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Generating Orally-Active Galanin Analogs with Analgesic Activities

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Abstract

The endogenous neuropeptide galanin has anticonvulsant and analgesic properties mediated by galanin receptors expressed in the central and peripheral nervous systems. Our previous work showed that combination of truncation of the galanin peptide along with N- and C-terminal modifications afforded analogs that suppressed seizures or pain following intraperitoneal administration. To generate orally-active galanin analogs, the previously reported lead compound Gal-B2 (NAX 5055) was redesigned by (1) central truncation, (2) introduction of D-amino acids, (3) and addition of backbone spacers. Analog D-Gal(7-Ahp)-B2, containing 7-amino heptanoic acid as a backbone spacer and oligo-D-lysine motif at the C-terminus, exhibited anticonvulsant and analgesic activity post intraperitoneal administration. Oral administration of D-Gal(7-Ahp)-B2 demonstrated analgesic activity with reduction in both acute and inflammatory pain in the mouse formalin model of pain at doses as low as 8 mg/kg.

Keywords

galanin; analgesic peptides; orally-active; backbone prosthesis; neuropeptide

Introduction

Peptide-based therapeutics generally exhibit high potency and receptor specificity, lower toxicity, and minimal drug-drug interactions. Over 60 peptide drugs or peptidomimetics are used by patients. Additionally, there are more in preclinical and clinical development.^[1,2] In developing peptide-based therapeutics, a systematic strategy for converting metabolically unstable peptides into drug leads is employed and often includes: (1) identification of the active fragment from the parent peptide, either by alanine-scans, deletions, or D-amino acid scan, (2) modification of the active fragment to confer metabolic stability, using *N*-

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methylation, cyclization, or non-natural amino acids, (3) and esterification or PEGylation to improve pharmacokinetic properties.^[2]

Orally-active peptides with various activities have been reported. A handful of these peptides are summarized in Table 1. Cyclosporin A, an immunosuppressant, is a peptide drug with remarkable systemic bioavailability that can be accounted for by the presence of *N*-methylation, backbone cyclization, and presence of non-natural amino acids. Desmopressin (DDAVP) belongs to the vasopressin/oxytocin family of peptides and is an FDA approved drug for the treatment of nocturnal enuresis. To generate orally-active somatostatin analogs, Kessler and coworkers applied *N*-methylation of the peptide backbone.^[3–5] Work towards developing orally-active peptide analgesics have resulted in the development of peptides based on opioid peptides such as dermorphin^[6] and Leu-enkephalin.^[7] Oral dermorphin incorporates many β -amino acids, D-methionine oxide and an *N*-terminal imine. Other examples of orally bioavailable peptides that have activity against neuropathic pain are RAP-103^[8] and cVc1.1, derived from *Conus* snail venoms.^[9] The parent toxin Vc1.1, is a 16 amino acid sequence with two disulfide bridges, and did not show activity when administered orally (p.o.) or subcutaneously (s.c.). This peptide, when cyclized from the *N*-terminus to the *C*-terminus using the amino acid linker sequence GGAAGG, exhibited a significant increase in analgesia in a dose dependent manner.^[9] Along with the listed peptides, work has been reported on developing oral insulin (Oral-LynTM)^[10], oral glucagon (liraglutide, Victoza®; NN9924)^[11] for blood-sugar management, and calcitonin for osteoporosis.^[12] Neuroprotective peptides have also been developed, with the synthesis of the all D-isomers of the D-NAP and D-SAL peptides as potential treatments for Alzheimer's disease.^[13]

Galanin is an endogenous neuropeptide that has been known to mitigate neuronal hyperexcitability associated with seizures and pain,^[14] as well as being implicated in other neurological diseases.^[15,16] As illustrated in Figure 1, galanin-based therapies may be useful for the treatment of assorted neurological diseases. The previously reported lead galanin compound, **Gal-B2** (NAX 5055) demonstrated anticonvulsant and analgesic activities for up to 2 h post intraperitoneal (i.p.) administration.^[19–22] As described further, the **Gal-B2** analog served as the starting point for further modifications to obtain an orally-active galanin analog **D-Gal(7-Ahp)-B2** (Figure 2), that maintains analgesic activities. This was achieved by using a combination of chemical modifications such as truncations, backbone prosthesis,^[23,24] and D-amino acids.

Results and Discussion

Rational design

Our previous efforts on generating systemically active galanin analogs that penetrate the blood-brain barrier focused on C-terminal lipidization and cationization of the active galanin fragment.^[19] Using **Gal-B2** (NAX 5055) as the starting point, we incorporated D-amino acids during peptide synthesis of the oligolysine motif to confer added metabolic resistance. We observed that the resulting **Gal-B9** analog displayed equivalent anticonvulsant activity to **Gal-B2**. Successive internal truncations of the LLGP amino acids of the galanin fragment showed a slight loss towards receptor affinity, however these analogs maintained

anticonvulsant activity. Next, the LLGP internal fragment was replaced with an aliphatic linker, e.g., 6-aminohexanoic (6-Ahx) or 7-aminoheptanoic acid (7-Ahp). The **D-Gal(7-Ahp)-B2** (Figure 2) analog exhibited increased metabolic stability, in vivo anticonvulsant activity (i.p. administration), and displayed analgesic activity post p.o. administration.

Peptide synthesis

A series of analogs contained the following modifications: cationization/lipidization, D-amino acids, truncations, and backbone spacers (Table 2). All analogs were synthesized using an automated peptide synthesizer using Fmoc chemistry protocols. Backbone prosthetic groups were introduced manually using the corresponding Fmoc protected amino acid. To keep the lysine-palmitoyl (K_P), 6-Ahx, and 7-Ahp soluble, NMP/THF (9:1 v/v) was used as the solvent. The peptides were then cleaved from resin using reagent K and purified by HPLC. Peptide masses were confirmed by MALDI-MS, and the peptides were quantitated by UV absorption (279.8 nm, $\epsilon = 7000 \text{ M}^{-1} \text{ cm}^{-1}$). Average yields of purified peptides were found to be 2% to 8% of resin mass used.

Receptor binding

Galanin receptor binding affinity (K_i) was determined by competitive binding assay, as previously reported.^[19,25] Europium-labeled galanin was used in competition with the analogs against human galanin receptors GalR1 and GalR2. The galanin receptor and Eu-galanin concentrations were held constant and the analog was tested at concentrations that ranged from 0.5 pM to 1.0 mM. Assays were performed in quadruplicate. Following a 90 min incubation and successive washings the remaining Eu-galanin was measured by time resolved fluorescence. The resulting data were analyzed using GraphPad Prism software to generate a binding curve. Three independent binding curves were used to determine the K_i , which are summarized in Table 2.

It has already been noted that with the introduction of the K_P motif, reduces galanin receptor affinity when compared to Gal(1-16);^[19] however, low nanomolar affinities were retained. With the introduction of D-amino acids in the oligolysine motif (**Gal-B9**), we found that the binding affinity was slightly increased. With the stereo chemistry of the unmodified lysines in the KKK_PK motif inverted, one can infer that steric bulk in the tail region has been altered to allow for better receptor binding.

Internal truncation of Pro13 resulted in lower receptor affinity; indeed, removal of the LLGP fragment nearly ablated receptor binding affinity. Since an overview of receptor-ligand interactions^[26–30] shows that these *N*-terminal amino acids are not requisite for high affinity binding, the loss in receptor affinity could potentially be attributed to the steric bulk of the lysine-palmitoyl motif encroaching on the *N*-terminal active fragment. Replacing these residues with an aliphatic backbone prosthetic group restored binding affinity. Combining the strategy of using of backbone prosthesis along with D-amino acids generated analog **D-Gal(7-Ahp)-B2**, which has sub-millimolar affinity for GalR1.

Anticonvulsant Activity

Each analog was assayed for its anticonvulsant activity and the results are summarized in Table 3. Based on our previous work, the galanin analogs were first administered intraperitoneally (i.p.) to CF-1 mice at dosages of 4 mg/kg ($n = 4$). At 15, 30, 60, 120, and 240 min post-injection, groups of mice were challenged with corneal stimulation (6 Hz, 32 mA).^[19,21] Analog **Gal-B2** and **Gal-B9** were exemplary in demonstrating anticonvulsant activities up to 4 h post i.p. administration. All of the analogs demonstrated some level of anticonvulsant activity following i.p. administration that lasted for at least 1 h.

Antinociceptive Activity

The analgesic activity of **Gal-B2** has been previously reported.^[22] Antinociceptive activity was determined using the formalin model for pain. For i.p. administration, mice were dosed at 4 mg/kg ($n = 8$). After the time to peak effect (TPE) of 1 h, the hind paw of the mouse was injected with a 5% formalin solution and the licking response was then monitored; 2 min of every 5 min epoch. The licking response was then integrated from 0–10 min for phase I (acute) and from 10–45 min for phase II (inflammatory) nociception and normalized to the saline response (100% licking = no antinociception), summarized in Table 4.

When compared to saline control, analgesic activity was seen in all of the analogs tested. Analog **Gal-B2** reduced licking in phase I and phase II by 46% and 26%, respectively, when compared to saline. Analog **Gal-B9**, which incorporates D-amino acids, had even greater effect with phase I at 29% and phase II at 7%, relative to the saline control. **Gal-B12** (internal deletion of LLGP motif) and **D-[des-LLGP]Gal-B2** (D-amino acids with internal deletion) both showed apparent analgesic properties.

Analog **D-Gal(7-Ahp)-B2**, containing D-amino acids and backbone prosthetic groups had antinociceptive activity equivalent to **Gal-B9** (containing just D-amino acids), with a phase I response at 25% and phase II at 7%, post i.p. administration. Though receptor binding of the analog **D-Gal(7-Ahp)-B2** was much more reduced, overall, increased metabolic stability of **D-Gal(7-Ahp)-B2** could potentially account for the improved in vivo activity.

The previous results were encouraging; therefore selected analogs were administered orally at a dosage of 8 mg/kg. Following a 2 h TPE, the mice were injected with formalin, and licking response was monitored, integrated area under the curves were calculated (Figure 3). **Gal-B2** showed a phase II response post p.o. administration (50%), but no phase I nociception was seen relative to the saline control. The modified **Gal-B9** analog also had reduced activity, showing modest phase I nociception and phase II nociception, similar to **Gal-B2**.

Analog **D-Gal(7-Ahp)-B2** had significant reduction in the phase I response (55%) and in the phase II response (30%). Previously, we demonstrated that i.p. administration of **D-Gal(7-Ahp)-B2** had equivalent activity to **Gal-B9**, however as we observe here, oral administration of **D-Gal(7-Ahp)-B2** had greater antinociception activity than either **Gal-B2** or **Gal-B9**. At this time, it is unclear how much of the analgesic effect is exerted by targeting centrally or peripherally expressed galanin receptors.^[14]

Physicochemical Properties

To test the analogs for their in vitro metabolic stability, the analogs were incubated at 37 °C in 25% rat serum, for up to 8 hours. The reactions were quenched at various times (0 min, 30 min, 1 h, 2 h, 4 h, and 8 h) with trichloroacetic acid (TCA) solution, and the amount of intact peptide remaining was determined by HPLC. The time-course of disappearance of intact peaks was used to determine the half-life of each analog.

Overall, incorporation of the KKK_pK motif extended the half-lives of the peptides significantly. When comparing **Gal-B2** to the galanin fragment Gal(1-16), the $t_{1/2}$ increases 80-fold, from ~7 min to 9.4 h.^[19] Incorporation of D-amino acids also had the same effect; i.e., it increased the metabolic stability, with a $t_{1/2}$ >10 h for analog **D-Gal(7-Ahp)-B2**. It is likely, however, that the peptide will be cleared from the system well before it undergoes metabolic degradation, as concluded from our previous pharmacokinetic and pharmacodynamic data.^[21]

A useful tool for predicting bioavailability of drugs is the octanol-water partitioning coefficient, log *D*. Classically, this was performed using the shake-flask technique between aqueous and organic (n-octanol) layers of test compound. Once a series of log *D*'s of similar compounds is determined, log *D*'s can then be determined by HPLC retention times, wherein the log *D* of a compound has a linear correlation with HPLC elution times, which is a good measure of a compound's lipophilicity.^[31] The combination of lipidization and cationization increased the lipophilicity of **Gal-B2** to 1.24 with respect to Gal(1-16)'s 0.69 value.^[19] As predicted, the loss of hydrophobic residues LLGP did show a decrease in lipophilicity, and subsequently a reduction in log *D*, 1.11 was observed for analog [**des-LLGP**]**Gal-B2**. Replacement of the LLGP sequence with 7-Ahp had a similar effect of reducing the log *D*, 1.11 for analog **D-Gal(7-Ahp)-B2**. Though the LLGP hydrophobic residues were replaced with an aliphatic carbon chain, the overall number of aliphatic carbons from the LLGP and amide bonds were not. Overall, these changes were nominal when comparing Gal-B2 to **D-Gal(7-Ahp)-B2**, and all of the analogs produced herein were greater than Gal(1-16).

Conclusion

Here we report a peptidic analog derived from the galanin neuropeptide, **D-Gal(7-Ahp)-B2**, that demonstrates in vivo analgesic and anticonvulsant activities, and maintains antinociception following oral administration. Previously we reported that cationization and lipidization of the Gal(1-16) fragment significantly increased metabolic stability and log *D*, thus resulting in analog **Gal-B2**, which was found to be systemically active post intraperitoneal administration.^[19] Incorporation of D-amino acids was also effective in producing metabolically stable analogs, and did not impact galanin receptor binding. Here, we further investigated internal truncations to the Gal(1-16) motif, and the incorporation of backbone prosthetic groups. Central truncation of the LLGP motif in combination with our lipidization/cationization motif caused significant loss to the receptor binding affinity. However, we also noted that regardless of the receptor affinity, antinociception activity was

seen in all of the analogs tested. This demonstrates that high affinity binding is not a requisite for in vivo activity for these analogs (Figure 4).

The approach taken to generate **D-Gal(7-Ahp)-B2** differs from some of the cyclic peptides seen in the in Table 1. Noteworthy, cyclized galanin analogs were described previously, but their in vivo activities and bioavailability are not known.^[32] The significance of the work presented is that the analogs described herein join the rare class of orally bioavailable peptides that demonstrate antinociceptive activity in vivo. This work also encourages an exploration of other systemically-bioavailable anticonvulsant neuropeptides, such as neuropeptide Y, neurotensin,^[33] neuropeptide W,^[34] and others,^[30] to develop them as orally-active leads for analgesia or epilepsy. We see a potential use for orally-active anticonvulsant neuropeptides as future drugs for postoperative treatment for pain that is an alternative to current opioid-based regimens. Patient-controlled analgesia^[36] of morphine has mixed results, with many factors to consider: e.g., dosage, efficacy, tolerance, addiction, sedation and other side-effects. Future orally-active, peptide-based therapeutics will allow patients alternative to morphine for self-administered, non-opioid based analgesia.

Experimental Section

Materials and methods

Fmoc protected amino acids, Rink amide resins, DIPEA, and PyBOP were purchased from Chem-Impex International, Inc. (Wood Dale, IL; USA). Other solvents, DMF, NMP, HPLC grade acetonitrile, and reagents TFA, piperidine, and aqueous trichloroacetic acid (20% w/v) were purchased from Sigma-Aldrich (St. Louis, MO; USA) and used without further purification.

HPLC analysis was performed on a Waters HPLC system using Waters Delta 600 pumps in line with a Waters 2487 Dual λ Absorbance Detector using reverse phase diphenyl columns. Time resolved fluorescence was measured on a Victor³ Multilable Reader (Perkin Elmer, Waltham, MA; USA) in a 96-well format.

Peptide synthesis

Peptides were synthesized on a Protein Technologies, Inc. (Tucson, AZ; USA) Symphony multichannel synthesizer using Fmoc solid phase peptide synthesis protocols. Coupling reagents PyBOP and DIPEA were used with Fmoc protected amino acids, using DMF or NMP as solvents. Fmoc-6-amino hexanoic acid and Fmoc-7-amino heptanoic acid (Chem-Impex) were used for introduction of aliphatic linkers where needed. Peptides were cleaved from resin using reagent K (82.5% TFA, 5% H₂O, 5% ethanedithiol, 2.5% thioanisole v/v, 75 mg/mL phenol), 1 mL per 75 mg resin, and resin beads were filtered off. Peptides in the flow-through were precipitated with 3x volume of MTBE (−20 °C, 1 h), and purified by HPLC.

Peptides were purified on linear gradients of solvent A (0.1% TFA in water) and solvent B (0.1% TFA, 90% acetonitrile, 10% H₂O), 15% solvent B to 70% solvent B in 30 min, on Vydac diphenyl columns (Grace, Deerfield, IL; USA, cat# 219TP1010). Purified peptides were quantified by measuring UV absorbance at 279.8 nm ($\epsilon = 7000 \text{ M}^{-1} \text{ cm}^{-1}$). All

peptides had 95% purity or greater. Peptide masses were confirmed by MALDI mass spectrometry analysis at the University of Utah core facility (See Supporting Information).

Receptor binding

Receptor affinity was determined using time-resolved fluorescence, competitive binding assays in a 96-well format. Peptide analogs were competed against Eu-labeled galanin (Perkin-Elmer) on AcroWell filter plates (Pall Life Sciences, Ann Arbor, MI; USA, cat# 5020) charged with receptor membrane preparations (hGAL1 or hGAL2), purchased from either Millipore (Billerica, MA; USA) or Perkin-Elmer, using the DELFIA assay buffers (Perkin-Elmer). Binding assay was carried out with 6 μ g of receptor membrane preparations (1.4 pmol/mg protein) and 2 nM Eu galanin in a volume of 100 μ L of the DELFIA L*R binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 25 μ M EDTA, and 0.2% BSA). Peptides of interest were then added ranging from 1 μ M to 0.5 pM concentration (ten-point curve). Samples were incubated at room temperature for 90 min, followed by washing (4x) with 200 μ L of DELFIA wash buffer (50 mM Tris-HCl, pH 7.5, and 5 mM MgCl₂). DELFIA enhancement solution (200 μ L) was added, and the plates were incubated at room temperature for 30 min. The plates were read on a VICTOR³ multilabel plate reader (Perkin-Elmer) using a standard Eu-TRF measurement (excitation at 340 nm, delay for 400 μ s, and emission at 615 nm for 400 μ s). Competition curves were analyzed with GraphPad Prism software (La Jolla, CA; USA) using the sigmoidal dose-response (variable slope) equation for nonlinear regression analysis. Samples were performed in quadruplicate to generate a single binding curve. Three independent binding curves were used to determine average K_i values.

Animal care

Adult male CF-1 albino mice (26–35 g) and male Sprague-Dawley rats (175–225 g) were obtained from Charles River (Portage, Michigan). Animals were housed, fed, and handled in a manner consistent with IACUC approved protocols (www.iacuc.org). The animals were maintained on an adequate diet (Prolab RMH 3000) and allowed free access to food and water, except during the short time they were removed from their cage for testing.

Anticonvulsant activity

Galanin analogs were administered intraperitoneally to five groups of CF-1 mice ($n = 4$ mice) at 4 mg/kg. At select time points (15, 30, 60, 120 and 240 min) post i.p. administration, the mice were challenged with a 6 Hz corneal stimulation, 32 mA for 3 seconds. Mice were considered protected if they did not display characteristic limbic seizure (e.g., jaw chomping, vibrissae twitching, forelimb clonus, Straub tail) and were graded all or none.

Antinociceptive activity

The formalin assay was used to evaluate the analgesic activity of select analogs.^[35] Male, CF-1 mice (Charles River Laboratories, Wilmington MA) 18–30 g were used. Mice ($n = 8$) were allowed to acclimate to a plexiglass observation chamber for 15 min prior to experiment. Conditioned mice were then dosed with vehicle or test peptide (either 4 mg/kg

for i.p., 4 mg/kg for s.c., or 8 mg/kg for p.o.), after which the mouse was returned to its home tube. At the TPE, a 5% formalin solution was then injected sub-dermally into the plantar surface of the hind paw. Following the formalin injection, each mouse was observed for the first 2 min of each 5 min epochs until 45 min had elapsed. The cumulative length of licking for each 2 min time period was recorded. The bi-modal response was plotted using GraphPad Prism software (La Jolla, CA; USA), and the area under curve (AUC) for each phase (Phase I or Phase II) was determined. Standard error between individual sample sets was determined using Microsoft Excel.

Rat serum stability

Trunk blood from sacrificed male Sprague-Dawley rats was obtained and kept at 0 °C. The whole blood was centrifuged at 4000 rpm for 5 min, the top serum was removed and stored at –80 °C, until use. Serum stored longer than 3 months was not used.

The stability of peptides was determined by incubating peptides in 25% rat serum at 37 °C for 0 min, 30 min, 1 h, 2 h, 4 h, and 8 h ($n = 3$). Lyophilized peptides were resuspended in H₂O, and 10 µg of peptide was added to a pre-warmed tube of 1 mL 25% rat serum in 0.1 M Tris-HCl buffer, pH 7.5. At the appropriate time points, the reaction was quenched with 100 µL of TCA quenching solution (45% *iso*-propanol, 40% water, 15% aqueous trichloroacetic acid [20% aq.], v/v) and the samples were incubated at –20 °C for 20 min. The samples were then centrifuged at 10,000 rpm for 3 min and the supernatant was analyzed by analytical HPLC using a YMC ODS-A 5 µm 120 Å column (Waters Corp., Milford, MA; USA) using a gradient ranging from 5% to 95% solvent B in 45 min including a 15 min pre-equilibration.

Metabolic stability was assessed by the 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 the following equation (1) (see Supporting Information):

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

Where m is the slope of the line and b is the y-intercept.

HPLC determination of log D

The log D values of a series of analogs were determined using shake-flask method.^[17, 23] HPLC retention times of 5 µg aliquots of peptides were run in triplicate on linear gradients ranging from 20% solvent B to 90% solvent B in 15 min, with an immediate return to initial conditions for 20 min. The retention times were then averaged. The capacity factors (k') of the peptides were calculated using the following formula (2):

$$k' = \frac{t_r - t_o}{t_o} \quad (2)$$

where t_o is the solvent front, and t_r is the retention time of the peptide. The log D values obtained from the shake-flask method were plotted against their peptides respective k' values, giving a linear plot. The log D values for all other peptides were calculated using this standard curve (see Supporting Information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ahp	7-amino heptanoic
Ahx	6-amino hexanoic
DIPEA	<i>N,N</i> -diisopropylethyl amine
GALR#	galanin receptor subtype (# = 1 or 2)
i.p	intraperitoneal
Kp	palmitoyl- N^{ϵ} -L-lysine
MTBE	methyl- <i>t</i> -butyl ether
p.o	per os (oral administration)
PyBOP	benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
s.c	subcutaneous
TCA	trichloroacetic acid (aq. 20%)
TFA	trifluoroacetic acid
TPE	time to peak effect

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Figure 1.

Potential for developing galanin-based peptides as possible therapeutics for epilepsy,^[14] pain,^[17] multiple sclerosis,^[18] and neuroprotective effects.^[15]

Figure 2.

Structure and sequence of analog **D-Gal(7-Ahp)-B2**. This analog incorporates an N-terminal sarcosine (N-methylglycine) to the Gal(2-9) fragment coupled to an 7-aminohexanoic acid motif. Cationization and lipidization is achieved by incorporating C-terminal K_DK_DK_pK_D moiety, using D-amino acids and K_p is palmitoylation of N^ε-lysine.

Figure 3.

Antinociceptive activity of galanin analogs in the formalin model for acute (Phase I) and inflammatory (Phase II) pain in mice. All analogs were administered orally at a dosage of 8 mg/kg ($n = 8$), with a time to peak effect of 2 h. Post administration, the mice were tested for analgesic activity, and the area under the curves are presented. Analog **Gal-B2**, demonstrated no acute nociception, whereas analog **D-Gal(7-Ahp-B2)** did have an effect. P-values are with respect to individual analogs against saline controls; error bars represent standard error.

Figure 4.

The antinociceptive response of analog **D-Gal(7-Ahp)-B2** with respect to routes of administration against saline control (black solid line). The analog was administered at 4 mg/kg with a 1 h time to peak effect (TPE) for intraperitoneal (i.p., dashed line) and subcutaneous (s.c., solid gray line). Oral administration (p.o., dash-dot-dash line) performed at 8 mg/kg followed by a 2 h TPE. The duration of licking was monitored for the first 2 min of every 5 min epoch. Regardless of the route of administration, the galanin analog demonstrated analgesic effects.

Table 1

Orally bioavailable peptides and their sequences.

Peptide	Structure ^[a]	Indication
cyclosporine A	$\text{HN}-\text{a}-(\text{Me})\text{L}-(\text{Me})\text{L}-(\text{Me})\text{V}-\text{Bmt}-\text{Abu}-(\text{Me})\text{G}-(\text{Me})\text{L}-\text{V}-(\text{Me})\text{L}-\text{A}-\text{C(=O)}$	Immunosuppressant
desmopressin	$\text{HN}-\text{Y}-(\text{D-MetO})-\text{F}-(\text{Me})\beta\text{A}-\text{OH}$	Nocturnal enuresis
(methylated)somatostatin	$\text{H}_3\text{C}-\text{N}-\text{A}-\text{Y}-(\text{Me})\text{w}-(\text{Me})\text{K}-\text{V}-(\text{Me})\text{F}-\text{C(=O)}$	Neurogenic inflammation
dermorphin	$\text{HN}-\text{Y}-(\text{D-MetO})-\text{F}-(\text{Me})\beta\text{A}-\text{OH}$	Analgesic
cVc1.1	$\text{HN}-\text{G}-\text{C}-\text{C}-\text{S}-\text{D}-\text{P}-\text{R}-\text{C}-\text{N}-\text{Y}-\text{D}-\text{H}-\text{P}-\text{E}-\text{I}-\text{C}-\text{G}-\text{G}-\text{A}-\text{A}-\text{G}-\text{G}-\text{C(=O)}$	Neuropathic pain
D-SAL	s-a-l-l-r-s-i-p-a	Neuroprotection
D-NAP	n-a-p-s-v-i-p-q	Neuroprotection

These peptides have many modifications that increase metabolic stability including: cyclization, N-methylation of peptide backbone (Me), isosteric replacement, and D-amino acids.

^[a] Abbreviations: Mpa = mercaptopropionic acid, Bmt = butenylmethyl threonine, Abu = aminobutyric acid, MetO = methionine oxide, βA = beta-alanine, lowercase letters denote D-AA's

Table 2

Galanin peptide analogs synthesized with various modifications.

Analog	Sequence	hGAL ₁ (nM)	hGAL ₂ (nM)	R2/R1
Gal-B2	(Sar)WTLNSAGYLLGPKKK _p K	3.5 ± 1.0	51.5 ± 34.4	14.71
Gal-B9	(Sar)WTLNSAGYLLGPK _D K _D K _p K _D	0.9 ± 0.2	15.0 ± 8.5	16.67
Gal-B11	(Sar)WTLNSAGYLLGKKK _p K	25.5 ± 4.9	75.0 ± 10.6	2.94
Gal-B12	(Sar)WTLNSAGYLLKKK _p K	306.5 ± 12.0	1,323.5 ± 27.6	4.32
[des-LLGP]Gal-B2	(Sar)WTLNSAGYKKK _p K	30,000	27,395 ± 6,229	–
D-[des-LLGP]Gal-B2	(Sar)WTLNSAGYK _D K _D K _p K _D	30,000	n.d.	n.d.
L-Gal(6-Ahx)-B2	(Sar)WTLNSAGY(6-Ahx)KKK _p K	1166 ± 300	n.d.	n.d.
D-Gal(6-Ahx)-B2	(Sar)WTLNSAGY(6-Ahx)K _D K _D K _p K _D	n.d.	n.d.	n.d.
L-Gal(7-Ahp)-B2	(Sar)WTLNSAGY(7-Ahp)KKK _p K	n.d.	n.d.	n.d.

K_D is the D-amino acid of lysine, K_p is palmitoylation of N^ε-L-lysine, 6-Ahx is 6-aminohexanoic acid, 7-Ahp is 7-aminoheptanoic acid. Competitive receptor binding assays were performed on human galanin receptor membrane preparations.

Table 3

The anticonvulsant activity of galanin analogs.

Analog	Sequence	6 Hz	AUC
Gal-B2	(Sar)WTLNSAGYLLGPKKK _p K	3/4 4/4 4/4 4/4 0/4	16313
Gal-B9	(Sar)WTLNSAGYLLGPK _D K _D K _p K _D	4/4 4/4 4/4 3/4 4/4	20250
Gal-B11	(Sar)WTLNSAGYLLGKKK _p K	1/4 2/4 2/4 0/4 0/4	3563
Gal-B12	(Sar)WTLNSAGYLLKKK _p K	1/4 4/4 4/4 1/4 0/4	9188
[des-LLGP]Gal-B2	(Sar)WTLNSAGYKKK _p K	1/4 4/4 3/4 1/4 1/4	9563
D-[des-LLGP]Gal-B2	(Sar)WTLNSAGYK _D K _D K _p K _D	3/4 4/4 2/4 0/4 0/4	5063
L-Gal(6-Ahx)-B2	(Sar)WTLNSAGY(6-Ahx)KKK _p K	3/4 2/4 2/4 0/4 0/4	3938
D-Gal(6-Ahx)-B2	(Sar)WTLNSAGY(6-Ahx)K _D K _D K _p K _D	2/4 1/4 2/4 0/4 0/4	3138
L-Gal(7-Ahp)-B2	(Sar)WTLNSAGY(7-Ahp)KKK _p K	3/4 2/4 3/4 0/4 0/4	5063

Anticonvulsant activity was assessed after CF-1 mice were treated with the analog (4 mg/kg) i.p. and challenged with corneal stimulation (6 Hz, 32 mA) at 15, 30, 60, 120, and 240 min post-injection.

The number of mice protected per group is shown; integrated area under the curve (AUC) is present for each analog.

Table 4

Antinociceptive activity of analogs in formalin assay.

Analogue	Sequence	Phase I (% of saline control)	Phase II (% of saline control)
Gal-B2	(Sar)WTLNSAGYLLGPKKK _p K	46.4 ± 12.8 ^[a]	26.2 ± 12.4 ^[a]
Gal-B9	(Sar)WTLNSAGYLLGPK _D K _D K _p K _D	29.2 ± 5.8 ^[b]	7.8 ± 5.6 ^[b]
Gal-B11	(Sar)WTLNSAGYLLGKKK _p K	n.d.	n.d.
Gal-B12	(Sar)WTLNSAGYLLKKK _p K	77.5 ± 12.4 ^[b]	2.2 ± .60 ^[a]
[des-LLGP]Gal-B2	(Sar)WTLNSAGYKKK _p K	n.d.	n.d.
D-[des-LLGP]Gal-B2	(Sar)WTLNSAGYK _D K _D K _p K _D	51.4 ± 12.5 ^[b]	20 ± 12.9 ^[a]
L-Gal(6-Ahx)-B2	(Sar)WTLNSAGY(6-Ahx)KKK _p K	64.1 ± 6.3 ^[a]	0 ^[a]
D-Gal(6-Ahx)-B2	(Sar)WTLNSAGY(6-Ahx)K _D K _D K _p K _D	n.d.	n.d.
L-Gal(7-Ahp)-B2	(Sar)WTLNSAGY(7-Ahp)KKK _p K	n.d.	n.d.

Mice were dosed i.p. at 4 mg/kg, followed by a 1 h TPE. Mice were then challenged with a 5% formalin solution injection, and the lick response was monitored. The bimodal response was recorded and the integrated area under the curves are reported for acute (Phase I) and inflammatory (Phase II) responses. Data are reported as the % response with respect to the saline control, with standard error.

^[a] $p < 0.01$

^[b] $p < 0.05$