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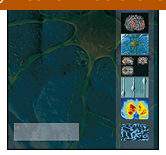

Reversal of morphine tolerance by a compound with NPFF receptor subtype-selective actions

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HIGHLIGHTS

- AC-263093 previously activated type 2, but not type 1, Neuropeptide FF receptors.
- Morphine infusion induced robust tolerance to morphine analgesia (tail flick test).
- AC-263093, 10 mg/kg i.p., totally reversed this tolerance to 5 mg morphine sulfate.
- AC-263093 did not induce an analgesic effect in rats never exposed to morphine.
- AC-263093 blocked activation of type 1 receptor, further altering balance between types 1 and 2.

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ABSTRACT

Neuropeptide FF (NPFF) modulates opiate actions. It has pro-nociceptive effects, primarily through the NPFF receptor 1 subtype, and anti-nociceptive effects, primarily through the NPFFR2 subtype. AC-263093 is a small l, organic, systemically active molecule that was previously shown to functionally activate NPFFR2, but not NPFFR1. It was hypothesized that AC-263093 would attenuate morphine tolerance. Rats were tested for radiant heat tail-flick latency before and after 5 mg/kg morphine sulfate s.c. They were then rendered morphine-tolerant by continuous subcutaneous infusion of 17.52 mg/kg/day morphine sulfate. On the seventh day of infusion, they were retested for analgesia 10 and 20 min after 5 mg/kg morphine sulfate s.c. Tolerance was indicated by reduction of morphine analgesia from the pre-infusion test. Fifty minutes prior to morphine challenge, rats received either 10 mg/kg i.p. AC-263093 or injection vehicle alone. AC-2623093-treated rats had far smaller tolerance scores than control rats. This drug effect was significant, $p = 0.015$. The same dose of AC-263093 had almost no analgesic effect in non-tolerant, saline-infused rats. *In vitro* experiments revealed that AC-263093 had equal affinity for NPFFR1 and NPFFR2, and functionally inactivated NPFFR1, in addition to its previously shown ability to activate NPFFR2. Thus, altering the balance between activation of NPFF receptor subtypes may provide one approach to reversing opiate tolerance.

1. Introduction

Chronic use of opiate narcotic drugs, such as morphine, causes drug tolerance: a profound loss of potency. These drugs are commonly used to alleviate chronic pain. Opiate tolerance necessitates repeated escalation of narcotic doses, creating major problems in pain management [1–3].

Chronic morphine administration in the rat results in significantly increased levels of NPFF in cerebrospinal fluid [4]. NPFF is sometimes considered an anti-opiate peptide since it antagonizes various acute effects of opiate drugs [5–7]. Conversely, antibodies and antagonists to NPFF administered i.c.v. or s.c. have attenuated morphine tolerance in the rat [8–11]. On the other hand, spinal administration of NPFF has been shown to potently intensify morphine analgesia [12]. Moreover, spinal NPFF administration has long-acting, opiate-like analgesic effects [13–15]. These actions

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are likely mediated by the release of met-enkephalin [16] and are naloxone-reversible [17], consistent with pro-opiate actions of NPFF.

Two NPFF receptor subtypes (NPFFR1 and NPFFR2) have been discovered [18]. NPFF binds to both, while a series of related RFamide C-terminal peptides, such as NPAF and NPSF, have varying affinities for these receptor subtypes. Both subtypes are distributed in the brain, but only NPFFR2 is readily detected in the rat spinal cord [18–20]. Several reports suggest that the NPFFR2 receptor is responsible for antinociceptive, or pro-opiate, activity [12–16]. Although its receptor binding affinities were not measured, Lameh et al. [21] demonstrated that the compound AC-263093 selectively stimulates functional activation of this receptor subtype. This was indicated by receptor modulation of cAMP levels in cells transfected with NPFFR1 or NPFFR2, as well as selection amplification technology (RSAT) which quantifies cellular proliferation dependent on receptor activation. As would be expected from this receptor selectivity, it also induced antinoceptive effects in multiple *in vivo* models of rodent hyperalgesia. The present study evaluated the hypothesis that this compound would restore the analgesic effect of morphine in morphine-tolerant rats. Another experiment determined whether AC-263093 might have reduced pain sensitivity by exerting an acute analgesic effect of its own rather than actually altering morphine tolerance. The receptor binding affinities of AC-263093 and related compounds were also measured, as well as AC-263093 functional inhibition of the NPFFR1 receptor, as distinguished from binding affinity and lack of functional activation.

2. Materials and methods

2.1. Approval

These experimental procedures were approved by the UHCL Animal Care and Use Committee.

2.2. Experiment 1: The ability of AC-263093 to reverse morphine tolerance

2.2.1. Materials

AC-263093 was synthesized as described previously as "compound 2" [22]. The chemical formula for AC-263093 is 2-(3,4-dibromobenzylidene) hydrazine carboximidamide hydrochloride. The structure is shown in Fig. 3.

2.2.2. Subjects

Eight male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 405 ± 27 g ($M \pm$ SD) were maintained on a 12-h light/dark cycle with food and water *ad lib*.

2.2.3. Apparatus

A radiant heat tail flick apparatus (Stoelting, Wood Dale, IL) was employed. The device was calibrated such that a normal, drug-free rat would remove its tail after approximately 3–4 s of heat exposure. The trial was terminated after a maximum of 12 s in order to prevent tissue damage.

2.2.4. Inducing morphine tolerance

Under isoflurane anesthesia, each rat was implanted s.c. with an Alzet 2ML1 osmotic pump filled with morphine sulfate in saline. Each rat was infused over seven days with 17.52 mg/kg/day of morphine sulfate. This treatment previously resulted in robust morphine tolerance [11,12].

2.2.5. Pre-infusion testing (before morphine sulfate infusion)

Rats were injected s.c. with 1.0 ml/kg saline as a control for the subsequent morphine sulfate injection. The average of three tail flick latencies was recorded at 10 and 20 min after saline injection and the average latency was computed for each test. Rats were then injected i.p. with 1.0 ml/kg of 20% dimethyl sulfoxide (DMSO)/80% saline. (This was a control for the injection vehicle subsequently used for AC-263093.) Fifty minutes after i.p. injections, rats received 5 mg/kg morphine in saline s.c. This dose was chosen as the smallest dose that induced near maximal analgesia (80–90% of the maximum possible effect), thereby providing optimal sensitivity to detecting tolerance. At 10 and 20 min after these injections, the rats were retested for tail flick latencies using the same protocol. Ten and twenty minutes morphine analgesia scores were calculated as the increase in average latency from pre- to post-morphine injection at each post-injection interval expressed as a percentage of the maximum possible increase, given the 12 s cutoff. Analgesia score as a percentage = 100 × (post-morphine latency − pre-morphine latency)/(12 s − pre-morphine latency).

2.2.6. Post-infusion testing (after morphine sulfate infusion)

On the seventh day of infusion, rats were pretested using the same method as the pre-infusion pretest. AC-263093 was dissolved in 0.2 ml DMSO and brought up to 1.0 ml with saline. An experimental group of four morphine-tolerant rats were given i.p. injections of 10 mg/kg AC-263093 while a control group of four morphine-tolerant rats were administered the injection vehicle (DMSO/saline) alone. This 10 mg/kg dose of AC-263093 was previously shown to produce analgesia in hyperalgesic carrageenan-treated rats and in hyperalgesic rats following spinal nerve ligation, but not in untreated rats [21]. Fifty minutes after i.p. injection, rats received morphine injections (5 mg/kg s.c.) and were tested for tail flick latencies 10 and 20 min later. The morphine analgesia scores in response to injection of 5 mg/kg morphine sulfate were computed as before. Each morphine tolerance score was calculated as the change in morphine analgesia score from pre-infusion testing to post-infusion testing. A negative tolerance score (decrease in morphine analgesia score) indicated morphine tolerance.

2.3. Experiment 2: Effect of 10 mg/kg AC-263093 on tail flick response in morphine-naïve rats

2.3.1. Subjects

Subjects included eleven male Sprague-Dawley rats with an average weight of 293 ± 29 g ($M \pm$ SD), maintained as in Experiment 1.

2.3.2. Procedure

The goal of this procedure was to evaluate any acute analgesic effect of AC-263093 by itself (without morphine infusion or injection) in opiate-naïve rats exposed to the same non-drug conditions as in Experiment 1. Therefore, the same procedures as in Experiment 1 were repeated with the following exceptions: all rats were infused s.c. for seven days with saline alone, to control for any effects of osmotic minipump implantation in Experiment 1; only saline injections were administered before each pre-infusion and post-infusion set of tail flick tests, so that the subjects were never exposed to morphine; although each rat experienced pre-infusion testing as before, the effect of AC-263093 was evaluated only by post-infusion testing since that compound had been administered post-infusion in Experiment 1; six rats were assessed for analgesia before and 60 and 70 min after i.p. injection of 10 mg/kg AC-263093 in DMSO/saline (10 and 20 min after s.c. saline injection); five control rats were assessed for analgesia at the same intervals after injection of DMSO/saline vehicle alone, followed 50 min later by injection by saline s.c.; each rat's AC-263093 analgesia scores were the change in tail flick latency from pre- to 60 and 70 min post-i.p injection as a percentage of the maximum possible change, given

the 12 s cutoff. Note that there were no tolerance scores, since only the acute effect of AC-263093 was evaluated.

2.4. Binding to and inhibition of NPFFR subtypes

2.4.1. Materials

The radioligand was [^{125}I]1DMe-NPFF (D-Tye[^{125}I]-Leu-MePhe-Gln-Pro-Gln-Arg-Phe-NH2) from American Radiolabeled Chemicals, Inc (St. Louis, MO; lot#131016, 159 μCi/ml, 2200Ci/mmol). Human NPFFR1 (RBHNF1M, lot 347-677-B) and human NPFFR2 membrane preparations (RBHNF2M, lot 624-755-A) were obtained from PerkinElmer (Boston, MA). AC-263093 was synthesized by Acadia Pharmaceuticals.

2.4.2. Radioligand binding protocol

Ninety-six well polypropylene plates (U-bottom, Falcon #351190) were prepared with serial dilutions of test compounds in binding buffer (50 mM Tris–HCL, pH 7.4, 1 mM $MgCl_2$, 60 mM NaCl, and 0.5% bovine serum albumin). Membranes (NPFFR1, 0.1 μg/well; NPFFR2, 1 μg/well) were thawed rapidly, diluted with binding buffer and added to the plates. Radioligand (0.016 nM ^{125}I-NPFF for NPFFR1, 0.033 nM ^{125}I-NPFF for NPFFR2) was added to make a final volume of 200 or 100 μl/well for NPFFR1 and NPFFR2, respectively. The plates were incubated at room temperature for 2 h with shaking. Binding was terminated by filtration through GF/B filters (presoaked with 0.1% polyethylenimine) with a 96-well harvester (Brandel Inc., Gaithersburg, MD). The filters were washed with ice-cold binding buffer (150 ml/plate) and allowed to air-dry for 30 min. MicroScint-20 cocktail (50 μl) was added to each dried well, and the plates were sealed and counted for 2 min/well using a TopCount scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA).

2.4.3. Functional inhibition of NPFFR1 receptor

To detect the ability of AC-263093 to inhibit functional g-protein linked receptor activation, RSAT assays of NIH/3T3 cells transfected with NPFFR1 were performed as described previously (Lameh et al. [21]) in the presence or absence of 1 μM NPAF (a high affinity ligand for this subtype) and 5 μM AC-263093.

3. Results

3.1. Experiment 1: AC-263093 reverses morphine tolerance

Fig. 1 shows the tolerance scores: change from pre- to post-morphine infusion in analgesia scores (increase in tail flick latency/maximum possible increase) in response to s.c. morphine injection. Control rats receiving injection vehicle only prior to retest responded 10 min after morphine injection with analgesia scores that were 39.1% ± 13.1% ($M \pm$ SEM) lower than their corresponding pre-infusion scores, a significant change, $p = 0.028$ according to a one-sample t-test. Their analgesia scores at 20 min post-morphine were 69.9% ± 23.1% lower than the corresponding pre-infusion scores, a significant difference, $p = 0.029$, according to one-sample t-test. In contrast, the group pre-treated with AC-263093 prior to retest decreased their morphine analgesia scores by only 12.4% ± 8.89% from their pre-infusion scores 10 min after morphine challenge, a non-significant change, $p = 0.257$. They actually increased their 20 min post-morphine scores by 19.6% ± 12.6%, which was also not a significant change from pre-infusion scores, $p = 0.217$. Analysis of variance of these morphine tolerance scores with one repeated measures variable (time post-morphine injection) revealed a significant effect of drug (AC-263093 *vs.* injection vehicle only), $F(1,6) = 11.40$, $p = 0.015$ The effect of time post-morphine injection was not significant, $F(1,6) = 0.002$, $p = 0.965$.

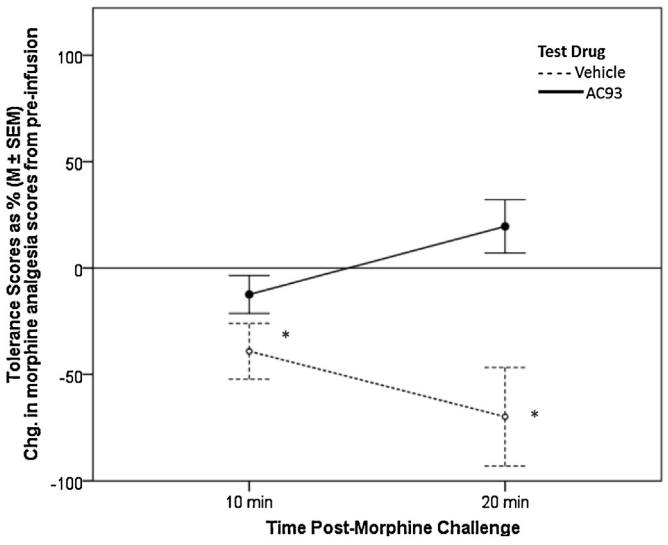

Fig. 1. Reversal of morphine tolerance by AC-263093. Morphine analgesia scores are the % of maximum possible increase in latency. Tolerance scores are the change in these % scores from before to after chronic morphine infusion and injection of AC263093 (solid line) or vehicle alone (dotted line) 50 min. prior to morphine challenge. Latency is retested 10 and 20 min after morphine s.c. $^*p = 0.028$ (10 min.) and 0.029 (20 min.) *vs.* corresponding pre-infusion morphine analgesia scores.

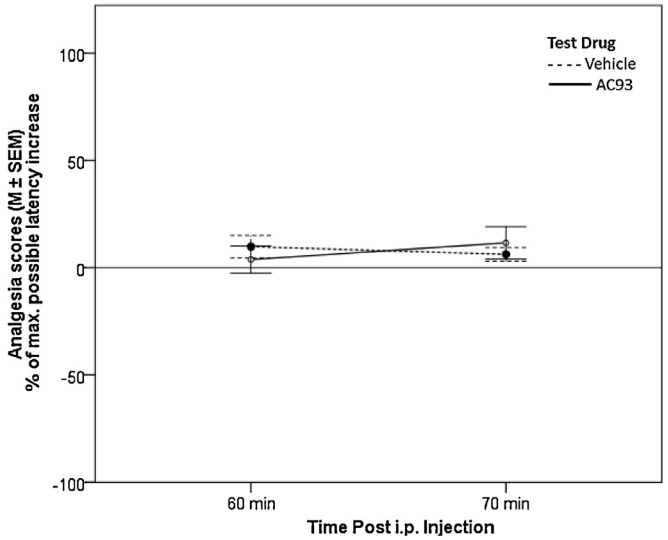

Fig. 2. AC-263093 (10 mg/kg) by itself does not induce analgesia in saline-infused, opiate-naïve rats. Analgesia scores (% of maximum possible increase in latency from pretest) 10 and 20 min after s.c. saline injection, 60 and 70 min after injection with AC263093 (solid line) or vehicle alone (dotted line).

The interaction effect (drug × time) did not reach significance, $F(1,6) = 5.57$, $p = 0.056$.

3.2. Experiment 2: 10 mg/kg AC-263093 does not induce analgesia in morphine-naïve rats

Fig. 2 shows the acute analgesic effects of AC-263093 or injection vehicle alone on opiate-naïve rats that were otherwise subjected to the same procedures as the rats in Experiment 1. As the figure shows, there were only analgesia scores observed at 60 and 70 min post-i.p. injections (10 and 20 min post-s.c. saline injections). At 60 min post-i.p. injection, the vehicle control rats increased their latencies by only 5.75% ± 6.45%, while the AC-263093 rats increased their latencies by only 2.37% ± 5.05%. At 70 min post-i.p. injection, the control rats had analgesia scores of 3.38% ± 4.22%, while the

Table 1
Binding affinities of NPFF-related compounds at NPFFR1 and R2 subtypes. pKi is the negative logarithm of Ki, so that a high value indicates high affinity. N is number of replicate samples, SD is standard deviation of pKi among those samples.

Ligand	hNPFF1			hNPFF2			Fold selectivity
	pKi	SD	*N*	pKi	SD	*N*	FF1/FF2
AC-263093	7.0	0.1	3	6.9	0.2	3	1
Dansyl-PQR-amide	5.2	0.2	3	4.1	0.3	3	11
Dansyl-PQRF-amide	8.5	0.2	3	8.3	0.3	3	2
Dansyl-RF-amide	8.1	0.1	3	6.0	0.1	3	143
NPFF	9.9	0.3	5	9.6	0.2	4	2

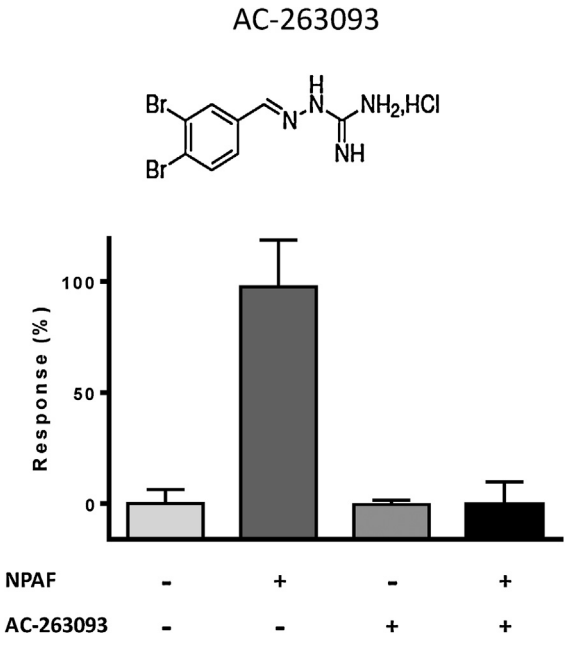

Fig. 3. AC-263093 blocks activation of NPFF1 receptors by neuropeptide AF. RSAT assays were performed using 1 μM NPAF or 5 μM AC-263093 where indicated by (+) or no drug where indicated by (−). Scores are decimal fractions indicating degree of receptor activation as indicated by levels β-galactosidase relative to that induced by NPAF stimulation in the absence of AC-263093. There was virtually zero receptor stimulation under the other conditions.

AC-263093 group had scores of 8.33% ± 6.40%. Two-way ANOVA with one repeated measures variable (time) revealed no significant effects of drug, $F(1,9) = 0.170$, $p = 0.900$, or of time, $F(1,9) = 0.379$, $p = 0.553$, or of interaction (drug × time), $F(1,9) = 2.036$, $p = 0.187$.

3.3. Receptor binding

Table 1 presents the binding affinities of various NPFF-related compounds to NPFFR1 and NPFFR2. Surprisingly, AC-263093 bound with approximately equal affinity to both receptor subtypes. Dansyl-PQRamide, and Dansyl-RFamide showed high selectivity for NPFFR1, while the natural peptide NPFF and Dansyl-PQRFamide had lower selectivity for NPFFR1.

3.4. Functional inhibition of NPFFR1

Fig. 3 illustrates the ability of 1 μM NPAF to functionally activate g-protein-linked NPFFR1 activity as indicated by increased levels of β-galactosidase [21]. It also shows the complete abolition of this effect by 5 μM AC-263093.

4. Discussion

Systemic administration of AC-263093 totally reversed robust morphine tolerance to 5 mg/kg morphine sulfate induced by seven days of continuous morphine sulfate infusion. This cannot be explained by an analgesic effect of AC-263093 in itself, since the same dose had no significant effect on tail flick latencies in saline-infused, opiate-naïve rats, consistent with its previously reported lack of analgesia in untreated rats [21]. This same dose previously produced analgesia in hyperalgesic carrageenan-treated rats and in rats with spinal nerve ligation [21]. Since only type 2 NPFF receptors are readily detected in the spinal cord, this suggests that AC-263093 likely activates NPFFR2 function *in vivo*. Thus, the present results raise the possibility that stimulation of NPFFR2 provides one approach for reversing opiate narcotic tolerance.

However, binding data in the current study indicates that AC-263093 binds with approximately equal affinity to NPFFR1 and NPFFR2. Despite this, AC-263093 previously failed entirely to functionally stimulate NPFFR1-mediated actions *in vitro*. At the same dose employed in the present study, it also failed to exert any pro-nociceptive actions that might be expected with NPFFR1 activation [21]. Compounds that bind receptors but do not produce agonist responses generally act as antagonists. Lameh et al. [21] did not test for AC-263093 functional antagonist activity at the NPFF1 receptor, but we now report that AC-263093 does have this effect. This suggests that AC263093 inhibits the pro-nociceptive NPFFR1 subtype, in addition to stimulating the anti-nociceptive NPFFR2. While AC-263093 has only moderate affinity for NPFF receptors, its affinity is far higher than the NPFF receptor affinities of dansyl-PQRamide, which exerted powerful actions against morphine tolerance [10].

The binding data presented in Table 1, in combination with results of earlier behavioral experiments, sheds light on the function of NPFFR1. The NPFFR1-selective systemically active agonists RFamide and dansyl-PQRFamide precipitated morphine abstinence syndrome [23,24], while the NPFFR1-selective systemically active antagonist dansyl-PQRamide attenuated morphine tolerance, restoring the analgesic effect of morphine [10]. Thus AC-263093 reversal of morphine tolerance coupled with its functional inhibition of NPFFR1 is consistent with the hypothesis that the NPFFR1 stimulation has anti-opiate effects in subjects that have been exposed to chronic opiate administration, while NPFFR1 inhibition may alleviate some effects of such opiate exposure. Whether through stimulation of NPFFR2 or antagonism of NPFFR1, altering the balance in favor of NPFFR2 over NPFFR1 stimulation appears to markedly attenuate morphine tolerance. The relative contribution of these two actions to tolerance reversal by AC-263093 could be clarified by adding a selective NPFFR2 antagonist. Unfortunately, to our knowledge, no such compound is currently available.

Opiate-like analgesic NPFF effects have been clearly demonstrated, especially with spinal administration [8,12]. This might appear inconsistent with reports that NPFF can inhibit morphine analgesia [7,25] and precipitate morphine withdrawal syndrome [26], or that antibodies [9] and antagonists against NPFF [10,11] restore sensitivity to morphine in opiate-tolerant rats. Research such as the present study, utilizing NPFF receptor subtype-selective compounds, may help resolve this seeming paradox. The failure of AC-263093 to produce an analgesic effect in non-tolerant rats also suggests that such receptor subtype-selective compounds might attenuate opiate tolerance without substituting opiate-like actions conducive to addiction liability. Thus, development of drugs with differential actions on NPFFR1 and NPFFR2 might provide one possible approach to the treatment of opiate narcotic tolerance and dependence. Further studies are warranted to evaluate the effects of such compounds on varying degrees of opiate tolerance assessed by differing brain-mediated and spinally mediated measures of analgesia.

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