NEUROPEPTIDE FF RECEPTORS HAVE OPPOSING MODULATORY EFFECTS ON NOCICEPTION

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Running Title: NPFF receptors have opposing roles

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Abbreviations: 1DMe = [D-Tyr¹,(N.Me)Phe³]NPFF, CI = confidence interval, DMSO = dimethylsulfoxide, dPQR = Dansyl-Pro-Gln-Arg-NH₂, IP = intraperitoneal, NPAF = neuropeptide AF, NPFF = neuropeptide FF, ONPG = ortho-Nitrophenyl-β-galactoside, PWT = paw withdrawal threshold, SNL = spinal nerve ligation
ABSTRACT

The role of Neuropeptide FF (NPFF) and its analogs in pain modulation is ambiguous. Although NPFF was first characterized as an anti-opioid peptide, both antinociceptive and pronociceptive effects have been reported depending on the route of administration. Currently, two NPFF receptors, termed FF1 and FF2, have been identified and cloned but their roles in pain modulation remain elusive due to the lack of availability of selective compounds suitable for systemic administration in in vivo models. Ligand binding studies confirm ubiquitous expression of both subtypes in brain, while only FF2 receptors are expressed spinally. This disparity in localization has served as the foundation of the hypothesis that FF1 receptors mediate the pronociceptive actions of NPFF. We have identified novel small molecule NPFF receptor agonists and antagonists with varying degrees of FF2/FF1 functional selectivity. Utilizing these pharmacological tools in vivo has allowed us to define the roles of NPFF receptor subtypes as it pertains to the modulation of nociception. We demonstrate that selective FF2 agonism does not modulate acute pain, but instead, ameliorates inflammatory and neuropathic pains. Treatment with a non-selective FF1/FF2 agonist potentiates allodynia in neuropathic rats and increases sensitivity to noxious thermal and to non-noxious mechanical stimuli in normal rats in an FF1 antagonist reversible manner. Treatment with FF1 antagonists reversed established mechanical allodynia indicating the possibility of increased NPFF tone through FF1 receptors. In conclusion, we provide evidence for the
opposing roles of NPFF receptors and highlight selective FF2 agonism and/or selective FF1 antagonism as potential targets warranting further investigation.
INTRODUCTION

The role of Neuropeptide FF (NPFF) and its analogs in pain modulation is ambiguous. Although “classically” NPFF is characterized as an anti-opioid peptide, it has been shown to also elicit robust antinociceptive effects depending on the dose and route of administration employed (for review see (Yang et al., 2008). For example, intrathecal administration of NPFF or of its stable analogue [D-Tyr\textsubscript{1},(NMe)Phe\textsuperscript{3}]NPFF (1DMe) either elicits antinociception or potentiates the antinociceptive effect of morphine (Kontinen and Kalso, 1995; Gouarderes et al., 1996; Xu et al., 1999). In contrast, intracerebroventricular administration of these agents antagonizes morphine-induced antinociception (Gicquel et al., 1992; Oberling et al., 1993; Dupouy and Zajac, 1995).

To date, two NPFF receptor subtypes, termed FF1 and FF2, have been identified and subsequently cloned (Bonini et al., 2000; Elshourbagy et al., 2000). Presently, the specific role of each NPFF receptor subtype, with respect to pain modulation, has not been adequately described. The lack of selective pharmacological tools suitable for systemic administration has made elucidating the in vivo pharmacology of these receptors challenging. Based on results from ligand binding studies in rodents, it is clear that both NPFF receptors are widely expressed in brain tissue, whereas, only the FF2 receptor is expressed spinaly (Bonini et al., 2000; Liu et al., 2001; Yang and Iadarola, 2006). The lack of spinal FF1 receptors has led to the hypothesis that the antinociceptive actions of NPFF are mediated via FF2 receptors, whereas, the pronociceptive actions of NPFF are mediated via FF1 receptors (Liu et al., 2001). We have identified several novel small
molecule, non-peptidic, ligands with varying degrees of functional selectivity for the NPFF receptor subtypes (Gaubert et al., 2009). The goal of this investigation was to utilize these pharmacological tools in order to define the roles of the NPFF receptors as they pertain to pain modulation.

Here, we present the in vitro and in vivo profiles for a representative set of ligands that have allowed us to unmask the putative roles of the NPFF receptor subtypes. Based on our in vitro profiling we have identified compounds that, based on in vitro functional assays, are: a) selective agonists for FF2 receptors (i.e., AC-263093); b) non-selective agonists for NPFF receptors (i.e., AC-262616); and c) selective FF1 receptor antagonists (i.e., AC-262620 and AC-262970). Systemic administration of a non-selective FF1/FF2 receptor agonist resulted in a pronociceptive phenotype as indicated by increased sensitivity to noxious thermal as well as, to non-noxious mechanical stimuli. In stark contrast, systemic administration of a selective FF2 receptor agonist resulted in an antinociceptive phenotype as indicated by a) attenuation of phase II of the formalin test; b) reversal of carrageenan-induced thermal hyperalgesia; and c) reversal of mechanical hypersensitivity induced by L5/L6 spinal nerve ligation (SNL). Similar results were obtained with FF1 receptor antagonists.

Taken together, these data suggest that the antinociceptive actions of NPFF are driven by activation of FF2 receptors while the pronociceptive, and by extension the anti-opioid, actions of NPFF, are mediated via activation of FF1 receptors. Therefore, selective FF2 receptor agonists and selective FF1 receptor antagonists, alone or in
combination, may hold promise for the treatment of various types of chronic pains whether inflammatory or neuropathic in origin.
METHODS

Compounds and dosing solutions. The novel small molecule, non-peptidic NPFF ligands AC-262616, AC-263093, AC-262620 and AC-262970 were synthesized by ACADIA Pharmaceuticals. Further details pertaining to the chemical properties and structures of these compounds have been disclosed elsewhere (Gaubert et al., 2009). NPFF (FLFQPFQRF-NH$_2$) and NPAF (AGEGLNSQFWSLAAPQRF-NH$_2$) were purchased from American Peptide Company (Sunnyvale, CA) and Bachem (King of Prussia, PA), respectively. BIBP-3226 ((R)-N[(diphenylacetyl)-N-[(4-hydroxyphenyl)-methyl]-argininamide) was purchased from Sigma (St. Louis, MO). The N-terminally modified tripeptide amide, and putative NPFF antagonist, dansyl-PQR-NH$_2$ (dPQR), was synthesized by Phoenix Pharmaceuticals (Burlingame, CA). Morphine sulfate was purchased from Sigma (St. Louis, MO). For intraperitoneal (IP) administration compounds were dissolved in dimethylsulfoxide (DMSO) and brought up to volume in sterile 0.9% saline and administered in a final volume of 1 mL/kg of body weight. Doses are expressed in terms of freebase content.

Receptor Selection and Amplification Technology (R-SAT). NIH-3T3 cells were co-transfected with a mixture of plasmid DNAs containing receptor of interest and β-galactosidase. The plates were incubated at 37°C in a humidified CO$_2$ incubator for 18-24 hours, the transfection media was removed and cells harvested and frozen for future use. On the day of the assay, cells were plated onto 96-well tissue culture plates containing varying concentrations of ligands. For agonist assays, only agonist was added to the
wells. For antagonist assays, antagonist was added to the wells and the cells were spiked with a fixed concentration of agonist (NPFF and NPAF for FF2 and FF1 receptors respectively) before addition to the wells. After 5 days of incubation, the cells were assayed for production of β-galactosidase by adding ortho-Nitrophenyl-β-galactoside (ONPG) in a 0.5% NP40 solution to the wells and measuring the absorbance at 420 nm (Burstein et al., 1997).

**Cyclic AMP Inhibition.** HEK-293T cells were transiently co-transfected with DNA for either human FF1 or FF2 receptors and the DNA for EP2 receptor in 10 cm² tissue culture plates. After 48 hours, cells were harvested and plated onto 96 well TC plates. Inhibition of cAMP production was measured in the presence of varying concentrations of agonist of interest after stimulation of cAMP production by PGE₂ (agonist of EP2 receptor). For antagonist assays, the ability of each compound to reverse the inhibitory effect of NPFF on cAMP production stimulated by PGE₂ was measured. Cyclic AMP levels were measured using DiscoverRx Hit Hunter™ cAMP CL kit following manufacturer’s instructions. Dose response curves were fitted to a nonlinear regression model with an equation for one site competition using Prism™ (GraphPad Software, La Jolla, CA).

**Binding Assays.** HEK-T cells were transfected with either FF1 or FF2 receptors. Cells were harvested 48 hours post transfection and cell homogenates prepared. The Kₐ of [¹²⁵I]-NPFF was determined to be 50 and 100 pM for FF2 and FF1 receptors respectively. Competition binding assays were carried out in the presence of 70 pM [¹²⁵I]-NPFF (GE healthcare; Piscataway, NJ) and varying concentration of the ligand in 96-well plates as
Incubations were terminated by filtration onto GF/B filters followed by 3 washes with ice-cold buffer. Radioactivity retained on the filters was quantitated by scintillation counting in a Perkin Elmer Top Count. Data were fitted to a nonlinear regression model with an equation for one site competition using Prism™ (GraphPad Software, La Jolla, CA).

Animals. Male, Sprague-Dawley rats (150-300 g; Harlan; Indianapolis, IN) were housed in groups of 3 in a climate-controlled room on a 12 hr light/dark cycle (lights on at 7:00 AM), with food and water available ad libitum. All testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines for the handling and use of laboratory animals and received approval from the Institutional Animal Care and Use Committee of ACADIA Pharmaceuticals.

Formalin Test. Naïve rats were injected with 50 μl of a 5.0% formalin solution into the dorsal surface of a hind paw and then placed in individual plastic cages for observation. The number of nociceptive responses (i.e., paw flinches/licks/bites) was counted for a period of 60 min following formalin injection. Vehicle or test compounds were administered 15 min prior to formalin injection.

Carrageenan-induced thermal hyperalgesia. Naïve rats were assessed for their responsiveness to a noxious thermal stimulus. Response latencies were measured using the hot plate test. Inflammatory pain was produced by injecting 0.1 mL of 2% λ-carrageenan (Sigma; St. Louis, MO) into to the left hind paw. Three hours after carrageenan injection, hot plate latencies were again obtained. A significant reduction in
the hot plate latency was interpreted as the presence of thermal hyperalgesia. Rats were injected with vehicle or test compound and then tested at various time-points following administration. To generate dose-response curves, raw data were converted to % Maximum Possible Effect (%MPE) by the formula, \( \% \text{MPE} = \left[ \frac{\text{experimental score} - \text{post-carrageenan score}}{\text{naïve score} - \text{post-carrageenan score}} \right]\times100 \), where the experimental score is the hot plate latency obtained after compound administration, the post-carrageenan score is the hot plate latency obtained 3 hr post-carrageenan treatment, and the naïve score is the hot plate latency obtained prior to any treatments. Each dose was assessed in separate groups of rats.

**Assessment of thermal hyperalgesia.** This test was performed by placing rats in a plexiglass enclosure on a thermostatically controlled metal plate maintained at 52°C. The time elapsed until the rat showed an obvious nociceptive response (i.e., licking/stomping/elevating hind paw) was measured. Animals were tested before and at various time-points following compound administration. A cut-off time of 40 sec was employed to prevent tissue damage.

**L5/L6 Spinal nerve ligation surgery.** Spinal nerve ligation (SNL) injury was produced as previously described (Kim and Chung, 1992) in 6 separate cohorts of rats, 5 of which had n=24 and the final had n=30 subjects. Anesthesia was induced with 2% isoflurane in O₂ at 2 L/min and maintained with 1.5% isoflurane in O₂. The dorsal vertebral column from L4 to S2 was exposed, and the L5 and L6 spinal nerves were identified and carefully isolated. The L5 and L6 spinal nerves were tightly ligated distal to the dorsal root ganglion with 6–0 silk suture. The incision was closed, and the animals were
observed for uneventful recoveries. Rats were allowed a period of at least 2 weeks prior to test compound evaluation.

**Assessment of mechanical hypersensitivity.** Paw withdrawal thresholds (PWTs) of the left hind paws (i.e., ipsilateral to SNL) of the rats were determined in response to probing with eight calibrated von Frey filaments (Stoelting; Wood Dale, IL) in logarithmically spaced increments ranging from 0.41 to 15 g (4 – 150 mN). Each filament was applied perpendicularly to the plantar surface of the paw of rats kept in suspended wire-mesh cages. PWT was determined in g by sequentially increasing and decreasing the stimulus intensity and estimated using a Dixon non-parametric test. A significant reduction in PWT from the pre-SNL baseline value indicated the presence of mechanical hypersensitivity. Rats were allowed to recover for a minimum of 2 weeks prior to test compound assessment. Any rat that exhibited motor deficiency or a lack of subsequent increased sensitivity to innocuous mechanical stimulation (PWT ≤ 4.3 g) was excluded from additional testing (12 out of a possible 186). To generate dose-response curves, raw data were converted to %MPE by the formula, %MPE = [(PWT after drug - PWT for vehicle-control)/(15 - PWT for vehicle-control)] * 100. Each dose was assessed in separate groups of rats.

**Statistical Analyses.** Statistical analyses were conducted using Prism™ 5 (GraphPad, San Diego, CA). The effects of test compounds were analyzed by carrying out two way ANOVAs. Planned comparisons of means (each group versus vehicle) were performed using Bonferroni’s post hoc test provided a significant main effect was detected. Significance was set at the level of p ≤ 0.05. The dose that elicits 50% efficacy (ED$_{50}$)
and the corresponding 95% confidence interval (95% CI) were determined using linear regression analysis. These calculations were performed with the pharmacological statistics package FlashCalc (Dr. Michael H. Ossipov, University of Arizona, Tucson, AZ).
RESULTS

In vitro characterization of non-peptidic small molecule NPFF agonists. The in vitro receptor profiles of several novel, non-peptidic, small molecule NPFF agonists were determined using recombinant human FF1 and FF2 receptors. Two functional assay formats, R-SAT and cAMP assays, in addition to binding assays using $[^{125}\text{I}]$-NPFF were evaluated (Figure 1; Table 1). As internal reference standards, the peptides NPAF and NPFF were used for FF1 and FF2 receptors, respectively. The pEC$_{50}$ values obtained for these reference peptides in the R-SAT and cAMP assays were consistent with those reported elsewhere using other functional assays (Kotani et al., 2001; Liu et al., 2001). In the R-SAT assay, test compound AC-263093 demonstrated full agonist activity (~100% efficacy) at FF2 receptors while having a potency value that is ~3-fold less than the reference peptide NPFF (EC$_{50} = 5.9 \pm 0$ vs. EC$_{50} = 6.5 \pm 0.3$, respectively). However, this compound only demonstrated minimal agonist activity (~12% relative efficacy) at FF1 receptors. Finally, AC-262616 showed full agonist activity at FF2 receptors while demonstrating partial agonist activity at FF1 receptors (Figure 1, panels A & B). In both cases AC-262616 was more potent than the reference peptides NPFF (3-fold) and NPAF (>6-fold) for FF2 and FF1 receptors, respectively. Thus, based on our functional assays, compound AC-263093 is a highly selective full agonist of FF2 receptors with little, if any, intrinsic efficacy at FF1 receptors in recombinant systems. However, AC-261616 has agonist activity at both FF1 and FF2 receptors with minimal selectivity.
The agonist activity and functional selectivity of the test compounds were confirmed in the cAMP assay. The selective FF2 agonist, AC-263093, was fully efficacious at FF2 receptors and only showed weak agonist activity at FF1 receptors (~23% relative efficacy) confirming that this compound is functionally selective for FF2 over FF1 receptors. AC-262616, which displayed agonist activity at both NPFF receptors in the R-SAT assay, behaved similarly in the cAMP assay. Moreover, as in R-SAT, AC-262616 demonstrated slightly higher potency at FF1 over FF2 receptors in the cAMP assay (Figure 1, panels C & D).

The selective FF2 agonist, AC-263093, retained its selectivity in the binding assay (~2 fold for FF2 over FF1), whereas, AC-262616 displayed equal or increased affinity for the FF1 receptor (Table 1). Furthermore, AC-263093 showed little to no binding across a broad screening panel (n = 50) of other receptors, channels and enzymes (CEREP; IC$_{50}$ ≥ 10 μM).

**In vitro characterization of non-peptidic small molecule FF1 antagonists.** The in vitro receptor profiles of several novel, non-peptidic, small molecule NPFF antagonists were determined using recombinant human FF1 and FF2 receptors. Two functional assay formats, including R-SAT and cAMP assays, as well as binding assays using $[^{125}\text{I}]-$NPFF were used for this characterization (Figure 2; Table 2). As internal reference standards, the peptides BIBP-3226 and dPQR were included for comparison in both functional assays (Table 2). The pK$_i$ values obtained for BIBP-3226 in the R-SAT and cAMP assays were consistent with those reported elsewhere (Mollereau et al., 2002). The lack of stability of dPQR precluded us from characterizing it in R-SAT, however, the pK$_i$ value
obtained in the cAMP format was concordant with previous binding studies (Prokai et al., 2001).

Test compounds AC-262970 and AC-262620 demonstrated potent antagonist activity at FF1 receptors in both RSAT and cAMP assay formats. While showing similar binding affinity for FF2 and FF1 receptors (Ki values of 17.3 ± 20.5 and 14.3 ± 3.0 nM, respectively) (Figure 2, panel C), AC-262970 was a selective antagonist of FF1 receptors. AC-262970 antagonized the effect of NPAF (R-SAT) and NPFF (cAMP) at FF1 receptors achieving pKᵢ values of 8.2 ± 0.3 and 7.4 ± 0.4, respectively (Figure 2, panels A & B). AC-262970 behaved as a full agonist at FF2 receptors with a pEC₅₀ value of 6.7 ± 0.3, representing a 30-fold lower activity at FF2 receptors based on functional activity in the R-SAT assay (Table 2). Similarly, AC-262620 showed functional selectivity as antagonist at FF1 receptor. This compound antagonized the effect of NPAF (R-SAT) and NPFF (cAMP) at FF1 receptors achieving pKᵢ values of 8.1 ± 0.3 and 7.7 ± 0.4, respectively (Figure 2, panels D & E). AC-262620 behaved as a full agonist at FF2 receptors with a pEC₅₀ value of 5.9 ± 0.5, representing a >150-fold lower activity at FF2 receptors based on functional activity in the R-SAT assay (Table 2). However, unlike AC-262970, selectivity of AC-262620 for FF1 receptors was further demonstrated by 20-fold higher binding affinity for FF1 receptors compared to FF2, Ki values of 16.4 ± 10.1 and 320.3 ± 84.8 nM, respectively (Figure 2, panel F). Furthermore, AC-262970 showed little to no binding to a broad screening panel (n = 50) of other receptors, channels and enzymes (CEREP; IC₅₀ ≥ 10 μM).
Effects of the selective FF2 receptor agonist AC-263093 in the formalin test. Given that antinociceptive effects of NPFF and 1DMe were unremarkable in the tail flick test following spinal administration (data not shown) we profiled AC-263093 (a selective FF2 agonist) in the formalin model head-to-head with morphine. The two-way ANOVA yielded; $F(2,15) = 217.6$, $p < 0.0001$ and $F(1,15) = 391.9$, $p < 0.0001$ for factors associated with Treatment and Phase, respectively. Further the Treatment x Phase interaction was statistically significant; $F(2,15) = 170.2$, $p < 0.0001$. Formalin administration produced the characteristic bi-phasic flinching response in vehicle-treated rats; $68.3 \pm 4.7$ and $562.8 \pm 17.6$ flinches across phase 1 (i.e., 0-10 min) and phase 2 (i.e., 10.1-60 min), respectively (Figure 3, panel A). Consistent with the pharmacology of mu opioid agonists, 10 mg/kg of morphine, significantly decreased flinching in both phases of the formalin test; values obtained were $18.0 \pm 6.0$ and $27.7 \pm 9.1$ for phase 1 (i.e., 0-10 min) and phase 2 (i.e, 10.1-60 min), respectively ($p$ values $< 0.05$ Bonferroni post-tests). In contrast to the effect of morphine, IP administration of 10 mg/kg of AC-263093 selectively inhibited flinching behavior in phase 2 (i.e., 11-60 min) of the formalin test; value obtained for phase 2 was $213.3 \pm 26.0$ ($p < 0.001$ Bonferroni post-test).

Effect of the selective FF2 receptor agonist AC-263093 on thermal hyperalgesia produced by carrageenan. Following 3 hr after intraplantar carrageenan administration, rats demonstrate significant reductions in hot plate latency; values obtained pre-carrageenan and post-carrageenan were $10.7 \pm 0.1$ and $6.8 \pm 0.1$ sec, respectively ($p \leq 0.05$, paired $t$-test). Administration of AC-263093 IP produced a significant dose- and time-dependent attenuation of thermal hypersensitivity produced by carrageenan.
treatment (Figure 3, panel B). The two-way ANOVA yielded; $F_{(3,120)} = 28.4, p < 0.0001$ and $F_{(6,120)} = 55.5, p < 0.0001$ for factors associated with Dose and Time, respectively. Further the Dose x Time interaction was statistically significant; $F_{(18,120)} = 5.4, p < 0.0001$. AC-263093 at a dose of 1 mg/kg was without effect. In contrast, both the 3 and 10 mg/kg doses of AC-263093 produced significant attenuation of carrageenan-induced thermal hypersensitivity and the levels of efficacy were maintained for 2-3 hr (p values < 0.05, Bonferroni post-tests). The effect of 10 mg/kg AC-263093 was absent by 3 hr as indicated by a return of hot plate latency to pre-treatment levels (p > 0.05, Bonferroni post-tests). The calculated ED$_{50}$ value for IP AC-263093 was 2.4 mg/kg (1.6-3.6; 95% CI) following 30 min post-treatment. No significant effects of 10 mg/kg AC-263093 were noted in non-inflamed rats (p values > 0.05, Bonferroni post-tests) suggesting that systemic administration of a selective FF2 agonist is unlikely to alter basal sensory thresholds (Figure 3, panel C).

**Effects of the selective FF2 receptor agonist AC-263093 on SNL-induced mechanical hypersensitivity.** Following L5/L6 SNL rats show significant reductions in PWTs; values obtained pre-SNL and post-SNL were 15 ± 0 and 2.7 ± 0.2 g, respectively (p ≤ 0.05, paired t-test). AC-263093 produced a dose-dependent reversal of SNL-induced mechanical hypersensitivity following IP administration (Figure 4, panel A). A two-way repeated measures ANOVA of these data revealed significant main effects for both Dose and Time, $F_{(4,150)} = 26.4, p < 0.0001$ and $F_{(6,150)} = 267.3, p < 0.0001$, respectively, as well as, a significant Dose x Time interaction, $F_{(24,150)} = 14.3, p < 0.0001$. AC-263093 at doses of 1 and 3 mg/kg produced small increases in PWTs which peaked at 30 min and steadily
declined with time; this effect was not significantly different from those values obtained for the vehicle-treated controls (p values > 0.05, Bonferroni post-tests). In contrast, both the 10 and 30 mg/kg doses of AC-263093 produced significant attenuation of SNL-induced mechanical hypersensitivity and the levels of efficacy were maintained for 1-2 hr (p values < 0.001, Bonferroni post-tests). The effect of 30 mg/kg AC-263093 was absent by 3 hr as indicated by a return of PWTs to pre-treatment levels (p values > 0.05, Bonferroni post-tests). The calculated ED$_{50}$ value for IP AC-263093 was 6.2 mg/kg (4.7-8.1; 95% CI) following 30 min post-treatment.

**Effects of the non-selective NPFF receptor agonist AC-262616 on SNL-induced mechanical hypersensitivity.** Following L5/L6 SNL rats show significant reductions in PWTs; values obtained pre-SNL and post-SNL were 15 ± 0 and 2.7 ± 0.1 g, respectively (p ≤ 0.05, paired t-test). AC-262616 produced a dose-dependent potentiation of SNL-induced mechanical hypersensitivity following IP administration (Figure 4, panel B). A two-way repeated measures ANOVA of these data revealed significant main effects for both Dose and Time, F$_{(3,160)}$ = 7.9, p = 0.001 and F$_{(8,160)}$ = 740.2, p < 0.0001, respectively, as well as, a significant Dose x Time interaction, F$_{(24,160)}$ = 2.5, p = 0.0004. AC-262616 at a dose of 1 mg/kg was without effect as the PWTs were not significantly different from those values obtained for the vehicle-treated controls (p values > 0.05, Bonferroni post-tests). In contrast, both the 3 and 10 mg/kg doses of AC-262616 produced a significant potentiation of SNL-induced mechanical hypersensitivity and this pronociceptive effect peaked 1 hr post-treatment and was maintained for an additional 0.5-1.5 hr (p values < 0.01, Bonferroni post-tests). To further characterize the pronociceptive actions of AC-
we administered a dose of 10 mg/kg IP of AC-262616 to sham-operated SNL and to naïve rats, and monitored PWTs and hot plate latencies, respectively.

Administration of AC-262616 (10 mg/kg, IP) to sham-operated SNL rats produced a marked and significant reduction in PWTs (Figure 4, panel C). A two-way repeated measures ANOVA of these data revealed significant main effects for both Treatment and Time, $F_{(1,10)} = 46.9, p < 0.0001$ and $F_{(8,80)} = 22.1, p < 0.0001$, respectively, as well as a significant Treatment x Time interaction, $F_{(8,80)} = 19.5, p < 0.0001$. The onset of action for AC262616 was approximately 1 hr and the pronociceptive action persisted at least 2 hr (p values < 0.01, Bonferroni post-tests). Similarly, IP administration of AC-262616 (10 mg/kg) to naïve rats significantly decreased hot plate latencies (Figure 4, panel D). A two-way repeated measures ANOVA of these data revealed significant main effects for both Treatment and Time, $F_{(1,10)} = 6.3, p = 0.031$ and $F_{(5,50)} = 9.9, p < 0.0001$, respectively, as well as, a significant Treatment x Time interaction, $F_{(5,50)} = 8.8, p < 0.0001$. The onset of action for AC262616 was approximately 90 min and the pronociceptive action persisted at least 30 min (p values < 0.001, Bonferroni post-tests). These data confirm the pronociceptive actions of AC-262616 across two different sensory modalities.

**Effects of the FF1 receptor antagonist dPQR on the pronociceptive effects of AC-262616.** To address whether the pronociceptive effects of AC-262616 were driven by FF1 receptors, we attempted to pharmacologically block the effects of AC-262616 with dPQR a putative NPFF receptor antagonist (Figure 5, panel A). Following IP administration of AC-262616 (10 mg/kg), to naïve rats, there was a significant decrease
in PWTs in response to von Frey filaments; values obtained pre-treatment (i.e., naïve) and post-treatment (i.e., baseline) were 14.7 ± 0.2 and 4.4 ± 0.4 g, respectively (p ≤ 0.05, paired t-test). Immediately following baseline testing, rats received either vehicle or dPQR (30 mg/kg) IP and then tested across a 2.5 hr period. A two-way repeated measures ANOVA of these data revealed significant main effects for both Treatment and Time, F(2,120) = 64.7, p < 0.0001 and F(8,120) = 51.1, p < 0.0001, respectively, as well as, a significant Treatment x Time interaction, F(16,120) = 23.9, p < 0.0001. AC262616-treated rats that received IP vehicle treatment continued to demonstrate significantly lower PWTs relative to vehicle-treated controls (p values < 0.001, Bonferroni post-tests). In contrast, AC262616-treated rats that received 30 mg/kg dPQR IP showed a significant reversal of mechanical hypersensitivity. The effect of 30 mg/kg dPQR was almost complete, but short-lived as the reversal was maintained for approximately 0.5 hr (p values < 0.001).

Effects of the FF1 receptor antagonist dPQR on SNL-induced mechanical hypersensitivity. Following L5/L6 SNL rats show significant reductions in PWTs; values obtained pre-SNL and post-SNL were 15 ± 0 and 2.7 ± 0.1 g, respectively (p ≤ 0.05, paired t-test). Administration of IP dPQR produced a dose-dependent reversal of SNL-induced mechanical hypersensitivity (Figure 5, panel B). A two-way repeated measures ANOVA of these data revealed significant main effects for both Dose and Time, F(3,160) = 17.4, p < 0.0001 and F(8,160) = 141.6, p < 0.0001, respectively, as well as, a significant Dose x Time interaction, F(24,160) = 6.5, p < 0.0001. A dose of 3 mg/kg of dPQR produced a small increase in PWTs which peaked at 15 min and declined over
time; this effect was not significantly different from those values obtained for the vehicle-treated controls (p values > 0.05, Bonferroni post-tests). In contrast, both the 10 and 30 mg/kg doses of dPQR produced significant attenuation of SNL-induced mechanical hypersensitivity and the levels of efficacy were maintained for 1 hr (p values < 0.05, Bonferroni post-tests). The effect of 10 and 30 mg/kg dPQR were absent by 1 hr as indicated by a return of PWTs to pre-treatment levels (p values > 0.05, Bonferroni post-tests). The short-lived effects of dPQR are consistent with its published pharmacokinetic parameters (Prokai et al., 2000). The calculated ED50 value for dPQR administered IP was 9.5 mg/kg (7.0-12.7; 95% CI) following 15 min post-treatment.

Effects of the NPFF receptor antagonists AC-262970 and AC-262620 on SNL-induced mechanical hypersensitivity. Following L5/L6 SNL rats show significant reductions in PWTs; values obtained pre-SNL and post-SNL were 15 ± 0 and 2.4 ± 0.2 g, respectively (p ≤ 0.05, paired t-test). AC-262970 produced a dose-dependent reversal of SNL-induced mechanical hypersensitivity following IP administration (Figure 6, panel A). A two-way repeated measures ANOVA of these data revealed significant main effects for both Dose and Time, F(3,90) = 12.8, p = 0.0001 and F(5,90) = 203.3, p < 0.0001, respectively, as well as, a significant Dose x Time interaction, F(15,90) = 6.4, p < 0.0001. AC-262970 at a dose of 1 mg/kg produced a small increase in PWTs which peaked at 30 min and declined over time; this effect was not significantly different from those values obtained for the vehicle-treated controls (p values > 0.05, Bonferroni post-tests). In contrast, both the 3 and 10 mg/kg doses of AC-262970 produced significant attenuation of SNL-induced mechanical hypersensitivity and the levels of efficacy were maintained
for up to 1 hr (p values < 0.05, Bonferroni post-tests). The effect of 3 and 10 mg/kg AC-262970 were absent by 1 hr as indicated by a return of PWTs to pre-treatment levels (p values > 0.05, Bonferroni post-tests). The calculated ED\textsubscript{50} value for IP AC-262970 was 3.5 mg/kg (2.1-5.7; 95% CI) following 30 min post-treatment. As previously described, the FF1 antagonists used herein also showed full agonist activity at FF2 receptors with potency values similar to that obtained for AC-263093. In order to ascertain whether the efficacy in the SNL model is driven primarily via FF1 receptor antagonism we next evaluated AC-262620. AC-262620 is a potent FF1 antagonist with 20-fold and >150-fold selectivity for FF1 receptors in binding and R-SAT assays, respectively.

Rats show significant reductions in PWTs following SNL; values obtained pre-SNL and post-SNL were 15 ± 0 and 2.5 ± 0.3 g, respectively (p ≤ 0.05, paired t-test). AC-262620 at a dose of 10 mg/kg produced a time-dependent reversal of SNL-induced mechanical hypersensitivity following IP administration (Figure 6, panel B). A two-way repeated measures ANOVA of these data revealed significant main effects for both Drug and Time, F\textsubscript{(1,54)} = 21.1, p = 0.0013 and F\textsubscript{(6,54)} = 38.1, p < 0.0001, respectively, as well as, a significant Drug x Time interaction, F\textsubscript{(6,54)} = 8.7, p < 0.0001. AC-262620 at a dose of 10 mg/kg produced a significant attenuation of SNL-induced mechanical hypersensitivity and the levels of efficacy were maintained for up to 1.5 hr (p values < 0.01, Bonferroni post-tests). The effect of 10 mg/kg AC-262620 was absent by 2 hr as indicated by a return of PWTs to pre-treatment levels (p values > 0.05, Bonferroni post-tests).
DISCUSSION

In the present investigation we report on the activity of a set of novel, small-molecule, NPFF receptor ligands which are exemplified by: a) AC-263093, a selective FF2 receptor agonist, which is fully efficacious in vitro in both R-SAT and cAMP functional assays at hFF2 receptors (relative to NPFF) while virtually devoid of intrinsic efficacy at hFF1 receptors (relative to NPAF); b) AC-262616, a pan FF1/FF2 receptor agonist, which is fully efficacious at both NPFF receptor subtypes in a cAMP assay; and c) AC-262970 and AC-262620, selective FF1 antagonists, which bind hFF1 receptors with low nM affinity and display functional selectivity values of 30- and 150-fold, respectively, for FF1 over FF2 receptors. Using these molecules we have, for the first time, been able to elucidate the pharmacology of NPFF receptors following systemic administration. Our results provide the first direct in vivo evidence for the opposing roles of NPFF receptor subtypes as it pertains to the modulation of nociception. Utilizing these compounds we have been able to directly show that FF1 receptor activation is pronociceptive while FF2 receptor activation is antinociceptive.

Intrathecal administration of NPFF elicits acute antinociception and enhances the efficacy of spinal opioids, whereas, intracerebroventricular administration NPFF antagonizes the effects of opioids and results in pronociceptive effects (for review see (Yang et al., 2008). Receptor localization studies have established that while NPFF receptors are expressed ubiquitously in the brain, only the FF2 receptor has been detected at the level of the spinal cord (Bonini et al., 2000; Liu et al., 2001; Yang and Iadarola,
2006). The apparent lack of FF1 receptor expression spinally has led to the idea that the pronociceptive actions of NPFF are mediated via FF1 receptors (Liu et al., 2001). However, this hypothesis has never been adequately addressed in vivo due to a lack of highly selective non-peptidic NPFF ligands suitable for systemic administration. Using our novel small molecule, non-peptidic, NPFF receptor ligands as pharmacological tools, we have clearly demonstrated that NPFF receptors act in an opposing manner to modulate nociceptive input.

We were unable to detect any significant antinociceptive effects of AC-263093, a selective FF2 selective agonist, following IP administration in the hot plate test. While it remains plausible that NPFF agonists can potentiate the antinociceptive actions of opioids (Kontinen and Kalso, 1995; Gouarderes et al., 1996; Xu et al., 1999), depending on the route of administration, this possibility was beyond the scope of the present investigation. Given the lack of activity of AC-263093 on acute sensory thresholds, we also profiled this compound in the formalin and carrageenan models. Like morphine, AC-263093 markedly attenuated flinching behavior, although this effect was specific to the second phase, suggesting that AC-263093 may be active in models of spinal sensitization. Consistent with this interpretation, AC-263093 dose-dependently alleviated thermal hyperalgesia produced by intraplantar carrageenan, an effect similar to that previously reported for 1DMe following intrathecal administration (Xu et al., 1999). Increases in NPFF immunoreactivity have been reported in the spinal cord dorsal horn following carrageenan treatment (Kontinen et al., 1997). Morphine pre-treatment completely blocks the increase in spinal NPFF immunoreactivity (Kontinen et al., 1997), suggesting that
enhanced release of NPFF may promote thermal hyperalgesia. NPFF binding sites are not significantly altered by either neonatal capsaicin treatment or by dorsal rhizotomy receptors, suggesting that these receptors are almost exclusively expressed postsynaptically in the spinal cord (Lombard et al., 1995). It is possible that spinal FF2 receptors may act as autoreceptors to negatively regulate NPFF release. A reduction in endogenous NPFF release could drive the antihyperalgesic effects of selective FF2 agonists as well as provide a mechanism by which the antinociceptive activity of intrathecal morphine may be enhanced by these agents.

In addition, systemic administration of AC-263093 dose-dependently attenuated mechanical hypersensitivity following SNL, without altering the response thresholds of sham-operated rats, consistent with previous studies using microinjections in discrete brain stem regions (Xu et al., 1999; Altier et al., 2000; Wei et al., 2001). In stark contrast to the effects observed with AC-263093, IP administration of the non-selective FF1/FF2 receptor agonist AC-262616 potentiated the severity of the SNL-induced hypersensitivity. Furthermore, IP administration of AC-262616 produced marked increases in sensitivity to both noxious thermal and to non-noxious mechanical stimuli in both naïve and sham-operated rats, respectively. Given that efficacy observed in the SNL model is correlated with the degree of FF2 functional selectivity, we hypothesized that the pronociceptive actions were a direct result of supraspinal FF1 receptor activation.

To date, the only well-characterized, and commercially-available, NPFF receptor antagonist is BIBP-3226 (Mollereau et al., 2002; Fang et al., 2006). While intracerebroventricular administration of BIBP-3226 has been shown to block effects
produced by NPFF and NPVF (Fang et al., 2006), BIBP-3226 remains a very potent and selective neuropeptide Y1 (NPY1) receptor antagonist (Doods et al., 1995). As a result, efficacy measured in vivo would be confounded as NPY1 antagonists effectively reverse SNL-induced mechanical hypersensitivity in their own right (Ossipov et al., 2002). Therefore we opted for dPQR, a putative NPFF receptor antagonist (Prokai et al., 2001) which crosses the blood-brain barrier (Prokai et al., 2000), for our initial in vivo assessments. Systemic administration of dPQR reversed the pronociceptive actions of AC-262616, confirming biological activity of this FF1 receptor antagonist.

To determine whether endogenous NPFF systems play a role on the maintenance of mechanical hypersensitivity following SNL, we administered dPQR to neuropathic rats. Systemic administration of dPQR dose-dependently alleviated SNL-induced allodynia with a duration of action consistent with its published pharmacokinetic profile (Prokai et al., 2000). These initial proof-of-concept data provided the impetus for the discovery of FF1 receptor antagonists. Like dPQR, both AC-262970 and AC-262620, two highly potent FF1 antagonists, with low nM affinity for FF1 receptors, attenuated SNL-induced mechanical hypersensitivity. It should be noted that these antagonists also elicited full agonist activity at FF2 receptors with potencies in the range of AC-263093. Therefore, one possible interpretation is that the FF2 agonist activity of AC-262970 and AC-262620 is sufficient to attenuate SNL-induced hypersensitivity. However, this possibility is unlikely given that a) administration of AC-262616 produced mechanical and thermal hypersensitivity in naïve rats; b) dPQR is devoid of FF2 agonist activity; and c) AC-262620 is >150-fold selective in the functional R-SAT assay and is 20-fold
selective for FF1 receptors in binding assays. Taken together, these data support the concept that, following peripheral nerve injury, FF1 receptor tone may be enhanced.

As previously mentioned, we are unaware of any reports confirming the presence of FF1 receptors in the spinal cord of rats, therefore, the pronociceptive actions of FF1 receptor activation would most likely originate in the brain. Since NPVF preferentially activates FF1 receptors, this endogenous peptide may be the source of pronociceptive drive, as in vitro studies have confirmed that it possesses anti-opioid activity (Liu et al., 2001; Kersante et al., 2006). The ability of AC-262616 to significantly decrease baseline sensory thresholds suggests the possibility that FF1 receptor activation, in addition to promoting morphine antinociceptive tolerance, may also contribute to opioid-induced paradoxical pain. This idea is supported by the observation that intracerebroventricular administration of an anti-NPFF antiserum completely restores the efficacy of intracerebroventricular morphine in tolerant rats (Lake et al., 1991). Additionally, administration of a pan FF1/FF2 receptor antagonist, RF9, prevents heroin-induced hyperalgesia and effectively blocks the development of antinociceptive tolerance following repeated heroin treatment (Simonin et al., 2006).

Mechanistically, opioid-induced paradoxical pain and neuropathic pain share common features, including the activation of descending pain facilitatory pathways (Porreca et al., 2001; Burgess et al., 2002; Gardell et al., 2002). The activation of descending pain facilitation by anti-opioid peptides is not without precedence. For example, microinjection of cholecystokinin (CCK) into the rostralventromedial medulla produces mechanical hypersensitivity in naive rats (Xie et al., 2005), an effect similar to
that observed following administration of AC-2626216. Further, CCK$_2$ receptor antagonists restore the antinociceptive potency of morphine in tolerant rats, reverse opioid-induced paradoxical pain (Xie et al., 2005), and diminish mechanical allodynia in SNL rats (Kovelowski et al., 2000). Given the similarities between the aforementioned effects with CCK and those reported herein with our FF1 receptor ligands, together with those reported elsewhere (Simonin et al., 2006), highlights the possibility that supraspinal FF1 receptor activation may drive descending pain facilitatory pathways, although further work in this area is warranted.

In addition to the activation of descending pain facilitatory systems, opioid-induced paradoxical pain and neuropathic pain share another common feature which is the upregulation of spinal dynorphin (Gardell et al., 2002; Gardell et al., 2003). While dynorphin is an endogenous kappa opioid receptor agonist, enhanced levels of this peptide promote neuropathic pain (Wang et al., 2001; Burgess et al., 2002), as well as opioid antinociceptive tolerance and opioid-induced paradoxical pain (Gardell et al., 2002). Critically, manipulations which inhibit the pronociceptive actions of spinal dynorphin alleviate neuropathic pain (Wang et al., 2001; Burgess et al., 2002; Gardell et al., 2003; Gardell et al., 2004) and reverses antinociceptive tolerance and the accompanying abnormal pain (Vanderah et al., 2000; Gardell et al., 2002). This is highly relevant as a recent study has shown that 1DMe suppresses spinal dynorphin release in anesthetized rats (Ballet et al., 2002). Since FF1 receptors are not localized spinally the effects of 1DMe must therefore be driven by FF2 receptors, and thus represents a mechanism by which selective FF2 agonists may alleviate neuropathic pain and restore
the antinociceptive potency of opioids. Moreover, the suppression of dynorphin release also would have beneficial effects in chronic inflammatory pain as this type of pain is associated with both descending pain facilitation as well as spinal dynorphin upregulation (Dubner and Ruda, 1992). Of course, this proposed mechanism of selective FF2 agonism does not preclude potential supraspinal effects, although further work in this area is warranted.

In conclusion, we have demonstrated the opposing roles of FF1 and FF2 receptors as they pertain to the modulation of nociception. We have shown that FF2 agonism is efficacious in various pain models, while FF1 agonism is pronociceptive. Selective FF2 agonists, FF1 antagonists or bi-functional ligands may represent novel approaches for the treatment of chronic pain whether of inflammatory or neuropathic in origin. However, identification of the selective tools necessary to validate these targets is only the first step.
ACKNOWLEDGMENTS

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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. In vitro activity of FF2 receptor agonists at human FF1 and FF2 receptors as measured in R-SAT and cAMP assays. Representative concentration-response curves for several NPFF ligands at human FF2 (panel A) and FF1 (panel B) receptors in R-SAT and human FF2 (panel C) and FF1 (panel D) receptors in cAMP assays are presented. Data are reported as average % values relative to responses obtained for the reference agonists NPFF (FF2 R-SAT; FF1 and FF2 cAMP) and NPAF (FF1 R-SAT) (i.e., controls), for FF2 and FF1 receptors, respectively. Compounds tested include controls (open circles), AC-262616 (filled circles) and AC-263093 (open squares). Similar concentration-response curves were used to generate pEC$_{50}$ values for each test compound which are summarized in Table 1.

Figure 2. In vitro activity of selective FF1 receptor antagonists at human FF1 and FF2 receptors as measured in R-SAT, cAMP and binding assays. Representative concentration-response curves for AC-262970 and AC-262620 at human FF1 receptors in R-SAT (panels A and D) and cAMP (panels B and E) assays are presented. Data are reported as average % of response obtained relative to the reference FF1 antagonist BIBP-3226 (open circles). Radioligand binding assays for AC-262970 (panel C) and AC-262620 (panel F) were also conducted in membranes isolated from cells expressing either human FF1 (open circles) or FF2 (filled circles) receptors. Similar concentration-
response curves were used to generate pEC$_{50}$ and pK$_{i}$ values for each test compound which are summarized in Table 2.

Figure 3. Effects of NPFF receptor agonists on various types of pain. Panel A. Data are expressed as average number of flinches over a 5 min period. Animals were administered IP vehicle (open circles), morphine 10 mg/kg (filled circles) or AC-263093 10 mg/kg (filled squares) and the number of flinches was tabulated across a 1 hr testing period. Panel B. Data are expressed as average hot plate latency in sec. Carrageenan treated rats received IP vehicle (open circles), or the selective FF2 receptor agonist AC-263093 at doses of 1 (filled circles), 3 (open squares) and 10 mg/kg (filled squares) and hot plate latencies were obtained at various time points across a 3 hr testing period. Panel C. Data are expressed as average hot plate latency in sec. Non-inflamed rats received IP vehicle (open circles), or 10 mg/kg of AC-263093 (filled circles) and hot plate latencies were obtained at various time points across a 3 hr testing period. Panel D. Data are expressed as average hot plate latency in sec. Naive rats received IP vehicle (open circles), or 10 mg/kg of AC-262616 (filled circles) and hot plate latencies were obtained at various time points across a 3 hr testing period. ***, ** and * denote p values < 0.001, < 0.01 and < 0.05, respectively vs. vehicle controls (two-way RM ANOVA followed by Bonferroni post-tests). n=6 rats per group.

Figure 4. Effects of AC-263093 and AC-262616 on L5/L6 SNL-induced mechanical hypersensitivity following IP administration. Data are expressed as average PWT of
the injured paw in SNL rats. Panel A. SNL rats were given IP vehicle (open circles) or the selective FF2 receptor agonist AC-263093 at doses of 1 (filled circles), 3 (open squares), 10 (filled squares) and 30 mg/kg (open triangles) and PWTs were obtained at various time points across a 3 hr testing period. Panel B. SNL rats received vehicle (open circle) or the non-selective FF1/FF2 receptor agonist AC-262616 at doses of 1 (filled circles), 3 (open squares) and 10 mg/kg (filled squares) and PWTs were obtained at various time points across a 3.5 hr testing period. Panel C. Sham-operated SNL rats were given either vehicle (open circles) or 10 mg/kg IP of the non-selective FF1/FF2 receptor agonist AC-262616 (filled circles) and PWTs were obtained at various time points across a 3.5 hr testing period. N denotes pre-surgical response thresholds. BL denotes baseline response prior to test compound administration. ***, ** and * denote p values < 0.001, < 0.01 and < 0.05, respectively vs. vehicle controls (two-way RM ANOVA followed by Bonferroni post-tests). n=6 rats per group.

Figure 5. Effects of the putative NPFF receptor antagonist dPQR on mechanical hypersensitivity following IP administration. Panel A. Data are expressed as average PWT of the left hind paw. After obtaining pre-drug PWTs (N), rats were immediately administered IP vehicle (open circles) or 10 mg/kg AC-262616 and 75 min later PWTs were again obtained (BL). Immediately following testing, rats received either IP vehicle (filled circles) or 30 mg/kg of dPQR (open squares) and PWTs were obtained at various time points across a 2.5 hr testing period. Panel B. Data are expressed as average PWT of the injured paw in rats. Animals were administered IP vehicle (open circle) or dPQR at
doses of 3 (filled circles), 10 (open squares) and 30 mg/kg (filled squares) and PWTs were obtained at various time points across a 3 hr testing period. N and BL denote pre-SNL and post-SNL PWTs, respectively. *** and * denote p values < 0.001 and < 0.05, respectively vs. vehicle controls (two-way RM ANOVA followed by Bonferroni post-tests). n=6 rats per group.

Figure 6. Effects of the FF1 receptor antagonists AC-262970 and AC-262620 on L5/L6 SNL-induced mechanical hypersensitivity following IP administration. Data are expressed as average PWT of the injured paw in rats. Panel A. Animals were administered IP vehicle (open circles) or the potent FF1 receptor agonist AC-262970 at doses of 1 (filled circles), 3 (open squares) and 10 mg/kg (filled squares) and PWTs were obtained at various time points across a 2 hr testing period. Panel B. Animals were administered IP vehicle (open circles) or the potent and selective FF1 receptor agonist AC-262620 at a dose of 10 mg/kg (filled circles) and PWTs were obtained at various time points across a 3 hr testing period. N and BL denote pre-SNL and post-SNL PWTs, respectively. ***, ** and * denote p values < 0.001, < 0.01 and < 0.05, respectively vs. vehicle controls (two-way RM ANOVA followed by Bonferroni post-tests). n=6 rats per group.
Table 1: *In vitro* activity of NPFF receptor agonists at human FF1 and FF2 receptors as measured by R-SAT, cAMP and Binding assays.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R-SAT</th>
<th></th>
<th>cAMP</th>
<th></th>
<th>Binding</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>hFF2</td>
<td></td>
<td>hFF1</td>
<td></td>
<td>hFF2</td>
<td></td>
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<tr>
<td></td>
<td>%Eff</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>%Eff</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>%Eff</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>NPAF</td>
<td>105 ± 16</td>
<td>8.7 ± 0.6</td>
<td>100</td>
<td>6.6 ± 0.4</td>
<td>–</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td>NPFF</td>
<td>100</td>
<td>6.5 ± 0.3</td>
<td>–</td>
<td>NT</td>
<td>100</td>
<td>8.2 ± 0.4</td>
</tr>
<tr>
<td>AC-262616</td>
<td>73 ± 13</td>
<td>7.0 ± 0.4</td>
<td>52 ± 3*</td>
<td>&lt; 7.4</td>
<td>114 ± 8</td>
<td>5.5 ± 0</td>
</tr>
<tr>
<td>AC-263093</td>
<td>90 ± 15</td>
<td>5.9 ± 0</td>
<td>12 ± 0*</td>
<td>ND</td>
<td>78 ± 24</td>
<td>5.2 ± 0.3</td>
</tr>
</tbody>
</table>

Efficacy values are expressed relative to the internal reference standards, NPAF and NPFF, for FF1 and FF2 receptors, respectively (R-SAT) and NPFF (cAMP). * % Eff values reported are those obtained at the highest testable concentration of compound (6µM for RSAT and 30 µM for cAMP assays). Due to toxicity of the compound, higher concentrations could not be tested for these compounds. Since proper dose-response could not be constructed, potency of these compounds has been estimated. NA = not active; ND= could not be determined; NT = not tested.
Table 2: *In vitro* activity of NPFF receptor antagonists at human FF1 and FF2 receptors as measured by R-SAT, cAMP and binding assays.

<table>
<thead>
<tr>
<th>Compound</th>
<th>hFF1</th>
<th>hFF2</th>
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<tbody>
<tr>
<td></td>
<td>R-SAT Antagonist</td>
<td>cAMP Antagonist</td>
</tr>
<tr>
<td></td>
<td>pKi</td>
<td>pKi</td>
</tr>
<tr>
<td>BIBP-3226</td>
<td>7.6 ± 0.6</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>(66 ± 0)</td>
<td></td>
</tr>
<tr>
<td>dPQR</td>
<td>ND*</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>AC-262620</td>
<td>8.1 ± 0.3</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC-262970</td>
<td>8.2 ± 0.3</td>
<td>7.4 ± 0.4</td>
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</table>

NPAF and NPFF were used as the FF1 agonists in the R-SAT and cAMP formats, respectively.

pKi (i.e., -log Ki) values are expressed relative to the internal reference standard BIBP-3226.

Ki values were calculated using the Cheng-Prusoff equation; 
\[ K_i = \frac{IC_{50}}{(1 + [\text{agonist}] / EC_{50 \text{ agonist}})} \]

*dPQR is not stable enough for R-SAT assays, thus not determined (ND).

NT = not tested
Figure 2.

A) % Inhibition of NPAF response vs. Log [Ligand] for BIBP-3226 and AC-262970.

B) Reversal of cAMP inhibition (% No Drug) vs. Log [Ligand] for BIBP-3226 and AC-262970.

C) Specific Binding (%) vs. Log [AC-262970] for different ligands.

D) % Inhibition of NPAF response vs. Log [Ligand] for BIBP-3226 and AC-262620.


F) Specific Binding (%) vs. Log [AC-262620] for different ligands.
Figure 3.

A. Flinches / 5 min vs. Time after formalin injection (min).
- Vehicle
- Morphine (10 mg/kg)
- AC-263093 (10 mg/kg)

B. Hot plate latency (sec) vs. Time (hr).
- Vehicle
- AC-263093 (1 mg/kg)
- AC-263093 (3 mg/kg)
- AC-263093 (10 mg/kg)

C. Hot plate latency (sec) vs. Time (hr).
- Vehicle
- AC-263093 (10 mg/kg)

D. Hot plate latency (sec) vs. Time (hr).
- Vehicle
- AC-262616 (10 mg/kg)
Figure 4.
Figure 5.

A

B

Paw withdrawal threshold (g)

Paw withdrawal threshold (g)

Time (hr)

Time (hr)

**Veh + Veh**

**AC-262616 (10 mg/kg) + Veh**

**AC-262616 (10 mg/kg) + dPQR (30 mg/kg)**

**Vehicle**

**dPQR (3 mg/kg)**

**dPQR (10 mg/kg)**

**dPQR (30 mg/kg)**
Figure 6.

A

Paw withdrawal threshold (g)

Time (hr)

N BL 0.5 1.0 1.5 2.0

Vehicle
AC-262970 (1 mg/kg)
AC-262970 (3 mg/kg)
AC-262970 (10 mg/kg)

B

Paw withdrawal threshold (g)

Time (hr)

N BL 0.5 1.0 1.5 2.0 2.5 3.0

Vehicle
AC-262620 (10 mg/kg)