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Original article

A new synthetic approach of *N*-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl)benzamide (JTC-801) and its analogues and their pharmacological evaluation as nociceptin receptor (NOP) antagonists

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Abstract

A series of 4-amino-2-methylquinoline and 4-aminoquinazoline derivatives, including the reference NOP antagonist JTC-801, were synthesized by an alternative pathway and their in vitro pharmacological properties were investigated. 3-Substitution of the quinoline ring resulted very critical for affinity. So 3-methyl derivative **4j** showed a similar potency compared with the reference **4h** while bulky lipophilic or electron withdrawing groups in the same position strongly decreased affinity. Structural and conformational requirements for affinity were outlined by NOE NMR and computational methods and suggestions for a pharmacophore model design were provided.

Keywords: Benzamidoquinolines; Benzamidoquinazolines; NOP receptor antagonists; Opioids

1. Introduction

The NOP (ORL1, OP_4) receptor is a G-protein-coupled receptor closely related to the OP_1, OP_2 and OP_3 opioid receptors but having poor affinity with the opioid peptides [1]. Since its discovery and the subsequent identification of its endogenous agonist, the heptadecapeptide nociceptin (NC) or orphanin FQ [2,3], many efforts have been made in order to elucidate the role of the NOP/NC system. The NOP receptor is widely distributed in both central and peripheral nervous system [4] and it is involved in many physiological effects [5] including nociception [6], attenuation of anxiety [7], inhibition of learning and memory [8], stimulation of food intake [9], diuresis [10], inhibition of reward pathways in drug addiction [11,12], inhibition of tachykinergic bronchoconstriction [13], hypotension and bradycardia [14], inhibition of colonic motility [15].

The hyperalgesic effect of NC [2,6] has led to pharmacological experiments about the use of NOP antagonists in animal models of pain [16–18], which show that these compounds could find therapeutical applications as analgesics devoid of the side effects of opioids [19]. Such compounds have been also proposed as memory enhancers [20].

At present, only a few classes of non-peptidic NOP antagonists with fairly good selectivity towards opioid receptors have been developed [5], namely benzimidazolylpiperidines (which include J-113397 [21,22], the first non-peptide pure NOP antagonist, and its analogues [23]), spiropiperidines [24,25] and 4-aminoquinolines such as JTC-801 [26], which was chosen as the candidate for clinical trials as analgesic, because of its good oral bioavailability [16].

In this paper, we report a new synthetical pathway starting from 2-amino-5-nitrobenzonitrile, which led to an easier and convenient preparation of JTC-801, also allowing some of its analogues to be obtained (Fig. 1).

The structural and pharmacological study of these compounds aimed at exploring the influence on NOP receptor binding of the following topics:

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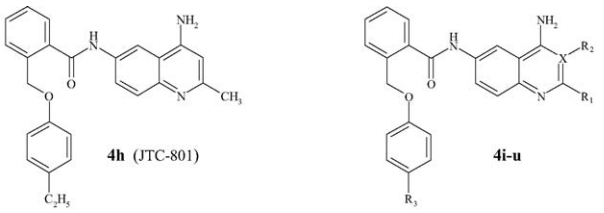


Fig. 1. Structures of JTC-801 and of the synthesized compounds.

- introduction in the 3-position of the quinoline of substituents exhibiting different electronic and steric effects, such as the hydrophobic methyl and phenyl groups and the hydrogen bond acceptor ethoxycarbonyl;
- 3-aza substitution on the quinoline moiety;
- enhancement of the molecular lipophilicity and size on respect to the reference compound, which was achieved by substituting 1,2,3,4-tetrahydroacridine for quinoline;
- presence of the electronegative fluorine atom in 4-position of the phenolic ring.

2. Chemistry

The synthetical pathways to the target compounds are outlined in Fig. 2.

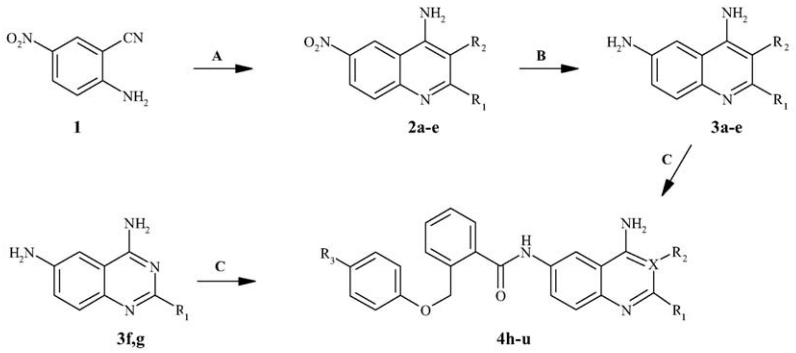
The reaction of 2-amino-5-nitrobenzonitrile **1** [27] with acetone, butanone, phenylacetone, ethyl acetoacetate or cyclohexanone and stannic chloride in boiling toluene gave the 4-amino-6-nitroquinolines **2a–e** in good yields. For the asymmetric ketones, only the products derived from cyclization between carbonyl and its α-methylene group were obtained. The reduction of the nitro group with nickel–aluminium alloy in hydroalcoholic potassium hydroxide at 60 °C (**2a–c**), or in refluxing 50% aqueous acetic acid (**2e**), gave the diamines **3a–c**,**e**. This method did not give good results in the case of the 3-phenyl derivative **2d** which was reduced to the diamino compound **3d** with sodium borohydride in methanol in the presence of a catalytic amount of 10% palladium on activated charcoal. Acylation of compounds **3a–e**,**f** [28] and **3g** [29] with 2-[(4-ethylphenoxy)methyl]benzoyl chloride or with 2-[(4-fluorophenoxy)methyl]benzoyl chloride (obtained from the corresponding acids and thionyl chloride) in pyridine at room temperature, gave the final compounds **4h–u** in good yields.

All the compounds were characterized by ^{1}H NMR and MS spectroscopies.

Calorimetric studies on the final compounds were carried out to ascertain the presence of water, the presence of different crystalline modifications (polymorphs and pseudopolymorphs), and to determine the melting temperature (see Section 6).

3. Pharmacology

The binding affinities for the NOP receptor of all synthesized compounds were first measured as competition for the binding of radiotracer concentrations of [^{125}I]-nociceptin. Those which displayed the best binding affinity (IC_{50} lower than 0.5 μM) were also tested on OP_1, OP_2 and OP_3 receptors



	X	R_1	R_2
a	C	CH_3	H
b	C	CH_3	CH_3
c	C	$(CH_2)_4$	
d	C	CH_3	C_6H_5
e	C	CH_3	$CO_2C_2H_5$
f	N	H	□
g	N	CH_3	□

	X	R_1	R_2	R_3
h	C	CH_3	H	C_2H_5
i	C	CH_3	H	F
j	C	CH_3	CH_3	C_2H_5
k	C	CH_3	CH_3	F
l	C	$(CH_2)_4$		C_2H_5
m	C	$(CH_2)_4$		F
n	C	CH_3	C_6H_5	C_2H_5

	X	R_1	R_2	R_3
o	C	CH_3	C_6H_5	F
p	C	CH_3	$CO_2C_2H_5$	C_2H_5
q	C	CH_3	$CO_2C_2H_5$	F
r	N	H	□	C_2H_5
s	N	H	□	F
t	N	CH_3	□	C_2H_5
u	N	CH_3	□	F

Reagents: A: $CH_3COCH_2R_2$ (for **2a**,**b**,**d**,**e**) or cyclohexanone (for **2c**), $SnCl_4$ / toluene, reflux; **B:** Nickel/Aluminium alloy, methanol / aq.KOH 1N, 60°C (for **2a-c**), Nickel/Aluminium alloy, refluxing 50% aq. acetic acid (for **2e**) or $NaBH_4$, Pd/C, methanol, 25°C (for **2d**); **C:** 2-[(4-ethylphenoxy)methyl]benzoyl chloride (for **4h**,**j**,**l**,**n**,**p**,**r**,**t**) or 2-[(4-fluorophenoxy)methyl]benzoyl chloride (for **4i**,**k**,**m**,**o**,**q**,**s**,**u**), pyridine, 25°C.

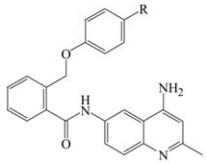
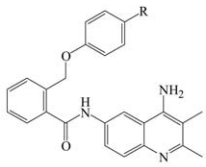
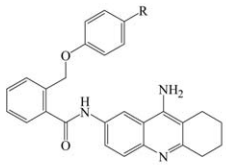
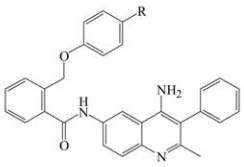
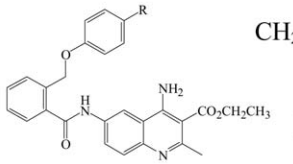
Fig. 2. Synthetic route to compounds **4h–4u**.

to estimate their selectivity towards different opioid receptor subtypes. We used as radioligands [^{3}H]-naltrindole for OP_1, and [^{3}H]-diprenorphine for OP_2 and OP_3 in such experiments. The results are shown in Tables 1, 2.

The biological activities of compounds **4h–m** were evaluated as their ability to enhance the binding of [^{35}S]GTPγS in the presence of GDP, using membranes isolated from cells transfected with NOP receptors. This assay is a direct measure of receptor-mediated G protein activation (Fig. 3). Since the parent compound (JTC-801) is an antagonist at the nociceptin receptor, it does not alter GTP/GDP exchange on the G protein α subunit. To verify whether our synthetic analogues could have acquired any level of agonistic efficacy, we used membranes prepared from a cell line expressing a tandem fusion protein between the NOP receptor and the α subunit of G_o. As previously reported [30], the forced 1:1 stoichiometry of expression of receptor and Gα subunit in such system greatly enhances the GTPγS response and allows to detect even small levels of ligand efficacy. Moreover, for the compounds with the greatest affinity (**4h** (JTC-801) and **4j**), we scanned the [^{35}S]GTPγS binding response at a wide range of GDP concentrations, because under such experimental conditions, the partial agonist properties of certain nociceptin peptide antagonists can be revealed (Fig. 4).

Compounds **4h** (JTC-801) and **4j** were also tested for their potency to inhibit nociceptin-stimulated GTPγS binding. The dose–response curves are reported in Fig. 5.

Table 1
In vitro affinity of compounds **4h–n**

	Compound	R	IC_{50} [a] (10^{-8}M)
4h		CH_2CH_3	**9.5**
4i		F	**20.9**
4j		CH_2CH_3	**8.2**
4k		F	**12.3**
4l		CH_2CH_3	**25.1**
4m		F	**21.8**
4n		CH_2CH_3	**96.7**
4o		F	**137.5**
4p		CH_2CH_3	**280.0**
4q		F	**309.5**

[a] IC_{50} from binding. ^{125}I-nociceptin used as radioligand
Data are a media of six (**4i-m**) or four determinations (**4h,n-q**).
Variability coefficent = 6% for **4h-m**
Variability coefficent = 10% for **4n-q**

Table 2
Selectivity of compounds **4h–m**

Compound	OP_1 [a]	OP_2 [b]	OP_3 [c]
4h (JTC-801)	>100 [d]	>100 [d]	3.4 [d]
4i	>100	20	5.0
4j	>100	16.4	4.0
4k	>100	12.5	5.3
4l	>100	4.8	2.8
4m	>100	5.1	2.6

Radioligands: [^{3}H]-naltrindole for OP_1, [^{3}H]-diprenorphine for OP_2 and OP_3. Data are a media of two determinations.
[a] Selectivity expressed as OP_1 IC_{50}/NOP IC_{50} ratio.
[b] Selectivity expressed as OP_2 IC_{50}/NOP IC_{50} ratio.
[c] Selectivity expressed as OP_3 IC_{50}/NOP IC_{50} ratio.
[d] Ref. [16].

4. Results and discussion

4.1. Pharmacological data

The 3-methyl derivative **4j** ($IC_{50} = 8.2 \times 10^{-8}$ M) was the only compound that showed comparable affinity with that of **4h** (JTC-801, $IC_{50} = 9.5 \times 10^{-8}$ M). Slightly lower affinities were displayed by their fluoro-analogues **4i** ($IC_{50} = 20.9 \times 10^{-8}$ M) and **4k** ($IC_{50} = 12.3 \times 10^{-8}$ M). The two tetrahydroacridine derivatives **4l**,**m** exhibited less than a half of

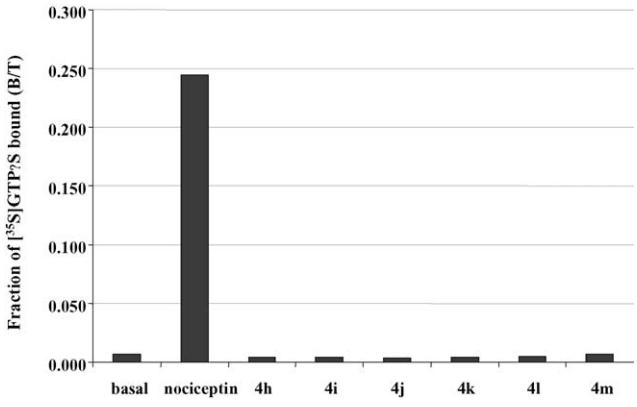


Fig. 3. Effect of compounds **4h–4m** on G protein α-subunit activation. The binding of [^{35}S]GTPγS was measured in the presence of 3 μM GDP as described in Section 6 using membranes prepared from cells transfected with NOP receptors. All compounds were present at a concentration of 10 μM. Data are expressed as bound/total ratio of the radioligand (1 nM) and are averages of triplicate determinations.

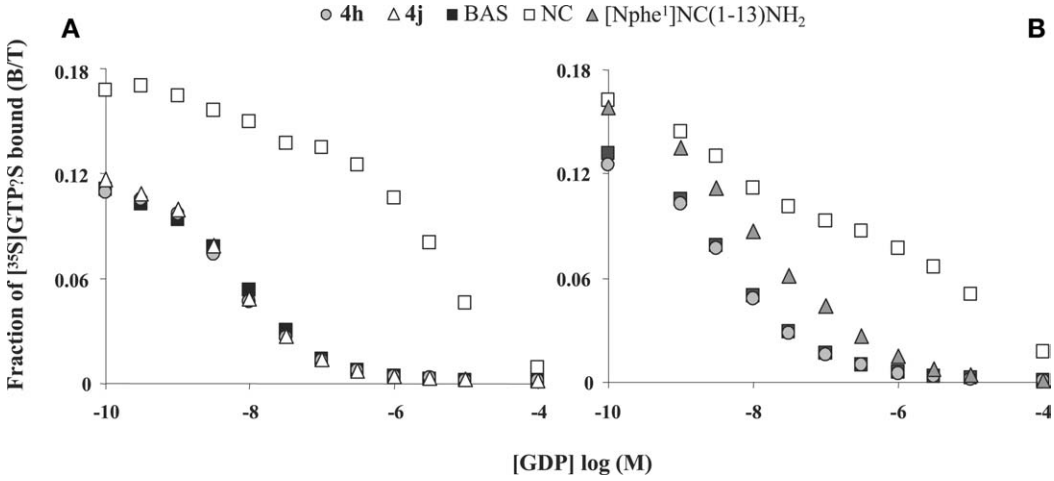


Fig. 4. Effects of compounds **4j** and **4h** on GPTγS binding in comparison with peptide ligands (nociceptin NC and [Nphe^1]NC(1-13)NH_2). The binding of [^{35}S]GTPγS was measured at increasing GDP concentrations as indicated on *x*-axis, in the absence (BAS) or in the presence of the ligands. Note that, unlike the peptide antagonist [Nphe^1]NC(1-13)NH_2, compounds **4j** and **4h** exhibit lack of stimulation at all concentrations of GDP.

JTC-801 affinity. A dramatic loss of receptor binding activity was displayed by the 3-phenyl derivatives **4n**,**o** and the 3-carbethoxy compounds **4p**,**q**, while the quinazolines **4r**–**u** had no detectable binding affinity.

These results indicate that the substitution in 3-position of the quinoline plays an important role in the structural requirements for receptor affinity. This role may be ascribed to the substituent capability to affect the functionality of the vicinal 4-amino group. As previously reported [26], not only an unsubstituted NH_2 is a very strict requirement for affinity but also the avoidance of any steric and electronic effects that may lower NH_2 complete accessibility. Therefore, compounds **4p** and **4q**, where the carbethoxy substituent can engage the 4-amino group by an intramolecular hydrogen bond and compounds **4r**–**u** bearing the strongly electron withdrawing aza group, all show a diminished activity. The 3-methyl substitution enhances NH_2 basicity, which apparently maintains a high binding affinity. On the other hand, the fused cyclohexane ring in **4l**,**m** and the phenyl group in **4n**,**o** may exert variable extents of steric hindrance to the amino group, which may explain the moderate or extreme losses of binding affinity observed, respectively, for such two sets of compounds.

In general, the fluoro derivatives resulted slightly less active than the corresponding ethyl compounds suggesting that the *para*-phenolic fluorine is tolerated, but probably not involved in direct interactions with the receptor subsites.

The selectivity study of compounds **4h**–**m** towards OP_1, OP_2 and OP_3 receptors, whose data have been reported in Table 2, led to the following considerations. The modifications introduced in the structure of the lead compound **4h** (JTC-801), did not influence its high selectivity towards OP_1 receptor (OP_1 IC_{50}/NOP IC_{50} > 100-fold for all the examined compounds). On the other hand, the poor selectivity of **4h** (JTC-801) towards OP_3 underwent a slight enhancement by introduction of a fluorine atom in the phenolic portion (OP_3 IC_{50}/NOP IC_{50} = 3.4 for **4h** and OP_3 IC_{50}/NOP IC_{50} = 5 for **4i**). This trend was observed also for the compounds **4j** and its fluorinated analogue **4k** (OP_3 IC_{50}/NOP IC_{50} = 4.0 for **4j** and OP_3 IC_{50}/NOP IC_{50} = 5.3 for **4k**).

Finally, compounds **4i**–**m** showed a consistent decrease in specificity towards OP_2 receptor compared with **4h**: both molecular size enhancement and the presence of the fluorine atom in the phenolic portion seem to contribute to the observed loss of selectivity.

Compounds **4h**–**m** showed no stimulation of GTPγS binding in the presence of GDP, indicating that none of the modifications made introduced any agonistic property in the JTC-801 pharmacophore, despite the wide differences in binding affinities (Fig. 3). A more in-depth study of the compound with the highest binding affinity (**4j**) demonstrated that the lack of agonist activity was maintained at all GDP concentrations. In contrast, the peptide antagonist [Nphe^1]NC(1-13)NH_2 exhibited detectable level of agonistic activity at low GDP concentrations (Fig. 4).

To analyze the mechanism of antagonism exerted by this class of ligands, we performed a classical Schild analysis [31] of compounds **4h** and **4j**. We measured the effects of increasing concentrations of the two antagonists on the concentration–response curves of nociceptin. Both analogues induced a right-ward shift of the nociceptin-stimulated binding of [^{35}S]GTPγS without changing the maximum agonist stimulation (Fig. 5). The K_i of the compounds **4h** and **4j**, determined by the Schild plots, were respectively 112 and 116 nM, in good agreement with binding results. However, both slopes of the Schild plots were significantly greater than unity, indicating that the two compounds do not act as true competitive antagonist. Although beyond the scope of this study, such results suggest that 4-aminoquinolines and related antagonists of the OP_4 receptor might be allosteric ligands, which inhibit nociceptin binding through a conformational effect mediated by an interaction at a site other than

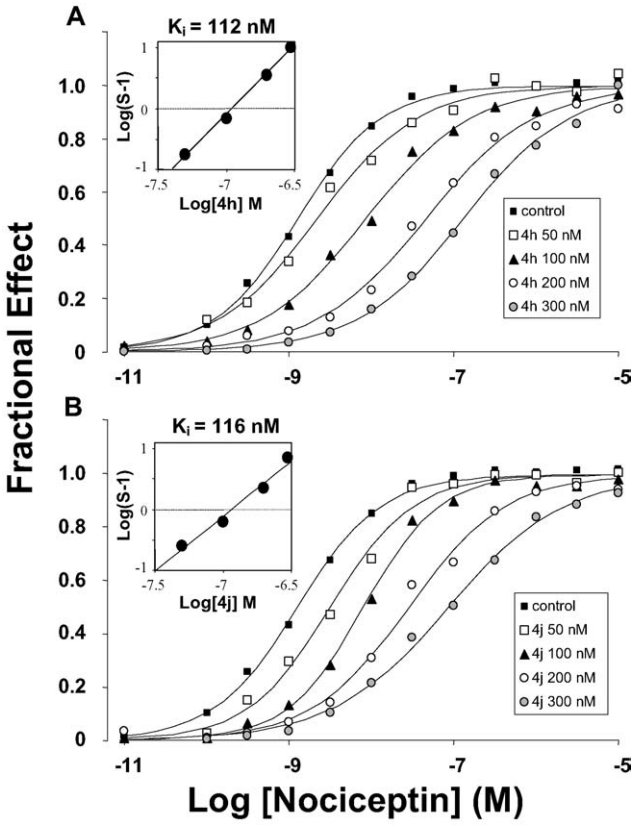


Fig. 5. Antagonism of nociceptin-mediated activation of $G\alpha_o$ induced by compounds **4j** and **4h**. Concentration–response curves for nociceptin-mediated stimulation of [^{35}S]GTPγS in the presence of 3 μM GDP were performed in membranes expressing the ORL_1R-$G\alpha_o$ fusion protein as described in Section 6. Curves were generated in the absence and presence of increasing concentrations of either **4h** (A) or **4j** (B), as indicated. Data points, after subtraction of basal activity (specific binding measured in the absence of nociceptin) were plotted as fractional effect by dividing all values for the net cpm stimulated by 10 mM nociceptin in the control curve of each experiment. Data were analyzed using ALLFIT (see Section 6), to compute the EC_{50} in the absence and presence of the various concentrations of antagonist. *Insets*: Schild analysis of the data. The log of the shift induced by each agonist (i.e. ratio of EC_{50} in the presence versus absence of antagonist minus one, S-1) is plotted as a function of the log of the antagonist concentration. Linear regression analysis of the data gave the following results ± standard error): A: slope 2.28 ± 0.01, *x* value at *y* = 0 (pA_2), 6.95 ± 0.29; B: slope 1.81 ± 0.19 , *x* value at *y* = 0 (pA_2), 6.93 ± 0.71.

the nociceptin binding pocket. Additional work will be necessary to investigate this interesting possibility.

4.2. Conformational studies

Compounds **4h–q** were studied by theoretical and experimental methods aiming at correlating their conformational features to biological activity.

4.2.1. NOE experiments

NOE difference experiments were performed to predict the dominant conformations in solution of **4h** (JTC-801) and its analogues. The spectra were run in DMSO-d_6 solution at 25 °C at different decoupler powers and irradiation times. All the spin systems resolved within 0.2 ppm were irradiated.

The most remarkable effect showed upon amidic NH irradiation: the overlapping quinoline H-7, H-8 signals underwent approximately a 20% enhancement, while no NOE was displayed by the equally neighbouring H-5 proton. It might be supposed that there are some predominant conformations with the NH oriented "anti" to the 4-amino substituent. As NH and NH_2 groups are both essential for activity, the NOE information may result useful for refining the pharmacophore model.

Irradiation of H-7 and H-8 produced an expected minor enhancement of NH (8%) as NH relaxation is dominated by the quadrupolar effect of nitrogen.

Irradiation of 2″ and 6″ phenol protons caused enhancement of OCH_2 methylene and vice versa according to the relative rather fixed distances of these groups.

However, the more intriguing and less predictable NOE effects were displayed only by those molecules exhibiting high affinity for the receptor.

In details, compounds **4h–k** and to a lesser extent compounds **4l**,**m** when submitted to NH irradiation showed NOE enhancement of the OCH_2 methylene (5–9%) and/or of the phenolic moiety (4–7%), and vice versa. Moreover, compounds **4j** and **4k** showed a NOE (3%) on the H-7/H-8 pattern when phenolic H-2″/H-6″ were irradiated.

In compounds **4n–q**, no Overhauser effect between structurally far hydrogens was observed.

These results suggest that folded conformations with the phenolic ring approaching the amidic portion are possible only in some of the examined compounds and are somehow related to activity.

4.2.2. Computational studies

The geometry of compounds **4h–q** was determined in order to find out some conformational differences and/or similarities between the most and the less active among them.

The structures were predicted using the semi-empirical molecular orbital approach. Both the PM3 and AM1 Hamiltonians were used and similar results were obtained. The computations were performed on a Silicon Graphics "Octane" Workstation with the MOPAC 2000 program package [32].

The presence of a peptide linkage was taken into account using the MMOK keyword. It allowed for a molecular mechanic correction to increase the barrier to rotation for the peptide linkage to about 15 kcal/mol.

The geometries were optimized simulating a water environment: solvation was taken into account using a modified version of the Miertus, Scrocco and Tomasi self-consistent reaction field method (MST/SCRF) [33–35].

The structures' prediction was improved using evidences by ^{1}H NMR NOE experiments.

Proximity between some hydrogen atoms suggested by NOE data evaluation, helped hypothesizing the preferred conformation for each molecule.

A common structural element was displayed by **4h–m** substances. The plane of the phenolic ring and the one of the

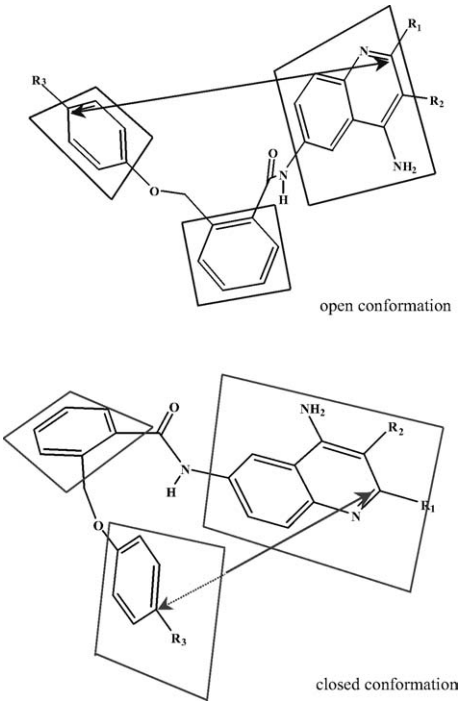


Fig. 6. "Closed" and "open" conformations. In the figure, the planes formed by the two aromatic systems are depicted.

quinoline moiety showed to be rather near and tended to face each other. In compounds **4n**–**q**, those planes are instead not parallel and quite far. These conformations named "closed" and "open" are showed in Fig. 6.

To describe this situation, the C2–C4″ distance for each optimized structure was calculated and reported versus the corresponding IC_{50}.

Quite a good correlation (Fig. 7) between this distance and the activity of the molecules was found. Closed structures **4h**–**m** with a shorter C2–C4″ distance (8.5 Å), showed the best receptor affinity (8.2×10^{-8} M ≤ IC_{50} ≤ 25×10^{-8} M).

A 10 Å distance was related to a five times decrease of potency (**4n**,**o**), and longer distances (12 Å) corresponded to the lowest activities (**4p**,**q**).

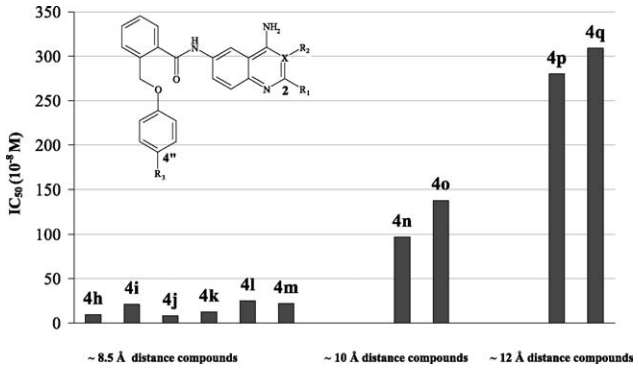


Fig. 7. Correlation of the C2–C4″ distance to affinity for NOP receptor for compounds **4h**–**4q**. Compounds are grouped according to the distances showed by their optimized structures. Compounds **4h**–**m** displayed $8.2 \times 10^{-8} < IC_{50} < 25 \times 10^{-8}$ M; **4n**,**o** displayed $96 \times 10^{-8} < IC_{50} < 137 \times 10^{-8}$ M and **4p**,**q** displayed $280 \times 10^{-8} < IC_{50} < 310 \times 10^{-8}$ M.

From these evidences, it can be envisaged that the molecules have to assume a "closed" structure to interact with the receptor. If for a given compound, such a "closed" structure is less probable (i.e. a too high energy is needed to reach the closed conformation), the compound will not be able to interact with the receptor in the proper way. The higher the C2–C4″ distance, the higher the energy needed to reach the "closed" conformation, the less the receptor inhibiting capability.

5. Conclusion

A number of 4-aminoquinolines derivatives besides their reference compound JTC-801 were synthesized through a new convenient pathway and their activity as NOP ligands was investigated.

The take home message emerging from this study is that the activity of these nociceptin antagonists depends strictly on molecular conformational features. NMR NOE measurements together with theoretical calculations provide useful suggestions to design the pharmacophore model.

6. Experimental

6.1. Chemistry

Melting points were determined on a Köfler hot stage apparatus, unless otherwise stated, and are uncorrected.

Column chromatographic separations were accomplished on Merck silica gel (70–230 mesh) or on Merck aluminium oxide 90.

The purity of each compound was checked on silica gel C. Erba 60 F_{254} or Merck aluminium oxide 60 F_{254} (type E) plates and spots were located by UV light. Sodium sulfate was used to dry organic solutions.

Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values.

Compounds **1** [27], **3f** [28], **3g** [29] and 2-[(4-ethylphenoxy)methyl]benzoic acid [26] were prepared according to literature methods.

The ^{1}H NMR spectra and NOE experiments were performed on a Varian Gemini 200 MHz instrument; all values are reported in ppm (δ) and standard abbreviations were used (a = apparent; b = broad; d = doublet; dd = doublet of doublets; m = multiplet; q = quadruplet; s = singlet; t = triplet; u = unresolved).

Electron Ionization Mass Spectra were recorded on a HP 59980 B spectrometer operating at 70 eV.

Calorimetric studies were accomplished using a Perkin Elmer Differential Scanning Calorimeter (model DSC7) and a Perkin Elmer Thermo Gravimetric Analyzer (model Pyris 1 TGA). The scanned temperature range was 20–350 °C with a scan speed of 10°C/min. Aluminium crucibles with a cover hole were used. Hot-stage FT-IR microscopy (a Perkin Elmer

i-series microscope linked to a Perkin Elmer System 2000 IR Spectrometer) was used to control variations in the IR spectrum during the temperature scan.

*6.1.1. General procedure for the preparation of 4-amino-6-nitroquinolines (**2a**,**b**,**d**,**e**) and 9-amino-7-nitro-1,2,3,4-tetrahydroacridine (**2c**)*

To a mixture of **1** (1.6 g, 0.01 mol) and the appropriate ketone in anhydrous toluene (50 ml), stannic chloride (1.2 ml, 0.01 mol) was slowly added, under cooling and stirring; the mixture was refluxed for 4 h with the Dean and Stark apparatus. After cooling, the toluene was removed by decantation, the solid phase was washed with a small amount of diethyl ether and suspended in aqueous sodium hydroxide. The product was filtered off and crystallized (**2a**–**c**) or extracted with ethyl acetate (**2d**,**e**), the organic phase was evaporated and the residue crystallized from an appropriate solvent.

*6.1.1.1. 4-Amino-2-methyl-6-nitroquinoline (**2a**).* Compound **2a** was obtained from acetone (5 ml, 0.07 mol) in 85% yield, m.p. 285–287 °C (ethyl acetate); ^{1}H NMR (DMSO-d_6): δ 9.23 (d, 1H, H-5, J_{meta} = 2.6 Hz), 8.28 (dd, 1H, H-7, J_{ortho} = 9.4 Hz, J_{meta} = 2.6 Hz), 7.79 (d, 1H, H-8, J_{ortho} = 9.4 Hz), 7.35 (s, 2H, NH_2), 6.56 (s, 1H, H-3), 2.45 (s, 3H, CH_3); MS: *m*/*z* 203 [M^+], 187, 173, 157, 145, 130. Anal. $C_{10}H_9N_3O_2$ (C, H, N).

*6.1.1.2. 4-Amino-2,3-dimethyl-6-nitroquinoline (**2b**).* Compound **2b** was obtained from butanone (5 ml, 0.055 mol) in 38% yield, m.p. 283–285 °C (ethyl acetate); ^{1}H NMR (DMSO-d_6): δ 9.28 (d, 1H, H-5, J_{meta} = 2.6 Hz), 8.22 (dd, 1H, H-7, J_{ortho} = 9.0 Hz, J_{meta} = 2.6 Hz), 7.78 (d, 1H, H-8, J_{ortho} = 9.0 Hz), 6.95 (s, 2H, NH_2), 2.53 (s, 3H, 2-CH_3, partially under DMSO signal), 2.17 (s, 3H, 3-CH_3); MS: *m*/*z* 217 [M^+] 201, 187, 171, 159, 144, 130. Anal. $C_{11}H_{11}N_3O_2$ (C, H, N).

*6.1.1.3. 9-Amino-7-nitro-1,2,3,4-tetrahydroacridine (**2c**).* Compound **2c** was obtained from cyclohexanone (2.1 ml, 0.02 mol) in 65% yield, m.p. 278–281 °C dec. (water); ^{1}H NMR (DMSO-d_6): δ 9.29 (d, 1H, H-8, J_{meta} = 2.2 Hz), 8.22 (dd, 1H, H-6, J_{ortho} = 9.2 Hz, J_{meta} = 2.2 Hz), 7.74 (d, 1H, H-5, J_{ortho} = 9.2 Hz), 7.20 (bs, 2H, NH_2), 2.85 (bt, 2H, 4-CH_2), 2.51 (m, 2H, 1-CH_2 partially under DMSO signal), 1.81 (m, 4H, 2-CH_2 and 3-CH_2); MS: *m*/*z* 243 [M^+], 228, 213, 198, 171. Anal. $C_{13}H_{13}N_3O_2$ (C, H, N).

*6.1.1.4. 4-Amino-2-methyl-6-nitro-3-phenylquinoline (**2d**).* Compound **2d** was obtained from phenylacetone (5 ml, 0.038 mol) in 51% yield, m.p. 228–230 °C (ethyl acetate); ^{1}H NMR (DMSO-d_6): δ 9.37 (d, 1H, H-5, J_{meta} = 2.2 Hz), 8.30 (dd, 1H, H-7, J_{ortho} = 9.4 Hz, J_{meta} = 2.2 Hz), 7.85 (d, 1H, H-8, J_{ortho} = 9.4 Hz), 7.63–7.40 (m, 3H, H-3, H-4 and H-5 phenyl ring), 7.30 (m, 2H, H-2 and H-6 phenyl ring), 6.49 (s, 2H, NH_2), 2.19 (s, 3H, CH_3); MS: *m*/*z* 279 [M^+] 263, 249, 233, 221, 206. Anal. $C_{16}H_{13}N_3O_2$ (C, H, N).

*6.1.1.5. Ethyl 4-amino-2-methyl-6-nitro-3-quinolinecarboxylate (**2e**).* Compound **2e** was obtained from ethyl acetoacetate (1.3 ml, 0.01 mol) in 28% yield, m.p. 190–191 °C (methanol); ^{1}H NMR (DMSO-d_6): δ 9.40 (d, 1H, H-5, J_{meta} = 2.6 Hz), 8.37 (dd, 1H, H-7, J_{ortho} = 9.4 Hz, J_{meta} = 2.6 Hz), 8.13 (bs, 2H, NH_2), 7.83 (d, 1H, H-8, J_{ortho} = 9.4 Hz), 4.37 (q, 2H, CH_2CH_3), 2.64 (s, 3H, CH_3), 1.35 (t, 3H, CH_2CH_3); MS: *m*/*z* 275 [M^+] 229, 199, 183, 155, 128. Anal. $C_{13}H_{13}N_3O_4$ (C, H, N).

*6.1.2. General procedure for the preparation of 4,6-diamino-2-methylquinoline (**3a**), 4,6-diamino-2,3-dimethylquinoline (**3b**) and 7,9-diamino-1,2,3,4-tetrahydroacridine (**3c**)*

A suspension of **2a**,**b** or **c** (0.01 mol) and nickel–aluminium alloy (10.0 g) in 100 ml of a 1:1 mixture of methanol and 1 M aqueous potassium hydroxide was heated at 60 °C for 2 h. The mixture was cooled and filtered, the residue was washed with methanol and the collected liquid phase was evaporated to remove methanol; the solid obtained was collected, washed with water and diethyl ether, then crystallized from an appropriate solvent.

*6.1.2.1. 4,6-Diamino-2-methylquinoline (**3a**).* Compound **3a** was obtained in 78% yield, m.p. 193–196 °C dec. (ethyl acetate) ^{1}H NMR (DMSO-d_6): δ 7.41 (d, 1H, H-8, J_{ortho} = 8.8 Hz), 6.98 (dd, 1H, H-7, J_{ortho} = 8.8 Hz, J_{meta} = 2.2 Hz), 6.91 (d, 1H, H-5, J_{meta} = 2.2 Hz), 6.30 (s, 1H, H-3), 6.05 (s, 2H, 4-NH_2), 5.06 (s, 2H, 6-NH_2), 2.31 (s, 3H, CH_3); MS: *m*/*z* 173 [M^+]. Anal. $C_{10}H_{11}N_3$ (C, H, N).

*6.1.2.2. 4,6-Diamino-2,3-dimethylquinoline (**3b**).* Compound **3b** was obtained in 77% yield, m.p. 102–104 °C (ethyl acetate); ^{1}H NMR (DMSO-d_6): δ 7.41 (d, 1H, H-8, J_{ortho} = 9.4 Hz), 6.94 (m, 2H, H-5 and H-7), 5.82 (s, 2H, 4-NH_2), 5.07 (bs, 2H, 6-NH_2), 2.39 (s, 3H, 2-CH_3), 2.08 (s, 3H, 3-CH_3); MS: *m*/*z* 187 [M^+]. Anal. $C_{11}H_{13}N_3$ (C, H, N).

*6.1.2.3. 7,9-Diamino-1,2,3,4-tetrahydroacridine (**3c**).* Compound **3c** was obtained in 80% yield, m.p. 98–100 °C (ethyl acetate); ^{1}H NMR (DMSO-d_6): δ 7.37 (d, 1H, H-6, J_{ortho} = 9.6 Hz), 6.93 (m, 2H, H-5 and H-8), 5.72 (s, 2H, 9-NH_2), 5.00 (s, 2H, 7-NH_2), 2.73 (t, 2H, 4-CH_2), 2.50 (m, 2H, 1-CH_2, under DMSO signal), 1.78 (m, 4H, 2-CH_2 and 3-CH_2); MS: *m*/*z* 213 [M^+]. Anal. $C_{13}H_{15}N_3$ (C, H, N).

*6.1.3. 4,6-Diamino-2-methyl-3-phenylquinoline (**3d**)*

To a solution of **2d** (2.8 g, 0.01 mol) in 170 ml of methanol was slowly added at room temperature sodium borohydride (2.8 g, 0.07 mol); after cessation of gas development a catalytic amount of 10% palladium on charcoal was added and the solution stirred for 2 h. The mixture was filtered, the catalyst was washed with methanol and the collected liquid phase evaporated. Water was added to the residue and the suspension was extracted with ethyl acetate; the organic phase was evaporated and the residue purified by crystalliza-

tion, 54% yield, m.p. 212–215 °C (ethyl acetate/hexane); ^{1}H NMR (DMSO-d_6): δ 7.50 (m, 4H, H-8 quinoline, H-3, H-4 and H-5 phenyl ring), 7.23 (dd, 2H, H-2 and H-6 phenyl ring, J_{ortho} = 6.6 Hz, J_{meta} = 1.6 Hz), 7.03 (dd, 1H, H-7, J_{ortho} = 8.8 Hz, J_{meta} = 2.2 Hz), 6.98 (d, 1H, H-5, J_{meta} = 2.2 Hz), 5.16 (s, 4H, 4-NH_2 and 7-NH_2), 2.09 (s, 3H, CH_3); MS: *m/z* 249 [M^+]. Anal. $C_{16}H_{15}N_3$ (C, H, N).

*6.1.4. Ethyl 4,6-diamino-2-methyl-3-quinolinecarboxylate (**3e**)*

A suspension of **2e** (2.75 g, 0.01 mol) and nickel–aluminium alloy (8.0 g) in 50% (v/v) aqueous acetic acid (80 ml) was refluxed for 1.5 h, then cooled and filtered; the solid was washed with methanol and the collected liquid phase evaporated to dryness; the residue was then treated with aqueous potassium carbonate. The precipitate was filtered and washed with ethyl acetate, 90% yield, m.p. 174–176 °C (ethyl acetate); ^{1}H NMR (DMSO-d_6 with trifluoroacetic acid): δ 9.36 (bs, 4H, 4-NH_2 and 6-NH_2), 8.24 (ud, 1H, H-8), 8.08–7.55 (m, 2H, H-5 and H-7), 4.35 (q, 2H, CH_2CH_3), 2.73 (s, 3H, CH_3), 1.31 (t, 3H, CH_2CH_3); MS: *m/z* 245 [M^+], 199, 171, 144. Anal. $C_{13}H_{15}N_3O_2$ (C, H, N).

*6.1.5. General procedure for the preparation of 4-amino-6-benzamidoquinolines (**4h–k,n–q**), 9-amino-7-benzamido-1,2,3,4-tetrahydroacridines (**4l,m**) and 4-amino-6-benzamidoquinazolines (**4r–u**)*

The 2-[(4-ethylphenoxy)methyl]benzoic acid (m.p. 112–114 °C, ethyl acetate/hexane) and the 2-[(4-fluorophenoxy)-methyl]benzoic acid (m.p. 147–152 °C, ethyl acetate) were prepared according to a literature method [26]. The corresponding acyl chlorides were obtained by stirring a suspension of the acid (0.01 mol) in thionyl chloride (20 ml) overnight at room temperature, then the excess of thionyl chloride was evaporated and the raw acyl chloride so obtained was directly used in the following reactions.

To a solution of diamine (**3a–g**) (0.01 mol) in pyridine (60 ml), the acyl chloride (0.01 mol) was added. The mixture was stirred at room temperature for 3 h, then poured in ice water. The suspension was kept in the refrigerator overnight and then the precipitate was separated by filtration, washed with water and diethyl ether, then crystallized from the appropriate solvent. In the case of compounds **4m**,**p** and **4q**, purification was accomplished by chromatography on a silica gel column or aluminium oxide **(4m)**, by eluting with ethyl acetate.

*6.1.5.1. 4-Amino-6-[2-(4-ethylphenoxymethyl)benzamido]-2-methylquinoline (**4h**).* Compound **4h** was obtained in 42% yield, m.p. >300 °C (ethanol) (hydrochloride m.p. 235 °C [26]); ^{1}H NMR (DMSO-d_6): δ 10.81 (s, 1H, NH amide), 8.65 (s, 1H, H-5), 8.20 (bs, 2H, NH_2), 7.95–7.78 (m, 2H, H-7 and H-8), 7.72–7.43 (m, 4H, toluic acid), 7.05 (d, 2H, H-2 and H-6 phenol, J_{ortho} = 8.7 Hz), 6.84 (d, 2H, H-3 and H-5 phenol, J_{ortho} = 8.7 Hz), 6.57 (s, 1H, H-3), 5.30 (s, 2H, CH_2O), 2.54 (s, 3H, CH_3), 2.49 (q, 2H, CH_2CH_3, partially under DMSO signal), 1.08 (t, 3H, CH_2CH_3); MS: *m/z* 411 [M^+], 290, 239, 210, 145, 118. Anal. $C_{26}H_{25}N_3O_2$ (C, H, N).

*6.1.5.2. 4-Amino-6-[2-(4-fluorophenoxymethyl)benzamido]-2-methylquinoline (**4i**).* Compound **4i** was obtained in 45% yield, m.p. 108 °C (DSC) (ethanol); ^{1}H NMR (DMSO-d_6): δ 10.88 (s, 1H, NH amide), 8.71 (bs, 3H, NH_2 and H-5), 8.00–7.82 (m, 2H, H-7 and H-8), 7.73–7.43 (m, 4H, toluic acid), 7.15–6.86 (m, 4H, phenol), 6.60 (s, 1H, H-3), 5.31 (s, 2H, CH_2O), 2.58 (s, 3H, CH_3); MS: *m/z* 401 [M^+], 290, 229, 145, 118. Anal. $C_{24}H_{20}FN_3O_2.2/3\ H_2O$ (C, H, N).

*6.1.5.3. 4-Amino-6-[2-(4-ethylphenoxymethyl)benzamido]-2,3-dimethylquinoline (**4j**).* Compound **4j** was obtained in 38% yield, m.p. 257–263 °C (ethanol); ^{1}H NMR (DMSO-d_6): δ 10.85 (s, 1H, NH amide), 8.77 (s, 1H, H-5), 8.14 (bs, 2H, NH_2), 7.99 (d, 1H, H-8, J_{ortho} = 9.0 Hz), 7.90 (d, 1H, H-7, J_{ortho} = 9.0 Hz), 7.74–7.44 (m, 4H, toluic acid), 7.06 (d, 2H, H-2 and H-6 phenol, J_{ortho} = 8.5 Hz), 6.85 (d, 2H, H-3 and H-5 phenol, J_{ortho} = 8.5 Hz), 5.31 (s, 2H, CH_2O), 2.66 (s, 3H, 2-CH_3), 2.48 (q, 2H, CH_2CH_3 partially under DMSO signal), 2.19 (s, 3H, 3-CH_3), 1.09 (t, 3H, CH_2CH_3); MS: *m/z* 425 [M^+], 304, 274, 239, 210, 118. Anal. $C_{27}H_{27}N_3O_2$ (C, H, N).

*6.1.5.4. 4-Amino-6-[2-(4-fluorophenoxymethyl)benzamido]-2,3-dimethylquinoline (**4k**).* Compound **4k** was obtained in 35% yield, m.p. >300 °C (methanol); ^{1}H NMR (DMSO-d_6): δ 10.85 (s, 1H, NH amide), 8.77 (s, 1H, H-5), 8.19 (bs, 2H, NH_2), 7.97 (d, 1H, H-8, J_{ortho} = 9.0 Hz), 7.89 (d, 1H, H-7, J_{ortho} = 9.0 Hz), 7.77–7.46 (m, 4H, toluic acid), 7.17–6.88 (m, 4H, phenol), 5.33 (s, 2H, CH_2O), 2.66 (s, 3H, 2-CH_3), 2.19 (s, 3H, 3-CH_3); MS: *m/z* 415 [M^+], 304, 274. Anal. $C_{25}H_{22}FN_3O_2$ (C, H, N).

*6.1.5.5. 9-Amino-7-[2-(4-ethylphenoxymethyl)benzamido]-1,2,3,4-tetrahydroacridine (**4l**).* Compound **4l** was obtained in 40% yield, m.p. 167–170 °C (ethanol); ^{1}H NMR (DMSO-d_6): δ 10.48 (s, 1H, NH amide), 8.39 (s, 1H, H-8), 7.76–7.43 (m, 6H, H-5, H-6 and toluic acid), 7.08 (d, 2H, H-2 and H-6 phenol, J_{ortho} = 8.5 Hz), 6.87 (d, 2H, H-3 and H-5 phenol J_{ortho} = 8.5 Hz), 6.09 (s, 2H, NH_2), 5.31 (s, 2H, CH_2O), 2.82 (bt, 2H, 4-CH_2), 2.51 (m, 4H, 1-CH_2 and CH_2CH_3 partially under DMSO signal), 1.82 (m, 4H, 2-CH_2 and 3-CH_2), 1.11 (t, 3H, CH_2CH_3); MS: *m/z* 451 [M^+], 330, 239, 210, 122, 118. Anal. $C_{29}H_{29}N_3O_2$ (C, H, N).

*6.1.5.6. 9-Amino-7-[2-(4-fluorophenoxymethyl)benzamido]-1,2,3,4-tetrahydroacridine (**4m**).* Compound **4m** was obtained in 35% yield, m.p. 102 °C (DSC) (ethanol); ^{1}H NMR (DMSO-d_6): δ 10.49 (s, 1H, NH amide), 8.39 (s, 1H, H-8), 7.78–7.42 (m, 6H, H-5, H-6 and toluic acid), 7.16–6.89 (m, 4H, phenol), 6.11 (s, 2H, NH_2), 5.32 (s, 2H, CH_2O), 2.82 (bt, 2H, 4-CH_2), 2.56 (bt, 2H, 1-CH_2 partially under DMSO signal), 1.82 (m, 4H, 2-CH_2 and 3-CH_2); MS: *m/z* 441 [M^+], 330, 112. Anal. $C_{27}H_{24}FN_3O_2.H_2O$ (C, H, N).

*6.1.5.7. 4-Amino-6-[2-(4-ethylphenoxymethyl)benzamido]-2-methyl-3-phenylquinoline (**4n**).* Compound **4n** was obtained in 32% yield, m.p. 139 °C (DSC) (ethanol); ^{1}H NMR

(DMSO-d_6): δ 10.89 (s, 1H, NH amide), 8.87 (s, 1H, H-5), 8.13 (d, 1H, H-8, J_{ortho} = 9.0 Hz), 7.97 (d, 1H, H-7, J_{ortho} = 9.0 Hz), 7.83–7.26 (m, 9H, toluic acid and phenyl ring), 7.06 (d, 2H, H-2 and H-6 phenol, J_{ortho} = 8.4 Hz), 6.85 (d, 2H, H-3 and H-5 phenol, J_{ortho} = 8.4 Hz), 5.31 (s, 2H, CH_2O), 3.37 (s, 2H, NH_2 under H_2O signal), 2.51 (q, 2H, CH_2CH_3 under DMSO signal), 2.32 (s, 3H, CH_3), 1.10 (t, 3H, CH_2CH_3); MS: *m/z* 487 [M^+], 366, 239, 210, 118. Anal. $C_{32}H_{29}N_3O_2.2/3\ H_2O$ (C, H, N).

*6.1.5.8. 4-Amino-6-[2-(4-fluorophenoxymethyl)benzamido]-2-methyl-3-phenylquinoline **(4o)**.* Compound **4o** was obtained in 40% yield, m.p. 100 °C (DSC) (methanol); ^{1}H NMR (DMSO-d_6): δ 10.88 (s, 1H, NH amide), 8.85 (s, 1H, H-5), 8.05 (d, 1H, H-8, J_{ortho} = 9.0 Hz), 7.95 (d, 1H, H-7, J_{ortho} = 9.0 Hz), 7.88–7.29 (m, 9H, toluic acid and phenyl ring), 7.22–6.87 (m, 4H, phenol), 5.33 (s, 2H, CH_2O), 3.39 (s, 2H, NH_2 under H_2O signal), 2.31 (s, 3H, CH_3); MS: *m/z* 477 [M^+], 366, 229, 118. Anal. $C_{30}H_{24}FN_3O_2.H_2O$ (C, H, N).

*6.1.5.9. Ethyl 4-amino-6-[2-(4-ethylphenoxymethyl)benzamido]-2-methyl-3-quinolinecarboxylate **(4p)**.* Compound **4p** was obtained in 35% yield, m.p. 154–156 °C (ethyl acetate/hexane); ^{1}H NMR (DMSO-d_6): δ 10.63 (s, 1H, NH amide), 8.56 (d, 1H, H-5, J_{meta} = 1.8 Hz), 7.75 (dd, 1H, H-7, J_{ortho} = 9.2 Hz, J_{meta} = 1.8 Hz), 7.72–7.43 (m, 7H, H-8, NH_2 and toluic acid), 7.06 (d, 2H, H-2 and H-6 phenol, J_{ortho} = 8.6 Hz), 6.85 (d, 2H, H-3 and H-5 phenol, J_{ortho} = 8.6 Hz), 5.30 (s, 2H, CH_2O), 4.35 (q, 2H, OCH_2CH_3), 2.62 (s, 3H, CH_3), 2.51 (q, 2H, CH_2CH_3 under DMSO signal), 1.35 (t, 3H, OCH_2CH_3), 1.10 (t, 3H, CH_2CH_3); MS: *m/z* 483 [M^+], 438, 362, 316, 288, 239, 210, 118. Anal. $C_{29}H_{29}N_3O_4$ (C, H, N).

*6.1.5.10. Ethyl 4-amino-6-[2-(4-fluorophenoxymethyl)benzamido]-2-methyl-3-quinolinecarboxylate **(4q)**.* Compound **4q** was obtained in 61% yield, m.p. 143–145 °C (diethyl ether); ^{1}H NMR (DMSO-d_6): δ 10.63 (s, 1H, NH amide), 8.55 (d, 1H, H-5, J_{meta} = 1.8 Hz), 7.78 (dd, 1H, H-7, J_{ortho} = 9.2 Hz, J_{meta} = 1.4 Hz), 7.73–7.44 (m, 7H, H-8, NH_2 and toluic acid), 7.16–6.89 (m, 4H, phenol), 5.32 (s, 2H, CH_2O), 4.37 (q, 2H, OCH_2CH_3), 2.62 (s, 3H, CH_3), 1.35 (t, 3H, OCH_2CH_3); MS: *m/z* 473 [M^+], 428, 362, 316, 288, 229, 118, 111. Anal. $C_{27}H_{24}FN_3O_4$ (C, H, N).

*6.1.5.11. 4-Amino-6-[2-(4-ethylphenoxymethyl)benzamido]-quinazoline **(4r)**.* Compound **4r** was obtained in 40% yield, m.p. 232–234 °C (methanol); ^{1}H NMR (DMSO-d_6): δ 10.66 (s, 1H, NH amide), 8.51 (d, 1H, H-5, J_{meta} = 2.2 Hz), 8.34 (s, 1H, H-2), 7.85 (dd, 1H, H-7, J_{ortho} = 10.0 Hz, J_{meta} = 2.2 Hz,), 7.75–7.42 (m, 7H, H-8, NH_2 and toluic acid), 7.06 (d, 2H, H-2 and H-6 phenol, J_{ortho} = 8.8 Hz), 6.85 (d, 2H, H-3 and H-5 phenol, J_{ortho} = 8.8 Hz), 5.30 (s, 2H, CH_2O), 2.51 (q, 2H, CH_2CH_3 under DMSO signal), 1.09 (t, 3H, CH_2CH_3); MS: *m/z* 398 [M^+], 277, 239, 210, 118. Anal. $C_{24}H_{22}N_4O_2$ (C, H, N).

*6.1.5.12. 4-Amino-6-[2-(4-fluorophenoxymethyl)benzamido]-quinazoline **(4s)**.* Compound **4s** was obtained in 68% yield, m.p. >300 °C (ethanol); ^{1}H NMR (DMSO-d_6): δ 10.90 (s, 1H, NH amide), 8.74 (as, 1H, H-5), 8.05 (s, 1H, H-2), 7.90 (ad, 1H, H-7, J_{ortho} = 8.8 Hz), 7.70–7.40 (m, 5H, H-8 and toluic acid), 7.12–6.80 (m, 6H, NH_2 and phenol), 5.30 (s, 2H, CH_2O); MS: *m/z* 388 [M^+], 292, 239, 210, 118. Anal. $C_{22}H_{17}FN_4O_2$ (C, H, N).

*6.1.5.13. 4-Amino-6-[2-(4-ethylphenoxymethyl)benzamido]-2-methylquinazoline **(4t)**.* Compound **4t** was obtained in 35% yield, m.p. 249–251 °C (methanol); ^{1}H NMR (DMSO-d_6): δ 12.18 (s, 1H, C_4=NH tautomeric form of NH_2), 10.72 (s, 1H, NH amide), 8.59 (d, 1H, H-5, J_{meta} = 2.2 Hz), 7.98 (dd, 1H, H-7, J_{ortho} = 8.8 Hz, J_{meta} = 2.2 Hz), 7.70–7.40 (m, 5H, H-8 and toluic acid), 7.05 (d, 2H, H-2 and H-6 phenol, J_{ortho} = 8.8 Hz), 6.83 (d, 2H, H-3 and H-5 phenol, J_{ortho} = 8.8 Hz), 5.28 (s, 2H, CH_2O), 2.47 (q, 2H, CH_2CH_3 partially under DMSO signal), 2.34 (s, 3H, CH_3), 1.10 (t, 3H, CH_2CH_3); MS: *m/z* 412 [M^+], 291, 239, 210, 118. Anal. $C_{25}H_{24}N_4O_2.1/3\ H_2O$ (C, H, N).

*6.1.5.14. 4-Amino-6-[2-(4-fluorophenoxymethyl)benzamido]-2-methylquinazoline **(4u)**.* Compound **4u** was obtained in 44% yield, m.p. 284–288 °C (ethanol); ^{1}H NMR (DMSO-d_6): δ 12.17 (s, 1H, C_4=NH tautomeric form of NH_2), 10.72 (s, 1H, NH amide), 8.58 (d, 1H, H-5, J_{meta} = 2.2 Hz), 7.97 (dd, 1H, H-7, J_{ortho} = 8.8 Hz, J_{meta} = 2.2 Hz), 7.70–7.40 (m, 5H, H-8 and toluic acid), 7.15–6.80 (m, 4H, phenol), 5.29 (s, 2H, CH_2O), 2.34 (s, 3H, 2-CH_3); MS: *m/z* 402 [M^+], 291, 118, 112. Anal. $C_{23}H_{19}FN_4O_2$ (C, H, N).

6.2. Calorimetric studies

The thermal behaviour of compounds **4h**–**u** is summarized in Table 3. A first group (**4j**,**q**,**r**,**u**) showed a neat melting peak followed by decomposition.

A second group (**4i**,**m**–**o**) showed a first melting peak in the 100–150 °C range and a second one in the 230–290°C range. Between the two, an exothermal peak due to recrystallization always appeared.

TGA and FT-IR demonstrated that these compounds lose crystallization water and undergo a transition from a hydrated to an anhydrous form, during the first melting. The various hydrates have different amount of water in their crystalline structure. The number of water molecules was calculated by TGA weight loss for each different substance and reported in Table 3.

A third group (**4h**,**k**) showed neither a melting peak nor water loss. These compounds readily undergo decomposition upon reaching a given temperature in the range from 250 to 300 °C.

Compound **4p** displayed a first melting followed by recrystallization and then a second melting. Compound **4t** loses water at about 70 °C and then shows a unique melting peak at about 240 °C.

Table 3
Thermal behaviour of compounds **4h–u**

Compound	First melting (°C)	Second melting (°C)	Water molecules per compound molecule [a]
Group I			
4j	250		0
4l	170		0
4q	140		0
4r	228		0
4u	286		0
Group II			
4i [b]	108	265	2/3 [c]
4m	102	292	1
4n	139	249	2/3
4o	100	270	1
Group III			
4h (JTC-801) [d]			0
4k [d]			0
4s [d]			0
Other compounds			
4p [e]	155	230	0
4t	72	242	1/3

[a] Determined by TGA.
[b] Crystallization exothermal peak at 180 °C.
[c] Determined by DSC.
[d] Decomposition without melting.
[e] It may exist in two anhydrous crystalline forms, the first one converting into the second at temperatures higher then 160 °C.

6.3. Pharmacology

6.3.1. NOP receptor binding assay in membrane preparations

Enriched plasma membranes from transfected cells were prepared by differential centrifugations [36] and stored at –80 °C (protein concentration 1–2 mg/ml) until used. The binding of ^{125}I–Tyr$_{14}$–nociceptin (Perkin Elmer Life Sciences) was measured in 1 ml reactions containing 50 mM Hepes–Tris pH 7.4, 0.2 mM DTT, 5 mM $MgCl_2$, 10 mM leupeptin, 10 mM bestatin, 0.1 mg/ml bacitracin, 0.1% (w/v) bovine serum albumin, and 3 μg of membrane proteins from HEK-293 cells transfected with the NOP receptor as previously described [30]. The concentration of radiotracer was maintained constant at 5–10 pM in the presence of increasing concentrations of compounds to be tested. Reactions lasted 90 min at room temperature and were terminated by rapid filtration onto GF/B glass fiber filtering microplates (Filtermate 196; Packard Instruments, Meriden, CT). Filters were washed three times with 1 ml of ice-cold 50 mM Tris–HCl pH 7.4 and allowed to dry for a few hours. The plates were counted in a Top Count (Packard Instruments) after the addition (50 μl) of Microscint 20 (Packard) to each well. IC_{50} values were obtained by fitting the competition curves according to a 4-parameter logistic model [37].

6.3.2. OP_1, OP_2, OP_3 receptors binding assay

The binding affinities of compounds **4h–m** for human OP_1, OP_2 and OP_3 receptors were determined with [^{3}H]-naltrindole for OP_1 receptor and with [^{3}H]-diprenorphine for OP_2 and OP_3 receptors and were accomplished by NovaScreen Biosciences Corporation, Maryland, USA [38,39].

Each membrane fraction of HEK-293 cells expressing OP_1 receptor and of CHO cells expressing OP_2 and OP_3 receptors was incubated in 50 mM Tris (pH 7.4) with radioligand at 25 °C for 2 h (OP_1), or 1 h (OP_2, OP_3). Nonspecific binding was determined in the presence of 10 μM naloxone. IC_{50} values were calculated as the concentration of the examined compound required to displace 50% inhibition of each ligand.

6.3.3. GTPγS binding

The [^{35}S] GTPγS binding was determined in a 1 ml reactions containing 50 mM Hepes–Tris, pH 7.4, 1 mM EGTA, 1 mM DTT, 100 mM NaCl, 5 mM $MgCl_2$, 1–2 nM [^{35}S] GTPγS (Perkin Elmer Life Sciences), 3 μM GDP (or concentrations varying between 0.1 nM and 100 μM) and 1–2 μg of membrane proteins, with or without compounds to be tested [30]. The dose–response curves of nociceptin were determined in the absence and presence of fixed concentrations of the tested compounds (50, 100, 200, 300 nM) [40]. Samples were incubated for 90 min at 20 °C, filtered onto GF/B glass fiber filtering microplates (Filtermate 196; Packard Instruments, Meriden, CT) and washed three times with 1 ml of ice-cold buffer prior to scintillation counting on a Packard Top Count. Nonspecific binding was determined in the presence of 10 μM GTPγS.

The data of dose–response experiments were analyzed using program ALLFIT to compute the EC_{50} in the absence and presence of the various concentrations of antagonist [37].

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