Bioconjugate Chemistry

Article

Cite This: *Bioconjugate Chem.* XXXX, XXX, XXX–XXX

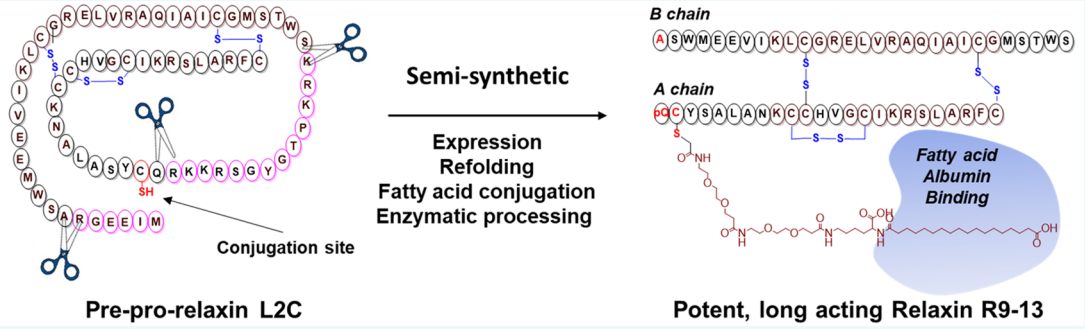
pubs.acs.org/bc

Design and Synthesis of Potent, Long-Acting Lipidated Relaxin-2 Analogs

Avinash Muppidi, Sang Jun Lee, Che-Hsiung Hsu, Huafei Zou, Candy Lee, Elsa Pflimlin, Madhupriya Mahankali, Pengyu Yang, Elizabeth Chao, Insha Ahmad, Andreas Crameri, Danling Wang, Ashley Woods, and Weijun Shen*

Calibr at the Scripps Research Institute, 11119 North Torrey Pines Road, La Jolla, California 92037, United States

Supporting Information



ABSTRACT: Peptide hormone relaxin-2, a member of the insulin family of peptides, plays a key role in hemodynamics and renal function and has shown preclinical efficacy in multiple disease models, including acute heart failure, fibrosis, preeclampsia, and corneal wound healing. Recently, serelaxin, a recombinant version of relaxin-2, has been studied in a large phase 3 clinical trial (RELAX-AHF-2) for acute decompensated heart failure patients with disappointing outcome. The poor *in vivo* half-life of relaxin-2 may have limited its therapeutic efficacy and long-term cardiovascular benefit. Herein, we have developed a semisynthetic methodology and generated potent, fatty acid-conjugated relaxin analogs with long-acting pharmacokinetic (PK) profile in rodents. The enhanced PK properties translated into improved and long-lasting pharmacodynamic effect in pubic ligament elongation (PLE) studies. The resultant novel relaxin analog, **R9-13**, represents the first long-acting relaxin-2 analog and could potentially improve the clinical efficacy and outcome for this important peptide hormone. This semisynthetic methodology could also be applied to other cysteine-rich peptides and proteins for half-life extension.

Relaxin initiates its functions through binding to LGR7, a member of the leucine-rich repeat-containing G-protein coupled receptor family, also known as RXFP1 (relaxin family peptide receptor 1).[1] Relaxin-2 plays a significant role in reproduction and various physiological and pathological processes. Its role in improving hemodynamics and renal function has been associated with reduced complications that arise during heart failure.[2] Serelaxin, a recombinant version of human relaxin-2 developed by Novartis/Corthera, is an investigational drug for the treatment of acute heart failure (AHF).[3] In phase II and first phase III clinical studies, serelaxin provided greater dyspnoea relief in patients with AHF compared to the placebo. The average length of the patient hospital stay and early worsening of heart failure symptoms at 6 months were also decreased up to 30% by serelaxin.[4] However, the second phase III study in more than 6000 patients (RELAX-AHF-2) showed that the short-term intravenous infusion of serelaxin does not improve cardiovascular mortality in patients with acute decompensated heart failure (ADHF) compared to placebo.[5] Besides these clinical studies in heart failure, serelaxin has also shown preclinical efficacy in fibrosis, preeclampsia, and corneal would healing.[6] Being a short-lived peptide with an *in vivo* half-life of approximately 10−20 min,[7] the mode of treatment for serelaxin in the hospitalized AHF patients is continuous infusion for 48 h. The poor *in vivo* half-life of serelaxin may have limited its therapeutic efficacy, and hence optimization of the pharmacokinetic (PK) properties could potentially change the clinical outcome and the fate of this important peptide hormone. People have reported relaxin-2 analogs, single chain relaxin (B-chain), and small molecule LGR7 agonists;[8−10] however, nothing has been evaluated in the clinical setting beyond serelaxin. Here we have developed a semisynthetic methodology where we recombinantly expressed prepro-relaxin in *E. coli* and refolded into pro-relaxin, conjugated with serum binding motifs, and enzymatically processed to generate long-acting relaxin-2 analogs with a PK profile superior to that of the native relaxin-2 when evaluated in rodents (Figure 1). The

Received: October 22, 2018
Revised: November 27, 2018

DOI: 10.1021/acs.bioconjchem.8b00764
Bioconjugate Chem. XXXX, XXX, XXX–XXX

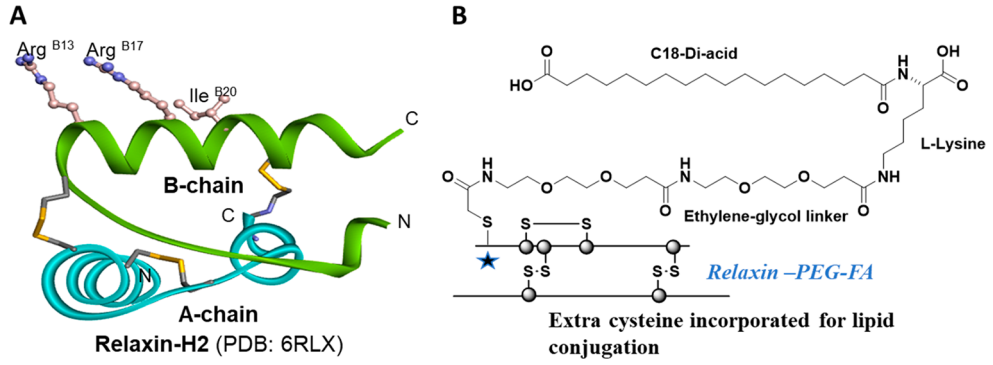


Figure 1. Strategy to generate long-acting versions of relaxin-2. (A) Structure of relaxin-2 depicted in the ribbon diagram highlighting the key residues (Arg, Arg, and Ile) vital for the interaction and activation of LGR7 receptors. (B) Long-acting relaxin-2 analogs through site-specific fatty acid conjugation through the cysteine introduced in recombinant relaxin-2.

enhanced PK properties translated into long-lasting pharmacodynamic (PD) effect in pubic ligament elongation (PLE) studies. These potent long-acting relaxin analogs could potentially translate into once weekly injectables in human, avoid patient admission into hospitals and improve patient compliance and potentially clinical efficacy in acute and chronic heart failure.

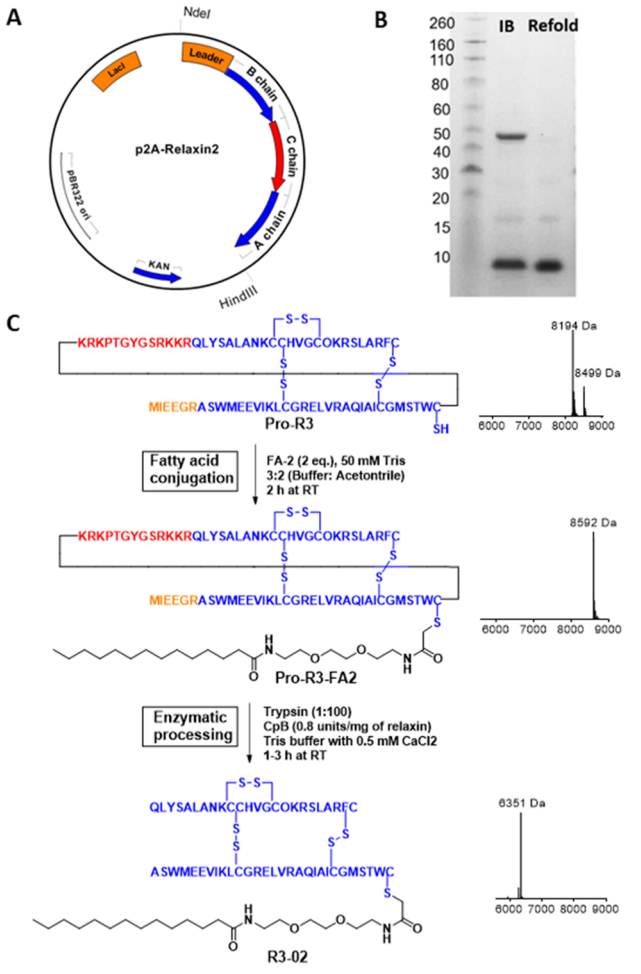
Wild type human relaxin-2 and analogs have been synthesized chemically by solid-phase peptide synthesis followed by random chain combination or regioselective disulfide bond formation,[11−15] while serelaxin is synthesized through recombinant expression in *E. coli*.[16] To generate wild type-like dual chain relaxin-2, we decided to pursue a recombinant approach and designed relaxin constructs (Figure 2Aand Figure S3) containing both a leader sequence (MIEEGR) at the N-terminus of the B-chain and a mini-C-chain linker (KRKPTGYGSRKKR) connecting B and A chains, which can eventually be cleaved off to produce mature wt relaxin.[17] The prepro-relaxin was refolded from purified inclusion bodies and the pro-relaxin was subjected to limited proteolysis with trypsin and carboxypeptidase B (Cp-B) in 0.1 M Tris buffer for 1−2 h. The reaction was quenched by acidifying the solution to pH < 3 with 0.1 N HCl and subsequently purified by RP-HPLC. To evaluate the activity of the relaxin-2 analogs, we generated a LGR7-overexpressing HEK-293 stable cell line equipped with a Cre-Luc reporter (cyclic AMP response element-driven firefly luciferase) and the EC_{50} of each relaxin analog was determined from 3 independent experiments. The resultant relaxin (**R1**) was found to have equivalent activity to that of the commercially available relaxin (Figure S4). During the enzymatic processing, it appears that the cleavage between the Arg and the Asp 1 of the B-chain with trypsin is rate limiting, though extending the incubation time led to decreased yield and overdigestion at undesired sites. The presence of Asp at the P1′ position slows down the digestion process with trypsin.[18] To increase the efficiency, we mutated Asp 1 of the B-chain (a P1′ site) to Ala, yielding B:D1A relaxin (**R2**) with much improved yield (overall 30% after refolding and enzymatic cleavage) but with similar *in vitro* potency to wt relaxin (Figure S4). Thus, D1A-relaxin was used as the basis for generation of all the other relaxin analogs.

To extend the *in vivo* half-life of relaxin-2, we used a semisynthetic approach to recombinantly express relaxin-2 and then chemically conjugated it to a multiple-ethylene glycol (MEG) linked fatty acid. Fatty acids have been widely exploited for their inherent affinity toward serum albumin, a carrier protein that protects its bound peptides from renal clearance and proteolysis.[19] Long-acting insulin, including detemir and degludec, was achieved through site specific conjugation of fatty acids onto lysine residues in insulin.[20] The presence of multiple lysine residues in relaxin-2 is expected to complicate the homogeneous conjugation onto the peptide; therefore, we decided to incorporate a cysteine residue for fatty acid conjugation. Since relaxin already contains three endogenous disulfides from 6 cysteines, the introduction of an additional cysteine for conjugation purposes may interfere with the refolding process. At first, we designed relaxin B:S29C construct (B chain Ser 29 replaced with Cys) generating relaxin **R3**. Refolding was carried out overnight using the conditions similar to wt relaxin. LC-MS analysis indicated that ∼50% of the refolded **R3** existed as free thiol (MW 8194 Da) and the rest existed as the glutathione adduct (MW 8499 Da) (Figure 2). Reducing the adduct with TCEP, following a similar approach employed for BMP-2,[21] was not successful. In fact, the relaxin-glutathione adduct formation was found to be dependent on the refolding time and buffer composition. Adduct formation is correlated to the refolding time and to the ratio of the oxidized and the reduced glutathione (GSSG:GSH) in the refolding buffer (Table S1). Refolding in a buffer containing 5 mM of GSH and 1 mM of GSSG for 4 h led to >70% of refolded relaxin with a free thiol (Figure 2 and Table S1).

To facilitate cysteine conjugation, a series of fatty acids functionalized with α-bromo-acetamide and multiethylene glycol (MEG) spacers were synthesized (Figure S6). While the fatty alkyl chain length modulates protein half-life by promoting albumin binding, the spacer keeps the alkyl chain away from the protein and prevents potential interference in its binding toward LGR7. The presence of a terminal carboxylic acid in the alkyl chain is known to enhance the binding affinity toward albumin.[22] Thus, FA-1 to FA-12 were designed and synthesized using solid-phase peptide synthesis (SPPS) or solution-phase synthesis (Supporting Information).

All conjugations were performed in 50 mM Tris buffer (pH 8.0) containing 20−50% acetonitrile in water, depending on the solubility of the fatty acid used. Conjugation was usually complete in 2 h and the progress of the reaction was monitored by analytical HPLC. Conjugation was carried out prior to enzymatic processing, as the reverse order led to disulfide scrambling and loss of the product. The conjugation mixture was then lyophilized and reconstituted in 0.1 M Tris

DOI: 10.1021/acs.bioconjchem.8b00764
Bioconjugate Chem. XXXX, XXX, XXX−XXX

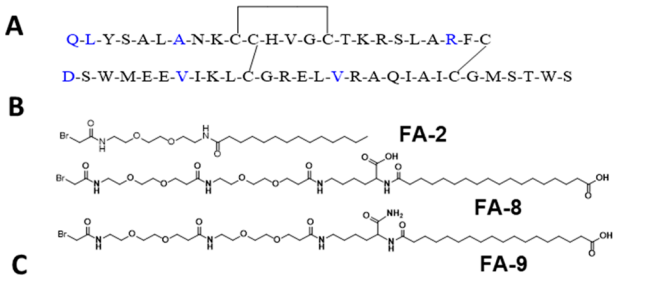


D

Analogue	Mutation	Fatty acid	MS		EC_{50} (nM)
			Theoretical	Found	
R2	B-D1A	none	5936.15	5936.92	0.025 ± 0.004
R3-01	B-S29C,D1A	FA-1	6205.14	6205.62	4.3 ± 0.55
R3-02	B-S29C,D1A	FA-2	6349.98	6350.52	0.028 ± 0.005
R3-08	B-S29C,D1A	FA-8	6752.87	6753.27	1.5 ± 0.18
R3-09	B-S29C,D1A	FA-9	6751.90	6752.49	0.8 ± 0.10

Figure 2. A representative scheme for generating relaxin analogs. Shown here is the process for generating the conjugate, **R3-02** from cloning to final product. (A) A desired relaxin gene was inserted into pBR322 and expressed in *E. coli* through arabinose induction. Inclusion bodies were isolated, solublized in 8 M guanidine-HCl and refolded using 5 mM GSH and 0.5 mM GSSG, in 0.1 M Tris buffer (pH 8.0). (B) Coomassie Blue-stained reducing SDS-PAGE of relaxin proteins before and after refolding. (C) The process of fatty acid conjugation and enzymatic processing with the corresponding deconvoluted masses; see Experimental Section and Supporting Information for detailed procedure. (D) LC-MS characterization, *in vitro* potencies of **R3**-based relaxin analogs in the aforementioned CRE-Luc reporter assay. EC_{50} values for activation of LGR7 were determined using a stable HEK-293 cell line overexpressing LGR7 and a CRE-Luc reporter. The assay was performed in triplicate in each experiment and the EC_{50} results were obtained from three independent experiments.

buffer (pH 8) for leader sequence and C-peptide cleavage with trypsin and Cp-B. Fatty acid-conjugated relaxin-2 analogs were



Analog	Cys Mutation	MEG-FA	Mass	EC_{50} (nM)
R4-02	A:Q1C;B:D1A	FA-2	6309.87	0.17 ± 0.03
R4-08	A:Q1C; B:D1A	FA-8	6712.45	0.33 ± 0.08
R4-09	A:Q1C; B:D1A	FA-9	6711.33	1.10 ± 0.13
R5-02	A:A5C; B:D1A	FA-2	6366.74	0.46 ± 0.08
R5-08	A:A5C; B:D1A	FA-8	6769.36	1.15 ± 0.20
R5-09	A:A5C; B:D1A	FA-9	6768.42	0.63 ± 0.08
R6-02	A:R18C; B:D1A	FA-2	6281.79	0.71 ± 0.07
R6-08	A:R18C; B:D1A	FA-8	6684.26	1.20 ± 0.14
R6-09	A:R18C; B:D1A	FA-9	6683.27	1.24 ± 0.34
R7-02	B:D1C	FA-2	6366.82	0.11 ± 0.02
R7-08	B:D1C	FA-8	6769.34	0.83 ± 0.14
R7-09	B:D1C	FA-9	6768.10	0.82 ± 0.13
	B: D1A, V7C	NA		
R8-02	B: D1A, V13C	FA-2	6338.86	0.31 ± 0.06
R8-08	B: D1A, V13C	FA-8	6741.48	0.38 ± 0.09
R8-09	B: D1A, V13C	FA-9	6740.55	0.47 ± 0.12
R9-02	A: L2C; B:D1A	FA-2	6324.72	0.16 ± 0.04
R9-08	A: L2C; B:D1A	FA-8	6727.34	0.21 ± 0.01
R9-09	A: L2C; B:D1A	FA-9	6726.39	0.72 ± 0.10

Figure 3. Cysteine residue variants of relaxin. (A) Cysteine positions (highlighted in blue) explored for conjugation. (B) Structures of fatty acids FA2, FA8, and FA9. (C) Table summarizing the LGR7 CRE-Luc reporter activity induced by relaxin-FA conjugates. EC_{50} values for activation of LGR7 were determined using a stable HEK-293 cell line overexpressing LGR7 and a CRE-Luc reporter. The assay was performed in triplicate for each experiment and the EC_{50} results were obtained from three independent experiments.

obtained at 7−10% overall yield from refolding, conjugation, and enzymatic cleavage, for subsequent biological testing (Figure 2). Various **R3** conjugates were evaluated in the CRE-Luc reporter assay and a particular trend in the potency of receptor activation was observed with respect to the fatty acids attached. Direct conjugation of a lipid chain without a spacer (FA-1) to relaxin was not tolerated (**R3-01**, 170-fold loss in potency compared to **R2**), while **R3-02**, generated through FA-2 conjugation, was equipotent to wt relaxin **R2** (Figure 2D and Table S2). A short spacer might avoid the interference of fatty acid with LGR7 receptor binding. To evaluate if the FA-2 conjugation protracts the *in vivo* half-life, we evaluated the PK profile of **R3-02** in mice. The concentrations of **R3-02** in plasma samples determined by luciferase assay were used to derive the half-lives. FA-2 conjugation onto relaxin extended the half-life from 0.3 h (wt) to 1 h (IV dosing, Table S2). In an attempt to further extend the half-life of the relaxin conjugates, we synthesized FA-8 and FA-9, with a C18 alkyl chain and a terminal acid and amide, respectively. FA-8 resembles the lipid component used for the once-weekly GLP-1 agonist semaglutide; therefore, we envisioned that conjugation of FA-8 onto relaxin would improve the half-life to a similar extent. FA-8 and FA-9 were conjugated onto relaxin S29C to generate **R3-08** and **R3-09**, which exhibited a 60- and 30-fold

DOI: 10.1021/acs.bioconjchem.8b00764
Bioconjugate Chem. XXXX, XXX, XXX−XXX

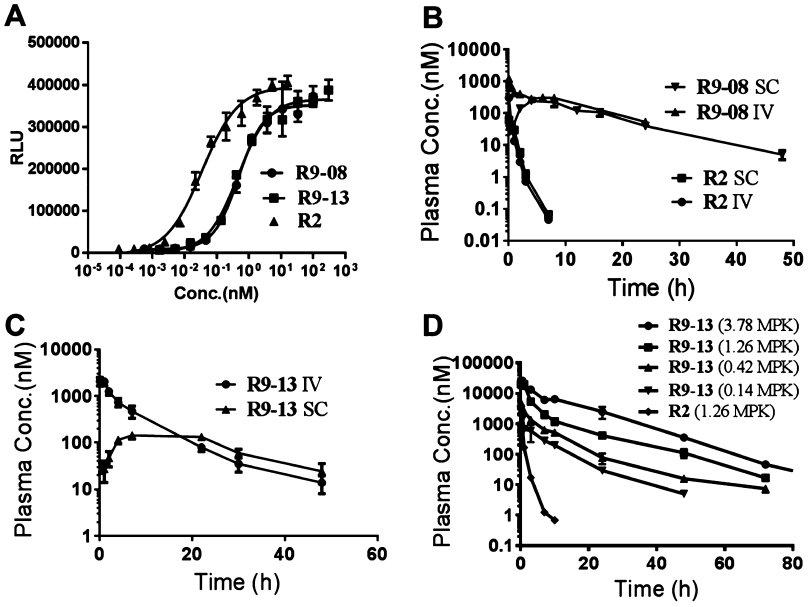


Figure 4. *In vitro* activity and PK profile of wild type relaxin (**R2**) and fatty acid conjugates (**R9-08** and **R9-13**). (A) *In vitro* activity of **R2**, **R9-08**, and **R9-13** in LGR7 (RXFP1) mediated CRE-Luc reporter assay (n = 3). (B) PK profiles of **R9-08** and **R2** in mice (n = 4) when administered via SC and IV routes. (C) PK profile of **R9-13** in rats (n = 4). (D) Dose−response rat PK study with **R9-13** (n = 4). Peptides (0.3 mg/kg) in PBS (pH 8.5) were administered by either IV or SC delivery into CD1 female mice or SD rats (n = 4 per group). Plasma samples were collected at the indicated time points and analyzed by using the in vitro LGR7 activity assay. The assay was performed in triplicate. Pharmacokinetic profiles were determined with WinNonLin using noncompartmental analysis.

drop in *in vitro* activity, respectively. We hypothesized the reduced binding affinity may be due to the enhanced affinity of FA-8 and FA-9 toward serum albumin present in the assay media.

We aimed to optimize the potency of the relaxin conjugates by designing other cysteine mutants. We used the program ASAview[23] to determine the solvent-exposed amino acid residues of relaxin (Figure S7). Together with crystal structure analysis,[24] selected cysteine mutations were designed, including hydrophobic residues of the A-chain (L2, A5, and V13) and B-chain (V7), and hydrophilic residues of A-chain (Q1 and R18) and B-chain (D1) (Figure 3A). All the mutants were expressed, refolded, conjugated, and digested as described previously. No significant variations in refolding yields between the constructs were observed, except for the V7C construct which was unable to refold. We explored the conjugation with FA-2, FA-8, and FA-9 (Table S3). **R4-02** and **R9-02** were evaluated *in vivo* in mice, and PK profiles similar to **R3-02** were observed, suggesting that the shorter lipid chain moiety (myristic acid, C14) might not be sufficient to achieve a half-life suitable for once-weekly dosing in human, irrespective of the conjugation site. Among all the FA-8 conjugates, **R4-08** (A:Q1C), **R8-08** (A:V13C), and **R9-08** (A:L2C) exhibited higher potency (Figure 3C) and were evaluated for their PK profile in mice. To our satisfaction, the half-lives of all these three analogs appear to be ∼8 h after subcutaneous administration (Figure S8), which is similar to that of semaglutide in rodents, indicating a potential translation into a once-weekly injectable drug in humans. **R9-08** retains the most potency compared to the other FA-8 conjugates, with the desired half-life.

To further optimize the synthetic process, we converted the glutamine at the N-terminus of the relaxin A-chain to pyroglutamate to avoid spontaneous cyclization and product heterogeneity (similar to serelaxin). By incubating **R9-08** in acetic acid for 4 h at 90 °C (Figure S10), **R9-13** with N-terminal pyroglutamate was obtained in quantitative yield and exhibited similar potency as that of **R9-08** (Figure 4A). Next, we optimized the downstream processing of **R9-13** by minimizing RP-HPLC purification steps. After refolding, the pro-relaxin was precipitated from the refolding concentrate using 80% ammonium sulfate (Figure S11) with 95% recovery. Furthermore, to confirm the site-specific conjugation onto the mutated cysteine without disulfide scrambling, we performed LC-MS/MS analysis. We conjugated pro-relaxin **R9** with simple FA1, to prevent the fragmentation of fatty acid linker in the MS/MS experiment. The pro-relaxin **R9**-FA1 conjugate was then fully reduced with DTT and digested with trypsin before subjecting to LC-MS/MS analysis. The result showed that the conjugation occurred only at the engineered free cysteine (L2C), demonstrating the refolding process is selective without disulfide mispairing (Figure S15). Next, we explored conjugations with various fatty acid chain lengths, with/without terminal carboxylate groups, and observed that as the length of the lipid chain increases, the *in vitro* potency decreases (potency trend for diacids: **R9-04** (C14) > **R9-06** (C16) > **R9-13** (C18) > **R9-10** (C20), Table S3). The analog with the shortest diacid (**R9-04**) is as potent as wt relaxin, whereas the analog with the longest diacid (**R9-10**) is 70-fold less potent. On the other hand, longer chain fatty acids bind better to serum albumin and may provide longer half-life in mouse. Though **R9-10** has the longest lipid chain among all the conjugates, we did not pursue PK analysis due to its weaker potency. Among all the **R9** conjugates, **R9-13** turned out to have the optimum balance between the *in vitro* potency and *in vivo* half-life (Figure 4). Consistent with its extended half-life in mice, **R9-13** showed similarly improved PK profile in rat (Figure 4C).

Finally, we evaluated whether the extended half-life of relaxin analog **R9-13** can translate into long-acting efficacy *in*

DOI: 10.1021/acs.bioconjchem.8b00764
Bioconjugate Chem. XXXX, XXX, XXX−XXX

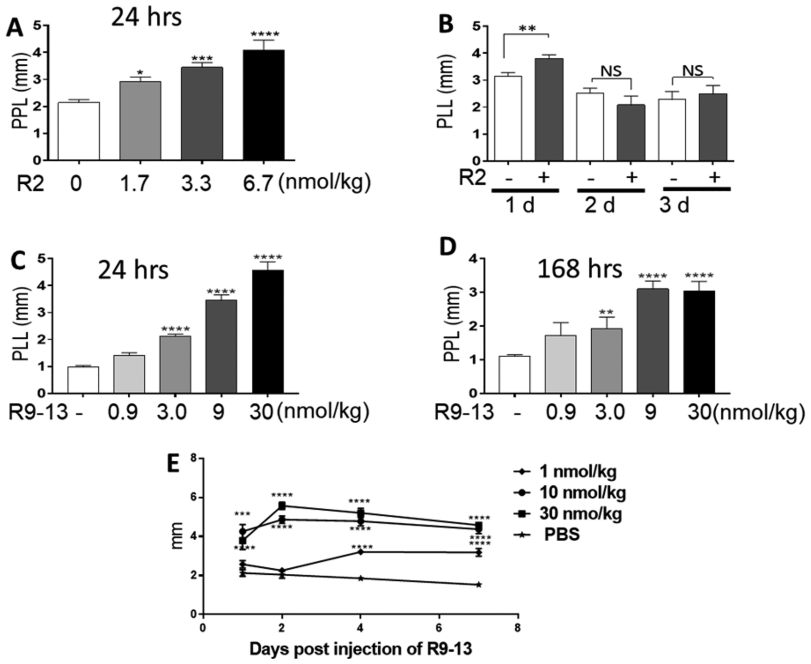


Figure 5. Pubic ligament elongation (PLE) study with wt relaxin (**R2**) and fatty acid conjugated relaxin (**R9-13**). Mice were treated with either PBS or relaxin, in addition to estradiol cypionate, PLE was measured by the end of treatment. (A) **R2** dose−response study, PLE was measured 24 h after the treatment. (B) PLE was measured after 1, 2, and 3 days after **R2** administration. (C) **R9-13** dose−response study, where pubic ligament elongation was measured at 24 h after treatment and (D) at 168 h after **R9-13** treatment. (E) PLE study at different days post **R9-13** injection. Results were compared to untreated and analyzed with one way ANOVA followed by Dunnett's multiple comparison test, NS = adjusted *P* value > 0.05, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

vivo. Relaxin was initially discovered and named for its relaxation effect on the pubic symphysis and its first confirmed pharmacodynamic (PD) effect is also to elongate the collagenous interpubic ligament to separate the pubic bones, thus facilitating parturition.[25−27] A pubic ligament elongation (PLE) model was established in mice with wt relaxin, **R2**: CD1 female mice were primed with estrogen for a week and administered with **R2** (1.7, 3.3, and 6.7 nmol/kg). The ligament length was measured 24 h post injection and dose-responsive elongation of the ligament was observed (Figure 5A). However, the effect of **R2** only lasted for 24 h (Figure 5B). We then evaluated **R9-13** in this model, and similarly, a dose-responsive PLE effect was observed at 24 h (Figure 5C). Excitingly, this pubic ligament elongation effect for relaxin **R9-13** was sustained even 1 week after dosing (Figure 5D). To further establish a clear time course and *in vivo* dose linearity, we conducted the PLE study at 1, 2, 4, and 7 days post dosing at multiple doses (Figure 5E). 10 and 30 nmol/kg doses reached saturation, and pubic ligament elongation by nearly 4 mm was observed at 2 days. Combining these *in vivo* studies, the ED_{50} of **R9-13** is estimated to be 5 nmol/kg in the PLE model, which translates into a very low dose of 30 μg/kg per week for **R9-13**.

In summary, we have developed a semisynthetic methodology for the synthesis of a series of potent fatty acid conjugated relaxin-2 analogs that demonstrate extended *in vivo* half-life and improved *in vivo* efficacies. These novel long-acting analogs warrant further preclinical and clinical development and provide new hope in translating the promising potential of relaxin-2 into innovative human therapies.

EXPERIMENTAL SECTION

Materials and Methods. Relaxin wt and its cysteine mutants were expressed in *E. coli* and processed according to the detailed procedure described. All peptides were purified using a Gilson or Shimadzu semipreparative reverse-phase HPLC system equipped with a Phenomenex C18 column with a flow rate of 5 mL/min and a gradient of 10−90% ACN/H_2O while monitoring at 220 and 254 nm. Analytical HPLC was performed using a Phenomenex Luna C18 or Kinetex C18 column (250 × 4.6 mm) with the flow rate set at 1.0 mL/min and UV detection set at 220 and 254 nm. Electrospray LC-MS analysis was performed using a Finnigan LCQ Advantage IonTrap mass spectrometry coupled with a Surveyor HPLC system or using an Agilent 6520 accurate-mass quadrupole-time-of-light (QTOF) instrument equipped with reverse phase liquid chromatography and an electrospray ionization (ESI) source.

Relaxin wt and Cys Mutant Cloning and Expression. The inserts containing relaxin wt and Cys mutant were prepared through polymerase cycling assembly, where forward and reverse primers were annealed to generate the DNA fragment which was further enriched by forward and reverse end primers. The amplified fragment was then digested with restriction digestion enzymes (NdeI/*Kpn*I). The prepared insert was then ligated into a vector with a PET backbone under the control of a T7 promoter and terminator. The encoded DNA fragment was then confirmed by DNA sequencing. For expression, relaxin constructs were transformed into K12 *E. coli* (derivative of W3110) with a genomic T7 RNA polymerase gene under the control of an araBAD promoter.

The transformed *E. coli* cells were cultured in Terrific Broth (TB) supplemented with 50 μg/mL kanamycin to OD_{600} = 0.8

DOI: 10.1021/acs.bioconjchem.8b00764
Bioconjugate Chem. XXXX, XXX, XXX−XXX

at 37 °C with vigorous shaking (250 rpm). To induce expression of the genes, L-arabinose stock solution (20% w/v in H_2O) was added to a final concentration of 0.2% (2 g per 1 L), and the cells were continuously incubated with shaking at 250 rpm at 37 °C for 5 h (OD_{600} ~5). Cells were then harvested by centrifugation (6000 rpm, 15 min) and the pellet was kept at −80 °C overnight.

Inclusion Body Purification. The cell pellet was resuspended in 20 mL lysis buffer (50 mM Tris, pH 8, 100 mM NaCl, 1% Triton-X100, 1 mM EDTA) per 1 L of bacterial culture and homogenized manually using Pipet Aid XP. The resuspended bacterial cells were lysed by passing through a French pressure cell press at 18 000 psi at least 5 times while the cell suspension was chilled to 4 °C after each pass.

Lysed cell suspension was clarified by centrifuging for 45 min at 15 000 rpm at 4 °C. The pellet containing the inclusion body was washed at least three times with wash buffer (50 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA) by resuspension through homogenization followed by centrifugation (15 000 rpm, 20 min, 4 °C).

Refolding. The pellet containing the purified inclusion body was stored at −80 °C and directly used for *in vitro* refolding of the relaxin analog without any further purification. Refolding of the relaxin analogs was carried out in the presence of oxidized glutathione $(GS)_2$ and reduced glutathione (GSH). Inclusion bodies were dissolved in 5−10 mL (for every 1 L of *E. coli* culture) at 37 °C for 1 to 1.5 h in 6 M guanidine hydrochloride (6 M guanidine hydrochloride in 100 mM, pH 8.0 Tris buffer with 2 mM EDTA). The pellet was resuspended multiple times until it was completely dissolved.

The denatured protein was then diluted in 200 mL (for every 10 mL inclusion body solution) of precooled fresh refolding buffer (containing 400 mM arginine, 100 mM Tris, pH 8.2 with 0.5 mM oxidized glutathione $(GS)_2$ and 5 mM reduced glutathione GSH). The refolding was continued for 4 h at 4 °C and quenched by adding conc. HCl until pH < 3. The precipitate was then centrifuged (5000 rpm, 15 min, 4 °C) and the supernatant (containing the relaxin precursor) was collected, transferred into a stirred cell unit (Amicon Stirred cells with 5K membrane) and concentrated to 30 mL.

Ammonium Sulfate Precipitation of Pro-Relaxin L2C Mutant. The concentrated protein mixture was then precipitated using ammonium sulfate (16.0 g solid added to a final 80% concentration) at 4 °C for 30 min. The resulting mixture was then centrifuged again (4000*g*, 30 min). The pellet was collected and lyophilized to obtain a pale yellowish foam. The weight of the whole solid was ~500 mg and the solid was estimated to contain 10% relaxin. The total yield of relaxin at this stage was around 50 mg/L of *E. coli* culture. The glutathione adduct is around 10−20% (Figure S11).

Conjugation of FA8 to Pro-Relaxin L2C Mutant Yielding L2C-Pro-Relaxin-FA8. The lyophilized crude protein solid (50 mg, assuming 10% yield from 500 mg crude, 6.1 μmol) was dissolved in 5.0 mL of 50 mM pH 8.0 Tris buffer/ACN (*v*/*v*, 3/1, 1.5 mL). The mixture was clarified by centrifugation (4000*g*, 10 min) and the solution containing the relaxin protein was collected. 2.0 eq (molar equivalent, 12.2 μmol, 10.7 mg, MW 880.44) of PEGylated lipid FA8 dissolved in 1 mL of acetonitrile/50 mM Tris buffer (1/3, *v*/*v*, pH 8.0) was added to the protein solution. The reaction mixture was agitated 5 h at RT. After completion of the reaction, the mixture was purified by RP-HPLC to obtain the relaxin product in 10 to 15 mg.

One-pot Enzymatic Processing to Yield L2C-Relaxin-FA8. The lyophilized powder of L2C-pro-relaxin-FA8 (10 mg) was resuspended in 10 mL of 25 mM Tris buffer containing 0.5 mM $CaCl_2$, pH 8.0 to prepare a final 1 mg/mL solution (1 mg/mL). 0.01 mg of trypsin per mg of protein + carboxy peptidase-B (0.8 units for 1 mg of protein) were added to the mixture. The reaction was completed in 1 h. After the cleavage was completed, the pH of the mixture was brought to 4 by adding acetic acid. The mixture was then heated to 95 °C for 2 h. The final product was purified by RP-HPLC to afford the 3.5 mg of L2C-Relaxin-FA8 final product (Figure S14). The final yield is around 3.5−5 mg/L of *E. coli* culture.

Cyclization of the N-terminal Glutamine of the A Chain to Pyroglutamate. After quenching with acetic acid, the resultant mixture was kept at 90 °C for 4 h and monitored for the loss of water through mass spectrometry. No obvious aggregate/decomposition was observed at 90 °C for 4 h incubation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.8b00764.

Detailed protocols for the synthesis and characterization of the linker, fatty acid conjugation onto various relaxin analogs, and *in vitro* and *in vivo* characterization (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: wshen@calibr.org.

ORCID

Avinash Muppidi: 0000-0001-8820-8444
Pengyu Yang: 0000-0001-7554-5281
Weijun Shen: 0000-0001-8388-729X

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank Jose Gonzalez, Dawna Caballero, Herlinda Quirino, Murali Surakattula, Brent Benish and Vanessa Nunez for technical assistance and helpful discussions and Dr. Sam Lear for proof of the manuscript.

■ REFERENCES

(1) Hsu, S. Y., Nakabayashi, K., Nishi, S., Kumagai, J., Kudo, M., Sherwood, O. D., and Hsueh, A. J. (2002) Activation of orphan receptors by the hormone relaxin. *Science 295*, 671−674.

(2) Teichman, S. L., Unemori, E., Dschietzig, T., Conrad, K., Voors, A. A., Teerlink, J. R., Felker, G. M., Metra, M., and Cotter, G. (2009) Relaxin, a pleiotropic vasodilator for the treatment of heart failure. *Heart Failure Rev. 14*, 321−329.

(3) Cannon, J. A., McKean, A. R., Jhund, P. S., and McMurray, J. J. (2015) What can we learn from RELAX-AHF compared to previous AHF trials and what does the future hold? *Open Heart 2*, No. e000283.

(4) Teerlink, J. R., Cotter, G., Davison, B. A., Felker, G. M., Filippatos, G., Greenberg, B. H., Ponikowski, P., Unemori, E., Voors, A. A., Adams, K. F., et al. (2013) Serelaxin, recombinant human relaxin-2, for treatment of acute heart failure (RELAX-AHF): a randomised, placebo-controlled trial. *Lancet 381*, 29−39.

DOI: 10.1021/acs.bioconjchem.8b00764
Bioconjugate Chem. XXXX, XXX, XXX−XXX

(5) Teneggi, V., Sivakumar, N., Chen, D., and Matter, A. (2018) Drugs' development in acute heart failure: what went wrong? *Heart Failure Rev. 23*, 667−691.
(6) McVicker, B. L., and Bennett, R. G. (2017) Novel Anti-fibrotic Therapies. *Front. Pharmacol. 8*, 1 DOI: 10.3389/fphar.2017.00318.
(7) Chen, S. A., Perlman, A. J., Spanski, N., Peterson, C. M., Sanders, S. W., Jaffe, R., Martin, M., Yalcinkaya, T., Cefalo, R. C., Chescheir, N. C., et al. (1993) The pharmacokinetics of recombinant human relaxin in nonpregnant women after intravenous, intravaginal, and intracervical administration. *Pharm. Res. 10*, 834−838.
(8) Xiao, J., Huang, Z., Chen, C. Z., Agoulnik, I. U., Southall, N., Hu, X., Jones, R. E., Ferrer, M., Zheng, W., Agoulnik, A. I., et al. (2013) Identification and optimization of small-molecule agonists of the human relaxin hormone receptor RXFP1. *Nat. Commun. 4*, 1953.
(9) Hossain, M. A., Kocan, M., Yao, S. T., Royce, S. G., Nair, V. B., Siwek, C., Patil, N. A., Harrison, I. P., Rosengren, K. J., Selemidis, S., et al. (2016) A single-chain derivative of the relaxin hormone is a functionally selective agonist of the G protein-coupled receptor, RXFP1. *Chemical science 7*, 3805−3819.
(10) Jayakody, T., Marwari, S., Lakshminarayanan, R., Tan, F. C., Johannes, C. W., Dymock, B. W., Poulsen, A., Herr, D. R., and Dawe, G. S. (2016) Hydrocarbon stapled B chain analogues of relaxin-3 retain biological activity. *Peptides 84*, 44−57.
(11) Barlos, K. K., Gatos, D., Vasileiou, Z., and Barlos, K. (2010) An optimized chemical synthesis of human relaxin-2. *J. Pept. Sci. 16*, 200−211.
(12) Bullesbach, E. E., and Schwabe, C. (1991) Total synthesis of human relaxin and human relaxin derivatives by solid-phase peptide synthesis and site-directed chain combination. *J. Biol. Chem. 266*, 10754−10761.
(13) Tang, J. G., Wang, Z. H., Tregear, G. W., and Wade, J. D. (2003) Human gene 2 relaxin chain combination and folding. *Biochemistry 42*, 2731−2739.
(14) Hossain, M. A., and Wade, J. D. (2014) Synthetic relaxins. *Curr. Opin. Chem. Biol. 22*, 47−55.
(15) Thalluri, K., Kou, B., Yang, X., Zaykov, A. N., Mayer, J. P., Gelfanov, V. M., Liu, F., and DiMarchi, R. D. (2017) Synthesis of relaxin-2 and insulin-like peptide 5 enabled by novel tethering and traceless chemical excision. *J. Pept. Sci. 23*, 455−465.
(16) Winslow, R. J., and Rinderknecht, B. E. (1995) Human relaxin: purification, characterization and production of recombinant relaxin for structure−function studies, *Progress in Relaxin Research. Proceedings of the 2nd International Congress on the Hormone Relaxin*; Global Publication Services; pp 59−72.
(17) Breece, T., Rinderknecht, E., Vandlen, R., and Yansura, D. (1998) *Process for producing relaxin*; Genentech, Inc.
(18) Lawless, C., and Hubbard, S. J. (2012) Prediction of missed proteolytic cleavages for the selection of surrogate peptides for quantitative proteomics. *OMICS 16*, 449−456.
(19) Kratz, F. (2008) Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. *J. Controlled Release 132*, 171−183.
(20) Koniev, O., and Wagner, A. (2015) Developments and recent advancements in the field of endogenous amino acid selective bond forming reactions for bioconjugation. *Chem. Soc. Rev. 44*, 5495−5551.
(21) Hu, J., Duppatla, V., Harth, S., Schmitz, W., and Sebald, W. (2010) Site-specific PEGylation of bone morphogenetic protein-2 cysteine analogues. *Bioconjugate Chem. 21*, 1762−1772.
(22) Madsen, K., Knudsen, L. B., Agersoe, H., Nielsen, P. F., Thøgersen, H., Wilken, M., and Johansen, N. L. (2007) Structure−Activity and Protraction Relationship of Long-Acting Glucagon-like Peptide-1 Derivatives: Importance of Fatty Acid Length, Polarity, and Bulkiness. *J. Med. Chem. 50*, 6126−6132.
(23) Ahmad, S., Gromiha, M., Fawareh, H., and Sarai, A. (2004) ASAView: database and tool for solvent accessibility representation in proteins. *BMC Bioinf. 5*, 51.
(24) Eigenbrot, C., Randal, M., Quan, C., Burnier, J., O'Connell, L., Rinderknecht, E., and Kossiakoff, A. A. (1991) X-ray structure of human relaxin at 1.5 A. Comparison to insulin and implications for receptor binding determinants. *J. Mol. Biol. 221*, 15−21.
(25) Kroc, R. L., Steinetz, B. G., and Beach, V. L. (1959) The effects of estrogens, progestagens, and relaxin in pregnant and nonpregnant laboratory rodents. *Ann. N. Y. Acad. Sci. 75*, 942−980.
(26) Hisaw, F. L. (1926) Experimental relaxation of the pubic ligament of the guinea pig. *Exp. Biol. Med. 23*, 661−663.
(27) Agoulnik, A. I. (2007) Relaxin and related peptides in male reproduction. *Advances in experimental medicine and biology 612*, 49−64.

DOI: 10.1021/acs.bioconjchem.8b00764
Bioconjugate Chem. XXXX, XXX, XXX−XXX