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Development and characterization of a long-acting recombinant hFSH agonist

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BACKGROUND: Fusion of the carboxyterminal peptide (CTP) of hCG to FSH results in a follitropin agonist with an extended half-life, presumably due to the four *O*-oligosaccharides on the CTP. Alternatively, an rhFSH analogue containing additional *N*-linked carbohydrate is described in this report. METHODS: A DNA sequence containing two *N*-oligosaccharide signal sequences was ligated into a vector containing hFSHβ- and α-subunit encoding cDNA, and expressed in CHO-K1 cells. In-vitro bioactivity of the single-chain hormone was assessed in CHO cells expressing the hFSH receptor. Pharmacokinetic values were derived from serial serum assays of the analogue in immature female rats following a single i.v. injection. In-vivo bioactivity was assessed by measuring ovarian weight gain 3 days post-injection. RESULTS: rhFSH-N2 and native rhFSH induced comparable levels of cAMP in vitro. $t_{1/2}$ for native rhFSH, rhFSH-CTP and rhFSH-N2 were 3.7, 7.1 and 7.3 h respectively. Rats receiving rhFSH-N2 had a mean ± SD ovarian weight 3 days post-i.v. injection (22 ± 3.6 mg) significantly greater than rats receiving rhFSH and saline (16.7 ± 1.5 and 15.3 ± 0.47 mg respectively, $P < 0.05$). CONCLUSIONS: rhFSH-N2 has prolonged half-life and increased bioactivity compared with native rhFSH. This rhFSH agonist, and other analogues containing additional *N*-oligosaccharides may have important clinical applications.

Key words: carboxyterminal peptide/FSH/hormone analogue/oligosaccharide/pharmacokinetics

Introduction

Exogenous gonadotrophin therapy remains a mainstay of treatment for couples having difficulty conceiving. Prolonged therapy is necessary to achieve a therapeutic effect, typically for 8–10 consecutive days to stimulate folliculogenesis in women and for several months as replacement therapy in hypogonadotrophic males to induce spermatogenesis. Recombinant hFSH is administered as an i.m. or s.c. daily injection, with consequent discomfort and potential for local injection site reaction. Decreasing the frequency of administration would facilitate therapy and render gonadotrophin administration more tolerable.

Two long-acting gonadotrophin agonists developed by fusing the carboxyterminal peptide of hCG (CTP) to native recombinant human FSH (rhFSH) either as a non-covalently bound heterodimer containing the common α- and β-FSH subunits (Fares *et al*., 1992) or as a contiguous peptide (Klein *et al*., 2002) have previously been reported. The increased half-life and biopotency of this analogue (rhFSH-CTP) is speculated to be due to the four *O*-linked glycosylation sites present on the CTP moiety (Matzuk *et al*., 1990). Alternatively, the biology of an FSH analogue containing additional *N*-linked oligosaccharides has not been reported.

Addition of carbohydrate to the peptide backbone is a post-translational event that may occur through *O*-linkage with serine or threonine residues, or *via N*-linkage to asparagine. Whereas the glycosylation signal sequence for *O*-linked sugars is poorly defined, addition of *N*-linked carbohydrates to asparagine occurs whenever the primary amino acid sequence contains the trimer Asn-X-Ser/Thr, where X denotes any amino acid except proline (Imperiali and O'Connor, 1999). The number of *N*-linked carbohydrates that are introduced in a synthetic polypeptide chain can thus be precisely defined. Consequently, this represents a potentially more powerful tool to refine the pharmacokinetic properties of rhFSH and perhaps other recombinant species in a clinically meaningful way, compared with adding CTP or other sequences containing clusters of *O*-linked sugars. To determine the feasibility of this approach, we designed a synthetic oligopeptide containing two copies of the *N*-linked glycosylation signal sequence, and tethered this sequence between the β- and α-subunits of hFSH, creating a single-chain fusion hormone analogue (rhFSH-N2). The ability of this novel construct to bind and activate the hFSH receptor *in vitro*, and the in-vivo activity and half-life of this analogue compared to native recombinant hFSH and hFSH-CTP, were examined.

Materials and methods

Molecular biology

All enzymes for recombinant DNA were purchased from New England Biolabs (Beverly, MA, USA). The Columbia University Core Laboratory synthesized DNA primers for PCR. cDNA for hFSHβ and the common α-subunit were provided by Dr William Moyle (Robert Wood Johnson Medical School, Piscataway, NJ, USA). PCR reactions were performed with Vent DNA polymerase (primer sequences are available upon request) and all products of the reactions were sequenced to ensure that no mutations were introduced during the amplification process. Ligation reactions were transformed into *E. coli* strain DH5α, and DNA clones grown in standard Luria–Bertani medium for large-scale plasmid preparation. Transformation of DH5α was performed according to standard techniques with calcium chloride.

Controls

Native rhFSH (follitropin-β) was generously provided by Serono, Inc. (Rockland, MD, USA). Single-chain rhFSH-CTP was produced in a CHO-K1 cell line as described previously (Klein *et al*., 2002).

Construction of a rhFSHβ-N2-α (rhFSH-N2) single-chain fusion clone

Two complementary DNA strands encoding the following polypeptide sequence were synthesized by Columbia's protein core: Ser-Gly-Ser-Asn-Ala-Thr-Gly-Ser-Gly-Ser-Asn-Ala-Thr-Ser-Gly-Ser, such that following annealing of the strands 5′ *Bam*HI and 3′ *Spe*I sticky ends were formed (DNA sequences available upon request). The synthetic DNA duplex was then ligated into a vector along with the rhFSHβ- and α-subunit cDNA. The in-frame ligation of these three DNA was accomplished by placing a *Xho*I site immediately preceding the start codon and replacing the terminator codon of the hFSH β-subunit with a *Bam*HI site. In addition a *Spe*I site was placed at the 5′ end and a *Sac*I site immediately following the terminator codon of the α-subunit. The three fragments were then inserted into an SV40-based expression vector at *Xho*I/*Sac*I sites to form the rhFSH-N2 expression construct (Figure 1).

Expression and purification of rhFSH-N2

An SV40 expression clone containing the fusion construct was co-transfected into CHO-K1 cells along with an SV2neo clone encoding resistance to G418. The CHO cell transformation was performed using a standard calcium phosphate precipitate technique. Selectable media containing G418 (Gemini Bioproducts, Woodland, CA, USA) were used to isolate colonies that were pooled and maintained in Ham's F-12 containing 500 μg/ml G418, 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 4 mmol/l glutamine. Pooled colonies were subcloned in 96-well microtitre dishes to isolate a high-secreting clone. To further increase yields cells, were grown in suspension cultures, with spinner bottles seeded at 10^5 cells/ml in CHO-S-SFM II (Life Technologies, Rockville, MD, USA) containing 400 μg/ml G418. Cultures generally reached a density of 2×10^6 cells/ml on day 6 or 7, and the cell supernatant was harvested on day 7 or 8.

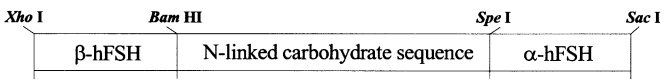

Figure 1. Schematic of rhFSH-N2 construct and associated restriction sites. A polypeptide-encoding DNA sequence containing two *N*-glycosylation signal sequences was used to tether the α- and β-subunits of hFSH.

Supernatants received 0.2 mmol/l phenylmethylsulphonyl fluoride and were filtered through a 0.2 μm membrane and kept at 4°C until the day of purification.

Affinity purification of rhFSH-N2 was accomplished using an α-specific monoclonal antibody column [A103 (O'Connor *et al*., 1994)]. The column was prepared by coupling purified A103 immunoglobulins to cyanogen bromide (CNBr)-Sepharose-4B according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at a concentration of 5 mg antibody/ml Sepharose. After applying the cell supernatant, the column was washed with 50 bed volumes of PBS followed by 2 bed volumes of distilled water. rhFSH-N2 was eluted with 3 or 4 bed volumes of 1 mol/l acetic acid and immediately dried on a Speed-Vac concentrator (Savant Instruments, Holbrook, NY, USA).

Electrophoresis and Western blotting

Sodium dodecyl sulphate–polycrylamide gel electrophoresis was performed and proteins were transferred to nitrocellulose using standard techniques (Laemmli, 1970; Towbin *et al*., 1979; Burnette, 1981). After blocking, the nitrocellulose was incubated overnight in a 1:10 000 dilution of a monoclonal antibody to FSH-β from Biodesign International (Saco, ME, USA). The membrane was then washed and incubated for 1 h in a 1:10 000 dilution of a peroxidase-conjugated polyclonal antibody to mouse immunoglobulins (Amersham–Pharmacia Biotech). After washing, the nitrocellulose was incubated in a chemiluminescent detection reagent according to the manufacturer's directions (Amersham Pharmacia). Bands were visualized by exposure to X-ray film.

Isoelectric focusing gel electrophoresis

Samples were electrophoresed on a Novex pre-cast IEF gel, with a pI range of pH 3–7 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and then visualized by silver staining.

In-vitro bioactivity

The rhFSH-N2 was quantified using an hFSH-β specific antibody (Biodesign International, Saco, ME, USA) radioimmunoassay. Biological activity was assessed using CHO cells transfected with the FSH receptor (CHO FSHr) as previously described (Kelton *et al*., 1992). A total of 2×10^4 cells was mixed with rhFSH-N2, rhFSH-CTP or rhFSH at varying concentrations in a total volume of 200 μl.

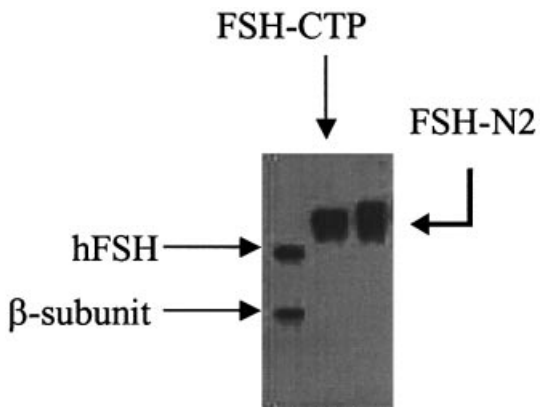

Figure 2. Non-reduced western Blot showing migration of rhFSH-N2, rhFSH-CTP and rhFSH in 10% sodium dodecyl sulphate–acrylamide gel. (Note the lack of dissociation of the β-subunit in single-chain species due to covalent linkage between subunits.)

Mixtures were incubated at 37°C for 15 min, and cAMP levels were measured using a radioimmunoassay kit (Perkin Elmer Life Sciences, Boston, MA, USA).

In-vivo bioactivity and half-life determinations

Approval was obtained from the Institutional Animal Care and Use Committee at Columbia University. Immature (21 day old) female Sprague–Dawley rats were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were housed three to a cage and given standard food and tap water *ad libitum*. Animals were randomly assigned to one of four treatment groups: rhFSH-N2, single-chain recombinant hFSH tethered to CTP (rhFSH-CTP), rhFSH, or saline. All hormones were diluted to 11 μg/ml using injection buffer containing bovine serum albumin (1 mg/ml). Hormone was administered as a single i.v. dose of 2800 ng/rat in 0.25 ml of solution *via* a dorsal tail vein. Serum was obtained by periorbital venipuncture at the following intervals post-injection: 0.5, 1.0, 3.0, 6.0 and 12 h. Anaesthesia with isoflurane was administered prior to injection and each bleed. Serum was assayed for hFSH by enzyme-linked immunosorbent assay (Diagnostic Systems Laboratory, Webster, TX, USA). Rats were killed 72 h post-injection by carbon dioxide inhalation followed by exsanguination *via* cardiac puncture. Ovaries were then extirpated and weighed.

Pharmacokinetic analysis was performed using Winnonlin 1.0 software (Pharsight Corporation, Mountain View, CA, USA). Mean values for each time-point within a group were used to estimate pharmacokinetic parameters for terminal (elimination) half-life ($t_{1/2}$) and area under the serum concentration–time curve (AUC). Clearance (Cl) was calculated using the relationship Cl = dose/AUC.

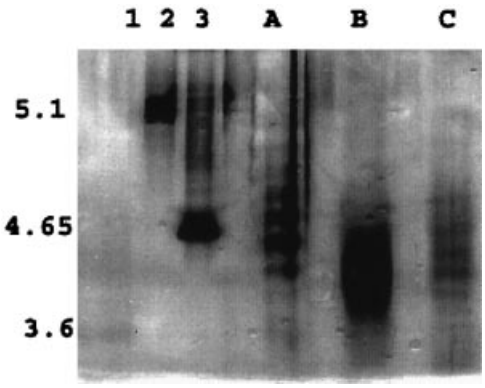

Figure 3. An isoelectric focusing gel, pH 6–3 silver-stained. Lanes 1–3 are pH standards; lane A: rhFSH; lane B: rhFSH-CTP; lane C: rhFSH-N2.

Statistical analysis

Mean ovarian plasma concentrations and ovarian weights were compared between groups in a one-way analysis of variance (ANOVA) model. Since groups with higher means tended to also have higher variances, the data were first log-transformed to stabilize the variance between groups and satisfy the model assumptions. The primary comparison of interest was between rhFSH-N2 and rhFSH, and this difference was tested using a single contrast within the one-way structure. In addition, mean serum plasma concentrations at all time-points were compared using a one-way ANOVA model. At each time-point, the comparisons of interest were rhFSH-N2 versus rhFSH and rhFSH-N2 versus rhFSH-CTP. These hypotheses were tested with simultaneous contrasts at each time-point using a Bonferroni adjustment to control for multiple comparisons, and the analysis for each time-point was considered separately.

Results

A CHO cell clone producing 0.4 pmol/ml rhFSH-N2 was isolated. Production increased to 3 pmol/ml when cells were grown in suspension culture. A Western blot revealed purified

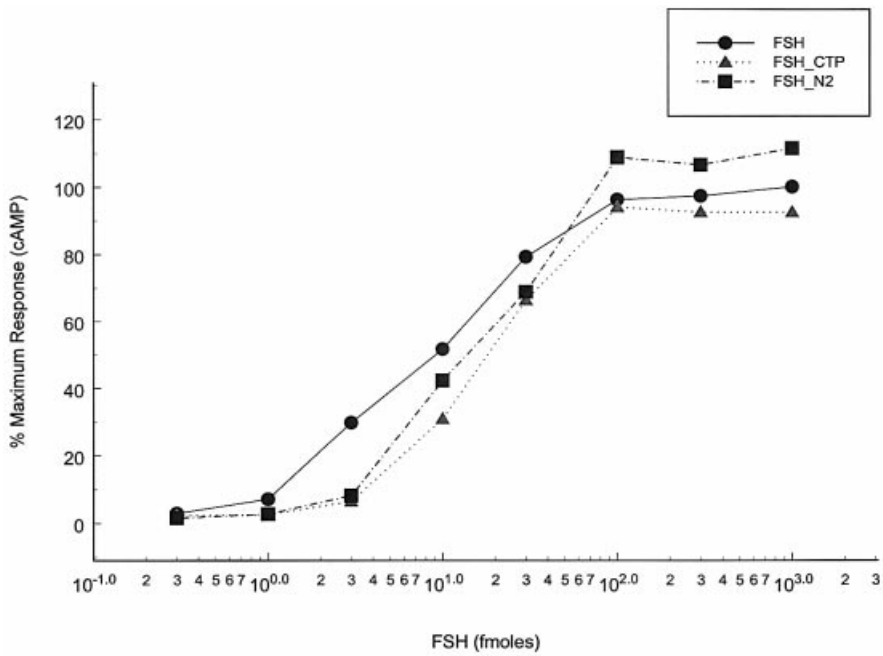

Figure 4. In-vitro bioactivity, as assessed by induction of cAMP, of rhFSH, rhFSH-CTP and rhFSH-N2 in CHO cells expressing the FSH receptor (CHO FSHr). Maximum response corresponds to highest level of cAMP induced by rhFSH in 2×10^4 cells (~3000–5000 fmol cAMP).

rhFSH-N2 migration corresponding to a molecular size of ~58 kDa. This species migrated at a similar size to rhFSH-CTP, yet larger than native rhFSH, consistent with increased mass due to additional glycosylation (Figure 2). Isoelectric focusing gel electrophoresis showed that both rhFSH-CTP and rhFSH-N2 have a more acidic profile than rhFSH. There were five major isoforms for rhFSH between pI 4.3 and 4.9 and for rhFSH-N2 between 4.05 and 4.8, while rhFSH-CTP focused predominately as a smear between pI 3.85 and 4.55 (Figure 3).

In-vitro bioactivity

Binding of rhFSH-N2 to the hFSH receptor and signal transduction was confirmed by induction of cAMP in CHO cells expressing the hFSH receptor. rhFSH-N2 induced a similar rise in cAMP levels *in vitro* compared with rhFSH and rhFSH-CTP (Figure 4).

In-vivo bioactivity and half-life determinations

Mean ovarian weights following a single injection of rhFSH-N2, rhFSH and saline are shown in Figure 5. The results of the simultaneous contrast testing indicated significant differences in the mean log ovarian weight in animals receiving rhFSH-N2 as compared with those receiving rhFSH ($P < 0.025$). There was no significant difference between ovarian weights from rats receiving rhFSH-N2 and rhFSH-CTP (Figure 6).

Mean serum concentration–time curves for rhFSH-N2, rhFSH-CTP and rhFSH are shown in Figure 7. Pharmacokinetic parameter estimates are listed in Table I. The elimination half-life of rhFSH-N2 (7.3 h) was comparable with that of rhFSH-CTP (7.1 h) and ~2-fold longer than rhFSH (3.7 h). The amount of hormone present in the circulation at all time-points post-injection was significantly higher for rhFSH-N2 compared with rhFSH. Mean plasma levels of rhFSH-N2 were not significantly different from those of rats receiving rhFSH-CTP, although *P*-values were near the usual significance level for

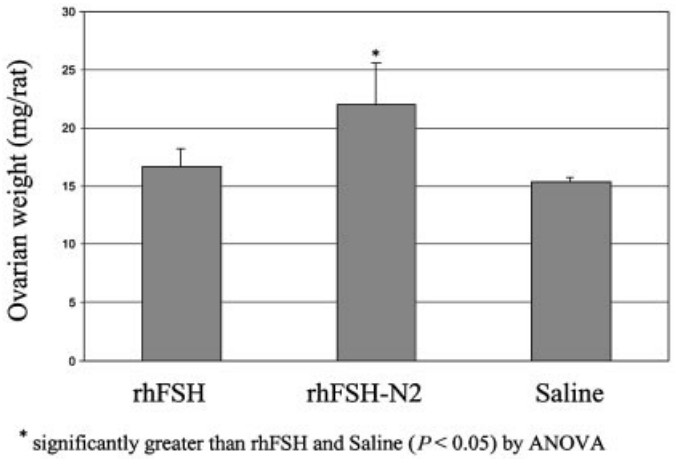

Figure 5. Mean ovarian weight from 21 day old female rats 3 days post-i.v. injection of rhFSH-N2, rhFSH and saline ($n = 3$ for each group).

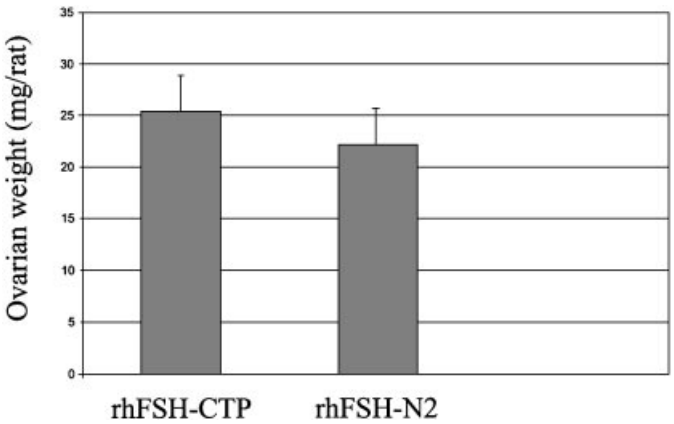

Figure 6. Mean ovarian weight from 21 day old female rats 3 days post-i.v. injection of rhFSH-N2 and rhFSH-CTP ($n = 3$ for each group).

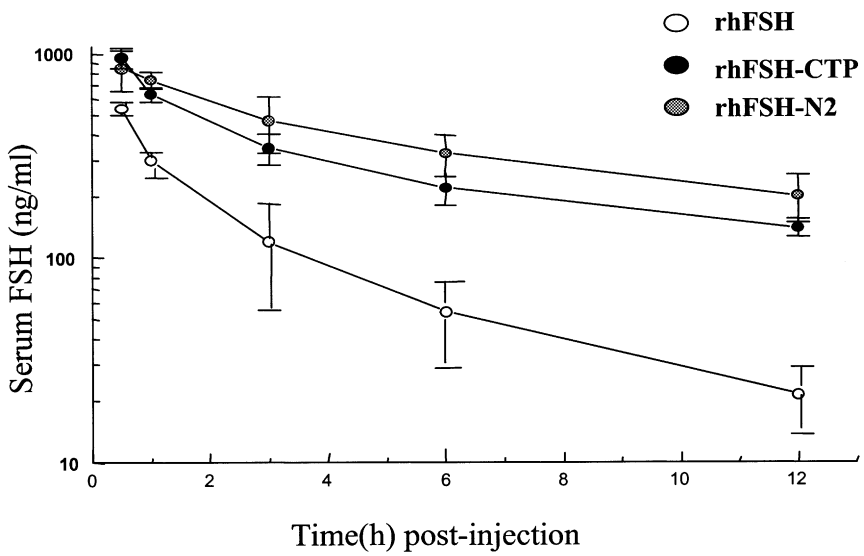

Figure 7. Mean ($n = 3$) serum concentration–time profiles following a single i.v. injection of rhFSH, rhFSH-CTP and rhFSH-N2 (2800 ng/rat) to 21 day old female rats.

later time-points (0.057 and 0.062 for time-points 6 and 12 h respectively) (Table II).

Discussion

Current recombinant gonadotrophin preparations are generally administered as daily injections due to their relatively short half-life. A long-acting follitropin agonist employing a CTP tether to the carboxyterminus of FSHβ (rhFSH-CTP) is currently under development to provide a more convenient dosing schedule for clinical use. The CTP contains a series of four tightly spaced *O*-oligosaccharides, which are presumed to be responsible for delaying hormone metabolism *in vivo* (Matzuk *et al*., 1990). rhFSH-CTP has been shown to have an elimination half-life 2–4-fold longer than native recombinant hFSH (Klein *et al*., 2002), and a phase I trial in 12 hypogonadotrophic men supported the safety of this product (Bouloux *et al*., 2001). However, the pharmacodynamic effect of rhFSH-CTP in women has not been reported and the utility of this fusion hormone in ovarian stimulation regimens remains uncertain. Ovarian response to gonadotrophin stimulation is highly variable, and is dependent on a number of factors including patient age, ovarian reserve, and presence of polycystic-appearing ovaries. rhFSH analogues with alternative half-lives may ultimately yield superior products for clinical use in certain patient populations. For example, patients with high sensitivity to gonadotrophins may require an analogue with a shorter half-life, while a hypogonadotrophic hypogonadal man requiring long-term replacement may benefit most from an extremely long-acting formulation.

Proteins are glycosylated by one of two distinct mechanisms: *N*-linkage *via* an amide group on asparagine, or *O*-linkage, in which the oligosaccharide is attached to the hydroxyl group on a serine or threonine residue. *N*-Oligosaccharides share a common trimannosyl-chitobiose core domain distinct from that of *O*-linked sugars, although overall size and composition is variable (Dell and Morris, 2001). In addition to structural disparities, differences in the function of native *O*-linked and *N*-linked oligosaccharides on glycoprotein biology exist. For example, removal or deglycosylation of the CTP of hCG, containing naturally occurring *O*-oligosaccharides, increases metabolism of the hormone *in vivo* (el-Deiry *et al*., 1989) but has no effect on proper folding of the molecule and receptor activation. (el-Deiry *et al*., 1989; Matzuk *et al*., 1990). In contrast, the native *N*-linked oligosaccharides of hCG and the other glycoprotein hormones play critical roles in both signal transduction and hormone metabolism (Matzuk and Boime, 1988; Van Zuylen *et al*., 1997; Imperiali and O'Connor, 1999).

Addition of the CTP, containing four *O*-linked oligosaccharides, to rhFSH has been shown to increase the half-life of the molecule without altering in-vitro bioactivity (and therefore proper folding of the protein) (Klein *et al*., 2002). Lack of an identifiable *O*-oligosaccharide consensus signal sequence, however, limits precise adjustments in carbohydrate content when designing hormone analogues. In an attempt to provide a more flexible, well-defined means of delaying gonadotrophin metabolism *in vivo*, we developed a recombinant follitropin agonist containing additional *N*-linked carbohydrates on the FSHβ-subunit. Unlike *O*-linked sugars, the number of *N*-linked moieties is easily modifiable in a synthetic oligopeptide sequence by inserting a specified number of consensus *N*-oligosaccharide signal sequences. Adjusting the number of added carbohydrates could theoretically result in FSH agonists with a spectrum of pharmacokinetic characteristics and bioactivity *in vivo*. These 'designer gonadotrophin' formulations may be tailored to meet the needs of specific patient populations.

In this report, we examined the pharmacokinetics and pharmacodynamics of a recombinant FSH analogue containing two additional *N*-oligosaccharides to test the utility of this approach. Stable, high-secreting CHO cell clones transfected with our FSH analogue were isolated, and proper folding of the fusion hormone was confirmed using an in-vitro FSH bioassay. Serum levels of rhFSH-N2 following i.v. administration were comparable with that of rhFSH-CTP and significantly

Table I. Mean pharmacokinetic parameter estimates for rhFSH, rhFSH-CTP and rhFSH-N2 following i.v. injection of material at a dose of 2800 ng/rat (n = 3 per group) to immature female rats (21 days old)

Parameter	rhFSH	rhFSH-CTP	rhFSH-N2
$AUC_{0-¥}$ (ng/h/ml)	1491	3887	4802
$t_{1/2}$ (elimination) (h)	3.7	7.1	7.3
Clearance (ml/h)	1.9	0.72	0.58

CTP = carboxyterminal peptide; AUC = area under the curve; N2 = tether containing two *N*-oligosaccharide sequences.

Table II. Comparison of mean serum concentrations of rats receiving rhFSH-N2 with those receiving rhFSH and rhFSH-CTP at all time-points post-i.v. injection of hormone (2800 ng), along with observed significance level using contrasts in the one-way layout with Bonferroni corrections for multiple comparisons[a]

Time (h)	rhFSH-N2 (ng/ml)	rhFSH (ng/ml)		rhFSH-CTP (ng/ml)	
	Mean ± SD	Mean ± SD	*P*	Mean ± SD	*P*
0.5	845 ± 187.8	540 ± 40.1	0.053	958.8 ± 111	NS
1	747 ± 73.2	299.2 ± 36.4	0.00012	635.8 ± 51	NS
3	467 ± 129	119.1 ± 77	0.0074	343.5 ± 55.6	NS
6	324 ± 65	53.6 ± 21	0.00059	220 ± 34.1	0.057
12	198 ± 42	21.2 ± 7.1	0.0036	137.7 ± 14.6	0.062

[a]Each time-period considered as a separate analysis.
CTP = carboxyterminal peptide; N2 = tether containing two *N*-oligosaccharide sequences.

increased relative to native recombinant FSH, confirming the ability of added *N*-oligosaccharides to decrease clearance of FSH in a manner analogous to a CTP moiety. Pharmacokinetic analysis of the data indicates rhFSH-N2 has a half-life ~2-fold that of native rhFSH and increased in-vivo bioactivity relative to native rhFSH following an i.v. bolus dose of hormone.

The altered in-vivo characteristics of rhFSH-N2 are most likely related to the increased carbohydrate load present on the peptide linker sequence used to covalently bind the two subunits. *N*-Linked oligosaccharides on native hFSH are known to confer protein stability, and FSH analogues containing added *O*-linked carbohydrates attached exclusively to the β-subunit (without covalent binding to the common α-subunit) have prolonged half-lives compared with native hFSH (Bouloux *et al*., 2001). Furthermore, while both single-chain rhFSH-CTP and rhFSH-CTP lacking a covalent linkage between the subunits have never been compared, the prolongation in serum half-life relative to control rhFSH appears comparable (2–3-fold increase) (Bouloux *et al*., 2001; Klein *et al*., 2002). Nevertheless, the covalent bond between the two subunits of rhFSH-N2 may play a contributory role in conferring increased biopotency to our hormone analogue, independent of any alteration in carbohydrate load. By stabilizing subunit interaction through covalent linkage, deactivation of the intact heterodimer due to subunit dissociation may theoretically be inhibited. This theory has yet to be examined. Most single-chain glycoprotein hormone analogues have typically employed a carbohydrate-rich sequence as a tether between subunits, confounding the effect of covalent binding *per se* on bioactivity. A single-chain rhFSH construct devoid of any linker sequence was able to bind and activate the hFSH receptor *in vitro* (Sugahara *et al*., 1996). A pharmacokinetic comparison between this analogue and native hFSH would help to clarify the role of single-chain assembly on hormone metabolism. The role of the peptide backbone of the linker sequence must also be resolved. We plan to perform a pharmacokinetic and pharmacodynamic comparison employing a single-chain FSH analogue containing a comparable tether but devoid of *N*-linked consensus sequences to clarify this issue.

rhFSH-N2, with two additional *N*-oligosaccharides, displayed similar in-vivo characteristics (pharmacokinetics and pharmacodynamics) compared with rhFSH-CTP, which contains four *O*-linked carbohydrates in the CTP tether. This is not unexpected, as *N*-oligosaccharides tend to be more complex and highly branched than *O*-linked moieties. However, mass spectrometric analysis is required to characterize the carbohydrates of these modified FSH species, as size (particularly of *O*-linked oligosaccharides) can be highly variable. The effect of carbohydrate mass, volume and/or spatial constitution on half-life and bioactivity of FSH analogues remains speculative, pending the outcome of comparative analyses of species with well-characterized carbohydrate constituents.

Others have shown that carbohydrate variability within native preparations of FSH can have an effect on half-life. More acidic, sialic acid-rich isoforms have longer half-lives (Blum and Gupta, 1985). In-vivo bioactivity of standard recombinant preparations is correlated with a more acidic pI (Mulders *et al*.,1997). Increased sialic acid content of rhFSH-N2, as suggested by a lower pI on IEF gel electrophoresis may be a factor in its increased half-life and in-vivo bioactivity. A complex carbohydrate characterization and further studies using desialylated hormone may provide a more definitive answer.

In conclusion, we report a novel long-acting recombinant follitropin agonist containing an *N*-oligosaccharide-rich tether between the β- and α-subunits of FSH. Further studies are required to ascertain the clinical potential of this analogue in patients in whom exogenous gonadotrophin therapy is indicated. The immunogenicity of our analogue, and its effects on clinical parameters such as folliculogenesis, oocyte quality, embryo development, implantation and pregnancy, and spermatogenesis remain to be defined. The utility of this approach in the development of additional FSH and other recombinant protein analogues is promising, and merits further investigation.

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