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Design, Synthesis, and Characterization of a Single-Chain Peptide Antagonist for the Relaxin-3 Receptor RXFP3

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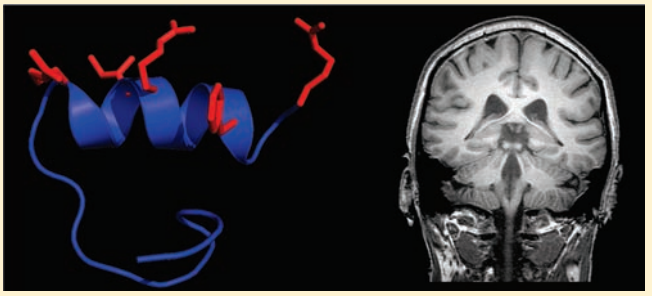
Supporting Information

ABSTRACT: Relaxin-3 is a two-chain disulfide-rich peptide that is the ancestral member of the relaxin peptide family and, together with its G protein-coupled receptor RXFP3, is highly expressed in the brain. Strong evolutionary conservation of relaxin-3 suggests a critical biological function and recent studies have demonstrated modulation of sensory, neuroendocrine, metabolic, and cognitive systems. However, detailed studies of central relaxin-3-RXFP3 signaling have until now been severely hampered by the lack of a readily available high-affinity antagonist for RXFP3. Previous studies have utilized a complex two-chain chimeric relaxin peptide, R3(BΔ23−27)R/I5, in which a truncated relaxin-3 B-chain carrying an additional C-terminal Arg residue was combined with the insulin-like peptide 5 (INSL5) A-chain. In this study we demonstrate that, by replacing the native Cys in this truncated relaxin-3 B-chain with Ser, a single-chain linear peptide of 23 amino acids that retains high-affinity antagonism for RXFP3 can be achieved. In vivo studies demonstrate that this peptide, R3 B1−22R, antagonized relaxin-3/RXFP3 induced increases in feeding in rats after intracerebroventricular injection. Thus, R3 B1−22R represents an excellent tool for biological studies probing relaxin pharmacology and a lead molecule for the development of synthetically tractable, single-chain RXFP3 modulators for clinical use.



1. INTRODUCTION

The relaxin peptide family consists of seven members in humans, namely, relaxins 1−3 and insulin-like peptides (INSL) 3−6. The relaxin peptides are structurally related to insulin and insulin-like growth factors (IGF) I and II and together form the insulin/relaxin superfamily.[1] Relaxins and insulin are expressed as preprohormones that are subsequently activated by proteolytic processing into two-chain (A and B) peptides that are cross-braced by two interchain and one intra-A-chain disulfide bonds.[2] This bracing stabilizes a conserved overall fold in which the A-chain adopts two helical segments that are separated by a central β-sheet and lie antiparallel to each other in a U-shaped arrangement, with the B-chain consisting of a central helix that lies across the face of the U[3] (Figure 1).

Despite the close structural relationship between members of the insulin/relaxin superfamily, they have widely different biological functions and pharmacological modes of action. In contrast to insulin and IGFs, which bind to tyrosine kinase receptors,[4] relaxin family peptides bind to G protein-coupled receptors (GPCRs). To date four receptors have been identified and are referred to as the relaxin family peptide receptors (RXFP) 1−4.[5] Interestingly, RXFP1−4 belong to two different subtypes of type I or Class A GPCRs. RXFP3 and RXFP4, the endogenous receptors for relaxin-3 and INSL5,[6,7] are classic peptide ligand GPCRs, whereas the relaxin-1/2 and INSL3 receptors RXFP1 and RXFP2 are characterized by a large extracellular N-terminal leucine-rich repeat domain.[8,9]

Relaxin-3, which is the product of the ancestral gene of the relaxin family,[10] is highly expressed in mammalian brain, in neurons of the nucleus incertus, and in other smaller populations of neurons in the brainstem.[11−16] The ascending nucleus incertus neural network, mapped comprehensively in the rat,[17,18] is suggested to have a role in sensory, emotional, and neuroendocrine processing, with a link to control of behavioral state,[17] stress responses,[19] metabolic regulation, circadian activity,[18] and important brain rhythms.[20] Consistent with these data, relaxin-3 or RXFP3-selective relaxin-3 analogues have been shown to increase feeding in rats after acute intracerebroventricular (icv) injection[21,22] or chronic injection/infusion,[22−24] and relaxin-3

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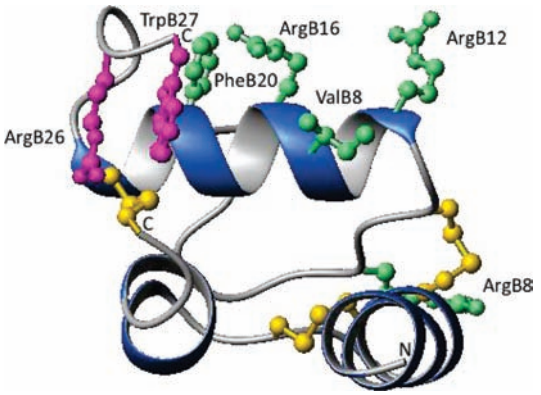

Figure 1. Relaxin-3 solution NMR structure illustrating the positions of key amino acid side chains involved in the binding interaction (green) and activation (magenta) of RXFP3. Disulfide bonds are shown in yellow.

expression was demonstrated to increase in response to neurogenic stressors via CRF_1 receptors.[13,25] More recently, RXFP3 modulation within the septohippocampal pathway revealed an effect of nucleus incertus relaxin-3 signaling on hippocampal θ rhythm and associated spatial memory.[15,26,27]

Relaxin-3 has the ability to bind and activate its endogenous receptor RXFP3 as well as both RXFP1 and RXFP4 with high affinity.[6,28,29] This cross-reactivity, together with an overlapping expression profile of RXFP3 and RXFP1 in the brain,[14,30,31] make a selective RXFP3 analogue critical for further precise investigations of RXFP3 signaling in vivo and for future exploration of RXFP3 as a drug target. It was recently shown that a selective agonist of RXFP3 and RXFP4 can be generated by combining the A-chain from INSL5 with the B-chain from relaxin-3 (R3/I5).[32] Structural studies of this chimera revealed an identical conformation of the relaxin-3 B-chain as in the native relaxin-3 peptide, confirming that this observation is not related to an overall structural change but that binding to RXFP1 requires the native characteristics of both chains.[33] This is in contrast to RXFP3 and RXFP4, for which no determinants of ligand activity have been identified in the A-chain,[34] and thus the binding sites for these receptors are thought to be located solely within the B-chain. Additional structure−activity studies on relaxin-3 and the R3/I5 relaxin chimera peptide have identified the key residues involved in binding and activation of the RXFP3 and RXFP4 receptors.[22] Whereas the primary binding site, comprising ArgB12, IleB15, ArgB16, and PheB20, is located in the B-chain helical region, the two C-terminal residues ArgB26 and TrpB27 at the end of a flexible tail are critical for activation of the receptors.[22] Importantly, in a recent study a R3/I5 chimeric peptide with a truncated B-chain and an additional C-terminal Arg, R3(BΔ23−27)R/I5, was created due to incomplete recombinant protein processing and it was found to be a high-affinity RXFP3 antagonist.[22] The C-terminal B-chain Arg "artefact" was subsequently shown to be critical for high-affinity binding to RXFP3, since truncated analogues lacking this residue show considerably lower affinity.[35] This is presumably the result of the creation of a non-native interaction point with the receptor that has the ability to compensate for the lack of the affinity contribution from the activation domain ArgB26 and TrpB27.

In this study we explored whether it was possible to create high-affinity single-chain antagonists of RXFP3 by using this key Arg interaction. Via solid-phase synthesis, a series of relaxin-3 B-chain analogues of various lengths was generated and characterized. We demonstrate that a single-chain analogue with potent RXFP3 antagonist activity both in vitro and in vivo can be obtained. This new analogue is a pharmacological tool for deciphering the role of relaxin-3/RXFP3 in neural signaling and is a lead molecule for the development of pharmaceuticals targeting RXFP3 for potential use in the treatment of psychiatric diseases involving this receptor.

2. EXPERIMENTAL SECTION

2.1. Solid-Phase Peptide Synthesis. All amino acids were purchased from either Auspep Pty. Ltd. (Melbourne, Australia) or GL Biochem (Shanghai, China). Solvents and chemicals were of analytical or peptide synthesis grade. Peptides were synthesized on either an automatic PerSeptive Biosystems Pioneer continuous-flow peptide synthesizer or a CEM Liberty automated microwave-assisted peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) peptide chemistry protocols as previously described,[36] with Fmoc-PAL-PEG-PS or Fmoc-L-Arg(Pbf)-PEG-PS as solid support [where PAL = [(aminomethyl)dimethoxyphenoxy]pentanoic acid linker, PEG = poly(ethylene glycol), PS = polystyrene, and Pbf = pentamentyldihydrobenzofuran]. After standard resin cleavage and side-chain deprotection, the crude peptides were purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC), freeze-dried, and stored at −20 °C. The purity of the peptides was determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and RP-HPLC. Peptide content was determined by amino acid analysis.

2.2. Cyclic Adenosine Monophosphate Assay. Chinese hamster ovary (CHO) cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and HAMS-F12 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/strepicillin and cultivated in a humidified chamber with 5% CO_2 at 37.8 °C. Twenty thousand cells per well were seeded into a precoated poly-L-lysine 96-well plate. Cells were incubated overnight. The next day the CHO cells were transfected with either human RXFP3 or RXFP4 by use of the mammalian expression vector pcDNA3.1(+)zeo (UMR cDAN Resource Center) together with a pCRE β-galactosidase reporter plasmid by using *Trans*IT-CHO, OptiMEM, and CHO-MOJO reagents. Cells were then incubated overnight in a humidified chamber with 5% CO_2 at 37.8 °C before cAMP assays were performed.

To measure the activity of the peptides, the cells were stimulated with 5 μM forskolin for RXFP3 and 1 μM forskolin for RXFP4, as previously described.[36] In brief, the cells were stimulated with peptide for 6 h in a humidified chamber before the medium was aspirated off and the cells were frozen at −80 °C. To determine the cAMP activity for RXFP1, HEK-293T cells were transfected with RXFP1 and a cAMP response element (CRE)−β-galactosidase reporter plasmid, as previously described.[37] For measurement of β-galactosidase activity cells were lysed by adding 25 μL of buffer A (5 mL of 10 mM sodium phosphate buffer, pH 8.0, 1 mL of 0.2 mM $MgSO_4$, and 50 μL of 0.01 mM $MnCl_2$ in 44 mL of H_2O) to each well and incubated on a shaker for 10 min at room temperature before an addition of 100 μL of buffer B (5 mL of 100 mM sodium phosphate buffer, pH 8.0, 1 mL of 2 mM $MgSO_4$, 50 μL of 0.1 mM $MnCl_2$, 2.5 mL of 0.5% Triton X-100, and 0.16 mL of 40 mM β-mercaptoethanol in 41.3 mL of H_2O) followed by a 10 min incubation. The substrate, chlorophenol red−β-D-galactopyranoside (CPRG), was then added to each well and the reaction was left at room temperature until a absorbance of <1 AU at 570 nm was reached. Each peptide concentration was tested in triplicate, and each peptide was tested at least three times. The results were analyzed and plotted on the basis of a sigmoidal dose−response curve with variable slope by using Graph Pad Prism 5 and presented as mean ± SEM.

2.3. Binding Assays. HEK-293T cells stably transfected with RXFP1, or CHO cells stably transfected with RXFP3[38] or RXFP4, were plated out onto a precoated poly-L-lysine 96-well viewplate with a density of 50 000 cells per well. Medium was aspirated off and the cells were washed with phosphate-buffered saline (PBS) before competition binding assays were performed with 1 nM Eu-DTPA-R3/I5,[39] 0.5 nM Eu-DTPA-H2 relaxin,[40] or 2.5 nM Eu-DTPA-mINSL5 as previously described. Data are presented as mean of triplicates ± SEM. Each experiment was repeated at least three times. Data analyses and pK_i were determined on the basis of one-site competition and 0.5 nM Eu-R3/I5 with a K_d value of 9.14 by using Graph Pad Prism 5. One-way analysis of variance (ANOVA) was used as the statistical analysis method.

2.4. Solution NMR Spectroscopy. R3 B1−22R (0.5 mg) was dissolved in 90% H_2O/10% D_2O and subjected to solution NMR. Two-dimensional (2D) ^{1}H homonuclear total correlation spectroscopy (TOCSY) and nuclear Overhauser enhancement spectroscopy (NOESY) were recorded with a mixing time of 200 ms at 298 K and at 283 K on a 600 MHz spectrometer. Spectra were processed by use of Topspin 2.1 (Bruker) and analyzed by CARA. Secondary shifts were calculated from the random coil chemical shifts described by Wishart et al.[41]

2.5. In Vivo Feeding Studies. Male Sprague-Dawley rats (n = 27; 250−300 g; supplied by Animal Resources Centre, Perth, WA, Australia) were housed under ambient conditions (21 °C) and maintained on a 12 h light/dark cycle (lights on 0700−1900), with access to food (laboratory chow) and water ad libitum. Rats were acclimatized to the animal facility for at least 1 week before further treatment. Experiments were conducted with the approval of the Florey Neuroscience Institutes Animal Welfare Committee and according to the ethical guidelines issued by the National Health and Medical Research Council of Australia. All efforts were made to minimize the number of animals used and any pain or suffering.

For the stereotaxic implantation of a cannula into the lateral cerebral ventricle, rats were anesthetized with isoflurane (Delvet, Seven Hills, NSW, Australia). Deep anesthesia was induced with 4% isoflurane in oxygen, 2 L/min, and maintained with 2−3% isoflurane in oxygen, 200 mL/min. The skull was positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and a stainless-steel guide cannula (22 gauge, cut 5 mm below pedestal; Plastics One, Roanoke, VA) was implanted with the cannula tip inserted into the lateral ventricle (coordinates: anteroposterior, −0.8 mm; mediolateral, −1.4 mm; dorsolateral, −3.6 mm).[42] The cannula was affixed to the skull by use of surgical screws and dental cement. Rats were placed under a heat lamp until they regained consciousness and were housed individually in clean cages. Meloxicam (3 mg/kg, i.p; Troy Laboratories, Smithfield, NSW, Australia) and 0.5 mg/mL paracetamol in 5% sucrose/water (for 3 days) were administered to provide acute and ongoing postoperative analgesia, respectively.

Rats were single-housed and allowed to recover for 7 days, during which they were handled and weighed daily to habituate them to the experimenter. A stylet was fashioned from stainless steel wire (30 gauge) and inserted into each cannula to maintain patency. The stylet extended no further than the base of the cannula.

Lateral cerebral ventricle infusions were made using a 30-gauge stainless steel hypodermic tubing injector (Small Parts Inc., Miramar, FL) connected to a 10-μL Hamilton microsyringe (Hamilton Instruments, Reno, NV) by polyethylene tubing (0.80 mm outer and 0.40 mm internal diameter; Microtube Extrusions, North Rocks, NSW, Australia). Correct positioning of the cannula was verified in each rat by injecting 2.5 μL of a 4 ng/μL (or 5 μL of a 2 ng/μL) solution of angiotensin II (Auspep, Parkville, VIC, Australia) in artificial cerebrospinal fluid [aCSF; made from stock of 10× aCSF (1470 mM NaCl, 40 mM KCl, 8.5 mM $MgCl_2$, and 23 mM $CaCl_2$)], and observing if this produced a positive dipsogenic response, defined as repeated drinking episodes of ≥5 s that commenced within 1 min of angiotensin II administration. Injectors that extended between 0.5 and 2 mm below the base of the cannula were tested as required on consecutive days and the length of the effective injector was recorded for each rat and used for all subsequent experiments. Those rats that failed to display a drinking response (n = 3) were subsequently given mock injections by removing and reinserting the stylet into their cannulas and were included in a control group.

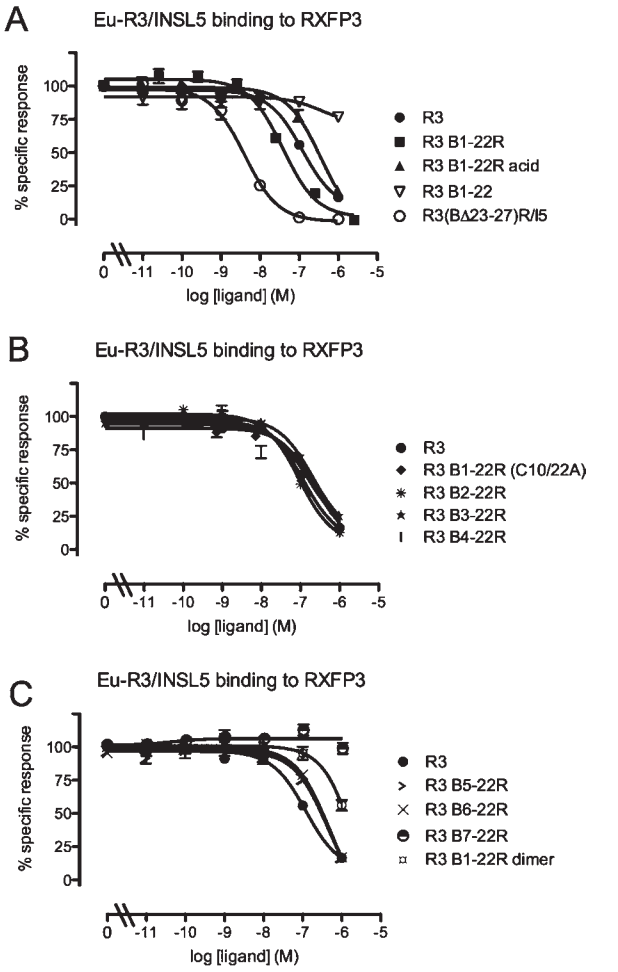

Figure 2. Competition binding curves for relaxin-3 B-chain analogues and R3(BΔ23−27)R/I5 in comparison to relaxin-3 using Eu-R3/I5 in RXFP3-expressing cells. Data points are shown as mean ± SEM of triplicate determinations from three independent experiments.

Previous studies have described significant increases in food intake following central administration of 54−180 pmol of relaxin-3 and 10 μg (∼2 nmol) of R3/I5, an RXFP3 agonist.[22,23] The latter effect was inhibited by prior injection of 10 μg (∼2 nmol) of R3(BΔ23−27)R/I5, an RXFP3 antagonist.[22] Therefore, in the current study, rats were allocated into four groups (n = 6−9 per group). Group 1 (control, n = 7) received an injection of 5 μL of vehicle or were mock-injected; group 2 (agonist, n = 9) received icv infusions of 5 μg (∼1 nmol) of R3/I5 (5 μL of 1 μg/μL solution in aCSF); group 3 (antagonist and agonist, n = 6) received icv infusions of 10 μg (∼4 nmol) of R3 B1−22R (2.5 μL of 4 μg/μL solution in aCSF) followed 10 min later by 2.5 μg (∼1 nmol) of R3/I5 (2.5 μL of 2 μg/μL solution in aCSF); group 4 (antagonist and vehicle, n = 6) received an icv infusion of 10 μg of R3 B1−22R (2.5 μL of 4 μg/μL solution in aCSF) followed 10 min later by 2.5 μL of aCSF.

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Table 1. Peptide Sequences and Apparent Equilibrium Constants for Competitive Binding to RXFP3[a]

Peptide	Sequence[b,c]	pK_i (mean±SEM)
R3	RAAPYGVRLCGREFIRAVIFTCGGSRW-NH_2 DVLAGLSSSCCKWGCSKSEISSLC-NH_2	6.93 ± 0.09
R3/I5	RAAPYGVRLCGREFIRAVIFTCGGSRW-NH_2 ZDLQTLCCTDGCSMTDLSALC-NH_2	7.80 ± 0.14
R3(BΔ23-27)R/I5	RAAPYGVRLCGREFIRAVIFTCR-NH_2 ZDLQTLCCTDGCSMTDLSALC-NH_2	8.49 ± 0.13
R3 B1-22R	RAAPYGVRL**S**GREFIRAVIFT**S**R-NH_2	7.44 ± 0.05
R3 B1-22R acid	RAAPYGVRL**S**GREFIRAVIFT**S**R-OH	6.47 ± 0.17
R3 B1-22R C10/22A	RAAPYGVRL**A**GREFIRAVIFT**A**R-NH_2	6.86 ± 0.13
R3 B1-22	RAAPYGVRL**S**GREFIRAVIFT**S**-NH_2	NA[d]
R3 B2-22R	AAPYGVRL**S**GREFIRAVIFT**S**R-NH_2	6.93 ± 0.13
R3 B3-22R	APYGVRL**S**GREFIRAVIFT**S**R-NH_2	6.13 ± 0.35
R3 B4-22R	PYGVRL**S**GREFIRAVIFT**S**R-NH_2	5.91 ± 0.56
R3 B5-22R	YGVRL**S**GREFIRAVIFT**S**R-NH_2	6.27 ± 0.25
R3 B6-22R	GVRL**S**GREFIRAVIFT**S**R-NH_2	5.81 ± 0.50
R3 B7-22R	VRL**S**GREFIRAVIFT**S**R-NH_2	NA[c]
R3 B1-22R dimer	RAAPYGVRLCGREFIRAVIFTCR-NH_2 RAAPYGVRLCGREFIRAVIFTCR-NH_2	<6

[a] Competition binding in relation to Eu-R3/I5 (K_d 9.14). [b] Ala or Ser residues replacing native Cys in the single-chain analogues are shown in boldface type. [c] Amidated C-termini are denoted by $-NH_2$ and free carboxyl termini by $-OH$. [d] NA indicates no detected binding under any concentration used in this study.

For measurements of food and water intake, rats were habituated for a minimum of 7 days to the holding room and behavioral studies were performed during the light phase beginning at 10.30−11.00 h. Rats were injected as described and then each rat was placed back into its home cage, where a preweighed amount of rat chow (10−14 g) was located in the food compartment of the wire cage lid. A preweighed water bottle was also placed in its usual compartment. Food and water were weighed at intervals of 1 h up to 4 h postinjection, with minimal disturbance to the rat. Some cannulated rats with no drinking response were given mock injections by inserting a stylet into their cannulas.

Food and water intake data were analyzed by use of GraphPad Prism 5 (GraphPad Software, San Diego, CA). Results are expressed as mean ± SEM. Statistical significance was evaluated by one-way ANOVA with Bonferroni's post-test: $^*P < 0.05$, significant; $^{**}P < 0.01$, highly significant. Food and water intake levels of rats that were vehicle- or mock-injected were compared and in a control group, as there was no significant difference between them.

3. RESULTS

3.1. Design and Synthesis of Linear Relaxin-3 B-Chain Analogues. Despite extensive mutational studies suggesting that all determinants of relaxin-3 activity are located in the B-chain, the isolated B-chain alone possesses poor affinity for RXFP3.[32] This observation is thought to be a result of the B-chain having a highly compromised structure without the support from the A-chain in the native peptide. As it was recently shown that addition of a non-native C-terminal Arg can create an additional interaction point, thereby increasing the affinity for a chimeric antagonist,[22] we decided to investigate whether incorporating this feature in a single-chain analogue in which the Cys residues were replaced by Ser or Ala would lead to a similar gain that would be sufficient to create a high-affinity single-chain analogue. Furthermore, to investigate the importance of the N-terminal region of this analogue, we synthesized a range of analogues in which the N-termini were truncated. Finally, in light of a recent observation that the related peptide INSL3, a B-chain homodimer in which the two half-cystines of the B-chain were used to couple two identical chains together, displayed improved affinity for the INSL3 receptor RXFP2,[43] we synthesized a dimer of two truncated relaxin-3 B-chains.

All peptides were synthesized by Fmoc solid-phase peptide synthesis using automated peptide synthesizers in high overall yields. The peptides were purified by reverse-phase high-performance liquid chromatography, and high final purity (>95%) was confirmed by MALDI-TOF MS and RP-HPLC analysis. The concentration of samples for pharmacological testing was confirmed by amino acid analysis as previously described.[44]

3.2. In Vitro Activity of Single-Chain Relaxin-3 Antagonist Peptides. The properties of the relaxin-3 B-chain analogues were evaluated in competition binding assays and cAMP activity assays in RXFP1-, RXFP3-, and RXFP4-expressing cells. Strikingly, although a truncated single-chain analogue R3 B1−22 showed no affinity for RXFP3, addition of a C-terminal Arg resulted in binding at the same or even increased affinity in comparison to native relaxin-3 (Figure 2A, Table 1). However, this analogue was 10-fold less potent than the chimeric two-chain R3(BΔ23−27)R/I5

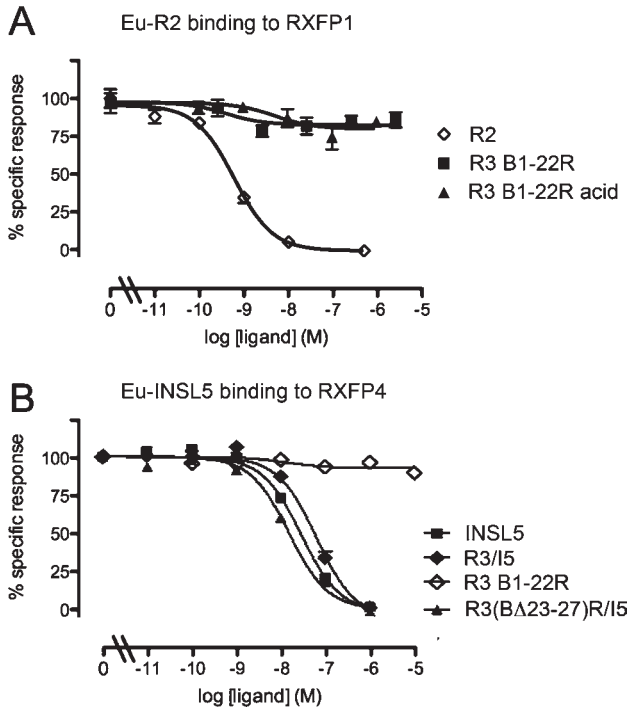

Figure 3. Competition binding curves for relaxin-3 B-chain analogues and R3(BΔ23−27)R/I5, (A) in comparison to Eu-relaxin-2 in RXFP1-expressing cells and (B) in comparison to Eu-mINSL5 in RXFP4-expressing cells. Data points are shown as mean ± SEM of triplicate determinations from three independent experiments.

antagonist. Although the most potent single-chain analogue was found to be the variant with an amidated C-terminus and with Ser residues in place of the native Cys, no statistically significant difference was seen between R3 B1−22R(C10/22S) and R3 B1−22R(C10/22A), in which Ala was used, or between R3 B1−22R amide and R3 B1−22R acid. Truncations of the R3 B1−22R amide analogue at the N-terminus resulted in a gradual decrease in affinity for RXFP3 until R3 B7−22R, where the affinity was lost. Interestingly, combining two R3 B1−22R peptide chains into a homodimer significantly decreased RXFP3 affinity, in contrast to the result seen for INSL3 binding to RXFP2[43] (Figure 2, Table 1).

To assess the degree of selectivity for RXFP3, the single-chain R3 B1−22R peptide was also tested for biding to RXFP1 and RXFP4. As expected, on the basis of the documented importance of an A-chain for activity at RXFP1, the single-chain peptide had no affinity for RXFP1 (Figure 3A). R3 B1−22R also showed no binding to RXFP4, which is in contrast to the two-chain chimeric agonist R3/I5 and antagonist R3(BΔ23−27)R/I5, which both bind strongly to RXFP4 (Figure 3B).

All peptides were tested for their ability to activate receptors in a functional cAMP assay by use of CHO or HEK-293T cells transfected with CRE-β-galactosidase and RXFP1, RXFP3, or RXFP4. Since RXFP3 and RXFP4 are coupled to $G_{\alpha i}$, the activity of the analogues was measured as inhibition of forskolin-induced cAMP activity. None of the truncated analogues displayed any ability to activate the RXFP3 receptor (Figure 4A). However, all B-chain analogues comprising the C-terminal Arg, except for the R3 B1−22R dimer, displayed antagonistic activity at RXFP3, evident from their ability to block 10 nM relaxin-3 inhibition of the forskolin-induced cAMP activity (Figure 4B−D). This competitive

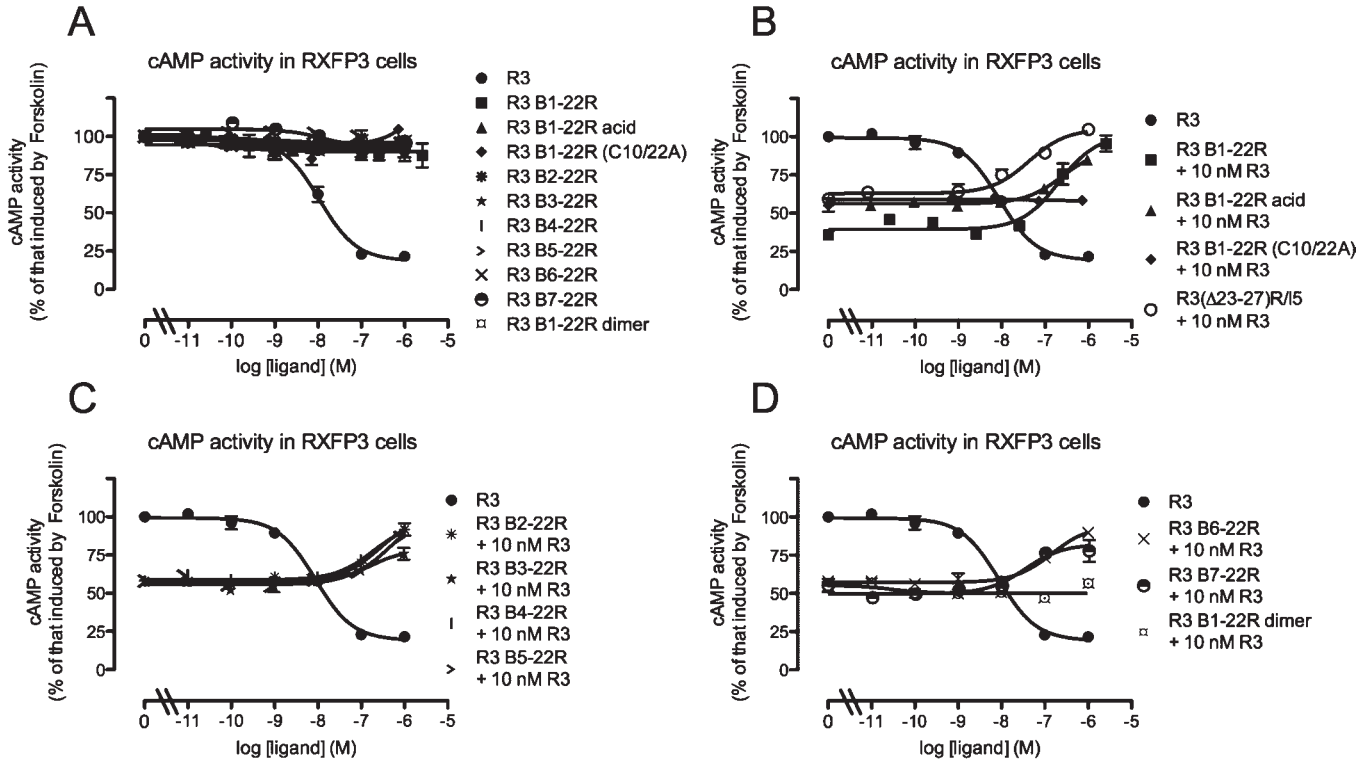

Figure 4. Agonist or antagonist activities of relaxin-3 B-chain analogues and R3(BΔ23−27)R/I5 compared to native relaxin-3 in RXFP3 transfected cells as measured by changes in cAMP activity. (A) Agonist activity in RXFP3-transfected cells. (B−D) Ability of R3(Δ23−27)R/I5 and relaxin-3 B-chain analogues to antagonize 10 nM relaxin-3-induced inhibition of cAMP activity in RXFP3-transfected cells.

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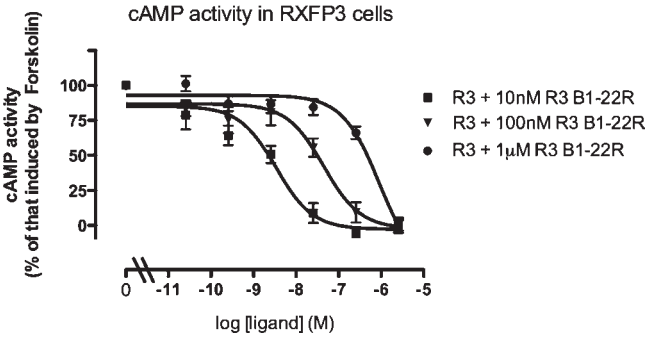

Figure 5. Ability of R3 B1−22R to antagonize relaxin-3 inhibition of cAMP activity in RXFP3-transfected cells. The antagonistic effect of R3 B1−22R is shown with a rightward shift of the H3 relaxin dose−response curve in the presence of 10 nM, 100 nM, or 1 μM R3 B1−22R. Data points are shown as mean ± SEM of triplicate determinations from three independent experiments.

antagonistic behavior was confirmed by monitoring activation of RXFP3 by relaxin-3 in the presence of various concentrations of R3 B1−22R, which resulted in a rightward shift of the activation curves (Figure 5). Consistent with the lack of observable affinity, R3 B1−22R is not able to activate either RXFP1 or RXFP4 (Figure 6 A,B) and, importantly, it does not antagonize the response of either relaxin-3 or INSL5 at the RXFP4 receptor; thus, unlike the two-chain R3(BΔ23−27)R/I5 antagonist, the peptide is fully selective for RXFP3 (Figure 6C).

3.3. In Vivo Activity of R3 B1−22R. Central administration of R3/I5 (1 nmol, icv) to satiated rats in the early light period significantly increased food intake within the first hour after injection, compared to control, and this was blocked by prior injection of R3 B1−22R (∼4 nmol, icv). By contrast, injection of R3 B1−22R alone prior to vehicle had no significant effect on food intake compared to control (3.30 ± 0.48 g for R3/I5, 1.20 ± 0.52 g for control, $P < 0.05$ vs R3/I5; 0.65 ± 0.35 g for R3 B1−22R + R3/I5, $P < 0.01$ vs R3/I5; 0.95 ± 0.46 g for R3 B1−22R + vehicle, $P < 0.01$ vs R3/I5; $n = 6$−9 per group; Figure 7).

3.4. Structural Analysis. To investigate whether the single-chain analogues were able to adopt a native-like conformation in solution without structural support from the A-chain, we examined the structural features of the most potent analogue, R3 B1−22R, using two-dimensional NMR spectroscopy. NOESY and TOCSY spectra were recorded at 600 MHz and the spectra were of good quality in terms of line width and signal-to-noise but had poor signal dispersion, as expected for an unstructured or helical peptide lacking a well-defined structural core. Resonance assignments were achieved by well-established sequential assignment strategies and an almost complete set of assignments was possible, with the exception of Arg1 and Arg23. Secondary Hα chemical shifts, that is, differences between observed Hα chemical shifts and shifts seen in random coil peptides, are good indicators of the presence of secondary structure. As shown in Figure 8, although there is a tendency toward negative shifts consistent with helical structure in the region Arg12−Ile19, which adopts a key helical structure in native relaxin-3, R3 B1−22R generally has secondary shifts close to zero, suggesting that it predominantly adopts a random-coil-like conformation in solution. This was confirmed by analysis of NOE data, which revealed the presence of very few medium-range NOEs that are typically observed in helical peptides, and by circular dichroic (CD) spectroscopic analysis (Supporting Information). Thus, it

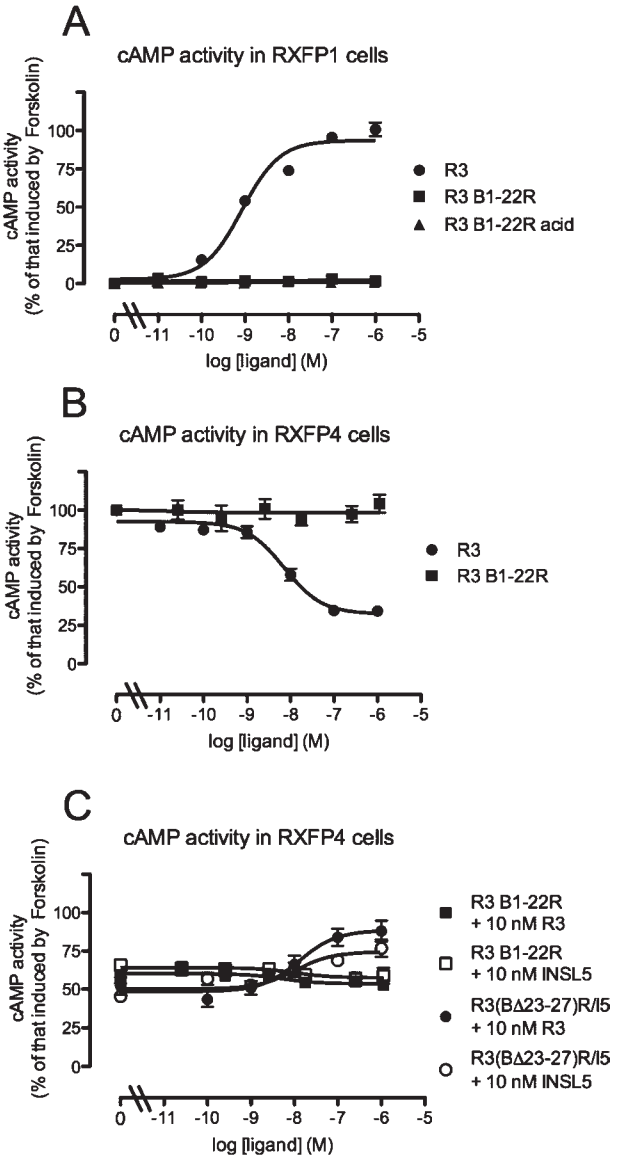

Figure 6. Agonist or antagonist activities of relaxin-3 B-chain analogues and R3(BΔ23−27)R/I5 compared to native relaxin-3 in RXFP1- and RXFP4-transfected cells as measured by changes in cAMP activity. (A) Agonist activity of R3 B1−22R in RXFP1-expressing cells. (B) Agonist activity of R3 B1−22R in RXFP4-expressing cells. (C) Ability of R3 B1−22R and R3(Δ23−27)R/I5 to antagonize 10 nM relaxin-3-induced and 10 nM INSL5-induced inhibition of cAMP activity in RXFP4-transfected cells. Data points are shown as mean ± SEM of triplicate determinations from three independent experiments.

appears that R3 B1−22R is flexible in solution and probably only adopts the correct conformation for binding upon interaction with the RXFP3 receptor.

4. DISCUSSION

The development of a potent and selective RXFP3 antagonist that is synthetically tractable, ideally a single-chain molecule, to facilitate in vivo characterization of the neurological role of relaxin-3/RXFP3 and realize the potential of RXFP3 as a pharmaceutical target, has been an important challenge for relaxin researchers over the past few years. This challenge has

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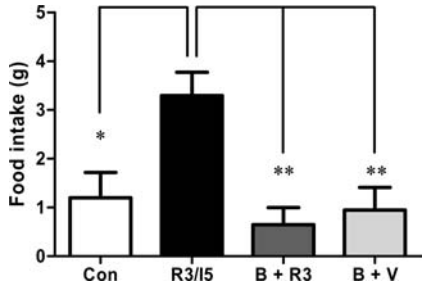

Figure 7. Effect of R3 B1−22R on R3/I5-induced feeding in adult Sprague-Dawley rats. R3/I5 (1 nmol, icv) significantly increased food intake in the first hour after injection in satiated rats. This increase was blocked by pretreatment with R3 B1−22R (~4 nmol, icv; 10 min prior), whereas injection of R3 B1−22R prior to vehicle had no significant effect on food intake. *P < 0.05, control vs R3/I5; **P < 0.01 R3 B1−22R + R3/I5 vs R3/I5; **P < 0.01 R3 B1−22R + vehicle vs R3/I5; n = 6−9 rats per group.

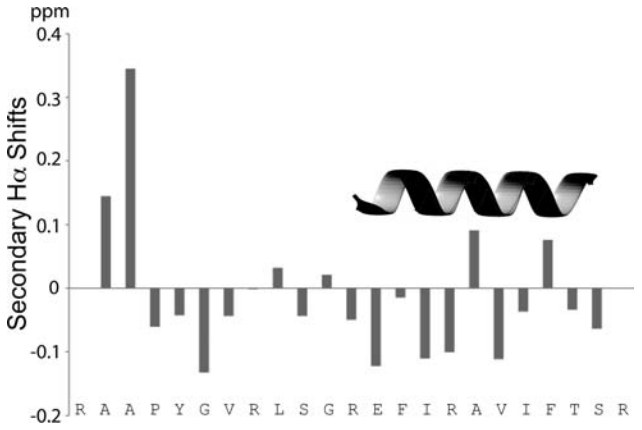

Figure 8. Secondary Hα-shift analysis for R3 B1−22R. Stretches of negative values indicate a helical region and positive values indicate β-sheet. R3 B1−22R displays only small secondary shifts, suggesting a mainly random coil structure. The position of the B-chain helix in native relaxin-3 is indicated by a helix.

been complicated by the fact that, in both in vitro and in vivo pharmacological studies, relaxin-3 is able to activate three different receptors (RXFP1, RXFP3, and RXFP4) and because its complex two-chain structure requires tedious multistep strategies for the synthesis of analogues.

In this study we synthesized a range of single-chain analogues based on modified relaxin-3 B-chain sequences and evaluated their pharmacological profiles, with the aim to develop a structurally minimized and potent antagonist. All peptides were truncated at the C-terminus and none was able to activate RXFP3, consistent with them lacking the critical Arg26-Trp27 activation domain.[22] By contrast, most single-chain analogues displayed some antagonistic activity at RXFP3, confirming that they still contain the main features for binding to the receptor and are thus able to compete with native relaxin-3 for the RXFP3 ligand-binding site. It is clear that addition of the non-native C-terminal Arg is the key feature providing the single-chain analogues with high affinity for RXFP3, as a variant lacking this residue displayed no binding to RXFP3 at any of the tested concentrations. Although addition of this Arg has been shown previously to be able to increase the affinity of two-chain truncated versions of relaxin-3,[22] it was not anticipated that this modification would be sufficient to provide a single-chain peptide with an affinity similar to that of native relaxin-3.

A characteristic response to intracerebroventricular (icv) administration of relaxin-3 is increased food intake in adult rats.[21,23] A similar effect was observed after icv administration of R3/I5, and this increased feeding was prevented by pretreatment with the RXFP3 antagonist R3(BΔ23−27)R/I5.[22] In studies to investigate whether the R3 B1−22R peptide was similarly effective as a RXFP3 antagonist in vivo as in vitro and capable of inhibiting the RXFP3-related feeding response, R3 B1−22R was administered centrally in adult rats prior to an effective dose of R3/I5. R3 B1−22R treatment prevented the increased feeding response to R3/I5 stimulation, confirming that the peptide is active in vivo and will be a valuable tool for further characterization of neural relaxin-3−RXFP3 signaling circuits.[15]

Selectivity for RXFP3 has been a key issue for analogue design. Numerous studies have confirmed that interaction with RXFP1 requires both the A- and B-chains of relaxin-3 or its cognate ligand relaxin.[32,34] Modification, truncation, or replacement of the relaxin-3 A-chain have therefore been used as strategies for removing activity at RXFP1.[32,34] Thus, undesired activity at RXFP1 was not predicted to be a problem for single-chain analogues, which lack the A-chain altogether. This was supported by our data illustrating that none of the analogues interacted with RXFP1. More complicated, however, has been the achievement of selectivity for RXFP3 over RXFP4. RXFP3 and RXFP4 are closely related and relaxin-3 appears to activate both receptors via a similar mechanism, using the same key residues.[22] Consequently, so far no analogue retaining high potency at RXFP3 has been shown to be selective for RXFP3 over RXFP4. We demonstrate here that single-chain analogues comprising the C-terminal Arg are not able to bind or antagonize RXFP4. A possible explanation is that the additional non-native binding site targeted by the Arg is present only in RXFP3 and not in RXFP4. Thus, the lead peptide R3 B1−22R is fully selective for RXFP3. It is tempting to speculate that introducing a different type of residue at this position might provide a different type of receptor contact point suitable for RXFP4 rather than RXFP3 and thus might be a way to create a selective RXFP4 antagonist.

With the synthesis of R3 B1−22R we have reduced the size of the peptide from 44 residues over two chains in R3(BΔ23−27)R/I5 to a more convenient 23 residues within a single chain, but further reduction in size would be desirable. Truncation studies on native relaxin-3 revealed that it was possible to truncate the B-chain N-terminus by up to 7 residues without significant loss of activity, suggesting that this region is not important for receptor interactions.[34] To determine whether it was also possible to minimize the single-chain analogues, we synthesized a series of N-terminally truncated analogues of R3 B1−22R. Although most analogues retained binding to RXFP3, the affinity progressively decreased with increased truncations until Val7 was removed, at which point the affinity for RXFP3 was lost. This progressive loss of activity suggests that although this region is not required for interactions with the receptor, it does appear to aid binding, presumably by supporting the overall structure of the single-chain analogues. This region thus appears to be an ideal target for the further development of the R3 B1−22R peptide as a potential pharmaceutical, as it could be used to introduce modifications that increase stability and/or sequences that assist the targeting of the peptide to the brain.

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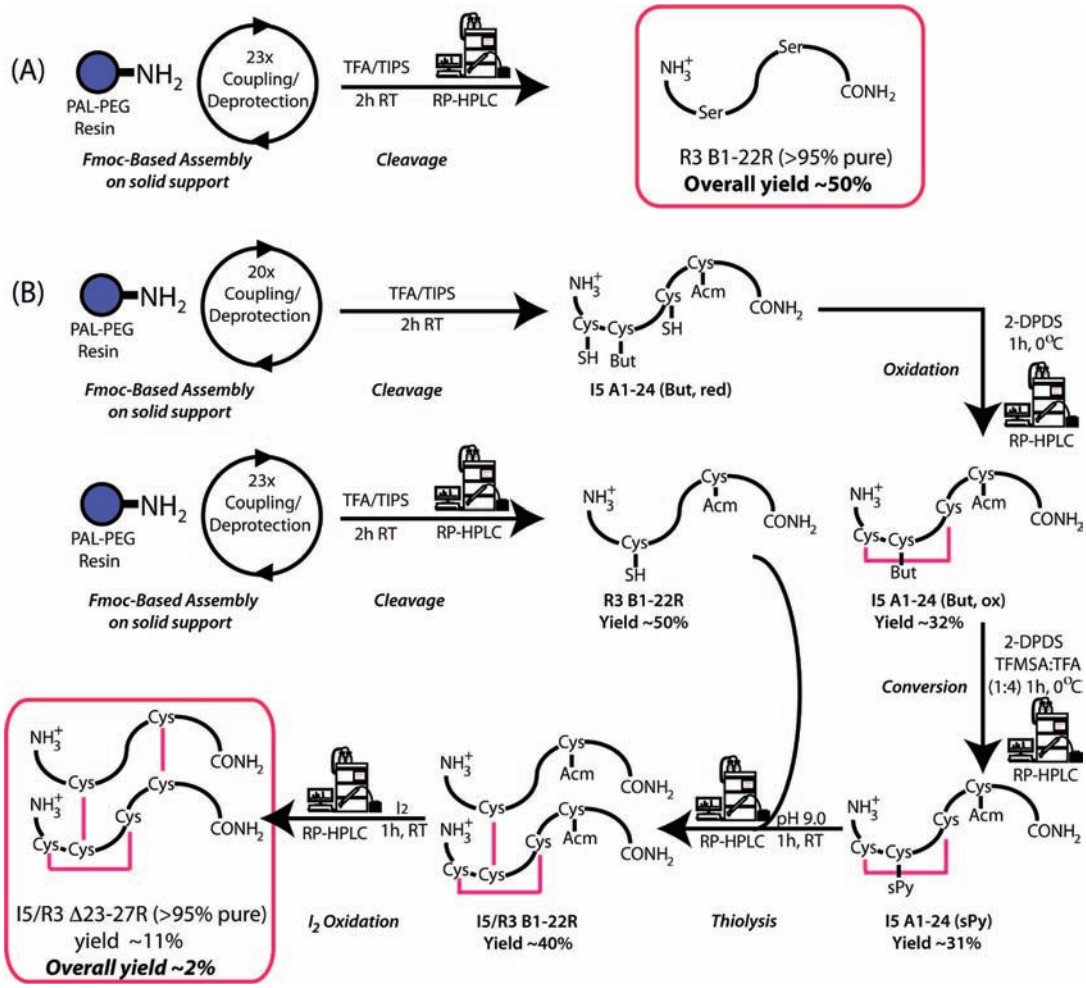

Figure 9. Comparison of synthetic routes to the antagonists (A) R3 B1−22R and (B) R3(BΔ23−27)R/I5. R3 B1−22R is directly assembled in its final form on resin, requiring a single purification step after cleavage. In contrast, generation of the two-chain R3(BΔ23−27)R/I5 antagonist requires assembly of two separate chains, four separate reactions for the regioselective formation of its disulfide bonds, and typically five purification steps, resulting in low overall yields.

The most potent analogue, R3 B1−22R, was structurally characterized by solution NMR spectroscopy. Despite its high potency, it was found to be mainly unstructured in solution. This indicates that a stable native fold is not a prerequisite for interacting with RXFP3, but rather the correct fold is adopted upon interaction with its receptor. Folding associated with binding to an interaction partner is commonly observed and the penalty associated with this does not have to be large if a propensity for the correct structure exists.[45] This finding is consistent with our previous studies in which the A-chain in the native two-chain peptide was truncated at the N-terminus.[34] Removal of nine residues, up to CysA10, was found not to significantly affect binding or activation of RXFP3. However, structural studies revealed this analogue to have a compromised overall fold.[34] Furthermore, analysis of NMR line shapes in data recorded on native full-length relaxin-3 suggests that although the peptide adopts a well-defined overall fold, it has a significant degree of internal motion.[46]

A homodimer of R3 B1−22R was created by retaining the two native Cys residues in the sequence and combining two molecules via formation of two disulfide bonds through oxidative folding. For the related peptide INSL3 this has been shown to create a structural constraint that supports formation of the key helical conformation of the B-chain, resulting in a high-affinity RXFP2 antagonist.[43] In contrast, the R3 B1−22R dimer, which also contains two possible receptor binding sites, binds poorly to RXFP3 and displays no antagonistic activity. Thus, it appears the relaxin-3 B-chain is not ideal for the formation and packing of two helical segments against each other, and the creation of such a chimera prevents rather than supports the formation of this key structural element.

From a synthetic point of view, development of the R3 1−22R peptide represents a dramatic improvement over the currently used R3(BΔ23−27)R/I5 chimeric peptide. Figure 9 provides a comparison of the synthetic routes toward the final peptides and highlights typical yields relative to the crude material obtained after resin cleavage. The R3 B1−22R peptide is assembled in its final form on resin and requires a single purification step after cleavage, giving yields of ∼50%. In contrast, although the B-chain with selectively protected Cys side chains can be assembled and purified with similar efficiency in the production of R3(BΔ23−27)-R/I5, it has to subsequently be cross-linked to the A-chain through a two-step process, resulting in an overall yield relative to the crude B-chain of ∼2%. Moreover, the process requires separate assembly of the INSL5 A-chain followed by oxidation and conversion prior to the chain combination step; thus, relative

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to the crude A-chain, the overall yield is only ~0.5%. In addition to the losses through the multiple steps, R3(BΔ23−27)R/I5 contains nearly twice as many residues as R3 B1−22R, increasing the number of coupling and deprotection reactions, and typically it requires five purification steps. Thus, in comparison, the cost of production of R3 B1−22R in terms of both time and chemicals is fractional.

In conclusion, we have developed a single-chain peptide that is a high-affinity and highly selective antagonist for the relaxin-3 receptor RXFP3. This peptide is now being used in our laboratories to characterize the nature of neural relaxin-3/RXFP3 signaling circuits in vivo and represents a valuable pharmacological tool as well as an important lead molecule for the development of drugs targeting RXFP3.

■ ASSOCIATED CONTENT

Supporting Information. Complete references 22 and 24; additional text and equations providing a theoretical framework for analysis of binding and activation of RXFP1, RXFP3, and RXFP4; and a figure showing the CD spectrum of R3 B1−22R. This material is available free of charge via the Internet at http://pubs.acs.org.

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