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Preclinical analgesic and safety evaluation of the GalR2-preferring analog, NAX 810-2

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Abstract

The potential clinical utility of galanin peptidic analogs has been hindered by poor metabolic stability, lack of brain penetration, and hyperglycemia. In addition to possessing potent anticonvulsant efficacy, galanin analogs are analgesic in various assays. The purpose of these studies was to evaluate the lead galanin receptor type 2 (GalR2)-preferring analog, NAX 810-2, in various pain assays, as well as determine any potential for insulin inhibition, growth hormone stimulation, and cognitive impairment. NAX 810-2 was evaluated in mouse (carrageenan, formalin, tail flick, plantar incision) and rat pain models (partial sciatic nerve ligation). NAX 810-2 dose-dependently increased paw withdrawal latency following plantar administration of carrageenan (ED₅₀ 4.7 mg/kg). At a dose of 8 mg/kg, NAX 810-2 significantly attenuated nociceptive behaviors following plantar administration of formalin, and this was observed for both phase I (acute) and phase II (inflammatory) components of the formalin behavioral response. NAX-810-2 was active at higher doses in the mouse tail flick model (ED₅₀ 20.2 mg/kg) and similarly, reduced mechanical allodynia following plantar incision in mice at a dose of 24 mg/kg. NAX 810-2 also reduced mechanical allodynia in the partial sciatic nerve ligation model at a dose of 4 mg/kg. In addition, NAX 810-2 did not impair insulin secretion at doses of 2.5 and 8 mg/kg (acutely) or at a dose of 8 mg/kg given daily for 5 days. Similarly, 8 mg/kg (twice daily, 5 days) of NAX 810-2 did not increase growth hormone levels. These results demonstrate that NAX 810-2 possesses a favorable pre-clinical profile as a novel and first-in-class analgesic.

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Author's Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Disclosure of Conflicts of Interest

GB, LZ, DRM, CM are co-inventors of patented and patent-pending galanin-based technology, owned by the University of Utah and licensed to NeuroAdjuvants, Inc. CM, BK and DRM were full-time employees at NeuroAdjuvants during the collection the data.

Keywords

galanin; analgesic; pain; toxicology; neuropeptide; drug development

INTRODUCTION

The neuropeptide galanin was found to possess anticonvulsant properties 25 years ago, when Mazarati and co-workers found that central administration of galanin reduced seizure behaviors [1–3]. Following this work, Mazarati and others further evaluated the role of galanin in seizure regulation using transgenic and pharmacologic approaches [4–11]. During this time, at the University of Utah, Dr. H. Steve White entered into collaboration with Dr. Grzegorz Bulaj to further study galanin in epilepsy using a novel peptide engineering approach. Initial development of galanin or galanin peptide fragments was hindered by poor metabolic stability and lack of blood-brain barrier (BBB) penetration, and small molecule analogs lacked selectivity and specificity for galanin receptors [12,6]. Therefore, the purpose at the outset of this endeavor was to design and synthesize galanin analogs that can penetrate the blood-brain barrier, attenuate seizure behaviors, and possess improved metabolic stability that supports systemic administration. Through the rational design, good luck and evaluation of several chemical motifs, White and Bulaj discovered that a judicious combination of lipophilicity and cationization was required to meet the goal of a centrally-acting galanin analog that could block seizure activity following systemic administration. This initial discovery triggered more in-depth analysis of the BBB-penetrant galanin analogs using the 6-Hz model of refractory epilepsy [13,14], as well as subsequent validation of this technology toward other neuropeptides such as NPY, NPW and neurotensin [15,16]. From the early studies, the novel galanin receptor type 1 (GalR1)-preferring analog, NAX 505-5 emerged, possessing potent antiseizure activity in several animal models [17,18]. Despite apparent efficacy in seizure models, it was observed that NAX 505-5 elevated glucose levels, an effect thought to be mediated by activating peripheral GalR1 [19,20]. Though inhibition of insulin is a known effect of galanin [21–25], compounds with preference for galanin receptor type 2 (GalR2) over GalR1 may avoid this limitation. Therefore, additional analogs were designed and synthesized that showed preference toward GalR2 over GalR1 and still maintained metabolic stability and anticonvulsant efficacy [26]. The lead analog NAX 810-2, a GalR2-preferring anticonvulsant, was selected and has since been further evaluated for pre-clinical efficacy and safety [27–29]. Both NAX 505-5 (also known as Gal-B2) and its GalR2-preferring derivative [N-Me, desSar]Gal-B2 have been recognized as pharmacological tools for studying the galanin receptors [30,31].

Dr. H Steve White's contribution to 12-year translational research on galanin was driven by rigorous science and in-depth knowledge of neuropharmacology. While continuing development of galanin analogs, Dr. White and colleagues also noted that galanin analogs possess analgesic activity in several animal models. Further work demonstrated the use of polyethylene glycol moieties to aid in peripheral restriction of galanin analogs [32], which possess analgesic activity and avoid side effects common to centrally-acting analgesic compounds (e.g. sedation). This led to the development of the lead peripherally-acting galanin analog, NAX 409-9 [33]. Additional work is ongoing to develop peripherally-

restricted, GalR2-preferring analogs that are effective in rodent preclinical models and possess a favorable tolerability profile that warrants clinical development.

GalR1- or GalR2-preferring and non-selective analogs have been shown to be effective in various models of pain [11,9] and a GalR2-preferring peptide, Gal(2–11), reversed nerve injury-induced allodynia [34]. In order to determine whether the anticonvulsant analog NAX 810-2 also possesses analgesic activity, we evaluated the compound in various rodent pain models: the mouse carrageenan model of inflammatory pain, the mouse formalin model (acute and inflammatory pain components), the mouse tail flick model (centrally-mediated pain response), the mouse plantar incision model (inflammatory and centrally-mediated pain components), and the partial sciatic nerve ligation (PSNL; Seltzer) model of neuropathic pain. Further, as discussed above, galanin analogs may inhibit insulin secretion and therefore we determined whether acute or sub-chronic administration of NAX 810-2 affected insulin and glucose levels. Finally, as galanin overexpression has been shown to elevate growth hormone (GH) secretion [35,36], we determined whether acute or sub-chronic administration of NAX 810-2 affected GH levels. Our findings support favorable preclinical properties of NAX 810-2 as analgesic lead compound.

METHODS

Animals

Male albino CF-1 mice (25–35g; Charles River, Kingston, NY, USA) and male Sprague Dawley rats (200–225g, Charles River, Raleigh, NC, USA) were used as experimental animals. All animals were allowed free access to food and water, except during testing procedures. Experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Utah and were carried out in accordance with the Guide for Care and Use of Laboratory Animals, as adopted by the National Institutes of Health (USA).

Peptide Preparation and Formulation

NAX 810-2 (GalR2-preferring) and NAX 505-5 (GalR1-preferring) (molecular weights 2,124 g/mol and 2,113 g/mol, respectively) was synthesized by PolyPeptide Laboratories (San Diego, CA, USA). NAX 505-5 was dissolved in a vehicle (Veh) solution of 1% Tween 20 (Sigma, St. Louis, MO, USA) in 0.9% NaCl. NAX 810-2 was supplied as a lyophilized powder (acetate salt, purity > 95%) that included a diastereoisomeric mixture (40/60) of the N-terminal amino acid, N-methyl-tryptophan (structure described previously [27]). NAX 810-2 was dissolved in a Veh solvent containing 20% hydroxyl-propyl-beta cyclodextrin (HP β CD; Sigma, St. Louis, MO, USA) in acetate buffer [0.1 M acetic acid (from glacial acetic acid stock; Sigma, St. Louis, MO, USA); 0.1 M sodium acetate (Sigma, St. Louis, MO, USA), pH 5–7].

Mouse Carrageenan

Localized inflammation in the hindpaw of mice was induced by injecting carrageenan (25 μ l, 2% in 0.9% NaCl, λ -carrageenan; Sigma-Aldrich, St. Louis, MO) subcutaneously into the plantar surface of the right (ipsilateral) hindpaw. Carrageenan-induced inflammation was

verified (3h post-carrageenan) using a digital caliper to assess paw edema across the dorso-ventral aspect of both the carrageenan-injected (ipsilateral) and the non-injected (left paw, contralateral) hindpaw. Paw withdrawal responses from thermal stimulation were assessed according to previously described methods [37–39,33]. After carrageenan injection, mice were placed in plexiglass chambers on top of a heated glass surface (30 °C) (IITC, Woodland Hills, CA). Thermal stimulation was applied with a projection bulb (IITC, Woodland Hills, CA; 53 mJ, 35% of maximal stimulus intensity) below the heated glass surface. Latency to paw withdrawal was measured from the onset of thermal stimulation until a full paw withdrawal occurred. Two measurements were obtained from each paw, with at least 1 minute between assessments, and subsequently averaged to obtain the mean paw withdrawal latency. NAX 810-2 was administered by intraperitoneal (IP) injection, 2h following carrageenan paw injection such that the time-to-peak effect (TPE) for NAX 810-2 would match the time of peak inflammation following carrageenan (1h post-NAX 810-2, 3h post-carrageenan). NAX 810-2 was administered at doses of 0.5, 1, 2, 4, and 8 mg/kg IP (N=10–18 per group). Separate groups of mice were also treated with Veh and Veh-treated mice were pooled (N=22) comprising individual groups of Veh-treated mice (N=2–4) that were treated alongside each NAX 810-2 group. Mice were considered “protected” if their ipsilateral paw latency was greater than 50% of the maximal latency observed in Veh treated animals (adapted from Tallarida, 1987 [40]), using the following formula: $[(\text{contralateral latency} - \text{ipsilateral latency})/2] + \text{ipsilateral latency}$. The number of protected/unprotected animals for each dose was then analyzed using a Probit analysis [41] to obtain a median effective dose (ED₅₀) and 95% Confidence Interval (CI).

Mouse Formalin Assay

NAX 810-2 was administered by IP injection (4 & 8 mg/kg) 1h prior to injection of 0.5% formalin (20 µl, 27 gauge needle) subcutaneously into the plantar region of the right hindpaw of mice, according to previously described methods [32]. As formalin elicits a biphasic behavioral response, mice were observed for 45 min following formalin injection, comprising phase I (acute; 0–15 min) and phase II (inflammatory; 15–45 min). Each animal was observed for 2 min of each 5 min epoch throughout the observation period, and the cumulative period of paw licking during each 2 min period was calculated. The area-under-the-curve (AUC) for NAX 810-2 treatment was compared to the AUC following Veh administration and data are reported as a percentage of Veh AUC for each formalin behavioral phase.

Mouse Tail Flick Assay

Tail flick responses were measured using a radiant beam focused on the tail while using an automated analgesic meter (IITC, Woodland Hills, CA), according to previously described methods [33]. Mice were placed in a restraint tube and a light beam (peak intensity 75 mJ, 50% of maximal intensity) was focused on the distal portion of the tail, 2–5 mm from the tip. The light beam included an automatic shutoff in response to movement of the tail or reached a maximal light exposure (time cutoff) of 25 seconds. The trigger temperature (pre-warming) was 25 °C and the idle intensity was set at 1% of maximum. The latency to tail flick was defined as the time from the onset of light beam until movement of the tail. Data are presented as mean latencies to tail flick for each treatment group. Dose-response studies

were analyzed as the number of mice demonstrating a latency of at least 12.5 sec (50% of maximal/cutoff) for each group (N=10) and were analyzed using a Probit analysis [41].

Mouse Plantar Incision Assay

The mouse plantar incision assay was performed in a similar manner to previously described methods [42]. Mice were anesthetized using 1.5–3% isoflurane and received a 0.5 cm incision on the plantar surface of the right hindpaw as well as separation and elevation of the underlying plantaris muscle. The muscle was replaced and the wound sutured. We previously determined that mechanical allodynia following plantar incision were comparable both one and two days following the initial injury but that responses were more consistent on the second day (data not shown). Therefore, two days following surgery, mice were placed on an elevated wire mesh rack (approx. 24 inches high) in plexiglass cages. This allowed for access of the plantar surface of the hindpaw and testing using von-Frey monofilaments. Filaments (10 g maximum fiber stimulation) were applied to the mid paw plantar surface near the incision. Individual paw responses to mechanical stimulation were quantified using the Dixon up-down method [43,44] and allowed for determination of the 50% paw withdrawal threshold (PWT). On the day of testing, each paw was evaluated prior to drug treatment for the purposes of obtaining a baseline PWT and for verification of a reduced PWT (at least 50% of left paw (non-incised) PWT) in the incised paw. Mice that did not demonstrate mechanical allodynia 1 day following incision were excluded (approx. 10–20%). Following, NAX 810-2 was administered at various doses (12, 16, and 24 mg/kg), IP, 1h prior to testing. Data are presented as mean $\pm$ standard error of baseline (pre-drug) PWT for the incised paw.

Mouse Partial Sciatic Nerve Ligation Model (Seltzer Model)

The Seltzer PSNL model has been described previously [45,33]. Anesthesia was induced in rats using 4–5% isoflurane followed by separation of the sciatic nerve and ligation of approximately one-third to one-half of the fibers. Approximately two weeks following surgery, withdrawal responses to mechanical stimulation were assessed by application of von-Frey fibers to each hindpaw. After verification of mechanical allodynia in the right hindpaw, on the day of testing, a baseline PWT was obtained using the Dixon up-down method [43,44]. NAX 505-5 was administered to rats at a dose of 2 mg/kg (IP), followed 2h later by PWT determination (N=8 per group). NAX 810-2 was administered to rats at a dose of 4 mg/kg by IP injection, followed 1h later by PWT determination (N=8 per group). Data are presented as mean $\pm$ standard error of baseline (pre-drug) PWT.

Mouse Glucose Tolerance Test (GTT)

To determine whether the GalR2-preferring analog NAX 810-2 affects glucose and insulin levels, mice were subjected to a GTT following compound administration. Mice were placed in metabolic cages containing wire platforms that prevented access to bedding material, and were fasted for 6 h prior to a glucose challenge (1 g/kg i.p.). Mice received NAX 810-2 (2.5 or 8 mg/kg i.p.), the GalR1-preferring NAX 505-5 (0.8 mg/kg i.p.) or vehicle (HP β CD in acetate buffer – Veh for NAX 810-2, or 1% Tw20 in PBS – Veh for NAX 505-5) one hour prior to glucose challenge. Individual groups of mice (N=4) were randomly assigned to be sacrificed at different time points (0, 15, 30, 60, 90, 120 min) following glucose

administration. At each time point, blood glucose was collected via tail nick, using a handheld glucometer. After glucose sampling, mice were sacrificed and trunk blood was collected into microvials containing ethylenediaminetetraacetic acid (EDTA). Plasma was separated by centrifugation, collected and stored at -80°C . Insulin levels were determined using an ultra-sensitive rodent enzyme-linked immunosorbent assay (ELISA) kit (CrystalChem, Downers Grove, IL, USA). Glucose and insulin samples were also collected from mice that received sub-chronic administration of NAX 810-2 or NAX 505-5 (for GH, analysis – see description below). Glucose and insulin samples were analyzed using the same methods as described above.

Growth Hormone Assay

Plasma GH was evaluated in animals treated with NAX 810-2 (in comparison with NAX 505-5). NAX 810-2 was administered at a dose of 8 mg/kg (IP) either acutely or following sub-chronic (twice daily, 5 days) administration. Trunk blood was collected 30 min after either acute or the final treatment (sub-chronic treatment), into EDTA-coated vials. After centrifugation, plasma was removed and stored at -80°C . Plasma GH levels were measured using an ELISA kit (Millipore, Billirica, MA, USA). Samples of plasma were diluted in assay buffer in a 96-well plate and incubated with agitation for 1.5 hours at room temperature. Following washing of the wells, the detection antibody was added for an hour incubation, followed by washing of the wells. The enzyme was added and incubated for 30 minutes. Then the substrate was added, incubated for 8–15 minutes, and a stop solution added. Absorbance was read at 450 and 590 nm. Baseline GH levels were determined from vehicle treated mice.

Morris Water Maze Assay

The Morris water maze test of spatial learning and memory was used to evaluate whether NAX 810-2 reduced cognitive abilities when administered at a therapeutic dose. The task is based on the principle that rodents are highly motivated to escape from a water environment by the quickest, most direct route [46] and the learning of the animal can be assessed by the time it takes to find a hidden platform (latency) over a number of trials. Mice were randomized into two groups, vehicle-treated or NAX 810-2 (2.5 mg/kg, IP) (N=9 per group). Four daily trials for each mouse began 1h after treatment and were completed within 2h of drug administration. After 5 treatment/trial days, mice were re-evaluated in the absence of drug for two additional days. Escape latency and distance traveled were recorded using an HVS (HVS Image, Ltd, Buckingham, UK) image tracking system. Group means for each of these parameters following daily trials and re-tests were compared between NAX 810-2-treated and vehicle-treated mice for statistical significance (results presented in supplemental materials).

Statistical Analysis

Data are generally presented as means $\pm$ standard error. Comparison between two means was performed using a Student's t-test whereas multiple comparisons were made using a one-way ANOVA followed by a Newman-Keuls or a Dunnett's test for *a posteriori* analysis of the difference between group means. A $P < 0.05$ was considered significant.

RESULTS

Analgesic Pharmacology: Evaluation of NAX 810-2 in Rodent Pain Models

In the mouse carrageenan model, carrageenan injected into the plantar hindpaw induced a lasting state of inflammation, with a peak inflammation 3–4 h after injection (confirmed by verification of paw edema, data not shown). As shown in Figure 1, NAX 810-2 dose-dependently increased paw withdrawal latency following carrageenan. Minimal or no efficacy was observed at doses below 2 mg/kg and maximal efficacy was observed at a dose of 8 mg/kg, as ipsilateral paw withdrawal latencies were comparable to the non-carrageenan-treated (contralateral) paw. An ED₅₀ (95% CI) was calculated to be 4.7 mg/kg (2.6 – 8.3) (see Table 2). In the mouse formalin assay, doses of 4 and 8 mg/kg IP of NAX 810-2 reduced pain responses in the mouse formalin assay for both Phase I and Phase II (see Table 2). In the mouse tail flick model, NAX 810-2 dose-dependently increased tail flick latency, reaching a mean maximal latency at a dose of 24 mg/kg. An ED₅₀ (95% CI) was calculated as 20.2 mg/kg (15.1 – 34.5) (see Figure 2 and Table 2). In the mouse plantar incision model, as shown in Figure 3, NAX 810-2 dose-dependently increased PWT, with a maximal effect at a dose of 24 mg/kg. In the rat PSNL model, NAX 810-2 at a dose of 4 mg/kg increased PWT to 307% of pre-drug PWT (see Table 1 & Table 2).

Toxicological Evaluation of NAX 810-2

Pre-clinical evaluation of safety and tolerability of NAX 810-2 has largely been performed at doses that correspond to the ED₅₀ and ED₉₇ (corresponding to Probit-derived dose predicted to have 97% efficacy) observed in the mouse 6 Hz (32 mA) seizure model [27,28] (2.5 and 8 mg/kg, IP, respectively). For comparative purposes, some studies included evaluation of the GalR1-preferring analog NAX 505-5. In these studies, ED₅₀ and ED₉₇ values for NAX 505-5 in the mouse 6 Hz (32 mA) model were used (0.8 and 2.6 mg/kg, respectively) [47,48,17].

Evaluation of NAX 810-2 in the Mouse Glucose Tolerance Test—To determine whether NAX 810-2 reduced insulin and/or increased glucose levels, we evaluated the compound in a GTT assay (note: a summary of these results has been described previously [28]). NAX 810-2 was administered by IP injection to male CF-1 mice at doses of 2.5 and 8 mg/kg, in comparison to NAX 505-5 at a dose of 0.8 mg/kg. In comparison to Veh-treated animals, NAX 505-5, increased blood glucose levels (Figure 4A,B) and inhibited insulin release (Figure 4C,D) (see also Table 3). By comparison, NAX 810-2 at both doses evaluated tested did not affect either blood glucose or insulin levels (Figure 4, Table 3)

Evaluation of blood glucose levels following sub-chronic (5-days, twice daily) administration of NAX 810-2—Blood samples collected following either acute or sub-chronic administration of NAX 810-2 were evaluated for glucose and insulin to determine whether sub-chronic administration induced changes in glucose levels (note – samples taken from the same cohort of animals were also used for GH evaluation, see below). As shown in Figure 5, five days of treatment with NAX 810-2 (8 mg/kg, IP, twice daily) did not increase blood glucose levels whereas the GalR1-preferring analog NAX 505-5 significantly increased glucose levels following twice daily administration of 2.6 mg/kg. Consistent with

GalR1-mediated insulin inhibition, NAX 505-5 sub-chronic administration significantly reduced insulin levels whereas NAX 810-2 5-day administration had no effect (see Figure 5 & Table 3).

Evaluation of plasma GH levels following acute or sub-chronic (5 days, twice daily) administration of NAX 810-2—Time course studies (0.25 – 2 hrs) showed that GH levels were not elevated any time point following treatment with NAX 810-2 (8 or 16 mg/kg)(data not shown). Whereas phenobarbital (50 mg/kg, IP; positive control) produced a marked increase in GH levels, NAX 810-2 did not lead to an increase in growth hormone secretion after acute or chronic treatment, compared to vehicle-treated mice (see Figure 6).

Evaluation of performance in the Morris water maze following treatment with NAX 810-2—Learning was evident by decreasing mean latency time and distance traveled through five daily trials with no significant separation between NAX 810-2 and Veh. Furthermore, treatment with NAX 810-2 (2.5 mg/kg, IP) did not change AUC for either escape latency or distance traveled (Figure S1, Table 3).

DISCUSSION

To advance preclinical studies on the galanin-based analogs, herein NAX 810-2 was evaluated in several pain models and demonstrates an analgesic profile that is consistent with both anti-inflammatory (e.g. diminished pain behaviors following carrageenan and in phase II of the formalin model) and centrally-mediated effects (efficacy in phase I of formalin, tail flick, and plantar incision models). In addition, efficacy of NAX 810-2 in the PSNL model suggests potential utility of galanin analogs against neuropathic pain. Further, NAX 810-2 did not elevate glucose levels or inhibit insulin following either acute or sub-chronic administration. Finally, NAX 810-2 did not elevate glucose levels. These studies show that NAX 810-2 is a novel, potentially first-in-class analgesic.

The mouse carrageenan model has previously been used to characterize novel analgesic compounds [49,50]. Further, galanin levels are endogenously increased following carrageenan insult [51], and intrathecal galanin improves paw withdrawal latency following carrageenan administration in rats [52]. Previously, we observed that the peripherally-acting and GalR2 preferring analog, NAX 409-9, is effective in the carrageenan model [33], and NAX 810-2 shows comparable efficacy in this model. Interestingly, central action on galanin systems by NAX 810-2 would be predicted to increase the potency of this compound relative to NAX 409-9 and therefore comparable ED₅₀ values between these two compounds is somewhat surprising. It is also noteworthy that efficacy was observed for this compound at doses that did not produce motor impairment or other untoward effects, which is consistent with a therapeutic index of ~6 (NAX 810-2 efficacy in seizure models described previously [27,28]). Future studies with NAX 810-2 will include evaluation of the pre-clinical safety profile of this compound.

In a similar manner, NAX 810-2 was effective at doses of 4 and 8 mg/kg in the mouse formalin model, in both acute and inflammatory phases. The biphasic behavioral response following formalin is thought to be mediated by distinct mechanisms, due largely to the

effect of various classes of analgesic drugs in reducing paw lickin in one or both phases [53]. NAX 810-2 was effective against the acute phase of the formalin response, which is mediated at least in part by a central mechanism [53]. Further, NAX 810-2 was also effective against the inflammatory phase of the formalin response, which is in agreement with our previous observations of the effect of galanin against inflammatory pain [33,32]. Interestingly, both centrally- and peripherally-acting galanin analogs are effective in the formalin model [32], suggesting that the acute behaviors observed following intraplantar formalin may be mediated by both central and peripheral mechanisms. It is noteworthy that responses at 4 and 8 mg/kg were similar for both phases of formalin. Additional doses will be necessary to confirm the dose-dependent efficacy of NAX 810-2 in this model.

NAX 810-2 was efficacious in the mouse tail flick model, but at higher doses than those observed in the mouse carrageenan and formalin models. Initial doses were selected for IP administration in pain models based on anticonvulsant efficacy observed for this compound [28,27]. Given efficacy observed in the mouse carrageenan and formalin models at doses that are effective in mouse seizure models, lack of effect in the mouse tail flick model is surprising, as NAX 810-2 can cross the blood-brain barrier and acts centrally to reduce seizures. Presumably, NAX 810-2 could be acting via a central mechanism to reduce pain behaviors, though this has not been evaluated directly. The mouse tail flick model is thought to be almost entirely centrally mediated, though this may vary depending on test conditions [54]. However, given greater potency of NAX 810-2 observed in inflammatory models, it is possible that the primary mechanism of action of this analog is via an anti-inflammatory mechanism. Galanin has been shown to possess anti-inflammatory effects, reducing inflammation in an experimental model of intestinal colitis [55]. In addition, evaluation in carrageenan and formalin models was performed following acute administration. Further evaluation of the role of NAX 810-2 in inflammation can include repeated administration and evaluation of cytokines and other mediators of inflammation as a means of determining whether NAX 810-2 reduces inflammation.

In a similar manner, NAX 810-2 was also effective in the mouse plantar incision model, but significant efficacy was only observed at a dose of 24 mg/kg (3-fold higher than maximal efficacy observed in the carrageenan model). Post-surgical pain can result from unique nociceptive mechanisms, thus differentiating this type of pain from others [56]. While efficacy in inflammatory pain (carrageenan, formalin phase II) and acute pain (formalin, phase I) might predict activity of NAX 810-2 in the plantar incision model, there was no apparent efficacy until a dose of 24 mg/kg. Results from the plantar incision and tail flick assays therefore suggest that galanin may not play a major role in post-surgical pain, and that it is effective only at higher doses, where a central analgesic effect of galanin is evident. Further, it is also possible that activation of GalR2 may not play a substantial role in post-surgical pain and that analgesia is only observed at higher doses, where both GalR1 and GalR2 are activated.

Numerous studies have evaluated the role of galanin following peripheral nerve injury. Galanin inhibits spinal excitability, particularly following nerve injury [57]. In a model of chronic constriction injury, galanin levels increased dramatically following the injury [58] and similarly, galanin mRNA increases dramatically in a model of PSNL [59]. Intrathecal

administration of galanin reduces signs of hyperalgesia following sciatic nerve ligation [60], and galanin over-expression can reduce pain-like behaviors following PSNL in mice [61]. NAX 810-2 was moderately effective in the Seltzer PSNL model, an effect that is comparable to that observed for NAX 409-9 (2 mg/kg), morphine (2 mg/kg), and gabapentin (40 mg/kg)[33]. The GalR1-preferring analog, NAX 505-5, was also effective in this model at a dose of 2 mg/kg, and taken together with NAX 810-2 efficacy in this model, these data are in agreement with an influential role of galanin following nerve injury. Therefore, NAX 810-2 is potentially beneficial in neuropathic pain, and additional doses are needed to confirm dose-dependent efficacy.

NAX 810-2 was evaluated for potential effects on glucose, GH, and cognition at doses that generally correspond to the previously established ED₅₀ and ED₉₇ values for this compound in the mouse 6 Hz (32 mA) seizure model. NAX 810-2 at a dose of 8 mg/kg did not elevate glucose levels or diminish insulin either acutely or following sub-chronic administration. This observation reaffirms the role of GalR1, and not GalR2, on inhibition of insulin. Further, this dose was efficacious in the mouse carrageenan and formalin models, suggesting that lack of effect on glucose or GH occurs at therapeutically relevant doses. In contrast to insulin inhibition observed following galanin administration in mice [22] and rats [24], galanin infusion in humans (at doses that stimulate GH release) were not associated with insulin inhibition [62–65]. This suggests that in humans, galanin or galanin analog administration is less likely to affect insulin levels. In the Morris water maze test, a dose of 2.5 mg/kg was used to verify whether, at a therapeutically effective dose, NAX 810-2 did not impair cognition. However, higher doses will be necessary to confirm lack of effect in this model.

These studies add to growing evidence that GalR2-preferring analogs possess analgesic effects in experimental pain models. We also demonstrate herein that the lead centrally- and peripherally-acting GalR2-preferring analog, NAX 810-2, possesses a favorable pre-clinical safety and tolerability profile that warrants further development efforts. To that end, ongoing efforts are directed at evaluation of novel centrally- and peripherally-acting GalR2-preferring analogs as well as continuing efforts in advancing NAX 810-2 as potentially first-in-class analogs for pain. In summary, our long-term collaboration with our colleague and friend Dr. H. Steve White has been a great experience, filled with fun, gratitude and inspiration, and yielding two lead compounds for the treatment of pain, namely NAX 409-9 and NAX 810-2. We are very thankful for the privilege to work with Dr. White, and we are looking forward to more contributions to translational research and neuropharmacology of anticonvulsant neuropeptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effect of NAX 810-2 in the mouse carrageenan model. Carrageenan was injected into the plantar surface of the right (ipsilateral) hindpaw, which produced a state of localized inflammation reaching maximal effect 3h after injection. Vehicle (Veh, N=22, white bar) or NAX 810-2 at various doses [0.5 mg/kg, N=12, red bar, left diagonal lines; 1 mg/kg, N=18, green bar, right diagonal lines; 2 mg/kg, N=10, orange bar, vertical lines; 4 mg/kg, N=18, blue bar, horizontal lines; 8 mg/kg, N=16, purple bar, crossed lines] were administered by intraperitoneal injection 2h following carrageenan. * $P < 0.05$, ** $P < 0.01$ compared to Veh on the same side (ipsilateral or contralateral). ## $P < 0.01$ compared to contralateral in the same treatment group.

Figure 2.

Effect of NAX 810-2 in the mouse tail flick model. Mice were placed in a restraint tube during testing procedures wherein a focused beam of light was centered on the distal portion of the tail. Latencies to tail flick were determined for Vehicle (Veh, N=8, white bar), or NAX 810-2 at various doses 8 mg/kg, N=10, red bar, left diagonal lines; 16 mg/kg, N=10, green bar, right diagonal lines; 24 mg/kg, N=10, orange bar, vertical lines) were administered by intraperitoneal injection 1h prior to testing. ** $P < 0.01$ compared to Veh.

Figure 3.

Effect of NAX 810-2 in the mouse plantar incision model. Two days following plantar incision, mice were evaluated for pre- and post-drug paw withdrawal threshold (PWT). Following a baseline (pre-drug) PWT assessment in both the incised and non-incised paw, Veh (white bar) or NAX 810-2 [12 mg/kg, red bar, left diagonal lines; 16 mg/kg, green bar, right diagonal lines; and 24 mg/kg, orange bar, vertical lines) were administered by IP injection followed by PWT 1h after treatment. * $P < 0.05$ compared to Veh. $N = 11-14$ per group.

Figure 4.

Effect of NAX 810-2 on blood glucose and insulin levels in a glucose tolerance test. NAX 810-2 was administered by IP injection to male CF-1 mice at doses of 2.5 and 8 mg/kg whereas the GalR1-preferring analog NAX 505-5 was administered at a dose of 0.8 mg/kg. One hour later, glucose was administered by IP injection and separate groups of mice were evaluated for blood glucose and plasma insulin levels. The time course of blood glucose levels (panel A, N=8–12 per group) was used to determine the mean glucose area-under-the-curve (AUC) for each treatment group (panel B). The coincident plasma insulin time course (panel C, N=4–8 per group) was also used for determination of insulin AUC levels for each treatment group (panel D). Panel A & C: Veh (A) – HP β CD, Veh for NAX 810-2, filled box, dotted line; NAX 810-2 2.5 mg/kg – filled red circle, red line; NAX 810-2 8 mg/kg, filled green triangle, green line; Veh (B) – Tween20/phosphate buffered saline, filled downward triangle, dotted line; NAX 505-5 0.8 mg/kg, orange filled diamonds, orange line. Panel B & D: Veh (A) – HP β CD, Veh for NAX 810-2, white bar; NAX 810-2 2.5 mg/kg – red bar, left diagonal lines; NAX 810-2 8 mg/kg, green bar, right diagonal lines; Veh (B) – Tween20/phosphate buffered saline, white bar; NAX 505-5 0.8 mg/kg, orange bar, vertical lines.

* $P < 0.05$, *** $P < 0.001$ compared to all other treatment groups.

Figure 5.

Effect of sub-chronic (5-day) administration of NAX 810-2 on blood glucose and insulin levels. NAX 810-2 was administered by IP injection to mice twice daily (8 mg/kg, IP, red bar, left diagonal lines) and NAX 505-5 was administered at a dose of 2.6 mg/kg (IP, twice daily; orange bar, vertical lines) (Veh – white bar). Blood glucose (panel A) was analyzed from tail clip sections using a glucometer whereas plasma insulin (panel B) was determined using an ELISA (trunk blood samples). ** $P < 0.01$, compared to all other treatment groups.

Figure 6.

Effect of acute or sub-chronic administration of NAX 810-2 on plasma GH levels. NAX 810-2 was administered at a dose of 8 mg/kg (IP) either acutely (red bar, left diagonal lines) or following sub-chronic (twice daily, 5 days; green bar, right diagonal lines) administration. Veh (HP β CD; white bars) was administered (IP) either acutely or sub-chronically (twice daily, 5 days). Phenobarbital sodium (50 mg/kg, IP; orange bar, vertical lines) was administered acutely. N=8–24. ***P<0.001 compared to all other groups.

Table 1

Comparative Efficacy of NAX 810-2 in the Rat Seltzer PSNL Model.

Compound (dose, IP)	Mechanical Allodynia Assessment % pre-drug PWT (mean $\pm$ standard error)	Notes (Literature References)
NAX 810-2 4 mg/kg	307 $\pm$ 98 *	
NAX 505-5 2 mg/kg	507 $\pm$ 123 **	
NAX 409-9	342 $\pm$ 98 *	Metcalf et al. 2015 [33]
Morphine 2 mg/kg	395 $\pm$ 103 *	Metcalf et al. 2015 [33]
Acetaminophen 300 mg/kg	124 $\pm$ 44	Metcalf et al. 2015 [33]
Gabapentin 40 mg/kg	404 $\pm$ 82 *	Metcalf et al. 2015 [33]

PWT times shown are means $\pm$ standard error. Pre-treatment times: NAX 810-2 (1h), NAX 505-5 (2h), NAX 409-9 (1h), morphine (2h), acetaminophen (1h), gabapentin (4h). N=8 for each group

**
P<0.01,

*
P<0.05 compared to baseline (pre-drug) PWT.

Table 2

Summary of Analgesic Efficacy of NAX 810-2.

Animal Model	Treatment Group	Efficacy	Notes
Mouse Carrageenan	0.5 – 8 mg/kg IP, 1h	ED ₅₀ (95% CI): 4.7 mg/kg (2.6 – 8.3)	Comparable to NAX 409-9 and morphine ^a
	4 mg/kg, IP, 1h	39.5 ± 6.5**	
Mouse Formalin <i>Phase I</i> <i>Phase II</i>	8 mg/kg, IP, 1h	45.6 ± 8.7**	P<0.01 vs. Veh AUC P<0.01 vs. Veh AUC
	4 mg/kg, IP, 1h	38.8 ± 9.3**	
	8 mg/kg, IP, 1h	38.2 ± 12.7**	
Mouse Tail Flick	8–24 mg/kg, IP, 1h	ED ₅₀ (95% CI): 20.2 mg/kg (15.1 – 34.5)	P<0.01 for 24 mg/kg vs. Veh
Mouse Plantar Incision	12–24 mg/kg, IP, 1h	845% of pre-drug PWT @ 24 mg/kg	P<0.01vs. Veh
Rat Partial Sciatic Nerve Ligation	4 mg/kg, IP, 1h	307% of pre-drug PWT	

^aMetcalf et al., 2015 [33]

Table 3

Summary of Toxicology Studies of NAX 810-2.

Animal Model	Treatment Group	Observations	Notes
Mouse Glucose Tolerance Test	2.5 and 8 mg/kg IP	No increase in glucose or inhibition of insulin	GalR1-preferring NAX 505-5 increased glucose and reduced insulin
Mouse Blood Glucose Following Sub-Chronic Administration	8 mg/kg IP, BID, 5 days	No increase in glucose or inhibition of insulin	GalR1-preferring NAX 505-5 increase glucose and reduced insulin
Mouse Growth Hormone (GH)	8 mg/kg, acute OR BID, 5 days	No increase in GH levels	
Mouse Morris Water Maze	2.5 mg/kg, IP during trial phase	No change in AUC on maze challenge	No evidence of behavioral impairment