Novel Mixed NOP/Opioid Receptor Peptide Agonists

Salvatore Pacifico, Valentina Albanese, Davide Illuminati, Erika Marzola, Martina Fabbri, Federica Ferrari, Victor A.D. Holanda, Chiara Sturaro, Davide Malfacini, Chiara Ruzza, Claudio Trapella, Delia Preti, Ettore Lo Cascio, Alessandro Arcovito, Stefano Della Longa, Martina Marangoni, Davide Fattori, Romina Nassini, Girolamo Calò, and Remo Guerrini

ABSTRACT: The nociceptin/orphanin FQ (N/OFQ) receptor (NOP) system controls different biological functions including pain and cough reflex. Mixed NOP/opioid receptor agonists elicit similar effects to strong opioids but with reduced side effects. In this work, 31 peptides with the general sequence [Tyr/Dmt,5]N/OFQ(1-13)-NH2 were synthesized and pharmacologically characterized for their action at human recombinant NOP/opioid receptors. The best results in terms of NOP versus mu opioid receptor potency were obtained by substituting both Tyr1 and Thr5 at the N-terminal portion of N/OFQ(1-13)-NH2 with the noncanonical amino acid Dmt. [Dmt1,5]N/OFQ(1-13)-NH2 has been identified as the most potent dual NOP/mu receptor peptide agonist so far described. Experimental data have been complemented by in silico studies to shed light on the molecular mechanisms by which the peptide binds the active form of the mu receptor. Finally, the compound exerted antitussive effects in an in vivo model of cough.

INTRODUCTION

Nociceptin/orphanin FQ (N/OFQ; FGGFTGARKSAR-KLANQ) is the endogenous ligand of the N/OFQ peptide (NOP) receptor. N/OFQ and the NOP receptor display high structural homology with peptides and receptors of the opioid family but distinct pharmacology. The N/OFQ-NOP receptor system controls several biological functions at both central and peripheral levels including pain transmission, mood and anxiety, food intake, learning and memory, locomotion, cough and micturition reflexes, cardiovascular homeostasis, intestinal motility, and immune responses.

The effects of N/OFQ and selective NOP agonists in analgesiometric assays are complex depending on the dose, administration route, type of pain, and animal species. On the contrary, strong and consistent experimental evidence suggests that the simultaneous activation of NOP and opioid receptors elicits synergistic analgesic effects. On these bases, mixed NOP/opioid receptor agonists (cebranopadol, AT-121, BU10038, and BPR1M97) have been developed and investigated for their antinociceptive properties. It was consistently demonstrated that these drugs elicit similar analgesic effects to strong opioids but with substantially reduced side effects including respiratory depression, tolerance, and abuse liability (see the recent review by Kiguchi et al.). Other ligands targeting multiple opioid receptors have been studied. For example, dual-acting mu agonist/delta antagonist peptidomimetics demonstrated to produce antinociception in vivo with reduced tolerance liability compared with morphine. Moreover, mixed kappa agonist/mu partial agonist ligands have been investigated as potential treatment agents for cocaine and other psychostimulant abuses. Finally, mixed kappa agonist/delta antagonist ligands have been developed as}

Cite This: J. Med. Chem. 2021, 64, 6656–6669

Read Online

© 2021 The Authors. Published by American Chemical Society

6656

https://doi.org/10.1021/acs.jmedchem.0c02062

J. Med. Chem. 2021, 64, 6656−6669
Figure 1. SAR investigation leading to a series of [Tyr/Dmt,Xaa]N/OFQ(1-13)-NH2 peptide derivatives as possible mixed NOP/opioid receptor ligands.

Table 1. Effects of Standard Agonists and a First Series of [Tyr1,Xaa5]N/OFQ(1-13)-NH2 Derivatives at NOP and mu Opioid Receptors in Calcium Mobilization Studies

|   | NOP                      | mu                      | NOP/mu                      |
|---|--------------------------|-------------------------|-----------------------------|
|   | pEC50 (CL95%)            | Emax ± S.E.M.           | pEC50 (CL95%)               | Emax ± S.E.M. | CR     |
| 1 | N/OFQ(1-13)-NH2          | 9.29 (9.12–9.46)        | 368 ± 11                    | inactive      |        |
| 2 | dermorphin               | inactive                |                             |               |        |
| 3 | [Tyr1]N/OFQ(1-13)-NH2    | 9.23 (9.07–9.39)        | 380 ± 11                    |               |        |
| 4 | [Tyr1,Asn]N/OFQ(1-13)-NH2| 8.70 (8.26–9.11)        | 327 ± 15                    |               |        |
| 5 | [Tyr1,Val]N/OFQ(1-13)-NH2| 8.70 (8.53–8.87)        | 334 ± 19                    |               |        |
| 6 | [Tyr1,Lys(Ac)]N/OFQ(1-13)-NH2 | 8.55 (7.88–9.22)       | 370 ± 16                    |               |        |
| 7 | [Tyr1,Abu]N/OFQ(1-13)-NH2 | 8.15 (7.61–8.69)        | 366 ± 20                    |               |        |
| 8 | [Tyr1,Lys]N/OFQ(1-13)-NH2 | 7.26 (6.74–7.78)        | 366 ± 6                     |               |        |
| 9 | [Tyr1,Dap]N/OFQ(1-13)-NH2 | 7.20 (6.95–7.45)        | 367 ± 9                     |               |        |
|10 | [Tyr1,Dab]N/OFQ(1-13)-NH2 | 6.43 (6.28–6.58)        | 340 ± 22                    |               |        |
|11 | [Tyr1,Leu]N/OFQ(1-13)-NH2 | 8.03 (7.46–8.60)        | 339 ± 24                    |               |        |
|12 | [Tyr1,Nle]N/OFQ(1-13)-NH2 | 8.77 (8.45–9.09)        | 375 ± 9                     |               |        |
|13 | [Tyr1,Nva]N/OFQ(1-13)-NH2 | 8.43 (7.35–9.31)        | 358 ± 14                    |               |        |
|14 | [Tyr1,Nal]N/OFQ(1-13)-NH2 | 7.07 (6.68–7.46)        | 332 ± 19                    |               |        |

In N/OFQ(1-13)-NH2 can be replaced with several different residues without loss of peptide efficacy and potency at the NOP receptor; (ii) the substitution of Phe1 in N/OFQ with Tyr2 increases affinity and potency at classical opioid receptors. Thus, in the present study, 31 peptide derivatives with the general sequence [Tyr1,Xaa5]N/OFQ(1-13)-NH2 were generated and tested for their action at NOP and opioid receptors (Figure 1).

Experimental data have been complemented by an in silico study of the binding of [Dmt1,5]N/OFQ with Tyr26 and particularly with Dmt19,27 increases affinity and potency at classical opioid receptors. Thus, in the present study, 31 peptide derivatives with the general sequence [Tyr1,Dmt,Xaa5]N/OFQ(1-13)-NH2 were generated and tested for their action at NOP and opioid receptors (Figure 1).

With the aim of generating a peptide acting as a nonselective NOP/opioid agonist, we investigated different approaches. On one hand, the peptide [Dmt1]N/OFQ(1-13)-NH2 has been identified as a nonselective agonist for NOP and opioid receptors and its tetrabranched derivative, generated using the peptide welding technology (PWT), was demonstrated to produce a robust analgesic effect; (ii) the replacement of Thr5 in N/OFQ(1-13)-NH2 with Thr5 in NOFQ(1-13)-NH2 can be replaced with several different residues without loss of peptide efficacy and potency at the NOP receptor; (iii) the substitution of Phe1 in N/OFQ with Tyr increases affinity and potency at classical opioid receptors. Thus, in the present study, 31 peptide derivatives with the general sequence [Tyr1,Dmt,Xaa5]N/OFQ(1-13)-NH2 were generated and tested for their action at NOP and opioid receptors (Figure 1).
NOP and mu Opioid Receptors in Calcium Mobilization Studies

Calcium Mobilization Studies

MD simulation of the DAMGO-mu receptor-Gi protein molecular dynamics (MD) simulations and compared with a successful nonselective NOP/opioid agonist. Finally, consider complex to identify the key interactions necessary for a guinea pigs.

Table 2. Effects of Standard Agonists and [Tyr1,Xaa5]/N/OFQ(1-13)-NH2 Derivatives with Different Aromatic Residues as Xaa at NOP and mu Opioid Receptors in Calcium Mobilization Studies

|   | NOP | mu | NOP/mu |
|---|-----|----|--------|
| 1 | N/OFQ(1-13)-NH2 | 9.40 (9.19–9.61) | 288 ± 15 | inactive |
| 2 | demorphin | inactive | 7.83 (7.56–8.11) | 306 ± 23 | >50 |
| 3 | [Tyr1]/N/OFQ(1-13)-NH2 | 9.13 (8.83–9.43) | 266 ± 14 | inactive |
| 15 | [Tyr1,His]N/OFQ(1-13)-NH2 | 7.79 (7.66–7.87) | 242 ± 11 | 6.41 (5.85–6.97) | 315 ± 32 | 0.05 |
| 17 | [Tyr1,Tyr]N/OFQ(1-13)-NH2 | 7.27 (7.19–7.35) | 288 ± 23 | inactive |
| 19 | [Tyr1,LPro]N/OFQ(1-13)-NH2 | 8.70 (8.24–9.16) | 301 ± 26 | inactive |
| 19 | [Tyr1,Phg]N/OFQ(1-13)-NH2 | 7.57 (7.12–8.02) | 293 ± 31 | inactive |
| 20 | [Tyr1,p(pOCH3)Pro]N/OFQ(1-13)-NH2 | 7.31 (6.81–7.81) | 311 ± 22 | inactive |
| 23 | [Tyr1,Dip]N/OFQ(1-13)-NH2 | 6.61 (6.13–7.10) | 240 ± 26 | inactive |
| 24 | [Tyr1,Bip]N/OFQ(1-13)-NH2 | 6.93 (6.63–7.22) | 272 ± 29 | inactive |
| 25 | [Tyr1,Ala]N/OFQ(1-13)-NH2 | 7.17 (6.82–7.52) | 245 ± 16 | inactive |
| 26 | [Tyr1,2Na]N/OFQ(1-13)-NH2 | 6.82 (6.60–7.04) | 266 ± 15 | inactive |
| 27 | [Tyr1,p(NH2)Pro]N/OFQ(1-13)-NH2 | 7.52 (7.11–7.93) | 274 ± 15 | inactive |
| 28 | [Tyr1,Dmt]N/OFQ(1-13)-NH2 | 7.75 (7.22–8.27) | 251 ± 22 | inactive |

Table 3. Effects of Standard Agonists and [Dmt1,Xaa5]/N/OFQ(1-13)-NH2 Derivatives at NOP and mu Opioid Receptors in Calcium Mobilization Studies

|   | NOP | mu | NOP/mu |
|---|-----|----|--------|
| 1 | N/OFQ(1-13)-NH2 | 9.59 (9.30–9.88) | 289 ± 34 | inactive |
| 2 | demorphin | inactive | 8.15 (7.97–8.33) | 359 ± 18 | >100 |
| 3 | [Tyr1]/N/OFQ(1-13)-NH2 | 9.15 (8.48–9.82) | 236 ± 13 | 5.80 (5.22–6.38) | 251 ± 34 | >100 |
| 29 | [Dmt1,N]N/OFQ(1-13)-NH2 | 8.57 (8.26–8.87) | 294 ± 22 | 7.37 (7.12–7.51) | 311 ± 20 | 0.06 |
| 30 | [Dmt1,Tyr,Tyr]N/OFQ(1-13)-NH2 | 6.91 (6.81–7.01) | 304 ± 21 | 7.81 (7.47–8.15) | 410 ± 25 | 7.94 |
| 31 | [Dmt1,Tyr]N/OFQ(1-13)-NH2 | 7.25 (6.69–7.81) | 277 ± 28 | 8.19 (7.71–8.66) | 335 ± 22 | 8.71 |
| 32 | [Dmt1,Phg]N/OFQ(1-13)-NH2 | 6.95 (6.78–7.12) | 282 ± 12 | 8.54 (8.13–8.96) | 339 ± 28 | 3.9 |
| 33 | [Dmt1,Nal]N/OFQ(1-13)-NH2 | 7.22 (6.91–7.54) | 286 ± 19 | 7.80 (7.51–8.09) | 349 ± 20 | 3.80 |
| 34 | [Dmt1,p(NH2)Pro]N/OFQ(1-13)-NH2 | 7.58 (7.37–7.79) | 258 ± 20 | 7.82 (7.35–8.29) | 358 ± 15 | 1.73 |
| 35 | [Dmt1,N]N/OFQ(1-13)-NH2 | 8.39 (8.05–8.72) | 270 ± 25 | 8.08 (7.74–8.42) | 354 ± 16 | 0.49 |

Table 4. Effects of Standard Agonists and [Dmt1,5]/N/OFQ(1-13)-NH2 at NOP and mu Opioid Receptors in DMR Studies

|   | NOP | mu | mu/NOP |
|---|-----|----|--------|
| 36 | N/OFQ | 9.37 (8.96–9.79) | 209 ± 14 | inactive |
| 2 | demorphin | inactive | 8.92 (8.74–9.10) | 151 ± 16 | >500 |
| 35 | [Dmt1,5]N/OFQ(1-13)-NH2 | 7.71 (6.65–8.76) | 223 ± 11 | 8.64 (8.28–9.01) | 202 ± 20 | 8.51 |

carried out to provide the best binding pose of the two peptides [Dmt1,5]/N/OFQ(1-9)-NH2 and N/OFQ(1-9)-NH2. This docking procedure was further challenged by long-lasting molecular dynamics (MD) simulations and compared with an MD simulation of the DAMGO-mu receptor-Gi protein complex to identify the key interactions necessary for a successful nonselective NOP/opioid agonist. Finally, considering that NOP receptor agonists have demonstrated antinociceptive effects in vivo33−37 and that opioids are effective drugs currently in use to treat cough,82 the most potent mixed NOP/opioid agonist has been evaluated in vivo for its antinociceptive effects in guinea pigs.

RESULTS

Chemistry. The peptide derivatives reported in Tables 1−4 were prepared through automated Fmoc/tBu-based solid-phase peptide synthesis (SPPS) on a Rink amide MBHA resin. Commercially available protected amino acids were employed as synthetic precursors of the target peptides except for Fmoc-2′,6′-dimethyl-tyrosine (Fmoc-Dmt-OH) that was instead synthesized in analogy to an approach previously published by Wang et al.39 (Scheme S1 of the Supporting Information). Specifically, H-Tyr-OH was first esterified to H-Tyr-OMe under standard conditions, and then, the phenolic hydroxyl was protected with a tert-butylmethylsilyl ether moiety before the following coupling with picolinic acid. The latter function worked as a directing group for the subsequent Pd(OAc)2-catalyzed C–H alkylation with CH2I2 and K2CO3 allowing the simultaneous and regioselective introduction of two methyl groups at the ortho-positions of the aromatic ring. Then, full deprotection under strongly acidic conditions, followed by treatment with Fmoc-Cl, led to the desired 2′,6′-dimethyl...
tyrosine scaffold (detailed procedures and analytical character-
izations of Fmoc-Dmt-OH and its precursors have been reported in the Supporting Information). The structures of other nonproteinogenic amino acids employed in this work have been depicted in Table S1.

In Vitro Structure–Activity Relationship. N/OFQ(1-
13)-NH2 stimulated calcium mobilization with high potency and maximal effects in cells coexpressing NOP receptors and chimeric G proteins, while being inactive in cells expressing the mu opioid receptor. On the contrary, dermorphin stimulated calcium mobilization with high potency and maximal effects in mu expressing cells, while it was inactive in NOP cells (Table 1). The substitution of Phe1 with Tyr as in [Tyr1]N/OFQ(1-13)-
NH2 did not affect NOP potency while promoting a minor increase in mu potency. Thr5 in [Tyr1]N/OFQ(1-13)-NH2 was replaced with a series of both proteinogenic and non-
proteinogenic amino acids with different polar/nonpolar, aliphatic/aromatic, linear/branched side chains with the aim to explore the effect of several structural parameters on the biological activity. The substitution of Thr5 with Asn, Val, Lys(Ac) caused a slight (<10-fold) reduction in NOP potency and no modification of mu potency. The same substitution with Abu, Lys, Dap, and Dab induced a larger loss (>10-fold) of NOP potency. The introduction in position 5 of Leu, Nle, and Nva promoted a moderate decrease in NOP potency associated with a significant increase in mu potency. A similar increase in mu potency was achieved with [Tyr1,5]N/OFQ(1-13)-NH2, which however displayed a larger decrease in NOP potency; thus, the NOP/mu concentration ratio for this peptide was near 1 (Table 1). None of the amino acid substitutions evaluated in Table 1 modified ligand efficacy at both NOP and mu receptors. Based on these results, aromatic residues were selected for further modifications of position 5 of [Tyr1]N/OFQ(1-13)-NH2.

As shown in Table 2, 14 compounds with an aromatic residue substituting Thr5 in [Tyr1]N/OFQ(1-13)-NH2 were assayed in NOP and mu receptor expressing cells. The different amino acids did not modify ligand efficacy but produced different effects on NOP and mu potency. In particular, the NOP potency of these derivatives was in the range of 8.70–6.61, while the mu potency of these compounds was <6 with the exceptions of peptides substituted with Phe, Phg, 1Nal, (pNH2)Phe, and Dmt (range 6.08–6.81). Then, for further investigation, we selected those sequences showing pEC50 values >7 for the NOP receptor and >6 for the mu receptor associated with an NOP/mu concentration ratio >0.05. These criteria were matched by [Tyr1]N/OFQ(1-13)-NH2 derivatives substituted in position 5 with Tyr, Phe, Phg, 1Nal, (pNH2)Phe, and Dmt.

The third series of peptides was generated by substituting Tyr1 with Dmt that is known to increase opioid receptor potency.40 In fact, as shown in Table 3, [Dmt1]N/OFQ(1-13)-
NH2 displayed a moderate (10-fold) decrease in NOP potency compared to [Tyr1]N/OFQ(1-13)-NH2 associated to a more pronounced increase (approx. 40-fold) in mu potency. The substitution of Thr5 of [Dmt1]N/OFQ(1-13)-NH2 with the above-mentioned amino acids generated results similar to those obtained with [Tyr1]N/OFQ(1-13)-NH2 that is, a slight to moderate decrease in NOP potency associated to a large increase in mu potency. The most exciting result has been obtained with [Dmt1,5]N/OFQ(1-13)-NH2 that displayed similar and high potency at both NOP and mu receptors.

[Dmt1,5]N/OFQ(1-13)-NH2 was further evaluated in dynamic mass redistribution (DMR) experiments performed on CHO cells expressing the human NOP and mu receptors. As summarized in Table 4, N/OFQ elicited a concentration-dependant positive mu receptor DMR signal in cells expressing the NOP receptor being inactive in mu expressing cells. Opposite results were obtained with dermorphin that behaves as a mu-selective agonist. [Dmt1,5]N/OFQ(1-13)-NH2 elicited a robust DMR response in both cell lines with similar maximal effects to standard agonists. [Dmt1,5]N/OFQ(1-13)-NH2 displayed nanomolar potency at both NOP and the mu receptor with a mu/NOP potency ratio of 8.51 (Table 4).

Finally, the agonist properties of [Dmt1,5]N/OFQ(1-13)-
NH2 were evaluated at delta and kappa opioid receptors in calcium mobilization experiments. As shown in Figure 2,

![Figure 2](https://doi.org/10.1021/acs.jmedchem.0c02062)

Figure 2. Effects of [Dmt1,5]N/OFQ(1-13)-NH2 at NOP and classical opioid receptors in calcium mobilization studies.
displayed and superimposed, that is, hydrophobic and polar average number of contacts (Figure 4A,B), percentage of formation of hydrogen bonds, and average “strength” of π−π stacking and π–cation interaction (Figure 4C–E, respectively). Accordingly, the representative conformation of [Dmt$$^{1-5}$$N]/OFQ(1-9)-NH$_2$ in the orthosteric site largely overlaps with that of DAMGO (Figure 3A). The N-terminus of Dmt$^1$ forms salt bridge/hydrogen bond contacts with D147 (a residue conserved all along the opioid family) similar to both DAMGO and the morphinan agonist BU72 (PDB code SC1M). MD simulations show that this important interaction is strongly stabilized by the presence of another conserved residue, Q$^{124}$ (TM2), whose nitrogen and oxygen side-chain atoms reinforce the hydrogen bond network by contacts with both the carboxyl oxygen of D$^{147}$ and the N-terminus of Dmt$^1$. Moreover, water bridges fill the small remaining volume between the D$^{147}$ and Q$^{124}$ side chains and the backbone donor/acceptors of Dmt$^1$, Gly$^5$, and Gly$^2$, with the latter being in direct H-bond with W$^{318}$ of TM7.
Dmt₁ is also in direct hydrophobic contact with TM6 residues (W²⁹³, H²⁹⁷, and especially I²⁹⁶, Figure 3B, C). Along the MD trajectories, its aromatic head moves alternating first- and second-order water bridges with H²⁹⁷ of TM6 (Figure 3C). Partial π−π stacking between the Dmt₁ and H²⁹⁷ rings is also observed during the simulations. Hydrophobic, π-stacking, and water bridge contacts between Dmt₁ and Y¹⁴⁸ (TM3), H²⁹⁷ and W²⁹³ (TM6) frequently occur (Figure 3C). The latter residue, in the so-called receptor polar cavity, is thought to be very important for the activation mechanism in many class A GPCRs, and these interactions, although not fully stable, could contribute to stabilize the receptor active state.

The formation of alternating second-order water bridges (along 78% of the trajectory) shows that the N-terminus of Dmt₁, together with D¹⁴⁷, is also in contact with N¹⁵⁰ (Figure 3C), an important conserved residue that in the reported high-

Figure 4. Maps of hydrophobic (A), polar (B), H-bond (C), π−π stacking (D), and π−cation (E) interactions between the mu receptor and the studied ligands along MD trajectories. Only residues 1–5 are considered for [Dmt¹⁵] N/OFQ(1-9)-NH₂ and N/OFQ(1-9)-NH₂.

6661
https://doi.org/10.1021/acs.jmedchem.0c02062
J. Med. Chem. 2021, 64, 6656–6669
resolution structure of the inactive delta receptor\textsuperscript{41} is shown to connect the orthosteric site to the sodium pocket in the central part of the receptor.

While Dmt\textsuperscript{1} interacts with both TM3 (more than 40 contacts with residues Y\textsuperscript{148} and M\textsuperscript{151}) and TM6 (about 80 contacts with residues W\textsuperscript{293}, I\textsuperscript{296}, and H\textsuperscript{297}), Phe\textsuperscript{4} is immersed in the same hydrophobic pocket as the phenyl group of DAMGO between TM2 (residues F\textsuperscript{123} and Q\textsuperscript{124}) and TM3 (residues V\textsuperscript{143} and I\textsuperscript{144}) (Figure 3B,D), still participating with its amidic nitrogen and water bridges to the main hydrogen bond network linking the peptide to D\textsuperscript{147} and Q\textsuperscript{124} (Figure 3D).

The Dmt\textsuperscript{5} peptide residue mainly interacts with residues not conserved within the opiate family, that is, E\textsuperscript{229} and K\textsuperscript{233} of TM5, V\textsuperscript{300}, and K\textsuperscript{303} of TM6, and W\textsuperscript{318} of TM7. Movements of this ring allow an alternation of nonpolar interactions with the aliphatic chains of K\textsuperscript{303} (TM6) and K\textsuperscript{233} (TM5) (Figure 3B) and of possible \(\pi\)–cation interactions with the positively charged amine of both the same K residues (Figure 3E). Similarly, the amidic oxygens of Gly\textsuperscript{3}, Dmt\textsuperscript{5}, and Gly\textsuperscript{8} alternate in H-bond or water bridge contacts with R\textsuperscript{211} (ECL2) and E\textsuperscript{229} (TM5) on two opposite sides of the receptor.

Details on the MD simulations of DAMGO and [Dmt\textsuperscript{1-5}]N/OFQ(1-9)-NH\textsubscript{2} in complex with the mu receptor are given in Figures S1 and S2, reporting the root-mean-square deviation (RMSD) analyses and clustering outcomes for each of the investigated peptides. Moreover, the RMSD analysis (Figure S3) and the representative conformation of residues 1–5 of N/OFQ(1-9)-NH\textsubscript{2} (purple) are shown, compared to DAMGO (yellow). MD shows that the interactions of N/OFQ(1-9)-NH\textsubscript{2} with TM6 are strongly diminished; in addition to the absence of polar contacts and the water density between residues 1–5 of the peptide and TM6, there are only about 20 nonpolar contacts (between Phe\textsuperscript{1} and W\textsuperscript{293} and between Phe\textsuperscript{1} and F\textsuperscript{236}), while both polar and nonpolar interactions with TM3 increase.

In Figure 4, the maps of hydrophobic, polar, hydrogen bond, \(\pi\)–\(\pi\) stacking, and \(\pi\)–cation interactions for the peptides under study are superimposed for an overall immediate comparison. The hydrophobic and polar interaction maps are widely superimposable on all peptides (Figure 4A,B), attesting the similarity of their conformation inside the orthosteric site, with an increase of nonpolar contacts between [Dmt\textsuperscript{1-5}]N/OFQ(1-9)-NH\textsubscript{2} and TM6 (I\textsuperscript{296}, V\textsuperscript{300}, and K\textsuperscript{303}) essentially due to the aromatic ring of Dmt\textsuperscript{5}. The N-terminus of all three peptides forms hydrogen bonds with D\textsuperscript{147} and Q\textsuperscript{124} (Figure 3D).

In Figure 5, the maps of hydrophobic, polar, hydrogen bond, \(\pi\)–\(\pi\) stacking, and \(\pi\)–cation interactions for the peptides under study are superimposed for an overall immediate comparison. The hydrophobic and polar interaction maps are widely superimposable on all peptides (Figure 4A,B), attesting the similarity of their conformation inside the orthosteric site, with an increase of nonpolar contacts between [Dmt\textsuperscript{1-5}]N/OFQ(1-9)-NH\textsubscript{2} and TM6 (I\textsuperscript{296}, V\textsuperscript{300}, and K\textsuperscript{303}) essentially due to the aromatic ring of Dmt\textsuperscript{5}. The N-terminus of all three peptides forms hydrogen bonds with D\textsuperscript{147} and Q\textsuperscript{124} (Figure 3D).

Figure 5. Effect of [Dmt\textsuperscript{1-5}]N/OFQ(1-13)-NH\textsubscript{2} on citric acid-induced cough in conscious guinea pigs. Schematic representation of the experimental procedure for the cough measurement in conscious guinea pigs and pooled data of cough number after [Dmt\textsuperscript{1-5}]N/OFQ(1-13)-NH\textsubscript{2} (1 mM) or vehicle (0.9% NaCl) nebulization, 30 min before the nebulization of the tussive agent, citric acid (0.4 M). Values are the mean ± SEM of the numbers of coughs/15 min, with data points overlaid (n = 6 guinea pigs for each condition). *p < 0.05 vs vehicle, Student’s t-test.
negligible hydrogen and water bond contacts with the inner side of the receptor, and the contacts between Phe<sup>4</sup>, Thr<sup>5</sup> of the peptide and T<sup>218</sup>, L<sup>219</sup> of extracellular loop 2 (ECL2) are stronger (Figure 4C). The N-terminus of the three peptides can form π–cation interactions with the aromatic ring of Y<sup>326</sup>, while as mentioned above, π–cation contributions due to interactions of K<sup>233</sup> and K<sup>305</sup> are exclusive of [Dmt<sup>1,5</sup>]N/OFQ(1-9)-NH<sub>2</sub> (Figure 4E).

*In Vivo* Experiments: [Dmt<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub> Effect on Citric Acid-Induced Cough in the Conscious Guinea Pig. To test the antitussive effect of [Dmt<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub>, we used a model of cough induced by citric acid in guinea pigs. Data showed that the coadministration of [Dmt<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub> with citric acid did not affect the cough response (coughs/15 min: vehicle = 17.83 ± 3.95 vs [Dmt<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub> 17.67 ± 1.73). However, the nebulization with [Dmt<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub> before (30 min) the challenge with the tussive agent significantly reduced the cough number induced by citric acid (Figure 5).

**DISCUSSION**

This structure activity investigation was aimed at the identification of novel peptides acting as mixed NOP/mu receptor agonists. To this aim, we substituted Phe<sup>3</sup> of N/OFQ(1-13)-NH<sub>2</sub> with amino acids containing a phenol moiety and Thr<sup>5</sup> with several proteinogenic and nonproteinogenic residues. Novel peptides were investigated in calcium mobilization experiments performed in cells expressing the human recombinant receptors and chimeric G proteins. The structure activity investigation led to the identification of the potent mixed agonist [Dmt<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub> whose NOP and mu agonist properties were confirmed in DMR studies. Moreover, [Dmt<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub> was also able to potently stimulate kappa but not delta opioid receptors. The capability of this peptide to bind the mu receptor has been also investigated in MD studies that suggested a similar active conformation for [Dmt<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub> and DAMGO and crucial interactions with D<sup>47</sup> and H<sup>297</sup>. Moreover, the binding of [Dmt<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub> is reinforced by additional polar interactions of Dmt<sup>1</sup> with K<sup>233</sup> and K<sup>305</sup>. Finally, [Dmt<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub> elicited a robust antitussive action in *vivo* in a model of cough induced by nebulization of citric acid in conscious guinea pigs.

Previous studies demonstrated that the substitution of Phe<sup>1</sup> in N/OFQ with Tyr reduces NOP selectivity over opioid receptors<sup>26,42</sup> and that Thr<sup>5</sup> of N/OFQ(1-13)-NH<sub>2</sub> can be substituted with different amino acids with no changes in peptide efficacy and relatively little modifications of potency. Thus, we selected a series of amino acids to substitute Thr<sup>5</sup> in [Tyr<sup>1</sup>]N/OFQ(1-13)-NH<sub>2</sub> in order to increase the mu receptor activity of the peptide derivatives. The results obtained with [Tyr<sup>1</sup>]N/OFQ(1-13)-NH<sub>2</sub> derivatives were similar to those previously obtained with N/OFQ(1-13)-NH<sub>2</sub> derivatives in terms of NOP receptor activity. As far as the mu receptor is concerned, an increase in potency has been obtained with Leu, Nle, Nva, and Tyr. These results are not unexpected since Leu in position 5 is found in naturally occurring opioid ligands (Leu-enkephalin and dynorphin) and Nle (and possibly Nva) may mimic methionine, which is also present in position 5 of other endogenous opioid peptides (Met-enkephalin, beta-endorphin). In addition, the same can be said for Tyr<sup>1</sup>, which is found in amphibian opioid peptides such as the mu-selective agonist dermorphin. Moreover, previous studies demonstrated that position 5 of enkephalin can be replaced with aromatic residues<sup>35</sup> or non-natural aliphatic residues<sup>44</sup> with no major changes of bioactivity. Interestingly, [Tyr<sup>1,5</sup>]N/OFQ(1-13)-NH<sub>2</sub> displayed very similar potency at NOP and the mu opioid receptor; thus, with the aim to identify potent mixed NOP/mu agonists, further studies were performed substituting position 5 with aromatic amino acids.

Despite the investigation of 14 chemically different aromatic residues, no clear structure activity information was obtained. In fact, with the exception of hPhe, little changes in NOP potency were measured and the same can be said for mu receptor activity. Thus, for further studies, we selected compounds matching the following criteria: pEC<sub>50</sub> > 7 for the NOP receptor and >6 for the mu receptor, with NOP/mu ratio > 0.05. This let us to select Tyr, Phe, Phg, 1Nal, (pNH<sub>2</sub>)Phe, and Dmt to be substituted in position 5 of [Dmt<sup>1</sup>]N/OFQ(1-13)-NH<sub>2</sub>.

The opioid receptor binding enhancing properties of Dmt in position 1<sup>19</sup>, 27, 28 were confirmed by the present results. In fact, compared to [Tyr<sup>1</sup>]N/OFQ(1-13)-NH<sub>2</sub>, [Dmt<sup>1</sup>]N/OFQ(1-13)-NH<sub>2</sub> displayed approximately 3-fold reduced potency at NOP associated with almost 100-fold increased potency at the mu receptor. The same pattern of effects, that is, no change or modest reduction of NOP potency associated with a large increase in mu potency was obtained with [Dmt<sup>1</sup>]N/OFQ(1-13)-NH<sub>2</sub> derivatives substituted in position 5 with Tyr, Phe, Phg, 1Nal, (pNH<sub>2</sub>)Phe, and Dmt to be substituted in position 5 of [Dmt<sup>1</sup>]N/OFQ(1-13)-NH<sub>2</sub>.

The opioid receptor binding enhancing properties of Dmt in position 1<sup>19</sup>, 27, 28 were confirmed by the present results. In fact, compared to [Tyr<sup>1</sup>,Dmt<sup>5</sup>]N/OFQ(1-13)-NH<sub>2</sub>, [Dmt<sup>1</sup>,5]N/OFQ(1-13)-NH<sub>2</sub> displayed approximately 3-fold reduced potency at NOP associated with almost 100-fold increased potency at the mu receptor. The same pattern of effects, that is, no change or modest reduction of NOP potency associated with a large increase in mu potency was obtained with [Dmt<sup>1</sup>]N/OFQ(1-13)-NH<sub>2</sub> derivatives substituted in position 5 with Tyr, Phe, Phg, 1Nal, (pNH<sub>2</sub>)Phe, and Dmt to be substituted in position 5 of [Dmt<sup>1</sup>]N/OFQ(1-13)-NH<sub>2</sub>.
residues that may favor mu and kappa interactions but are detrimental for delta receptor binding. To get insights into the mechanisms by which [Dmt<sup>1,5</sup>]/OFQ(1-13)-NH<sub>2</sub> binds the mu receptor, MD studies were performed using the recently solved DAMGO-mu receptor-G<sub>i</sub> complex. The results obtained with [Dmt<sup>1,5</sup>]/OFQ(1-9)-NH<sub>2</sub> were compared with those of DAMGO and N/OFQ(1-9)-NH<sub>2</sub> used as the positive and negative control, respectively. These studies show that beyond the pivotal and expected interaction between the [Dmt<sup>1,5</sup>]/OFQ(1-9)-NH<sub>2</sub> N-terminus and D<sup>147</sup>, the phenol oxygen of Dmt<sup>1</sup> can make first- or second-order water bridges with H<sup>297</sup> (Gln in NOP) of TM6 of the mu receptor. This is in good agreement with the observation of a water bridge between the agonist BU72 and H<sup>297</sup> in the active mu receptor and other small molecules or peptide mimetic agonists of kappa and delta receptors<sup>51</sup> and can account for the reduction of NOP selectivity and the increase of mu potency as simply induced by the presence of the phenol groups of Tyr<sup>1</sup> or Dmt<sup>1</sup> instead of the phenyl group of Phe<sup>1</sup>. Partial π−π stacking between Dmt<sup>1</sup> (or Tyr<sup>1</sup>) and H297 could further contribute to peptide stabilization in the orthosteric site, thus enhancing these effects. Analogous contacts have been reported for cocystallized mu<sup>51</sup> and delta<sup>52</sup> but not NOP<sup>53</sup> antagonists. Importantly, Phe<sup>1</sup> of the N/OFQ sequence cannot form water bridges with H<sup>297</sup>, and this is most probably the reason for the lack of mu affinity of the peptide.

Interestingly, as stated above, Dmt<sup>1</sup> mainly interacts with residues that differ within the opioid family, that is, E<sup>229</sup> (G in NOP, D in kappa and delta) and K<sup>233</sup> (A in NOP) of TM5, V<sup>300</sup> (I in kappa), and K<sup>303</sup> (W, E, and Q in delta, kappa, and NOP receptor, respectively) of TM6, and W<sup>318</sup> (L in NOP) of TM7. While the carbonyl oxygen of Dmt<sup>1</sup> is in water bridge contact with E<sup>229</sup>, its aromatic bulky head is stacked between the aliphatic chains of K<sup>303</sup> and K<sup>233</sup>, making possible π−cation interactions with the positively charged amine of both lysines (Figure 3B,E). In the reported crystal structure of mu-DAMGO<sup>30</sup> (PDB code 6DDF), the K<sup>303</sup> positive charge is found at a 3.3 Å distance of the carbonyl oxygen of N(Me)-Phe of DAMGO, compatible with a weak H-bond, whereas K<sup>233</sup> does not appear to contribute to the stabilization of the mu active state induced by both DAMGO and BU72<sup>28</sup>-<sup>29</sup> the K<sup>233</sup> amine group is found covalently linked to the antagonist β-fumaltrexamine in the crystal structure of the inactive mu receptor. As K<sup>303</sup> and K<sup>233</sup> are present in the mu but not the NOP receptor, the above-mentioned interactions between Dmt<sup>1</sup> and the two lysine residues could contribute to explain the mu-selective increase of affinity of [Dmt<sup>1,5</sup>]/N/OFQ(1-13)-NH<sub>2</sub> compared to [Dmt<sup>1</sup>]/N/OFQ(1-13)-NH<sub>2</sub>, thus making [Dmt<sup>1,5</sup>]/N/OFQ(1-13)-NH<sub>2</sub> a mixed mu/NOP agonist. Last, as observed along the MD runs, the indole nitrogen of W<sup>318</sup> in TM7 does not interact with N/OFQ but can form H-bond contact with Gly<sup>2</sup> of [Dmt<sup>1,5</sup>]/N/OFQ(1-9)-NH<sub>2</sub> as well as with DAMGO (49 and 63% of the trajectory, respectively). Thus, beyond differences in steric hindrance of Tyr<sup>1</sup> and Dmt<sup>1</sup> that may generically contribute to a larger hydrophobic core for the last one, the entity of the interaction between W<sup>318</sup> and Gly<sup>2</sup> could also contribute to explain the enhanced potency of [Dmt<sup>1,5</sup>]/N/OFQ(1-13)-NH<sub>2</sub>. Data obtained from this molecular modeling investigation are in agreement with those reported by recent studies performed on a series of cyclic opioid peptides.<sup>54</sup>

The role of the N/OFQ-NOP receptor system has been widely reported in several biological functions at the central and peripheral levels, including the cough reflex. Previous studies showed that NOP receptor agonists given centrally or peripherally suppress capsaicin and acid inhalation-induced cough in guinea pigs.<sup>33−35</sup> Moreover, opioid drugs are widely used as antitussive agents,<sup>38</sup> and inhalation of encephalin was shown to be effective in reducing cough reflex in vivo.<sup>55</sup> The novel mixed NOP/opioid agonist [Dmt<sup>1,5</sup>]/N/OFQ(1-13)-NH<sub>2</sub> showed an inhibitory activity against citric acid-induced cough in guinea pigs, thus demonstrating the in vivo activity of the compound. However, further studies are needed to investigate the receptor mechanism involved in the antitussive action of the molecule.

**CONCLUSIONS**

In this study, starting from the NOP-selective sequence of N/OFQ(1-13)-NH<sub>2</sub>, we developed a structure activity investigation focused on positions 1 and 5. Regarding position 1, a phenol moiety is required to increase mu receptor binding, and regarding position 5, aromatic residues generated the best results in terms of similar potency at NOP and mu receptors. This study led to the identification of [Dmt<sup>1,5</sup>]/N/OFQ(1-13)-NH<sub>2</sub> as the most potent mixed peptide agonist for NOP and mu receptors so far described in the literature. MD studies shed light on the molecular mechanisms adopted by this peptide to bind the active form of the mu receptor: some features of the mode of binding of [Dmt<sup>1,5</sup>]/N/OFQ(1-9)-NH<sub>2</sub> are superimposable to those of DAMGO, that is, the ionic bond with D<sup>147</sup> of TM3 and the H-bond network with H<sup>297</sup> of TM6, while others are peculiar of [Dmt<sup>1,5</sup>]/N/OFQ(1-9)-NH<sub>2</sub> that is, polar interactions of Dmt<sup>1</sup> with K<sup>223</sup> and K<sup>303</sup> of TM5 and TM6, respectively.

[Dmt<sup>1,5</sup>]/N/OFQ(1-13)-NH<sub>2</sub> is a novel mixed agonist for NOP and mu receptors that exerted antitussive effects in an in vivo model of cough. The compound will be evaluated in future studies for its antinociceptive properties. In fact, mixed NOP/mu agonists of both peptide and nonpeptide structures have been consistently demonstrated in preclinical studies to promote antinociceptive effects similar to those of morphine being however better tolerated particularly in terms of respiratory depression, tolerance, and abuse liability.<sup>13</sup> Importantly, phase II and III clinical studies performed with the mixed NOP/mu agonist cebranopadol have confirmed this favorable profile in pain patients.<sup>55</sup> Nowadays, the availability of safer analgesic drugs is particularly needed for facing the opioid epidemic that leads to a progressive increase of fatal overdoses over the past 2 decades.<sup>57</sup>

**EXPERIMENTAL SECTION**

**Chemistry. Materials and Methods.** All solvents and reagents were purchased from Sigma-Aldrich and Fisher Scientific. Enantipure Fmoc-protected amino acids and the resins for SPPS were purchased from AAPPTec. Peptides were synthesized using a standard Fmoc/t-butylic strategy<sup>58</sup> with a Syro XP multipptide synthesizer (MultiSynTech GmbH, Witten Germany) on a Rink amide MBHA resin (4-(2-hydroxyethyl)pyridine-1-carboxylic acid resin, diethyl ether was added to the cleavage cocktail to obtain the peptides from the resin, diethyl ether was added to...
the filtrate to promote precipitation of the peptide products that were finally isolated by centrifugation. Reverse-phase purification of crude peptides was carried out on a Waters Prep 600 high-performance liquid chromatography (HPLC) system with a Jupiter column C18 (250 × 30 mm, 300 Å, 15 μm spherical particle size) using a gradient, programmed time by time, of acetonitrile/water (with 0.1% trifluoroacetic acid (TFA)) at a flow rate of 20 mL/min. Nonpeptide derivatives were purified through flash column chromatography using a Biotage System Isolera One. Analytical HPLC was performed with a Beckman 110 liquid chromatograph furnished of a UV detector. The purity of peptides in Table 1 was assessed with a Symmetry C18 column (4.6 × 75 mm, 3.5 μm particle size, SYSTEM GOLD) at a flow rate of 0.5 mL/min using a linear gradient from 100% of A (water + 0.1% TFA) to 100% of B (acetonitrile + 0.1% TFA) over a period of 25 min. The purity of peptides in Tables 2 and 3 was assessed with an Agilent Zorbax C18 column (4.6 × 150 mm, 3.5 μm particle size, KARAT32) at a flow rate of 0.7 mL/min using a linear gradient from 100% of A (water + 0.1% TFA) to 100% of B (acetonitrile + 0.1% TFA) over a period of 25 min. All final compounds were monitored at 220 nm showing ≥95% purity, and their molecular weights were confirmed using an ESI Micromass ZQ Waters (HPLC chromatograms and ESI mass spectra of the final peptide derivatives have been reported in the Supporting Information).13,14 H and 13C NMR spectra were recorded for nonpeptide derivatives on a Varian 400 MHz instrument, and all experiments were performed in deuterated DMSO using its residual shifts as reference (s: singlet, δ: doublet, d: double doublet, t: triplet, m: multiplet). In Vitro Pharmacological Studies. Drugs and Reagents. (D-Pen2,D-Pen5)enkephalin (DPPDE) and naltrexone were purchased from Tocris Bioscience (Bristol, UK). Concentrated solutions (1 mM) were made in bidistilled water and kept at −20 °C until use. The medium and reagents for cell culture were from Euroclone (Milan, Italy). Fluo-4 AM and pluronic acid were purchased from Invitrogen/Thermo Fisher Scientific (Waltham, USA). N-(2-Hydroxyethyl)piperazine-N'-ethanesulfonic acid (HEPES), probenecid, brilliant black, and bovine serum albumin (BSA) fraction V were from Sigma-Aldrich (St. Louis, USA).

Calcium Mobilization Assay. CHO cells stably coexpressing the human NOP or kappa or the mu receptor and the C-terminally modified Gpr55 and CHO cells coexpressing the delta receptor and the Gpr55G669D5 protein were generated and cultured as described previously.35,46 Cells were maintained in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (DEMEM/F-12) supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, 100 μg/mL hygromycin B, and 200 μg/mL G418 and cultured at 37 °C in 5% CO2 humidified air. Cells were seeded at a density of 50,000 cells/well in a 96-well black, clear-bottom plate. The following day, the cells were incubated with Hanks’ balanced salt solution (HBSS) supplemented with 2.5 mM probenecid, 3 μM of the calcium-sensitive fluorescent dye Fluo-4 AM, and 0.1% pluronic acid for 30 min at 37 °C. After that, the loading solution was aspirated and 100 μL of HBSS supplemented with 20 μM HEPES, 2.5 mM probenecid, and 500 μM brilliant black was added. Serial dilutions were carried out in HBSS/HEPES supplemented with 20 μM HEPES, 2.5 mM probenecid, and 500 μM brilliant black was added. Serial dilutions were carried out in HBSS/HEPES (20 μM) buffer (containing 0.02% BSA fraction V). After placing both plates (cell culture and master plate) into the fluorometric imaging plate reader FlexStation II (Molecular Devices, Sunnyvale, CA), fluorescence changes were measured. On-line additions were carried out in a volume of 50 μL/well. To facilitate drug diffusion into the wells, the present studies were performed at 37 °C. Maximum change in fluorescence, expressed as percent over the baseline fluorescence, was used to determine agonist response.

DMR Assay. CHO cells stably expressing the human NOP or kappa or the mu receptor were kindly provided by D.G. Lambert (University of Leicester, UK). Cells were cultured in DMEM/F-12 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. The medium was supplemented with 20 μg/mL G418 to maintain expression. Cells were cultured at 37 °C in 5% CO2 humidified air. For DMR measurements, the label-free EnSight Multimode Plate Reader (Perkin Elmer, MA, US) was used. Cells were seeded 15,000 cells/well in a volume of 30 μL onto fibronectin-coated 384-well DMR microplates and cultured for 20 h to obtain confluent monolayers. Cells were starved in the assay buffer (HBSS with 20 mM HEPES, 0.01% BSA fraction V) for 90 min before the test. Serial dilutions were made in the assay buffer. After reading the baseline, compounds were added in a volume of 10 μL; then, DMR changes were recorded for 60 min. Responses were described as picometer (pm) shifts over time (sec) following subtraction of values from vehicle-treated wells. Maximum picometer (pm) modification (peak) was used to generate concentration response curves. All the experiments were carried out at 37 °C.

Data Analysis and Terminology. The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations.15 All data are expressed as the mean ± standard error of the mean (SEM) of at least three experiments performed in duplicate. For potency values, 95% confidence limits (CI95%) were indicated. Agonist potencies are given as pEC50, that is, the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal effect of that agonist. Concentration-response curves to agonists were fitted to the classical four-parameter logistic nonlinear regression model:

EFFECT = Baseline + (EASY – Baseline) / (1 + 10[^LogIC50 – Log(ECmax)] × Hillslope).

Curve fitting was performed using PRISM 6.0 (GraphPad Software Inc., San Diego).

Molecular Dynamics. The setup of an in silico model of the non-peptide [Dmt19,Nle20]-NOFPQ(1-9)-NH2 and [Nle20]-NOFPQ(1-9)-NH2 in complex with the human mu receptor has been described in the Supporting Information. Classical MD simulations of these two receptor-peptide complexes were performed and compared with an MD simulation of the experimental system DAMGO-mu receptor-G protein complex as derived by the PDB file 6DDE.50 The GROMACS 2018.3 package51 was used under the AMBER parm99bs force field52 at the full atomic level using a TIP3P water solvent and an explicit pre-equilibrated phospholipid bilayer of 128 POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) molecules obtained by the Prof. Tieleman website (http://moose.bio.ucalgary.ca). All the MD sessions were performed in a water–membrane system prepared as previously described.31,52 The receptor-peptide-membrane systems were solvated in a triclinic water box (having basis vector lengths of 7.74, 9.3, and 9.9 nm) under periodic boundary conditions for a total number of about 45,000 atoms (6400 solvent molecules). The total charge of the system was neutralized by randomly substituting water molecules with Na+ ions and Cl− ions to obtain neutrality with a 0.15M salt concentration. Following a steepest descent minimization algorithm, the system was equilibrated under canonical ensemble (NVT) conditions for 300 ps using a V-rescale, modified Berendsen thermostat with position restraints for both the receptor-peptide complex and the lipids and thereafter in a isothermal–isobaric ensemble (NPT) for 500 ps, applying position restraints to the heavy atoms of the protein-peptide complex, and using a Nose–Hoover thermostat and a Parrinello-Rahman barostat at 1 atm with a relaxation time of 2.0 ps. The MD simulation of the mu receptor-DAMGO-G protein was carried out on the whole ternary complex without positional restraints. On the other hand, in order to reduce the computational time, in the two mu receptor-peptide complexes, the G protein was not included in the system, but all residues within 5 Å of the G protein interface were restrained to the initial structure of the activated receptor using 5.0 kcal mol−1 Å−2 harmonic restraints applied to non-hydrogen atoms. Using such restraints ensures that the receptor maintains an active conformation throughout the simulation. MD runs were performed under NPT conditions at 300 K with a T-coupling constant of 1 ps, van der Waals interactions were modeled using a 6–12 Lennard-Jones potential with a 1.2 nm cutoff. Long-range electrostatic interactions were calculated, with a cutoff for the real space term of 1.2 nm. All covalent bonds were constrained using the LINCS algorithm. The time step employed was 2 fs, and the coordinates were saved every 5 ps for analysis.

The MD analysis of the DAMGO-mu receptor-G protein complex (Figure S1) shows an overall stability of the starting configuration (corresponding to the crystal structure) with some motion of the phenolic head toward the intracellular side of the receptor, still conserving the water bridge contact with H397. A non-negligible rearrangement is observed (Figures S2 and S3) along the MD sessions,
starting from the docked conformations of \([\text{Dmt}^{1,5}]\text{N/OFQ(1-9)-NH}_2\) and \(\text{N/OFQ(1-9)-NH}_2\), probably due to the limitations of the docking procedures applied to molecules with a large number of torsions, and confirms the importance of performing long-lasting MD sessions. Analysis of MD trajectories was performed using state-of-the-art computational tools, as described in the Supporting Information.

In Vivo Pharmacological Studies. Animals. Guinea pigs (Dunkin Hartley, male, 400–450 g, Charles River, Milan, Italy) were used. The group size of \(n = 6\) animals was determined by sample size estimation using G*Power (v3.1)' to detect the size effect in a post-hoc test with type 1 and 2 error rates of 5 and 20%, respectively. Allocation concealment to the vehicle(s) or treatment group was performed using a randomization procedure (http://www.randomizer.org/). The assessors were blinded to the identity (allocation to the treatment group) of the animals. Guinea pigs were housed in a temperature- and humidity-controlled vivarium (12 h dark/light cycle, free access to food and water) for at least 1 week before the start of the experiments. Cough experiments were done in a quiet, temperature-controlled (20–22 °C) room between 9 am and 5 pm and were performed by an operator blinded to the treatment. All experiments were carried out according to the European Union (EU) guidelines for animal care procedures and the Italian legislation (DLgs 26/2014) application of the EU Directive 2010/63/EU. All animal studies were approved by the Animal Ethics Committee of the University of Florence and the Italian Ministry of Health (permit #450/2019-PR) and followed the animal research reporting in vivo experiment (ARRIVE) guidelines.

Measurement of Cough in Conscious Guinea Pigs. Cough experiments were performed using a whole-body plethysmography system (Buxco, Wilmington, NC, USA, upgraded version 2018). The apparatus consists of four plethysmographs (four transparent Perspex chambers) ventilated with a constant airflow and each provided by a nebulizing head (Aerogen) and adjustable bias flows were 1750 mL/min during the acclimation phase and 800 mL/min during nebulization. These rates were previously found in the literature to be representative of clinical aerosols. The particle size presents an aerodynamic mass median diameter of 6 μm, and the output of the nebulizing heads can be set in the range between 0 and 0.4 mL per minute. The number of elicited coughs was automatically counted using the instrument. The nebulization rate used in the following experiments was 0.15 mL/min, and the airflows were 1750 mL/min during the acclimation phase and 800 mL/min during nebulization. These rates were previously found in our lab to elicit a significant number of cough events in the citric acid-induced cough model.

On the day of experiments, guinea pigs were individually placed into the chambers and let to acclimate for 10 min. To test the antitussive effect of \([\text{Dmt}^{1,5}]\text{N/OFQ(1-13)-NH}_2\), two different protocols were used. Protocol 1: after acclimation, a mixture of \([\text{Dmt}^{1,5}]\text{N/OFQ(1-13)-NH}_2\) (1 mM) or its vehicle (0.9% NaCl) and the tussive agent, citric acid (0.4 M), was nebulized for 10 min. During the 10 min of nebulization and for 5 min immediately post challenge (recovery period), the number of elicited coughs was automatically recorded using the BUXCO system. Protocol 2: after acclimation, \([\text{Dmt}^{1,5}]\text{N/OFQ(1-13)-NH}_2\) (1 mM) or its vehicle (0.9% NaCl) was nebulized for 10 min. After 20 min of recovery, the tussive agent, citric acid (0.4 M), was delivered by aerosol via a nebulizer for 10 min. During the 10 min of the citric acid challenge and 5 min immediately post challenge (recovery period), the number of elicited coughs was automatically recorded using the BUXCO system.

For the in vivo experiment, the statistical significance of differences between groups was assessed using Student’s t-test.
**REFERENCES**

(1) Meunier, J.-C.; Mollerreau, C.; Toll, L.; Suauveau, C.; Moisand, C.; Alvinerie, P.; Butour, J. L.; Guillemot, J. C.; Ferrara, P.; Monsarrat, B.; Mazarguil, H.; Vassart, G.; Parmentier, M.; Costentin, J. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature 1995*, 377, 532–535.

(2) Reinscheid, R. K.; Nothacker, H. P.; Bousron, A.; Ardati, A.; Henningsen, R. A.; Bunzow, J. R.; Grandy, D. K.; Langen, H.; Monsma, F. J., Jr.; Civelli, O. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science 1995*, 270, 792–794.

(3) Toll, L.; Bruchas, M. R.; Calò, G.; Cox, B. M.; Zaveri, N. T. Nociceptin/Orphin FQ receptor structure, signaling, ligands, functions, and interactions with opioid systems. *Pharmacol. Rev.* 2016, 68, 419–457.

(4) Lambert, D. G. The nociceptin/orphin FQ receptor: a target with broad therapeutic potential. *Nat. Rev. Drug Discovery* 2008, 7, 694–710.

(5) Schröder, W.; Lambert, D. G.; Ko, M. C.; Koch, T. Functional plasticity of the N/OFQ-NOP receptor system determines analgesic properties of NOP receptor agonists. *Br. J. Pharmacol.* 2014, 171, 3777–3800.

(6) Toll, L.; Ozawa, A.; Cippitelli, A. NOP-related mechanisms in pain and analgesia. *Handb. Exp. Pharmacol.* 2019, 254, 165–186.

(7) Kiguchi, N.; Ko, M. C. Effects of NOP-related ligands in nonhuman primates. *Handb. Exp. Pharmacol.* 2019, 254, 323–343.

(8) Lina, K.; Christoph, T.; Tschentscher, T. M.; Koch, T.; Schiene, K.; Gautois, M.; Schroder, W.; Kogel, B. Y.; Beier, H.; Englberger, W.; Schunk, S.; De Vry, J.; Jahnel, U.; Frosch, S. Cebranopadol: a novel potent analgesic nociceptin/orphin FQ peptide and opioid receptor agonist. *J. Pharmacol. Exp. Ther.* 2014, 349, 535–548.

(9) Calò, G.; Lambert, D. G. Nociceptin/orphin FQ receptor ligands and translational challenges: focus on cebranopadol as an innovative analgesic. *Br. J. Anaesth.* 2018, 121, 1105–1114.

(10) Ding, H.; Kiguchi, N.; Yasuda, D.; Daga, P. R.; Polgar, W. E.; Lu, J. J.; Czoty, P. W.; Kishioka, S.; Zaveri, N. T.; Ko, M.-C. A bifunctional nociceptin and mu opioid receptor agonist is analgesic without opioid side effects in nonhuman primates. *Sci. Transl. Med.* 2018, 10, eaar3483.

(11) Kiguchi, N.; Ding, H.; Cami-Kobeci, G.; Sukhtankar, D. D.; Czoty, P. W.; DeLoid, H. B.; Hsu, F.-C.; Toll, L.; Husbands, S. M.; Ko, M.-C. BU10038 as a safe opioid analgesic with fewer side-effects after systemic and intrathecal administration in primates. *Br. J. Anaesth.* 2019, 122, e146–e156.

(12) Chao, P. K.; Chang, H. F.; Chang, W. T.; Yeh, T. K.; Ou, L. C.; Chuang, J. Y.; Tsu-An Hsu; J.; Tao, P. L.; Loh, H. H.; Shih, C.; Ueng, S. H.; Yeh, S. H. BPR1M97, a dual mu opioid receptor/nociceptin/orphin FQ peptide receptor agonist, produces potent antinociceptive effects with safer properties than morphine. *Neuropharmacology 2020*, 166, 107678.

(13) Kiguchi, N.; Ding, H.; Ko, M.-C. Therapeutic potentials of NOP and NOP receptor coactivation for the treatment of pain and opioid abuse. *J. Neurosci. Res. 2020*, DOI: 10.1002/jnr.24624.

(14) Azzam, A. A. H.; McDonald, J.; Lambet, D. G. Hot topics in opioid pharmacology: mixed and biased opioids. *Br. J. Anaesth.* 2019, 122, e136–e145.

(15) Anand, J. P.; Kochan, K. E.; Fastace, A. F.; Montgomery, D.; Griggs, N. W.; Traynor, J. R.; Mosberg, H. I.; Jutkiewicz, E. M. In vivo effects of μ-opioid receptor agonist/δ-opioid receptor antagonist peptideidemetics following acute and repeated administration. *Br. J. Pharmacol.* 2018, 175, 2013–2027.

(16) Aceto, M. D.; Harris, L. S.; Negus, S. S.; Banks, M. L.; Hughes, L. D.; Akgun, E.; Portoghesi, P. S. MDAN-21: a bivalent opioid ligand containing mu-agonist and delta-agonist pharmacophores and its effects in rhesus monkeys. *Int. J. Med. Chem.* 2012, 2012, 327257.

(17) Greedy, B. M.; Bradbury, F.; Thomas, M. P.; Grivas, K.; Cami-Kobeci, G.; Archambeau, A.; Bosse, K.; Clark, M. J.; Aceto, M.; Lewis, J. W.; Traynor, J. R.; Husbands, S. M. Orvinols with mixed kappa/mu opioid receptor agonist activity. *J. Med. Chem.* 2013, 56, 3207–3216.

**ACKNOWLEDGMENTS**

FAR (Fondo di Ateneo per la Ricerca Scientifica) grants from the University of Ferrara support D.P., G.C., and R.G. C.R. is supported by an FIR (Fondo per l’Incentivazione alla Ricerca) grant from the University of Ferrara. S.P., F.F., D.P., G.C., and R.G. are supported by the grant PRIN 2015 (Prot. 2015WX8YSB 002) from the Italian Ministry of Research and Education.

**ABBREVIATIONS**

CHO cells, chinese hamster ovary cells; CL$_{95\%}$, 95% confidence limits; CR, concentration ratio; crc, concentration—response curve; DAMGO, [D-Ala$_2$, N-MePhe$_4$, Gly-$\omega$]-enkephalin; DIC, N,N’-disopropylcarbodiimide; Dipea, N,N-disopropylethylamine; DME/F-12, Dulbecco’s modified Eagle’s medium/nutrient mixture F-12; DMR, dynamic mass redistribution; DPPDE, [D-Pen$_2$, D-Pen$_2$]-enkephalin; EtOAc, ethyl acetate; FBS, fetal bovine serum; FMocCl, FMoc chloride; HATU, hexafluorophosphate azabenzotriazole tetramethyl uronium; HBSS, Hanks’ balanced salt solution; LINCS, LINear Constraint Solver; MBHA resin, 4-methylbenzhydrolamine resin; N/OFQ, nociceptin/orphin FQ; NPT, isomolar—isoaric ensemble; NVT, canonical ensemble conditions; PWT, peptide welding technology; SEM, standard error of the mean; SPPS, solid-phase peptide synthesis.
(18) Daniels, D. J.; Kulkarni, A.; Xie, Z.; Bhushan, R. G.; Portoghese, P. S. A bivalent ligand (KDN-18) containing δ-agonist and κ-agonist pharmacophores bridges δ2 and κ1 opioid receptor phenotypes. J. Med. Chem. 2005, 48, 1713–1716.

(19) Molinari, S.; Camarda, V.; Rizzi, A.; Marzola, G.; Salvadori, S.; Marzola, E.; Molinari, P.; McDonald, J.; Ko, M.; Lambert, D.; Calò, G.; Guerini, R. [Dmt'N/OFQ(1-13)-NH2]: a potent nociceptin/orphanin FQ and opioid receptor universal agonist. Br. J. Pharmacol. 2013, 168, 151–162.

(20) Guerini, R.; Marzola, E.; Trapella, C.; Pelà, M.; Molinari, S.; Cerlesi, M. C.; Malafanci, D.; Rizzi, A.; Salvadori, S.; Calò, G. A novel and facile synthesis of tetra branched derivatives of nociceptin/orphanin FQ. Bioorg. Med. Chem. 2014, 22, 3703–3712.

(21) Cerlesi, M. C.; Ding, H.; Bird, M. F.; Kiguchi, N.; Ferrari, F.; Malafanci, D.; Rizzi, A.; Ruzza, C.; Lamberti, D. G.; Ko, M. C.; Calò, G.; Guerini, R. Pharmacological studies on the NOP and opioid receptor agonist PWT2-[Dmt'N/OFQ(1-13)]. Eur. J. Pharmacol. 2017, 794, 115–126.

(22) Pacifico, S.; Albanese, V.; Illuminati, D.; Fantinati, A.; Marzola, E.; Ferrari, F.; Neto, J. A.; Sturaro, C.; Ruzza, C.; Calò, G. A novel nociceptin receptor induced by cebranopadol: hints from microsecond molecular dynamics. J. Chem. Inf. Model. 2019, 59, 818–831.

(15) McLeod, R. L.; Bolser, D. C.; Jia, Y.; Parra, L. E.; Mutter, J. C.; Wang, X.; Tulshian, D. B.; Egan, R. W.; Hey, J. A. Antittusive effect of nociceptin/orphanin FQ in experimental cough models. Pulm. Pharmacol. Ther. 2002, 15, 213–216.

(16) McLeod, R. L.; Jia, Y.; Fernandez, X.; Parra, L. E.; Wang, X.; Tulshian, D. B.; Kiseløf, E. J.; Tan, Z.; Fawzi, A. B.; Smith-Torhan, A.; Zhang, H.; Hey, J. A. Antittusive profile of the NOP agonist Ro-64-6198 in the guinea pig. Pharmacology 2004, 71, 143–149.

(17) McLeod, R. L.; Tulshian, D. B.; Ho, G. D.; Fernandez, X.; Bolser, D. C.; Parra, L. E.; Zimmer, J. C.; Erickson, C. H.; Fawzi, A. B.; Jayappa, H.; Lehr, C.; Erskine, J.; Smith-Torhan, A.; Zhang, H.; Hey, J. A. Effect of a novel NOP receptor agonist (SCH 225288) on guinea pig irritant-evoked, feline mechanically induced and canine infectious tracheobronchitis cough. Pharmacology 2009, 84, 153–161.

(18) McLeod, R. L.; Parra, L. E.; Mutter, J. C.; Erickson, C. H.; Carey, G. J.; Tulshian, D. B.; Fawzi, A. B.; Smith-Torhan, A.; Egan, R. W.; Cuss, F. M.; Hey, J. A. Nociceptin inhibits cough in the guinea-pig by activation of ORL1 receptors. Br. J. Pharmacol. 2001, 132, 1175–1178.

(19) Lee, M.-G.; Undem, B. J.; Brown, C.; Carr, M. J. Effect of nociceptin in acid-evoked cough and airway sensory nerve activation in guinea pigs. Am. J. Respir. Crit. Care Med. 2006, 173, 271–275.

(20) Gibson, P. G.; Ryan, N. M. Cough pharmacotherapy: current and future status. Expert Opin. Pharmacother. 2011, 12, 1745–1755.

(21) Wang, X.; Niu, S.; Xu, L.; Zhang, C.; Meng, L.; Zhang, X.; Ma, D. Pd-catalyzed dimethylation of tyrosine-derived picolinamide for synthesis of (S)-N-Boc-2,6-dimethyltyrosine and its analogues. Org. Lett. 2017, 19, 246–249.

(22) Bryant, S. D.; Jinsmaa, Y.; Salvadori, S.; Okada, Y.; Lazarus, L. H. Dmt and opioid peptides: a potent alliance. Biopolymers 2003, 71, 86–102.

(23) Fenalti, G.; Giguere, P. M.; Katrich, V.; Huang, X.-P.; Thompson, A. A.; Cherezov, V.; Roth, B. L.; Stevens, R. C. Molecular control of δ-opioid receptor signalling. Nature 2014, 506, 191–196.

(24) Shimohigashi, Y.; Hatano, R.; Fujita, T.; Nakashima, R.; Nose, T.; Sujaku, T.; Saigo, A.; Shinjo, K.; Nagahisa, A. Sensitivity of opioid receptor-like receptor ORL1 for chemical modification on nociceptin, a naturally occurring nociceptive peptide. J. Biol. Chem. 1996, 271, 23642–23645.

(25) Morley, J. S. Structure-activity relationships of enkephalin-like peptides. Annu. Rev. Pharmacol. Toxicol. 1980, 20, 81–110.

(26) Moshal, K.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(27) Garcia, J. U.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(28) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(29) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(30) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(31) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(32) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(33) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(34) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(35) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(36) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(37) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.

(38) Cadeddu, G.; Aebischer, P.; Sanghera, S.; Pankratov, S. P.; Padmanabhan, R.; Mauerer, Y. L. Exploration of the third intracellular loop of the NOP receptor for drug delivery. Neuropharmacology 2004, 46, 858–869.
(51) Manglik, A.; Kruse, A. C.; Kobilka, T. S.; Thian, F. S.; Mathiesen, J. M.; Sunahara, R. K.; Pardo, L.; Weis, W. I.; Kobilka, B. K.; Granier, S. Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. *Nature* 2012, 485, 321−326.

(52) Granier, S.; Manglik, A.; Kruse, A. C.; Kobilka, T. S.; Thian, F. S.; Weis, W. I.; Kobilka, B. K. Structure of the δ-opioid receptor bound to naltrindole. *Nature* 2012, 485, 400−404.

(53) Thompson, A. A.; Liu, W.; Chun, E.; Katritch, V.; Wu, H.; Vardy, E.; Huang, X.-P.; Trapella, C.; Guerrini, R.; Calò, G.; Roth, B. L.; Cherezov, V.; Stevens, R. C. Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature* 2012, 485, 395−399.

(54) Stefanucci, A.; Dimmito, M. P.; Macedonio, G.; Ciarlo, L.; Pieretti, S.; Novellino, E.; Lei, W.; Barlow, D.; Houseknecht, K. L.; Streicher, J. M.; Mollica, A. Potent, efficacious, and stable cyclic opioid peptides with long lasting antinociceptive effect after peripheral administration. *J. Med. Chem.* 2020, 63, 2673−2687.

(55) Adcock, J. J. Peripheral opioid receptors and the cough reflex. *Respir. Med.* 1991, 85, 43−46.

(56) Tzschentke, T. M.; Linz, K.; Koch, T.; Christoph, T. Cebranopadol: A novel first-in-class potent analgesic acting via NOP and opioid receptors. *Handb. Exp. Pharmacol.* 2019, 254, 367−398.

(57) Volkow, N. D.; Blanco, C. The changing opioid crisis: development, challenges and opportunities. *Mol. Psychiatry* 2021, 26, 218−233.

(58) Benoiton, N. L. *Chemistry of Peptide Synthesis*; Taylor & Francis: London, 2006; pp 125−155.

(59) Neubig, R. R.; Spedding, M.; Kenakin, T.; Christopoulos, A. International union of pharmacology committee on receptor nomenclature and drug classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol. Rev.* 2003, 55, 597−606.

(60) Abraham, M. J.; Murtop, T.; Schulz, R.; Páll, S.; Smith, J. C.; Hess, B.; Lindahl, E. GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* 2015, 1−2, 19−25.

(61) Hornak, V.; Abel, R.; Okur, A.; Strockbine, B.; Roitberg, A.; Simmerling, C. Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins* 2006, 65, 712−725.

(62) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera: A visualization system for exploratory research and analysis. *J. Comput. Chem.* 2004, 25, 1605−1612.

(63) Faul, F.; Erdfelder, E.; Lang, A.-G.; Buchner, A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* 2007, 39, 175−191.

(64) Belvisi, M. G.; Patel, H. J.; Freund-Michel, V.; Hele, D. J.; Crispino, N.; Birrell, M. A. Inhibitory activity of the novel CB2 receptor agonist, GW833972A, on guinea-pig and human sensory nerve function in the airways. *Br. J. Pharmacol.* 2008, 155, 547−557.