

Immunogenicity, toxicology, pharmacokinetics and pharmacodynamics of growth hormone ligand–receptor fusions

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A B S T R A C T

A fundamental concern for all new biological therapeutics is the possibility of inducing an immune response. We have recently demonstrated that an LR-fusion (ligand–receptor fusion) of growth hormone generates a potent long-acting agonist; however, the immunogenicity and toxicity of these molecules have not been tested. To address these issues, we have designed molecules with low potential as immunogens and undertaken immunogenicity and toxicology studies in *Macaca fascicularis* and pharmacokinetic and pharmacodynamic studies in rats. Two variants of the LR-fusion, one with a flexible linker (GH–LRv2) and the other without (GH–LRv3), were tested. Comparison was made with native human GH (growth hormone). GH–LRv2 and GH–LRv3 demonstrated similar pharmacokinetics in rats, showing reduced clearance compared with native GH and potent agonist activity with respect to body weight gain in a hypophysectomized rat model. In *M. fascicularis*, a low level of antibodies to GH–LRv2 was found in one sample, but there was no other evidence of any immunogenic response to the other fusion protein. There were no toxic effects and specifically no changes in histology at injection sites after two repeated administrations. The pharmacokinetic profiles in monkeys confirmed long half-lives for both GH–LRv2 and GH–LRv3 representing exceptionally delayed clearance over rhGH (recombinant human GH). The results suggest that repeated administration of a GH LR-fusion is safe, non-toxic, and the pharmacokinetic profile suggests that two to three weekly administrations is a potential therapeutic regimen for humans.

INTRODUCTION

A major challenge for recombinant DNA-based protein drugs is the generation of long-acting molecules that are non-toxic, non-immunogenic and provide appropriate

pharmacokinetics for the drug in question. We have recently described a technology to make long-acting cytokines by fusing cytokine to its cognate extracellular domain receptor at the DNA level: an LR-fusion (ligand–receptor fusion) [1]. The advantages of this technology

Key words: growth hormone (GH), immunogenicity, ligand, pharmacokinetics, pharmacodynamics, receptor fusion, toxicity.

Abbreviations: GH, growth hormone; GHBP, GH-binding protein; GHRExt, growth hormone extracellular domain; GLP, Good Laboratory Practice; IGF-1, insulin-like growth factor-1; i.v., intravenous; LR-fusion, ligand–receptor fusion; mAb, monoclonal antibody; PEG, poly(ethylene glycol); rh, recombinant human; s.c., subcutaneous.

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include fusion at the DNA level, no requirement for chemical modification and delayed clearance. We have made a GH (growth hormone, 1–191) to GHRext (growth hormone extracellular domain, 1–238) LR-fusion that has a long circulating half-life, greatly reduced clearance and potent agonist activity [1]. However, a key question in the development of this new therapeutic technology is whether such a LR-fusion is immunogenic or toxic.

The original LR-fusion was generated using a peptide linker, (Gly₄Ser)₄, between the C-terminus of GH and the N-terminus of GHRext. This long linker, with a predicted length of 80 Å (1 Å = 0.1 nm), was chosen as a relatively flexible tether between GH and GHRext, such that the GH moiety could still interact with cell surface GHR. Similar Gly₄Ser linkers have been used in recombinant single chain Fv antibody production because of their stability and lack of immunogenicity [2]. *In silico* screening of the GH LR-fusion showed no new T-cell epitopes, and the only non-native sequence was the (Gly₄Ser)₄ linker. As the N-terminal residues of the GHR molecule are disordered in the crystal structure [3] and therefore likely to be relatively flexible, we questioned whether there was a need for the (Gly₄Ser)₄ linker in this particular case. We have therefore generated a new variant GH LR-fusion, which is a direct fusion between the GH C-terminus and the N-terminus of the GHRext.

Antibodies develop to varying extents during treatment with most human proteins, including insulin, GH, GM-CSF (granulocyte/macrophage colony-stimulating factor), Factor VIII, erythropoietin and interferons [4]. These antibodies may reduce clinical efficacy. In 30–60% of patients, antibodies against pituitary-derived human GH were detected, and in 5% of treated patients, these antibodies blocked the therapeutic activity of GH [5]. Antibody formation to GH has been reported in 1–75% of patients treated with recombinant human GH [6–16], but these antibodies have not been inhibitory or harmful. Antibodies are found whether GH is produced in *Escherichia coli* or mammalian cells [15]. Antibody formation probably relates to a small amount of denatured GH in the preparation [17], although antibody production is generally greater with pituitary-derived GH and recombinant GH with an N-terminal methionine residue; recent studies suggest similar immunogenicity when GH is presented in a depot preparation [18].

There is no established format or single model for defining immunogenicity for new biological molecules. It is generally accepted that the only final proof is use of the new molecule in humans. However, low immunogenicity and lack of toxicity in non-human primates provides strong reassurance for a non-toxic effect in humans. In rhesus monkeys, a comparison of pituitary-derived GH with recombinant methionyl-GH and natural sequence GH showed GH antibodies occurring in 69%, 81%

and 23% of animals, respectively [19]. In most animals, maximal antibody titres were detected before 40 days of treatment. There was no dose effect of GH on antibody production.

To address the question of immunogenicity with LR-fusions, we have designed two LR-fusion molecules, tested them for biological activity and undertaken an immunogenicity study in *Macaca fascicularis*.

MATERIALS AND METHODS

Materials

All the materials were purchased from Sigma unless otherwise stated. *E. coli*-derived recombinant GH, Nutropin AQ (5 mg/ml in 2 ml of diluent), was purchased from Ipsen.

LR-fusions and recombinant hGH

The methodology used has been previously described [1]. Two LR-fusions were designed, one with a flexible (Gly₄Ser)₄ linker, GH–LRv2, and the other a direct fusion of the C-terminus GH to the N-terminus of the GHRext, GH–LRv3. Stable clones were made in CHO (Chinese-hamster ovary cells) Flp-In cells (Invitrogen), adapted to protein-free medium and grown in suspension culture. LR-fusion expression was confirmed by an in-house ELISA. Affinity purification was performed using a GH mAb (monoclonal antibody) column. Recombinant hGH was also made under the same conditions.

In vivo bioactivity

The rat experiments were conducted in compliance with French laws (Council Directive N° 86/609/EEC of 24 November 1986). Normal Sprague–Dawley rats (7 weeks old) from Janvier were used for pharmacokinetic studies. s.c. (subcutaneous) administration and blood withdrawal were conducted under isoflurane anaesthesia. The rats ($n = 4–6$ /group) were injected subcutaneously with the test molecule or control molecule [rhGH (recombinant human GH)]. Blood samples were collected from the retro-orbital plexus. Serum was harvested and stored at -70°C until assayed using an ELISA against GH. The growth studies used hypophysectomized rats and were performed on Sprague–Dawley rats from Charles River Laboratories. Rats were hypophysectomized under isoflurane anaesthesia at 4 weeks of age by the breeder. The injection solutions of excipient, rhGH and LR-fusion never exceeded 2 ml/kg. The rats were given a single dose of the LR-fusions or excipient on day 1 and then retained without further dosing for 10 days; the rats given rhGH were dosed daily over the 10-day period. The rats were weighed daily. Terminal bleeds were collected and were assayed using the GH ELISA and an IGF-1 (insulin-like growth factor-1) ELISA (Octeia Rat/Mouse IGF-1 Assay Kit; iDS).

In vivo immunogenicity and extended pharmacokinetic study

The monkey study was conducted in a GLP (Good Laboratory Practice) compliant facility [U.K. GLP Regulations 1999 (S.I. 1999 No. 3106), as amended by the S.I. 2004 No. 994, which are in accordance with the OECD (Organisation for Economic Co-operation and Development) Principles of GLP [ENV/MC/CHEM (98) 17], and which are accepted by the U.S. FDA (Federal Drug Administration) and Japanese authorities]. Male *M. fascicularis* ($n = 19$), aged 2–3 years, weighing approximately 2.5 kg, were acclimatized for a minimum of 2 weeks prior to study (Bioculture, Mauritius Ltd). Animals were assigned to five treatment groups (group 1: vehicle, group 2: GH–LRv2 test group, group 3: GH–LRv3, group 4: GH control and group 5: Nutropin control), comprising three males in the vehicle group and four males per group in the remaining four groups. All animals from the vehicle control were dosed on two occasions (days 0 and 14), by s.c. injection. GH–LRv2- and GH–LRv3-treated groups were dosed on two occasions (days 0 and 14), by s.c. injection with 1 mg of protein/kg of body weight and then retained without dosing for a further 4-week period. All animals from the rhGH and Nutropin-treated groups were dosed daily, for 27 days, by subcutaneous injection with $0.1 \text{ mg} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{day}^{-1}$ and then retained without dosing for a further 2-week period. Blood samples were collected, and serum was harvested and stored at -70°C . The sampling times for groups 1–3 were at predose, 1-h postdose, then at approx. 6, 12, 24, 48, 72, 144, 192, 288 and 336 h (for day 1, predose for day 15) and 1 h postdose, then at approx. 6, 12, 24, 48, 72, 144, 192, 288, 360, 648 and 696 h after administration. The sampling times for groups 4 and 5 were at predose and 1 h postdose, then at approx. 3, 6, 12, 24, 48, 72, 144, 192, 288 and 336 h after dose administration, then predose on day 27 and 1 h postdose, then at approx. 3, 6, 12, 24, 48, 72, 144, 192, 288 and 360 h after administration. The serum samples were analysed by GH ELISA and immunogenicity ELISA. Blood samples, for haematology, coagulation assays and blood chemistry, were obtained from all animals before the start of treatment, then during week 6 of treatment. Urine samples were obtained for analysis before the start of treatment, then during week 6 of treatment. After necropsy, external and internal examinations of the monkeys were performed, which included examinations of the major organs and the sites of injection.

Quantitative assay of GH-containing molecules

An in-house GH and LR-fusion ELISA has been established based on the sandwich ELISA format. In the assay, standards (GH or LR-fusion), controls and unknowns are incubated with biotin-labelled mouse

antibody to human GH (mAb 7F8) in wells precoated with a mouse antibody to human GH antibody (mAb 10A7). The detection limit for the assay is 2.5 pg/ml and the intra- and interassay CV is $<10\%$.

Assessment of immunological response to GH-containing molecules

An ELISA-based method was used to analyse serum samples from *M. fascicularis* for the presence of antibodies against the respective injected molecule. The serum samples were diluted in PBS and incubated on plates coated with the relevant antigen. The presence of bound antibodies was then analysed by the addition of Protein A–peroxidase followed by TMB (3,3',5,5'-tetramethylbenzidine) solution. The positive control for the assays was a mAb to hGH (mAb 10A7). Samples were assayed at a 1 in 400 dilution, at which dilution the negative control showed acceptable background levels, and there was clear differentiation from the positive control. The cut-off for a positive result was the mean absorbance measured spectrophotometrically at 450 nm (A_{450}) for the negative control in the individual assay plus 3 S.D. Serum samples giving a positive reading were further analysed using an inhibition assay to determine if the result was a false positive or a true positive.

Statistics and pharmacokinetics

Comparisons of groups of treatment regimes were done using one-way ANOVA; if $P < 0.5$ then the Dunnett's test was performed. Pharmacokinetic parameters were estimated by fitting values of hormone concentration compared with time to compartmental models using non-linear least-squares regression analysis. Clearance values were normalized to animal weight. Clearance rate per animal weight and $t_{1/2}$ (terminal half-lives) were calculated using the coefficient and exponents obtained from the i.v. (intravenous) bolus model fits.

RESULTS

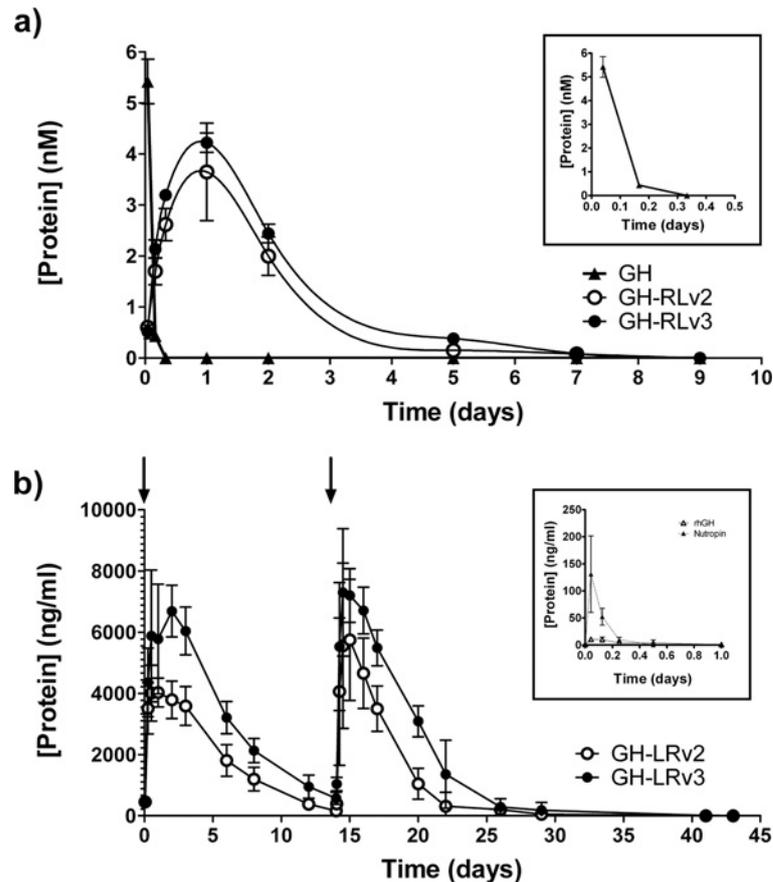
Pharmacokinetic analysis

The GH LR-fusions showed greatly delayed clearance compared with rhGH in both rats and monkeys (Table 1 and Figure 1). Clearance was even more delayed in monkeys than in rats. The monkeys received two injections of the GH LR-fusions within an interval of 14 days. The kinetics of GH–LRv2 and GH–LRv3 showed no appreciable accumulation or reduction in systemic exposure over the dosing period. Systemic exposure ($\text{AUC}_{0-\tau}$) to GH–LRv3 was, on average, approx. 1.7-fold greater than GH–LRv2 on days 0 and 14. Both molecules gave continuous exposure appropriate for the subsequent immunogenicity study.

Table 1 Pharmacokinetic parameters in rats and *M. fascicularis* given a single administration of hGH and GH LR-fusion

Preparation	Rat		<i>M. fascicularis</i>	
	Clearance s.c. ($\text{ml} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ of body weight)	$t_{1/2}$ s.c. (h)	Clearance s.c. ($\text{ml} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ of body weight)	$t_{1/2}$ s.c. (h)
hGH	$820 \pm 94^*$	$0.66 \pm 0.1^*$	$154 \pm 4.4^*$	$1.7 \pm 0.4^*$
GH-LRv2	2.8 ± 0.2	25 ± 6.4	1.7 ± 0.2	54 ± 6.8
GH-LRv3	2.7 ± 0.2	26 ± 6.8	0.96 ± 0.2	76 ± 4.9

*From Osborn et al. [28].

**Figure 1** Delayed clearance of rhGH, GH-LRv2 and GH-LRv3 in (a) rats and (b) *M. fascicularis*

(a) Delayed clearance of rhGH, GH-LRv2 and GH-LRv3 in rats, following subcutaneous delivery of protein at time = 0 days; the inset shows the clearance for rhGH alone (N.B. units for protein concentration given in nM to enable direct comparison of GH with the GH-LR proteins). (b) Delayed clearance of GH-LRv2 and GH-LRv3 in *M. fascicularis*, following subcutaneous delivery of protein at time = 0 and 14 days (vertical arrows); the inset shows the clearance for rhGH and Nutropin.

LR-fusions and growth promotion

To test biological activity, the GH LR-fusions and GH were administered to hypophysectomized (GH-deficient) rats. Daily administration of rhGH induced continuous growth over 10 days. The LR-fusion was then compared with rhGH with a single injection. For all experiments, equimolar doses of rhGH and LR-fusion were used with the same total dose being given over the 10-day period: $220 \mu\text{g} \cdot \text{kg}^{-1}$ of body

weight $\cdot \text{day}^{-1}$, approx. 10 nmol over 10 days similar to the dose previously used to obtain a maximal growth response [13]. The LR-fusions promoted an increase in weight gain, which was similar to that seen after daily GH injections (Figure 2). There was no difference in weight gain between GH-LRv2 and GH-LRv3. IGF-I levels were all elevated in the treatment groups compared with controls ($P < 0.05$), but not different between the treatment groups (Figure 3).

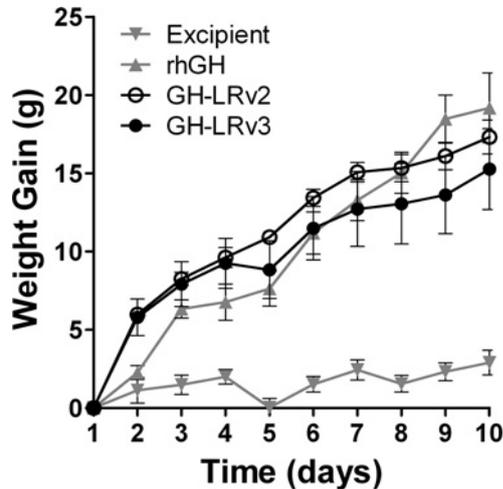


Figure 2 Weight gain in hypophysectomized rats due to LR-fusions compared with rhGH

The LR-fusions and excipient were injected subcutaneously only on day 1, and the rhGH was given daily.

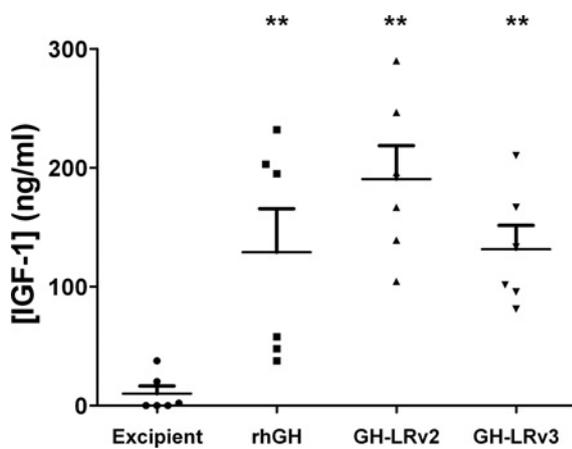


Figure 3 IGF-1 measurement in the terminal bleeds of rats given LR-fusions compared with rhGH

The LR-fusions and excipient were injected subcutaneously only on day 1, and the rhGH was given daily. ** $P < 0.05$ compared with excipient.

Immunogenicity and toxicology

No anti-test substance antibodies were detected in the serum of any animals, with the exception of anti-GH-LRv2 antibodies, which were detected in the serum obtained from one animal on day 44 (Table 2). The single positive result observed ($A_{450} = 0.210$) was only just above the cut-off ($A_{450} = 0.157$). One animal treated with GH-LRv3 had high background reading before drug administration, and this appeared to be non-specific and did not change after drug administration. The serum GH-LRv2 concentration in the animal with a single positive did not appear to be appreciably different compared with concentration data from the other animals in the

same group. There were no treatment-related effects on clinical signs, bodyweights or bodyweight gains during the course of the study. There were no treatment-related effects on haematology, blood chemistry or urinalysis observed during the course of the study. No treatment-related effects were noted on organ weights or necropsy in any of the groups tested, both on inspection and histology. There was no reaction at the injection sites and no evidence of lipoatrophy.

DISCUSSION

The use of LR-fusions as a therapeutic is an attractive target for a number of reasons. Since the therapeutic molecule is produced as a single amino acid chain and does not require further modification, both the heterogeneity of the final product and the costs required for production are greatly reduced compared with PEGylated [where PEG is poly(ethylene glycol)] or depot preparations. The efficacy of the GH LR-fusion has already been reported [1] and has been shown to have a superior pharmacokinetic profile compared with GH, with a terminal half-life 100 times that of GH when both are given intravenously. However, a concern with any protein-based therapy is the generation of antibodies against the therapeutic protein. Such a response may reduce the efficacy of the treatment or *in extremis* cause inhibition of the native protein.

The immunogenicity of protein therapeutics can be influenced by many factors, including the amino acid sequence, the glycosylation patterns, degradation of the protein, aggregation or aberrant folding of the protein, chemical modification (such as oxidation), the processing of the protein, product formulation and even the route of administration [20]. The GH LR-fusion proteins were designed to minimize the immunogenic proclivity of the therapeutic protein. GH and GHBP (GH-binding protein), the extracellular domains of the GH receptor, form a natural non-covalent complex in the blood circulation. This 'natural depot' is thought to prolong the circulation time and stability of GH. GH-LRv2 consists of GH and the extracellular domain of its receptor with the only non-natural sequence being the $(\text{Gly}_4\text{Ser})_4$ linker; this was further modified to remove the linker all together in GH-LRv3. Additionally, the LR-fusions were expressed in CHO cells to provide suitable glycosylation of the receptor domain.

The lack of an immunogenic response in *M. fascicularis* is not definitive proof that antibodies against the protein therapeutic will not occur in humans. However, the fact that the LR-fusion proteins, given at 10 times the human pharmacological dose, do not initiate an immune response in these animals, gives a strong reassurance of its non-immunogenicity when used as a biopharmaceutical in humans. One animal injected with GH-LRv2 gave a

Table 2 Immunogenicity results for GH-LRv2, GH-LRv3 and hGH

The A_{450} for the sera from *M. fascicularis* given GH-LRv2, GH-LRv3 or hGH is shown. Positive immunogenic results are shown in bold, false positives are shown in parentheses. The cut-offs for positive reactions were ≥ 0.157 for GH-LRv2, ≥ 0.156 for GH-LRv3 and ≥ 0.210 for hGH; these cut-off values were determined during the qualification of the respective assays.

(a) GH-LRv2

Day	Time	Animal number . . .	Vehicle			GH-LRv2 (1 mg · kg ⁻¹ of body weight · dose ⁻¹)			
			101	103	105	201	203	205	207
Day 1	Predose		0.060	0.066	0.052	0.067	0.053	0.046	0.132
Day 7	144 h		0.047	0.064	0.068	0.071	0.072	0.051	0.119
Day 15	Predose		0.060	0.046	0.050	0.064	0.077	0.080	0.106
Day 21	144 h		0.058	0.077	0.065	0.059	0.050	0.077	0.129
Day 27	288 h		0.067	0.054	0.055	0.049	0.078	0.068	0.118
Day 44	696 h		0.068	0.071	0.055	0.071	0.134	0.210	0.078

(b) GH-LRv3

Day	Time	Animal number . . .	Vehicle			GH-LRv3 (1 mg · kg ⁻¹ of body weight · dose ⁻¹)			
			101	103	105	301	303	305	307
Day 1	Predose		0.058	0.065	0.052	0.056	(0.371)	0.069	0.085
Day 7	144 h		0.062	0.060	0.067	0.060	(0.196)	0.064	0.082
Day 15	Predose		0.062	0.058	0.065	0.065	(0.305)	0.064	0.086
Day 21	144 h		0.066	0.075	0.070	0.073	(0.403)	0.068	0.083
Day 27	288 h		0.062	0.057	0.054	0.068	(0.408)	0.065	0.073
Day 44	696 h		0.074	0.068	0.058	0.065	(0.325)	0.063	0.081

(c) GH

Day	Time	Animal number . . .	Vehicle			GH (0.1 mg · kg ⁻¹ of body weight · dose ⁻¹)			
			101	103	105	401	403	405	407
Day 1	Predose		0.062	0.071	0.079	0.059	0.066	0.049	0.060
Day 7	144 h		0.063	0.074	0.070	0.070	0.067	0.059	0.058
Day 15	Predose		0.062	0.075	0.069	0.066	0.180	0.064	0.083
Day 21	144 h		0.064	0.069	0.081	0.132	0.185	0.081	0.109
Day 27	288 h		0.063	0.083	0.062	0.167	0.060	0.109	0.085
Day 44	696 h		0.062	0.096	0.065	0.147	0.136	0.076	0.074

low-level immunological response just above the established cut-off point at day 43 (Table 2). The group injected with GH-LRv3 did not show any immunological response at all, although one individual gave an apparent positive response in the predose sample, which remained high throughout the course of the study, the response for this individual was regarded as a false positive. These results compare favourably with results previously obtained for treatment with natural sequence recombinant GH, which showed a 23% immunogenic response in 22 treated rhesus monkeys [19].

The duration and dose of treatment may influence the probability of inducing an immunogenic response. Either too low a dose or too high a dose may reduce

immunogenic potential. In human treatment in the clinic, the GH dose ranges from 0.01 mg · kg⁻¹ of body weight · day⁻¹ for adult replacement to 0.055 mg · kg⁻¹ of body weight · day⁻¹ in children with renal failure. For our immunogenicity study, we chose to use a GH dose 10 times greater than the lower dose used in human adults. This equated to 0.1 mg · kg⁻¹ of body weight · day⁻¹ given daily for 28 days in the GH-treated control group. For LR-fusions, it was expected that the duration of action was 10 days or greater, and therefore, we gave a single total dose 10 times that of GH daily dose, which equated to 1 mg · kg⁻¹ of body weight · dose⁻¹ given on days 1 and 14. In a study of methionyl-GH immunogenicity in monkeys, where up to 81% of animals developed

detectable antibody titres, the majority of animals who developed an immunogenic response had done so by 35 days [19]. On the basis of that study, it is unlikely that we have missed a major immunogenic reaction to the LR-fusion proteins.

In humans, GH therapy has been variably reported to initiate an immune response between 1 to 75% of patients [6–16], and the degree of immune response is due to the source of the GH, its purity and the formulation [4]. The presence of anti-GH antibodies did not appear to have a detrimental effect on the therapeutic efficacy of GH in most of these cases. PEGylation has been used as a means of reducing the immunogenic profile of protein molecules; this has the additional advantage of extending the half-life of the molecule *in vivo*. In a short 12-week study, antibodies against the GH receptor antagonist Pegvisomant, a PEGylated form of mutated GH, low titres of anti-GH antibodies were detected in 10% of patients [21].

Sustained-release formulations of GH, where the protein is encapsulated in a microsphere, are also associated with an immunogenic response. This is dependent on the formulation, GH encapsulated in PLGA [poly(lactic-co-glycolic acid)] microspheres has been shown to have an immunogenic response in rhesus monkeys when delivered as microspheres; in contrast, none of the animals receiving GH as a protein solution gave an immunogenic response [18].

The GH LR-fusions did not show any toxicological effects, there were no treatment-related changes in the blood analysis, urine analysis and clinical signs; on inspection and histology, there was no reaction at the injection sites or evidence of lipoatrophy. Bruising and pain at the injection site is associated with other long-acting GH therapies, such as PEGylated GH (Pegvisomant) [22] and Nutropin Depot [23]; the latter is also associated with small erythematous nodules at the injection site [23,24]. Lipoatrophy has been of concern with long-acting GH molecules and has recently been reported with PEGylated GH [25].

A more serious side effect of Pegvisomant therapy is that of hepatic dysfunction due to the hepatotoxic effects of the PEGylated protein [22,26]. It has been postulated that PEGylated proteins can accumulate to toxic levels due to their unsuitability for renal clearance [27]. Theoretically, high levels of GH in a fat depot for a prolonged period might directly affect fat metabolism. This has not been reported with other long-acting formulations of GH. The LR-fusion appears to be well absorbed, and its delayed clearance is related to reduced clearance from the intravascular space, which may be an advantage in preventing local reactions.

The LR-fusions showed prolonged pharmacokinetic profiles in comparison with rhGH in both rats and monkeys; however, as the main focus of these studies was on immunogenicity, no comparison with i.v. dosing was

made, so at this stage, it is not possible to comment on bioavailability or rate of absorption. In *M. fascicularis*, the half-lives of the fusions are approx. 35 times and 45 times greater than GH alone for GH–LRv2 and GH–LRv3 respectively. In comparison, Pegvisomant shows a similar pharmacokinetic profile in humans with a half-life of 64.7 ± 10.9 h [28]. Another putative GH-fusion therapeutic, Albutropin, a fusion of GH with albumin, has a half-life six times that of GH when administered subcutaneously in monkeys [29]. Based on previous studies of allometric scaling, the clearance of hGH is 4-fold less in monkeys compared with rats and 2-fold less in humans compared with monkeys [30]. We found a similar difference for the LR-fusions with 1.6–2.8-fold reduced clearance in monkeys compared with rats, and if there is a similar reduction of clearance in humans, the LR-fusions could provide a 2–3 weekly GH replacement therapy.

The removal of the linker does not seem to affect the activity of the LR-fusion, as the pharmacokinetics and the pharmacodynamics of GH–LRv2 and GH–LRv3 are all very similar. The clearance profile of GH–LRv2 and GH–LRv3 in rat is similar and is also comparable with the clearance profile of GH–LRv1, the GH LR-fusion reported previously [1]. The IGF-1 levels post-treatment with the LR-fusions are also similar for both the LR-fusions tested and the previously reported value of 198 ± 6 ng/ml [1]. The pharmacokinetic profile in monkeys is also alike with values of 60 ± 5 and 78 ± 17 h for GH–LRv2 and GH–LRv3, respectively. The single injection of the LR-fusions gave a different growth profile to daily rhGH, with greater growth over the first 5 days and lesser growth over the second 5 days. Over the 10 days, the LR-fusions produced a weight gain lower than, but not significantly different from, a daily dose of rhGH. Neither GH–LRv2 nor GH–LRv3 gave an immunogenic response or adverse side effects during this study.

The GH LR-fusions demonstrate a long-acting GH therapy, which is simple to manufacture and shows good pharmacokinetic and pharmacodynamic profiles with low immunogenicity and no treatment-related effects on haematology, blood chemistry or urinalysis. Other long-acting GH therapies do not have all the advantages of the GH LR-fusions. PEGylation is effective at delaying the clearance of the proteins, but requires chemical modification and reduces the affinity of the ligand for its receptor [31]. Sustained-release formulations or depot formulations require encapsulation of the GH into microspheres; this may require excess hormone and may result in some protein instability due to the inefficiencies of the encapsulation process [32]. Microsphere delivery of GH has also been associated with an initial high peak of GH followed by a slow decline in GH concentration over the dosing period; the initial release of GH was associated with dissociation of GH from the surface of the microsphere and the prolonged release

phase associated with GH release as the microsphere disintegrated [33].

Fusion of GH with albumin (Albutropin) [29] has similar advantages to the GH LR-fusions in that they do not need to be further processed after purification. Albutropin has a 6-fold longer terminal half-life than GH when given subcutaneously to *M. fascicularis* [30]; in comparison, GH-LRv2 and GH-LRv3 have 35- and 45-fold longer terminal half-lives, respectively, compared with native GH when given subcutaneously to *M. fascicularis*. Additionally, the GH LR-fusions should produce a more native-like protein, since GH is naturally found bound to GHBP, thus one would expect the GH LR-fusions to be less immunogenic than the GH-albumin fusion.

The LR-fusion technology has been shown to be efficacious and to have the characteristics required for a good therapeutic agent, i.e. low immunogenicity and toxicity. A clear benefit of this technology is that it can be applied to other ligand-receptor systems for the therapy of a wide range of diseases.

AUTHOR CONTRIBUTION

All authors helped with data analysis, interpretation and manuscript writing. Eric Ferrandis and Richard Ross contributed to the study design and co-ordination of study; and Sarbendra Pradhananga, Caroline Touvay, Carol Kinoshita, Ian Wilkinson, Kevin Stafford, Zida Wu, Christian Strasburger, Jon Sayers and Peter Artymiuk generated the molecules tested and were responsible for development and performance of analytical techniques. All authors have seen and approved the final version.

ACKNOWLEDGEMENTS

The non-human primate studies were undertaken by Aptuit.

FUNDING

The study was supported by Ipsen and Asterion.

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Received 30 April 2010/14 June 2010; accepted 2 July 2010

Published as Immediate Publication 2 July 2010, doi:10.1042/CS20100241