Carbohydrate-Mediated Polyethylene Glycol Conjugation of TSH Improves Its Pharmacological Properties

Genzyme Corporation, a Sanofi Company, Framingham, Massachusetts 01701

Thyrogen (thyrotropin alfa for injection), recombinant human TSH (rhTSH), has been successfully used to enhance diagnostic radioiodine scanning and thyroglobulin testing in the follow-up of patients with thyroid cancer and as an adjunctive treatment for radioiodine thyroid remnant ablation. However, the short half-life of rhTSH in the circulation requires a multidose regimen. We developed novel sialic acid-mediated and galactose-mediated conjugation chemistries for targeting polyethylene glycol (PEG) to the three N-linked glycosylation sites on the protein, to prolong plasma half-life by eliminating kidney filtration and potential carbohydrate-mediated clearance. Conjugates of different PEG sizes and copy numbers were screened for reaction yield, TSH receptor binding, and murine pharmacokinetics/pharmacodynamics studies. The best performing of these products, a 40-kDa mono-PEGylated sialic acid-mediated conjugate, exhibited a 3.5-fold longer duration of action than rhTSH in rats, as a 5-fold lower affinity was more than compensated by a 23-fold extension of circulation half-life. Biochemical characterization confirmed conjugation through the sialic acids. Correlation of PEG distribution on the three N-linked glycosylation sites and the PEG effect on receptor binding supported the previously reported structure-function relationship of rhTSH glycosylation. This long-acting rhTSH has the potential to significantly improve patient convenience and provider flexibility while reducing potential side effects associated with a sudden elevation of serum TSH. (Endocrinology 154: 1373–1383, 2013)

Thyrorgen (recombinant human [rh] TSH, rhTSH) has been used clinically since 1998 for diagnostic monitoring in patients with well-differentiated thyroid cancer and more recently for thyroid remnant ablation (1). Two im injections of 0.9 mg Thyrorgen are given on day 1 and day 2, followed by radioiodine administration on day 3 and diagnostic scanning and thyroglobulin testing on day 5. This multidose regimen is necessitated by the relatively short plasma half-life (25 ± 10 h) of rhTSH (2). A higher single dose would lead to undesirable side effects resulting from a high ratio of peak-to-trough plasma exposure. Thus, a long acting TSH with sustained exposure over 1 wk after a single injection may offer a significant improvement over the current treatment for patient convenience and provider flexibility as well as potential improvements in treatment and diagnosis.

TSH is a member of the heterodimeric gonadotropin family (human chorionic gonadotropin [hCG], FSH, LH, TSH) and is composed of an α-subunit common to gonadotropins and a TSH-specific β-subunit. It is produced by thyrotrope cells in the anterior pituitary gland and stimulates the thyroid gland to secrete thyroid hormones, T4 and T3, which are essential for regulation of metabolism. Similar to the other gonadotropins, TSH is a glycosylated

Abbreviations: AUC, area under the curve; cv, column volume; GAD, galactose oxidase; GaINAc, N-acetylgalactosamine; GAM+, galactose-mediated conjugation with sialidase pretreatment of rhTSH; GAM−, galactose-mediated conjugation without sialidase pretreatment of rhTSH; hCG, human chorionic gonadotrophin; multi-SAM, SAM conjugate with multiple numbers of PEG; PD, pharmacodynamics; PEG, polyethylene glycol; PK, pharmacokinetics; rhTSH, recombinant human TSH; RP-HPLC, reversed-phase HPLC; SAM, sialic acid-mediated conjugation; TFA, trifluoroacetic acid; TSHR, TSH receptor.
protein, and the role of the N-linked oligosaccharides in the function of TSH is well established (3). The α-subunit of TSH has two N-glycosylation sites (Asn-52 and Asn-78) and the β-subunit has one site (Asn-23). In the pituitary form of human TSH, complex N-glycans are predominantly terminated with sulfated N-acetylgalactosamine (GalNAc) in addition to a small amount of sialic acid, due to the expression of both GalNAc-transferase and sulfotransferase in the pituitary thyrotrhops (4). However, rhTSH produced in Chinese hamster ovary cells is primarily sialylated and not sulfated because Chinese hamster ovary cells lack GalNAc-transferase and sulfotransferase (5, 6).

A number of studies have shown that more sialylation of rhTSH results in lower in vitro bioactivity but longer plasma half-life and higher in vivo bioactivity (7). The relationship between sialylation of rhTSH and in vitro half-life and higher in vivo bioactivity (7). The relationship between sialylation of rhTSH and in vitro activity has been further examined with hybrids of α- and β-subunits from the pituitary form of human TSH, rhTSH, and enzymatically desialylated rhTSH (8). Studying subunit-specific sialylation with these hybrids led to the conclusion that terminal residues of α-subunit oligosaccharides have a major impact on TSH intrinsic potency because desialylation of the α-subunit, but not the β-subunit, resulted in higher in vitro bioactivity. Another study with site-specific deglycosylation mutants reported that αN52Q/TSHβ showed a 6-fold increase in vitro bioactivity whereas αN78Q/TSHβ and α/TSHβN23Q showed an approximately 2- to 3-fold increase (9). Therefore, sialylated oligosaccharides in TSH, in particular at αAsn52, attenuate in vitro bioactivity, in contrast to the stimulatory role in HCG and FSH (9–11). Interestingly, for in vivo potency, sialylation of TSH has an opposite effect due to a longer plasma half-life presumably caused by reduced clearance through the hepatic mannose/GalNAc-sulfate receptor and/or asialoglycoprotein receptor (12, 13).

PEGylation is the covalent conjugation of chemically activated polyethylene glycol (PEG) molecules to a protein, peptide, small molecule, or oligonucleotide. PEGylation of therapeutic proteins has been shown to result in a number of benefits including increased circulating half-life and reductions in immunogenicity, aggregation, and proteolysis (14, 15). Although PEGylation frequently reduces in vitro activity due to a loss of target-binding affinity from steric hindrance, this effect is often more than compensated by an improved pharmacokinetic (PK) profile, which yields a net increase in in vivo efficacy. In recent years, PEGylation of biotherapeutics has emerged as an effective drug modification strategy to improve the therapeutic potentials through less frequent dosing, which results in greater patient compliance. Today, there are 10 Food and Drug Administration-approved PEGylated biotherapeutics. The most commonly used PEGylation chemistries have been conjugation to the ε-side chain amine of lysines using N-hydroxysuccinimide-activated PEGs (PegIntron, Pegaysys) or to the unprotected N-terminal amine using aldehyde PEGs (Neulasta). Alternatively, engineered cysteine residues have been targeted using thiol-reactive PEGs (Cimzia). The conjugation at oxidized carbohydrates with aldehyde-reactive hydrazide PEGs has been used (16), but not widely adopted. In one case, PEGylation of the ricin A-chain by this chemistry was shown to improve its pharmacokinetic profile (17). In our case, this approach is particularly attractive because of the beneficial in vitro effect of desialylation or deglycosylation of rhTSH.

In the current study, we have explored three different carbohydrate PEGylation strategies based on the structure-activity relationship of TSH glycosylation/sialylation and the oligosaccharide profiles. These include 1) conjugation to sialic acid residues after oxidation (sialic acid-mediated [SAM] chemistry), 2) conjugation to galactose residues after oxidation (galactose-mediated without neuraminidase treatment [GAM−] chemistry), and 3) conjugation to galactose residues after the removal of sialic acid and oxidation (galactose-mediated with neuraminidase treatment [GAM+] chemistry). Instead of hydrazide-PEGs, resulting in acid-labile hydrazone bonds, used by others (16, 17), aminooxy-PEGs were employed to form a more stable oxime linkage with the oxidized carbohydrate aldehydes. The effect of PEG size and mono- vs multi-PEGylation were also compared both in vitro and in vivo. The results of these studies are presented here.

Materials and Methods

Materials

Neuraminidase from Clostridium perfringens, biotinylated anti-rhTSH monoclonal detection antibody (clone TS8) and rhTSH were produced by Genzyme Corp (Framingham, Massachusetts). Galactose oxidase (GAO) was obtained from Worthington Biochemicals (Lakewood, New Jersey). Aminooxy PEG reagents were purchased from NOF America Corp. (White Plains, New York). The TSH receptor (TSHR) autoantibody second-generation ELISA kit from RSR Ltd (Pentwyn, Cardiff, Wales, UK) was purchased through Kronus, Inc. (Star, Idaho). Other reagents were purchased from Sigma (St Louis, Missouri) unless otherwise stated.

Sialic acid mediated (SAM) PEGylation of rhTSH

Sodium periodate was added to rhTSH in 100 mM NaOAc, pH 5.6, to a final concentration of 0.2–2 mM in a glass vial wrapped in aluminum foil. The vial was gently rocked on a shaker table in the dark for 30 minutes at 4°C. The reaction was then quenched by adding glycerol to 1.5% and shaking for 15
min before the buffer exchange to 100 mM NaOAc, pH 5.6, to remove excess sodium periodate and formaldehyde byproduct. Aminoxy-PEG was then added to varying PEG:TSH molar ratios. The reaction volume was adjusted to a final rhTSH concentration of 4 mg/ml and incubated either at 25°C or 8°C for 16 hours with gentle shaking. After incubation, a 50-fold molar excess of hydroxylamine was added and incubated at 25°C for 6 hours to block any remaining sugar aldehydes.

Galactose mediated (GAM) PEGylation of rhTSH and desialylated rhTSH

For desialylation, rhTSH was incubated with 20 mU Clostridium perfringens neuraminidase per mg rhTSH for 6 hours at 37°C. Desialylated rhTSH or rhTSH was treated with 4 μg of G A O and 2 U of catalase (18) per mg rhTSH at 37°C for 16 hours and buffer exchanged into 100 mM NaOAc, pH 5.6. The same procedure described under SAM PEGylation of rhTSH was followed for PEGylation.

Mono S-ÄKTA purification of PEGylated rhTSH

The PEGylated rhTSH reaction mixture was purified over a Mono S 10/100 GL column (GE Healthcare, Piscataway, New Jersey) using an ÄKTApurifier (GE Healthcare) at 280 nm and at a flow rate of 4 ml/min. The elution gradient increased from 0% buffer B (10 mM NaOAc, 1 M NaCl, pH 5.0) to 50% buffer B over 25 cv (column volume), followed by a column wash at 100% buffer B for 5 cv and equilibration at 100% buffer A (10 mM NaOAc, pH 5.0) for 5 cv.

Determination of the relative amount of PEGylation on each subunit

Subunit-specific PEGylation was calculated by measuring the relative amount of un-PEGylated α- and β-subunits using two consecutive reversed-phase HPLC runs. Samples were denatured in 6 M guanidine hydrochloride, 10 mM NaHPO₄, 100 mM NaCl, pH 7.0, at 25°C for 18 hours, loaded onto a Poroshell column as above using a linear gradient of 0.1% TFA in water, B: 0.08% TFA in CH₃CN over 15 min at 0.3 ml/min at 50°C. The relative percentage corresponding to un-PEGylated TSH subunits (~9.5–12.5 min) was collected and then reduced in 46 mM dithiothreitol, 4.4 M guanidine hydrochloride, 0.1 M Tris, pH 8.5, at 25°C overnight. Free thiols were alkylated by adding 87 mM iodoacetamide for 2 hours at 25°C before quenching it with 0.1% tris(2-carboxyethyl)phosphine (TCEP). Reduced and alkylated un-PEGylated TSH subunits were run on the same HPLC column as above using a linear gradient of 2%–75% B (A: 0.1% TFA in water, B: 0.08% TFA in CH₃CN) over 15 min at 0.3 ml/min at 50°C. The relative percentage of un-PEGylated α- vs β-subunits was determined by integration of the resulting A214 nm chromatograms from triple injections per sample. The relative percentage of PEGylated α- vs β-subunits was then taken as the inverse of these values.

In vitro TSHR binding assay

rhTSH was biotinylated using the ChromaLink Biotin Labeling Reagent according to the manufacturer’s protocol (SoluLink, San Diego, California) and buffer exchanged into 150 mM NaCl and 50 mM NaHPO₄, pH 7.0. In vitro measurement of receptor binding was performed by competition between biotinylated rhTSH and purified PEG-rhTSH conjugates for binding to porcine TSHR immobilized onto 96-well plates, which was supplied in the TSHR Autoantibody Second-Generation ELISA kit (R SR Ltd) PEG-rhTSH conjugates were serially diluted 1:5 from 16 μM to 41 pM in assay buffer (100 mM HEPES [pH 7.5], 20 mM EDTA, 1% BSA, 0.5% Triton X-100) and mixed 1:1 with 0.7 μg/ml biotinylated rhTSH in assay buffer. The mixture was added to each receptor-coated well and incubated at 25°C for 25 minutes before washing away unbound biotinylated or PEGylated rhTSH and determining the amount of biotinylated rhTSH bound to the plate according to the RSR Ltd ELISA protocol. The absorbance of each well was read at 450 nm using a SpectraMax 340pc plate reader (Molecular Devices, Sunnyvale, California). The data were fit using a sigmoidal dose response equation with GraphPad Prism software (GraphPad Software, Inc, San Diego, California) to generate IC₅₀ values.

Animal studies

Animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, NIH Publication No. 86-23) and were approved by Genzyme’s Animal Care and Use Committee. Animals were euthanized with CO₂ after the last sample collection. For the pharmacokinetic studies, PEGylated rhTSH conjugates were tested in two separate studies in comparison to rhTSH. A single dose (0.5 mg/kg) of rhTSH or PEGylated rhTSH conjugate was administered im to male and female jugular vein-cannulated Sprague Dawley rats. Blood was collected from the single port jugular cannula and processed for serum to analyze rhTSH or PEGylated rhTSH concentration by the ACE clinical chemistry T4 assay. The pharmacodynamics (PD) of 10-kDa SAM conjugate with multiple numbers of PEG (multi-SAM) and 40-kDa SAM conjugates were evaluated in male mice (ICR strain, 6 weeks of age; Taconic Farms, Inc., Germantown, New York) or in male and female Sprague Dawley rats. Animals were anesthetized with isoflurane before blood sample collection. For the retro-orbital plexus. For the rat PD study, a single dose (0.4 mg/kg) of rhTSH or PEGylated rhTSH was administered ip at 3 days after pellet implantation for the mouse pharmacodynamic (PD) study. Mice were anesthetized with isoflurane before blood sample collection from the retro-orbital plexus. For the rat PD study, a single dose (0.4 mg/kg) of rhTSH or PEGylated rhTSH was administered im to jugular vein-cannulated rats at 3 days after pellet implantation and blood samples were collected from the single-port jugular cannula. Blood samples were processed for serum to analyze T₄ concentrations by the ACE clinical chemistry T₄ assay (Alfa Wassermann Diagnostic Technologies, Caldwell, New Jersey).

ELISA measuring protein concentration in serum samples (PK assay)

ELISA plates were coated with murine anti-hCG capture antibody (clone 1–04, part no. 3AT022; Scantibodies, California). A standard rhTSH or PEGylated rhTSH curve was prepared from purified protein serially diluted in 1× plate wash (NE ENDI plate wash; PerkinElmer, Wellesley, Massachusetts) with 1.0% (wt/vol) BSA, according to the prequalified linear range of
each species. rhTSH controls were prepared in the dilution buffer at 0.5 ng/ml and in the 100- and 10-fold diluted normal rat serum at 5 and 2.5 ng/ml, respectively. Serum samples were diluted at no less than a 1:10 dilution. After incubation of samples, standards and controls for 1 hour at 37°C, plates were washed and incubated with the biotinylated anti-rhTSH monoclonal detection antibody (clone TSB) for 1 hour at 37°C, followed by successive incubation with Streptavidine-horseradish peroxidase (Pierce Chemical Co, Rockford, Illinois), tetramethylbenzidine substrate (KPL, Inc, Gaithersburg, Maryland), and tetramethylbenzidine stop buffer before the plate was read at 450 nm.

Results

Conjugation strategies

Applying conventional lysine PEGylation to rhTSH would lead to a highly heterogeneous product profile because there are 13 lysine residues in the sequence. Consequently, we targeted the three carbohydrate sites of rhTSH for PEGylation instead, aiming to generate a more homogeneous product profile. We investigated SAM, GAM−, and GAM+ conjugation strategies to fully explore the effect of PEG distribution at the three carbohydrate sites on receptor binding (Figure 1, A and B). Monosaccharide analysis of rhTSH indicated that glycan termini at each of the three N-glycosylation sites have a different degree of sialylation. The proportion of galactose residues “capped” with sialic acid at α-Asn-52, α-Asn-78, and β-Asn-23 is 60%, 69%, and 87%, respectively (6). These results suggest that the three conjugation strategies may result in different patterns of PEG distribution at these sites. A homology model of the TSH-TSHR complex (Figure 1C), based on the crystal structure of the FSH-FSHR complex (19), shows that the proximity of the three glycosylation sites to the receptor binding interface also varies. The two glycosylation sites on the α-subunit (green balls in Figure 1C) are located proximally to the receptor interface, whereas the β-Asn-23 site is on the opposite face of TSH away from the receptor. Of the two α-subunit sites, the α-Asn-52 site is closer to the interface than the α-Asn-78 site. Distances from the O4 atom of the core GlcNAc to the closest amino acids on the receptor measure 17 Å and 23 Å for the sugars at α-Asn-52 and α-Asn-78 sites, respectively. Therefore, the three conjugation strategies were predicted to influence the receptor binding of the PEGylated rhTSH conjugates to a different extent.

PEGylation and analytical characterization

The terminal sialic acids of rhTSH are oxidized with sodium periodate to generate aldehydes, which react with aminooxy-PEGs to produce SAM conjugates of TSH (Figure 1A). Linear 10-kDa, 20-kDa, or two-arm branched 40-kDa aminooxy-PEGs were compared to assess their rel-
ative effect on receptor binding and PK. Because attachment of a single high molecular weight PEG can achieve a substantial improvement in PK, we first determined the effect of the PEG:TSH molar ratio during conjugation to maximize the yield of mono-PEGylated species. Figure 2A shows a size exclusion chromatogram of the 40-kDa SAM reaction at the optimal PEG:TSH molar ratio (2:1), which was found to produce the highest yield (47%) of mono-PEGylated species. We also explored a strategy of attaching multiple copies of smaller PEGs to the protein because this, in principle, could result in a similar improvement in PK while increasing the yield of a bulk mixture of PEGylated products. By this approach using a 10-kDa linear aminoxy PEG (10-kDa multi-SAM conjugate), we found that a (4:1) ratio is the lowest PEG:TSH ratio that produced highly PEGylated products without leaving any unmodified rhTSH. A higher PEG:TSH ratio would not increase the yield any further but may result in greater loss of receptor binding.

The exposed terminal galactose residues of rhTSH either without (GAM−) or with (GAM+) neuraminidase treatment were oxidized with GAO and conjugated with linear 20-kDa or two-arm branched 40-kDa aminoxy-PEG to produce GAM conjugates of TSH. Temperature-controlled SDS-PAGE analysis of the reaction products using GAM− or GAM+ chemistry (Figure 2, B and C) demonstrated the difference of these two conjugation strategies. GAM− conjugation saturated at a low PEG:TSH molar ratio (2:1 to 5:1, Figure 2B), whereas GAM+ conjugation saturated only at high PEG:TSH molar ratios (>5:1, Figure 2C), consistent with the increase in terminal galactose available for conjugation after the neuraminidase treatment.

Reaction products were fractionated by cation-exchange chromatography using gradient elution to resolve the mono-PEGylated species from unmodified rhTSH, multipegylated side products, and unconjugated PEG. Selective pooling of eluate fractions yielded almost exclusively mono-PEGylated products, as shown by Coomassie-stained nonreducing SDS-PAGE (Figure 2D). Staining for PEG (20, 21) demonstrated that all of the products also had PEG contents consistent with the PEG number and molecular weights (Figure 2E). As expected, rhTSH did

Figure 2. Analytical Characterization of PEGylation Reaction and Purified PEG-rhTSH Conjugates. A, Analytical size-exclusion HPLC (SEC-HPLC) profile of 40-kDa SAM reaction. The reaction mixture was analyzed on a Superdex 200 10/300 GL column (13 μm, 10 × 300 mm; GE Healthcare) at 280 nm using Agilent 1100 HPLC system (Agilent Technologies) and eluted at an isocratic flow rate of 0.4 ml/min with 150 mM sodium chloride and 50 mM sodium phosphate, pH 7.0, for 60 min. Each peak was identified by fractionation and SDS-PAGE analysis. B and C, SDS-PAGE analysis of 20-kDa GAM− (B) and GAM+ (C) reaction on NuPAGE Novex 4%–12% Bis-Tris gel (Life Technologies, Gaithersburg, Maryland). D and E, Coomassie staining (D) and PEG staining (E) of nonreducing SDS-PAGE analysis of purified PEG-rhTSH conjugates. PEG staining was done according to a modified procedure of Kurfürst (20, 21) before Coomassie staining. kD, kilodalton.
not stain for PEG (Figure 2E). The intensity of PEG staining was more pronounced than Coomassie staining with higher molecular weight PEGs. As judged by size-exclusion chromatography, each of the mono-PEGylated conjugates comprised at least 96% of a single-sized product, with higher purities typically obtained with higher molecular weight PEGs (Supplemental Figure 1 published on The Endocrine Society’s Journals web site at http://endo.endojournals.org).

MultiPEGylation using SAM chemistry generated a mixture comprising a series of products (Figure 2, D and E) beginning with a conjugate containing a single 10-kDa PEG and extending upward to six copies of PEGs, as indicated by comparison to mono-PEGylated 10-kDa SAM conjugates and the mobility shifts by nonreducing SDS-PAGE. Conjugates with two and three copies of PEGs appear to be the most dominant species in this mixture, suggesting the estimated average number of PEGs in the 10-kDa multi-SAM conjugate to be approximately 2.5 mol per mol protein. Total purification yield was significantly improved to about 85% compared with approximately 15%–30% for most mono-PEGylated conjugates because no species had to be removed other than unconjugated PEG. Size-exclusion chromatography could not completely resolve the heterogeneous 10-kDa multi-SAM species (Supplemental Figure 1).

PEGylation sites

The distribution of PEGs on the α- and β-subunit of mono-PEGylated conjugates obtained by the three chemistries was examined by reducing SDS-PAGE (Figure 3A). Depletion of the unmodified subunit was used to determine its extent of coupling to PEG. Both 20-kDa and 40-kDa GAM– conjugates showed the highest ratio of PEGylation at the α-subunit (~80%) whereas no significant difference was observed between GAM+ and SAM conjugates. An equal probability of PEGylation at each of the three glycans would result in about 67% PEGylation at the α-subunit, approximately in concert with these values except for a somewhat higher ratio for the GAM– conjugates. Because of the technical limitations for quantitation by densitometry of Coomassie-stained gels, a two-step reversed-phase HPLC (RP-HPLC) method was developed to quantitate subunit-specific PEGylation. First, PEGylated TSH was separated into unmodified and PEGylated subunits by RP-HPLC after guanidine hydrochloride denaturation of the dimer (Figure 3B). RP-HPLC of the recovered earlier eluting peak under more stringent conditions separated α- and β-subunits into two clearly resolved doublets (Figure 3C). Integration of these peaks provided values for the relative proportion of each subunit in non-PEGylated form and a more precise value for the subunit-specific distribution of PEG. This analysis showed the fraction of α-subunit modified in the 40-kDa mono-PEGylated GAM–, GAM+, and SAM conjugates to be 77%, 66%, and 58%, respectively, in agreement with the results obtained by SDS-PAGE.

A method for determining the sites of PEGylation was developed and applied to the 40-kDa SAM conjugate. Trypsin digestion of reduced and alkylated 40-kDa SAM conjugate produced a reversed-phase peptide map similar to that obtained with unmodified rhTSH, except with the addition of a relatively broad peak around 24 min (Supplemental Figure 2). This peak was collected and subjected to N-terminal amino acid sequencing, which showed the presence of the three tryptic fragments containing the known glycosylation sites (AT6, AT9, and BT3) and absence of any other peptide, strongly suggesting that these are the exclusive sites of conjugation. Consistent with this finding, desialylation of rhTSH by neuraminidase treat-
ment before SAM PEGylation resulted in only 5% of PEGylated products by size-exclusion-HPLC, as expected for the specific attachment of PEG to sialic acids using this chemistry (data not shown).

In vitro TSHR binding

The purified conjugates were tested for binding to porcine TSHR in vitro by a competition assay using biotinylated rhTSH as a probe and immobilized receptor provided as a component of a commercial diagnostic ELISA kit for TSHR autoantibody testing. Binding of biotinylated rhTSH to the TSHR was competitively inhibited by unmodified rhTSH or PEGylated rhTSH conjugates. The amount of bound biotinylated rhTSH probe at equilibrium was determined by a colorimetric assay using streptavidin-horse radish peroxidase. The concentration to inhibit 50% of biotinylated rhTSH binding (IC\textsubscript{50}) was obtained by nonlinear regression analysis of the binding data. The results showed IC\textsubscript{50} values for all of the conjugates and unmodified rhTSH were within a 10-fold range (Figure 4 and Table 1), indicating that all of the conjugates retained the function of TSH. Of the same size PEG conjugates, GAM+ chemistry retained the highest affinity for the receptor (Table 1), possibly due to the enhancement of receptor binding upon desialylation (22–24). Although, in general, conjugates with multiple number of PEGs (eg, 10-kDa multi-SAM conjugate) or higher molecular weight PEGs consistently resulted in greater loss of affinity com-

![Figure 4](image-url)  
Figure 4. In Vitro Porcine TSHR Binding Assay. Binding of biotinylated rhTSH to porcine TSHR was inhibited by different concentrations of either rhTSH control or PEGylated rhTSH conjugates, and percent inhibition was plotted to calculate IC\textsubscript{50}. The curves shown represent the average of three independent experiments. A, Comparison of SAM, GAM+, and GAM− with the same size PEG (40 kDa). B, Comparison of SAM conjugate with different size PEG (10, 20, and 40 kDa). kD, kilodalton.

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Data from three independent experiments were used to calculate IC\textsubscript{50} with GraphPad Prism software.

![Figure 5](image-url)  
Figure 5. Pharmacokinetic Study Results of rhTSH Control and PEGylated rhTSH Conjugates. A, rhTSH, 20-kDa SAM, 20-kDa GAM−, 20-kDa GAM+, and 40-kDa GAM+ conjugates. B, rhTSH, 10-kDa SAM, 10-kDa multi-SAM, 40-kDa SAM, and 40-kDa GAM− conjugates. Jugular vein-cannulated rats (n = 6 per group) were administered with 0.5 mg/kg of rhTSH or PEGylated rhTSH conjugate by im injection. The protein concentration was determined using TSH ELISA with the blood samples collected at 0, 0.5, 1, 2, 4, 8, 24, and 48 hours for panel A and 0, 0.5, 1, 3, 6, 24, 48, 72, and 96 hours for panel B. Calculated parameters are shown in Table 2. kD, kilodalton.
pared with those with a single PEG (10-kDa SAM conjugate) or lower molecular weight PEGs, the difference in IC\textsubscript{50} was moderate and within less than 3-fold.

**Pharmacokinetics (PK) of PEGylated rhTSH conjugates in the rat**

Pharmacokinetics of purified mono-PEGylated conjugates and 10-kDa multi-SAM conjugate were determined after im injection at 0.5 mg/kg to Sprague Dawley rats. Data from the two PK studies are shown in Figure 5 and summarized in Table 2. Plasma half-life increased proportionally relative to the size of PEG conjugated to rhTSH, consistent with data on other PEGylated proteins (25). There was no detectable difference between SAM and GAM− conjugates for the same size PEG, suggesting that the effect on plasma half-life is only PEG size dependent and not PEGylation site dependent. For example, both the 40-kDa SAM and 40-kDa GAM− conjugates showed a 23-fold increase in plasma half-life compared with rhTSH. The overall amount of PEG conjugated to TSH had a strong impact on plasma half-life. For example, the 10-kDa multi-SAM conjugate had a 14-fold increase in half-life, whereas the 10-kDa SAM conjugate had only a 5-fold prolongation in half-life compared with rhTSH control. The half-life of the 10-kDa multi-SAM conjugate was only 1.6-fold lower than the 40-kDa SAM or 40-kDa GAM− conjugate, roughly in keeping with the average of 2.5 copies of 10-kDa PEGs coupled to each TSH for the 10-kDa conjugate, suggesting that the effect on plasma half-life is only PEG size dependent and not PEGylation site dependent. For example, both the 40-kDa SAM and 40-kDa GAM− conjugates were also 2- to 9-fold higher than that of the rhTSH control, presumably due to PEG-mediated reduction of proteolysis and kidney filtration (14). The enhanced half-life coupled with higher peak concentration results in dramatically greater overall exposure area under the curve ([AUC]) for the SAM and GAM− conjugates. The clearance rates of the GAM+ conjugates were higher than that of the SAM and GAM− conjugates. Moreover, a significantly larger volume of distribution (Vz) was observed for the GAM+ conjugates compared with that of the SAM or GAM− conjugates, suggesting increased distribution of the GAM+ conjugates into tissues possibly through asialoglycoprotein receptor-mediated uptake.

**Pharmacodynamics of PEGylated rhTSH conjugates in the mouse and rat**

The 40-kDa SAM and 10-kDa multi-SAM conjugates were chosen for evaluation in a mouse PD model based on superior receptor binding and rat PK data. In this mouse PD model, endogenous T\textsubscript{4} was suppressed during the study treatment period by implantation of a slow-release T\textsubscript{3} pellet 3 days before dosing (Figure 6A, vehicle group) so that only the amount of T\textsubscript{4} release mediated by exogenous administration of rhTSH control or PEGylated rhTSH conjugates was measured. Although a high level of T\textsubscript{4} induction was initially observed with the rhTSH control, circulating T\textsubscript{4} levels were undetectable over baseline at 48 hours postdose. In stark contrast, robust effects of both conjugates were sustained even at 72 hours postdose, and the T\textsubscript{4} levels were comparable in all of the animals within each group (Figure 6A). These two conjugates could not be differentiated, however, because differences between the two were not apparent out to the 72-hour postdose period. An animal model with a longer window

| Table 2. Pharmacokinetic Parameters of PEGylated rhTSH Conjugates From the Rat PK Study |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                               | rhTSH            | 20-kDa SAM      | 20-kDa GAM−     | 20-kDa GAM+     | 40-kDa GAM+     |
| t\textsubscript{1/2} (hr)                      | 5.7 ± 2.3        | 30 ± 6.5        | 27 ± 3.9        | 12.6 ± 3.5      | 39 ± 20         |
| Cl (ml/h/kg)                                  | 131 ± 27.7       | 4.7 ± 0.5       | 3.3 ± 0.4       | 156 ± 26        | 12 ± 1.9        |
| Vz (ml/kg)                                    | 1051 ± 386       | 203 ± 39        | 128 ± 23        | 2818 ± 843      | 650 ± 233       |
| C\textsubscript{max} (µg/ml)                   | 0.4 ± 0.1        | 2.1 ± 0.6       | 3.9 ± 0.8       | 0.2 ± 0.1       | 0.8 ± 0.2       |
| T\textsubscript{max} (hr)                     | 2.0 ± 0.0        | 16 ± 8.8        | 5.3 ± 3.0       | 1.5 ± 0.6       | 7.0 ± 8.7       |
| AUC\textsubscript{inf} (µg • h/kg)             | 3.9 ± 0.7        | 107 ± 11        | 153 ± 21        | 3.3 ± 0.7       | 42 ± 6          |

|                                               | rhTSH            | 10-kDa SAM      | 10-kDa Multi-SAM | 40-kDa SAM      | 40-kDa GAM−     |
| t\textsubscript{1/2} (hr)                      | 3.4 ± 0.6        | 16 ± 1.2        | 47 ± 10          | 78 ± 17         | 80 ± 16         |
| Cl (ml/h/kg)                                  | 167 ± 31         | 16 ± 1.4        | 2.4 ± 0.8        | 1.0 ± 0.2       | 1.3 ± 0.2       |
| Vz (ml/kg)                                    | 810 ± 129        | 358 ± 19        | 159 ± 60         | 114 ± 9         | 146 ± 25        |
| C\textsubscript{max} (µg/ml)                   | 0.4 ± 0.1        | 1.0 ± 0.1       | 3.1 ± 1.0        | 3.8 ± 0.3       | 3.2 ± 0.6       |
| T\textsubscript{max} (hr)                     | 1.5 ± 1.2        | 5.4 ± 1.3       | 15 ± 18          | 30 ± 12         | 28 ± 10         |
| AUC\textsubscript{inf} (µg • h/kg)             | 3.1 ± 0.5        | 32 ± 3          | 228 ± 59         | 492 ± 83        | 397 ± 47        |

AUC\textsubscript{inf}, area under the curve from time zero to infinity.
for evaluation might be useful to differentiate effects of long-acting PEGylated conjugates.

A rat PD model was developed to monitor a longer postdose period. Similar to the mouse model, we implanted slow-release T₃ pellets 3 days before dosing to suppress the endogenous T₄ during the study period. Experiments to optimize this model identified a 1.5-mg pellet as the ideal T₃ dose to achieve sustained suppression of T₄ levels. T₄ levels in the vehicle group were suppressed for up to 13 days after pellet implantation (data not shown). The 40-kDa SAM and 10-kDa multi-SAM PEG conjugates were compared with the rhTSH control in the rat model throughout a 168-hour evaluation window (Figure 6B). The vehicle group showed successful endogenous T₄ suppression throughout the study period up to 168 hour postdose. As in the mouse, administration of the PEG conjugates produced elevated T₄ levels well after the decline in the response to rhTSH. Although we could not differentiate between the two PEGylated conjugates in the mouse PD model, cumulative effects of 40-kDa SAM conjugate on T₄ levels were substantially higher relative to 10-kDa multi-SAM conjugate in the rat PD model as observed by nearly 2-fold higher average area under the effect curve (AUEC, Figure 6B) of 429 vs 245 μg·h/dL, respectively. Whereas T₄ levels with the 10-kDa multi-SAM conjugate were near baseline by 96 hours, significantly elevated T₄ levels were still observed with the 40-kDa SAM conjugate even after 168 hours postdose. Therefore, the duration of time with T₄ release above the vehicle level was 3.5-fold longer for the 40-kDa SAM conjugate compared to the rhTSH control (168 hr vs 48 hr), and approximately 2-fold longer for the 10-kDa multi-SAM conjugate (96 hr vs 48 hr).

Discussion

In this study, we have successfully conjugated PEGs specifically to the three carbohydrate sites on rhTSH. The product profile of these novel conjugates would be simpler to characterize than that of traditional amine-based conjugates because TSH has 13 lysines and two N-terminal amines. Furthermore, at least half of the lysines are located on the same side as the receptor interface, whereas all three carbohydrate sites are relatively distant from the receptor interface (26), allowing the conjugation of high molecular weight PEGs without completely eliminating receptor binding. The carbohydrate chemistries we developed would also be easier to control compared with the more recently developed site-specific conjugation through the introduction of a single cysteine mutation. The selective uncapping of the introduced cysteine required for efficient cysteine conjugation (27, 28) would be particularly challenging given that...
TSH is a cysteine knot protein. Finally, our novel carbohydrate PEGylation chemistry relying on the more stable oxime linkage (29, 30) is a significant improvement over the previously reported conjugates generated by hydrazide-PEGs (16, 17).

Biochemical characterization of the PEG conjugates generally supports previously reported structure-function analysis of rhTSH. Higher distribution of the PEG to the α-subunit of rhTSH for the GAM – conjugates compared with the SAM conjugates is consistent with lower sialylation of the α-subunit (6). Lower receptor affinity observed for the GAM – conjugates than the SAM conjugates (Table 1) supports our structure model showing that the α-subunit is closer to the receptor than the β-subunit (Figure 1C) so that a bulky PEG moiety stemming from the α-subunit can more easily interfere with receptor binding, consistent with the observation that removal of sialic acid or the entire N-glycans from the α-subunit glycosylation sites increases in vitro bioactivity more than similar modifications on the β-subunit (8, 9). The GAM + conjugates displayed higher receptor affinity than the SAM and GAM – conjugates presumably due to desialylation of rhTSH before PEGylation, which have been reported to improve receptor binding (9, 22, 23).

The effect of PEG on rhTSH is consistent with what has been observed for other proteins (14). The amount of PEG conjugated to rhTSH correlates negatively with in vitro receptor binding (Table 1), but this is more than compensated by the positive correlation with in vivo half-life extension (Table 2), resulting in a dramatic improvement in PD. The overall increase in the hydrodynamic radius as a result of conjugating rhTSH to high molecular weight PEGs would eliminate kidney filtration and limit the volume of distribution in vivo. Moreover, conjugating to the carbohydrate sites on rhTSH would reduce potential carbohydrate-mediated hepatic elimination (12). The combined effect of slower clearance and higher volume of distribution led to a massive increase in overall exposure due to hepatic uptake of the hydroxylamine-modified galactose aldehyde generated at a higher level in the GAM + than the GAM – reaction.

In summary, we have developed novel carbohydrate-based PEGylation chemistries and successfully applied them to prolong the in vivo effect of an important therapeutic protein. We have identified the long-acting 40-kDa SAM conjugate of rhTSH through iterative screening of multiple conjugation strategies and PEG sizes and structures by receptor binding, PK, and PD studies in rodents. The 3.5-fold increase in the duration of action in rats observed for our lead candidate may significantly improve patient convenience, compliance, provider flexibility, and pharmacoeconomics if it is translated into the clinic. Furthermore, the lower peak-to-trough T₄ effect induced by PEGylated rhTSH (Figure 6B) will likely minimize any side effect associated with a sudden increase in circulating TSH. Finally, the overall much higher exposure observed for the PEGylated rhTSH can potentially increase therapeutic potency due to enhanced radiiodine uptake although this needs to be tested rigorously through clinical trials.

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Address all correspondence and requests for reprints to: Anna Park, Genzyme Corp., a Sanofi company, 1 The Mountain Road, Framingham, Massachusetts 01701. E-mail: anna.park@genzyme.com.

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References

7. Szkudlinski MW, Thotakura NR, Bucci I, et al. Purification and characterization of recombinant human thyrotropin (TSH) isoforms produced by Chinese hamster ovary cells: the role of sialylation and