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Cyclic analogs of galanin and neuropeptide Y by hydrocarbon stapling

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ABSTRACT

Hydrocarbon stapling is an effective strategy to stabilize the helical conformation of bioactive peptides. Here we describe application of stapling to anticonvulsant neuropeptides, galanin (GAL) and neuropeptide Y (NPY), that are implicated in modulating seizures in the brain. Dicarba bridges were rationally introduced into minimized analogs of GAL and NPY resulting in increased α-helical content, in vitro metabolic stability and *n*-octanol/water partitioning coefficient (log*D*). The stapled analogs retained agonist activities towards their respective receptors and suppressed seizures in a mouse model of epilepsy.

1. Introduction

Anticonvulsant neuropeptides act as potent modulators of seizure activity and nociception. Galanin (GAL) and neuropeptide Y (NPY) elicit their anticonvulsant properties through activation of galanin and neuropeptide Y receptors in the brain.[1–4] Direct hippocampal administration of full-length GAL or NPY was previously shown to inhibit seizure activities.[5,6] Despite promising biological activities, the relatively large size of GAL and NPY (29 and 36 amino acids, respectively) and a susceptibility for proteolytic degradation, have hampered development of these peptides as anticonvulsant drugs. Early efforts focused on identification of peptide-minimized structures of both GAL and NPY which elicited similar biological activities to those of the full-length peptides.[7,8] The N-terminal fragment of GAL and the C-terminal fragment of NPY also suppressed seizures following icv administration.[9,10] However, these truncated analogs were still prone to rapid degradation in an in vitro serum stability assay. As such, analogs of the minimized structures of GAL and NPY, possessing improved physicochemical and pharmacological profiles, have garnered recent interest.[11–13]

Peptide cyclization is one approach which has shown promise to improve biological activity of peptide analogs by limiting proteolysis and stabilizing secondary peptide structures.[14] One cyclizaton strategy that was previously applied to both Gal(1–16) and NPY(25–36) is lactam-bridge formation between amino acid side chains.[15–18] The previously described lactamized analogs demonstrated nanomolar receptor binding affinities similar to those of the unmodified peptides.[15,16] Another cyclization strategy which has shown recent promise is dicarba bridge formation, also known as hydrocarbon stapling.[19,20] The potential advantage of this method is the formation of a covalent C–C bond between side chains which was previously shown to contribute to increased proteolytic resistance.[21] It was hypothesized in this work that incorporation of dicarba bridges via hydrocarbon stapling motifs would result in peptide-minimized GAL and NPY analogs with comparable binding affinities, and increased α-helical character, with the added benefit of increased metabolic stability.

Previous efforts in hydrocarbon stapling have focused on the development of peptide analogs with increased α-helical content for modulation of protein-protein interactions within the cell.[22–25] Stapled peptide analogs have been shown to be effective in the modulation/control of a number of biological processes such as: regulation of estrogen receptors, inhibition of apoptotic pathways (MCL-1), activation of tumor suppressor pathways, and others.[22–24] Until recently, hydrocarbon stapling had not been reported for helical stabilization of peptides which possess receptor targets on the cell surface. Efforts by Platt et al. have recently illustrated the application of stapled peptides for extracellular targets, such as *N*-methyl-D-aspartate receptors (NMDAR).[26] Stapled analogs of conantokin G (ConG), a potent inhibitor of the NR2B subtype isolated from the venom of *Conus geographus* exhibited increased helical content and improved in vivo pharmacological activity of one analog.[26] This report describes the design, synthesis and characterization of

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stapled peptide-minimized active fragments of GAL and NPY towards the creation of neuropeptide analogs that possess increased helical content and improved physicochemical properties while maintaining their pharmacological activities.

2. Results

2.1. Design strategy

The agonist activities of galanin are mediated through the N-terminal residues of the 30 amino acid full-length peptide, with α-helical character centered around Tyr9.[27] Structure–activity studies of the Gal(1-16) active fragment previously identified Trp2, Tyr9, and Asn5 and Leu10 as integral for receptor binding and activation.[28] Carpenter et al. previously constructed a cyclized analog of Gal(1-16) which possessed a lactam bridge between the side-chains of Asp4 and Lys8.[16] This resulted in an analog that exhibited low nanomolar receptor binding affinity and a low nanomolar EC_{50} for the GalR2 receptor subtype.

In this work, two analogs of galanin were designed which incorporated hydrocarbon stapling motifs and oligo-cationization of the peptide active fragments (Fig. 1). The first stapled analog (Gal-S1) was patterned after the NMR model of transportan which maintains sequence identity to full-length galanin at the N-terminus.[29] This model showed Ala7 and Leu11 in an (*i*), (*i*+4) configuration on the opposing face of the α-helix, away from important receptor binding residues. Based on these findings, these positions were selected for replacement by (*S*)-2-(4-pentenyl)alanine to form the dicarba bridge. The second analog (Gal-S2) was patterned after the previously described systemically-active galanin analog, Gal-B2, which incorporated both oligo-cationization and lipidization at the N-terminus.[9] Our previous efforts showed that the combination of multiple modification strategies resulted in systemically-active peptide analogs with enhanced biological activities. The current work combined the strategies of hydrocarbon stapling and increased cationization towards development of anticonvulsant neuropeptide analogs with increased α-helical content and improved metabolic stability. Consistent with our previous efforts, stapled galanin analogs also possessed N-terminal methylation of Gly1 through replacement with sacrosine (Sar). This modification was previously shown to increase metabolic stability while leaving receptor binding unaffected.[9,30]

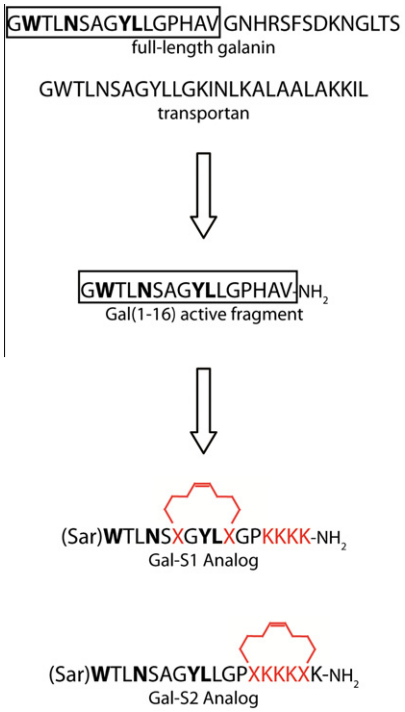

Figure 1. Design strategy for stapled galanin analogs. Analogs were designed and synthesized based on the Gal(1-16) active fragment, Gal-S1 was designed to stabilize α-helical content by substitution of Ala-7 and Leu-11 with (*S*)-2-(4-pentenyl) alanine (X). This analog also possessed increased cationization through attachment of oligo-Lys at the C-terminus. Gal-S2 was patterned after the previously described Gal-B2 analog and contained both stapling and cationization motifs at the C-terminus. Critical residues are listed in bold font.

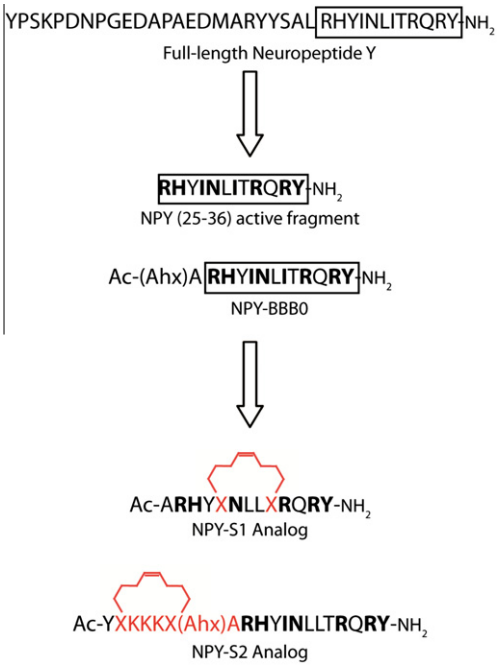

Figure 2. Design strategy for stapled neuropeptide Y analogs. NPY analogs were designed which retained the C-terminal active fragment NPY(25–36). NPY-S1 is the hydrocarbon-stapled active fragment of NPY. NPY-S2 replaces internal residues with both stapling and cationization motifs, in addition to the 6-aminohexanoic acid (Ahx) spacer. Critical residues are listed in bold font.

The lactamization strategy was also applied to the endogenous neuropeptide, NPY. Previous structure–activity studies identified the C-terminal fragment of NPY as essential for potent interaction with NPY-receptor subtypes.[8,31,32] N-terminally truncated analogs of NPY[15,17], with the lactam bridge between Lys28 and Glu32, stabilized α-helical character of the N-terminus and resulted in analogs with low nanomolar binding affinities for the NPY2R receptor subtype. As with lactam-stabilized NPY analogs, incorporation of hydrocarbon stapling motifs was expected to stabilize helicity while improving metabolic stability through removal of the protease-susceptible amide bond. Similar to the lactamized NPY analogs described by Yao,[17] the first stapled analog (NPY-S1) was designed to incorporate (*S*)-2-(4-pentenyl)alanine at Ile28 and Thr32.[17] The second analog, NPY-S2, was patterned after the structure of the previously described centrally-truncated analog, NPY-BBB1.[10] NPY-S2 incorporated both the poly-Lys and hydrocarbon stapling motifs between the N- and C-terminal fragments of the centrally-truncated peptide (Fig. 2).

2.2. Chemical synthesis

All analogs were synthesized on solid support using an automated peptide synthesizer and standard Fmoc-protocols as described in Section 3. Dicarba bridge formation was accomplished through on-resin ring-closing metathesis (RCM) using Grubb's first

generation ruthenium catalyst in a manner consistent with hydrocarbon stapling of ConG by Platt.[26] Peptides were cleaved from solid support resin by treatment with reagent *K* and were purified to near homogeneity (>95% purity) by preparative reversed-phase HPLC. Peptides were quantified by UV absorbance at using either tryptophan for Gal-analogs (λ_{max} = 279.8 nm; ε = 5600 $M^{-1}cm^{-1}$) or tyrosine for NPY-analogs (λ_{max} = 274.6 nm; ε = 1420 $M^{-1}cm^{-1}$).[33] Peptides were then aliquoted and dried by speed-vac. Masses of all compounds were confirmed by MALDI-TOF MS. Retention times were calculated from the average of three independent HPLC separations for each analog and are summarized in Table 1.

2.3. Partitioning coefficient (log*D*)

The log*D* values for each of the modified analogs were determined and compared to those of the unmodified active fragments (Fig. 3). This was accomplished either by HPLC capacity factor (k') or *n*-octanol/water shake-flask methods. Analogs of Gal(1-16) exhibited log*D* values similar to that of the unmodified peptide. The log*D* value for Gal-S1 was calculated as 0.71 ± 0.01, while Gal-S2 had a log*D* of 0.78 ± 0.01, compared to 0.69 for the unmodified peptide. For NPY analogs, the previously described parent compound (NPY-BBB0) possessed a log*D* of −2.13 ± 0.68.[10] The partitioning coefficients of the stapled analogs were −0.71 ± 0.12 (NPY-S1) and 0.08 ± 0.03 (NPY-S2).

2.4. Metabolic stability

To determine how hydrocarbon stapling influenced peptide stability, an in vitro serum stability assay was carried out, where analogs were incubated in 25% rat blood serum at 37 °C (Fig. 4). Time-course experiments were conducted, in triplicate, over a range of 0–8 h. At the appropriate time points, aliquots were removed and reactions were 'quenched' by acidification using a mixture of TCA/isopropanol/nH_2O (15:45:40, v/v/v). The degradation of the peptide was measured by the decrease in peak area over time using analytical HPLC methods. As described in Figure 4, $t_{1/2}$-values were increased for both hydrocarbon stapled GAL and NPY analogs in comparison to the peptide active fragments Gal(1-16) and NPY-BBB0. Half-lives for Gal-S1 and Gal-S2 were dramatically increased with calculated $t_{1/2}$-values of 6.4 ± 2.5 h and 15.7 ± 4.6 h compared to 7.8 min for the unmodified peptide (Fig. 4, Panels A and C). Serum stabilities of stapled NPY analogs were also improved, though not to the same extent of stapled galanin analogs. Calculated half-lives were 2.7 ± 0.4 h (NPY-S1) and 1.8 ± 0.4 h (NPY-S2) compared to 0.57 ± 0.03 h for NPY-BBB0 (Fig. 4, Panels B and D).

2.5. Structural characterization

Circular dichroism (CD) analysis was performed in order to evaluate the effects of hydrocarbon stapling on the α-helical character of stapled GAL and NPY analogs (Fig. 5). In comparison to the unmodified active fragments Gal(1-16) and NPY-BBB0, all stapled analogs exhibited increased α-helical content under aqueous conditions (Fig. 5). Compared to the unmodified galanin active fragment ($[\Theta_{222}]$ = 1.19%), Gal-S1 and Gal-S2 possessed α-helical contents of 8.50% and 22.8%, respectively. Similarly, compared to the unmodified neuropeptide Y active fragment NPY-BBB0 ($[\Theta_{222}]$ = 3.60%), stapled NPY analogs exhibited increased α-helicities of 19.7% (NPY-S1) and 9.4% (NPY-S2). This trend continued under helix-stabilizing conditions (50% v/v trifluoroethanol); with the exception of the Gal-S1 analog which actually exhibited slightly lower helical content compared to the unmodified Gal(1-16) fragment ($[\Theta_{222}]$ = 16.0% versus 21.8%).

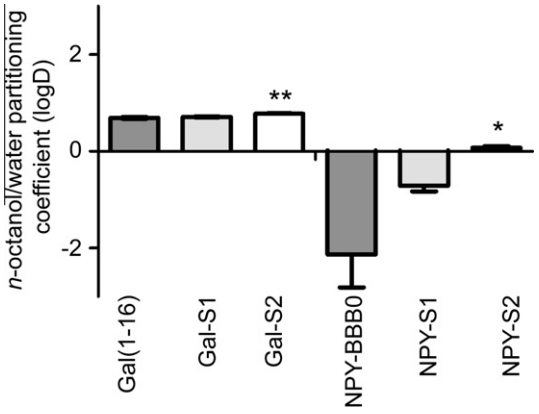

Figure 3. Summary of log*D* values for stapled GAL and NPY analogs. log*D* for stapled GAL analogs were calculated by comparison of HPLC retention times with those of peptides possessing experimentally-determined log*D* values using the capacity factor (k') method. log*D* values for stapled NPY analogs were calculated directly using the shake-flask method. Values were obtained from the average of three independent experiments. Statistical comparisons between stapled and unmodified peptides were performed using a standard two-sample *t*-significance test in GraphPad software. *P*-value< 0.05 (*), *P*-value< 0.005 (**).

2.6. Receptor binding & functional activity studies

Affinities of stapled GAL analogs were evaluated using competitive fluorescence-based binding assays. We previously reported binding affinities for the Gal(1–16) active fragment against GalR1 (k_I = 0.5 nM) and GalR2 (k_I = 13.0 nM).[9] The stapled analogs exhibited slightly lower binding affinities than the unmodified active fragment in this assay. Both Gal-S1 and Gal-S2 maintained low nanomolar affinities for galanin receptors. The k_I values for Gal-S1 were 37.5 nm and 119 nM for GalR1 and GalR2, respectively. The experimentally determined k_I values for Gal-S2 were 3.8 nM and 15.6 nM for GalR1 and GalR2, respectively (Fig. 6, Panels A and B).

Table 1
Sequences, mass spectrometry data, HPLC retention times, and partitioning coefficients for stapled galanin and neuropeptide Y analogs

Analog	Sequence [a]	Molecular Mass $[M+H]^+$ (calc/exp)	HPLC retention time (min) [b]	% Yield	Partitioning coefficient (log*D*) [c]
Galanin					
Gal-S1	(Sar) WTLNS**X**GYL**X**GPKKKK#	1940.15/1940.93	18.09 ± 0.03	19.9%	0.71 ± 0.01
Gal-S2	(Sar) WTLNSAGYLLGP**X**KKK**X**K#	2124.27/2124.10	18.66 ± 0.01	37.3%	0.78 ± 0.01
Neuropeptide Y					
NPY-S1	Ac-ARHY**X**NLL**X**RQRY#	1781.10/1782.22	18.22 ± 0.03	47.6%	−0.71 ± 0.12
NPY-S2	Ac-Y**X**KKK**X** (Ahx) ARHYINLLTRQRY#	2655.57/2656.66	18.67 ± 0.01	49.9%	0.08 ± 0.03

(Sar) represents sarcosine (e.g. N-methyl glycine).
[a] (#) denotes amidation of the C-terminus. (X) represents (S)-2-(4-pentenyl)alanine. (Ahx) is 6-aminohexanoic acid.
[b] Average retention times determined using a linear gradient of H_2O/acetonitrile.
[c] Log*D* values were calculated from the average of three independent experiments and were determined using either capacity factor (k') or shake-flask methods.

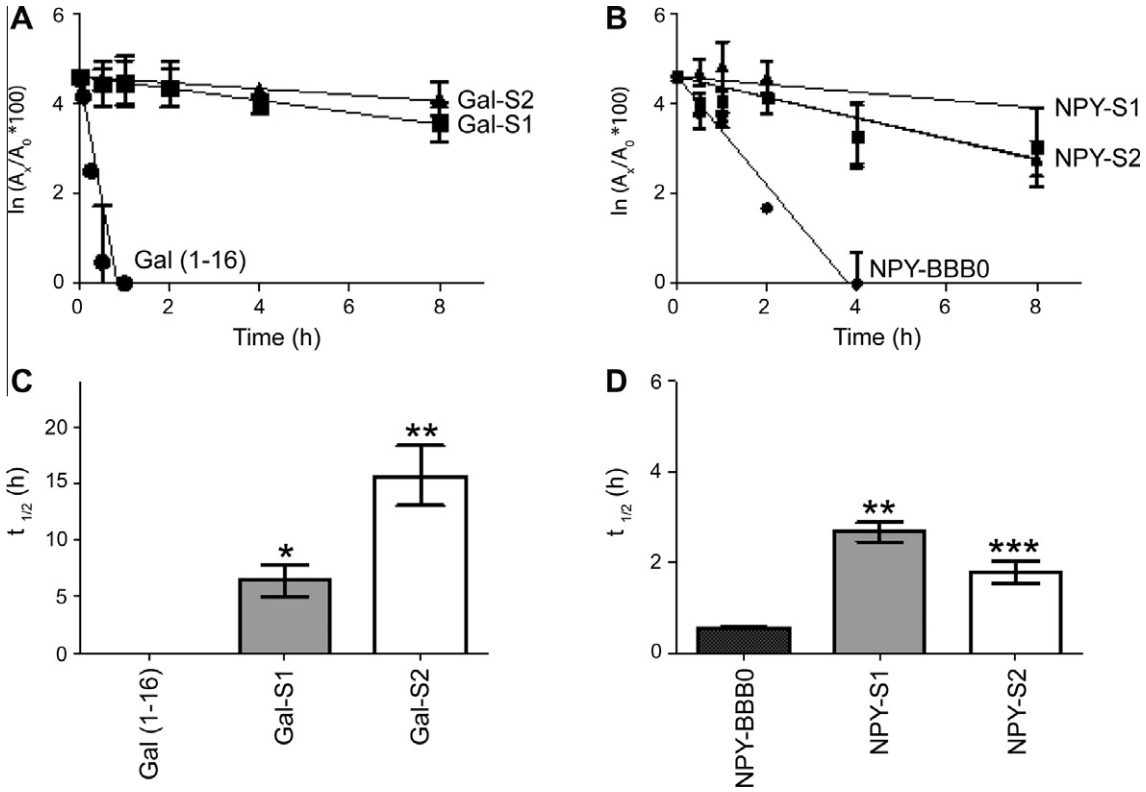

Figure 4. Metabolic stability of stapled neuropeptides in an in vitro serum stability assay. (A) Time course of the disappearance of stapled GAL analogs ranging from 0 to 8 h. (B) Time-course of the disappearance of stapled NPY analogs ranging from 0 to 8 h. Half-lives for all analogs were calculated by comparison of HPLC peak area at each time point (A_x) versus time zero (A_0) ($n \geqslant 3$ independent time course experiments). (C) Half-lives for Gal(1-16), Gal-S1, and Gal-S2 were calculated as 7.8 min, 6.4 h and 15.7 h, respectively. (D) Half-lives for NPY-BBB0 and its analogs were calculated as 0.7 h, 2.7 h and 1.8 h. Statistical comparisons of the stapled analogs against the unmodified active fragments were performed using a standard two sample t-significance test in GraphPad software. Statistical significance was noted as *P*-value< 0.05 (*), *P*-value< 0.005 (**), and *P*-value< 0.001 (***)

Due to the motor toxic effects of NPY-S1, and technical limitations, we selected NPY-S2 for further characterization in the receptor functional assay. Concentration-response curves for full-length hNPY and NPY-S2 were generated in CHO-K1 cells expressing human NPY2R (Fig. 6, Panel C). NPY-S2 inhibited the forskolin-mediated cAMP production with potencies in the nanomolar range (EC_{50} = 402.8 ± 52.3 nM), while the potency of full-length hNPY was in the low nanomolar range (EC_{50} = 2.5 ± 0.4 nM).

2.7. Anticonvulsant activity

Anticonvulsant activities for the stapled peptides were evaluated in the 6 Hz seizure model at a current intensity of 32 mA in mice following bolus i.c.v. injection of each analog (Table 2). Protection from seizures was tested at 0.5 h post-drug administration. As shown in Table 2, groups of 8 mice were injected with 1 nmol/5 μL peptide and the number of animals which did not exhibit seizure activities were classified as 'protected'. Injections of the unmodified active fragment, Gal(1–16) resulted in 5/8 animals protected with 0/8 exhibiting motor toxicity. Both stapled galanin analogs were efficacious in suppressing seizure with 8/8 mice protected for Gal-S1 with 0/8 exhibiting motor impairment and 7/8 mice protected with 1/8 exhibiting motor impairment for Gal-S2. Injections of the unmodified NPY(13-36) resulted in 4/8 animals protected with 0/8 exhibiting motor impairment. Stapled NPY analogs were also effective in suppressing seizures using this model with 8/8 (NPY-S1) and 7/8 (NPY-S2) exhibiting seizure protection. Of note, i.c.v. administration of NPY-S1 resulted in 7/8 animals manifesting motor toxicity as evidenced by tremor that commenced upon injection of the compound. The reasoning for the motor impairment is still not yet understood. It is important to note that the dose-dependent effect of NPY-S1 on motor toxicity below the 1 nmol screening dose was not determined in this study. NPY-S2 resulted in 1/8 animals displaying motor impairment.

3. Materials and methods

3.1. Chemical synthesis

Synthesis of Gal/NPY analogs was carried out using a Symphony automated peptide synthesizer (Protein Technologies, Inc). Syntheses were performed at 100 μmol scale using rink Amide-AM resin (subs. 0.4 mmol/g). Triple couplings of each amino acid (30, 30, and 40 min) were performed using 0.2 M PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate), 0.4 M DIPEA (*N*,*N*-diisopropylethylamine), and 200 mM of each amino acid. Manual coupling of Fmoc-(*S*)-2-(4-pentenyl) alanine was accomplished by two 1 h coupling reactions using 20 mM amino acid in the presence of 0.2 M PyBOP and 0.4 M DIPEA. Fmoc deprotection was carried out by 20 min treatment with 20% piperidine in dimethylformamide (DMF). Fmoc-protected amino acids were obtained commercially from Chem-Impex International. Fmoc-(*S*)-2-(4-pentenyl) alanine was obtained from AAPPTec.

On-resin ring closing metathesis (RCM) was performed on the fully-protected peptides using 40 mol% Grubb's first generation catalyst in dichloromethane at room temperature under nitrogen for 48 h. At the conclusion of the reaction, resins were washed with the following: *N*-Methyl-2-pyrrolidone (5 × 1 mL × 1 min), MeOH (5 × 1 mL × 1 min), and DCM (5 × 1 mL × 1 min). Peptides were cleaved from resin by treatment with reagent *K* (82.5% TFA, 5%

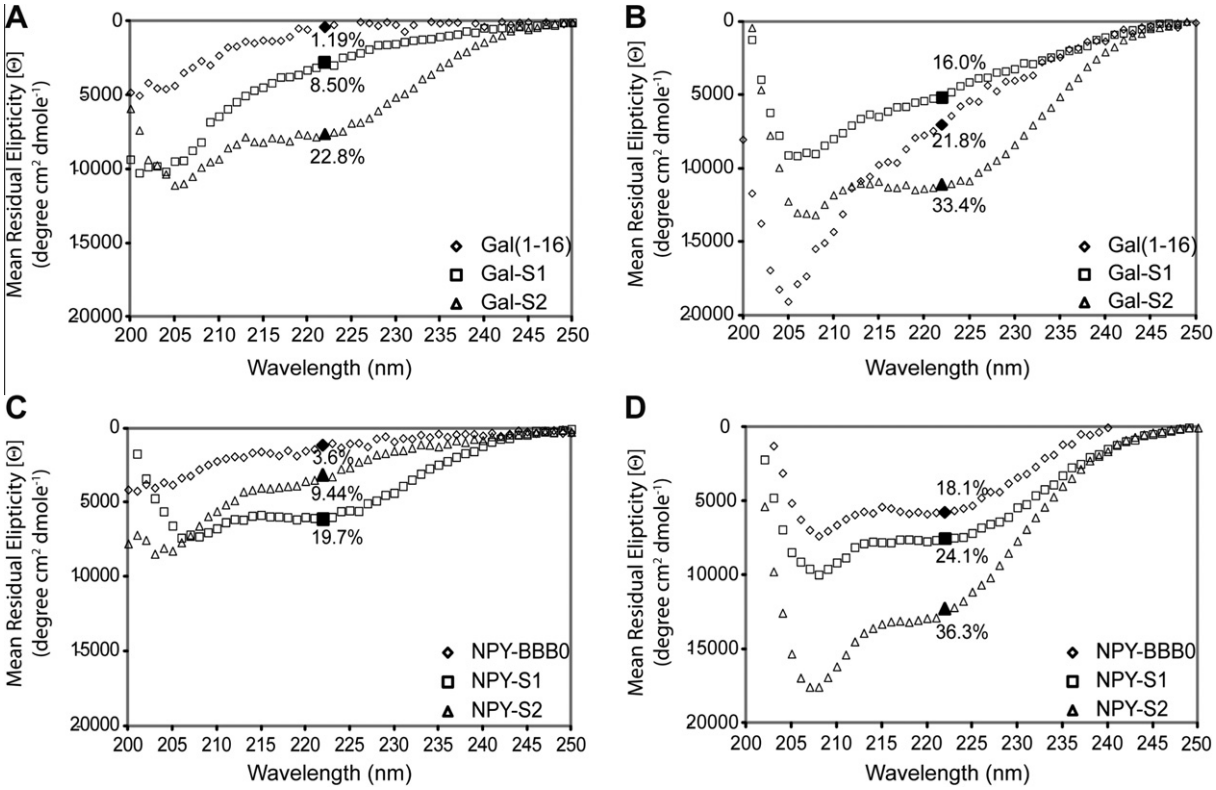

Figure 5. Helical properties of hydrocarbon stapled galanin and NPY analogs in comparison to peptide minimized structures. (Panels A and B) Spectra of Gal(1-16) (◇), Gal-S1 (□), and Gal-S2 (Δ) collected in aqueous (150 nM NaF phosphate buffer, pH 7.4) and helix-stabilizing conditions (150 mM NaF phosphate buffer, pH 7.4 in 50% v/v TFE). α-helical content was also determined for NPY-BBB0 (◇), NPY-S1 (□), and NPY-S2 (Δ) under the same conditions (Panels C and D). Percent helicity was calculated at 222 nm and is highlighted by filled data points.

water, 5% ethanedithiol, 2.5% thioanisole v/v, 75 mg/mL phenol) followed by vacuum filtration to separate peptide from resin, and finally precipitated with chilled methyl-*tert*-butyl ether. Crude peptides were then purified by preparative reversed-phase HPLC over a linear gradient of solvent B (90% acetonitrile in 0.1% TFA) ranging from 5% to 60% in 55 min. Solvent A was 0.1% TFA in water. Purities of separated fractions were assessed by analytical reversed-phase HPLC using a linear gradient ranging from 5% to 95% of solvent B in 30 min. HPLC fractions with peptide purities greater than 95% were pooled and quantified by measuring UV absorbance at either 279.8 nm (Gal) or 274.6 nm (NPY). Molecular masses of all analogs were confirmed by MALDI-TOF MS.

3.2. Partitioning coefficient, log*D*

3.2.1. HPLC capacity factor (*k*′) method

For galanin analogs, log*D* values were calculated by comparison to HPLC retention times with experimentally-determined samples. Briefly, 5 μg standards of each peptide were injected, in triplicate, onto a Vydac diphenyl HPLC column over a linear gradient ranging from 5 to 95% solvent B in 30 min. The average retention time for each peptide standard was then used to calculate capacity factors (k') using Eq. (1) seen below. In this equation t_0 is the solvent front and t_r is the retention time of each peptide standard. log*D* values obtained experimentally from the shake-flask method were then plotted against their respective *k*′ values, yielding a linear plot. This plot was then used to determine the log*D* values for remaining analogs using this standard curve:

$$k' = \frac{(t_r - t_0)}{t_0} \quad (1)$$

3.2.2. Shake-flask method

For stapled NPY analogs, log*D* values were calculated directly using the *n*-octanol/water shake-flask method, as previously described.[34] Briefly, water–saturated *n*-octanol was prepared by mixing equal volumes of *n*-octanol and water for 24 h at room temperature. Lyophilized peptides (0.1 mg) were then reconstituted in 1 mL phosphate-buffered saline (PBS), followed by addition of an equal volume of water–saturated *n*-octanol. Samples were then allowed to mix on a rotary mixer for 24 h. Following mixing, samples were allowed to equilibrate for 15 min. Aqueous phases were carefully removed and analyzed by analytical HPLC over a gradient of solvent B ranging from 5% to 95% in 30 min. The amount of peptide in the aqueous phase was determined by HPLC using a standard curve constructed for each peptide. log*D* values for each analog were then calculated using the Eq. (2):

$$\log D = \log\left(\frac{[\text{Peptide}_{\text{Octanol}}]}{[\text{Peptide}_{\text{Aqueous}}]}\right) \quad (2)$$

3.3. Serum stability assay

The stability of peptides in the presence of 25% rat blood serum was evaluated for each analog by incubation at 37 °C for 0 min, 30 min, 1 h, 2 h, 4 h, and 8 h.[35,36] Samples were prepared by adding 5 μg of a peptide, dissolved in nH_2O, to pre-heated tubes containing 25% rat blood serum and 0.1 M Tris–HCl, pH 7.5. At the appropriate time points, reactions were quenched by precipitation of serum proteins through addition of 100 μL isopropanol/water/trichloroacetic acid (45%:40%:15% v/v/v). Quenched samples were incubated at −20 °C for 20 min, followed by centrifugation at

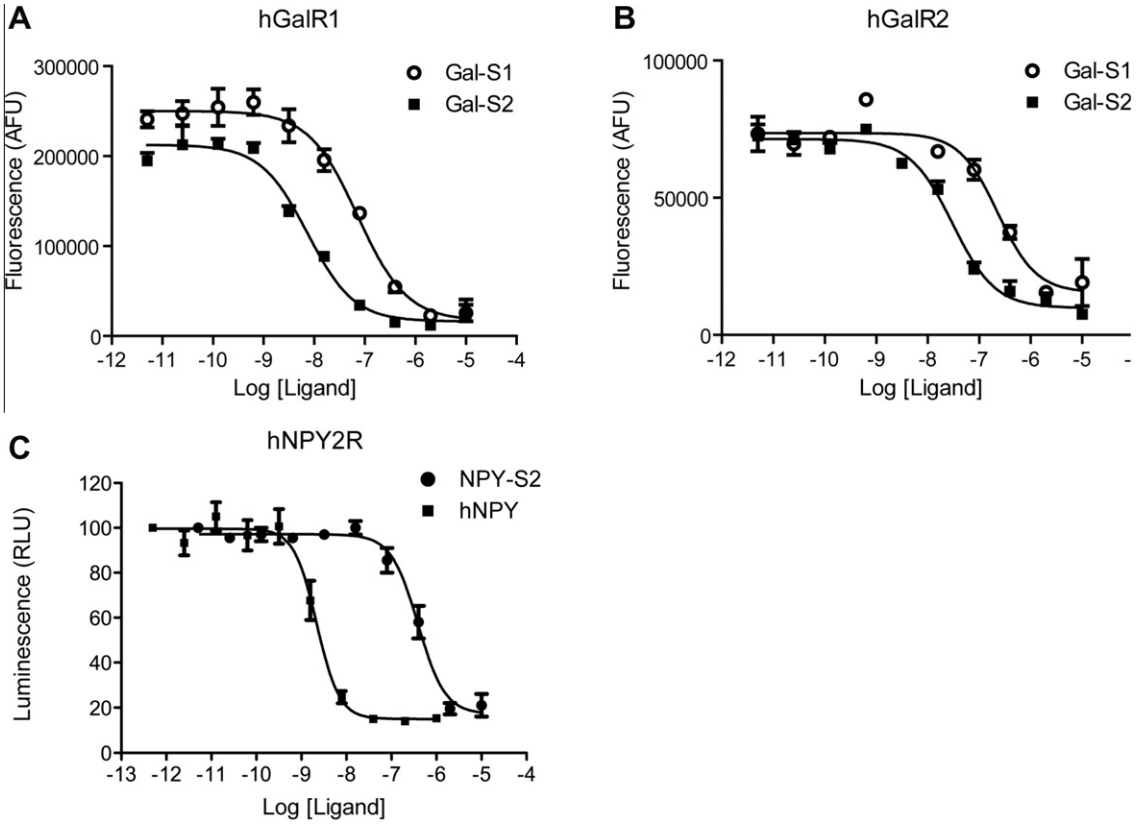

Figure 6. Receptor binding studies and functional studies for stapled neuropeptide analogs. Competitive fluorescence-based binding assays were performed using europium-labled galanin with human galanin receptor 1 (hGalR1) (A) and hGalR2 (B) membrane preparations. The binding assays were performed in triplicate. (Panel C) Representative curves illustrating the inhibition of forskolin-mediated cAMP production by NPY-S2 and full-length hNPY. Cells (30,000 cells/well) were incubated in a 96-well format for 24 h. Increasing concentrations (up to 10 μM) of NPY-S2 were co-incubated with 20 μM of forskolin at 37 °C for 30 min. EC_{50} values were obtained using data from three independent experiments. Data points were collected as the percentage of relative luminescence units (RLU).

Table 2
Summary of 6 Hz (32 mA) data for stapled neuropeptides. Analogs were administered icv to groups of 8 mice at a concentration of 1 nmol/5 μL. Protection and motor impairment were determined 0.5 h post administration. Data represent the number of mice which did not exhibit seizure activity or that exhibited motor impairment in each test group ($n = 8$).

Analog	Anticonvulsant activity (# mice protected)	Motor impairment
Galanin		
Gal(1-16)	4/8	0/8
Gal-S1	8/8	0/8
Gal-S2	7/8	1/8
Neuropeptide Y		
NPY(13-36)	5/8	0/8
NPY-S1	8/8	7/8
NPY-S2	7/8	1/8

10,000 rpm for 3 min to remove serum proteins. Supernatants were then removed and were analyzed by analytical HPLC equipped with a Waters YMC ODS-A 5 μm 120 Å column over a linear gradient ranging from 5 to 95% solvent B in 45 min including a 15 minute pre-equilibration. Metabolic stability was assessed by determining a time-course of the disappearance of an intact peptide. Half-lives ($t_{1/2}$) for each peptide were determined from at least three independent time-course experiments using Eq. (3) below (where m is the slope of the line and b is the y-intercept):

$$t_{1/2}(\mathrm{h}) = \frac{[\ln(50) - b]}{m} \tag{3}$$

3.4. Circular dichroism

To determine the effects of hydrocarbon stapling on the α-helical content of galanin and neuropeptide Y analogs, CD experiments were conducted. 0.2 mg of peptide was reconstituted in 1.0 mL NaF/phosphate buffer (50 mg KH_2PO_4, 1.55 g NaF, 250 mL nH_2O, pH 7.4 with Na_2HPO_4) or in a 1:1 mixture of NaF/phosphate buffer and trifluoroethanol (TFE). After 1 h, 250 μL of each sample was loaded into a 0.1-cm quartz cuvette and analyzed using an Aviv 410 spectropolarimeter set to 25 °C. Scans were collected over the range of 250–200 nm in 1.0 nm steps with 1 s dwell time. Data were collected as the average of three scans and were processed using Eq. (4), where M_r is the molecular weight, c is the peptide concentration (mg/mL), d is the path length (cm), and n is the number of peptide bonds.[37] Percent helicity was determined using Eqs. (5) and (6).[38,39]

$$[\Theta] = \frac{\Theta \times 100 \times M_r}{c \times d \times n} \tag{4}$$

$$[\Theta]_{\max} = -39500\left[1 - \left(\frac{2.57}{n}\right)\right] \tag{5}$$

$$\%\alpha - \mathrm{helix} = \frac{[\Theta_{222}]}{[\Theta_{\max}]} \times 100 \tag{6}$$

3.5. Receptor binding studies

Binding affinities of stapled GAL analogs towards the GalR1 and GalR2 subtypes were determined using a competitive fluores-

cence-based binding assay. The binding assays were performed on AcroWell 96-well filter plates (Pall Life Sciences) using purchased human GaR1 and GalR2 receptor membrane preparations (Millipore and Perkin-Elmer), europium-labeled galanin (Perkin-Elmer), and DELFIA binding and wash buffers (Perkin-Elmer). The binding assays were performed in triplicate with 6 μg of membrane protein (1.4 *p*mol/mg protein) and 2 nM of europium-galanin, in a volume of 100 μL of the binding buffer (50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 25 μM ethylenediaminetetraacetic acid, and 0.2% bovine serum albumin). The galanin analogs were incubated at room temperature for 1.5 h, followed by 4 rinsing steps with wash buffer (50 mM Tris–HCl, pH 7.5 and 5 mM $MgCl_2$) using a vacuum manifold. DELFIA enhancement solution (200 μL) was added and the plates were incubated at room temperature for 0.5 h. The plates were read on a VICTOR™ spectrofluorometer using a standard time-resolved fluorescence measurement for europium-based compounds (excitation at 340 nm, delay for 400 μs, and emission at 615 nm). Competition binding curves were analyzed with GraphPad Prism software using a sigmoidal dose-response (variable slope) equation for nonlinear regression analysis.

3.6. Inhibition of forskolin-mediated cAMP production by NPY-S2

CHO-K1 cells transfected with human NPY2R (DiscoveRx, CA) were plated at 30,000 cells/well in a 96-well format. The inhibition of forskolin-mediated cAMP production was determined using cAMP detection kit (DiscoveRx, CA) according to the manufacturer's instruction. The assay was performed in triplicate. Briefly, after 24 h of incubation, the medium was removed from the well, and increasing concentrations (up to 10 μM) of NPY-S2 were co-incubated with 20 μM of forskolin at 37 °C for 30 min. The amount of cAMP in the cell was determined by measuring the luminescence on a Victor™ plate reader (Perkin-Elmer). The results were analyzed with GraphPad Prism using the sigmoidal dose–response (variable slope) classical equation for nonlinear regression analysis. Three independent experiments were performed to generate the average EC_{50}.

3.7. Anticonvulsant activity

The anticonvulsant activity of the analogs was studied in the 6 Hz (32 mA) partial psychomotor seizure model of pharmacoresistant epilepsy following bolus intracerebroventricular (icv) administration of 1 nmol peptide in 5 μL 0.9% saline vehicle to adult male CF-1 mice ($n = 8$). At 0.5 h post-administration (time-to-peak anticonvulsant effect), mice were challenged with a 6 Hz corneal stimulation (32 mA for 3 s delivered via corneal electrodes). Typically, the seizure phenotype is characterized by a minimal clonic phase that is followed by stereotyped, automatistic behaviors described originally as being similar to the aura of human patients with partial seizures.[40] Animals not displaying this behavior were considered protected.

3.8. Rotorod testing

The rotorod procedure was used to determine minimal muscular or neurological impairment. Briefly, the mouse was placed on a rod that rotated at a speed of 6 rpm, in order for the animal to maintain its equilibrium for extended periods of time. The animal was considered motor impaired (toxic) if it fell off the rotating rod three times during a 1-min observation period.

3.9. Animal care

Adult male CF-1 albino mice (26–35 g), obtained from Charles River (Portage, Michigan), were utilized for behavioral testing in both the 6 Hz seizure test and the rotorod procedure. The animals were allowed free access to food (Prolab RMH 3000) and water, except during the short time they were removed from their cage for testing. The animals were housed, fed, and handled in a manner consistent with the recommendations in the National Research Council Publication, 'Guide for the Care and Use of Laboratory Animals' All animals were euthanized in accordance with the Institute of Laboratory Resource policies on the humane care of laboratory animals.

4. Discussion and conclusions

The efforts described in this work focused on the application of RCM-mediated hydrocarbon-stapling, in conjunction with increased cationization (i.e., oligo-Lys motifs), to the peptide active fragments Gal(1-16) and NPY(25-36). The analogs described here were shown to be metabolically more stable than their respective peptide active fragments in an in vitro serum stability assay, exhibited increased α-helical content, possessed greater *n*-octanol/water partitioning, and were active in suppressing seizure in a mouse model of epilepsy following icv administration.

log*D* determination is of interest for the development of peptide analogs which can be administered peripherally to act on their receptor targets in the brain. There exists an apparent sequence and context dependence of the effects of hydrocarbon stapling on log*D* values of the neuropeptide analogues. Only modest differences were observed for the stapled galanin analogs Gal-S1 and Gal-S2, in comparison to Gal(1-16). In contrast to our previous efforts, which incorporated lipoamino acids,[9] these results suggested that the hydrocarbon staple did not significantly increase the hydrophobic character of the galanin active fragment. Stapled NPY analogs exhibited significant increase in log*D*, particularly for NPY-S2. The highly negative values of NPY analogs could be attributed to the abundance of positively-charged residues in the peptide active fragment. It should be noted that even the bioactive stapled analogs did not exhibit sufficiently high log*D* values that would implicate them as systemically active; however, in combination with our previous findings,[9,10] it is conceivable that systemically-active stapled analogs could be engineered by combining different modification strategies.

Hydrocarbon stapling improved serum stability of GAL and NPY analogs over the unmodified active fragments. These improvements were likely due to the increased α-helical character of the analogs and by the physical presence of the dicarba bridge itself which would be expected to sterically affect access to protease-sensitive peptide bonds. To reduce the N-terminal degradation, methylation (GAL) or acetylation (NPY) were also included for the stapled analogs. The N-terminus of GAL was previously shown to accommodate such modification without adversely affecting receptor binding. The Gal(1-16) fragment was rapidly degraded in an in vitro serum stability assay ($t_{1/2} = 7.8$ min).[9] C-terminal extension of Gal(1-16) with oligo-Lys motifs improved the half-life to 4.6 h.[9] Hydrocarbon stapling at either the N- or C-terminus, in the context of oligo-cationization, significantly increased proteolytic stability for both Gal-S1 and Gal-S2. The NPY-based analogs also exhibited greater metabolic stability compared to the active region of full-length NPY ($t_{1/2} = 0.67$ h). One unexpected outcome of these experiments was that NPY-S1 was more stable than NPY-S2. This observation was puzzling since NPY-S2 incorporated

an Ahx-spacer which would be expected to confer greater metabolic stability. Regardless of possible hypotheses to explain those differences, our results showed that through incorporation of hydrocarbon stapling motifs, metabolic stability of endogenous neuropeptides was improved. By increasing the half-life of peptide analogs in the serum, one may also improve the pharmacokinetic and pharmacodynamic properties by allowing more time for the intact peptide to reach the intended molecular target. Side-by-side comparison of hydrocarbon stapled analogs against other cyclization methods (e.g. lactams, disulfides, etc.) are recommended to conclude whether this method is more effective.

Helical contents of the stapled analogs were compared to the peptide active fragments by circular dichroism over a range from 200 to 250 nm. Under aqueous conditions all stapled analogs exhibited greater helicity at 222 nm. The data were also collected in the presence of 50% v/v TFE. Under helix-stabilizing conditions, the Gal-S1 analog actually exhibited lower helical content as compared to Gal(1-16). As mentioned previously, α-helical content may be important for proper interaction with the receptor target. As such, we hypothesized that stabilization of the helix by hydrocarbon stapling could improve binding and/or activation of galanin or neuropeptide Y receptors.

Receptor binding studies revealed that despite increased α-helicity, stapled analogs possessed lower affinities for their respective receptors than the unmodified fragments. However, the stapled galanin analogs still maintained low nanomolar affinities for GalR1 and GalR2. Similar to the Gal(1-16) fragment, both analogs exhibited a preference for the GalR1 subtype. Interestingly, the preference for GalR1 over GalR2 is decreased from 26-fold for Gal(1-16) to three or fourfold for Gal-S1 and Gal-S2, respectively. This is an interesting observation towards developing GalR2 preferring analogs which are of interest due to the location of these receptors in the brain: our recent efforts showed that N-terminal modification of galanin could shift preferences between receptor subtypes.[41] It is possible that application of these findings to stapled galanin analogs could result in metabolically stable peptide analogs that have a greater selectivity for GalR2 receptors. Full-length NPY and NPY-S2 were screened against the NPY2-receptor, which has been implicated in seizure activity. Though NPY-S2 exhibited nanomolar affinity for the receptor, binding was again lower than the peptide's active fragment. Due to limited resources and the significant motor toxic effects of NPY-S1, this analog was not tested in the receptor functional assay.

Despite lower binding affinities, stapled GAL and NPY analogs exhibited a notable activity in suppressing seizure in the 6 Hz mouse model of epilepsy. Approximately 50% of the animals were considered 'protected' following icv administration of the unmodified peptides. In contrast, the stapled analogs suppressed seizure in nearly all animals tested, an improvement that could be, at least in part, accounted for by their increased metabololic stability. More pharmacological studies will determine the consequences and potential benefits of increased helical content of stapled peptides. Nonetheless, our present data suggest that hydrocarbon stapling may be an effective design strategy for helical peptide analogs that target extracellular receptors.

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