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ORIGINAL RESEARCH

Preclinical Investigations of a Novel PEGylated Long-Acting Human Growth Hormone

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Purpose: The daily administration of recombinant human growth hormone (rhGH) replacement therapies via subcutaneous (SC) injections can be burdensome for patients. Long-acting Growth hormone (GH) formulation could reduce injection frequency, and it will be expected to increase treatment adherence. ZHB111 is a PEGylated rhGH that requires sc injection every two weeks. This study aims to examine the pharmacokinetics (PK), pharmacodynamics (PD), safety, and tolerability of multiple sc doses of ZHB111.

Design and Methods: In the PK study, naïve cynomolgus monkeys were divided into four groups with 3/sex/group. Each group received single subcutaneous administration at 0 (control article, Sodium chloride injection), 0.3, 1.0 and 3.0 mg/kg, respectively. To access safety and PD profile, cynomolgus monkeys were randomly assigned to 4 groups of 5/sex/group, and were dosed with ZHB111 once weekly for 4 times by subcutaneous injection at 0 (control article, Sodium chloride injection), 1, 5 or 20.6 mg/kg/dose. Tissue distribution was accessed by administering a single subcutaneous dose of ZHB111 to Sprague-Dawley Rats.

Results: ZHB111 exhibits a significant difference in serum half-life (in monkeys) across varying dosages, with a trend of decreasing half-life as the dosage increases, ranging from 110–30 h. No significant differences were observed between different genders at the same dosage level. After subcutaneous administration of 0.3mg/kg of ZHB111, the mean values of IGF-1 in male animals ranged among 594.93~1294.98ng/mL at various time points, while those in female animals were within the range of 394.15~770.01ng/mL.

Conclusion: The tolerability of ZHB111 was demonstrated in both rat and monkey models, with only minimal adverse effects observed. These findings support the continued clinical development of ZHB111 as a novel treatment for growth hormone deficiency (GHD) in both children and adult patients.

Keywords: long-acting, growth hormone, PEGylated, growth hormone deficiency, GHD

Introduction

Growth hormone (GH), a pituitary protein comprised 191 amino acids, promotes the hepatic synthesis and secretion of insulin-like growth factor-1 (IGF-1) into the systemic circulation. IGF-1 significantly promotes the linear growth of children and plays an important role in regulating metabolism and body-mass composition of adults. Growth hormone deficiency (GHD) can manifest as a standalone disorder or as part of a spectrum of hormone deficiencies. GH deficiency is characterized by insufficient circulating IGF-1 levels, leading to impaired linear growth in children, and further consequences such as diminished lean body mass, increased fat mass, weakness, and reduced exercise capacity, muscle mass/strength, cardiac performance and bone density. Additionally, it can cause neuropsychological disturbances in adults.[1]

Treating patients with GHD in a method of recombinant human growth hormone (rhGH) has been in use since the 1980s.[2] This therapy has been demonstrated to be both secure and efficacious in promoting height growth in pediatric patients and improving clinical and metabolic parameters in those with GHD. Meanwhile, rhGH can also be used to treat short stature associated with Noonan syndrome, Turner syndrome and other indications.[3,4] However, current rhGH

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therapy (0.024–0.034mg/kg) necessitates daily subcutaneous (SC) injections, which may reduce compliance, particularly among children and adolescents.

Over time, researchers have concentrated their efforts on the creation of long-acting GH formulations with the aim of extending the half-life of the GH molecule, thereby reducing the frequency of administration.

Many LAGH formulations have been developed but a few have been approved and marketed for treatment of adults or children with GHD. Those marketed formulations include PEGylated rhGH (Jintrolong®),[5] modified rhGH with increased albumin binding (somapacitan-beco, Sogroya®),[6] prodrug formulation (lonapegsomatropin-tcgd, Skytrofa®)[7] and rhGH fusion proteins (somatrogon-ghla, NGENLA®).[8]

Jintrolong® is a PEGylated long-acting rhGH. The 40 kDa hydrophilic polyethylene glycol (PEG) residue attached to rhGH serves to prolong the serum half-life of GH, whilst simultaneously reducing its immunogenicity.

Jintrolong® was approved for marketing in China in 2014 for pediatric patients affected by GHD, and its weekly dosage is 0.2 mg/kg.

In 2020, Sogroya® was approved for marketing in the US for adults with GHD. Its weekly dosage is 1.5 mg-8 mg. Somapacitan-beco is a human growth hormone (hGH) analog that has undergone a modification to its amino acid backbone (L101C) through the attachment of an albumin-binding moiety. This albumin-binding moiety (side-chain), which is connected to position 101 of the protein, is composed of an albumin binder and a hydrophilic spacer.

Skytrofa® is a human growth hormone which was approved for marketing in the US in 2021. The recommended dose is 0.24 mg/kg based on actual body weight administered once weekly. It has been indicated for the treatment of pediatric patients over one year old who weigh at least 11.5 kg and have growth failure due to inadequate secretion of endogenous growth hormone (GH). Lonapegsomatropin-tcgd is a long-lasting prodrug of a human growth hormone (somatropin) which is produced via recombinant DNA technology with E. coli. Lonapegsomatropin-tcgd is composed of a parent drug, somatropin, which is coupled to a methoxypolyethylene glycol carrier (4 x 10 kDa mPEG) through a proprietary TransCon Linker. The resulting molecule possesses a molecular weight of 63 kDa (the released somatropin exhibits a molecular weight of 22 kDa).

The human growth hormone, NGENLA, has been indicated for the treatment of pediatric patients aged 3 years and older with growth failure due to insufficient secretion of endogenous growth hormone, which was approved for marketing in the US in 2023. The recommended dosage is 0.66 mg/kg based on actual body weight administered once weekly. Somatrogon-ghla, a fusion protein, is produced in Chinese Hamster Ovary (CHO) cells using recombinant DNA technology. The amino acid sequence of human growth hormone (hGH) is incorporated into Somatrogon-ghla, along with one copy of the C-terminal peptide (CTP) derived from the beta chain of human chorionic gonadotropin (hCG) at the N-terminus, as well as 2 copies of CTP (in tandem) at the C-terminus. The molecular weight of Somatrogon-ghla is approximately of 40 kDa.

Existing literature documents that inadequate adherence to the prescribed daily GH treatment regimen is a critical factor affecting growth response.[9] Over time, patients often become non-adherent to GH treatment, which requires strict adherence for optimal outcomes. LAGH has the potential to assist in the reduction of non-adherent patients and the optimization of the benefits derived from GH therapy.[10,11]

ZHB111 is a PEGylated long-acting rhGH, with a 60 kDa hydrophilic polyethylene glycol (PEG) residual attached. PEGylation prolongs the serum half-life of GH and reduces its immunogenicity.[12,13] Protein PEGylation is a clinically proven approach that enables protein therapeutics to be better medicines. PEGylation involves synthesizing a protein and then modifying it through a conjugation reaction with a PEGylation reagent. As a widely accepted approach, PEGylation is utilized for the enhancement of the pharmaceutical properties of biopharmaceuticals, and has been employed increasingly in the development of new large-molecule drug candidates.[14,15] ZHB111 has been developed with the aim of facilitating treatment convenience for patients requiring GH administration.

This study aims to characterize the pharmacokinetic and pharmacodynamic profile of ZHB111 in Cynomolgus monkeys in vivo. Furthermore, the results of toxicological and safety assessments conducted on ZHB111 following repeated long-term administration are also presented herein. According to these outcomes, ZHB111 has the promise of being developed as a new LAGH drug for the treatment of GHD.

Materials and Methods

Preparation of ZHB111

ZHB111, developed by ZONHON BIOPHARMA INSTITUTE, INC., is a polyethylene glycol-modified recombinant human growth hormone (PEG-rhGH). The rhGH molecule is covalently attached via its amino terminus to a 60 kDa branched hydrophilic PEG residue. The molecular weight of ZHB111 is around 82 kDa, with 60 kDa PEG and 22 kDa rhGH.

Animals

Most animal experiments were conducted at JOINN (Suzhou) Laboratories Co. Ltd, and the short-term weight gain assay in hypophysectomized rats was carried out at ZONHON BIOPHARMA INSTITUTE, INC. All procedures were conducted in accordance with the relevant regulations that govern the care and use of laboratory animals. The number of animals used is the minimum needed to ensure the validity and statistical significance of the science data.

Thirty-six Sprague-Dawley rats (18 males and 18 females), obtained from JOINN (Suzhou) Laboratories, were used in the tissue distribution of ZHB111 study (30 were actually used, the rest were used for blank tissue collection in methodology). A total of 600 male Wistar rats, obtained from Shanghai Laboratory Animal Center (SLAC), were used in the short-term weight gain assay of hypophysectomized rats. The body weight of 60–80 g male Wistar rats were selected, prepared of hypophysectomized rats by surgery (parapharyngeal method can be used to make stable hypophysectomized model rats), and their weight was monitored for 2–3 weeks after the surgery. From the outset, any animal that had incomplete hypophysectomy, as indicated by weight gain over 10% during the monitoring period, were excluded from the further participation in the experiment. Prior to being considered for this study, all animals were housed in an environmentally controlled room and underwent a comprehensive clinical examination.

A total of 64 Cynomolgus monkeys, 32 of each sex, were purchased by JOINN (Suzhou) Laboratories and placed in an environmentally controlled facility.

Distribution

The tissue distribution of ^{125}I-ZHB111 after a single subcutaneous administration in Sprague-Dawley Rats was studied by a radioisotope (^{125}I) labeled tracer method.

Thirty Sprague-Dawley rats (15 females and 15 males) were injected subcutaneously with a single dose (1.2 mg/kg) of ^{125}I-ZHB111. Body fluid and tissue samples were collected at 4 h, 8 h, 24 h, 48 h, and 72 h, respectively, after administrations (3 females and 3 males per time point). The total radioactivity and the precipitated radioactivity after TCA precipitation were measured in a γ-counter to calculate the corresponding concentration and exposure of ^{125}I-ZHB111 in each tissue.

Efficacy Evaluation in Rats

The effect of ZHB111 on weight gain was evaluated in male hypophysectomized rats. ZHB111 (0.3, 0.6, and 1.2 mg/kg, n = 10/group) was injected subcutaneously with a single dose. The positive control (Jintrolong) group was dosed with Jintrolong injection by single subcutaneous administration at 1.2 mg/kg. During the experiment, clinical observation and body weight measurement were carried out on animals daily.

Pharmacokinetic and Pharmacodynamic Analyses in Monkeys

In the pharmacokinetic study, 24 (12/sex) naïve cynomolgus monkeys were divided into four groups with 3/sex/group. Monkeys in groups 1, 2 and 3 were dosed with ZHB111 injection by single subcutaneous administration at 0.3, 1.0 and 3.0 mg/kg, respectively. Monkeys in group 4 were dosed with ZHB111 injection by single intravenous bolus administration at 1.0 mg/kg. Serum samples of groups 1 to 3 were collected at pre-dose (0), 30 min, 4 h, 8 h, 24 h (Day 2), 48 h (Day 3), 72 h (Day 4), 96 h (Day 5), 144 h (Day 7), 192 h (Day 9), 288 h (Day 13), 360 h (Day 16) and 480 h (Day 26) post-dose. And for group 4, the serum samples were collected at pre-dose (0), 1 min, 2 h, 4 h, 8 h, 24 h (Day 2), 48 h (Day 3), 72 h (Day 4), 96 h (Day 5), 144 h (Day 7), 192 h (Day 9), 288 h (Day 13), 360 h (Day 16) and 480 h (Day

26) post-dose. Concentrations of ZHB111 in serum samples were determined by an enzyme-linked immunosorbent assay method. Concentrations of IGF-1 in group 1 of serum samples were determined by the Quantikine Human IGF-1 Immunoassay kit.

In the pharmacodynamic study, 40 (20/sex) cynomolgus monkeys were randomly assigned to 4 groups with 5/sex/group and were dosed with ZHB111 once weekly for 5 times by subcutaneous injection at 0 (control article, Sodium chloride injection), 1, 5 or 20.6 mg/kg/dose. The dosing volume was 2.5 mL/kg. The first dosing day of every group was Day 1. The parameters evaluated included cageside observations, detailed clinical observations, body weight, food consumption, ophthalmoscopy, electrocardiography, clinical pathology, ZHB111 antibodies, IGF-1, gross pathology, organ weights, concentrations of ZHB111 and histopathology.

Quantitation of ZHB111 Serum Levels by ELISA

This assay employed the quantitative immunoassay technique. Coating GH20 was coated onto a microplate. After washing away any unbound substances, samples were added to the wells to bind with the coated GH20. After a wash to remove any unbound samples, HRP (SA-HRP) was added to the wells. Following a wash to remove any unbound samples, TMB substrate solution was added to the wells and color development occurred in proportion to the amount of ZHB111 injection bound in the initial step. The determination of the color of the samples was conducted at 450 nm, with a wavelength correction set at 630 nm using a microplate reader. The quantitation range for this assay was 0.75–48 ng/mL.

Immunogenicity Analysis

This Enzyme-Linked Immunosorbent Assay (ELISA) was designed to analyze the antibodies levels against ZHB111 in cynomolgus monkey serum samples. In this ELISA method, firstly, ZHB111 injection was coated on 96-well plate, the samples were prepared at 1μg/mL using assay buffer, then antibodies of ZHB111 in samples were bound to the ZHB111 ADA capture-coated plate. Bound antibodies were detected using biotinylated ZHB111 and SA-HRP. Upon addition of tetramethylbenzidine (TMB)-peroxidase substrate solution to the plate, TMB in the substrate solution reacted with the HRP conjugates, and the reaction produced a colorimetric signal that was proportional to the amount of ZHB11 antibodies present in the samples. The color development was stopped by addition of 2M sulfuric acid and the intensity of the color (optical density, OD) was measured at 450 nm. The method confirmatory sensitivity was 6.25ng/mL.

An ELISA assay for detecting antibodies against ZHB111 in cynomolgus monkey serum has been validated. The samples were valid to be stable after being stored for 4 h at RT & 2°C~8°C, with 5 freeze/thaw cycles. This method has been validated for sample analysis in screening, confirmatory, and titer analyses of immunogenicity samples.

Measurement of IGF-1 Levels

In the case of the rats, the Quantikine Mouse/Rat IGF-1 Immunoassay kit was employed, whereas the Quantikine Human IGF-1 Immunoassay kit was utilized for the monkeys. The kit employed a quantitative sandwich ELISA technique. A monoclonal antibody specific for IGF-1 was pre-coated onto microplates, followed by the addition and incubation of standard solutions and samples. Any IGF-1 present in the sample was captured by the immobilized antibody. After washing, an enzyme-linked polyclonal antibody specific for IGF-1 was added. A substrate solution was added to the wells after a second wash to remove unbound antibody-enzyme reagent. Color development was proportional to the amount of IGF-1 bound in the first step. A450 was measured after stopping the color development.

Results

Efficacy Evaluation in Rats

A single dose investigation was conducted on hypophysectomized rats to evaluate the impact of ZHB111 on weight gain and a primary activity of growth hormone. ZHB111 (0.3, 0.6, and 1.2 mg/kg) was injected once during the experiment (n = 10 per dose/group) and the weight gain of animals was compared to those receiving Jintrolong injections (single dose, 1.2mg/kg, n = 10). After 7 days (on D8), as shown in Figure 1, the value of weight gain (g, Mean ± SD) of each

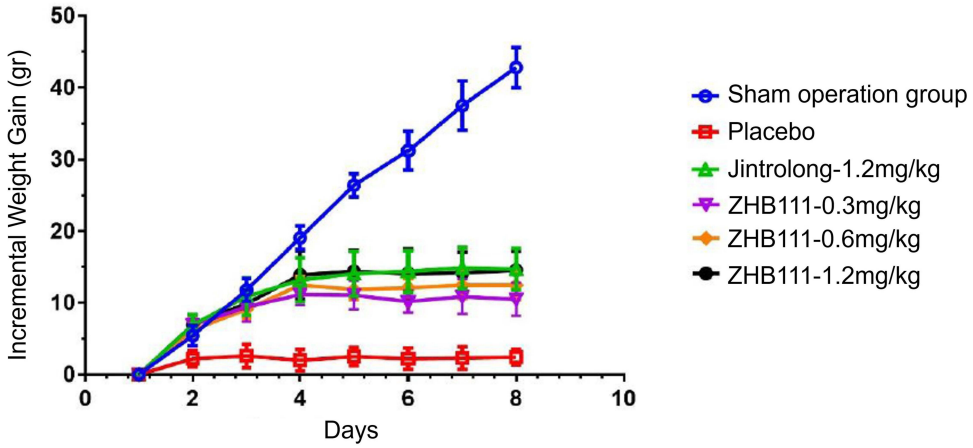

Figure 1 Effect of ZHB111 on weight gain in hypophysectomized rats compared to Jintrolong. Incremental weight gain (g) is shown for hypophysectomized rats (n = 10/group) injected with placebo, ZHB111(0.3 0.6, and 1.2mg/kg) or Jintrolong (1.2mg/kg).

group was: sham operation group: 42.80 ± 2.82; Placebo group: 2.40 ± 1.07; Jintrolong (1.2mg/kg) group: 14.70 ± 2.91; ZHB111 (0.3mg/kg) group: 10.50 ± 2.32; ZHB111 (0.6mg/kg) group: 12.50 ± 2.07; ZHB111 (1.2mg/kg) group: 14.60 ± 2.67. At 1.2mg/mL, an extremely significant weight gain effect was observed in Jintrolong and ZHB111 group. ZHB111 consistently displayed distinct therapeutic effects at three administered doses, indicating a discernible dose-response relationship. At the same dose, ZHB111 showed efficacy comparable to Jintrolong®. Taking into account the considerable species variations between rodents and humans, it is essential to undertake a systematic comparison of the PK/PD profiles between ZHB111 and Jintrolong® within upcoming clinical investigations.

Single-Dose PK Study in Cynomolgus Monkeys

Female and male cynomolgus monkeys (3/sex/group) received ZHB111 by single IV bonus and IM injection at doses of 1 mg/kg and 0.3, 1, 3 mg/kg, respectively. ZHB111 concentrations were determined in serum. The PK parameters were summarized in Table 1. All animals tolerated ZHB111 injection at the dosing levels during the entire course of the study. No adverse effect was observed during the in-life phase of the study. ZHB111 showed no sex difference at all dose levels when comparing the $AUC_{0\text{-last}}$ and C_{max} in female and male cynomolgus monkeys. The systemic exposure (AUC_{last} and C_{max}) of ZHB111 increased proportionally as dose levels increased from 0.3 mg/kg to 1 mg/kg, 1 mg/kg to 3 mg/kg and 0.3 mg/kg to 3 mg/kg. The absolute bioavailability of male animals was 93.92%, and that of female animals was 71.58%.

Table 1 The Average PK Parameters in Cynomolgus Monkeys After Single Administration

Group		Parameter	$t_{1/2}$ (hr)	T_{max} (hr)	C_{max} (μg/mL)	AUC_{last} (hr*μg/mL)	AUC_{INF_obs} (hr*μg/mL)	V (mL/kg)	Cl (mL/hr/kg)	MRTlast (hr)
Low dose group (0.3mg/kg)	Male	Mean	110.20	32.00	5.08	508.18	508.61	92.27	0.60	63.31
		SD	41.27	13.86	0.76	73.86	74.10	27.43	0.08	3.00
	Female	Mean	66.58	18.67	5.07	456.92	457.27	59.22	0.68	57.23
		SD	53.64	9.24	1.06	102.74	103.03	36.71	0.17	4.29
	P		0.33	0.99	0.52	0.52	0.28	0.48	0.11	0.33
Medium dose group (1.0mg/kg)	Male	Mean	33.13	32.00	19.93	2694.88	2695.42	17.98	0.37	92.23
		SD	6.12	13.86	1.05	222.28	221.90	4.53	0.03	4.23
	Female	Mean	55.81	24.00	17.73	2227.13	2227.82	38.15	0.46	82.77
		SD	33.17	0.00	1.97	351.30	351.16	26.66	0.07	10.21
	P		0.31	0.16	0.12	0.12	0.27	0.12	0.21	0.31

(*Continued*)

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Table 1 (Continued).

Group		Parameter	$t_{1/2}$ (hr)	T_{max} (hr)	C_{max} (μg/mL)	AUC_{last} (hr*μg/mL)	AUC_{INF_obs} (hr*μg/mL)	V (mL/kg)	Cl (mL/hr/kg)	MRTlast (hr)
High dose group (3.0mg/kg)	Male	Mean	30.11	24.00	66.93	9542.15	9548.43	13.64	0.31	109.82
		SD	7.32	0.00	2.96	511.81	515.34	3.08	0.02	7.13
	Female	Mean	42.12	24.00	62.73	10,428.5	10,466.56	17.16	0.29	118.35
		SD	14.53	0.00	6.97	1692.66	1722.83	3.89	0.05	5.89
	P		0.27	0.39	0.43	0.43	0.29	0.53	0.19	0.27
Intravenous injection group (1.0mg/kg)	Male	Mean	42.82	2.67	38.17	2869.28	2870.01	21.5	0.35	71.58
		SD	9.42	2.30	2.41	255.98	256.60	4.15	0.03	8.43
	Female	Mean	46.96	0.68	36.77	3111.20	3111.95	22.59	0.33	77.69
		SD	20.02	1.14	1.17	437.55	437.51	11.71	0.04	7.42
	P		0.76	0.25	0.42	0.45	0.46	0.89	0.46	0.40

The results showed that the intravenous injection group had slightly higher exposure levels than the subcutaneous injection group at the same dose.

After subcutaneous administration of 0.3mg/kg of ZHB111, the mean values of IGF-1 in male animals ranged among 594.93~1294.98ng/mL at various time points, while those in female animals were within the range of 394.15~770.01ng/mL. The IGF-1 levels in male animals were consistently slightly higher than those in female animals across all time points. Furthermore, a significantly statistical increase in IGF-1 levels was observed in both male and female animals at 96 h post-dose compared to their respective pre-dose levels (as shown in Table 2).

Repeat-Dose Range Finding Toxicity and Toxicokinetics Study in Cynomolgus Monkeys

The purpose of this study was to determine the potential toxicity of ZHB111, when administered once weekly (in total 5 times) by subcutaneous injection to cynomolgus monkeys for 29 days. In addition, the toxicokinetics (TK, as shown in Table 3) of ZHB111were determined.

Female and male cynomolgus monkeys received ZHB111 by subcutaneous injections once weekly at doses of 0, 1, 5, 20.6 mg/kg/dose for 28 days. No unscheduled death was noted in this study. And there was no test article-related change

Table 2 The IGF-1 Levels Statistics in PK Study (in Cynomolgus Monkeys)

Gender	Animal ID / Time(h)	Concentration(ng/mL)												
		0	30min	4h	8h	24h	48h	72h	96h	144h	192h	288h	360h	480h
Male	1	584.88	560.78	497.2	473.58	538.37	546.39	669.78	793.03	1015.49	860.3	601.46	541.92	592.89
	2	793.35	753.77	741.88	711.3	723.7	723.41	767.25	887.45	915.13	854.41	895.51	704.46	794.24
	3	934.86	854.84	652.5	599.92	613.42	566.78	682.59	865.57	1954.33	1026.29	880.37	760.03	855.08
	Mean	771.03	723.13	630.53	594.93	625.16	612.19	706.54	848.68	1294.98	913.67	792.45	668.8	747.4
	SD	176.1	149.4	123.8	118.9	93.2	96.9	53	49.4	573.2	97.6	165.6	113.3	137.2
	CV%	22.8	20.7	19.6	20	14.9	15.8	7.5	5.8	44.3	10.7	20.9	16.9	18.4
Female	1	492.71	446.72	373.45	330.45	460.7	563.61	612.29	691.39	894.79	564.75	629.34	591.59	579.12
	2	643.07	552.52	476.35	456.73	421.4	406.13	374.86	510.11	606.5	495.07	459.84	388.79	467.7
	3	526.89	516.37	452.48	395.28	361.4	442.1	489.85	600.02	808.74	661.57	502.69	445.38	456.28
	Mean	554.22	505.2	434.09	394.15	414.5	470.61	492.33	600.51	770.01	573.8	530.62	475.25	501.03
	SD	78.8	53.8	53.9	63.1	50	82.5	118.7	90.6	148	83.6	88.1	104.6	67.9
	CV%	14.2	10.6	12.4	16	12.1	17.5	24.1	15.1	19.2	14.6	16.6	22	13.5

Table 3 The Average TK Parameters in Cynomolgus Monkeys

Duration	Dose mg/kg	Parameter	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (μg/mL)	AUC_{last} (hr μg/mL)	AUC_{INF_obs} (hr μg/mL)
D1	1 (n=10)	Mean	67.79	26.4	15.82	1647.62	2096.4
		SD	19.08	7.59	2.01	286.29	461.44
		p	0.03	0.35	0.43	0.55	0.16
	5 (n=10)	Mean	99.82	31.20	92.92	11,048.14	16,623.21
		SD	23.89	11.59	16.08	1583.14	1938.63
		p	0.12	0.54	0.33	0.62	0.43
	20.6 (n=10)	Mean	104.64	38.4	362.3	42,789.01	68,135.17
		SD	25.01	12.39	37.43	5370.78	16,707.9
		p	0.09	0.00	0.72	0.42	0.15
D22	1 (n=10)	Mean	77.78	24.00	21.98	2583.77	3491.85
		SD	18.74	0.00	2.66	433.02	917.15
		p	0.54	0.15	0.47	0.38	0.35
	5 (n=10)	Mean	125.29	28.8	157.9	19,782.47	34,241.48
		SD	19.37	10.12	31.47	4443.16	11,024.26
		p	0.42	0.14	0.58	0.41	0.33
	20.6 (n=10)	Mean	140.05	38.40	673.50	87,062.6	163,328.63
		SD	34.85	12.39	85.55	13,221.03	45,195.54
		p	0.37	0.24	0.26	0.23	0.24

Notes: *p* value is the comparison of different genders in the same group, $p>0.05$ indicates that there is no significant difference between male and female animals, $p<0.05$ there are significant differences between male and female animals.

in clinical signs, body weight, food consumption, body temperature, clinical pathology (hematology, coagulation and serum chemistry), organ weights and gross observations.

Male animals in the 5 mg/kg dose group (1/5) and female animals in the 1, 5, 20.6mg/kg dose group (1/5, 5/5, 5/5) showed breast enlargement and white discharge at the nipple. Due to the dose-dependent incidence of symptoms, the mechanism of action of growth hormone and pathological examination results of the breast, the pharmacological effect of ZHB111 was believed to be the main reason.

For the toxicokinetic profiles of ZHB111, there was no marked sex difference in systemic exposure. As the dosage increased from 1 to 20.6mg/kg/dose, the systemic exposure increased proportionally on Day 1 and Day 22. At the same dose, the systemic exposure on Day 22 increased more than on Day 1, which demonstrated noticeable drug accumulation in all doses. The accumulation factors were 1.57, 1.79 and 2.03 respectively.

A notable escalation in IGF-1 levels was observed following the fourth administration of ZHB111 across all dosage regimens relative to the first administration. Moreover, these concentrations exceeded those recorded in the contemporaneous placebo control group. This phenomenon was considered stemming from the inherent pharmacological effects exerted by the ZHB111.

Under the condition of this study, the NOAEL of ZHB111 was considered to be 20.6 mg/kg/dose.

Distribution

As shown in Figure 2, the precipitated radioactivity concentrations in serum and tissues reached the highest at 8 h and then gradually decreased.

Except thyroid, the radioactivity was mainly distributed in the serum, lung stomach, bladder. It is least distributed in the brain. The order of AUC of ^{125}I-ZHB111 in various tissues calculated based on the radioactivity found in the precipitated tissues and body fluids was as follows (Figure 3): serum > stomach > lung > bladder > kidney > small intestine > liver > spleen > mesenteric lymph nodes > genital gland > adrenal gland > thymus > heart > muscle > fat > brain. The low radioactivity uptake in brain tissue suggested that the test substance could not enter the brain through the blood brain barrier.

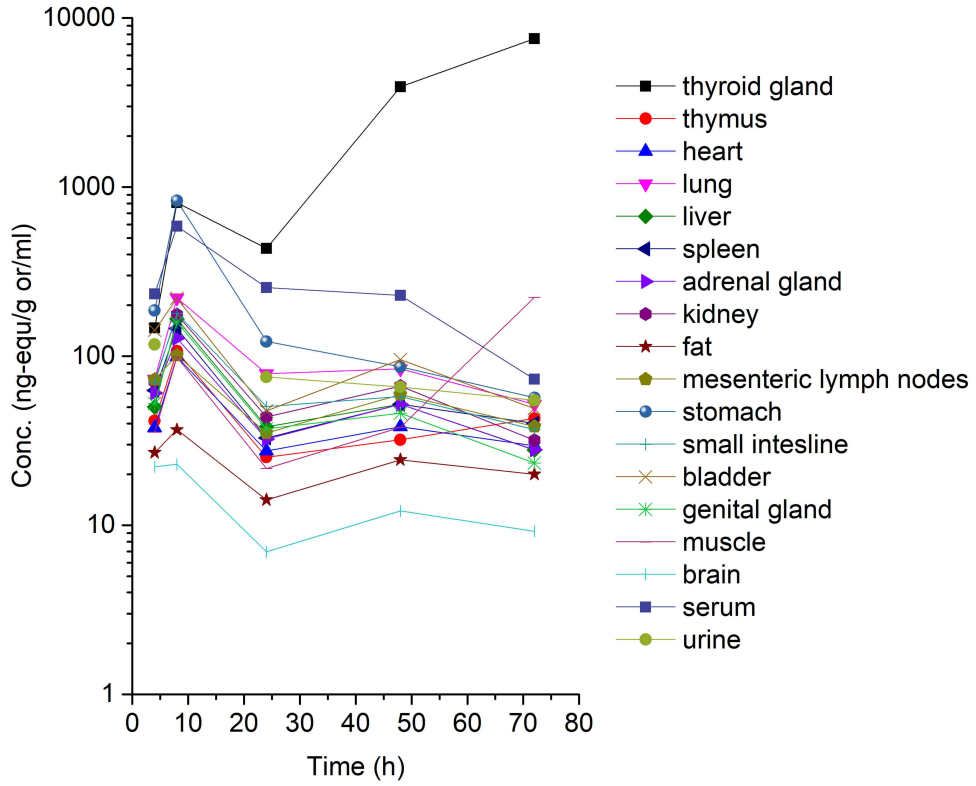

Figure 2 The concentration of precipitated radioactivity in different tissues of SD rats at different time points after single SC administration of ^{125}I-ZHB111.

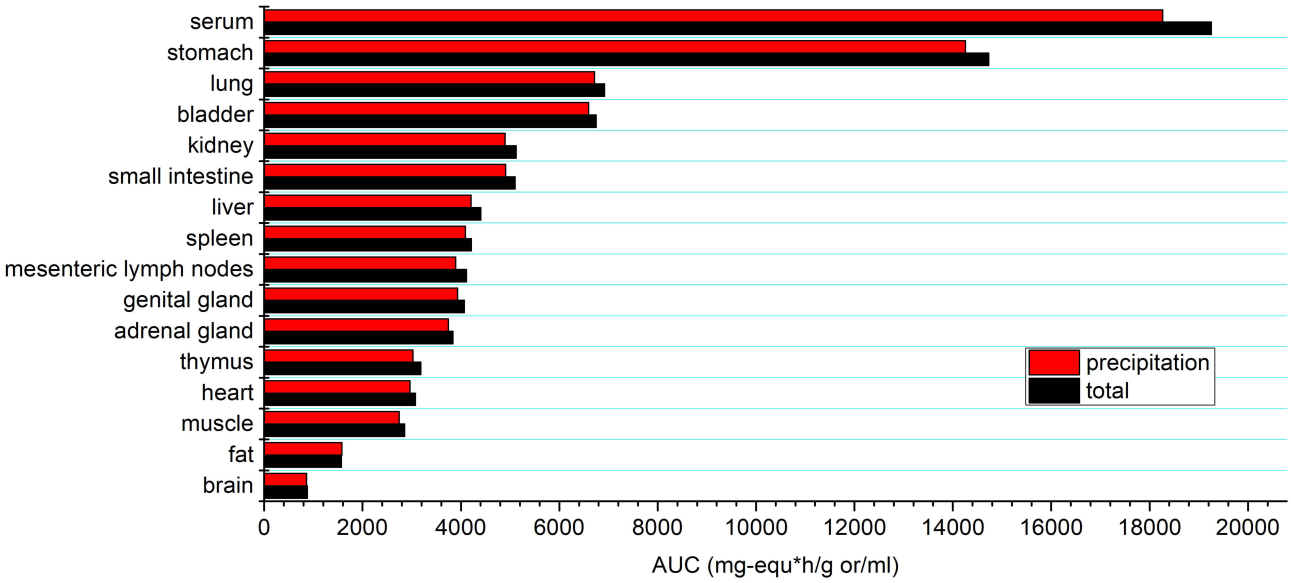

Figure 3 The AUC in different tissues of SD rat after single SC administration of ^{125}I-ZHB111.

Immunogenicity

The immunogenicity of ZHB111 was evaluated in 4-week repeated-dose toxicity studies in cynomolgus monkeys.

Four groups of monkeys (5/sex/group) received 0, 1, 5 or 20.6 mg/kg ZHB111 once a week by subcutaneous injection. Serum samples were collected at Day 1, Day 15, Day 29 pre-dose and Day 57 (end of recovery period) for ADA analysis. No ADA positive was confirmed in all groups.

Discussion

Daily GH administration has become a clinical treatment for GHD in adults and pediatric patients for over 30 years. Low-frequency GH injection is hypothesized to improve adherence and many LAGH formulations have been developed and four formulations have been approved and marketed. These four drugs are developed using different long-acting strategies, which is dosed once a week in clinical use. We hope to develop a LAGH with an extended dosing interval by utilizing PEGylation technology.

Among the four currently marketed LAGH drugs, Jintrolong® is also a PEGylated drug, where the employed PEG is conjugated randomly to the GH through ester bonds, resulting in multiple isomers. In contrast to Jintrolong®, a higher molecular weight PEG is conjugated to the N-terminal primary amino group of the GH via a propionaldehyde moiety, thereby providing greater control over the quality of the final product.[16] This design holds promise for the development of a PEGylated LAGH drug with an even longer half-life and more controllable quality characteristics.

ZHB111 exhibited a significant dose-dependent variation in serum half-life in monkeys, with a decreasing trend as the dosage increased, ranging from 110 to 30 h, which was considerably longer than that of native hGH (3.4 h after SC injection in humans).

In contrast, during pharmacokinetic study conducted in cynomolgus monkeys for NGENLA, the serum half-life was notably shorter, ranging only 12 to 20 h across administered doses of 1.5 mg/kg, 15 mg/kg and 30 mg/kg.

In Phase I clinical trials, Jintrolong® had a serum half-life of 33 h when administered at a dose of 0.2 mg/kg. Under similar dosing conditions, ZHB111 displayed a substantially longer half-life (66–110 h), indicating superior pharmacokinetics compared to Jintrolong®. Upon conversion to an equivalent dose, ZHB111 also displayed higher C_{max} and AUC compared to Jintrolong®. In addition, in the toxicokinetics study of ZHB111 in cynomolgus monkeys, the accumulated AUC for ZHB111 significantly increased during the first and the fifth weekly administration. These findings suggest that ZHB111 may have a longer duration of therapeutic action in clinical use, allowing for potential extension of the dosing interval.

In preclinical toxicology studies of many PEGylated drugs, PEG-related histological changes, described as cellular vacuolation, have been identified in specific tissues and cell types. Macrophage vacuolation is generally regarded as a normal physiological response that involves scavenger phagocytes to remove foreign bodies without affecting cellular function or viability.

As the number of marketed PEGylated drugs increases, there is a growing need for research focused on their long-term safety profiles after prolonged administration of these formulations. Assessing and characterising tissue vacuolation remains an important part of the toxicological evaluation of PEGylated biopharmaceuticals.

In our study of ZHB111 toxicity in cynomolgus monkeys, no tissue or organ vacuolation was observed. However, in the 28-Day repeated-dose range study of ZHB111 toxicity in SD rats, PEGylation-related vacuolation was observed in hepatic cells and sinusoidal macrophages of the submandibular lymph nodes at intermediate to high doses, although no accompanying functional impairments stemming from these histopathological changes were discernible. Upon cessation of administration, indicative manifestations of reabsorption or a recuperative tendency were noted. No tissue or organ vacuolation was observed in cynomolgus monkeys treated with Jintrolong® for 6 months or in young rats treated with Jintrolong® for 13 weeks in long-term toxicity studies of Jintrolong®.

The observed difference may be attributed to the disparity in molecular weight of PEG molecules employed in ZHB111 and Jintrolong®. Both ZHB111 and Jintrolong® utilize branched PEG molecules; however, ZHB111 employs 60 kDa PEG, while Jintrolong® uses 40 kDa PEG. It has been documented in literature that larger PEG molecules may have a greater tendency to induce vacuolation. Moreover, PEG molecules with higher molecular weight tend to exhibit longer metabolic durations within the body, raising the question of whether the length of metabolism is related to the induction of vacuolation.

Despite the growing number of PEGylated drugs on the market and their preliminary safety validation, it should be noted that no currently marketed PEGylated product incorporates a 60 kDa PEG molecule. Therefore, special attention must be given to the inherent safety profile of the PEG molecule in ZHB111's long-term preclinical toxicity studies and clinical research.

Protein-based drugs typically adopt an injectable formulation, and during clinical administration, protein injectables can readily elicit an immune response leading to the development of ADAs, particularly neutralizing antibodies, which can significantly impair drug efficacy. Consequently, evaluating both immunogenicity and immunotoxicity is critical during both preclinical and clinical stages of protein-based pharmaceuticals development.

Compared to Jintrolong®, ZHB111 utilizes a more larger PEG molecule. Theoretically, due to its increased steric hindrance effect, ZHB111 could demonstrate a superior capacity in reducing immunogenicity. During our repeated-dose toxicity study in cynomolgus monkeys, no ADAs were detected during the administration and the recovery period. Within the framework of repeated-dosing toxicity study, a concurrent immunotoxicological evaluation was carried out. Across various dosage cohorts, comprehensive assessments encompassed lymphocyte subtyping, cytokine profiling, relative organ weights of immune tissues, macroscopic dissections, and histopathological evaluations of these immune organs. Notably, no treatment-related animals were detected in any of these parameters following administration of ZHB111. This data collectively suggests that ZHB111 does not exert significant immunotoxic effects under the conditions of this study.

ZHB111 showed good rat and monkey tolerance with only minimal adverse effects. The observed changes were expected, either on the basis of the pharmacological activity of the drug or in relation to local effects at the injected site. For the PEGylated drugs, PEG has a simple repeating structure, and it is chemically inert and has low toxicity.[17,18] It is generally observed that findings, including the adverse effects, identified in preclinical studies of licensed PEGylated biopharmaceuticals are typically associated with the targeted pharmacological actions of the active drug substance, rather than the PEG moiety.[19,20]

In conclusion, the preclinical pharmacology, pharmacokinetic, and toxicity profile of ZHB111 supports the initiation of clinical studies a potential therapeutic agent for the treatment of GHD.

Recently, in the preliminary Phase I clinical results, ZHB111 has been found to be safe and well tolerated, and the PK data indicated that ZHB111 exhibits an appropriate profile for once-every-two-week treatment regimen.

Data Sharing Statement

Due to the nature of the research and commercial supporting, research data is not available.

Ethic Approval

The efficacy evaluation research was approved by the institutional animal care and use committee of QIANHONG BIOPHARMA Co., Ltd. / ZONHON BIOPHARMA INSTITUTE, INC. (IACUC No. 2020-003).

The rest research (including the distribution research, efficacy evaluation research, pharmacokinetic and pharmacodynamic analyses) was approved by the institutional animal care and use committee of JOINN (Suzhou) Laboratories Co. Ltd. (IACUC No. ACU20-2106, ACU20-1802, ACU20-1602).

All the experiments and animal housing were conducted in strict accordance with the institutional guide for care and use of laboratory animals.

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Disclosure

Dr Jun Wang reports a patent CN202111395675.7 issued to ZONHON BIOPHARMA INSTITUTE, INC. Dr He Wang reports a patent CN202111395675.7 issued to ZONHON BIOPHARMA INSTITUTE, INC. Dr Chenyang Jiang reports a

CN201711327350.9 issued to ZONHON BIOPHARMA INSTITUTE, INC., a patent CN202111395675.7 issued to ZONHON BIOPHARMA INSTITUTE, INC. Dr Bruce Ma reports a patent CN201711327350.9 issued to ZONHON BIOPHARMA INSTITUTE, INC., a patent CN201911190141.3 pending to ZONHON BIOPHARMA INSTITUTE, INC., a patent CN202111395675.7 issued to ZONHON BIOPHARMA INSTITUTE, INC. The authors report no other conflicts of interest in this work.

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