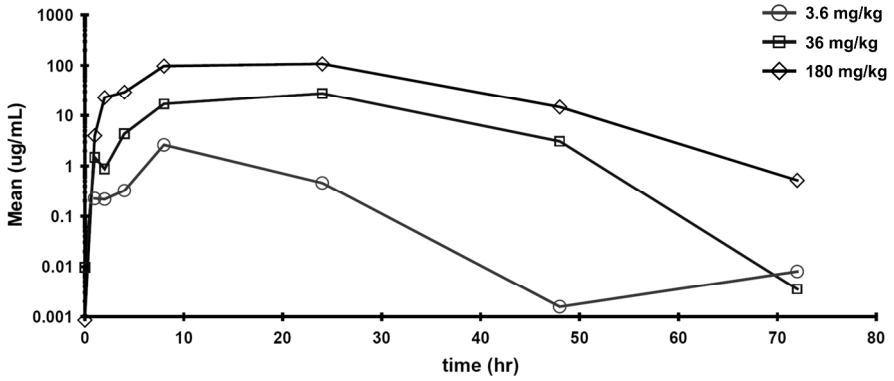
A.


3.6 mg/kg
36 mg/kg
180 mg/kg
Mean (ug/mL)
time (hr)

B.


3.6 mg/kg
36 mg/kg
180 mg/kg
Mean (ug/mL)
time (hr)

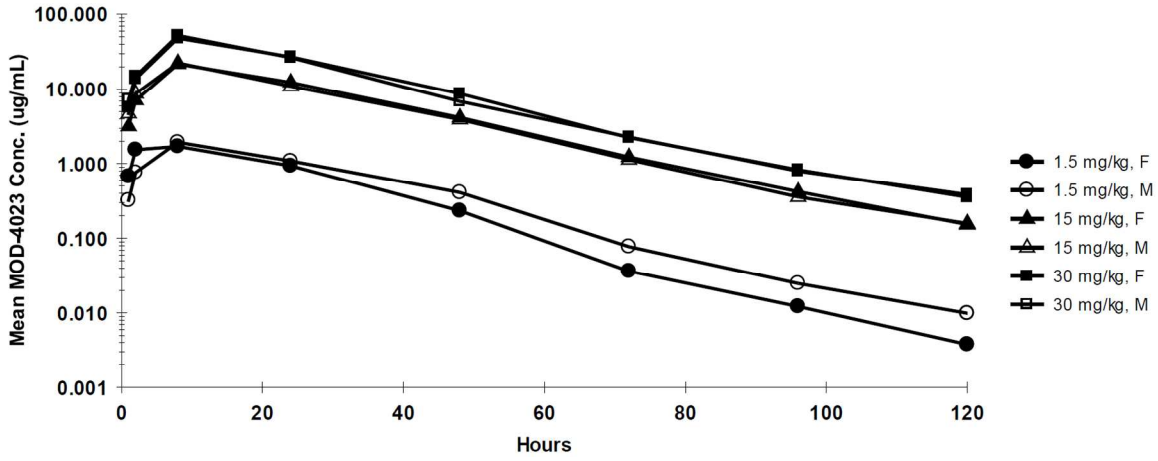
C.


3.6 mg/kg
36 mg/kg
180 mg/kg
Mean (ug/mL)
time (hr)

D.

Figure 2. Pharmacokinetic Analysis Following Repeated SC Injections of MOD-4023 in Rats.

Serum concentrations vs. time profiles in Sprague-Dawley rats following SC injections of 3.6, 36 and 180 mg/kg of MOD-4023. The upper panels show results from Day 1 (A, males, and B, females); the lower panels relate to Day 26 (C, males, and D, females).


100.000
10.000
1.000
0.100
0.010
0.001
Mean MOD-4023 Conc. (ug/mL)
0
20
40
60
80
100
120
Hours
1.5 mg/kg, F
1.5 mg/kg, M
15 mg/kg, F
15 mg/kg, M
30 mg/kg, F
30 mg/kg, M

A.

B.

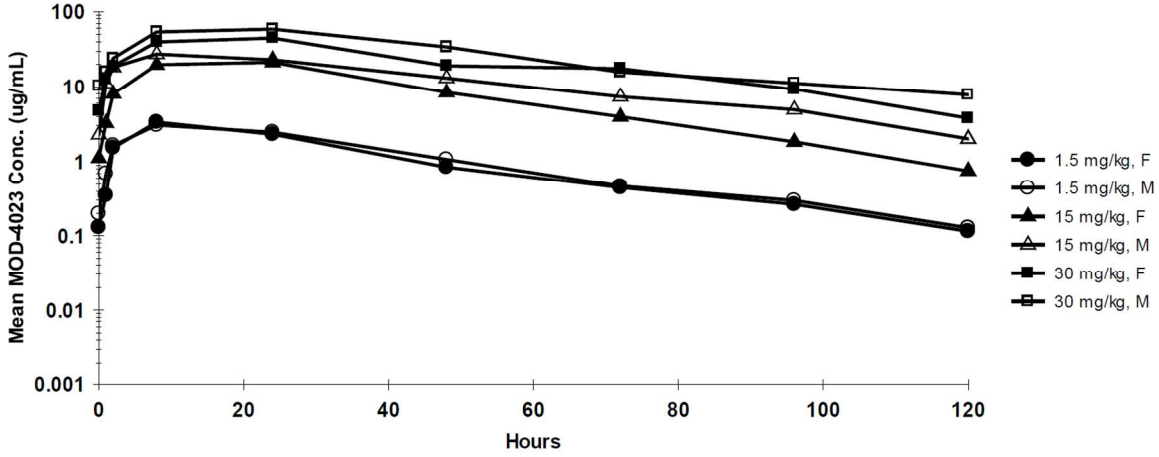

100
10
1
0.1
0.01
0.001
Mean MOD-4023 Conc. (ug/mL)
0
20
40
60
80
100
120
Hours
1.5 mg/kg, F
1.5 mg/kg, M
15 mg/kg, F
15 mg/kg, M
30 mg/kg, F
30 mg/kg, M

Figure 3. Pharmacokinetic Analysis Following Repeated SC Injection of MOD-4023 in Rhesus Monkeys.

Serum concentration vs. time profile for male (M) and female (F) Rhesus monkeys following SC injection of 1.5, 15 and 30 mg/kg MOD-4023. The data shown relates to Day 1 (panel A) and Day 181 (panel B).

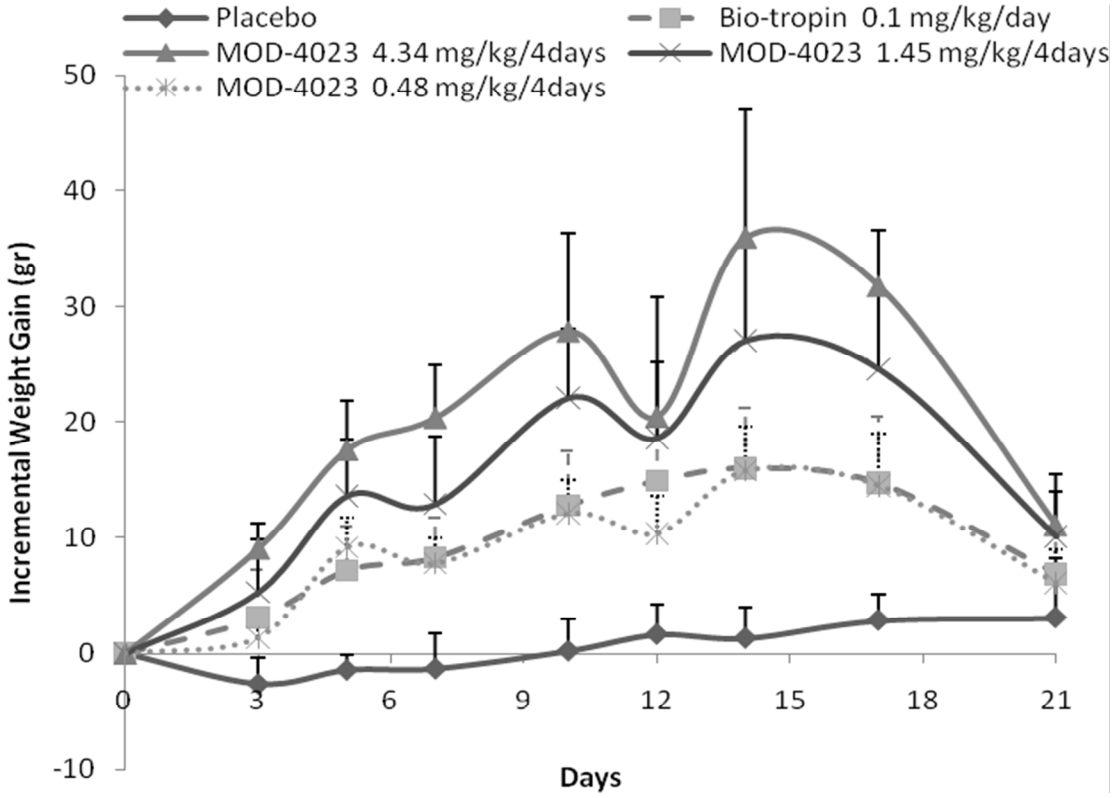
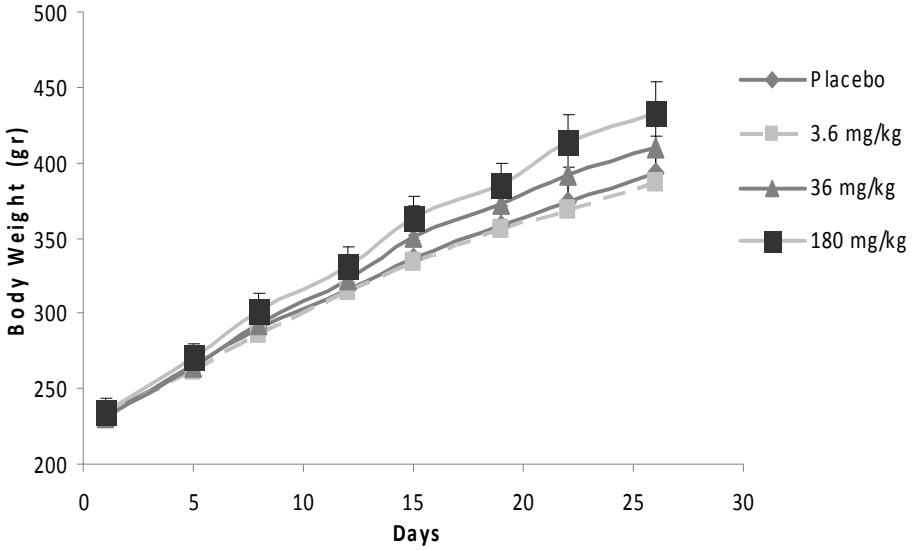

Placebo
Bio-tropin 0.1 mg/kg/day
MOD-4023 4.34 mg/kg/4days
MOD-4023 1.45 mg/kg/4days
MOD-4023 0.48 mg/kg/4days
Incremental Weight Gain (gr)
50
40
30
20
10
0
-10
0
3
6
9
12
15
18
21
Days

Figure 4. The effect of repeated dosing of MOD-4023 on weight gain in hypophysectomized rats compared to Biotropin. Incremental weight gain (gr) is shown for hypophysectomized male rats (n=10/group) injected with placebo, MOD-4023 every 4 days (0.48, 1.45 and 4.32 mg/kg every 4 days) or hGH daily (Biotropin, 0.1 mg/ml).

A.


Body Weight (gr)
500
450
400
350
300
250
200
0
5
10
15
20
25
30
Days
Placebo
3.6 mg/kg
36 mg/kg
180 mg/kg

B.

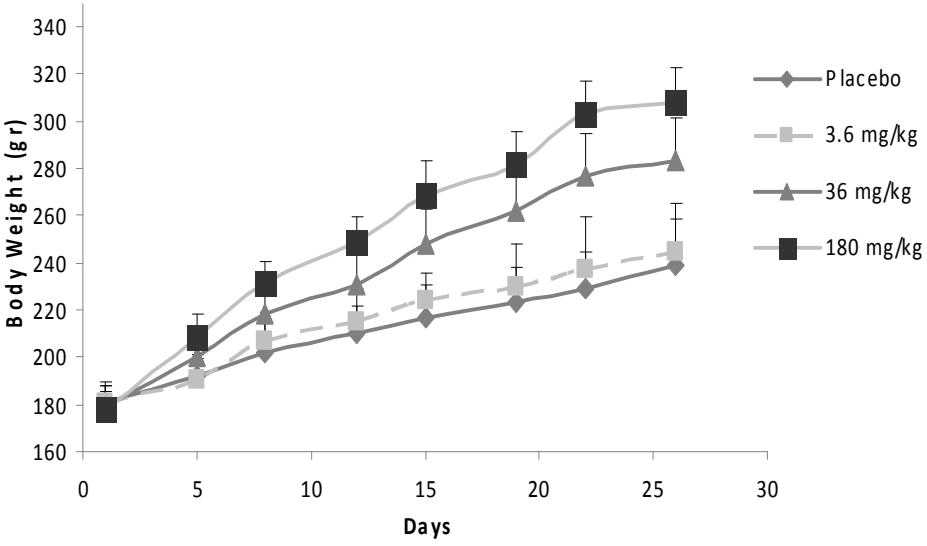
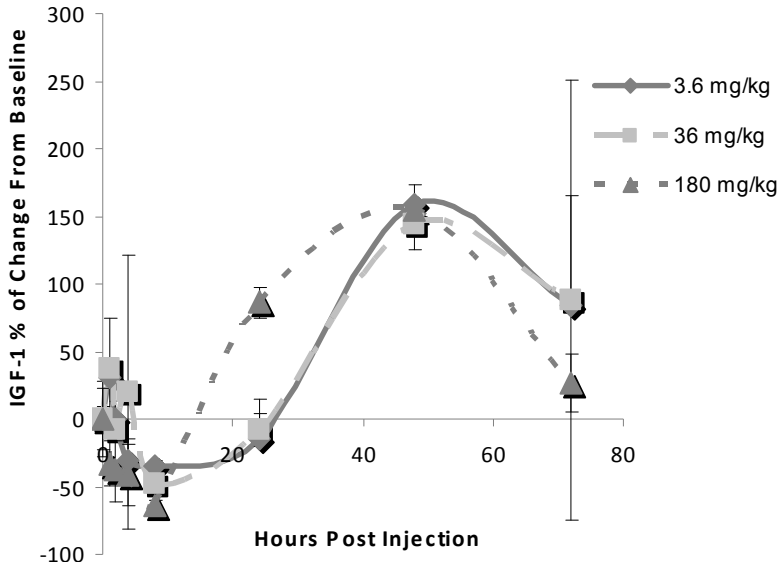

Body Weight (gr)
340
320
300
280
260
240
220
200
180
160
0
5
10
15
20
25
30
Days
Placebo
3.6 mg/kg
36 mg/kg
180 mg/kg

Figure 5. Weight gain in naïve, intact rats following repeated dosing of MOD-4023. Male (A) and female (B) SD rats received SC injection of placebo or MOD-4023 (3.6, 36, or 180 mg/kg) twice a week for four weeks.

A.


IGF-1 % of Change From Baseline
300
250
200
150
100
50
0
-50
-100
0
20
40
60
80
Hours Post Injection
3.6 mg/kg
36 mg/kg
180 mg/kg

B.

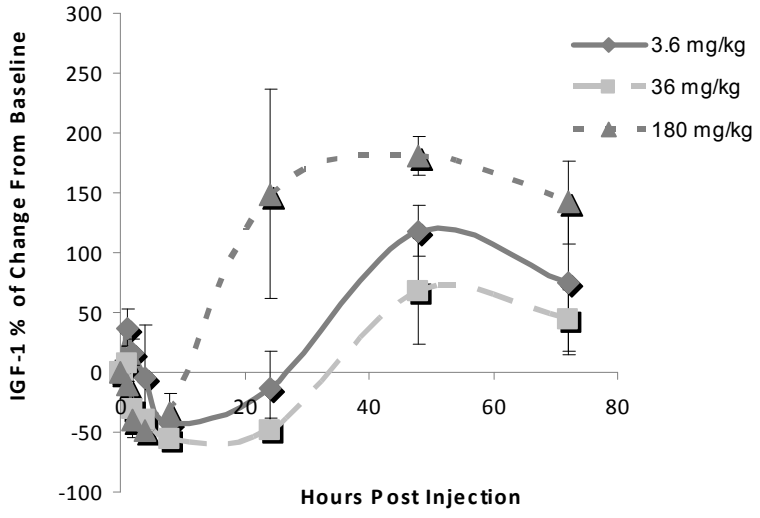

IGF-1 % of Change From Baseline
300
250
200
150
100
50
0
-50
-100
0
20
40
60
80
Hours Post Injection
3.6 mg/kg
36 mg/kg
180 mg/kg

Figure 6. IGF-1 % change from baseline in a repeat dose experiment in SD rats.

The results show the IGF-1 % change (±SD) at Day 1 relative to T = 0 for male (A) and female rats (B) injected with 3.6, 36 or 180 mg/kg MOD-4023 twice a week for four weeks.

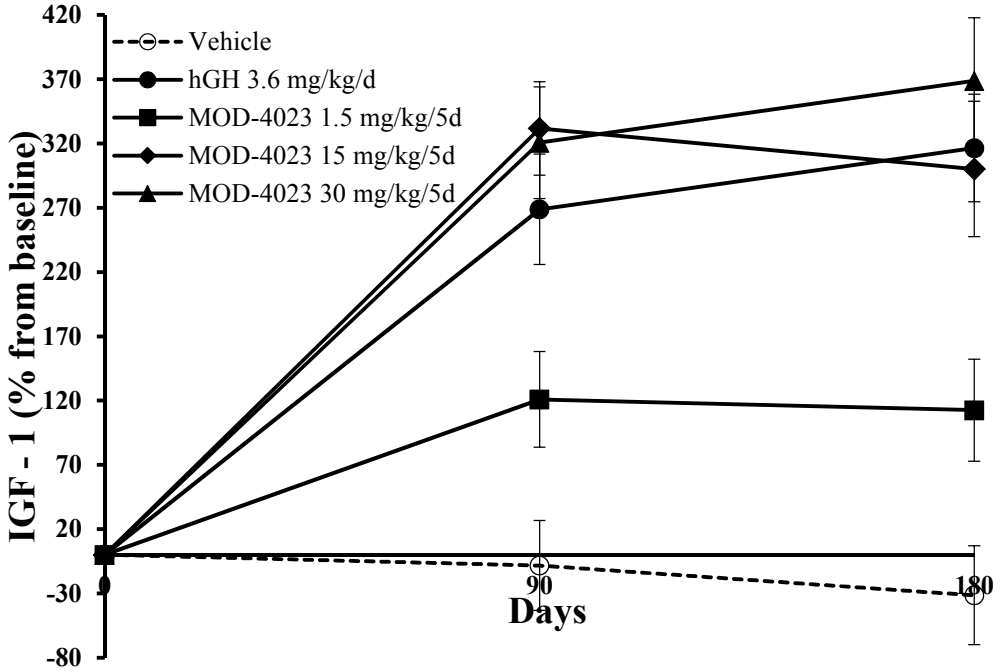

Vehicle
hGH 3.6 mg/kg/d
MOD-4023 1.5 mg/kg/5d
MOD-4023 15 mg/kg/5d
MOD-4023 30 mg/kg/5d
IGF - 1 (% from baseline)
420
370
320
270
220
170
120
70
20
-30
-80
90
180
Days

Figure 7. Change from Baseline in Serum IGF-1 Concentrations in Rhesus Monkeys Following SC Injection for 26 Weeks.

Average IGF-1 change from baseline in monkeys, following repeated dose administration of MOD-4023 every 5 days, or hGH/vehicle daily, for 26 weeks. N = 6 animals/sex/group. Error bars represent SEM.

Table 1. Binding Affinity (K_D) of MOD-4023 and hGH to GHR

Binding affinity to raGHR (nM)	
MOD-4023 (n = 3)	**rhGH (n = 3)**
5.12 ± 0.50	0.54 ± 0.19
Binding affinity to maGHR (nM)	
MOD-4023 (n = 11)	**rhGH (n = 11)**
11.46 ± 5.24	2.09 ± 1.33
Binding affinity to hGHR (nM)	
MOD-4023 (n = 12)	**rhGH (n = 9)**
6.38 ± 3.47	0.91 ± 0.79

Table 2. The Effect of MOD-4023 and rhGH on BAFB2B2 Cell Proliferation

Assay	**MOD-4023**	**rhGH**
Proliferation of BAFB2B2 cells	EC_{50} = 15.8 ± 2.0 ng/ml	EC_{50} = 0.36 ± 0.06 ng/ml
Inhibition of proliferation by maGHR	IC_{50} = 212.6 ± 25.4 ng/ml	IC_{50} = 13.62 ± 5.97 ng/ml
Inhibition of proliferation by hGHR	IC_{50} = 67.3 ± 13.2 ng/ml	IC_{50} = 3.74 ± 1.68 ng/ml

Table 3. PK Parameters of MOD-4023 Following SC Injections in Rats

Dose (mg/kg)	Sex	C_{max} (µg/ml)	T_{max} (hr)	$AUC_{0-\infty}$ (µg•hr/ml)	CL/F (ml/h/kg)	$T_{1/2}$ (hr)
Day 1						
3.6	F	1.30 ± 0.278	4	14.2	254	3.12
	M	1.47 ± 0.172	8	22.2	162	4.33
36	F	12.9 ± 1.14	8	250	144	3.69
	M	12.5 ± 0.659	8	306	118	5.18
180	F	45.7 ± 2.38	8	1390	129	4.79
	M	47.7 ± 4.26	24	1580	114	5.50
Day 26						
3.6	F	2.62 ± 1.35	8	37.1	97.0	6.60
	M	2.35 ± 1.80	8	52.6	68.4	7.75
36	F	27.9 ± 18.2	24	819	44.0	3.71
	M	23.6 ± 5.45	24	682	52.8	5.36
180	F	108 ± 15.1	24	3630	49.5	6.24
	M	83.3 ± 12.9	24	2940	61.2	6.20

Table 4. PK Parameters of MOD-4023 Following SC Injections in Monkeys

Dose mg/kg	Sex	C_{max} (µg/ml)	T_{max} (hr)	$AUC_{0-\infty}$ (µg•hr/ml)	CL/F (ml/h/kg)	$T_{1/2}$ (hr)	Vz/F ml/kg
Day 1							
1.5	F	2.50 ± 1.11	5 ± 2	53.04 ± 9.53	29.14 ± 5.77	12.76 ± 2.06	537 ± 144
	M	2.10 ± 0.60	7 ± 2	60.38 ± 11.98	25.60 ± 4.64	16.17 ± 2.30	592 ± 102
15	F	21.76 ± 6.24	8 ± 0	667.00 ± 156.19	23.40 ± 4.73	15.88 ± 0.98	539 ± 131
	M	22.89 ± 8.66	11 ± 7	648.06 ± 69.65	23.37 ± 2.51	15.47 ± 1.84	522 ± 82
30	F	48.19 ± 12.92	8 ± 0	1438.49 ± 173.68	21.11 ± 2.50	16.66 ± 2.41	513 ± 125
	M	53.54 ± 16.83	7 ± 2	1432.88 ± 195.63	21.23 ± 2.55	16.93 ± 2.01	517 ± 84
Day 181							
1.5	F	3.76 ± 2.18	9 ± 8	135.38 ± 154.51	20.57 ± 11.87	15.40 ± 7.39	375 ± 189
	M	3.71 ± 1.62	9 ± 8	142.43 ± 132.74	18.58 ± 10.87	17.01 ± 6.91	373 ± 174
15	F	21.75 ± 11.31	21 ± 7	1050.33 ± 678.85	19.55 ± 10.15	16.57 ± 6.32	398 ± 110
	M	35.89 ± 10.63	12 ± 9	1576.77 ± 1345.14	14.85 ± 8.77	19.94 ± 8.77	345 ± 117
30	F	48.54 ± 10.77	18 ± 9	2753.74 ± 1398.35	13.91 ± 7.52	22.44 ± 12.53	359 ± 67
	M	60.79 ± 66.57	16 ± 9	4123.58 ± 6684.93	19.29 ± 10.29	22.54 ± 19.55	422 ± 184

Table 5. Baseline (Day 1) IGF-1 Levels in Rats and Monkeys

Sex	Dose (mg/kg)	IGF-1 Levels on Day 1 (ng/ml)
Rats		
F	3.6	504 ± 54
M		561 ± 53
F	36	843 ± 32
M		711 ± 160
F	180	572 ± 32
M		1038 ± 32
Monkeys		
F	1.5	359 ± 181
M		224 ± 86
F	15	468 ± 301
M		297 ± 77
F	30	398 ± 131
M		316 ± 154

Acknowledgement

The authors wish to thank Prof. Angel Porgador (Ben Gurion University) and Dr. Hagit Zer (Hebrew University) for their assistance with the Biacore experiments. Dr. Doron Calo (OPKO Biologics) provided assistance in writing and technical editing of the manuscript.

Abbreviations

AUC, area under curve; CL/F, apparent total clearance; C_{max}, maximum concentration; CTP, carboxyl-terminal peptide; EC_{50}, concentration of half-maximal response; FSH, follicle-stimulating hormone; IGF-1, insulin-like growth factor 1; GH, growth hormone; GHD, growth hormone deficiency; hCH, human chorionic gonadotropin; hGHR, human growth hormone receptor; IC_{50}, half-maximal inhibitory concentration; maGHR, monkey growth hormone receptor; NOAEL, no-observed-adverse-effect level; PEG, polyethylene glycol; raGHR, rat growth hormone receptor; rhGH, recombinant human growth hormone; SC, subcutaneous; SD, Sprague-Dawley; T1/2, half-life; T_{max}, time of maximal concentration; TMB, tetramethylbenzidine; Vz/F, apparent volume of distribution.

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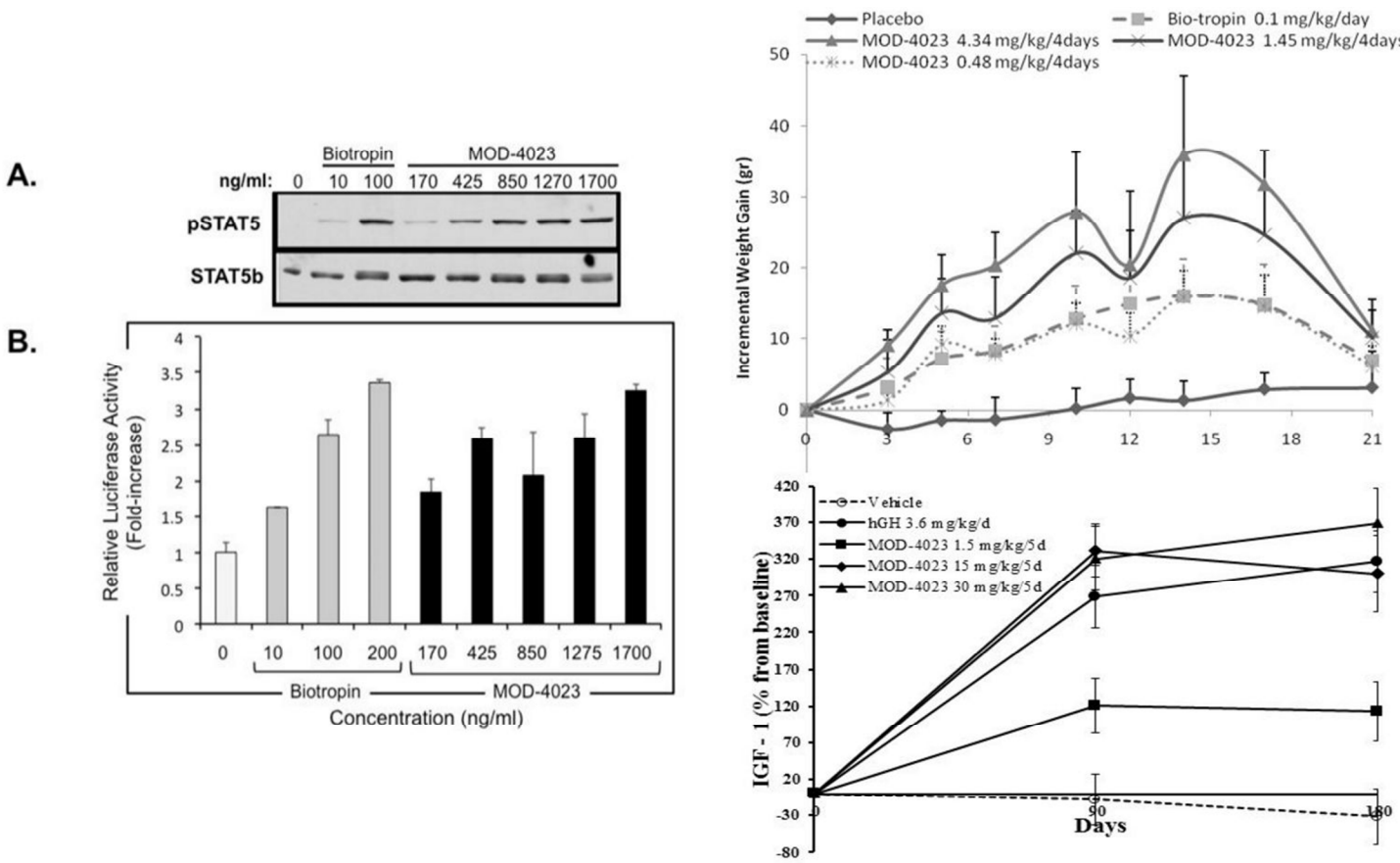

Abstract Graphic

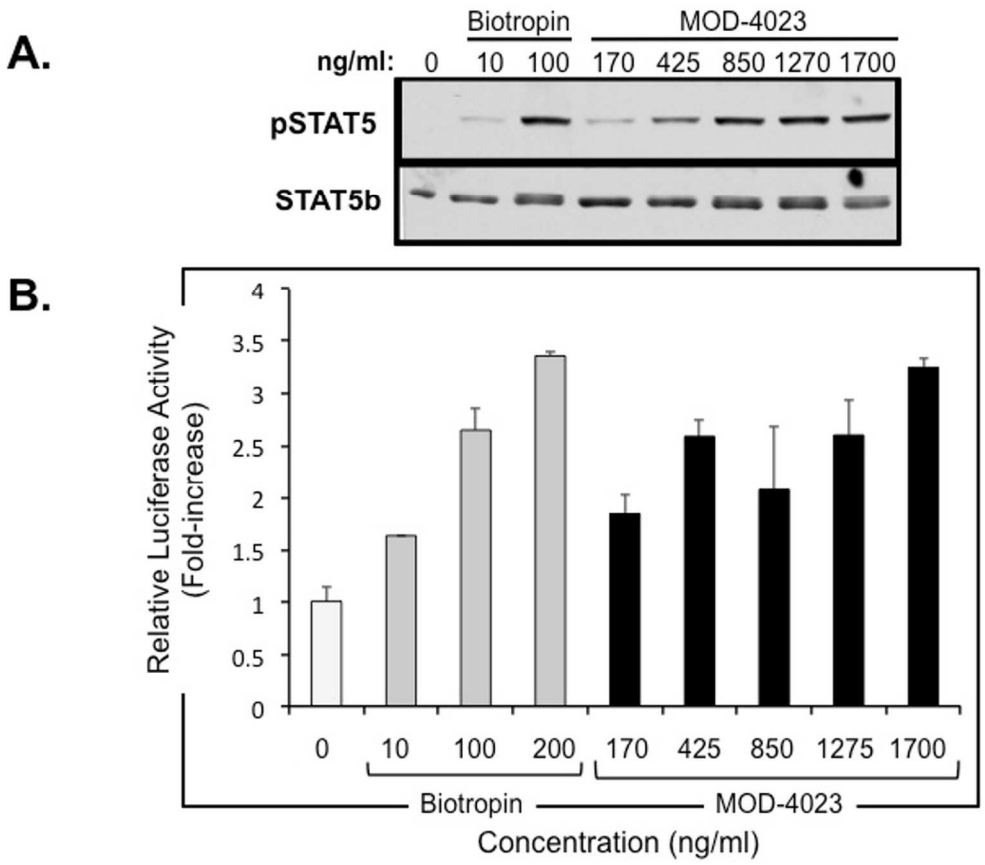

Figure 1. GH-induced STAT5b Phosphorylation and Luciferase Activity.
HEK293 cells expressing hGHR were treated (20 min) with concentrations of MOD-4023 and Biotropin as indicated. A. Representative western immunoblot of induced STAT5b phosphorylation. B. Relative luciferase activity of MOD-4023 (black bars) and rhGH (Biotropin; dark grey bars), compared to untreated conditions.

169x145mm (300 x 300 DPI)

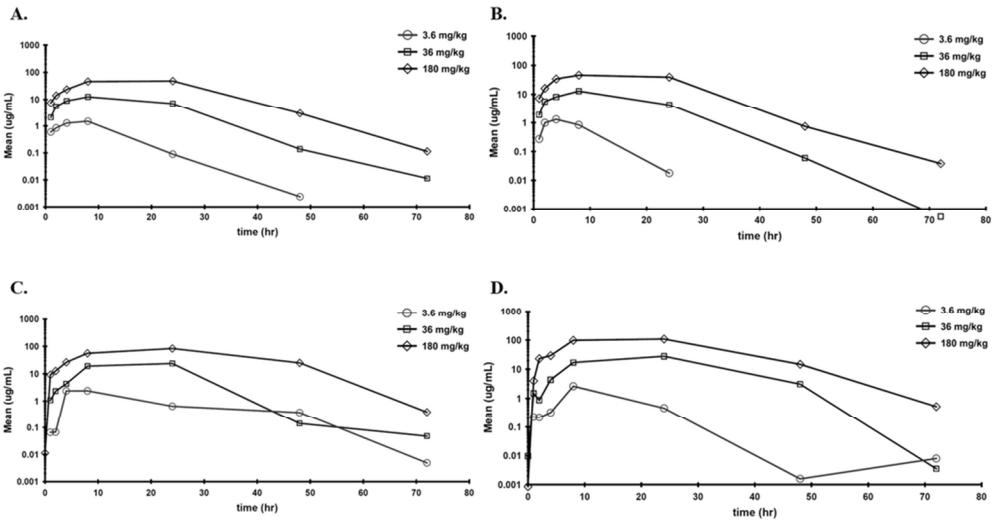

Figure 2. Pharmacokinetic Analysis Following Repeated SC Injections of MOD-4023 in Rats.
Serum concentrations vs. time profiles in Sprague-Dawley rats following SC injections of 3.6, 36 and 180 mg/kg of MOD-4023. The upper panels show results from Day 1 (A, males, and B, females); the lower panels relate to Day 26 (C, males, and D, females).
43x22mm (600 x 600 DPI)

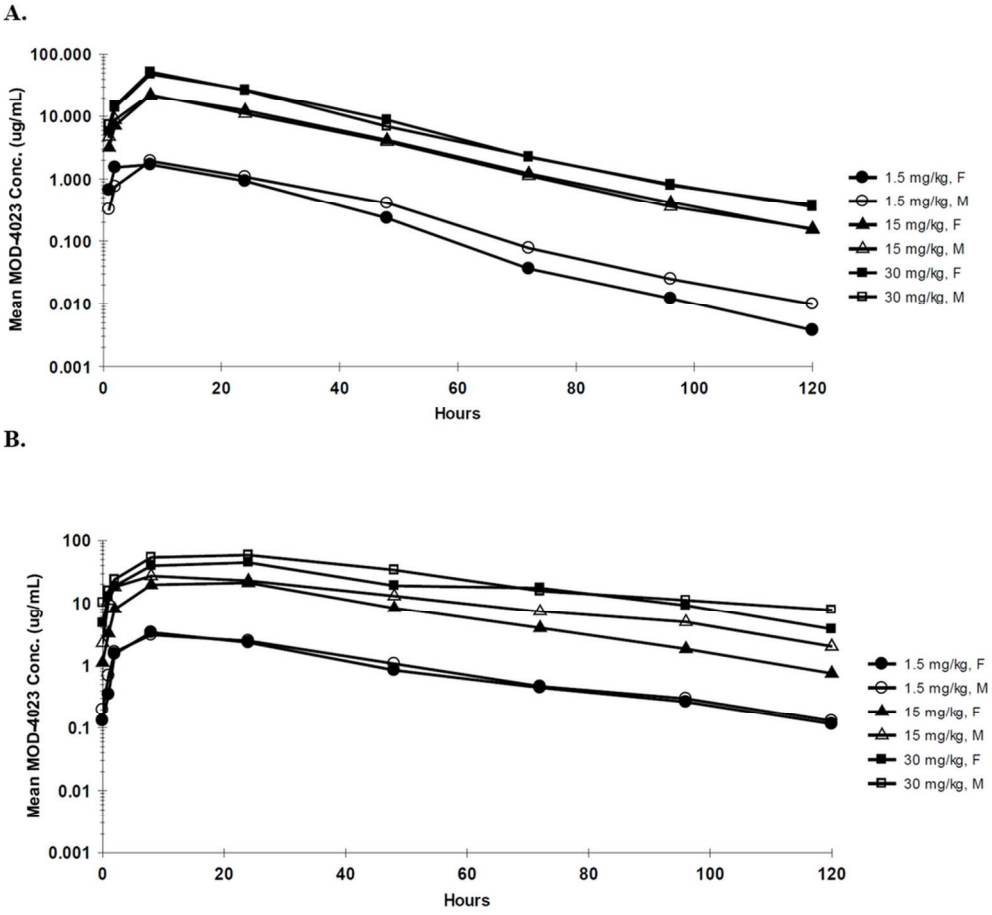

Figure 3. Pharmacokinetic Analysis Following Repeated SC Injection of MOD-4023 in Rhesus Monkeys. Serum concentration vs. time profile for male (M) and female (F) Rhesus monkeys following SC injection of 1.5, 15 and 30 mg/kg MOD-4023. The data shown relates to Day 1 (panel A) and Day 181 (panel B).
58x53mm (600 x 600 DPI)

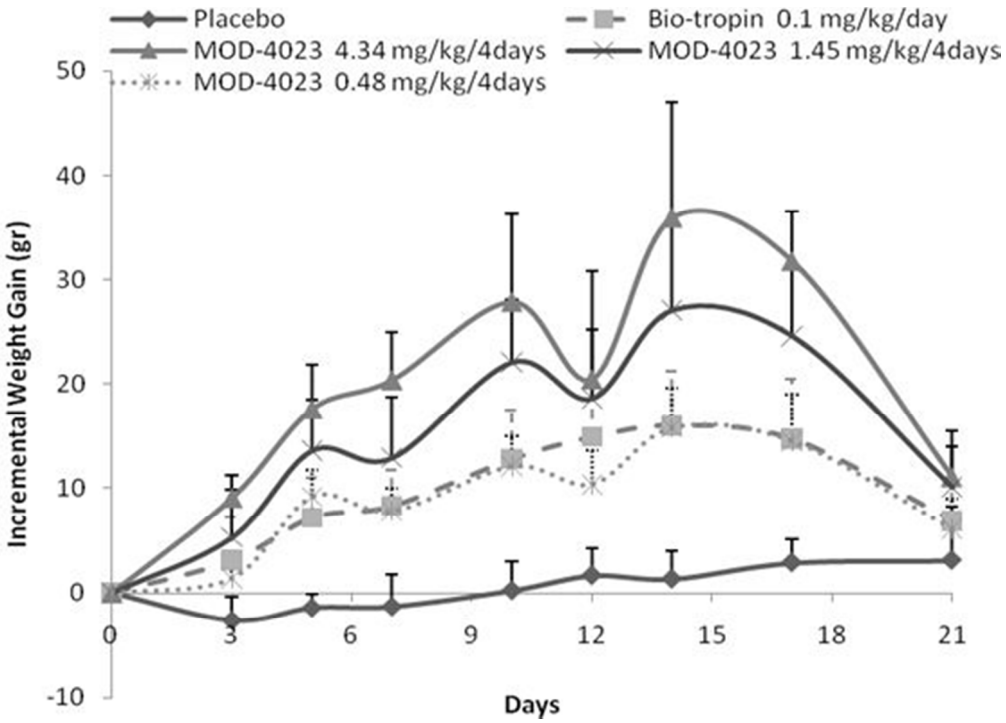

Figure 4. The effect of repeated dosing of MOD-4023 on weight gain in hypophysectomized rats compared to Biotropin. Incremental weight gain (gr) is shown for hypophysectomized male rats (n=10/group) injected with placebo, MOD-4023 every 4 days (0.48, 1.45 and 4.32 mg/kg every 4 days) or hGH daily (Biotropin, 0.1 mg/ml).

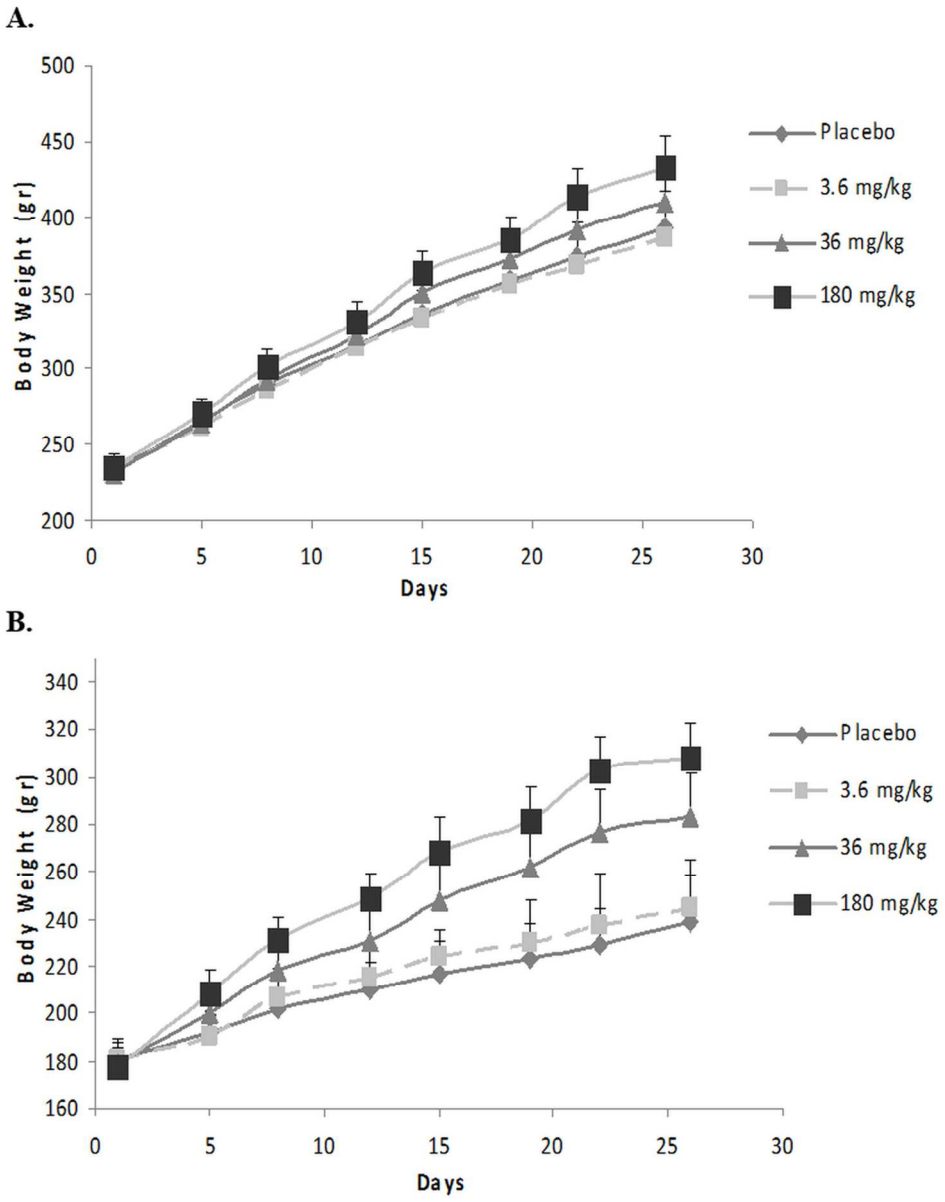

Figure 5. Weight gain in naïve, intact rats following repeated dosing of MOD-4023. Male (A) and female (B) SD rats received SC injection of placebo or MOD-4023 (3.6, 36, or 180 mg/kg) twice a week for four weeks.
107x137mm (300 x 300 DPI)

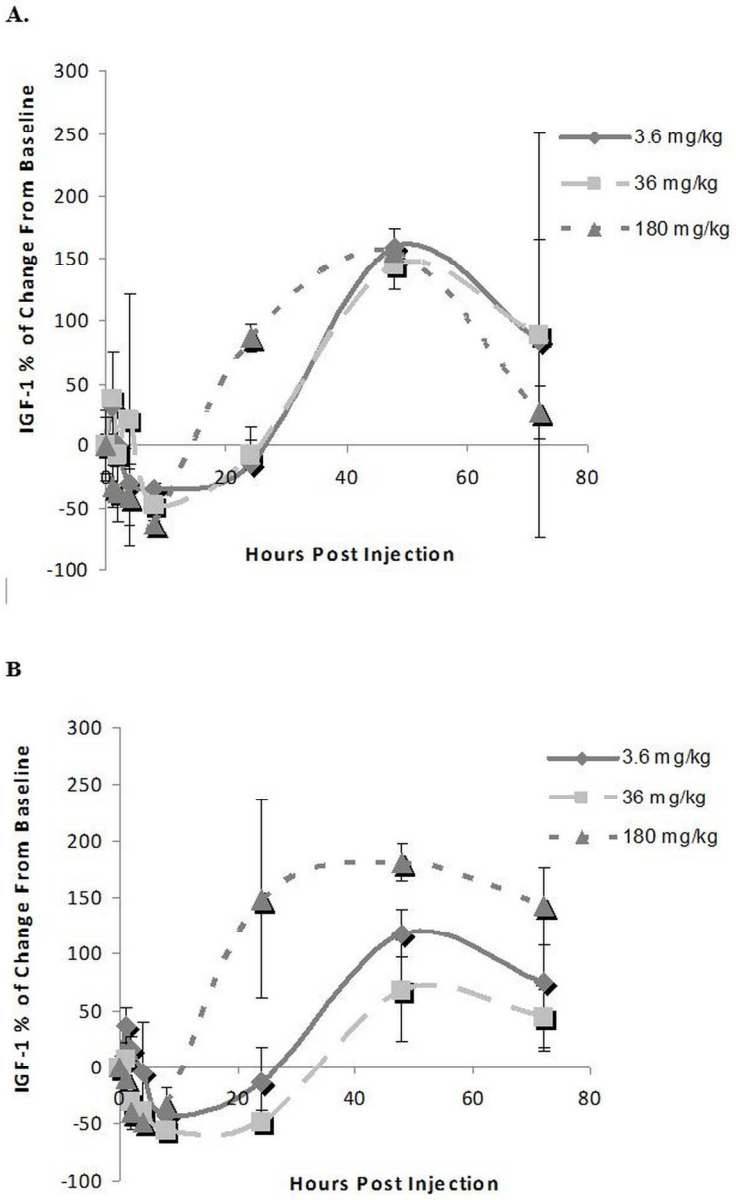

Figure 6. IGF-1 % change from baseline in a repeat dose experiment in SD rats.
The results show the IGF-1 % change (±SD) at Day 1 relative to T = 0 for male (A) and female rats (B) injected with 3.6, 36 or 180 mg/kg MOD-4023 twice a week for four weeks.

254x410mm (300 x 300 DPI)

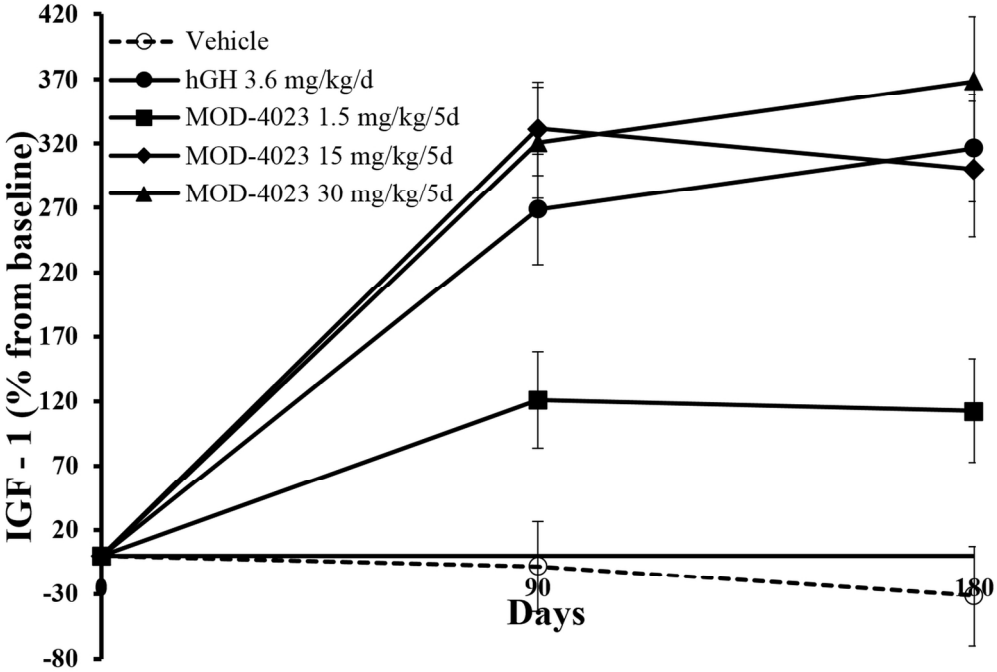

Figure 7. Change from Baseline in Serum IGF-1 Concentrations in Rhesus Monkeys Following SC Injection for 26 Weeks.
Average IGF-1 change from baseline in monkeys, following repeated dose administration of MOD-4023 every 5 days, or hGH/vehicle daily, for 26 weeks. N = 6 animals/sex/group. Error bars represent SEM.

169x113mm (300 x 300 DPI)