ACS OMEGA

Article

Cite This: ACS Omega 2019, 4, 5517−5525

http://pubs.acs.org/journal/acsodf

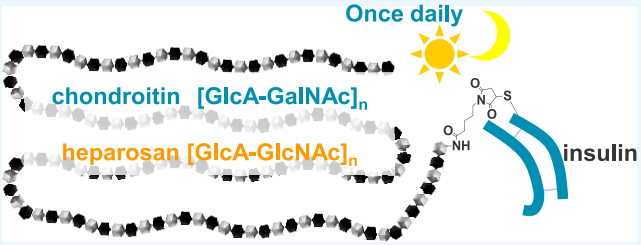
Glycosaminoglycan-Conjugated Insulin Derivatives Suitable for Once-Daily Formulations

Masanobu Fukushima, Tetsuya Hirayama, Megumi Ichikawa, Ikue Kitazawa, Kazuhiro Kojima, Tokiko Sakai, Yoshihiro Takatsu,*,† and Tetsuya Ohtaki

Central Research Laboratories, Seikagaku Corporation, 3-1253 Tateno, Higashiyamato-shi, Tokyo 207-0021, Japan

Supporting Information

ABSTRACT: A novel, long-acting insulin conjugate was developed by modification with a glycosaminoglycan (GAG; i.e., chondroitin [CH] or heparosan [HPN]) at different positions (Gly^{A1}, Lys^{B29}, or both) via different linkers with varying arm lengths. The in vitro glucose uptake-enhancing activity of conjugates inversely correlated with the circulatory in vivo half-life in mice and was affected primarily by the conjugation position rather than linker arm length. Conjugation at Gly^{A1}, which provided the best balanced profile of in vitro activity and circulation period in mice, also provided the strongest glucose-lowering efficacy, exhibiting 12 h durability, which was superior to that of insulin glargine and comparable to that of insulin degludec in streptozotocin (STZ)-treated mice. The use of different GAGs did not significantly affect blood circulation or efficacy in mice; however, blood levels of CH-conjugated insulin and efficacy were lower in rats, indicating species differences in the performance of CH. Finally, optimal activity was obtained by conjugation with HPN at Gly^{A1} using a C3 linker. These results demonstrate the applicability of GAG modification for prolonging the efficacy of peptide drugs, similar to our previous application to glucagon-like peptide 1.



INTRODUCTION

Patients with type 1 diabetes require insulin treatment because they are absolutely deficient in insulin. To mimic physiologic secretion of insulin, patients are often treated with basal−bolus therapy. Insulin treatment is also recommended for patients with type 2 diabetes as a means of controlling glycemia. Patients with type 2 diabetes are treated with once-daily injection of long-acting insulin or twice-daily injections of mixed insulin. Efforts to improve the efficacy of insulins have led to the development of many long-acting insulin analogues, such as neutral protamine Hagedorn, insulin glargine, insulin detemir, and insulin degludec, which have been shown to reduce overnight hypoglycemia.[1−3] Despite such improvements, a number of challenges remain to be overcome to further enhance glycemic control.[4−7] Many diabetes patients have serious fears of nocturnal hypoglycemia, but preventing this dangerous condition will require the development of insulin analogues exhibiting a peak-free and flat duration of activity.

The use of polyethylene glycol (PEG) to modify peptide/protein drugs is well established.[8−11] PEG conjugation increases the mass of a drug, thereby reducing renal excretion. In addition, PEG conjugation shields the drug surface, thus suppressing recognition by the immune system and proteolytic degradation. Conjugation with PEG has been used to enhance the therapeutic performance of several drugs, including Krystexxa, Oncaspar, and PEG-Intron.[12−16] However, as PEG is not biodegradable, it accumulates intracellularly, leading to the formation of so-called "foam cells".[17,18] Moreover, some reports indicate that PEG conjugates are deactivated by anti-PEG antibodies.[12,19−21]

Glycosaminoglycans (GAGs) such as hyaluronan and heparosan (HPN) are also known as drug delivery materials.[22−24] GAGs, which are composed of repeating disaccharide units, are ubiquitous in living cells. The high-molecular-mass sugar chains of GAGs are highly hydrophilic and biodegradable, features that have led to increased interest in the use of GAGs as drug delivery materials. We recently developed a long-acting glucagon-like peptide-1 conjugate using GAG modification.[25]

In the present study, we first explored nine different chondroitin (CH)-conjugated insulins prepared by conjugating CH at one or both of two sites using different heterobifunctional linkers to determine the optimal conjugation site and linker length. We then prepared an HPN-conjugated insulin using the same optimal linker design identified in the CH-conjugate study. Finally, we found that the HPN-conjugated insulin exhibits the best efficacy in streptozotocin (STZ)-treated rats and mice and has a BG-lowering effect comparable to a once-a-day insulin formulation, insulin degludec.

RESULTS

Synthesis of CH-Conjugated Insulins. Maleimide (Mal)-modified insulins were prepared by reacting recombi-

Received: January 7, 2019
Accepted: March 7, 2019
Published: March 19, 2019

DOI: 10.1021/acsomega.9b00059
ACS Omega 2019, 4, 5517−5525

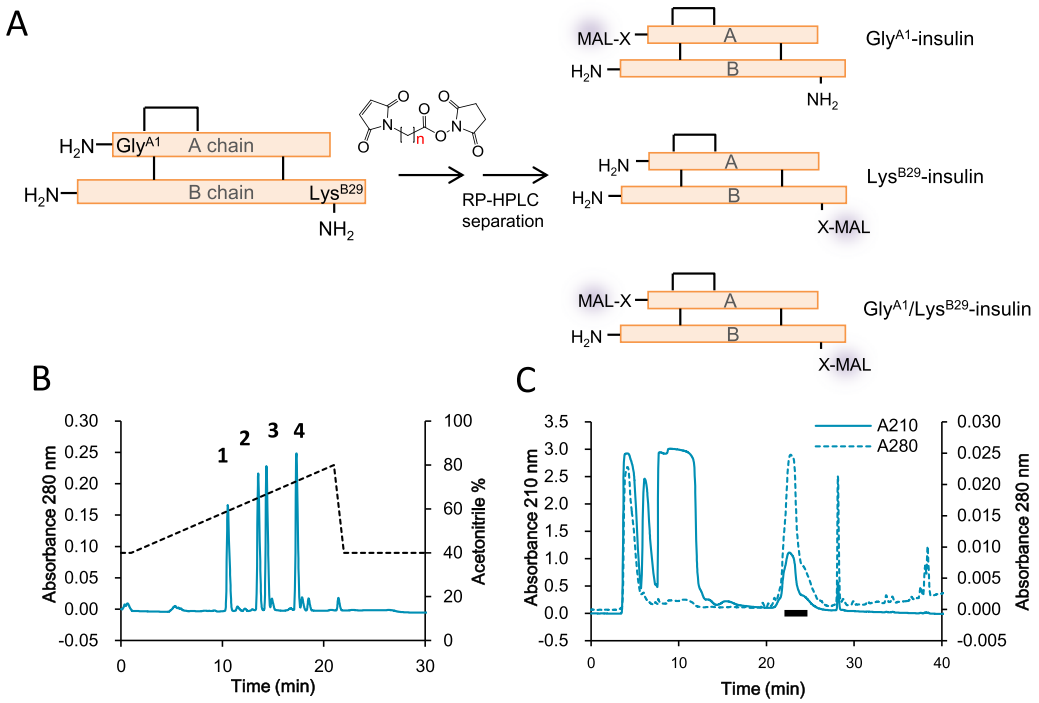


Figure 1. (A) Route of CH-conjugated insulin synthesis. (B) RP-HPLC purification of the reaction mixture of insulin and KMUS. Peak 1: insulin; peak 2: Mal-C11-Gly^{A1}-insulin; peak 3: Mal-C11-Lys^{B29}-insulin; and peak 4: Mal-C11-Gly^{A1}/Lys^{B29}-insulin. (C) RP-HPLC purification of CH-C3-Gly^{A1}/Lys^{B29}-insulin. Fractions of peak 2, not including the shoulder fractions denoted with a solid bar, were pooled and lyophilized for further characterization.

nant human (rHu) insulin with three different heterobifunctional linkers: C3 (*N*-[*β*-maleimidopropyloxy] succinimide ester, BMPS), C6 (*N*-[*ε*-maleimidocaproyloxy] sulfosuccinimide ester, EMCS), or C11 (*N*-[*κ*-maleimidoundecanoyloxy]-sulfosuccinimide ester, KMUS) in the tethering arm (alkyl chain) and carbonyl group (Figure 1A). Reversed-phase high-performance liquid chromatography (RP-HPLC) purification of each reaction mixture provided four major peaks, which were identified by liquid chromatography−mass spectrometry analysis (Figures S1−S16, Tables S1−S3) as intact insulin, Mal-X-Gly^{A1}-insulin (where X = C3, C6, or C11), Mal-X-Lys^{B29}-insulin, and Mal-X-Gly^{A1}/Lys^{B29}-insulin in HPLC elution order (Figure 1B).

Each Mal-modified insulin was reacted with thiolated chondroitin (CH-SH) to prepare nine CH-modified insulins (Table 1), as follows: CH-X-Gly^{A1}-insulin, CH-X-Lys^{B29}-insulin, and CH-X-Gly^{A1}/Lys^{B29}-insulin (where X = C3, C6, or C11). A typical HPLC elution profile is shown in Figure 1C. Shoulder fractions were not included in the purified samples. Yields in the conjugation reaction ranged between 13.2 and 98.3% (average 63.4%, Table S4) for insulin and between 3.1 and 15.7% (average 6.9%, Table S4) for CH and HPN. The relatively low yields obtained for CH and HPN were probably due to the use of excessive amounts (insulin/thiol molar ratio = 1:3) and incomplete amino modification of the reducing end of the sugars, as described previously.[25]

Modification at the *α*-amino group of Phe^{B1}, which is known to be less reactive, was not attainable under the conditions used.[26,27] The reaction of properly protected insulin with an increased amount of reagent is required for specific modification at Phe^{B1}, but we did not pursue this and left the Phe^{B1}-conjugated insulin untested.

In Vitro Bioactivity of CH-Modified Insulins. CH-conjugated insulins were characterized in vitro by measuring the bioactivity to enhance the uptake of [1-^{14}C] 2-deoxy-D-glucose by 3T3-L1 MBX adipocytes (Table 1). CH-conjugated insulins showed 9.2−616 times lower bioactivity (EC_{50} = 1.29−86.25 nM) compared to rHu insulin (EC_{50} = 0.14 nM), which varied depending primarily on the site of CH modification. The rank order of bioactivity was Lys^{B29}-conjugation (EC_{50} = 1.29−2.48 nM) > Gly^{A1}-conjugation (EC_{50} = 4.84−11.01 nM) > Gly^{A1}/Lys^{B29}-dual conjugation (EC_{50} = 6.32−86.25 nM). The effect of the tethering arm on bioactivity differed depending on the modification site; the longer C11 linker caused a slight potentiation in the bioactivity of the Lys^{B29}-conjugated insulin, a slight attenuation in the Gly^{A1}-conjugated insulin, and a significant reduction in the Lys^{B29}/Gly^{A1}-dual conjugated insulin.

These results indicate that Gly^{A1} is involved in receptor interaction to a greater extent than Lys^{B29}, and these data were consistent with crystallographic information showing close contact between the N-terminal *α*-helix (Gly^{A1}-Tyr^{A8}) and the *α*CT domain of the insulin receptor.[28,29]

Clearance of CH-Conjugated Insulins from the Circulation in Mice. Mice were injected with CH-conjugated insulins intravenously at a dose of 340 nmol/kg (=2 mg/kg), and serum levels of the conjugated insulins were determined over time to evaluate blood retention. As shown in Figure 2A, the C3-forms of the Gly^{A1}- and Gly^{A1}/Lys^{B29}-modified insulins exhibited a fairly good half-life in the circulation ($T_{1/2}$ = 5.6 and 7.5 h, respectively), whereas the C3-form of the Lys^{B29}-modified insulin decayed more rapidly ($T_{1/2}$ = 3.4 h). The rank order of half-lives found with the C3-forms, Gly^{A1}/Lys^{B29} > Gly^{A1} > Lys^{B29}-modification, was also true for the C6- and C11-forms (Table 1). Intriguingly, this rank order was

DOI: 10.1021/acsomega.9b00059
ACS Omega 2019, 4, 5517−5525

Table 1. Bioactivity and PK Profile of CH-/HPN-Conjugated Insulins in Mice[a,b]

GAG	linker	site	EC_{50} (nM)	$T_{1/2}$ (h)	C_{max} (ng/mL)	$AUC_{0-\infty}$ ((h·ng)/mL)	V_d (mL/kg)	BA (%)	ID
CH	C3 (BMPS)	Gly^{A1}	4.84	5.6	73 533	450 631	38	47	**1**
				5.5	19 571	213 290	90		
		Lys^{B29}	2.48	3.4	47 302	191 431	52	15	
				4.4	2302	29 072	433		
		Gly^{A1}/Lys^{B29}	6.32	7.5	35 331	261 869	82	8	**3**
				7.1	988	21 055	958		
	C6 (EMCS)	Gly^{A1}	9.76	5.6	74 932	375 880	58	7	
				6.9	1542	24 680	835		
		Lys^{B29}	1.52	2.2	330 550	1 690 143	4	4	
				2.8	7410	75 896	108		
		Gly^{A1}/Lys^{B29}	32.87	9.4	37 368	383 457	72	15	
				11.8	3811	56 056	609		
	C11 (KMUS)	Gly^{A1}	11.01	4.8	60 505	497 613	33	19	
				5.8	8403	96 856	204		
		Lys^{B29}	1.29	4.9	117 857	825 405	28	2	**2**
				4.4	1084	14 793	981		
		Gly^{A1}/Lys^{B29}	86.25	14.0	68 969	428 405	107	16	
				12.1	6308	69 608	591		
HPN	C3 (BMPS)	Gly^{A1}	6.54	7.3	11 124	164 435	38	NA	**4**
		Lys^{B29}	0.93	6.1	10 977	59 486	112	NA	**5**
		Gly^{A1}/Lys^{B29}	24.16	16.9	39 297	366 854	43	NA	**6**
	C11 (KMUS)	Gly^{A1}	8.68	5.6	21 883	284 120	28	NA	
		Lys^{B29}	1.25	6.9	29 179	150 814	59	NA	
		Gly^{A1}/Lys^{B29}	88.23	12.9	17 474	254 460	43	NA	

[a]NA, not available; BA, bioavailability; AUC, area under the curve. [b]Values of $T_{1/2}$, C_{max}, and $AUC_{0-\infty}$ with subcutaneous (s.c.) injection are shown in red.

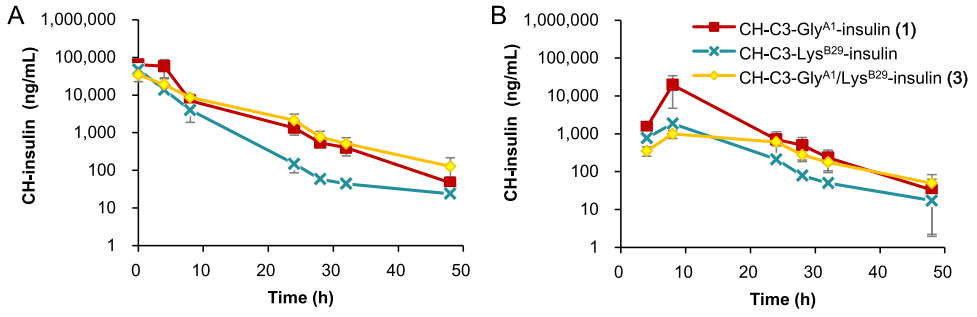


Figure 2. Serum levels of CH-conjugated insulins in ICR mice after (A) i.v. and (B) s.c. injection at 340 nmol/kg. All data are shown as mean ± SD of triplicate determinations.

opposite to that of the in vitro bioactivity (Figure 3). It should also be noted that the half-lives of the Gly^{A1}/Lys^{B29}-modified insulins were markedly affected by the alkyl chain length of the heterobifunctional linker and became longer in the following order: C11- > C6- > C3-form. These results also suggest that there is a close relationship between bioactivity and half-life: higher bioactivity is associated with shorter circulation period.

Based on the bioactivity and serum half-life data (Figure 3), we selected CH-C3-Gly^{A1}-insulin (**1**), CH-C11-Lys^{B29}-insulin (**2**), and CH-C3-Gly^{A1}/Lys^{B29}-insulin (**3**) from each group as candidates for evaluation of blood glucose (BG)-lowering efficacy. Prior to this analysis, conjugates **1**−**3** and related C3- and C11-forms of conjugates were subjected to pharmacokinetic (PK) analyses in mice by s.c. injection at 340 nmol/kg.

DOI: 10.1021/acsomega.9b00059
ACS Omega 2019, 4, 5517−5525

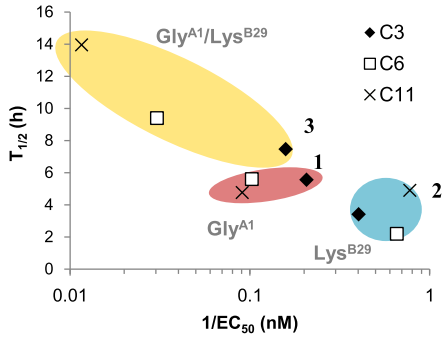


Figure 3. Inverse correlation between the half-life and bioactivity of CH-insulin conjugates. The *Y*-axis shows the $T_{1/2}$ (h) value after i.v. administration in ICR mice. The *X*-axis shows the inverse of the EC_{50} (nM) value for glucose uptake-enhancing activity in 3T3-L1 cells.

Serum levels peaked (T_{max}) at 8 h (or 12 h for **2** due to occasional changes in sampling time from 8 to 12 h) and then decreased at the same rates as observed after the respective intravenous (i.v.) injections (Figure 2B). Of the three conjugates, **1** exhibited the most optimal C_{max} and AUC values and thus exhibited the greatest BA (Table 1). In contrast, the BAs of **2** and **3** were much inferior to that of **1** (Table 1). In addition to **1**, the C11-form of Gly^{A1}-modified insulin exhibited the second best C_{max}, AUC, and BA. These data thus suggest that with s.c. injection, C_{max}, AUC, and BA tended to vary depending on the manner of modification. Enhanced BA was associated primarily with the Gly^{A1} modification, as demonstrated by the C3- (**1**) and C11-linker forms. With respect to peak shape, **3** provided the flattest one, remaining nearly constant for 24−28 h, although it was inferior to **1** and **2** in terms of C_{max}.

Decrease in BG Level in Mice with Injection of CH-Conjugated Insulins. CH-conjugated insulins **1**, **2**, and **3** were evaluated in terms of their BG-lowering efficacy in mice with STZ-induced diabetes in comparison with insulins glargine and degludec at a dose of 100 nmol/kg (Figure 4).

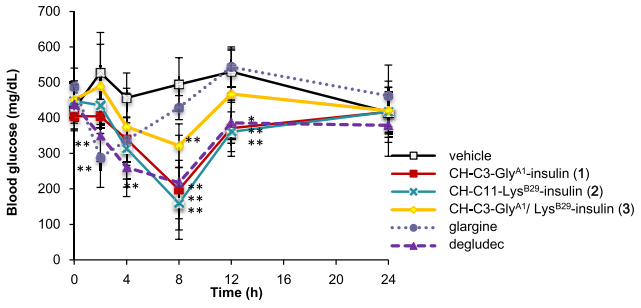


Figure 4. BG-lowering efficacy of CH-conjugated insulins in ICR mice with STZ-induced diabetes at a dose of 100 nmol/kg. All data are shown as mean ± SD (n = 6). $^{**}P < 0.01$ vs vehicle (Dunnett's test).

Insulin glargine decreased BG levels at 2 h, but its efficacy disappeared by 8 h postadministration. Insulin degludec significantly decreased BG levels for a longer period (from 2 to 12 h postadministration), with peak efficacy observed at 8 h. CH-conjugated insulin **1** did not change BG levels at 2 h postadministration, tended to decrease BG levels at 4 h, and significantly decreased BG levels at 8 and 12 h. The peak efficacy was observed at 8 h, which was in good accordance with results of the PK analysis (Figure 2B). CH-conjugated insulin **2** showed robust efficacy similar to that of **1**. In contrast, **3** exhibited much weaker efficacy at 8 h, and the relative ineffectiveness of **3** was probably due to its attenuated in vitro activity and decreased C_{max} (determined at 340 nmol/kg), suggesting that higher doses would be required. Taken together, these data indicate that CH-conjugated insulins **1** and **2** provide robust glucose-lowering efficacy similar to that of insulin degludec, but **1** and **2** function somewhat slower than insulin degludec.

HPN-Conjugated Insulins in Mice. Next, we prepared the C3-forms of HPN-conjugated insulin, HPN-C3-Gly^{A1}-insulin (**4**), HPN-C3-Lys^{B29}-insulin (**5**), and HPN-C3-Gly^{A1}/Lys^{B29}-insulin (**6**) by substituting HPN for CH. The bioactivities of **4** and **5** were similar to those of the respective CH-conjugated insulins, whereas the bioactivity of **6** was markedly more attenuated than that of **3** (Table 1). The rank order of bioactivity (Lys^{B29}- > Gly^{A1}- > Gly^{A1}/Lys^{B29}-modified insulin) was the same as that for the CH-conjugated insulins. The half-lives of **4** and **5** determined after i.v. injection at 100 nmol/kg were comparable to those of the respective CH-conjugated insulins (Figure 5A). It should be noted, however, that the decay curve of **6** was very flat, and its half-life (=16.9 h) was much longer than those of other GAG-conjugated insulins.

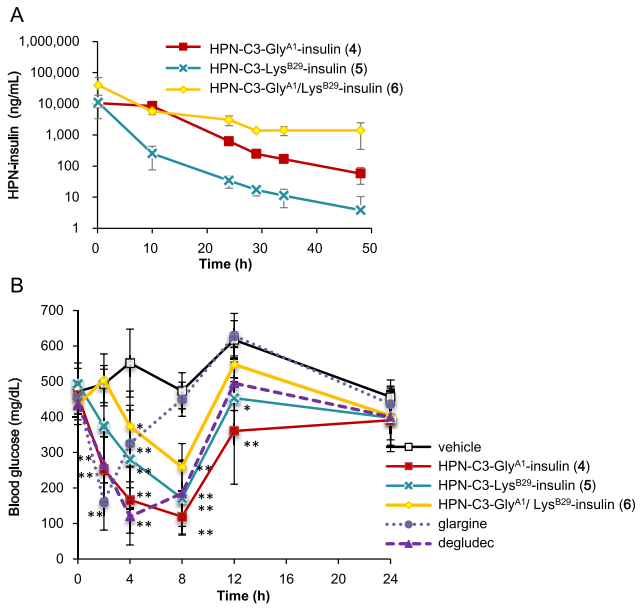


Figure 5. (A) Serum levels of HPN-conjugated insulins in ICR mice after i.v. injection at 100 nmol/kg. All data are shown as mean ± SD of triplicate determinations. (B) BG-lowering efficacy in ICR mice with STZ-induced diabetes of HPN-conjugated insulins at a dose of 100 nmol/kg. All data are shown as mean ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle (Dunnett's test).

Among insulins **1**−**6**, HPN-conjugated insulin **4** exhibited the greatest BG-lowering efficacy in mice with STZ-induced diabetes at 2−12 h postadministration, comparable to that of insulin degludec in terms of the extent and duration of activity. HPN-conjugated insulin **5** also exhibited fairly potent efficacy at 4−12 h postadministration (Figure 5B). HPN-conjugated insulin **6** partially but significantly decreased BG levels at 4−8 h postadministration (Figure 5B). These results indicated a clear rank order in terms of BG reduction: Gly^{A1}- > Lys^{B29}- >

DOI: 10.1021/acsomega.9b00059
ACS Omega 2019, 4, 5517−5525

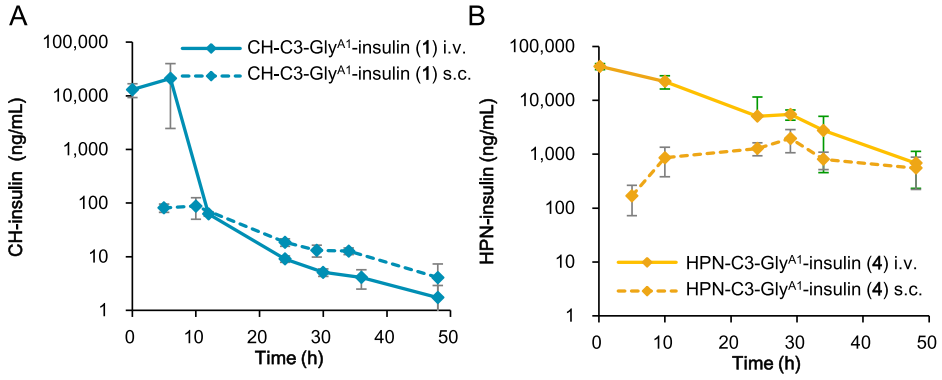


Figure 6. Serum levels of (A) CH- and (B) HPN-conjugated insulins after i.v. and s.c. injection in rats at 90 nmol/kg. The dose of **1** for i.v. injection was increased to 170 nmol/kg. All data are shown as mean ± SD (n = 3).

Table 2. Bioactivity and PK Profile of CH-/HPN-Conjugated Insulins in Rats[a,b]

GAG	linker	site	EC_{50} (nM)	$T_{1/2}$ (h)	C_{max} (ng/mL)	$AUC_{0-\infty}$ ((h·ng)/mL)	V_d (mL/kg)	BA (%)	ID
CH	C3 (BMPS)	Gly^{A1}	4.84	5.7	12 986	78 828	113	4	**1**
				9.5	94	1708	4385		
HPN			6.54	8.0	42 477	597 383	1	9	**4**
				11.7	2179	52 603	10		

[a]BA, bioavailability; AUC, area under the curve. [b]Values of $T_{1/2}$, C_{max}, and $AUC_{0-\infty}$ with s.c. injection are shown in red.

Gly^{A1}/Lys^{B29}-HPN-conjugated insulins. In general, HPN-conjugated insulins exhibited an earlier onset of activity relative to the CH-conjugated insulins, whereas the activity of the HPN-conjugated insulins was very similar to that of the CH-conjugated insulins in terms of the peak time, peak efficacy, and duration of activity. These results also demonstrated that HPN-conjugated insulin **4** has a slightly better profile compared to CH-conjugated insulin **1**, which has the same linker design.

CH- and HPN-Conjugated Insulins in Rats. We further evaluated the PK of CH-conjugated insulin **1** and HPN-conjugated insulin **4** in F344 male rats. CH-conjugated insulin **1** injected intravenously produced a two-phase decay curve consisting of distribution and elimination phases, as found in mice (Figure 6). The initial decrease in the distribution phase was more rapid than that in mice, however, resulting in lower serum levels of **1** in the elimination phase compared with mice. As such, the dose of **1** for measurement of serum levels was set at about twice (170 nmol/kg) that used for other measurements. Following s.c. injection at 90 nmol/kg, **1** exhibited extraordinarily low serum levels, approximately 1/100 of the levels observed after injecting mice at a dose of 340 nmol/kg (Figure 2B) and comparable to levels observed after injection at a dose of 20 nmol/kg (data not shown). Collectively, these data indicate that two major issues affect the PK of **1**: (i) very rapid disappearance from the circulation after i.v. injection and (ii) very poor BA after s.c. injection.

In contrast, HPN-conjugated insulin **4** exhibited a very slow decrease in serum level, with an apparent single-phase decay curve upon i.v. injection at 90 nmol/kg (Figure 6), indicating that distribution to the tissues was very slow, which was reflected by the very small volume of distribution (V_d = 10 mL/kg, Table 2). The half-life of **4** was 8.0 h with i.v. injection. After s.c. injection at the same dose, the serum levels of **4** began to plateau (around 1000 ng/mL) at 10 h postinjection and remained at that level until 48 h. The serum levels of **4** at the plateau were high enough to suggest good efficacy based on the mouse study of CH-conjugated insulins. The half-life of 11.7 h was difficult to determine, however, because of the flatness of the peak.

BG-Lowering Activity of CH- and HPN-Conjugated Insulins in Rats. Finally, we evaluated the BG-lowering activity of CH- and HPN-conjugated insulins for SD rats with STZ-induced diabetes at a dose of 100 nmol/kg in comparison with insulin glargine and insulin degludec (Figure 7). Glargine exhibited significant lowering of BG only at 2 and 4 h after administrations as was observed in the mouse model. Degludec exhibited durable efficacy until 12 h, with two peaks, at 6 and 12 h; however, the latter was probably a false peak associated

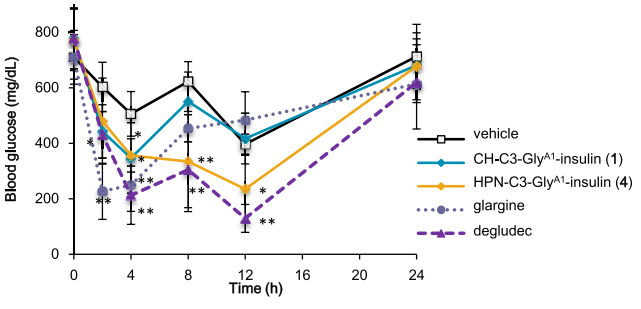


Figure 7. BG-lowering efficacy in SD rats with STZ-induced diabetes of CH- and HPN-conjugated insulins at a dose of 100 nmol/kg. All data are shown as mean ± SD (n = 6). *P < 0.05, **P < 0.01 vs vehicle (Dunnett's test).

DOI: 10.1021/acsomega.9b00059
ACS Omega 2019, 4, 5517−5525

with decreased control levels. HPN-conjugated insulin **4** also exhibited durable efficacy at 4−12 h after s.c. injection; however, CH-conjugated insulin **1** significantly lowered BG only at 4 h. The difference in efficacy between CH- and HPN-conjugated insulins was in good accordance with the findings of the PK study. The very low efficacy of **1** in rats is thus clearly due to its low blood levels.

DISCUSSION

The in vitro bioactivity of CH-/HPN-conjugated insulins was primarily affected by the insulin modification site. The potency of CH-/HPN-conjugated insulins decreased in the order Lys^{B29} > Gly^{A1} > Gly^{A1}/Lys^{B29} based on the site of modification. In contrast, the effect of the length of the heterobifunctional linker arm significantly affected only Lys^{B29}/Gly^{A1}-modified insulin. We speculate that the tendency toward decreased bioactivity with longer linkers in Gly^{A1}-modified insulin is exaggerated with the Lys^{B29}/Gly^{A1} dual modification, which appears to render the binding site "busy" and "congested". Modification in the C-terminus of the B-chain has been used in several marketed insulin derivatives. Insulin peglispro, PEGylated at Lys^{B28} of [Lys^{B28}, Pro^{B29}] insulin, reportedly exhibits bioactivity comparable to insulin in terms of autophosphorylation of the insulin receptor and lipogenesis in 3T3-L1 adipocytes, whereas the bioactivity of insulin detemir, myristoylated at Lys^{B29} of [ΔThr^{B30}] insulin, was approximately 100 times lower, although the bioactivity was potentially underestimated due to the presence of serum albumin in the assay medium.[7] In our adipocyte assay, GAG modification at Lys^{B29} or Gly^{A1} decreased the bioactivity from ten to several tens of times, although it remained in the same range as that of insulin glargine and insulin degludec.

Our PK studies in mice demonstrated that CH-/HPN-conjugated insulins remain in circulation for long periods, ranging from 2.2 to 16.9 h. The half-lives of these insulins are comparable or superior to those of the approved long-acting insulins glargine and degludec ($T_{1/2}$ of 4.3 h in rats and 2.7 h in mice after s.c. injection, respectively). The half-life rank order of the CH-/HPN-conjugated insulins in the circulation was Gly^{A1}/Lys^{B29}- > Gly^{A1}- > Lys^{B29}-modification, which was opposite to the rank order of bioactivity. This result is due in part to the fact that insulin modified with two GAG strands is more thoroughly shielded from immune cells and proteases. Also, the possibility cannot be excluded that a GAG-conjugated insulin with higher affinity for its receptor is more rapidly removed from the circulation via insulin receptors distributed throughout the body.

Taken together, our data highlight the difficulty of developing GAG-conjugated insulin derivatives maximized in terms of both bioactivity and half-life, at least with the current conjugation design. Conjugates in the Lys^{B29}-modification group exhibited the most optimal bioactivity but the poorest half-life, whereas those in the Gly^{A1}/Lys^{B29}-modification group exhibited the longest half-life but the lowest bioactivity. Conjugates in the Gly^{A1}-modification group were intermediate, exhibiting the best balance in terms of bioactivity and half-life.

The BA of GAG-conjugated insulin is generally poor in comparison with peglispro (PEGylated insulin, BA = 20−30%)[30] or PEGylated interferon (BA = 22−42%)[15] in rodents, most likely due to the different characteristics (anionic charge, biodegradability, etc.) of PEG and GAG. Also, structural differences between the GAG conjugates could affect BA, as suggested by the greater BA of Gly^{A1}-modified insulin in mice.

In mouse PD studies, Gly^{A1}-modified insulins exhibited the best efficacy among the three different modifications examined. The superiority of Gly^{A1}-modified insulins was more marked with HPN-conjugated insulins than CH-conjugated insulins. It should also be noted that the efficacy of CH-/HPN-conjugated insulins in mice was roughly comparable to that of insulin degludec at the same dose (100 nmol/kg); however, they differed in terms of peak efficacy time (8 h for CH-/HPN-conjugated insulins and 4 h for insulin degludec).

We hypothesized that the mechanism by which GAG improves PK and PD characteristics of conjugated peptides is probably related to attenuation of renal clearance due to the increase in the molecular mass of the peptide. Also, GAG modification could isolate the peptide from the reticuloendothelial system and plasma proteases. These hypotheses are based on the assumption of the inertness and stability of GAG in vivo. Quite unexpected were the observed differences in the PK characteristics and glucose-lowering activity of CH-conjugated insulins between rats and mice. Our results with i.v. injection suggest that CH-conjugated insulin is rapidly removed from the circulation or degraded by enzymes in rats. The poor BA in rats after s.c. injection suggests very slow transfer to the circulation or instability of the drug in s.c. injection areas. The underlying mechanism, which could differ from species to species, has not been reported in the literature. Hyaluronic acid receptor for endocytosis[31] and hyaluronidase-1[32,33] for possible uptake and breakdown mechanisms are subjects for future studies. In contrast, no differences in activity between rats and mice were observed with HPN-conjugated insulins. In this context, HPN-conjugated insulin **4** is the most promising candidate for use in humans.

To predict efficacy as a "once-a-day insulin formulation", we have shown that the duration of efficacy of conjugate **4** is comparable to that of insulin degludec in rodents. Also, the half-lives of the conjugate appear similar to that of insulin peglispro ($T_{1/2}$ = 5−20 h in rats).[30] However, these insulin derivatives may be differently affected by different species, because they exploit different mechanisms (degludec: self-assembly in the injection site and albumin binding in the circulation) or different materials (peglispro: conjugation with a synthetic polymer) for improving PK. In future studies, we need to evaluate the efficacy of conjugate **4** in nonhuman primates.

In conclusion, we evaluated the PK and PD characteristics of CH- and HPN-conjugated insulins in mice and rats. Eventually, we found that HPN-C3-Gly^{A1}-insulin exhibited a BG-lowering effect comparable to insulin degludec in both its extent and duration of efficacy.

METHODS

Animals. Male ICR mice (5 weeks old), F344/DuCr1Cr1j rats (5 weeks old), and SD rats (5 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan) and housed in groups of 3−5 animals at 23 ± 3 °C and 50 ± 20% humidity under 12 h light/12 h dark conditions. All animals were allowed ad libitum access to water and certified diet CRF-1 (Oriental Yeast, Tokyo, Japan).

All experiments were approved by the Institutional Animal Care and Use Committee of Seikagaku Corporation and carried out in accordance with relevant guidelines and regulations.

Preparation and Purification of Maleimide-Derivatized Insulin (Mal-Insulin). rHu insulin (Thermo Fisher

DOI: 10.1021/acsomega.9b00059
ACS Omega 2019, 4, 5517−5525

Scientific, Waltham, MA) was reacted with an equivalent amount of heterobifunctional linker, BMPS (Tokyo Chemical Industry, Tokyo, Japan), EMCS (Dojindo Laboratories, Kumamoto, Japan), or KMUS (Dojindo Laboratories) in *N*,*N*-dimethylformamide in the presence of three equivalents of triethylamine at room temperature for 1 h. The reaction mixture was diluted with aqueous 0.1% trifluoroacetic acid (TFA); injected onto a CAPCELL PAK C18 column (Shiseido, Tokyo, Japan) at a flow rate of 8 mL/min; and eluted using a linear gradient increase of acetonitrile concentration from 32 to 36% over 20 min (for reaction mixtures with BMPS), 33.6 to 38.5% over 24 min (reaction mixtures with EMCS), or 32 to 64% over 20 min (reaction mixtures with KMUS) in aqueous 0.1% TFA. Each peak fraction was collected, lyophilized, and stored at −20 °C until use.

Preparation and Purification of GAG-SH. CH (90 kDa) and HPN (50 kDa) were produced by fermentation and purified as described elsewhere.[25] CH-NH_2 and HPN-NH_2 were obtained by reductive amination of the respective GAG with ethylene diamine using sodium cyanoborohydride as a reducing agent, followed by purification by ethanol precipitation and gel-filtration.[25] CH-NH_2 or HPN-NH_2 dissolved at 40 mg/mL in 50% dimethylformamide (DMF), 30−300 mM 3-(2-pyridyldithio) propionic acid *N*-hydroxysuccinimide ester (Sigma-Aldrich, St. Louis, MO) dissolved in DMF, and 50 mM bicine buffer (pH 8.3) were mixed at a volumetric ratio of 50:1:4. After 1 h of reaction, dithiothreitol was added, and the reaction mixture was incubated for additional 1 h. The reaction mixture was then diluted with 0.1 M ammonium formic acid, injected onto two tandem-connected HiPrep desalting 26/10 columns (GE Healthcare, Chicago, IL), and eluted with the same buffer. The void-volume fractions containing CH-SH or HPN-SH were collected, lyophilized, and stored at −20 °C until use.

Preparation and Purification of GAG-Conjugated Insulins (GAG-Insulins). Mal-conjugated insulin dissolved in DMF and GAG-SH dissolved in distilled water were mixed at a molar ratio of 1:3. After adjusting the pH to 7.0 with 50 mM HEPES buffer (pH 7.0) and the DMF concentration to 50% by adding DMF, the conjugation reaction was performed at room temperature overnight. The reaction mixture was then diluted with aqueous 0.1% TFA, injected onto a CAPCELL PAK C18 column (Shiseido), and eluted using a linear gradient increase of acetonitrile concentration from 8 to 64% over 30 min in aqueous 0.1% TFA. The peak fractions of GAG-insulins were collected and lyophilized.

Preparation of 3T3-L1 MBX Adipocytes. Differentiation of 3T3-L1 MBX murine fibroblasts (American Type Cell Culture, Manassas, VA) to adipocytes was performed according to a previously published procedure.[34] 3T3-L1 MBX cells were plated at 20 000 cells/well in collagen-coated 96-well plates (Corning, Corning, NY) and cultured for 4 days. To induce differentiation, the cells were cultured in differentiation medium I containing 10% fetal bovine serum (FBS), 1 μg/mL rHu insulin, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 0.4 μg/mL dexamethasone (Sigma-Aldrich), and 2 μM rosiglitazone (Tokyo Chemical Industry) for 2 or 3 days and then cultured in differentiation medium II containing 10% FBS and 1 μg/mL rHu insulin for another 2 or 3 days. The cells were then maintained in a medium containing 10% FBS. Glucose uptake assays were performed within 7−21 days after induction of differentiation.

Glucose Uptake Test. Prior to the experiment, 3T3-L1 MBX adipocytes were serum-starved overnight in glucose-depleted Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific). Adipocytes were challenged with varying concentrations of insulin or insulin derivatives in glucose-depleted DMEM for 1 h at 37 °C and incubated with 100 nCi/20 μL/well of [1-^{14}C] 2-deoxy-D-glucose (2-DG; Japan RadioIsotope Association, Tokyo, Japan) for 30 min. After incubation, the cells were washed three times with cold PBS and lysed with 0.2 N sodium hydroxide. The amount of [1-^{14}C] 2-DG internalized by cells was measured in lysates using a liquid scintillation counter (model no. B291001, PerkinElmer, Waltham, MA). EC_{50} values were calculated according to the 4-parameter logistic model using Origin ver. 9.1 (OriginLab Corp., Northampton, MA).

PK in Rodents. ICR mice and F344/DuCr1Cr1j rats were acclimated for 7−14 days before drug administration. Mice (6 weeks old) were injected with CH-conjugated insulins or HPN-conjugated insulins at 340 and 100 nmol/kg, respectively. Rats (6 weeks old) were also injected with conjugates at 90 nmol/kg. Blood samples were collected immediately before injection and at various time points up to 48 h after injection. The serum concentration of each conjugate was determined using a sandwich ELISA kit (YK060; Yanaihara Institute Inc., Shizuoka, Japan) with some modifications (Figure S17). Samples were loaded into the wells of the plate supplied with the kit, and insulin conjugates were allowed to bind to the immobilized insulin antibody. Following a washing step, the bound conjugates were detected. Instead of the biotin-labeled antihuman insulin antibody included in the kit, we used biotinylated hyaluronic acid−binding protein (Seikagaku Corp.) for detecting CH-insulin. Alternatively, anti-HPN antibody (NAH46; Seikagaku Corp.) and HRP-conjugated goat antimouse IgM/G/A (H + L) (Merck, Kenilworth, NJ) were used for detecting HPN-insulin. Conjugate levels in serum were calculated according to the 4-parameter logistic model using Origin ver. 9.1, and PK parameters were calculated using Phoenix WinNonlin ver. 6.4 (Pharsight Corp. Sunnyvale, CA). The $T_{1/2}$ was that for the elimination phase.

PD in Rodents. STZ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was prepared in 20 mmol/L sodium citrate buffer (pH 4.5) and injected into ICR mice or SD rats at 200 mg/kg via the tail vein. After 5 days, blood samples were collected from the tail vein and used to measure BG levels using a self-monitoring BG meter (Life Check; Eisai, Tokyo, Japan). Mice in which BG levels were within the range of 360−650 mg/dL were used for experiments. Rats in which BG levels were <250 mg/dL were additionally treated with 65 mg/kg of STZ, and all STZ-treated rats were used for experiments.

Mice and rats with STZ-induced diabetes received a single s.c. injection of rHu insulin, insulin glargine (Eli Lilly, Indianapolis, IN), insulin degludec (Novo Nordisk, Bagsvaerd, Denmark), or GAG-conjugated insulin at 100 nmol/kg. BG levels were measured before administration and up to 24 h after administration. Dunnett's test was used to compare the control groups to groups treated with insulin derivatives. Differences with *P*-values <0.05 were considered statistically significant.

DOI: 10.1021/acsomega.9b00059
ACS Omega 2019, 4, 5517−5525

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00059.

Identification of the modification site of Mal-insulin; yield calculation for GAG-conjugated insulin; schematic illustration of GAG-insulin ELISA assay (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yoshihiro.takatsu@seikagaku.co.jp. Tel: +81-3-5220-8559. Fax: +81-3-5220-8594.

ORCID

Megumi Ichikawa: 0000-0001-8916-0251

Yoshihiro Takatsu: 0000-0001-9581-1313

Present Address

†Seikagaku Corporation, 6-1, Marunouchi 1-chome, Chiyoda-ku, Tokyo 100-0005, Japan (Y.T.).

Funding

All authors are employees of Seikagaku Corporation, which funded the entire research budget.

Notes

The authors declare no competing financial interest.

Some of the data were previously presented as a poster at the American Diabetes Association 2017 Annual Meeting.

■ ACKNOWLEDGMENTS

We thank Kiyoshi Suzuki, Kiwamu Yamagishi, Kazuhiko Tsujita, and Makoto Takashima for providing CH and HPN and Drs. Masaomi Miyamoto and Hiroshi Maeda for helpful discussions and encouragement throughout this work.

■ ABBREVIATIONS

2-DG, 2-deoxy-D-glucose; AUC, area under the curve; BA, bioavailability; BG, blood glucose; BMPS, *N*-[*β*-maleimidopropyloxy] succinimide ester; CH, chondroitin; DMEM, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; EMCS, *N*-[*ε*-maleimidocaproyloxy] sulfosuccinimide ester; FBS, fetal bovine serum; GAG, glycosaminoglycan; HPLC, high-performance liquid chromatography; HPN, heparosan; HRP, horseradish peroxidase; i.v, intravenous; KMUS, *N*-[*κ*-maleimidoundecanoyloxy]-sulfosuccinimide ester; Mal, maleimide; NA, not available; PEG, poly(ethylene glycol); PD, pharmacodynamics; PK, pharmacokinetics; RP, reversed-phase; s.c., subcutaneous; -SH, thiolated; STZ, streptozotocin; TFA, trifluoroacetic acid

■ REFERENCES

(1) Porcellati, F.; Lucidi, P.; Cioli, P.; Candeloro, P.; Andreoli, A. M.; Marzotti, S.; Ambrogi, M.; Bolli, G. B.; Fanelli, C. G. Pharmacokinetics and pharmacodynamics of insulin glargine given in the evening as compared with in the morning in type 2 diabetes. *Diabetes Care* **2015**, *38*, 503−512.

(2) Rodbard, H. W.; Cariou, B.; Zinman, B.; Handelsman, Y.; Philis-Tsimikas, A.; Skjøth, T. V.; Rana, A.; Mathieu, C. Comparison of insulin degludec with insulin glargine in insulin-naive subjects with type 2 diabetes: a 2-year randomized, treat-to-target trial. *Diabetic Med.* **2013**, *30*, 1298−1304.

(3) Zinman, B.; Fulcher, G.; Rao, P. V.; Thomas, N.; Endahl, L. A.; Johansen, T.; Lindh, R.; Lewin, A.; Rosenstock, J.; Pinget, M.; et al. Insulin degludec, an ultra-long-acting basal insulin, once a day or three times a week versus insulin glargine once a day in patients with type 2 diabetes: a 16-week, randomised, open-label, phase 2 trial. *Lancet* **2011**, *377*, 924−931.

(4) Garber, A. J. Will the next generation of basal insulins offer clinical advantages? *Diabetes, Obes. Metab.* **2014**, *16*, 483−491.

(5) Woo, V. C. New insulins and new aspects in insulin delivery. *Can. J. Diabetes* **2015**, *39*, 335−343.

(6) Muñoz-Garach, A.; Molina-Vega, M.; Tinahones, F. J. How can a good idea fail? Basal insulin peglispro [LY2605541] for the treatment of type 2 diabetes. *Diabetes Ther.* **2017**, *8*, 9−22.

(7) Owens, R. A.; Hansen, R. J.; Kahl, S. D.; Zhang, C.; Ruan, X.; Koester, A.; Li, S.; Qian, H.-R.; Farmen, M. W.; Michael, M. D.; et al. In vivo and in vitro characterization of basal insulin peglispro: a novel insulin analog. *J. Pharmacol. Exp. Ther.* **2016**, *357*, 459−465.

(8) Veronese, F. M.; Pasut, G. PEGylation, successful approach to drug delivery. *Drug Discovery Today* **2005**, *10*, 1451−1458.

(9) Jevševar, S.; Kunstelj, M.; Porekar, V. G. PEGylation of therapeutic proteins. *Biotechnol. J.* **2010**, *5*, 113−128.

(10) Turecek, P. L.; Bossard, M. J.; Schoetens, F.; Ivens, I. A. PEGylation of biopharmaceuticals: a review of chemistry and nonclinical safety information of approved drugs. *J. Pharm. Sci.* **2016**, *105*, 460−475.

(11) Pisal, D. S.; Kosloski, M. P.; Balu-Iyler, S. V. Delivery of therapeutic proteins. *J. Pharm. Sci.* **2010**, *99*, 2557−2575.

(12) Sundy, J. S.; Baraf, H. S. B.; Yood, R. A.; Edwards, N. L.; Gutierrez-Urena, S. R.; Treadwell, E. L.; Vázquez-Mellado, J.; White, W. B.; Lipsky, P. E.; Horowitz, Z.; et al. Efficacy and tolerability of pegloticase for the treatment of chronic gout in patients refractory to conventional treatment: two randomized controlled trials. *JAMA, J. Am. Med. Assoc.* **2011**, *306*, 711−720.

(13) Sattui, S. E.; Gaffo, A. L. Treatment of hyperuricemia in gout: current therapeutic options, latest developments and clinical implications. *Ther. Adv. Musculoskeletal Dis.* **2016**, *8*, 145−159.

(14) Avramis, V. I.; Sencer, S.; Periclou, A. P.; Sather, H.; Bostrom, B. C.; Cohen, L. J.; Ettinger, A. G.; Ettinger, L. J.; Franklin, J.; Gaynon, P. S.; et al. A randomized comparison of native *Escherichia coli* asparaginase and polyethylene glycol conjugated asparaginase for treatment of children with newly diagnosed standard-risk acute lymphoblastic leukemia: a children's cancer group study. *Blood* **2002**, *99*, 1986−1994.

(15) Basu, A.; Yang, K.; Wang, M.; Liu, S.; Chintala, R.; Palm, T.; Zhao, H.; Peng, P.; Wu, D.; Zhang, Z. Z.; et al. Structure−function engineering of interferon-*β*-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. *Bioconjugate Chem.* **2006**, *17*, 618−630.

(16) Bailon, P.; Palleroni, A.; Schaffer, C. A.; Spence, C. L.; Fung, W.-J.; Porter, J. E.; Ehrlich, G. K.; Pan, W.; Xu, Z.-X.; Modi, M. W.; et al. Rational design of a potent, long-lasting form of interferon: a 40 kDa branched polyethylene glycol-conjugated interferon alpha-2a for the treatment of hepatitis C. *Bioconjugate Chem.* **2001**, *12*, 195−202.

(17) Rudmann, D. G.; Alston, J. T.; Hanson, J. C.; Heidel, S. High molecular weight polyethylene glycol cellular distribution and PEG-associated cytoplasmic vacuolation is molecular weight dependent and does not require conjugation to proteins. *Toxicol. Pathol.* **2013**, *41*, 970−983.

(18) Zhang, F.; Liu, M.-R.; Wan, H. Discussion about several potential drawbacks of PEGylated therapeutic proteins. *Biol. Pharm. Bull.* **2014**, *37*, 335−339.

(19) Hasirci, V.; Changes, M.; Armstrong, J. K.; Hempel, G.; Koling, S.; Chan, L. S.; Fisher, T.; Meiselman, H. J.; Garratty, G. Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer* **2007**, *110*, 311−322.

(20) Schellekens, H.; Hennink, W. E.; Brinks, V. The immunogenicity of polyethylene glycol: facts and fiction. *Pharm. Res.* **2013**, *30*, 1729−1734.

(21) Hershfield, M. S.; Ganson, N. J.; Kelly, S. J.; Scarlett, E. L.; Jaggers, D. A.; Sundy, J. S. Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-week dosing of pegloticase for

DOI: 10.1021/acsomega.9b00059
ACS Omega 2019, 4, 5517−5525

refractory gout, including in organ transplant recipients. *Arthritis Res. Ther.* **2014**, *16*, R63.
(22) Lee, F.; Chung, J. E.; Kurisawa, M. An injectable hyaluronic acid-tyramine hydrogel system for protein delivery. *J. Controlled Release* **2009**, *134*, 186−193.
(23) Yoshioka, K.; Kisukeda, T.; Zuinen, R.; Yasuda, Y.; Miyamoto, K. Pharmacological effects of *N*-[2-[[2-[2-[(2,6-Dichlorophenyl)-amino]phenyl]acetyl]oxy]ethyl]hyaluronamide (Diclofenac etalhyaluronate, SI-613), a novel sodium hyaluronate derivative chemically linked with diclofenac. *BMC Musculoskeletal Disord.* **2018**, *19*, No. 157.
(24) Jing, W.; Roberts, J. W.; Green, D. E.; Almond, A.; DeAngelis, P. L. Synthesis and characterization of heparosan-granulocyte-colony stimulating factor conjugates: a natural sugar-based drug delivery system to treat neutropenia. *Glycobiology* **2017**, *27*, 1052−1061.
(25) Ichikawa, M.; Hirayama, T.; Fukushima, M.; Kitazawa, I.; Kojima, K.; Sakai, T.; Takatsu, Y.; Ohtaki, T. Glycosaminoglycan conjugation for improving the duration of therapeutic action of glucagon-like peptide-1. *ACS Omega* **2018**, *3*, 5346−5354.
(26) Uchio, T.; Baudyš, M.; Liu, F.; Song, S. C.; Kim, S. W. Site-specific insulin conjugates with enhanced stability and extended action profile. *Adv. Drug Delivery Rev.* **1999**, *35*, 289−306.
(27) Harris, J. M.; Struck, E. C.; Case, M. G.; Paley, M. S.; Yalpani, M.; Van Alstine, J. M.; Brooks, D. E. Synthesis and characterization of poly(ethylene glycol) derivatives. *J. Polym. Sci., Polym. Chem. Ed.* **1984**, *22*, 341−352.
(28) Huang, K.; Xu, B.; Hu, S. Q.; Chu, Y. C.; Hua, Q. X.; Qu, Y.; Li, B.; Wang, S.; Wang, R. Y.; Nakagawa, S. H.; et al. How insulin binds: the B-chain α-helix contacts the L1 β-helix of the insulin receptor. *J. Mol. Biol.* **2004**, *341*, 529−550.
(29) Menting, J. G.; Yang, Y.; Chan, S. J.; Phillips, N. B.; Smith, B. J.; Whittaker, J.; Wickramasinghe, N. P.; Whittaker, L. J.; Pandyarajan, V.; Wan, Z.; et al. Protective Hinge in Insulin Opens to Enable Its Receptor Engagement. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, E3395−E3404.
(30) Knadler, M. P.; Ellis, B. B.; Brown-Augsburger, P. L.; Murphy, A. T.; Martin, J. A.; Wroblewski, V. J. Disposition of basal insulin peglispro compared with 20-kDa polyethylene glycol in rats following a single intravenous or subcutaneous dose. *Drug Metab. Dispos.* **2015**, *43*, 1477−1483.
(31) Harris, E. N.; Weigel, P. H. The ligand-binding profile of HARE: hyaluronan and chondroitin sulfates A, C, and D bind to overlapping sites distinct from the sites for heparin, acetylated low-density lipoprotein, dermatan sulfate, and CS-E. *Glycobiology* **2008**, *18*, 638−648.
(32) Gushulak, L.; Hemming, R.; Martin, D.; Seyrantepe, V.; Pshezhetsky, A.; Triggs-Raine, B. Hyaluronidase 1 and beta-hexosaminidase have redundant functions in hyaluronan and chondroitin sulfate degradation. *J. Biol. Chem.* **2012**, *287*, 16689−16697.
(33) Honda, T.; Kaneiwa, T.; Mizumoto, S.; Sugahara, K.; Yamada, S. Hyaluronidases have strong hydrolytic activity toward chondroitin 4-sulfate comparable to that for hyaluronan. *Biomolecules* **2012**, *2*, 549−563.
(34) Zebisch, K.; Voigt, V.; Wabitsch, M.; Brandsch, M. Protocol for effective differentiation of 3T3-L1 cells to adipocytes. *Anal. Biochem.* **2012**, *425*, 88−90.

DOI: 10.1021/acsomega.9b00059
ACS Omega 2019, 4, 5517−5525