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# Development of a Peptidomimetic Antagonist of Neuropeptide FF Receptors for the Prevention of Opioid-Induced Hyperalgesia

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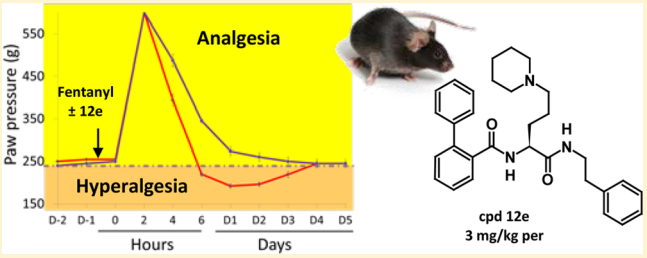
Supporting Information

**ABSTRACT:** Through the development of a new class of unnatural ornithine derivatives as bioisosteres of arginine, we have designed an orally active peptidomimetic antagonist of neuropeptide FF receptors (NPFFR). Systemic low-dose administration of this compound to rats blocked opioid-induced hyperalgesia, without any apparent side-effects. Interestingly, we also observed that this compound potentiated opioid-induced analgesia. This unnatural ornithine derivative provides a novel therapeutic approach for both improving analgesia and reducing hyperalgesia induced by opioids in patients being treated for chronic pain.




**KEYWORDS:** *NPFF, GPCR, GPR147, GPR74, peptidomimetic, unnatural amino acid, ornithine, arginine, bioisoster, opioid-induced hyperalgesia*

Development of drugs that can effectively and safely treat both acute and chronic pain (resulting from postoperative surgery, cancer, neuropathies, etc.) remains a major unmet challenge of pharmacotherapy. Opiate analgesics, such as morphine and fentanyl, continue to be the cornerstones for treating moderate to severe pain. However, with chronic administration, their usefulness is limited by prominent side effects such as tolerance and dependence. These side effects have been proposed to result from adaptive modifications in cellular responsiveness, primarily involving desensitization and down-regulation of opioid receptors.[1]

An intriguing hypothesis is that the decreased effectiveness of opioids with long-term use may, in part, result from activation of antiopioid systems that produce hyperalgesia, thus diminishing the net analgesic effect of opioid agonists.[2−4] This phenomenon is termed opioid-induced hyperalgesia (OIH). In rats, both acute and prolonged opioid treatment induces a long lasting OIH that persists for several days after opioid administration is stopped.[5−8] In humans, several reports indicate that both acute and chronic opioid treatments may be associated with paradoxical hyperalgesia and/or allodynia.[9−11] Enhancements in pain sensitivity have been also reported in heroin addicts.[12] Several neuromodulators have antiopioid properties, including NMDA, cholecystokinin (CCK), nociceptin/orphanin FQ and neuropeptide FF (NPFF).[3,13−17]

The NPFF system is composed of two GPCR subtypes, NPFFR1 (GPR147) that binds the mammalian neuropeptides VF (NPVF: VPNLPQRF-$NH_2$) whereas NPFFR2 (GPR74) has a greater affinity for neuropeptide FF (FLFQPQRF-$NH_2$). These two receptors belong to the GPCR subfamily called the RF-amide receptors, which also includes GPR10, GPR54, and GPR103. This subfamily is known to bind endogenous neuropeptides that share the C-terminal sequence Arg-Phe-$NH_2$. Originally identified as an inducer of hyperalgesia and a modulator of opiate analgesia,[18] subsequent in vitro and in vivo studies have implicated NPFF in the regulation of the cardiovascular system,[19−21] body temperature,[22,23] feeding behavior,[24] metabolism,[25] and water balance.[26,27]

Based on the observation that the C-terminal sequence Arg-Phe-$NH_2$ is common to all of the neuropeptide ligands of the RF-amide GPCR family,[28−31] we previously investigated the N-acylated dipeptide **RF9** as a nonselective antagonist of both

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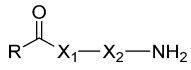
hNPFFR1 and hNPFFR2 (Table 1).[32] Stable enough to be subcutaneously coadministrated with opiates, **RF9** completely blocked the delayed and long lasting paradoxical hyperalgesia induced by chronic opiate treatment, and prevented the development of associated tolerance.[16,32] Moreover, in agreement with its antagonist function at both NPFF receptor subtypes, **RF9** was shown to prevent hypothermia and hypertension induced by NPFF or NPVF.[23,32,33] Starting from the N-acylated dipeptide Arg-Phe-$NH_2$, here we describe the design of an NPFF antagonist derived from an unnatural ornithine amino acid that is able to prevent opioid-induced hyperalgesia after oral administration in rats.

**Table 1. Binding affinity of N-Acylated Dipeptides toward NPFF1 and 2 Receptors**[a]



| Cpd | R | $X_1$-$X_2$ | Ki hNPFF1 (nM)[b] | Ki hNPFF2 (nM)[b] |
|---|---|---|---|---|
| **RF9** |  | Arg-Phe | 22 ± 5 | 43 ± 1 |
| **1** | | Cit-Phe | 880 ± 120 | > 13 μM |
| **2** |  | Arg-Phe | 0.32 ± 0.02 | 920 ± 50 |
| **3** | | Arg-Ala | 2 400 ± 440 | > 10 μM |

[a][$^3$H]-FFRF-$NH_2$ was used as radioligand for competition assays with both hNPFF1 and 2 receptors. [b]Values are expressed as the mean ± SEM

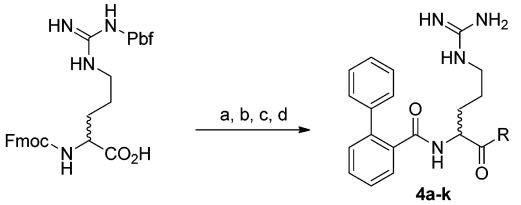
## RESULTS AND DISCUSSION

**Structure−Activity Relationships (SARs) of the Dipeptide RF-$NH_2$.** In a recent study, we reported on an extensive optimization of the N-terminal region of the amidated dipeptide Arg-Phe-$NH_2$, which allowed for the identification of compound **2** bearing the 2-phenylbenzoyl moiety at the N-terminus as a selective subnanomolar ligand of NPFFR1 (Table 1).[34] Starting with **RF9** and compound **2** as compounds of reference, we performed a structure−activity relationship analysis of the dipeptide series. All dipeptide compounds were synthesized by standard Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS) on Rink amide resin.

As reported previously, we have shown that the natural amino acid configuration of the sequence L-Arg-L-Phe of **RF9** was optimal for both receptors. We then evaluated the mode of interaction of the arginine residue with NPFF receptors. The guanidine moiety can establish two types of interaction within the active site. The first one is electrostatic through its ability to be protonated at physiological pH and the second one involves a set of H-bond acceptor−donor interactions through the various amino groups. To address the importance of the electrostatic interaction, we replaced the arginine amino acid of **RF9** by a citruline residue (**1**), which bears a urea moiety in place of a guanidine on the side chain. Like guanidine, urea can establish H-bond acceptor−donor interactions, but lacks electrostatic interactions. This change led to a drastic loss in affinity toward both NPFF receptors, confirming the requirement for an electrostatic interaction at this position. Indeed, replacement of arginine by citruline in the NPFF sequence was recently reported as leading to a dramatic loss in potency for both NPFF receptors.[28] To determine the importance of the aromatic ring close to the C-terminus, the phenylalanine in compound **2** was replaced by an alanine (**3**). This resulted in a significant loss of affinity, emphasizing the requirement of an aromatic ring close to the C-terminal end. This result is consistent with the fact that [Ala-8]NPFF was reported as not active toward both NPFF receptors.[28]

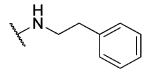
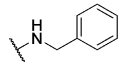
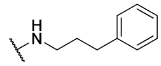
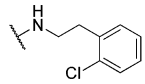
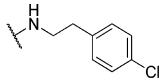
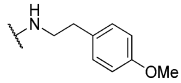
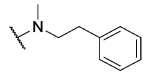
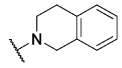
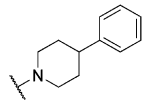
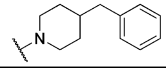
**SAR of the Arginine Derivatives.** In 1989, Allard et al. reported that replacing the C-terminal carboxamide by a carboxylic acid led to a complete loss of activity.[35,36] However, more recently, it has been reported that the NPY antagonist BIBP3226, based on a single arginine core resulting from the deletion of the C-terminal carboxamide of the Arg-Tyr sequence, was a potent antagonist of both NPFF receptors.[30] Based on this latter result, the C-terminal carboxamide function was deleted from dipeptide **2**, leading to arginine derivative **4a** (Table 2). The

**Table 2. Binding Affinity of Arginine Derivatives toward NPFF1 and 2 Receptors**[a]




**4a-k**

Conditions: (a) amine (1.1 eq.), BOP (1.1 eq.), NMM (2 eq.) DMF, rt; (b) DBU (2 eq.), DCM, rt; (c) 2-Phenylbenzoic acid (1.1 eq.), BOP (1.1 eq.), NMM (2 eq.), DCM, rt; (d) TFA, DCM, rt.

| Cpd | C* | R | Ki NPFF1 (nM)[b] | Ki NPFF2 (nM)[b] |
|---|---|---|---|---|
| **RF9** | | | 22 ± 5 | 43 ± 1 |
| **2** | | | 0.32 ± 0.02 | 920 ± 50 |
| **4a** | L |  | 58 ± 18 | 250 ± 43 |
| **4b** | D | | 99 ± 35 | 97 ± 5 |
| **4c** | L |  | 74 ± 4 | nd[c] |
| **4d** | L |  | 15 ± 3 | nd |
| **4e** | L |  | 53 ± 12 | 84 ± 19 |
| **4f** | L |  | 21 ± 1 | nd |
| **4g** | L |  | 69 ± 2 | nd |
| **4h** | L |  | 11 ± 2 | 25 ± 1 |
| **4i** | L |  | 320 ± 11 | 190 ± 19 |
| **4j** | L |  | 340 ± 45 | 130 ± 20 |
| **4k** | L |  | 53 ± 13 | nd |

[a]C*: amino acid configuration. [$^3$H]-FFRF-$NH_2$ was used as radioligand for competition assays with both hNPFF1 and 2 receptors. [b]Values are expressed as the mean ± SEM; [c]nd: not determined.



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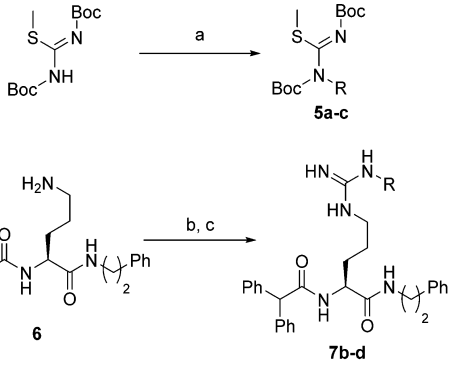
deletion of the C-terminal amide (**4a**) led to a two-order of magnitude loss in affinity toward hNPFFR1 ($K_i$ = 58 nM) and a significant gain in affinity toward hNPFFR2 ($K_i$ = 250 nM), in comparison with the dipeptide **2**. This result highlights the critical role played by the C-terminal carboxamide as a H-bond acceptor/donor system in the binding to hNPFFR1 but not to hNPFFR2. The corresponding D-enantiomer **4b** led to slight decrease of affinity for hNPFFR1, but interestingly, a gain of affinity for hNPFFR2 was observed, suggesting that the D-configuration would be more favorable to hNPFFR2. However, these negative or positive effects of the configuration on the affinity toward hNPFFR1 or hNPFFR2 respectively, are still moderate, maybe because of the spatial flexibility of the arginine side chain which compensates the configuration effect. By increasing the length of the alkyl chain (**4d**) or by adding a chlorine atom in the para-position (**4f**), the affinity for hNPFFR1 was further improved. These results suggest the presence of a hydrophobic pocket. The introduction of a *N*-methyl group at the C-terminal part of **4a** led to **4h**, which showed a potent affinity toward both receptors, similar to that observed with the reference compound **RF9**. This result highlights the fact this C-terminal amide is not involved in a H-bond donor interaction with either of the receptors. Based on this result, we applied a rigidification of the C-terminal part through the introduction of cyclic molecules such as tetrahydroisoquinoline (**4i**) or piperidine (**4j**, **4k**) moieties, resulting in a drop of affinity for hNPFFR1 and a slight gain of affinity for hNPFFR2 with compounds **4i** and **4j**. **4k**, bearing a 4-benzylpiperidine moiety, showed a similar affinity for hNPFFR1 as **4a**. At the difference of **4i** and **4j**, the phenyl group at the C-terminus of **4k** is in free rotation around a methylene moiety. This last result indicates that a flexible aromatic group is preferred for occupying the hydrophobic pocket.

We next explored the side chain of the arginine moiety, by introducing on the guanidine moiety an aromatic moiety using several alkyl spacers (Table 3). The $N^{\gamma}$-substituted argininamide derivatives **7b–d** were prepared by guanidinylation of the corresponding ornithinamide precursor **6** bearing a diphenylmethyl group on the terminal part. The guanidinylation reagents **5a–c** were synthesized by alkylation of *N*,*N*′-bis(*tert*-butoxycarbonyl)-*S*-methyl isothiourea under standard Mitsunobu conditions (Table 3). Taken as a reference, compound **7a** (no substituent on the guanidine) exhibited an affinity toward both NPFF receptors similar to that of **4a**. The introduction of an aromatic moiety on the guanidine group (compounds **7b–d**) did not improve their affinities toward both hNPFFR1 and hNPFFR2. Indeed, there seemed to be neither steric hindrance nor additional interaction, so we can hypothesize that the guanidine moiety does not interact with a residue buried into the transmembrane domain, but more likely with an acidic residue located at the surface of the receptor. This result is consistent with the model of binding proposed by Beck-Sickinger et al.,[37] who hypothesized an interaction between the guanidine moiety of Arg with an anionic residue on a loop of the GPCR. Using site-directed mutagenesis, two anionic residues (Asp6.59 located near the extracellular end of the TMD6, and Glu5.27 located on the TMD5 of both NPFF receptors) were postulated to form the negatively charged pocket responsible for the binding of the guanidinium moiety of the endogenous ligands.

**Identification and SAR of Ornithine Derivatives.** The presence of a salt bridge led us to investigate the effect of replacing the guanidine moiety by other cationic species, such as secondary or tertiary amines, that exist as salts at physiological pH. Primary amine **8** was easily obtained starting from the commercially available ornithine (Scheme 1). Reductive amination between the ornithine derivative **8** and benzaldehyde using $NaBH_4$ led to the corresponding secondary amine **9**. To introduce a tertiary amine, we developed an efficient strategy starting from the commercially available Fmoc-L-Glu(OtBu)–OH (Scheme 2). After introducing the C- and N-terminus using the classical Fmoc-strategy, the *tert*-butyl ester was cleaved with trifluoroacetic acid in DCM, affording compound **10**. The

**Table 3. Synthesis and Binding Affinity of Arginine Derivatives toward NPFF1 and 2 Receptors**[a]

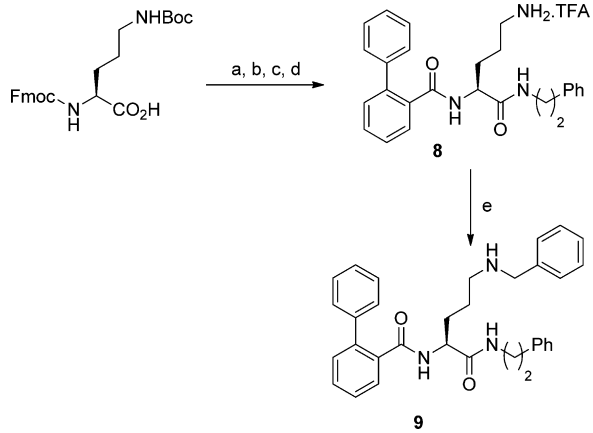



Conditions: (a) ROH (2 eq.), DIAD (2.2 eq.), $PPh_3$ (2.2 eq.), THF; (b) **5a-c** (1.2 eq.), TEA (3 eq.), DMF; (c) TFA, DCM.

| compd | R | $K_i$ NPFF1 (nM)[b] | $K_i$ NPFF2 (nM)[b] |
|---|---|---|---|
| **RF9** | | 22 ± 5 | 43 ± 1 |
| **2** | | 0.32 ± 0.02 | 920 ± 50 |
| **7a** | H | 53 ± 4 | 284 ± 40 |
| **7b** | $-CH_2-Ph$ | 43 ± 33 | 180 ± 46 |
| **7c** | $-(CH_2)_2-Ph$ | 49 ± 12 | 165 ± 52 |
| **7d** | $-(CH_2)_3$-Ph | 27 ± 4 | 162 ± 49 |

[a]$[^3H]$-FFRF-$NH_2$ was used as radioligand for competition assays with both hNPFF1 and 2 receptors. [b]Values are expressed as the mean ± SEM

**Scheme 1. Synthesis of an Ornithine Derivative Bearing a Benzylamine on the Side Chain**[a]




[a]Conditions: (a) Phenethylamine (1.1 equiv), BOP (1.1 equiv), NMM (2 equiv), DMF, rt; (b) DBU (2 equiv), DCM, rt; (c) 2-phenylbenzoic acid (1.1 equiv), BOP (1.1 equiv), NMM (2 equiv), DCM, rt; (d) TFA, DCM, rt; (e) PhCHO (2 equiv), TEA (4 equiv), DCM/MeOH, then $NaBH_4$ (3 equiv).



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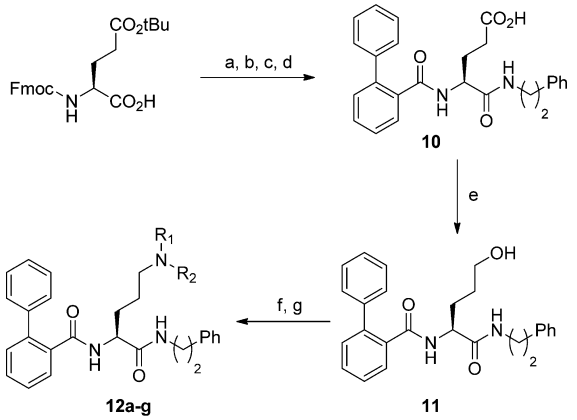
**Scheme 2. General Synthesis of Ornithine Derivatives Bearing Tertiary Amines on the Side Chain**[a]



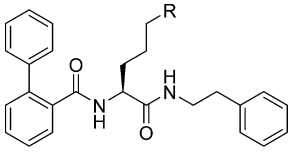

[a]Conditions: (a) Phenethylamine (1.1 equiv), BOP (1.1 equiv), NMM (2 equiv), DMF, rt; (b) DBU (2 equiv), DCM, rt; (c) 2-phenylbenzoic acid (1.1 equiv), BOP (1.1 equiv), NMM (2 equiv), DCM, rt; (d) TFA, DCM, rt; (e) *iBu*COCl (2.1 equiv), $NaBH_4$ (5 equiv), NMM (2.1 equiv), THF, −20 °C; (f) MsCl (2.5 equiv), TEA (2.5 equiv), DCM, rt; (g) amines (3.5 equiv), NaI (0.1 equiv), MeCN, 40 °C.

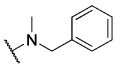
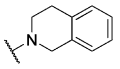
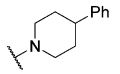
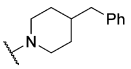
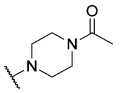
resulting carboxylic acid was activated with isobutylchloroformiate, and then reduced by $NaBH_4$ to the corresponding alcohol derivative **11**. After a mesylation step, a series of ornithine derivatives bearing tertiary amines were obtained by nucleophilic substitution with various secondary amines. Using piperidine in the last step, this seven-step synthesis was performed at a gram-scale, with up to 40% overall yield.

As shown in Table 4, replacement of arginine by ornithine led to a full loss of affinity toward both NPFF receptors. However, with the introduction of a benzyl group on the ornithine side chain (**9**), some affinity was recovered, especially toward NPFF1 ($K_i$ = 400 nM). Similar results were obtained for the tertiary amine **12a**, and with the other moieties bearing a benzene ring (**12b**−**d**). While as constrained tertiary amine derivatives **12b**−**d** did not improve the affinity for hNPFFR1 in comparison with the more flexible **12a**, their affinity toward hNPFFR2 was enhanced. **12d** showed a 30-fold improvement of affinity for hNPFFR2 in comparison with **12a**. Interestingly, **12d** appeared to be slightly more selective toward NPFF2 ($K_i$ = 34 nM) vs NPFF1 ($K_i$ = 400 nM). This result is remarkable as **12d** constitutes the first example of a reversed selectivity profile, and is likely due to the establishment of an hNPFFR2-specific interaction between the benzyl moiety and an aromatic amino acid located at proximity of the anionic pocket formed by Asp6.59 and Glu5.27. In contrast, the introduction of a piperidine moiety on the side chain led to compound **12e**, which exhibited a slight increase in affinity and a preference for hNPFFR1 ($K_i$ = 170 nM). The presence of a second protonatable nitrogen was well tolerated (**12f**) for hNPFFR1, but did not improve the affinity. A similar result was obtained with the H-bond acceptor group of the piperazine derivative **12g**. In contrast, both compounds (**12f**, **12g**) shown no affinity for hNPFFR2 ($K_i$ > 10 $\mu$M), highlighting the hypothesis of an anionic pocket less lipophilic for hNPFFR1 than for hNPFFR2, which is consistent with the better affinity of **12d** toward hNPFFR2.

**Functional Activities toward Both NPFF Receptors.** We examined the activity of a selection of compounds representative of each chemical series through in vitro functional experiments

**Table 4. Binding Affinity of Ornithine Derivatives toward NPFF1 and 2 Receptors**[a]




| Cpd | R | Ki NPFF1 (nM)[b] | Ki NPFF2 (nM)[b] |
|---|---|---|---|
| **RF9** | | 22 ± 5 | 43 ± 1 |
| **2** | | 0.32 ± 0.02 | 920 ± 50 |
| **8** | $NH_2$ | > 10 μM | > 10 μM |
| **9** | NH-Bn | 400 ± 38 | 2300 ± 66 |
| **12a** |  | 680 ± 160 | 1100 ± 510 |
| **12b** |  | 500 ± 99 | 320 ± 99 |
| **12c** |  | 485 ± 35 | nd[c] |
| **12d** |  | 400 ± 71 | 34 ± 12 |
| **12e** |  | 172 ± 13 | 563 ± 164 |
| **12f** |  | 232 ± 17 | > 10 μM |
| **12g** |  | 319 ± 13 | > 10 μM |

[a]$[^3H]$-FFRF-$NH_2$ was used as radioligand for competition assays with both hNPFF1 and 2 receptors. [b]Values are expressed as the mean ± SEM; [c]nd: not determined.

performed on CHO cells expressing NPFF receptors. Given the preferential coupling of hNPFFR1 and hNPFFR2 to Gi/o proteins,[38,39] the agonist activity of the selected compounds was inferred from their ability to inhibit forskolin-stimulated cAMP accumulation in NPFFR-expressing cells. In agreement with the already reported antagonist properties of **RF9**,[32] no agonistic activity was found for either the arginine derivatives (**4d**, **4h**, **7a**, **7b**) or the unnatural ornithine derivatives (**12b**, **12e**) at concentrations up to 10 $\mu$M.

**In Vivo Activity on Fentanyl-Induced Hyperalgesia.** As previously described, fentanyl administration in rats induces a short-lasting analgesia (few hours), followed by a hyperalgesic state that remains for several days.[6] The coadministration of an NPFF antagonist (e.g., **RF9**, **2**) with an opiate (e.g., fentanyl, morphine) was found to prolong analgesia, while strongly reducing hyperalgesia (Figure 1).[16,32,34] Using the Randall-Selitto test (paw pressure) in rats, the ornithine derivatives **12b** and **12e** were selected along with the arginine derivative **4h** to be coadministrated (0.5 mg/kg, s.c.) with fentanyl and evaluated for their ability to inhibit fentanyl-induced hyperalgesia (Figure 1).

The dipeptides **RF9** and **2** were previously reported to be strongly active in this model.[34] In contrast, **4h** surprisingly was found to have little effect in vivo, despite its high affinities toward both NPFF1 and 2 receptors (Table 2, $K_i$ = 11 and 25 nM, respectively). Although less potent in terms of affinity for both NPFFRs, the ornithine derivatives **12b** and **12e** strongly counteracted fentanyl-induced hyperalgesia at low doses.



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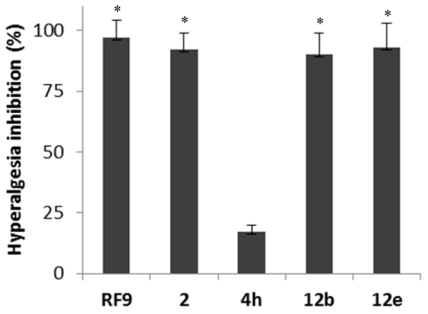


**Figure 1.** Inhibition of fentanyl-induced hyperalgesia by selected compounds in rats. On day 0 (D0), fentanyl injections (4 × 80 μg/kg, s.c.) were performed every 15 min for 1 h, resulting in a total dose of 320 μg/kg. NPFF antagonists (0.5 mg/kg, s.c.) or saline injections were performed 30 min before the first administration of fentanyl. Nociceptive threshold was measured by the paw-pressure test. Hyperalgesia inhibition percent was evaluated on day two (D2). Values are expressed as the mean ± SEM. *Dunnett test $P < 0.001$ for comparison between saline group and tested compounds.

**Metabolic Stability and Physicochemical Properties.** The lack of in vivo activity of **4h** may be due to rapid metabolic degradation. To test this possibility, intrinsic clearance in rat liver microsomes and half-life time in rat serum were evaluated for compounds **2**, **4h**, and **12e** (Table 5). Dipeptide **2** was quite

**Table 5. Metabolic Stability and Physicochemical Properties of a Set of NPFFR Antagonists**

| | RF9 | 2 | 4h | 12e | 12b |
|---|---|---|---|---|---|
| MW (g/mol) | 482 | 500 | 471 | 483 | 531 |
| $pK_a$[a] | 11.87 | 11.90 | 11.97 | 8.94 | 8.32 |
| log $D_{7.4}$[a] | −0.87 | 0.02 | 1.40 | 3.43 | 5.05 |
| PSA (Å$^2$)[a] | 163 | 165 | 113 | 63 | 63 |
| HBD[a,f] | 6 | 6 | 5 | 3 | 3 |
| $Cl_{int}$ | NT[e] | 8 | 46 | 51 | NT[e] |
| $t_{1/2}$ (min) | NT[e] | 106 | stable[d] | stable[d] | NT[e] |

[a]Calculator Plugins (Marvin 6.0.2, 2013, http://www.chemaxon.com) were used for structure−property prediction and calculation. [b]Intrinsic clearance in rat liver microsomes as a measure of metabolic stability. Values are expressed in μL/min/mg of protein. [c]Half-life time in rat serum as a measure of metabolic stability. [d]No degradation detected after 2 h in serum. [e]NT = Not tested. [f]Calculated with pH 7.4.

stable in liver microsomes ($Cl_{int}$ = 8 μL/min/mg protein), but was hydrolyzed in rat serum to the corresponding C-terminal carboxylic acid (detected by LC-MS-MS, $t_{1/2}$ = 1 h 46 min). In contrast, arginine and ornithine derivatives **4h** and **12e** showed good stability in rat serum, but a significant clearance in rat liver microsomes (Table 5). Using LC-MS-MS, we were able to detect an oxidation at the C-terminal phenethyl moiety. As **4h** and **12e** exhibit the same metabolic profile, the difference of in vivo efficacy may be explained by their ability to cross the blood-brain barrier (BBB) after systemic administration. Many reports have highlighted the critical role of a small subset of physicochemical data on BBB permeability.[40−43] According to Hitchcock, the application of four simple physicochemical rules may help to identify the compounds bearing favorable BBB permeability: (a) PSA < 90 Å$^2$; (b) HBD < 3; (c) 2 < log $D_{7.4}$ < 5; (d) MW < 450 Da.[43] Table 5 shows a comparison of these four physicochemical properties applied to the five compounds tested in vivo. First, in spite of their in vivo efficacy, both dipeptide Arg-Phe-$NH_2$ derivatives (**RF9** and **2**) do not respect any of the four rules. One way to explain the central efficacy of these compounds would be to consider a specific transport across the BBB through a carrier-mediated transport system. Recently, dipeptides such as Arg-Leu were shown to be substrates of the transporter Pept1, which is known to play an important role in the active transport of dipeptides through the intestinal epithelium.[44] In contrast, the arginine derivative **4h** exhibits slightly better physicochemical properties than **RF9** and **2**, but only one rule is fulfilled. In vivo, this compound demonstrated poor activity. Following our hypothesis of an active transport of dipeptide ligands, we may consider that **4h** is not recognized by any specific transporter, impairing its ability to reach the CNS. Finally, the switch from a guanidine group (**4h**) to tertiary amines (**12b** and **12e**) led to a great improvement of the probability for these compounds to cross the BBB through passive diffusion. Indeed, **12e** follows most of the Hitchcock rules, with a molecular weight just above the threshold of 450 Da. Notably, there is a great improvement of the polar surface area, which is reported as a critical property for passive BBB diffusion.[45] Therefore, **12e** appears in vivo as efficient as **RF9**, in spite of a lower affinity toward both NPFF receptors. A low polar surface area is also a dominating determinant for oral absorption,[45] making of **12e** a good candidate to be an orally available NPFF antagonist able to block opioid-induced hyperalgesia.

**Preventive Effect of 12e on Fentanyl-Induced Hyperalgesia after Oral Administration.** With an aqueous solubility greater than 24 mM, **12e** was easily solubilized in physiological solution before being administered per os to rats. Figure 2 shows

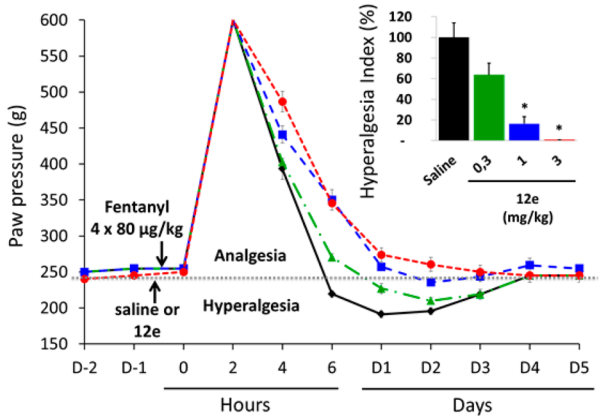


**Figure 2.** Preventive effect of **12e** on fentanyl-induced hyperalgesia. On day 0 (D0), four successive injections of fentanyl (80 μg/kg, s.c.) were performed every 15 min for 1 h, resulting in a total dose of 320 μg/kg. **12e** (0.3 (green up triangle), **1** (blue square), **3** (red circle) mg/kg), or saline (black tilted square) was orally administered 30 min before the first administration of fentanyl. Nociceptive threshold was measured by the paw-pressure test. Inset: Hyperalgesic index (HI) is the area between the baseline and the curve for nociceptive threshold on the days after fentanyl administration, normalized to control. Values are expressed as the mean ± SEM. *Dunnett's test $P < 0.001$ for comparison between saline group and compound **12e** group.

that the preventive effect of **12e** on fentanyl-induced hyperalgesia is dose-dependent, with a complete reversal at a dose of 3 mg/kg. Interestingly, **12e**-fentanyl treated rats still exhibited analgesia after 6 h, which was not the case for the saline-fentanyl group. Thus, **12e** seems to prolong the opioid-induced analgesia, similar to what was observed with **RF9**.[16] These results are in good agreement with the fact that NPFF receptors are known to reduce the analgesic effect of exogenous opioids, while


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ACS Chem. Neurosci. 2015, 6, 438−445

promoting tolerance to analgesics. Although it has been proposed that the NPFFR1 receptor subtype is mainly involved,[46] the respective roles of both NPFFR1 and NPFFR2 receptor subtypes in these functions is still unclear. Indeed, both NPFFR1 and NPFFR2 endogenous agonists, RFRP3 and NPFF (respectively), have been shown to display hyperalgesic or antimorphine properties and localization in the CNS of both receptors is consistent with a potential role in the modulation of nociception and antiopioid effects.[17] As **12e** displays a low selectivity between these two NPFF receptor subtypes, it is therefore difficult to say from our data whether one or both subtypes are responsible for the antihyperalgesic activity of this compound. In the future, development of highly selective compounds (antagonists and agonists) for NPFFR1 and NPFFR2 receptors should greatly help us to decipher the respective role of each NPFF receptor subtype in the modulation of the effects associated with acute and chronic opiate treatments.

**Selectivity of 12e toward a Set of Protein-Targets.** We next evaluated the ability of **12e** to bind to a set of 45 protein-targets, including GPCRs (opioid, dopamine, muscarinic receptors, etc.), amine transporters (SERT, NET, DAT), and ion channels (e.g., 5HT3, NMDA, and hERG) (Table S1 in the Supporting Information). Tested at 1 $\mu$M, **12e** exhibited no or very low affinity for any of these targets, except for the $\mu$-opioid receptor (MOR). **12e** had a significant affinity for MOR ($K_i$ = 1020 ± 50 nM SEM), but no agonistic effect of **12e** was observed on CHO cells expressing MOR (cAMP assay). In vivo, **12e** did not block the analgesia induced by fentanyl (Figure 2) and when administered alone did not lead to any analgesic or hyperalgesic effect (not shown), indicating that at the doses used, this compound did not display any agonist or antagonist activity at MOR. More investigations are required to see if this modest affinity toward MOR is correlated with the potentiation of the opioid-induced analgesia observed in our experiments. Finally, chronic administration of **12e** to mice (up to 30 mg/kg, i.p., once per week for 6 weeks) did not exhibit any visible toxicity, side effects, or any negative impact on muscular strength, locomotor activity or body mass evolution.

In conclusion, using drug design and bioisosterism concepts applied to the dipeptide **RF9**, we have developed unnatural analogues of ornithine as antagonists of NPFF receptors. Tested in vivo, compound **12e** is the first orally active NPFF antagonist capable of preventing opioid-induced hyperalgesia at low doses. Although we cannot exclude that the development of highly selective NPFFR1 or NPFFR2 antagonists could be an interesting strategy for the treatment of chronic pain, the lack of selectivity of **12e**, as well as its good pharmacological profile in terms of anti-OIH efficacy, selectivity, and preclinical observations, suggest that **12e** is a potential lead for preclinical studies for the treatment of acute and chronic pain. A lead optimization program for the further development of this compound is now in progress.

## METHODS

**Compound Synthesis.** See the Supporting Information.

**Binding Assays.** *hNPFFR1 and hNPFFR2 Receptors.* Membranes from CHO cells stably expressing hNPFFR1 or hNPFFR2 N-terminally fused to a Flag sequence (SF-hNPFFR1 and SF-hNPFFR2, respectively) were prepared as described.[47] SF-hNPFFR1 or SF-hNPFFR2 membranes (10 $\mu$g) were incubated for 30 min at 25 °C in a final volume of 0.5 mL containing 50 mM HEPES pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% bovine serum albumin, 10 nM and 3 nM [$^3$H]-FFRFamide (CEA, Paris; specific activity, 13.6 Ci/mmol) for SF-hNPFFR1 and SF-hNPFFR2, respectively, and the ligands to be tested. Nonspecific binding was determined in the presence of 1 $\mu$M neuropeptide FF for SF-hNPFFR2 and 1 $\mu$M neuropeptide VF for SF-hNPFFR1. Typical total and nonspecific binding were 1200 and 200 dpm, respectively, for SF-hNPFFR1 and SF-hNPFFR2. Incubation mixtures were rapidly filtered and washed with 50 mM HEPES pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, on 96 wells GF/B unifilter (PerkinElmer). Unifilter plates where then dried for 1 h at 65 °C. Bound radioactivity was determined by scintillation counting with 30 $\mu$L of scintillation cocktail (O-scint, PerkinElmer) per well on a Topcount scintillation apparatus (PerkinElmer).

*MOR Receptor.* Membrane preparations from HEK293 cells expressing human MOR and receptor binding assay were performed as described by Becker et al.[47]

**cAMP Measurements in Cells Expressing hNPFFR1 or hNPFFR2.** The inhibition by compounds of forskolin-stimulated cAMP accumulation in CHO cells expressing hNPFFR1 and hNPFFR2 was assessed according to Gicquiaux et al.[48] with minor modifications. CHO cells stably expressing SF-hNPFFR1 or SF-hNPFFR2 were harvested, seeded in 96-well plates (10 000 cells per well) in DMEM/Ham's F12 (1/1) supplemented with 5% FCS, penicillin (100 U/mL), and streptomycin (100 $\mu$g/mL), and grown at 37 °C in a humidified atmosphere with 5% $CO_2$ for 1−2 days until 90% confluency. Cells were washed with 0.2 mL of PBS and then incubated for 10 min at 37 °C with 0.2 mL of assay buffer (150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM HEPES, 10 mg/mL BSA, pH 7.4). The buffer was then replaced for 10 min with 0.06 mL of fresh buffer containing 1 mM IBMX before cell exposure to 10 mM forskolin, without or with compounds to be tested ($10^{-10}$−$10^{-5}$ M), for 10 min. The incubation medium (0.1 mL final volume) was maintained at 37 °C during the whole procedure. The reaction was stopped by adding 40 mL of ice-cold 0.5 M HCl and freezing at −80 °C. After 1 h, cells were disrupted by sonication and centrifuged at 2000*g* for 15 min. The resulting supernatants were stored at −20 °C until quantitation of cAMP levels by radioimmunoassay.

**In Vitro Metabolic Stability in Rat Liver Microsomes.** All incubations were performed individually for each test compound. Compounds (50 $\mu$M) were incubated at 37 °C under standard incubation conditions: phosphate buffer (pH 7.4), NADPH (1 mM), and rat liver microsomes (0.1 mg protein). The final concentration of DMSO was 0.5%. At different times (0, 5, 15, 30, and 45 min), aliquots (35 $\mu$L) of the reaction mixture were stopped with 100 $\mu$L of cold acetonitrile. Upon centrifugation of the resultant mixture, supernatants were analyzed by a generic HPLC method performed on a Dionex Ultimate 3000 (Waters XSelect CSH C18 column, 5 $\mu$m, 4.6 × 50 mm) with the following parameters: Flow rate, 1 mL/min; column temperature, 30 °C; solvent system: A (MeOH) and B (0.05% TFA in H2O), $t$ = 0 to 12 min, 20 to 100% of A then $t$ = 12 min to $t$ = 15 min, 100% of A. The ratio of product was determined by integration of UV spectra recorded at 202 nm. Metabolic stability was determined by the disappearance of the tested compound over time. The ln-linear plots of the percent of compound remaining based on chromatographic peak area versus time were plotted, and the slope was calculated by linear fitting of the curve. The microsomal intrinsic clearance ($Cl_{int}$, expressed in $\mu$L/min/mg protein) was calculated using the equation $Cl_{int} = 0.693/(t_{1/2})$ (mg of microsomal protein/volume of incubation).[49]

**In Vitro Metabolic Stability in Rat Serum.** All incubations were performed individually for each test compound. Compounds (50 $\mu$M) were incubated at 37 °C in rat serum (200 $\mu$L). Final concentration of DMSO was 0.5%. At different times (0, 15, 30, 60, and 120 min), aliquots (35 $\mu$L) of the reaction mixture were stopped with 100 $\mu$L of cold acetonitrile. Upon centrifugation of the resultant mixture, supernatants were analyzed by a generic HPLC method performed on a Dionex Ultimate 3000 (Waters XSelect CSH C18 column, 5 $\mu$m, 4.6 × 50 mm) with the following parameters: Flow rate, 1 mL/min; column temperature, 30 °C; solvent system: A (MeOH) and B (0.05% TFA in $H_2O$), $t$ = 0 to 12 min, 20 to 100% of A then $t$ = 12 min to $t$ = 15 min, 100% of A. The ratio of product was determined by integration of UV spectra recorded at 202 nm. Metabolic stability was determined by the disappearance of the tested compound over time. The ln-linear plots of the percent of compound remaining based on chromatographic peak


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area versus time were plotted, and the slope was calculated by linear fitting of the curve. The in vitro half-life time ($t_{1/2}$, expressed in minutes) was estimated by using $0.693/k$ where $k$ is the biotransformation rate constant and corresponds to the slope of the ln-linear curve.

**Solubility in HEPES pH 7.4.** An amount of 3 mg of compound **12e** was added to 200 $\mu$L of buffer (HEPES pH 7.4), and the heterogeneous mixture was agitated for 24 h at 20 °C. After centrifugation (15 000*g*, 10 min), the supernatant was diluted with buffer and analyzed via HPLC (Gilson) on a Kinetex C18 column (100 A, 50 × 4.6 mm) using the following parameters: Flow rate 2 mL/min; solvent system: A (MeCN) and B (0.1% TFA in H2O), $t$ = 0.2 to 3 min, 5% to 95% of A. The ratio of product was determined by integration of UV spectra recorded at 235 nm.

**Animals.** Experiments were performed on male Sprague−Dawley rats (Charles River, France), weighing 250−350 g, housed five per cage and maintained under a 12:12 light/dark cycle (lights on at 08:00 am) at a constant room temperature of 23 ± 2 °C. The animals had free access to food and water. Pharmacological tests and care of the animals were performed in accordance with the Guide for Animal Care and Use (NIH, 2011).

**Drug Administration.** Fentanyl citrate was obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). All drugs were dissolved in physiological saline (NaCl 0.9%) and injected subcutaneously or by oral route. Control animals were administered with an equal volume of saline.

**Measurement of the Nociceptive Threshold.** Nociceptive thresholds in rats were determined by a modification of the Randall-Selitto method, the paw-pressure vocalization test in which a constantly increasing pressure is applied to the hind paw until the rat squeaks. The Basile analgesimeter (Apelex, Massy, France; stylus tip diameter, 1 mm) was used. A 600 g cutoff value was employed for preventing tissue damage.

Experiments were performed essentially as described.[6] Briefly, the effect of fentanyl on nociceptive threshold was studied using an injection procedure designed to partly mimic its use in surgery. Fentanyl (or saline) was injected 4 times at 15 min intervals (80 $\mu$g/kg, s.c. per injection resulting in a total dose of 320 $\mu$g/kg). On day 0, nociceptive threshold was measured every 2 h for a period of 6 h after the first fentanyl injection and once daily during the 5 following days (D1 to D5). Pharmacological compounds (RF9, 2, 4h, 12b and 12e) were administered (30 min before the first fentanyl injection.

**Data and Statistical Analyses.** Data were expressed as the mean nociceptive threshold ± SEM. Student's paired $t$ test was used to assess paired comparisons of nociceptive threshold values to evaluate changes of the nociceptive threshold before the beginning of experiments ($D_{-1}$ and $D_0$ basal values). To evaluate the time-course effects of treatments on nociception, ANOVAs were performed on the nociceptive threshold values and followed by post hoc analysis using Dunnett's test. A difference was considered as significant for $p < 0.05$. Hyperalgesia index (HI) values, represented by the area above the curve for the days following fentanyl treatment, were calculated for each rat via the trapezoidal method[6] and were expressed as a mean percentage ± SEM of the reference index (100%: HI associated with hyperalgesia observed in the control group). $K_i$ values are expressed as means ± SEM from at least two separate experiments performed in duplicate.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Compound synthesis and analytical characterization; selectivity of **12e** against 45 protein targets. This material is available free of charge via the Internet at http://pubs.acs.org/.

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▽F.S. and J.-J.B. contributed equally to this work.



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### Notes

The authors declare no competing financial interest.

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