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Preparation and Characterization of Albumin Conjugates of a Truncated Peptide YY Analogue for Half-Life Extension

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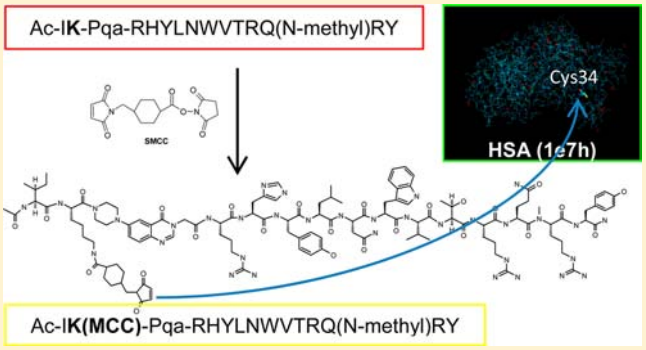
ABSTRACT: Recombinant human serum albumin (HSA) conjugates of a 15-amino-acid truncated peptide YY (PYY) analogue were prepared using three heterobifunctional linkers [succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC), 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (MHS), and *N*-[γ-maleimidobutyryloxy]-sulfosuccinimide ester (GMBS)] in 2 synthetic steps involving (1) reaction of succinimidyl ester on linker with ε-amine of Lys2 on the peptide and (2) reaction of maleimide on peptide linker with free thiol of Cysteine 34 (Cys34) on albumin. In-process controls using ESI LC-MS were used to follow reactions and identify reaction products. Proteolytic digests of the conjugate revealed that peptide conjugation occurs at Cys34 on HSA. Conjugates were assayed in cell-based assays to determine potency at the human Y2-receptor, and selectivity at the human Y1-, Y4-, and Y5-receptors using a calcium flux assay. All three conjugates assayed were selective agonists of the Y2-receptor, and displayed nanomolar potencies. MCC and MH conjugates were selected for acute PK/PD studies in DIO mice. Significant reduction in food intake was observed with the MH conjugate, which lasted for 24 h at the 10 mg (or 4 μmol)/kg dose. While the MCC conjugate exhibited greater potency *in vitro*, it was slightly less effective than the MH conjugate *in vivo* with respect to reduction in food intake. Both conjugates were significantly less active than the peptide coupled to a 30 kDa PEG. The observed $T_{1/2}$ (8−9 h) for both conjugates was significantly lower than that observed for the PEGylated peptide (~25 h). These results suggest that, as compared with the unmodified and PEGylated peptide, the extended circulation half-life of albumin conjugates is mediated through uptake and recirculation by FcRn, and allometric scaling methods are necessary to account for interspecies variation in pharmacokinetic properties.



INTRODUCTION

Peptide YY (PYY) is a 36-amino acid peptide released by the L-cells of the gastrointestinal tract following food intake. This peptide is rapidly processed by the enzyme DPP4 to Peptide Tyrosine Tyrosine 3−36 (PYY3−36). PYY3−36 displays more selectivity toward the neuropeptide Y2-receptor (Y2R) as compared with PYY, which activates Y1R, Y2R, and Y5R. Y2R localized in the hypothalamus and peripheral nervous system (e.g., in the gut) has been implicated in regulating food intake and gastric emptying. A robust reduction in food intake following PYY3−36 administration has been unequivocally demonstrated in rodents and humans. For this reason, Y2R is considered a validated target for the treatment of obesity and potentially type 2 diabetes. The pharmaceutical application of PYY3−36 as an anti-obesity drug is significantly limited due to (1) short half-life in circulation due to poor protease stability and rapid clearance[1−3] and (2) lack of selectivity for the Y2-receptor.[4]

A 30 kDa PEG conjugate of PYY3−36 designed for half-life extension was administered subcutaneously to diet-induced obese (DIO) mice, and was observed to have a profound effect on reducing food intake.[5] In addition, this PEG-conjugate robustly lowered glucose levels in diabetic *db/db* mice.[5]

A truncated PYY3−36 peptide agonist containing 15 amino acids was found to be highly selective for Y2R with subnanomolar potency (Figure 1A and B);[6,7] however, the half-life in DIO mice was only 0.1 h (unpublished data). A 30 kDa PEG-modified conjugate of this potent Y2R-selective peptide was observed to behave like PEG-PYY3−36 on food intake in treated DIO mice. Another 12 amino acid truncated 20 kDa PEG-PYY agonist reduced food intake by as much as 42% within 4 h and lasted up to 48 h (24%) on a single

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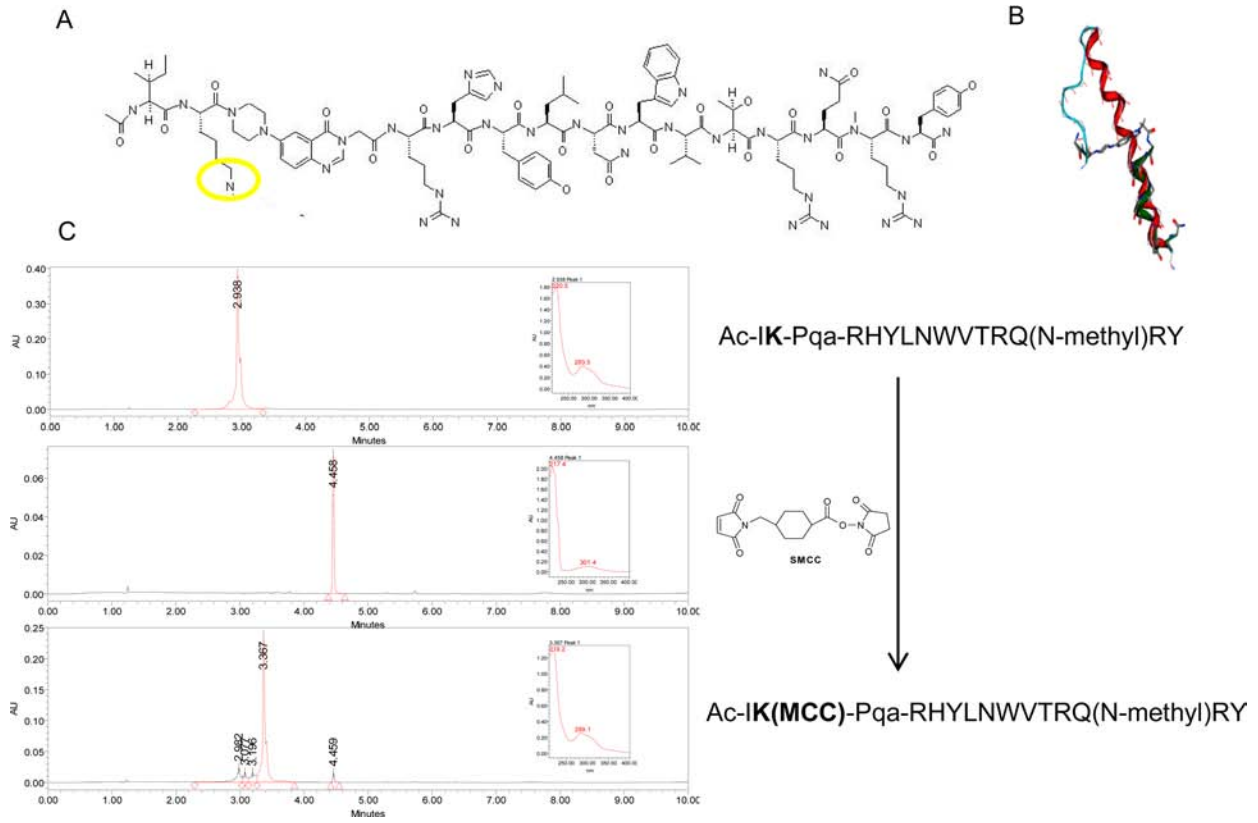

Figure 1. A. Structure of Y2R peptide with modification site circled in yellow. B. Overlay of truncated Y2R peptide and PYY3−36. C. Schematic of peptide linker synthesis using SMCC. RP-UPLC chromatograms illustrate the shift in retention times and wavelength (inset) observed in peptide-linker synthesis. Retention time (λ_{max}) of (A) Y2R std (0.2 mg/mL) = 2.94 min (283.5 nm), (B) SMCC linker (0.5 mg/mL) = 4.46 min (301.4 nm), and (C) Y2R-SMCC in reaction mixture containing 1:2 molar ratio of peptide to linker (and/or following SEC) = 3.37 min (289.1 nm).

subcutaneous injection of 5.6 μmol/kg in C57BL/6 mice.[8] However, some PEG-conjugates at higher doses have been reported to produce renal lesions,[9] and the safety of newer "biodegradable" polymers, like HES, for half-life extension remains unknown.[10] Thus, there is a desire to replace PEG with a suitable biocompatible polymer for pharmaceutical application of selective Y2R-peptides.

HSA is an example of a 66.4 kDa molecule that has a circulation half-life of 21 days in humans.[11] In addition to size, HSA, like IgG, has the added feature of FcRn receptor binding which is involved in recirculation.[12,13] At the molecular level, HSA has a free cysteine (Cys34), which makes it an ideal molecule for site-specific conjugation.

The circulation half-life of albumin in mouse is ∼24 h and increases accordingly in higher species on a weight basis.[14] As such, interspecies variation of albumin circulation allows weight-based allometric scaling to predict the pharmacokinetic properties of serum albumin conjugates.[14] However, this scaling does not account for variation in affinity of human albumin for the mouse FcRn-receptor, which unlike size-based PEG scaling, is crucial for albumin recycling and recirculation.

In this study, three HSA−Y2R conjugates were prepared and characterized for conjugate purity, conjugation site specificity, *in vitro* potency, and receptor selectivity. Two of these well-characterized conjugates were examined for food intake reduction in DIO mice, to establish proof of concept for further studies.

EXPERIMENTAL PROCEDURES

Materials. Y2R peptide (MW = 2259 g/mol, 1:4 TFA salt = 2715 g/mol) and 30 kDa mPEG-Y2R were synthesized in-house.[6,7] Recombinant HSA, [Recombumin (∼60% free thiol) and Albucult (∼90% free thiol)] were purchased from Novozymes (UK). Heterobifunctional linkers SMCC (MW = 334.3 g/mol) and Sulfo-GMBS (382.3 g/mol) and polyacrylamide desalting columns were purchased from Thermo-Fisher. MHS (MW = 308.3 g/mol) was purchased from Sigma-Aldrich (St. Louis, MO). Male C57Bl/6J mice were purchased from Janvier (Saint Berthevin, France). Anti-Y2R mAb was provided by Roche Diagnostics (Penzberg, Germany). Anti-HSA-HRP mAb was purchased from Thermo-Scientific (Pittsburgh, PA).

Preparation of HSA−Y2R Conjugates. *Conjugation of Heterobifunctional Linkers to Y2R Peptide.* Y2R peptide was dissolved in PBS, pH 7.2, containing 2 mM EDTA (PBS/EDTA) at 5−10 mg/mL or dimethylformamide (DMF) at 20 mg/mL. Heterobifunctional linkers were dissolved in DMF or PBS/EDTA at 50 mg/mL and added to Y2R peptide solutions to achieve a 1:0.5−2 peptide:linker molar ratio. Some reactions performed in DMF were initiated using a 1.1 mol equiv of triethylamine. Reaction mixtures were stirred at room temperature for 1−2 h. Reaction products were analyzed by RP-UPLC (see Analytical Methods below). Peptide-linkers were purified by size exclusion chromatography.

Isolation of Peptide-Linker by Size Exclusion Chromatography. Peptide-linker product was separated from *N*-hydroxysuccinimide and hydrolyzed linker by size exclusion

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chromatography on a Sephadex peptide (GE Healthcare, Uppsala, Sweden) or polyacrylamide desalting column (1800 Da MW cutoff) equilibrated with PBS/EDTA buffer, pH 6.8, or 20 mM sodium acetate buffer (NaOAc), pH 4.5. Fractions were analyzed by RP-UPLC and LC ESI-MS. Maleimide-linked Y2R peptide fractions were collected and subsequently used for HSA conjugation.

Conjugation of Maleimide-Linked Y2R Peptides to HSA. Peptide linkers were reacted with equimolar amounts of HSA overnight at 4 °C or at room temperature for 2 h in PBS/EDTA, pH 6.8 or 7.2. Reaction product was monitored by LC-ESI-MS. HSA−Y2R conjugates were dialyzed and/or concentrated by diafiltration (10 kDa MW cutoff membrane, Millipore, Billerca, MA) in PBS, pH 6.8, and stored at 4 °C.

Analytical Methods. *Reversed Phase Chromatography.* Reversed phase chromatography was performed on a Waters Acquity Ultraperformance liquid chromatography system using a 2.1 × 100 mm, 1.7 μm Acquity BEH-C8 column (Waters) monitored at 280 nm on a photodiode array (PDA) detector. Separations were performed at ambient temperature using a 5−75% linear gradient in mobile phase B (0.05% TFA in acetonitrile (v/v)) at a flow rate of 0.25 mL/min for 5 min. Elution at 75% mobile phase B [25% mobile phase A (0.05% TFA in deionized water (v/v) was held for 2.5 min before returning to initial conditions by 10 min.

Molecular Weight Determination of HSA and HSA-Conjugates. Molecular weight measurements were performed on an 1100 Series LC/MSD single quadrupole instrument (Agilent) using a reversed phase column (Ascentis Express C8, 3 × 150 mm) heated to 60 °C with UV detection at 214 and 280 nm. Samples were eluted with a 28 min linear gradient of 0% to 60% in mobile phase B (0.1% formic acid in acetonitrile (w/w)) at 0.6 mL/minute. Mobile phase A was 0.1% formic acid in water (w/w). ESI mass spectra were recorded on the single quadrupole instrument and the data was analyzed using the manufacturer's software.

Enzymatic Digestion of HSA and HSA-Conjugates. Reductions, carboxyamidomethylations, and digestions were done as previously described using Endoproteinase Lys-C (Wako Biochemicals, Richmond, VA) instead of trypsin.[15] Samples were digested at 37 °C for 20 h at an enzyme to substrate ratio (w/w) of 1 to 100.

LC ESI Mass Spectrometry. Liquid chromatography was performed using an Acquity UPLC system (Waters) equipped with a reverse phase column (Acquity UPLC BEH C18 1.7 μm, 2.1 × 150 mm) in mobile phase A (0.05% TFA in water (v/v) and mobile phase B (0.045% TFA in acetonitrile (v/v)) monitored at 214 nm. A solvent gradient was used for elution of the proteolytic peptides at a flow rate of 0.25 mL/minute. Mobile phase B was kept constant at 4% for 5 min after sample injection. The actual separation was done with a gradient of 4% to 55% B over 70 min. ESI-MS detection was done with an LTQ Orbitrap system (Thermo) and data was analyzed using the manufacturer's software.

Calcium Flux Assay. HEK-293 cells stably transfected with the G protein chimera Gαqi9 and the hygromycin-B resistance gene were further transfected with the various human NPY receptors (NPY1-, NPY2-, NPY4-, and NPY5-receptors) and placed under G418 antibiotic selection. The transfected cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 50 μg/mL hygromycin-B, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 μg/mL G418. Cells were harvested with trypsin-EDTA and dispensed into 384-well poly(D-lysine) coated black/clear microplates (Falcon) and the microplates were placed in a CO_2 incubator overnight at 37 °C.

Aliquots (25 μL) of dye (Calcium-3 Assay Kit, Molecular Devices) were dispensed into the cell plates, and the plates were incubated for 1 h at 37 °C. After incubation, 20 μL of compounds were transferred to the plates and fluorescence readings were taken simultaneously from all 384 wells of the plate every 1.5 s. Five readings were taken to establish a stable baseline, and then 20 μL of sample was rapidly (30 μL/s) and simultaneously added to each well of the plate. The fluorescence was continuously monitored before, during, and after sample addition for a total elapsed time of 100 s. Responses (increase in peak fluorescence) in each well following addition were determined. The initial fluorescence reading from each well, prior to ligand stimulation, was used as a zero baseline value for the data from that well. The responses are expressed as a percentage of maximal response of the positive control.

Formulation of Conjugates for *In Vivo* Study. PEG and HSA vehicles were prepared at PEG and HSA concentration equivalent to those estimated in the conjugates such that [vehicle] = $MW_{conjugate}/MW_{peptide}$ × [conjugate]. All conjugates and vehicles were prepared at the desired Y2R-based concentration of 2.5 mg/mL in isotonic buffer (PEG-Y2R in NaOAc, pH 5.5 and HSA-Y2R in PBS, pH 6.8), filtered through a 0.2 μm sterile PVDF filter, and stored at 4 °C until testing.

Albumin-Conjugate Experiment in DIO Mice. Male C57Bl/6J mice were given access to high fat SSNIFF diet (EF M D12492 with 60% energy from fat and 21% from sugar) from 9 weeks of age to induce obesity. Approximately 8-month-old nonfasted diet-induced obese (DIO) mice were sorted by body weight, and housed 1 per cage in standard caging at 22 °C in a 12-h light/12-h dark cycle. Mice were acclimated at least 6 days to reversed light−dark cycle before use. Food (HFD) and water were provided *ad libitum* throughout the study.

Vehicle and various drug treatments were administered as single subcutaneous (SC) injections (dosing volume 4 mL/kg) to 24 h fasted DIO mice (~54 g; N = 8 mice/treatment arm). Food (HFD) was returned to the cage and food intake was measured at 1 h, 2 h, 6 h, 24 h, 48 h, 72 h, and 96 h time points post-dosing. Data was analyzed by using ANOVA followed by Tukey's posthoc test. An additional four mice per albumin conjugate were included for the PK arms of the study. Blood sampling was performed in a composite manner, with samples taken from two animals each at 1 h, 6 h, 24 h, 48 h, 72 h and 96 h after dosing. PK parameter were estimated by non-compartmental analysis using WinNonlin.

ELISA of PK Serum Samples. Serum concentrations of conjugates were determined by sandwich ELISA using anti-HSA mAb (for HSA−Y2R) and anti-Y2R mAb. Briefly, the capture molecule, anti-Y2R monoclonal Ab, was added to a microtiter plate (Nalge Nunc, NY) and incubated for one hour at room temperature. After a washing step, standards, controls, and samples were diluted 10-fold with assay buffer, and were added to the plate. Following a one hour incubation period, anti-HSA−HRP conjugate was added to the plate for detection. TMB peroxide substrate was added forming a colored reaction product. The reaction was stopped with 2 N HCl and absorbance was read at 450 nm with a reference at 650 nm. All steps were consecutive and followed by washing. The color intensity was proportional to the analyte concentration and the

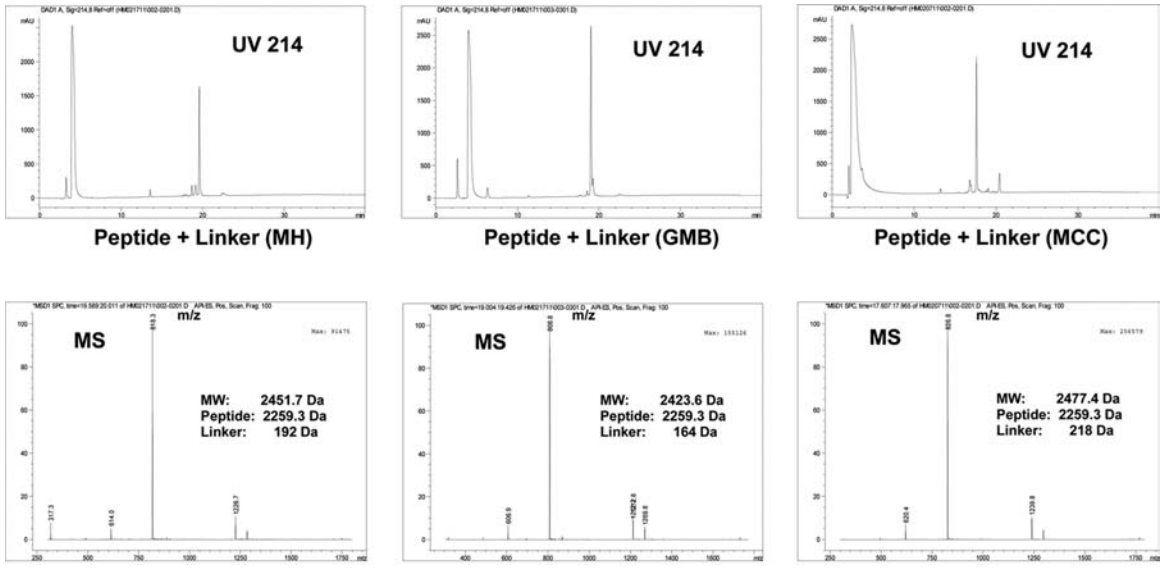

Figure 2. UV traces at 214 nm wavelength (above) and LC ESI-MS (below) of reaction mixtures containing linker-Y2R. MW_{linker} was determined by difference analysis ($MW_{linker\text{-}peptide} - MW_{peptide}$) and used to confirm the identity of the desired linker peptide product.

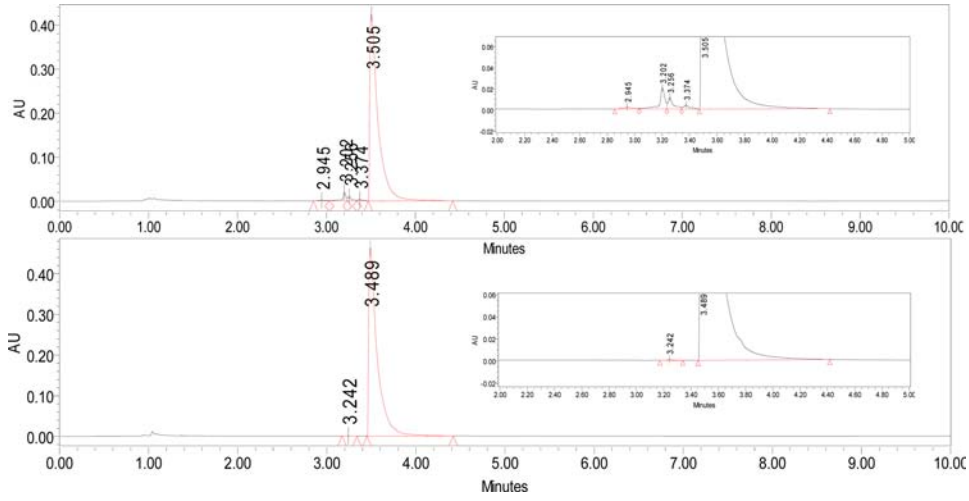

Figure 3. RP-HPLC chromatogram of HSA-MCC-Y2R reaction mixture before (above) and after (below) dialysis. Insets are magnifications of peptide related impurity peaks in the chromatograms. % Area of conjugate containing total impurity peaks before and after dialysis is 3.6 and 0.07, respectively.

serum concentrations of the albumin−Y2R conjugates were extrapolated from the standard curve.

RESULTS

Preparation and Analytical Characterization of HSA−Y2R Conjugates. Three heterobifunctional linkers (GMBS, SMCC, and MHS) were used to prepare maleimide-activated Y2R peptides through an amide bond with the ε-amine of lysine on the peptide. Synthesis of Y2R-linker was monitored by RP-UPLC. In one example, conversions of 44% and 83% were achieved using SMCC at 1:0.5 and 1:2 peptide:SMCC molar ratios. A UV shift from 283 nm in the peptide reactant to 289 nm in the peptide linker product was also observed when monitoring the reaction mixture with a photodiode array detector (Figure 1C). LC ESI-MS was used to confirm the identity of all three Y2R-linker peptides in the reaction mixtures (Figure 2). The determined molecular weights of all three Y2R-linker peptides were consistent with their respective theoretical values.

Y2R-linker peptides were separated from unreacted or hydrolyzed linker and NHS by size exclusion chromatography in PBS/EDTA buffer, pH 6.8. Fractions containing Y2R-linker peptide were then reacted with HSA in equimolar amounts. Reaction mixtures were dialyzed to remove unreacted peptide (Figure 3). In process testing was performed to identify desired albumin conjugate by LC ESI-MS. (HSA-MCC-Y2R) as illustrated in Figure 4.

Molecular Weight and Purity Determination of HSA and HSA-Conjugates. LC ESI-MS was used as needed for in-process control of conjugation chemistry and for the quality assessment (identity and purity) of the chemically synthesized HSA-conjugates. A representative comparison between the unmodified HSA and the HSA−peptide conjugate is illustrated in Figure 4. The measured molecular weights were in excellent agreement with the theoretical ones (Table 1). A noticeable increase in the average molecular weight of unmodified HSA indicated an increase in the formation of oxidized HSA species which occurred during the conjugation reaction. The highest

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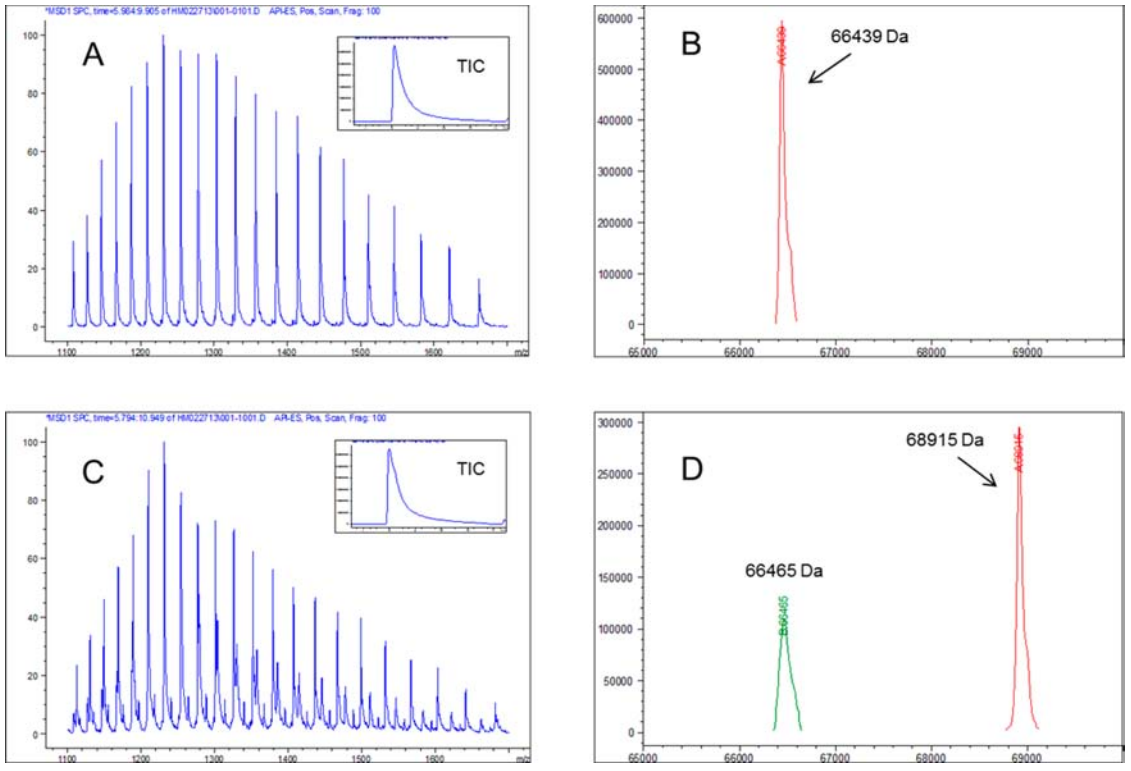

Figure 4. LC ESI-MS of HSA and HSA-MCC-Y2R conjugate. Reconstructed and deconvoluted mass spectra of unmodified HSA (A,B) and HSA-MCC-Y2R conjugate (C,D). Total ion currents (TIC) are shown as insets. The observed molecular weight difference of 26 Da does not represent one distinct modified form of HSA. The measured molecular weight of 66 465 Da most likely includes the unmodified HSA as well as one, two, or more modified forms (e.g., singly and doubly oxidized).

Table 1. Comparison of Measured with Calculated Molecular Weights

Sample	Measured MW	Calculated MW
HSA	66439 Da	66437 Da
HSA-MCC-Y2R	68915 Da	68914 Da
HSA-GMB-Y2R	68864 Da	68861 Da
HSA-MH-Y2R	68891 Da	68888 Da

mass difference measured at the end of the chemical reaction typically leveled at approximately 32 Da, which could represent the addition of two oxygens onto the HSA Cys34 thiol (HSA-SO_2H).[16] The chosen mass spectrometric method as well as the resolution capability of the single quadrupole mass spectrometer did not allow the clear distinction of these multiple modified HSA species. Further characterization of the exact nature of these modified forms was not attempted. These oxidized HSA species were treated as impurities in the overall quantitative assessment of the final HSA conjugate whereby purity was assessed by comparison of integrated ion currents representing the individual species of HSA (data not shown).

The conjugation yields varied between 50% and 80% and were mainly dependent on the quality of the starting HSA (e.g., percent free Cys34 thiol), the conditions for the conjugation reaction and the type of the linker. Yields for two of the conjugates (HSA-MCC-Y2R and HSA-HM-Y2R) were 56% and 52%, respectively. Although data obtained from proteolytic digests would provide some information with respect to conjugation yield, RP-HPLC ESI-MS with a single quadrupole mass spectrometer was used to the quantitate the total content of conjugated and unconjugated HSA. The mass readout for the mass spectrometer was limited but was chosen to include at least two prominent multiply charged ion species for every observed impurity and the desired conjugate. For these calculations, only one each of the prominent multiply charged ion species was used for the quantitative evaluation. Ion current traces for each observed species were integrated and percent values were calculated from the respective area counts.

Modification Site Determination. Cys34 was confirmed as the modification site in HSA. Both nonmodified HSA as well as the HSA peptide conjugate were digested with endoproteinase Lys-C. Subsequently, the resulting proteolytic peptide mixture was analyzed with RP LC ESI mass spectrometry. Proteolytic peptide maps obtained from unmodified HSA and peptide modified HSA are illustrated in Figures 5 and 6, respectively, where the expected peptide containing unmodified Cys34 elutes at 45.5 min (denoted with arrow in upper panel of Figure 5) and the expected peptide containing the peptide modified Cys34 elutes as a cluster of peaks at 45.0 min (denoted with arrow in the lower panel of Figure 6). The measured molecular weights of the respective proteolytic peptides were in excellent agreement with the calculated ones (Table 2).

***In Vitro* and *In Vivo* Testing of Albumin Conjugates.** Albumin conjugates were assayed for activation of NPY1-, NPY2-, NPY4-, and NPY5-receptors using the calcium flux assay. The potencies (EC_{50} values) were determined for the conjugates and parent peptide in three separate experiments. While none of the conjugates tested were active at NPY1R, NPY4R, and NPY5R in this assay, all of them activated NPY2R at nanomolar concentrations: MCC (6.8 ± 3.7 nM), ≥ MH (13.6 ± 6.1 nM), ≥ GMB (24.7 ± 8.1 nM). These values were 3.6−13-fold lower than that for PEG−Y2R (1.9 ± 0.29 nM)

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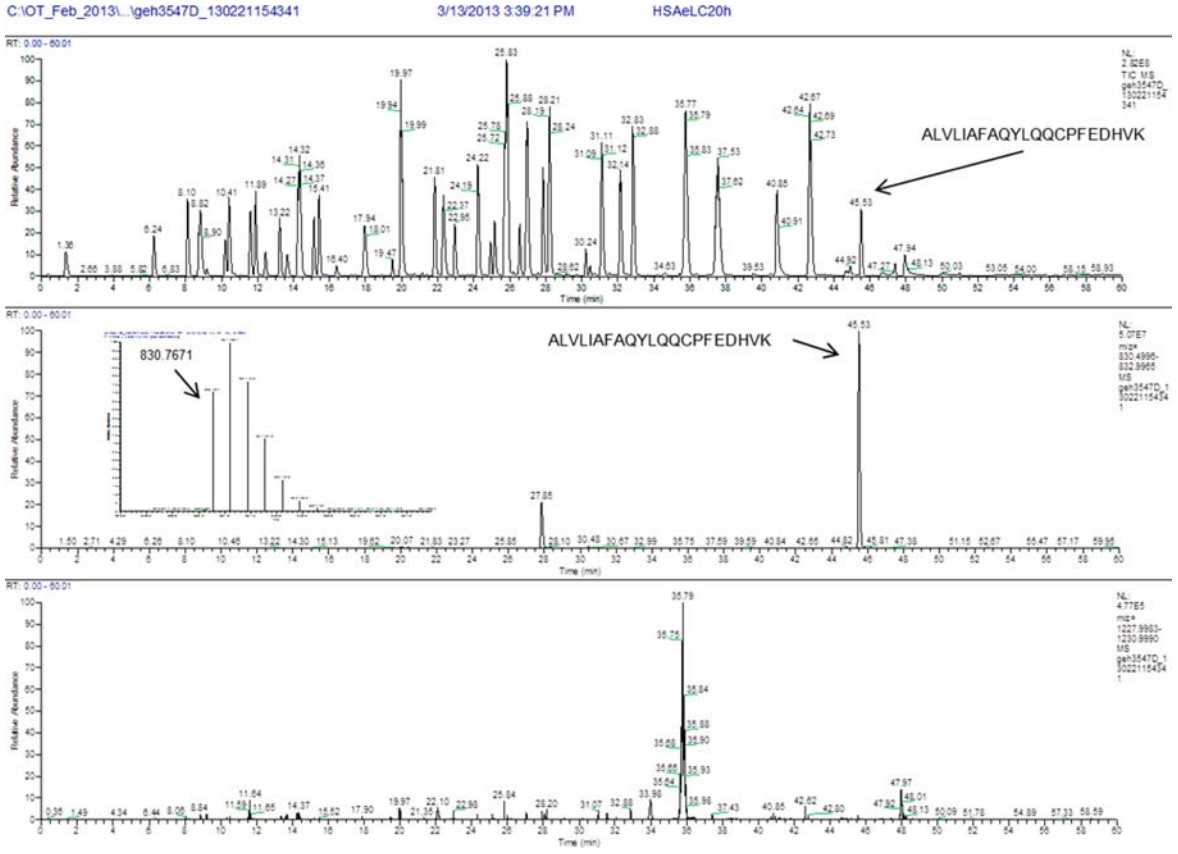

Figure 5. LC ESI-MS of endo Lys-C digested HSA (unmodified) illustrating the ion chromatogram from the total digest (upper panel), the individual ion current representing the proteolytic peptide containing the unmodified Cys34 (middle panel), the reconstructed mass spectrum (inset), and the individual ion current representing the proteolytic peptide containing the peptide modified Cys34 (lower panel).

and they were 29−107-fold lower than that of the parent peptide (Table 3).

The two most active albumin conjugates *in vitro* (HSA-MCC-Y2R and HSA-MH-Y2R) were selected along with the PEG-Y2R analogue for acute *in vivo* testing in DIO mice to determine their effect on food intake at equivalent peptide-based doses (10 mg/kg). The effect of a single subcutaneous treatment on food intake was measured over a 96 h period (Figure 7). Significant reduction (58%) in food intake was observed with the HSA-MH-Y2R conjugate in the 2−6 h time interval. A 39% food intake reduction was observed in the 6−24 h interval. The HSA-MCC-Y2R conjugate showed a trend toward reduction in food intake, but the effect was not determined to be statistically significant. The PEG-Y2R had a greater and longer-lasting effect on reducing food intake. PEG-Y2R reduced food intake by 80% in both the 2−6 and 6−24 h time intervals. Additionally, the effect was still reduced by 32% during the 24−48 h interval.

Serum concentrations of HSA-Y2R conjugates were also measured during the course of the study to determine pharmacokinetic parameters and were observed to be similar for both conjugates (Figure 8 and Table 4). While the more *in vivo* active HSA-MH-Y2R exhibited slightly greater $T_{1/2}$ and C_{max} than HSA-MCC-Y2R (8.7 h versus 8.2 h and 34.3 μg/mL versus 29.9 μg/mL, respectively), exposure of HSA-MH-Y2R based on area under curve (AUC_{96h} = 1077 μg.h/mL) was about 82% of that for HSA-MCC-Y2R (AUC_{96h} = 1321 μg.h/mL). Whereas HSA-MCC-Y2R was of similar potency to HSA-MH-Y2R *in vitro*, HSA-MH-Y2R displayed greater biological effect *in vivo*.

DISCUSSION

Significant retardation of renal clearance typically occurs in molecules larger than 60−70 kDa. As such, conjugation to recombinant HSA (molecular weight = 66.4 kDa) is considered to be a possible alternative to PEGylation for half-life extension, since it is biodegradable and has a long circulation half-life (21 days in humans). HSA contains one free thiol at Cys34, which makes it ideal for use in site-specific conjugation. An HSA−PYY3−36 conjugate tested for feeding behavior in rats was found to reduce food intake for up to 24 h.[17]

Unlike recombinant fusion proteins, conjugation of peptides to biological carriers has the advantage of using synthetic peptides that may include unnatural amino acids or other modifications, which can stabilize the peptide and/or confer selectivity to it. These properties are particularly important when half-life extension of the conjugate is the primary objective. Hence, this research was initiated to establish proof of concept using a 15 amino acid truncated analogue of PYY3−36 as an active ingredient conjugated to HSA through three heterobifunctional linkers.

The 15-amino-acid peptide (**IK-Pqa**-RHYLN**W**VTRQ(n-methyl)**RY**) used for conjugation in this study consisted of the 12-amino-acid Y2R pharmacophore taken from the α-helical carboxy terminal end of PYY3−36 (**IK**PEAPGEDAS-PEELNRYYASL**RHYLNLVTRQRY**), amidated and annealed to the carboxy terminus of acetylated amino terminal Ile3-Lys4

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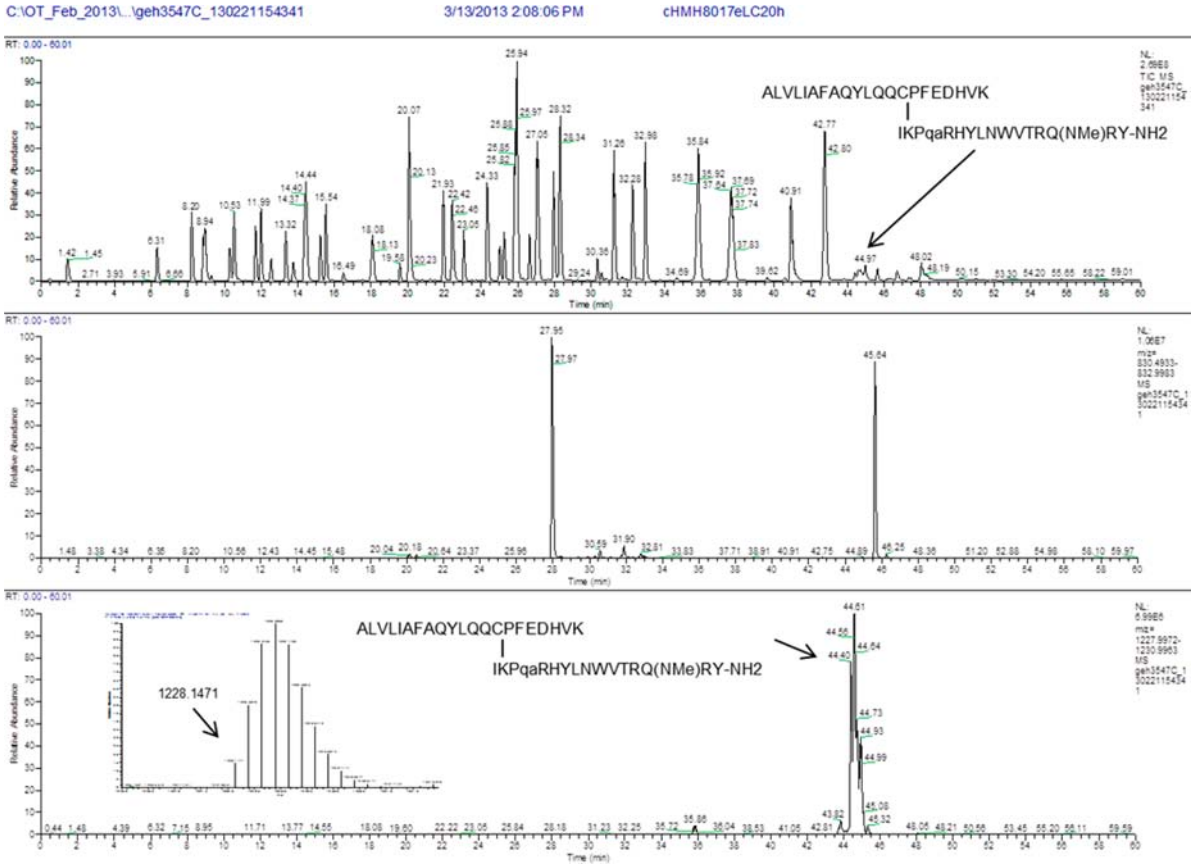

Figure 6. LC ESI-MS of endo Lys-C digested peptide modified HSA illustrating the ion chromatogram from the total digest (upper panel), the individual ion current representing the proteolytic peptide containing the unmodified Cys34 (middle panel), the individual ion current representing the proteolytic peptide containing the peptide modified Cys34 (lower panel), and the reconstructed mass spectrum (inset).

Table 2. Comparison of Experimental with Calculated *m/z* Values

Proteolytic peptide	Measured *m/z* (total MW)	Calculated *m/z* (total MW)	Chemical composition of proteolytic peptide
ALVLIAFAQYLQQCPFEDHVK	830.7671[a] (2492.301)	830.7665[a] (2492.300)	$C_{116}H_{179}N_{28}O_{31}S_1$
ALVLIAFAQYLQQCPFEDHVK IKPqaRHYLNWVTRQ(NMe)RY-NH2	1228.1471[b] (4912.588)	1228.1471[b] (4912.588)	$C_{232}H_{346}N_{62}O_{55}S_1$

[a] *m/z* of triply charged ions MH_3^{3+}. [b] *m/z* of quadruply charged ions MH_4^{4+}.

Table 3. *In Vitro* NPY2R Activation of Y2R and HSA−Y2R Conjugates Using Calcium Flux Assay[a]

Peptide	Average EC_{50}[b] (nM)	Relative Activity (Fold Loss)
Y2R	0.23 ± 0.10	1
HSA-MH-Y2R	13.6 ± 6.1	59
HSA-GMB-Y2R	24.7 ± 8.1	107
HSA-MCC-Y2R	6.8 ± 3.7	29
PEG-Y2R	1.9 ± 0.29	8.2

[a] Conjugates were not active at NPY1R, NPY4R, and NPY5R. [b] Values are means of 3 experiments ± standard deviation.

through an unnatural amino acid spacer, 4-oxo-6-(1-piperazinyl)-3(4*H*)-quinazoline-acetic acid (Pqa). The Pqa contained in this peptide was identified using the solution NMR structure of PYY3−36, whereby the spatial orientation of the two N-terminal amino acids (IK) and the 12-amino acid pharmacophore was found to be retained upon removal of 20 amino acids with slight loss in Y2R activity.[18] Two additional modifications were also included in the truncated peptide: (1) N-methylation of Arg19 and (2) tryptophan substitution of Leu24. The effect of these modifications was added stability and an approximate 10-fold reduction in *in vitro* Y2R activity relative to PYY3−36.[6,7] More significant loss of activity at Y1R, Y4R, and Y5R indicated greater Y2R selectivity, which is important because activation of Y1R and Y5R subtypes has been implicated in stimulating feeding behavior in animal models.[19,20]

The PEG-Y2R analogue was used as a benchmark in the current study. Localization of the PEG to the ε-amine of lysine was found to be optimal for *in vitro* binding and *in vivo* biological effect. Pharmacokinetic studies in DIO mice and mini-pigs demonstrated circulation half-lives of 25 and 191 h, respectively. The 30 kDa PEG size was selected to achieve a once-weekly profile in humans, where the circulation half-lives ranged between 115 and 200 h between a dose range of 0.1 and 20 mg/kg administered subcutaneously in a single ascending dose study.[21]

HSA−Y2R conjugates were prepared in two synthetic steps. The first reaction involved maleimide activation of the peptide

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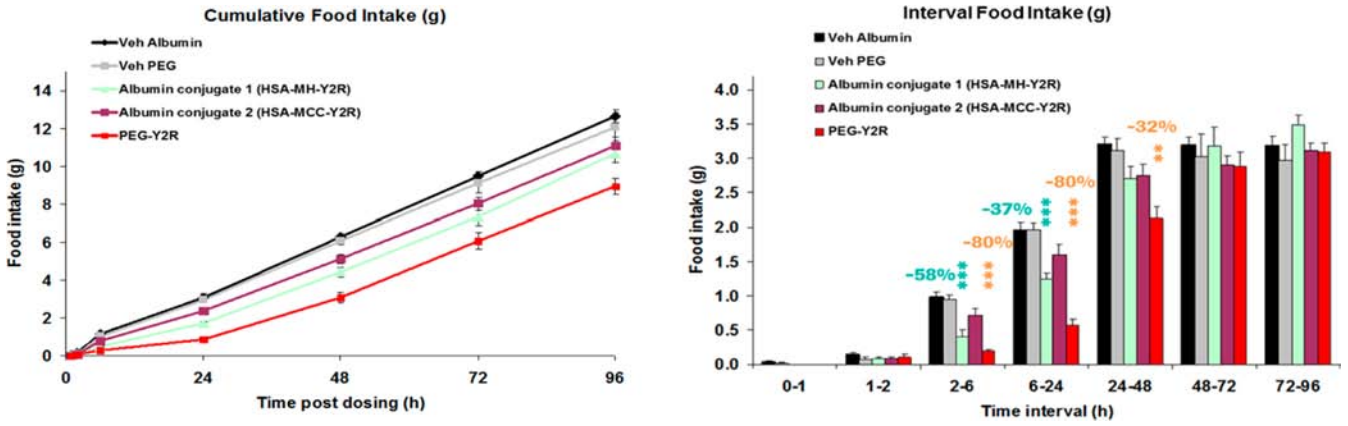

Figure 7. Effect of albumin−Y2R conjugates on cumulative and interval food intake in comparison to PEG−Y2R. Data are expressed as mean ± SEM; (N = 4−8 per group). $^{**}p < 0.01$; $^{***}p < 0.001$ vs vehicle albumin for albumin conjugates, and vehicle PEG for PEG−Y2R, ANOVA followed by Tukey's test. Enumerated as % of decrease versus vehicle albumin for albumin conjugates, and vehicle PEG for PEG−Y2R.

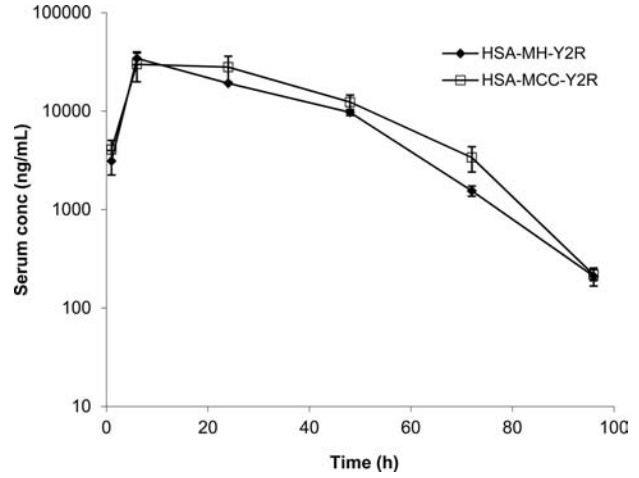

Figure 8. Pharmacokinetic profile of albumin conjugates in DIO mice treated subcutaneously at 10 mg/kg (4 μmol/kg). Serum concentrations of albumin conjugates over time were determined by ELISA.

Table 4. Pharmacokinetic Properties of Albumin Conjugates Tested at 10 mg/kg in DIO Mice

	HSA-MH-Y2R	HSA-MCC-Y2R	PEG-Y2R[a]
AUC_{96h} (μg.h/mL)	1077	1321	208[b]
C_{max} (μg/mL)	34.3	29.9	4.6
$T_{1/2}$ (h)	8.7	8.2	25.9

[a]PEG-Y2R was dosed at 1 mg/kg in a separate study. [b]Value represents $AUC_{0-120hr}$.

using the succinimidyl functionality of the heterobifunctional linker, resulting in an amide bond between the peptide at Lys2 on the ε-amine and the carbonyl group on the linker. Conversion of peptide reactant to peptide linker product was initially monitored by reversed phase HPLC. During the course of the reaction, a notable increase in retention time with a concomitant blue shift in wavelength was observed between the peptide and the peptide linker. The peptide linker was separated from excess linker by size exclusion chromatography to avoid undesirable linker albumin side product in the subsequent reaction. The size excluded peptide linker was quantitated using RP-HPLC and was then reacted with an equimolar amount of HSA in the second synthetic step. Unreacted peptide and peptide linker could be removed by extensive dialysis and/or diafiltration. LC ESI-MS was used successfully to establish identity and quality of reaction products in both synthetic steps and workup procedures. Measured molecular weights of reactants and products were all in excellent agreement with their theoretical molecular weights.

Cys34 was determined as the site of modification using LC-ESI-MS to compare proteolytic maps of HSA and HSA conjugate digested with endo-Lys C. The overall abundance of the unmodified Cys34 containing proteolytic peptide was reduced to approximately 20% after the conjugation and a "new" cluster of peaks was recovered in the sample conjugate which was not present in the unmodified form. Measured molecular weights of these "new" peaks were in excellent agreement with calculated molecular weights, hereby confirming Cys34 as major modification site.

The potencies of the three albumin conjugates tested *in vitro* at Y2R were significantly lower (1−2 orders magnitude) than the unmodified peptide yet more comparable to the EC_{50} for PEG-Y2R. HSA-MH-Y2R and HSA-MCC-Y2R were equipotent within error *in vitro* (6.8 ± 3.7 nM EC_{50} vs 13.6 ± 6.1 nM EC_{50}). Interestingly, HSA-MH-Y2R had greater effect on food intake lasting up to 24 h despite the observations that C_{max}, $T_{1/2}$, and exposure of HSA-MH-Y2R based on area under curve (AUC) were similar to those of HSA-MCC-Y2R.

While the effect of HSA-MH-Y2R on food intake was significant, it was not nearly as strong or durable as that of PEG-Y2R in this study. The elimination half-life of HSA-MH-Y2R (8.7 h) in DIO mice was significantly less than that observed for PEG-Y2R, which was 25 h (31 h in *db*/*db* mice). However, it was similar to that reported for an albumin−insulin fusion protein, i.e., 7 h in normoglycemic mice.[22] Moreover, the half-life of an HSA-PEG-exendin conjugate in *db*/*db* mice was found to be 11 h,[23] which is also close to that observed for HSA-MH-Y2R.

Complexation of albumin through albumin binding domains is another way of prolonging *in vivo* half-life.[24−26] One high-affinity albumin binding peptide, identified by phage display, was fused to a Fab fragment and found to have a 26-fold extended half-life (11 h) when compared to the unmodified fragment in mice.[24] Interestingly, this result was comparable to that observed for PEGylated Fab fragments. Nguyen et al. were first to account for albumin affinity in an adapted allometric scaling method to predict pharmacokinetic properties of phage derived albumin binding "AB.Fabs".[14]

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These studies suggest that the long circulation half-life of human albumin conjugate requires uptake and recirculation by human FcRn. Since mouse FcRn has low affinity for HSA, the conjugate is unlikely to be bound in circulation where there are high concentrations of mouse serum albumin competing for mouse FcRn. Interspecies variations of amino acids within DIII of albumin have recently been identified that are involved in binding to FcRn.[11]

While the free thiol on Cys34 in HSA is located within a hydrophobic crevice 10−12 Å from the surface,[27] the heterobifunctional linker lengths used in these conjugates were all less than 10 Å. This observation suggests the possibility of improving the binding activity by incorporation of longer spacers, such as miniPEGs, to reduce steric hindrance.

The studies above suggest the general utility of albumin conjugation for therapeutic use. Careful translation of interspecies pharmacokinetic properties of albumin conjugates with circulation half-lives between hours in mice to weeks in humans is needed to predict therapeutic doses. Albumin conjugate studies using variable length linkers on transgenic animals containing human FcRn are in progress to examine the effect on activity and circulation half-life.

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Notes

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ABBREVIATIONS

HSA, human serum albumin; PYY, Peptide YY; SMCC, succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate; GMBS, *N*-[γ-maleimidobutyryloxy]sulfosuccinimide ester; MHS, 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester; ESI, electro spray ionization; LC-MS, liquid chromatography mass spectrometry; PK/PD, pharmacokinetic/pharmacodynamics; PEG, polyethylene glycol; Y2R, Peptide Y2 receptor; Y1R, Peptide Y1 receptor; Y4R, Peptide Y4 receptor; Y5R, Peptide Y5 receptor; DPP4, Dipeptidyl peptidase-4; db/db, diabetic; FcRn, Neonatal Fc receptor; HRP, horseradish peroxidase; PBS, Phosphate buffered saline; TEA, triethylamine; DMF, Dimethylformamide

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