



HAL
open science

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

Eric Ferrandis, Sarbendra L Pradhananga, Caroline Touvay, Carol Kinoshita, Ian R Wilkinson, Kevin Stafford, Zida Wu, Christian Strasburger, Jon R Sayers, Peter J Artymiuk, et al.

► **To cite this version:**

Eric Ferrandis, Sarbendra L Pradhananga, Caroline Touvay, Carol Kinoshita, Ian R Wilkinson, et al.. Immunogenicity, toxicology, pharmacokinetics and pharmacodynamics of growth hormone ligand-receptor fusions.. *Clinical Science*, 2010, 119 (11), pp.483-491. 10.1042/CS20100241 . hal-00614844

HAL Id: hal-00614844

<https://hal.science/hal-00614844>

Submitted on 17 Aug 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Full Title:

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

Authors:

*Eric Ferrandis, *Sarbendra L. Pradhananga, Caroline Touvay, Carol Kinoshita, Ian R. Wilkinson, Kevin Stafford, Zida Wu, Christian J. Strasburger, Jon R. Sayers, Peter J. Artymiuk, and Richard J. Ross.

Institut Henri Beaufour (EF, CT), Ipsen, Les Ulis, France; Department of Human Metabolism (IRW, RJR) and Department of Infection & Immunity (SLP, JRS), University of Sheffield, Sheffield, S10 2RX, UK; Department of Molecular Biology and Biotechnology (PJA), University of Sheffield, UK; Ipsen Biomeasure (CK, KS), MA, USA; Department of Medicine (ZW, CJS), Campus Mitte Charite-Universitätsmedizin, 10117 Berlin, Germany.

* These authors made an equal contribution

Key Words:

Growth Hormone, GH, ligand, receptor, fusion, immunogenicity, toxicity, pharmacokinetics, pharmacodynamics.

Short title:

Immunogenicity of GH LR-fusions.

Author for Correspondence:

Professor R.J.M. Ross,
University of Sheffield, Room 112 Floor M, Royal Hallamshire Hospital, Glossop Road, Sheffield, S10 2JF, United Kingdom.

Telephone No: 00 44 (0)114 2713305;

Fax No.: 00 44 (0)114 27118

E-mail: r.j.ross@sheffield.ac.uk

Disclosure Statement:

EF, CT, CK & CT are employed by Ipsen. SLP, IRW, JRS, PA & RJR hold stock in Asterion Ltd. ZW & CJS have nothing to disclose.

Acknowledgements: The NHP studies were undertaken by Aptuit

Submission:

Main Manuscript (3694 words)

Abstract (228 words)

ABSTRACT

A fundamental concern for all new biological therapeutics is the possibility of inducing an immune response. We have recently demonstrated that a ligand-receptor fusion (LR-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.

GH-LRv2 and GH-LRv3 demonstrated similar pharmacokinetics in rats, showing reduced clearance compared to native GH and potent agonist activity with respect to body weight gain in a hypophysectomised 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. 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 administration 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; a ligand-receptor fusion (LR-fusion) [1]. The advantages of this technology include fusion at the DNA level, no requirement for chemical modification, and delayed clearance. We have made a growth hormone (GH, 1-191) to growth hormone extracellular domain (GHRext, 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, 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 degrees during treatment with most human proteins, including insulin, growth hormone, 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 growth hormone 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 *E. 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; 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 man. However, low immunogenicity and lack of toxicity in non-human primates provides strong reassurance for a non-toxic effect in man. 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 titers 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 *M. fascicularis*.

MATERIALS AND METHODS

Materials

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

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 GHR extracellular domain, GH-LRv3. Stable clones were made in CHO Flp-In cells (Invitrogen, Paisley, UK), adapted to protein free media and grown in suspension culture. LR-fusion expression was confirmed by an in-house ELISA. Affinity purification was performed using a GH mAb 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). Seven weeks old normal Sprague Dawley rats from Janvier (Le Genest Saint Isle, France) were used for pharmacokinetic studies. Subcutaneous (sc) 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). 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 (Larbesle, France). 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 these assayed using the GH ELISA and an IGF-1 ELISA (Octeia Rat/Mouse IGF-1 Assay Kit, iDS).

In vivo immunogenicity and extended pharmacokinetic study

The monkey study was conducted in a Good Laboratory Practice (GLP) compliant facility [United Kingdom GLP Regulations 1999 (S.I. 1999 No. 3106), as amended by the S.I. 2004 No. 994, which are in accordance with the Organisation for Economic Co-operation and Development (OECD) Principles of GLP (ENV/MC/CHEM (98) 17), and which are accepted by the US FDA and Japanese authorities]. Male *M. fascicularis* (n=19) aged 2 to 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 5 treatment groups (Group 1: vehicle, Group 2: GH-LRv2 test group, Group 3: GH-LRv3, Group 4: growth hormone control and Group 5: Nutropin control), comprising 3 males in the vehicle group and 4 males per group in the remaining 4 groups. All animals from the vehicle control were dosed on two occasions (Days 0 and 14), by subcutaneous injection. GH-LRv2, and GH-LRv3 treated groups were dosed on two occasions (Days 0 and 14), by subcutaneous injection with 1 mg/kg protein, 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 mg/kg/day, 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 pre-dose, 1 hour post-dose, then at approximately 6, 12, 24, 48, 72, 144, 192, 288 and 336 hours (for Day 1, pre-dose for Day 15) and 1 hour post-dose, then at approximately 6, 12, 24, 48, 72, 144, 192, 288, 360, 648 and 696 hours after administration. The sampling times for groups 4 and 5 were at pre-dose and 1 hour post-dose, then at approximately 3, 6, 12, 24, 48, 72, 144, 192, 288 and 336 hours after dose administration, then pre-dose on Day 27 and 1 hour post-dose, then at approximately 3, 6, 12, 24, 48, 72, 144, 192, 288 and 360 hours 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-labeled mouse antibody to human GH (mAb 7F8) in wells pre-coated 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 inter assay CV is <10%.

Assessment of immunological response to GH-containing molecules

An ELISA based method was used to analyze serum samples from the *M. fascicularis* for the presence of antibodies against the respective injected molecule. The serum samples were diluted in phosphate buffered saline and incubated on plates coated with the relevant antigen. The presence of bound antibodies was then analyzed by the addition of protein-A peroxidase followed by 3, 3', 5, 5'-tetramethylbenzidine (TMB) 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 cutoff for a positive result was the mean absorbance measured spectrophotometrically at 450nm (A_{450}) for the negative control in the individual assay plus 3 standard deviations. Serum samples giving a positive reading were further analyzed 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 analysis, if $P < 0.5$ then the Dunnett's test was performed. Pharmacokinetic parameters were estimated by fitting values of hormone concentration versus time to compartmental models using non-linear least-squares regression analysis. Clearance values were normalized to animal weight. Clearance rate per animal weight and terminal half lives ($t_{1/2}$) were calculated using the coefficient and exponents obtained from the intravenous bolus model fits.

RESULTS

Pharmacokinetic analysis

The GH LR-fusions showed greatly delayed clearance compared to 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 ($AUC_{0-\tau}$) to GH-LRv3 was, on average, approximately 1.7-fold greater than GH-LRv2 on Days 0 and 14. Both molecules gave continuous exposure appropriate for the subsequent immunogenicity study.

LR-fusions and growth promotion

To test biological activity, the GH LR-fusions and GH were administered to hypophysectomised (GH-deficient) rats. Daily administration of rhGH induced continuous growth over 10 days. The LR-fusion was then compared to 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 $\mu\text{g}/\text{kg}/\text{day}$, approximately 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 to controls ($P < 0.05$) but not different between the treatment groups (Figure 3).

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 to 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 to pegylated 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 to 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 GH binding protein (GHBP), 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 (Gly₄Ser)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 man. However the fact that the LR-fusion proteins, given at ten 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 man. One animal injected with GH-LRv2 gave a 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 pre-dose sample which remained high throughout the course of the study, the response for this individual was regarded as a false positive. These results compare favorably 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/day for adult replacement to 0.055 mg/kg/day 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/day 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/dose given on days 1 and 14. In a study of methionyl-GH immunogenicity in monkeys, where up to 81% of animals developed detectable antibody titers, the majority of animals who developed an immunogenic response had done so by 35 days [19]. Based on this study it is unlikely that we have missed a major immunogenic reaction to the LR-fusion proteins.

In man, GH therapy has been variably reported to initiate an immune response in 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 titers 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 dependant on the formulation, GH encapsulated in poly(lactic-co-glycolic acid) (PLGA) microspheres has been shown to have an immunogenic response in rhesus monkeys when delivered as microspheres, in contrast none of 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 nor 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 approximately 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 man with a half-life of 64.7 +/- 10.9 hours [28]. Another putative GH-fusion therapeutic, Albutropin, a fusion of GH with albumin, has a half-life 6 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 to rats and 2 fold less in humans compared to monkeys [30]. We found a similar difference for the LR-fusions with 1.6-2.8 fold reduced clearance in monkeys compared to rats and if there is a similar reduction of clearance in humans, the LR-fusions could provide a two to three 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 to 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 hours and 78 +/- 17 hours 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 five days and lesser growth over the second five days. Over the 10 days the LR-fusions produced a weight gain lower but not significantly different to 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 the all 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 six-fold longer terminal half-life than GH when given subcutaneously to *M. fascicularis* [30]; in comparison GH-LRv2 and GH-LRv3 have 35-fold and 45-fold longer terminal half-lives, respectively, compared to 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.

Accepted Manuscript

REFERENCES

1. Wilkinson, I. R., Ferrandis, E., Artymiuk, P. J., Teillot, M., Soulard, C., Touvay, C., Pradhananga, S. L., Justice, S., Wu, Z., Leung, K. C., Strasburger, C. J., Sayers, J. R. and Ross, R. J. (2007) A ligand-receptor fusion of growth hormone forms a dimer and is a potent long-acting agonist. *Nat Med* **13**.1108-1113
2. Huston, J. S., Tai, M. S., McCartney, J., Keck, P. and Oppermann, H. (1993) Antigen recognition and targeted delivery by the single-chain Fv. *Cell Biophys* **22**.189-224
3. de Vos, A. M., Ultsch, M. and Kossiakoff, A. A. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **255**.306-312
4. Schellekens, H. and Casadevall, N. (2004) Immunogenicity of recombinant proteins: causes and consequences. *J Neurol* **251**.114-119
5. Frasier, S. D. (1983) Human pituitary growth hormone (hGH) therapy in growth hormone deficiency. *Endocr Rev* **4**.155-170
6. Gunnarsson, R. and Wilton, P. (1987) Clinical experience with Genotropin worldwide: an update March 1987. *Acta Paediatr Scand Suppl* **337**.147-152
7. Job, J. C., Rochiccioli, P., Rappaport, R., Raux-Demay, M. C., Ponte, C., Juif, J., Colle, M., Lecornu, M., Leheup, B. and Garandeau, P. (1988) 1-year treatment with recombinant somatotropin in prepubertal and pubertal growth hormone deficient patients: results from a French multicentre trial. *Acta Paediatr Scand Suppl* **347**.187-190
8. Takano, K., Shizume, K., Hibi, I., Okuno, A., Hanyu, K., Suwa, S., Nakajima, H., Kondo, T., Kato, K., Iwatani, N. and al., e. (1988) Treatment of hypopituitarism with recombinant somatotropin for 1 year. *Acta Paediatr Scand Suppl* **347**.169-175
9. Buzi, F., Buchanan, C. R., Morrell, D. J. and Preece, M. A. (1989) Antigenicity and efficacy of authentic sequence recombinant human growth hormone (somatotropin): first-year experience in the United Kingdom. *Clin Endocrinol (Oxf)* **30**.531-538
10. Chang, T. C., Kuo, H. F., Liu, P. C., Shih, K. C., Zeisel, H. J. and Ho, L. T. (1991) Recombinant DNA produced somatotropin in the treatment of prepubertal growth hormone deficient children. *Zhonghua Yi Xue Za Zhi (Taipei)* **47**.7-12
11. Rougeot, C., Marchand, P. M., Dray, F., Girard, F., Job, J. C., Pierson, M., Ponte, C., Rochiccioli, P. and Rappaport, R. (1991) Comparative study of biosynthetic human growth hormone immunogenicity in growth hormone deficient children. *Horm Res* **35**.76-81
12. Lundin, K., Berger, L., Blomberg, F. and Wilton, P. (1991) Development of anti-GH antibodies during therapy with authentic human growth hormone. *Acta Paediatr Scand Suppl* **372**.167-168
13. Massa, G., Vanderschueren-Ledeweyckx, M. and Bouillon, R. (1993) Five-year follow-up of growth hormone antibodies in growth hormone deficient children treated with recombinant human growth hormone. *Clin Endocrinol (Oxf)* **38**.137-142
14. Shih, K. C., Ho, L. T., Kuo, H. F., Chang, T. C., Liu, P. C., Chen, C. K. and Tiu, C. M. (1994) Linear growth response to recombinant human growth hormone in children with growth hormone deficiency. *Zhonghua Yi Xue Za Zhi (Taipei)* **54**.7-13
15. Pirazzoli, P., Cacciari, E., Mandini, M., Cicognani, A., Zucchini, S., Sganga, T. and Capelli, M. (1995) Follow-up of antibodies to growth hormone in 210 growth hormone-deficient children treated with different commercial preparations *Acta Paediatr* **84**.1233-1236
16. Li, S., Yu, Y., An, Z., Xiong, Z. and Yu, H. (2002) The immunogenicity and effect of domestic recombinant human growth hormone: serum GH-antibody determination and evaluation. *Hua Xi Yi Ke Da Xue Xue Bao* **33**.77-79
17. Jones, A. J. (2002) The use of an animal immunogenicity model in the development of Protropin somatrem (methionyl human growth hormone). *Dev Biol (Basel)* **109**.107-118
18. Lee, H. J., Riley, G., Johnson, O., Cleland, J. L., Kim, N., Charnis, M., Bailey, L., Duenas, E., Shahzamani, A., Marian, M., Jones, A. J. S. and Putney, S. D. (1997) In vivo characterization of sustained-release formulations of human growth hormone. *J Pharmacol Exp Ther* **281**.1431-1439
19. Zwickl, C. M., Cocke, K. S., Tamura, R. N., Holzhausen, L. M., Brophy, G. T., Bick, P. H. and Wierda, D. (1991) Comparison of the immunogenicity of recombinant and pituitary human growth hormone in Rhesus monkeys. *Fund Appl Toxicol* **16**.275-287
20. Covic, A. and Kuhlmann, M. K. (2007) Biosimilars: recent developments. *Int Urol Nephrol*

39.261-266

21. Trainer, P. J., Drake, W. M., Katznelson, L., Freda, P. U., Herman-Bonert, V., Lely, A. J. v. d., Dimaraki, E. V., Stewart, P. M., Friend, K. E., Vance, M. L., Besser, G. M. and Scarlett, J. A. (2000) Treatment of acromegaly with the growth hormone-receptor antagonist Pegvisomant. *New Engl J Med* **342**.1171-1177
22. Hodish, I. and Barkan, A. (2008) Long-term effects of pegvisomant in patients with acromegaly. *Nat Clin Pract* **4**.324-332
23. Silverman, B. L., Blethen, S. L., Reiter, E. O., Attie, K. M., Neuwirth, R. B. and Ford, K. M. (2002) A long-acting growth hormone (Nutropin Depot): efficiency and safety following two years of treatment in children with growth hormone deficiency. *J Pediatr Endocrinol Metab* **15**.715-722
24. Cook, D. M., Biller, B. M. K., Vance, M. L., Hoffman, A. R., Phillips, L. S., Ford, K. M., Benzinger, D. P., Illeperuma, A., Blethen, S. L., Attie, K. M., Dao, L. N., REimann, J. D. and Fielder, P. J. (2002) The pharmacokinetic and pharmacodynamic characteristics of a lon-acting growth hormone (GH) preparation (Nutropin Depot) in GH-Deficient Adults. *J Clin Endocr Metab* **87**.4508-4514
25. Touraine, P., D'Souza, G.A., Kourides, I., Abs, R., Barclay, P., Xie, R., Pico, A., Torres-Vela, E., Ekman, B., and the GH Lipoatrophy Study Group (2009) Lipoatrophy in GH deficient patients treated with a long-acting pegylated GH. *Eur J Endocrinol* **161**, 533-540
26. Bernabeu, I., Cameselle-Teijeiro, J., Casanueva, F. F. and Marazuela, M. (2009) Pegvisomant-induced cholestatic hepatitis with jaundice in a patient with Gilbert's syndrome. *Eur J Endocrinol* **160**.869-872
27. Lou, K.-J. (2009) Growing a PEG-like alternative. *SciBX* **2**.1-2
28. Yin, D., Vreeland, F., Schaaf, L. J., Millham, R., Duncan, B. A. and Sharma, A. (2007) Clinical pharmacodynamic effects of the growth hormone receptor antagonist pegvisomant: Implications for cancer therapy. *Clin Cancer Res* **13**.
29. Osborn, B. L., Sekut, L., Corcoran, M., Poortman, C., Sturm, B., Chen, G., Mather, D., Lin, H. L. and Parry, T. J. (2002) Albutropin: a growth hormone-albumin fusion with improved pharmacokinetics and pharmacodynamics in rats and monkeys. *Eur J Pharmacol* **456**.149-158
30. Mordenti, J., Chen, S. A., Moore, J. A., Ferraiolo, B. L. and Green, J. D. (1991) Interspecies scaling of clearance and volume of distribution data for five therapeutic proteins. *Pharmaceut Res* **8**.1351-1359
31. Clark, R., Olson, K., Fuh, G., Marian, M., Mortensen, D., Teshima, G., Chang, S., Chu, H., Mukku, V., Canova-Davis, E., Somers, T., Cronin, M., Winkler, M. and Wells, J. A. (1996) Long acting growth hormones produced by conjugation with polyethylene glycol. *J Biol Chem* **271**.21969-21977
32. Pai, S. S., Tilton, D. T. and Przybycien, T. M. (2009) Poly(ethylene glycol)-modified proteins: Implications for ply(lactide-co-glycolide)-based microsphere delivery. *AAPS Journal* **11**.88-98
33. Kemp, S. K., Fielder, P. J., Attie, K. M., Blethen, S. L., Reiter, E. O., Ford, K. M., Marian, M., Dao, L. N., Lee, H. J. and Saenger, P. (2004) Pharmacokinetic and pharmacodynamic characteristics of long-acting growth hormone (GH) preparation (Nutropin Depot) in GH-deficient children. *J Clin Endocr Metab* **89**.3234-3240

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20100241

ACCEPTED MANUSCRIPT

TABLES AND FIGURES

	Rat		<i>M. fascicularis</i>	
	Clearance s.c. (ml hrs ⁻¹ kg ⁻¹)	T _{1/2} s.c. (hrs)	Clearance s.c. (ml hrs ⁻¹ kg ⁻¹)	T _{1/2} s.c. (hrs)
hGH	820 +/- 94 *	0.66 +/- 0.1 *	154 +/- 4.4 *	1.7 +/- 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)

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

Accepted Manuscript

Animal Number:		Vehicle			1 mg/kg/dose GH-LRv2			
		101	103	105	201	203	205	207
Day 1	Pre-dose	0.060	0.066	0.052	0.067	0.053	0.046	0.132
Day 7	144 hr	0.047	0.064	0.068	0.071	0.072	0.051	0.119
Day 15	Pre-dose	0.060	0.046	0.050	0.064	0.077	0.080	0.106
Day 21	144 hr	0.058	0.077	0.065	0.059	0.050	0.077	0.129
Day 27	288 hr	0.067	0.054	0.055	0.049	0.078	0.068	0.118
Day 44	696 hr	0.068	0.071	0.055	0.071	0.134	0.210	0.078

Animal Number:		Vehicle			1 mg/kg/dose GH-LRv3			
		101	103	105	301	303	305	307
Day 1	Pre-dose	0.058	0.065	0.052	0.056	(0.371)	0.069	0.085
Day 7	144 hr	0.062	0.060	0.067	0.060	(0.196)	0.064	0.082
Day 15	Pre-dose	0.062	0.058	0.065	0.065	(0.305)	0.064	0.086
Day 21	144 hr	0.066	0.075	0.070	0.073	(0.403)	0.068	0.083
Day 27	288 hr	0.062	0.057	0.054	0.068	(0.408)	0.065	0.073
Day 44	696 hr	0.074	0.068	0.058	0.065	(0.325)	0.063	0.081

Animal Number:		Vehicle			0.1 mg/kg/dose GH			
		101	103	105	401	403	405	407
Day 1	Pre-dose	0.062	0.071	0.079	0.059	0.066	0.049	0.060
Day 7	144 hr	0.063	0.074	0.070	0.070	0.067	0.059	0.058
Day 15	Pre-dose	0.062	0.075	0.069	0.066	0.180	0.064	0.083
Day 21	144 hr	0.064	0.069	0.081	0.132	0.185	0.081	0.109
Day 27	288 hr	0.063	0.083	0.062	0.167	0.060	0.109	0.085
Day 44	696 hr	0.062	0.096	0.065	0.147	0.136	0.076	0.074

* The table shows the A₄₅₀ for the sera from *M. fascicularis* given GH-LRv2, GH-LRv3 or hGH. Positive immunogenic results are shown in **bold**, false positives are shown in brackets. 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.

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

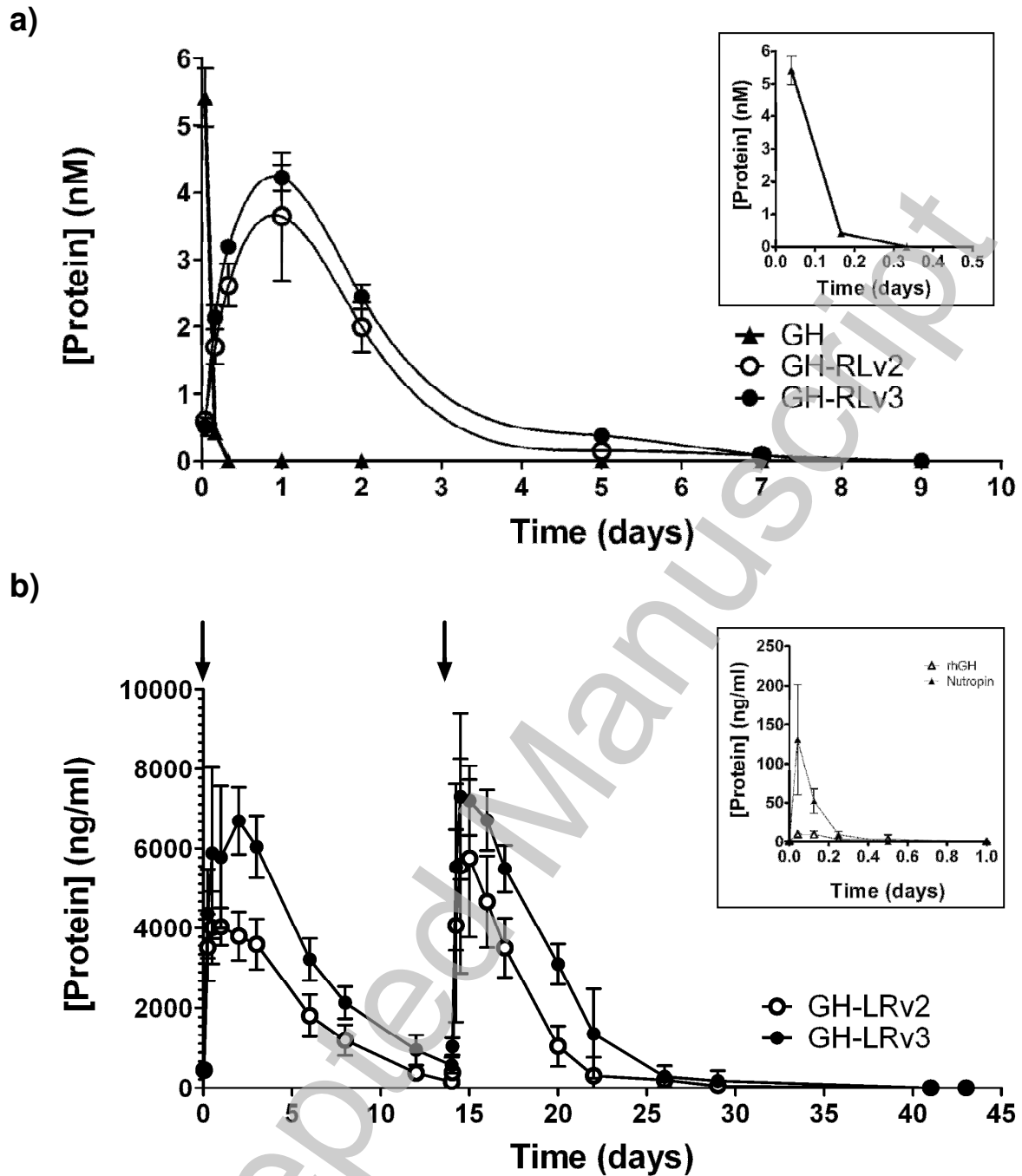


Figure 1: (a) Delayed clearance of rhGH, GH-LRv2 and GH-LRv3 in rats, following subcutaneous delivery of protein at time = 0 days; the inset shows the clearance for rhGH alone. [N.B. Units for protein concentration given in nM to enable direct comparison of GH with the GH-LR proteins]. (b) Delayed clearance of GH-LRv2 and GH-LRv3 in *M. fascicularis*, following subcutaneous delivery of protein at time = 0 days and 14 days (vertical arrows); the inset shows the clearance for rhGH and Nutropin.

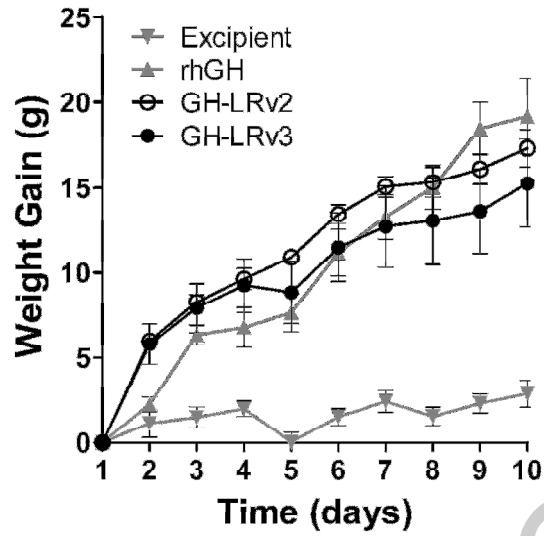


Figure 2: Weight gain in hypophysectomized rats due to LR-fusions compared with rhGH. The LR-fusions and excipient were injected subcutaneously only on day 1 and the rhGH was given daily.

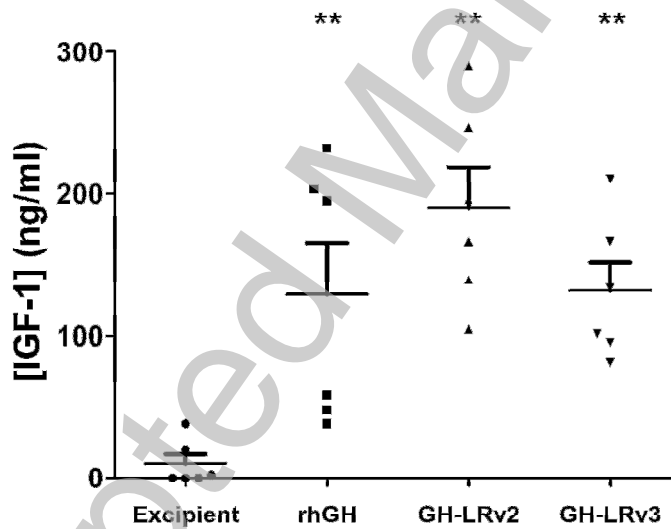


Figure 3: IGF-1 measurement in the terminal bleeds of rats (n=6) given LR fusions compared to rhGH. The LR-fusions and excipient were injected subcutaneously only on day 1 and the rhGH was given daily. ** = $P < 0.05$ versus Excipient.