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Preparation and Structural, Biochemical, and Pharmaceutical Characterizations of Bile Acid-Modified Long-Acting Exendin-4 Derivatives

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To develop an effective long-acting antidiabetic, the GLP-1 analogue of exendin-4 was modified with three different bile acids (BAs; cholic, deoxycholic, or lithocholic acid), at its two lysine residues. The biological, pharmaceutical, and physicochemical characteristics of these exendin-4 analogues were carefully investigated. Biological activity tests demonstrated that the monobile acid substitutions of exendin-4 showed well preserved receptor binding efficacy without noticeable insulinotropic or antidiabetic activity loss. However, physicochemical and pharmacokinetic studies revealed that the albumin-binding properties and in vivo elimination half-lives of BAM1-Ex4s (Lys^{27}-BA-Ex4s) were significantly enhanced by increasing the hydrophobicities of the conjugated BAs. Furthermore, the protracted antidiabetic effects of the BAM1-Ex4s were also verified by the prolonged restoration of normoglycemia in type 2 diabetic mice. Accordingly, the present study suggests that the derivatization of exendin-4 with BAs offers a means of producing long-acting GLP-1 receptor agonists for type 2 diabetic therapy.

Introduction

Type 2 diabetes is a chronic, deteriorative metabolic disease, characterized by progressive hyperglycemia followed by dysfunctional insulin secretion and/or its impaired utilization. Although several traditional hypoglycemic agents have been widely utilized for glycemic control, there is a continuing demand for improved diabetic therapies, not only for precise glycemic control but also for the maintenance and/or improvement of pancreatic endocrine functions.[1−3] Among the various developing strategies, incretin-based diabetic therapies are considered to be one of the most effective approaches because of the unique biological activities of the incretin hormones such as their multiple gluco-regulatory functions and their amelioration of pancreatic endocrine deficiencies, which are due to their promotion of β-cell proliferation and/or neogenesis.[3,4] Two distinct approaches to incretin-based diabetic therapies have been intensively developed, namely, the external administration of glucagon-like peptide-1 (GLP-1[a]) receptor agonists (also known as incretin mimetics) and the blockade of incretin inactivating enzymes by small molecular inhibitors (incretin enhancers).[2,5]

Because of rapid enzymatic inactivation by dipeptidyl peptidase-4 (DPP IV), incretin hormones, especially GLP-1 have extremely short biological half-lives (∼2 min).[4] Therefore, initial studies focused on the development of DPP IV resistant GLP-1 receptor agonists, which resulted in the successful clinical application of exendin-4 (natural GLP-1 receptor agonist isolated from lizard saliva) for type 2 diabetic therapies.[4−7] However, its therapeutic utility is limited because of the frequent injections required (twice daily) and the associated inconvenience to patients. Although the enzymatic inactivation by DPP IV does not limit its therapeutic potential, exendin-4 has a relatively short-lived euglycemic effect (4−6 h), presumably due to its rapid renal clearance by glomerular filtration caused by its low molecular weight (∼4.2 kDa).[7,8] Therefore, there still exists a demand for incretin mimetics with prolonged gluco-regulatory effects for type 2 diabetes.

To address this issue, numerous research efforts have focused on the development of long-acting incretin mimetics and/or the sustained delivery of its mimetics.[9−17] The chemical and/or structural modification of incretin mimetics appears to be one of the more effective approaches. These strategies are specifically aimed at preventing rapid renal clearance by increasing molecular size or by facilitating physical interactions with biological molecules, such as serum albumin.[9−16] More specifically, these strategies include modifications of GLP-1 receptor agonists by poly(ethylene glycol) conjugation (PEGylation)[9−11] and chemical or genetic modifications using serum albumin.[12,13] The resulting PEGylated or albumin-conjugated GLP-1 receptor agonists showed typical long-acting characteristics of protracted antidiabetic potentials and enhanced circulation half-lives.[9−13] Furthermore, incretin mimetics have been modified with fatty

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[a] Abbreviations: GLP-1, glucagon-like peptide-1; BA, bile acid; CA, cholic acid; DA, deoxycholic acid; LA, lithocholic acid; BAM1-Ex4, Lys^{27}-BA-exendin-4; BAM2-Ex4, Lys^{12}-BA-exendin-4; BADi-Ex4, $Lys^{12,27}$-DiBA-exendin-4; CAM1-Ex4, Lys^{27}-CA-exendin-4; CAM2-Ex4, Lys^{12}-CA-exendin-4; CADi-Ex4, $Lys^{12,27}$-DiCA-exendin-4; DAM1-Ex4, Lys^{27}-DA-exendin-4; DAM2-Ex4, Lys^{12}-DA-exendin-4; DADi-Ex4, $Lys^{12,27}$-DiDA-exendin-4; LAM1-Ex4, Lys^{27}-LA-exendin-4; LAM2-Ex4, Lys^{12}-LA-exendin-4; LADi-Ex4, $Lys^{12,27}$-DiLA-exendin-4; DPP IV, dipeptidyl peptidase IV; IPGTT, intraperitoneal glucose tolerance test; AUC, area under the curve; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time-of-flight mass spectroscopy.

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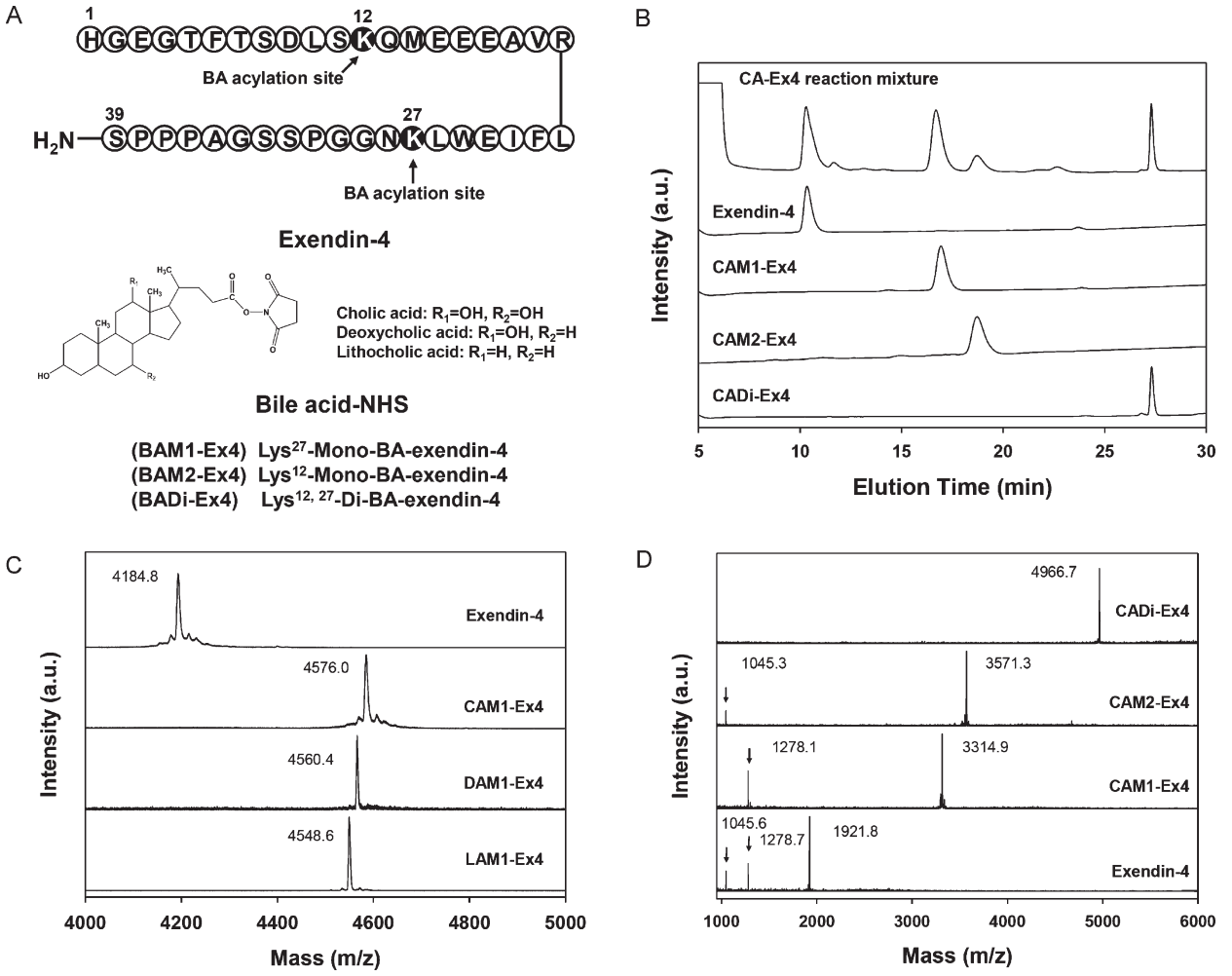

Figure 1. Preparation, purification, and structural characterizations of BA-Ex4s. (A) Chemical structures of BA-NHSs, the amino acid sequence of exendin-4, and of its BA acylated derivatives. (B) Reversed phase HPLC chromatograms of a CA acylation reaction mixture (CA/exendin-4 feed ratio of 1/1) and of purified CA-Ex4s. (C) Total mass spectra of exendin-4 and BAM1-Ex4s, obtained by MALDI-TOF MS. (D) Mass spectra of CA-Ex4s after Lys-C enzymatic digestion.

acids to facilitate the physical interaction with albumin, and the resulting long-acting GLP-1 derivative obtained by palmitic acid acylation has been clinically evaluated as an efficient long-acting antidiabetic agent.[14−16] In addition, an alternative approach utilizes the sustained-release of a GLP-1 receptor agonist from biodegradable microspheres.[17]

We hypothesized that the covalent coupling of exendin-4 to bile acids (BAs) followed by lead compound selection based on their antidiabetic efficacy might offer a practical means of developing long-acting exendin-4 derivatives. We believed that this might be a possibility because of the unique physicochemical characteristics of BA-conjugated peptides, such as their effective physical interactions with serum albumin and their self-aggregation behaviors in aqueous environments.[18,19] Accordingly, we designed site-specific BA conjugations on two lysine residues of exendin-4 (Lys^{12} and/or Lys^{27}). In addition, these lysine-specific conjugations might be free from the serious reductions in the biological activities of GLP-1 receptor agonists, which are accompanied by N-terminal modifications.[9,20,21]

Using this strategy, we prepared three different BA-derivatized exendin-4 adducts at Lys^{12} and/or Lys^{27}, and then explored their structural, biological, and physicochemical characteristics. The pharmacokinetic and antidiabetic characteristics of these three BA-conjugated exendin-4s were investigated using animal models to identify those with efficient long-acting antidiabetic properties.

Results

Preparation and Structural Characterization of Site-Specific BA-Acylated Exendin-4 Derivatives. Chemical conjugations to lysine residues of exendin-4 by BA-NHSs (*N*-hydroxysuccinimide activated BAs) were carried out using optimized reaction conditions (BA/exendin-4 molar ratio 1.2−1.5; Supporting Information, Figure S1). These conjugations resulted in the production of four different products, as illustrated in the summary diagram and the HPLC chromatogram (Figure 1A and B, respectively). The four peaks in the HPLC chromatogram corresponded to unreacted exendin-4 (elution time 9.8 min), Lys^{27}-CA-Exendin-4 (CAM1-Ex4, 16.4 min), Lys^{12}-CA-Exendin-4 (CAM2-Ex-4, 18.1 min), and $Lys^{12,27}$-CA-Exendin-4 (CADi-Ex-4, 27.1 min). The chromatograms of purified BA-Ex4s showed successful separations and high purity (Figure 1B and Supporting Information, Figure S2). In addition, the quantitative HPLC analysis revealed that the purities of BA-Ex4s

Table 1. Structural Characterizations of Exendin-4 and BA-Ex4s by MALDI-TOF Mass Spectrometry (before and after Lys-C Digestion)

	total mass		Lys-C digested fragments		
samples	calculated mass	observed mass	fragments	calculated mass	observed mass
exendin-4	4186.0	4184.8	His^{1}-Lys^{12}	1278.3	1278.7
			Gln^{13}-Lys^{27}	1921.7	1921.8
			Asn^{28}-Ser^{39}	1024.1	1045.6[a]
CAM1-Ex4	4576.6	4576.0	His^{1}-Lys^{12}	1278.3	1278.1
			Gln^{13}-(Lys^{27}-CA)-Ser^{39}	3316.3	3314.9
CAM2-Ex4	4576.6	4575.7	His^{1}-(Lys^{12}-CA)-Lys^{27}	3571.6	3571.3
			Asn^{28}-Ser^{39}	1024.1	1045.3[a]
CADi-Ex4	4967.2	4966.3	His^{1}-(Lys^{12}-CA)-(Lys^{27}-CA)-Ser^{39}	4967.2	4966.7
DAM1-Ex4	4560.6	4560.4	His^{1}-Lys^{12}	1278.3	1278.6
			Gln^{13}-(Lys^{27}-DA)-Ser^{39}	3300.3	3301.0
DAM2-Ex4	4560.6	4560.7	His^{1}-(Lys^{12}-DA)-Lys^{27}	3555.6	3556.1
			Asn^{28}-Ser^{39}	1024.1	1045.5[a]
DADi-Ex4	4935.2	4934.9	His^{1}-(Lys^{12}-DA)-(Lys^{27}-DA)-Ser^{3}	4935.2	4935.0
LAM1-Ex4	4544.6	4543.9	His^{1}-Lys^{12}	1278.3	1278.5
			Gln^{13}-(Lys^{27}LA)-Ser^{39}	3284.3	3284.8
LAM2-Ex4	4544.6	4544.7	His^{1}-(Lys^{12}-LA)-Lys^{27}	3539.6	3540.4
			Asn^{28}-Ser^{39}	1024.1	1045.7[a]
LADi-Ex4	4903.2	4902.4	His^{1}-(Lys^{12}-LA)-(Lys^{27}-LA)-Ser^{39}	4903.2	4904.2

[a] Mis-matchings on the observed mass to the calculated mass of this fragment originated because of adduct formation with the sodium ion.

Table 2. Summary of GLP-1 Receptor Binding Assay of Exendin-4 and BA-Ex-4s

samples	potency (IC_{50}, nM)	efficacy (at 10 nM, %)	samples	potency (IC_{50}, nM)	efficacy (at 10 nM, %)
Ex4	0.14 ± 0.02	98.51 ± 0.56	DAM1-Ex4	0.55 ± 0.05	87.35 ± 1.40
CAM1-Ex4	0.48 ± 0.05	93.26 ± 1.11	DADi-Ex4	10.10 ± 0.56	49.81 ± 0.52
CAM2-Ex4	0.37 ± 0.08	92.43 ± 1.13	LAM1-Ex4	0.58 ± 0.03	93.80 ± 1.30
CADi-Ex4	3.18 ± 0.51	70.50 ± 1.48	LADi-Ex4	71.96 ± 5.34	13.74 ± 5.49

were over 95% (95.2−99.0%, Supporting Information, Table S1 and Figure S2C.)

HPLC-purified exendin-4 and BA-Ex4s were further identified by MALDI-TOF MS. As shown in Figure 1C and Table 1, the experimental mass-to-charge (m/z) ratios of the BA-Ex4s closely matched the theoretical calculated values. Positional isomers were also confirmed by MALDI-TOF MS after the Lys-C digestion of BA-Ex4s. The monosubstituted BAM1-Ex4s and BAM2-Ex4s produced different MS spectra that closely corresponded to expectations (Figure 1D, Table 1, and Supporting Information, Figure S3).

Biological Activities of the BA-Ex4s. The chemical conjugation of BAs to exendin-4 resulted in a reduction of their GLP-1 receptor binding affinity. As shown in Figure 2A and Table 2, mono-BA substitutions reduced the binding affinity for the GLP-1 receptor by 3−4-fold versus that of exendin-4 (mean potency values (IC_{50} values) of exendin-4, CAM1-Ex4, and CAM2-Ex4 were 0.14, 0.48, and 0.37 nM, respectively). However, disubstitutions resulted in serious reductions in receptor binding affinities (e.g., 3.18 nM for CADi-Ex4, a 23-fold reduction). In addition, although the BA type did not significantly alter the receptor bindings of the BAM1-Ex4 series, disubstitution caused marked receptor binding reduction by increasing hydrophobicity (Supporting Information Figure S4). Interestingly, the efficacy values of BAM1-Ex4s were similar to that of exendin-4 (at 10 nM, Table 2). For this reason, given the high conjugation yields obtained, the following pharmaceutical experiments were performed using BAM1-Ex4s.

Chemical modifications with BAs altered the biological activities of exendin-4. Insulinotropic activity testing revealed that similar amounts of insulin were secreted from rat islets in the presence of 16.7 mM glucose in the presence of 10 nM of exendin-4, CAM1-Ex4, DAM1-Ex4, or LAM1-Ex4 (131.13 ± 6.89, 142.49 ± 21.45, 120.52 ± 13.18, and 125.68 ± 20.45 ng/mL/20 islets, respectively, after stimulation for 2 h) and that all showed significant increases versus that of untreated controls (58.32 ± 6.35 ng/mL/20 islets; $p < 0.01$) (Figure 2B). In addition, the insulinotropic activities of exendin-4 and BAM1-Ex4s clearly disappeared under hypoglycemic conditions (2.2 mM glucose).

The in vivo antidiabetic (hypoglycemic) activities of BAM1-Ex4s were examined by intraperitoneal glucose tolerance testing (IPGTT). As illustrated in Figure 2C, the administration of exendin-4 or BAM1-Ex4s (10 nmol/kg, s.c.) resulted in a significant increase in glucose tolerance patterns. Mean blood glucose level in the control group (PBS injection) rapidly increased to 20.96 ± 2.20 nM at 30 min after a 1 g/kg glucose challenge (i.p.) and showed a slow reduction in the hyperglycemic state at 120 min after the glucose challenge (13.14 ± 0.90 nM). However, exendin-4 and BAM1-Ex4 treatments (30 min before glucose injection) dramatically enhanced glucose tolerances and reduced average glucose levels to ∼9 and ∼5 nM at 30 and 120 min after the glucose challenge, respectively. Furthermore, calculated glucose AUC values revealed that the antidiabetic effects of BAM1-Ex4s and exendin-4 were similar in vivo (Figure 2D).

Albumin Binding and in Vivo Pharmacokinetics. Derivatizations at the lysine residues of exendin-4 by BAs were found to change its physicochemical properties dramatically, and one of the most promising of these was that they enhanced its affinity for serum albumin. As illustrated in Figure 3A, 31.5 ± 5.5% of exendin-4 was found to associate with albumin resin under physiological conditions (in PBS pH 7.4). However, BA conjugation increased this association between BAM1-Ex4s and albumin resin to 49.0 ± 3.2, 56.0 ± 6.3, and 78.8 ± 4.2% by CAM1-Ex4, DAM1-Ex4, and LAM1-Ex4, respectively.

BA conjugations also significantly enhanced pharmacokinetic profiles in vivo (SD rat), as illustrated in Figure 3B

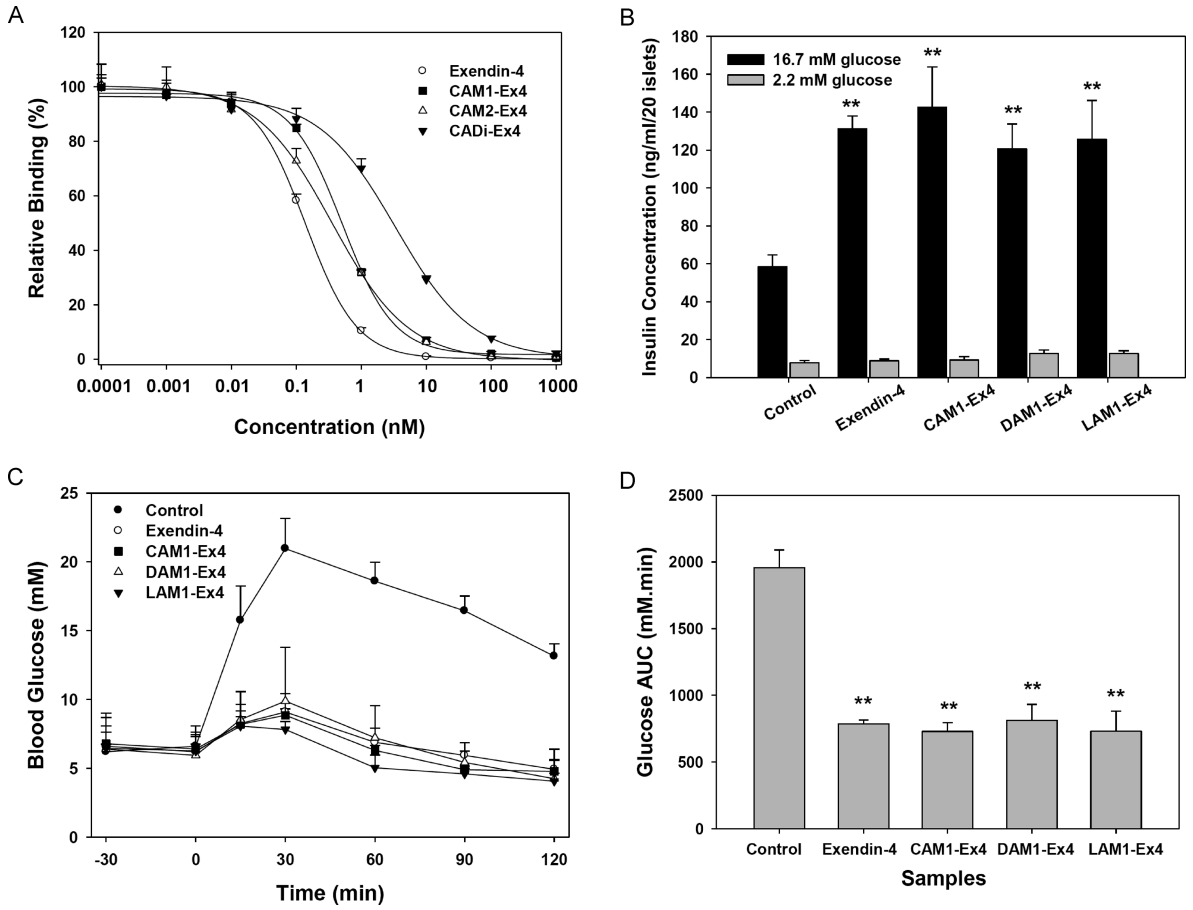

Figure 2. In vitro and in vivo biological activity tests on BA-Ex4s. (A) Competitive GLP-1 receptor binding characteristics of exendin-4 and of its CA conjugated derivatives on RIN-m5F insulinoma cells ($n = 3$). (B) Insulinotropic activities of exendin-4 and BAM1-Ex4s (10 nM) on isolated rat islets ($n = 6$). (C) The glucose lowering effects of exendin-4 and BAM1-Ex4s (10 n mol/kg) as determined by IPGTT experiments in db/db mice. (D) Hypoglycemic effects of exendin-4 and BAM1-Ex4s expressed as glucose $AUC_{0-120\ min}$ ($n = 4$). Means ± SDs, ** $p < 0.01$ compared with untreated controls.

Table 3. Pharmacokinetic Parameters after s.c. Injection of Exendin-4 or BAM1-Ex4s on SD Rats[a]

samples	T_{max} (h)	C_{max} (ng/mL)	AUC (ng·hr/mL)	$t_{1/2}$ (h)	Vd/F (L)	CL/F (L/h)	MRT (h)
exendin-4	0.63 ± 0.14	152.22 ± 16.88	291.88 ± 47.90	0.55 ± 0.06	0.11 ± 0.01	0.15 ± 0.02	1.24 ± 0.07
CAM1-EX4	1.00 ± 0.40	463.01 ± 61.67	994.65 ± 45.73	1.65 ± 0.33	0.10 ± 0.02	0.04 ± 0.00	2.57 ± 0.35
DAM1-EX4	1.31 ± 0.38	434.15 ± 85.36	3286.46 ± 554.42	5.01 ± 0.55	0.10 ± 0.03	0.01 ± 0.00	7.31 ± 0.70
LAM1-EX4	2.13 ± 0.63	414.23 ± 43.56	4780.40 ± 958.50	7.95 ± 1.27	0.10 ± 0.03	0.01 ± 0.00	11.84 ± 1.72

[a] Data are the mean ± SD ($n = 4$). T_{max}, time to reach maximum plasma concentration; C_{max}, maximum plasma concentration, AUC, area under the curve; $t_{1/2}$, elimination half-life; Vd/F, volume of distribution; CL/F, clearance; MRT, mean residence time.

and Table 3. After s.c. injections, the plasma concentration of exendin-4 rapidly increased and peaked at levels within 1 h ($t_{max} = 0.6 \pm 0.1$ h), after which they rapidly declined to baseline at 4 h postinjection with a calculated elimination half-life ($t_{1/2}$) of 0.55 ± 0.1 h. However, the BAM1-Ex4s showed slightly delayed absorption patterns postinjection, and t_{max} values increased according to the hydrophobicities of BAs (1.0 ± 0.4, 1.3 ± 0.4, and 2.1 ± 0.6 h by CAM1-Ex4, DAM1-Ex4, and LAM1-Ex4, respectively). Furthermore, dramatic enhancements of pharmacokinetic parameters such as elimination half-lives and areas under the curve (AUC) were observed for BAM1-Ex4s. In terms of elimination half-lives, 3.0-, 9.1-, and 14.5-fold enhancements were observed versus CAM1-Ex4, DAM1-Ex4, and LAM1-Ex4, respectively (Table 3). AUC calculations revealed similar increases.

Antidiabetic Effects of BAM1-Ex4s. Subcutaneous injections of exendin-4 or of BAM1-Ex4s into diabetic mice normalized blood glucose levels, and normoglycemia durations were found to be strongly dependent on BA conjugations. As illustrated in Figure 4A, blood glucose levels in control mice (PBS injected) maintained a hyperglycemic state (average >15 mM), independent of measuring time. However, exendin-4 injections (15 nmol/kg) rapidly normalized blood glucose for 6 h postinjection, and the glucose level later increased at 8 h postinjection. In the case of BAM1-Ex4s, normoglycemic durations were found to be closely related with their physicochemical and pharmacokinetic characteristics. The injections with BAM1-Ex4s induced normoglycemia at 8, 12, and >24 h after CAM1-Ex4, DAM1-Ex4, and LAM1-Ex4 injections, respectively. The blood glucose levels in LAM1-Ex4 injections showed that glycemic control was achieved over 24 h by a single injection (average 9.3 mM blood glucose at 24 h postinjection). Calculated glucose AUC values also revealed that BAM1-Ex4s had greater antidiabetic effects than exendin-4

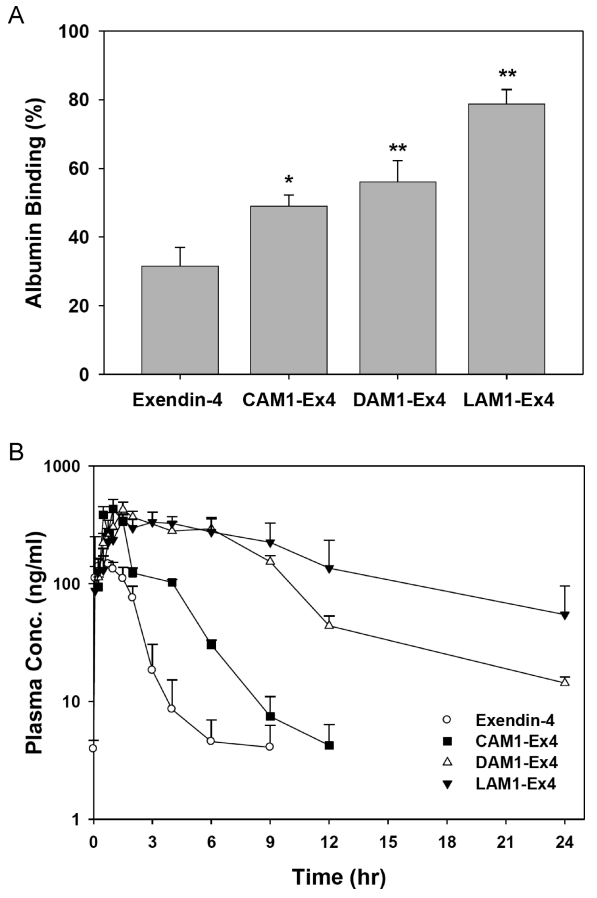

Figure 3. Physicochemical and pharmacokinetic characterizations of BAM1-Ex4s. (A) The albumin binding capacities of exendin-4 and BAM1-Ex4s ($n = 3$). (B) Pharmacokinetic profiles of exendin-4 and BAM1-Ex4s after subcutaneous administration (10 nmol/rat, $n = 4$). Means ± SDs, * $p < 0.05$ and ** $p < 0.01$ as compared with exendin-4.

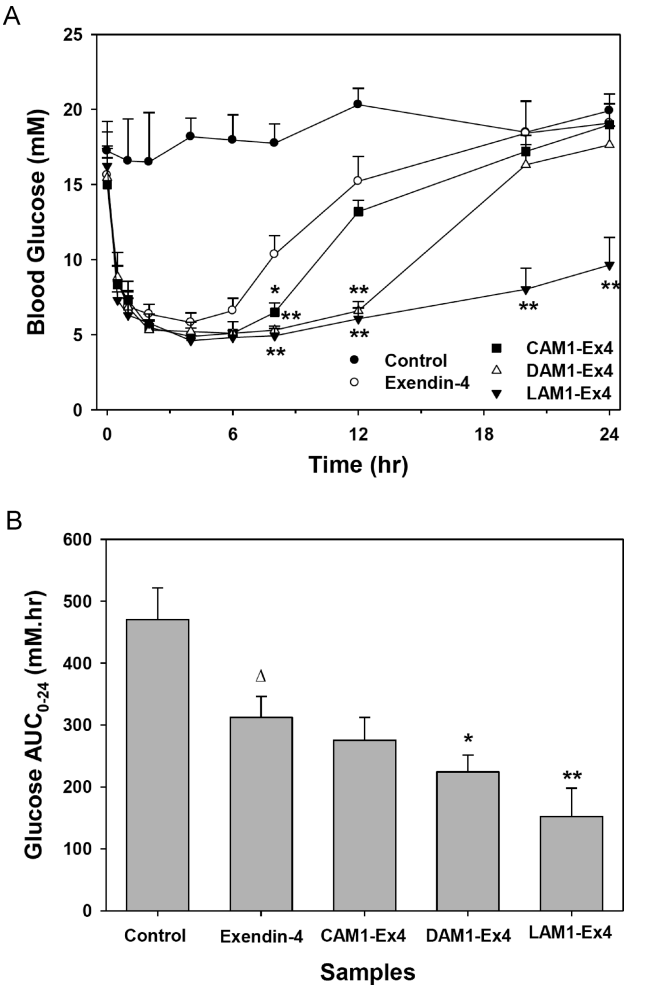

Figure 4. Glucose lowering and stabilizing effects of exendin-4 and BAM1-Ex4s. (A) Time-course average blood glucose levels of db/db mice after an s.c. injection of exendin-4 or BAM1-Ex4s (15 nmol/kg, $n = 6$). (B) Hypoglycemic effects of exendin-4 and BAM1-Ex4s based on the calculated glucose AUC_{0-24} values. Means ± SDs, * $p < 0.05$ and ** $p < 0.01$ compared with exendin-4 treatment. $^{\Delta}p < 0.05$ compared with the control group.

(Figure 4B, $p < 0.05$ and < 0.01 for DAM1-Ex4 and LAM1-Ex4 versus exendin-4, respectively).

Discussion

GLP-1 is a type of incretin hormone that is released by intestinal L cells after a meal and has attracted much interest in the context of type 2 diabetes because of its multiple antidiabetic effects.[3−5] GLP-1 and its receptor agonists share several antidiabetic actions such as the stimulation of glucose-dependent insulin secretion, the suppression of glucagon secretion, the reduction of gastric mobility and food intake, and the improvement of pancreatic endocrine functions through β-cell proliferation and/or neogenesis.[3−5] Intensive research and developmental efforts have resulted in the clinical application of the DPP IV resistant GLP-1 receptor agonist of exendin-4, which was first isolated from lizard saliva.[6,7,22]

Although the coadministrations of exendin-4 and traditional hypoglycemic agents achieve better glycemic control with significant reductions in serum glycated hemoglobin (HbA_{1c}) levels and body weight,[7,22] the demand for more efficient incretin-based antidiabetics has not been satisfied because the short biological half-life of exendin-4 caused its rapid renal clearance.[8,13] In addition, recent research on long-acting incretin mimetics or sustained release formula exendin-4 systems have achieved more efficient glycemic control than twice daily exendin-4 injections.[16,23]

With the specific aims of developing long-acting incretin mimetics, we designed exendin-4 derivatives with lysine-residue-specific BA conjugations. In addition, we considered that the use of BAs with different physicochemical characteristics might allow the tailoring of BA-Ex4s. Nine different BA-modified exendin-4 adducts were successfully prepared using lysine-residue-specific coupling reactions using three BAs (CA, DA, and LA) (Figure 1). Consistent with our previous findings,[24] conjugation reactions were found to occur predominantly at Lys[27]. The BAM1-Ex4s produced eluted faster than BAM2-Ex4s by HPLC. Interestingly, lysine-specific conjugations prevented the serious activity reductions caused by N-terminal conjugation (the N-terminal domain is known to play a crucial role during the cellular response to GLP-1 signaling).[9,20,21]

The following biological experiments provide direct evidence of the relevant structure−activity relationships. As illustrated in Figure 2, the monosubstitution of lysine residues resulted in 3−4-fold reductions in receptor binding potentials, independent of attached bile acid type and conjugated position. However, BA disubstitutions (BADi-Ex4) showed slightly different receptor binding patterns, and in particular,

receptor affinity reduced on increasing the hydrophobicity of the bile acid, presumably because of greater inter- and/or intramolecular hydrophobic interactions between the conjugated bile acid moieties.[25,26] Like the negligible reductions observed in receptor binding efficacy (based on 10 mM bindings), the insulinotropic activities (BAM1-Ex4s at 10 nM) and hypoglycemic efficacies (BAM1-Ex4s, 10 nmol/kg) of BAM1-Ex4s showed well-preserved biological activities versus those of exendin-4 (Figure 2B−D).

The most interesting findings of this study concerned albumin binding by BAM1-Ex4s and their pharmacokinetic and antidiabetic characteristics. Consistent with previous successful reports regarding the protracted in vivo circulation half-lives of fatty acid-modified insulin or GLP-1 analogues, attributed to their physical interactions with serum albumin,[16,18,27] bile acid conjugation was also found to enhance the interactions between exendin-4 adducts and albumin. Moreover, the strength of this interaction increased with BA hydrophobicity (Figure 3A). Detailed pharmacokinetic analysis revealed that BA conjugations not only extended plasma circulation characteristics (LAM1-Ex4 had an elimination half-life 14.5-fold that of exendin-4) but also dramatically increased drug utilizations, as represented by AUC values (the AUC value of LAM1-Ex4 was 16.4-fold that of exendin-4). Thus, LAM1-Ex4 was found to extend normoglycemia by 3-fold duration and to have an in vivo hypoglycemic effect (calculated by glucose AUC reduction) twice that of exendin-4, despite the fact that LAM1-Ex4 had less (4.1-fold lower) receptor binding potency than exendin-4. Furthermore, these albumin binding, pharmacokinetic, and antidiabetic characteristics of LAM1-Ex4 were also found for CAM1-Ex4 and DAM1-Ex4, and this included the strong relationship between antidiabetic efficacies and the hydrophobic nature of the conjugated BAs. In addition, the obtained successful glycemic control (> 24 h) by a single s.c. injection of 15 nmol/kg LAM1-Ex4 is comparable to that of the other long-acting incretin mimetics such as palmitic acid-acylated GLP-1 (Liraglutide, 300 μg/kg twice daily injections in db/db mice),[27] fatty acid-acylated GIP (> 24 h normoglycemia by a single 12.5 nmol/kg i.p. injection in ob/ob mice),[14] and albumin-conjugated GLP-1 (CJC-1131, ~10 h glucose normalization by 100 nmol/kg s.c. injection).[12]

In summary, the present study demonstrates that the substitutions of Lys^{12} and/or Lys^{27} residues of exendin-4 by BAs offer a useful approach to the development of long-acting incretin-based antidiabetics. In addition, monosubstitution at Lys^{27} by BAs appears to offer a better approach in terms of minimizing biologic and antidiabetic activity loss. Furthermore, the extended efficacy of LAM1-Ex4 in vivo, as determined by pharmacokinetic and antidiabetic evaluations, suggests that it has strong clinical potential as an efficient GLP-1 receptor agonist and as a hypoglycemic agent in type 2 diabetes.

Experimental Section

Materials and Animals. Exendin-4 was purchased from American Peptide, Inc. (Sunnyvale, CA). The cholic acid derivatives of cholic acid (CA), deoxycholic acid (DA), and lithocholic acid (LA) were purchased from Sigma-Aldrich Co. (Saint Louis, MO) and converted into the *N*-hydroxysuccinimide ester forms as described previously.[28] Insulin EIA kits were purchased from Mercodia (Uppsala, Sweden). All other reagents, unless indicated, were purchased from Sigma-Aldrich Co. (Saint Louis, MO) and were used as received. Type 2 diabetic C57BL/6 db/db mice (male, 6−8 weeks old) were obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejon, Korea). Male Sprague−Dawley rats (SD rat, 200−250 g) used for pancreatic islet isolation and pharmacokinetic studies were purchased from the Hanlim Experimental Animal Laboratory (Seoul, Korea). All animals were cared for according to the guidelines issued by the National Institutes of Health (NIH) for the care and use of laboratory animals (NIH publication 85-23, revised 1985). The animals were maintained on a 12-h light/12-h dark cycle with access to food and water ad libitum, except where mentioned.

Preparation of Bile Acid (BA)-Acylated Exendin-4s. BA acylations at the lysine residues of exendin-4 were performed using a coupling reaction between NHS-activated BAs (BA-NHS) and the lysine residues (Lys^{12} and Lys^{27}) of exendin-4 in organic solvent.[24] Briefly, 100 μL, 75 μL, and 50 μL of CA-NHS (1.2 mg/mL) in dimethylsulfoxide (DMSO) containing 3% triethylamine (TEA) were mixed with 100 μL of exendin-4 (5 mg/mL in DMSO) (BA-NHS/Exendin-4 molar ratios of 2, 1.5, and 1, respectively) with gentle mixing at room temperature for 1 h. The reaction was then stopped by adding 100 μL of stop solution (deionized water containing 1% trifluoroacetic acid (TFA)). Other bile acid derivatives of exendin-4 with DA or LA were prepared in the same manner.

Site-specific positional isomers, such as Lys^{27}-BA-Exendin-4 (denoted as BAM1-Ex4), Lys^{12}-BA-Exendin-4 (BAM2-Ex4), and $Lys^{12,27}$-DiBA-Exendin-4 (BADi-Ex4) were purified from their reaction mixtures by semipreparative reversed phase HPLC (RP-HPLC) using a Capcell-pak RP-18 column (250 × 10 mm, 5 μm, Shiseido, Japan) at room temperature at a constant flow rate of 5.0 mL/min under UV absorbance monitoring at 215 nm. The mobile phase consisted of 0.1% TFA in deionized water (eluent A) and acetonitrile containing 0.1% TFA (eluent B) linear gradient (35−90% B over 30 min). HPLC fractions corresponding to respective peaks were collected separately, acetonitrile was flushed off with nitrogen, and solutions were concentrated using Centricon-10 (MW cut off 3000, Millipore Corp., Billerica, MA). The products obtained were characterized by analytical HPLC and MALDI-TOF mass spectroscopy, and stored at −70 °C until required.

Characterizations of BA-Ex4s. To confirmed BA acylations of exendin-4 and their purities, the BA-Ex4s produced were further analyzed by analytical RP-HPLC using a Capcell-pak RP-18 column (250 × 4 mm, 5 μm) and the above-mentioned binary gradient system. Further characterizations regarding the numbers and positions of acylations were carried out by MALDI-TOF MS before and after lysyl endoproteinase Lys-C digestion. First, BA bioconjugations were confirmed using MALDI-TOF MS molecular weight measurements using a Voyager-RP Biospectrometry Workstation (PerSeptive Biosystems, Cambridge, MA). Briefly, sample−matrix solutions were prepared by mixing a 1 μL aliquot with 2 μL of matrix solution (a saturated solution of α-cyanohydroxycinnamic acid (α-CHCA) in 50% of water/ACN containing 0.1% (v/v) TFA). One microliter of the sample−matrix solution was then deposited into a well of a sample plate and dried rapidly by vacuum evaporation. Data generated by 2 ns pulses of a 337 nm nitrogen laser were averaged for each spectrum in reflective mode; positive ion TOF detection was performed using an accelerating voltage of 25 KV.

To confirm the position of BA acylation sites, Lys-C digestion experiments were performed. Briefly, 5 μL of Lys-C (10 μg/mL, pH 8.5) was added to 10 μL of sample solution (ca. 500 μg/mL, 50 mM Tris-HCl, and buffer at pH 8.5) containing a BA-Ex4 positional isomer, and the reaction mixture were then incubated at 37 °C for 60 min. After Lys-C digestion, samples were analyzed by MALDI-TOF-MS, as described above.

Biological Activity Tests. Two different in vitro activity methods, namely, insulinotropic activity testing and a GLP-1 receptor binding assay were employed. Receptor binding assays

were performed using rat insulinoma of RIN-m5F cells (ATCC, Manassas, VA) using a slight modification of a previously described method.[24] Briefly, RIN-m5F cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator. They were seeded in 12 well plates at 3×10^5 cells per well and grown for 48 h. They were washed twice with binding buffer (120 mM NaCl, 1.2 mM $MgSO_4$, 13 mM sodium acetate, 5 mM KCl, 1.2 g/L TRIS, 2 g/L bovine serum albumin, and 1.8 g/L glucose, pH 7.6) and cotreated with unlabeled BA-Ex4 samples (final concentration range $10^{-4}-10^3$ nM) and 30 pM of ^{125}I-Exendin-4 (exendin-4 (9−39), PerkinElmer, Boston, MA) for 2 h at room temperature. The cells were washed three times with chilled PBS containing 1 mg/mL of bovine serum albumin, lysed with cell lysis buffer (0.5 N NaOH with 1% SDS) for 15 min, and ^{125}I contents in lysates were measured using a γ-counter (GMI, Inc., Ransey MN).

The insulinotropic activities of exendin-4 and BAM1-Ex4s on rat pancreatic islets were determined as previously described.[9,24] Briefly, male Sprague−Dawley rats (SD rats, 250 g body weight) were anesthetized with ketamine and xylazine (90/10 mg/kg, i.p.). After opening the abdominal cavity, the pancreas was expanded by injecting cold Hank's balanced salt solution (HBSS) containing 1.5 mg/mL of type V collagenase, disintegrated by enzyme activation (37 °C, 15 min), and islets were isolated and purified by discontinuous Ficoll (Amersham Biosciences AB, Uppsala, Sweden) gradient centrifugation at 1900*g* for 25 min. Purified islets were cultured in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA) and 1% penicillin−streptomycin (Gibco) at 37 °C in a 95% air/5% CO_2 atmosphere. After a 2-day maintenance period, islets were washed and incubated in Krebs−Ringer HEPES (KRH) buffer (119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgCl_2$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, and 1 g/L BSA), seeded at 20 islet/well on 24-well plates in 1 mL of KRH buffer containing 16.7 mM glucose or 2.2 mM glucose, and then were incubated with 10 nM of exendin-4, CAM1-Ex4, DAM1-Ex4, or LAM1-Ex4 for 2 h. Insulinotropic activities were evaluated by measuring the amount of insulin released to media using an insulin EIA kit.

The in vivo bioactivities of exendin-4 and BAM1-Ex4s were evaluated by intraperitoneal glucose tolerance testing (IPGTT) in type 2 diabetic db/db mice after subcutaneous drug administration.[24] In brief, 18 h-fasted mice were randomly allocated to five groups (4 mice per group) and then administered normal saline, exendin-4, or one of the three BAM1-Ex4s (10 nmol/kg, s.c., 30 min prior to glucose administration). A 1.0 g/kg dose of glucose was then administered intraperitoneally to each mouse. At predetermined times, blood glucose levels were monitored using a glucometer (Accu-Chek Sensor, Roche Diagnostics Corp., Mannheim, Germany).

Physicochemical and Pharmacokinetic Characterizations of the BAM1-Ex4s. The albumin binding characteristics of the BAM1-Ex4s were investigated using a modified albumin-binding assay using albumin-conjugated Sepharose resin, according to the manufacturer's instructions. Briefly, human serum albumin (HSA; 30 mg in 10 mM PBS, pH 7.4) and NHS-activated Sepharose 4 fast flow (4 mL, wet resin volume, Amersham Bioscience, Sweden) were mixed and allowed to react by gentle shaking at room temperature for 4 h. The albumin-conjugated resin was then recovered by centrifugation, followed by triple PBS washing. The HSA content in the resin used was 6−7 mg/mL of wet resin. An albumin-free control resin was prepared by inactivating the resin with hydrolysis of the NHS-active ester. For the albumin binding study, exendin-4 or BAM1-Ex4s (100 μg/mL in PBS, 50 μL) were mixed with HSA resin and incubated for 2 h at room temperature. The resin and supernatant were separated by centrifugation (1000 *g*, 10 min), and unbound peptide contents were determined using a Micro BCA Protein assay kit (PIERCE, Rockford, IL). In a similar manner, the nonspecific absorptions of the peptides on albumin-free resin were determined using NHS-inactivated resin.

The pharmacokinetic profiles of s.c. exendin-4 and BAM1-Ex4s were assessed as previously described.[24,29] Briefly, the jugular veins of male Sprague−Dawley rats (body weight 200 g, 4 rats per group) were cannulated one day before the experiment. Exendin-4 or its derivatives (10 nmol/rat, 200 μL, s.c.) were then administered. Blood samples, drawn at predetermined times, were placed in chilled polyethylene tubes containing anticoagulant (heparin solution, 5000 U/mL, 1/100 volume of blood), and plasma samples were obtained by centrifugation and stored at −70 °C until required. The plasma concentrations of exendin-4 and its derivatives were determined using exendin-4 EIA kits (Phoenix Pharmaceuticals, Inc., Burlingame, CA). Data were analyzed for individual rats using noncompartment models.

In Vivo Antidiabetic Activities of BA-Ex4s. Six-week-old male C57BL/6 db/db mice were used for the acute antidiabetic activity tests, after being acclimatized for 1-week in our animal facility. Under nonfasting conditions with free access to water and food, mice were administered a single subcutaneous injection of exendin-4 or BAM1-Ex4s (15 nmol/kg, 200 μL, s.c., 6 mice per group), and blood glucose levels were then monitored using a glucometer and tail-tip blood samples (0, 0.5, 1, 2, 4, 6, 8, 12, 20, and 24 h after administration).

Statistical Analysis. Data are expressed as the means ± SDs. The student's *t*-test was used throughout, and values of $p < 0.05$ were considered statistically significant.

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Supporting Information Available: Purity table, HPLC chromatography and reaction optimization, HPLC analysis of DA-Ex4s and LA-Ex4s, MALDI-TOF MS spectra of BA-Ex4s and their Lys-C digested fragments, and the receptor binding characteristics of BAM1-Ex4s and BADi-Ex4s. This material is available free of charge via the Internet at http://pubs.acs.org.

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