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Synthesis and pharmacological studies of new hybrid derivatives of fentanyl active at the μ-opioid receptor and I_2–imidazoline binding sites

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Abstract—Two series of fentanyl-derived hybrid molecules bearing potent I_2–imidazoline binding site (IBS) ligands (i.e., guanidine and BU224 moieties) linked with an aliphatic (m = 2, 3, 4, 6, 7, 8, 9 and 12 methylene units) or aromatic spacer were prepared. Their affinities for the μ-opioid receptors and for the I_2–IBS were determined through competition binding studies on human postmortem brain membranes. Whereas the BU224 hybrid molecules bound to the μ-opioid receptor and the I_2–IBS in the micromolar to low micromolar range, the alkaneguanidine series exhibited remarkable affinities in the nanomolar range for both receptors. [^{35}S]GTPγS functional assays were performed on human postmortem brain membranes with selected ligands from each series (**4f** and **8g**) showing the highest dual affinity for the μ-opioid receptor and I_2–IBS affinities. Both compounds displayed agonist properties: at the μ-opioid receptor for the alkaneguanidine derivative **4f** (spacer: six methylene units) and at a G-protein coupled receptor (GPCR) which remains to be determined for **8g**. The lack of analgesic properties of **4f** in vivo (i.e., hot plate and writhing tests in mice), discordant with the good in vitro binding data (K_i μ = 1.04 ± 0.28 nM, K_i I_2 = 409 ± 238 nM), may possibly be due to the low intrinsic efficacy of the compound. Alternatively, a low access to the central nervous system for this kind of hybrid molecules cannot be ruled out. Two new compounds reported here (**9f** and **13**), which were not dual acting, are worth mentioning for their outstanding binding affinities; **9f** bound to the μ-opioid receptor with a picomolar affinity (K_i = 0.0098 ± 0.0033 nM), whereas **13** presented an I_2–IBS affinity (K_i = 18 ± 11 nM) similar to the reference compound BU224.

1. Introduction

Different studies have revealed that some agents that do not interact directly with opioid receptors are able to regulate the pharmacological actions of opioid ligands. In particular, involvement of imidazoline binding site (IBS) ligands[1] in the modulation of analgesia[2] and withdrawal effects of morphine has been observed in mice and rats.[3–7] This is the case, for example, of the putative endogenous IBS ligand agmatine (Chart 1) which enhances analgesic action of morphine and inhibits tolerance and dependence to opioids.[8] Our interest in a possible new therapy that aims at reducing tolerance developed along with the treatment by opioid drugs led us to investigate a new series of dual acting drugs that interact with μ-opioid receptor and IBS simultaneously albeit separately. Among the advantages of the multiple target ligand approach compared with the co-administration of multiple drugs are a better patient compliance, a more practical use in the clinical settings and a lower risk of drug–drug interactions.[9]

The existence of a chemical entity displaying concomitant opioid receptor and I_2–IBS affinities was unknown in the literature at the start of our studies.[9,10] In our previous work, two series of hybrid molecules incorporating a fentanyl core (i.e., opioid moiety) and guanidine-like moieties (i.e., IBS ligand) were synthesized and pharmacologically evaluated (Chart 1).[10,11] All of these hybrid structures showed moderate to high affinity towards μ-opioid receptor, whereas only few of them

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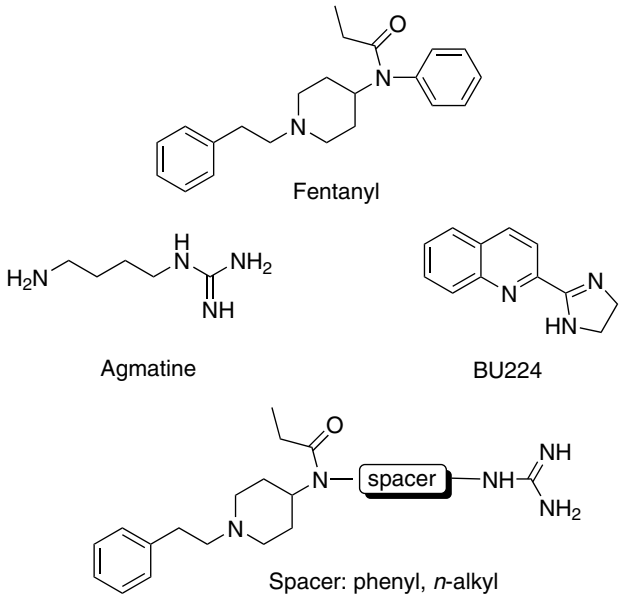
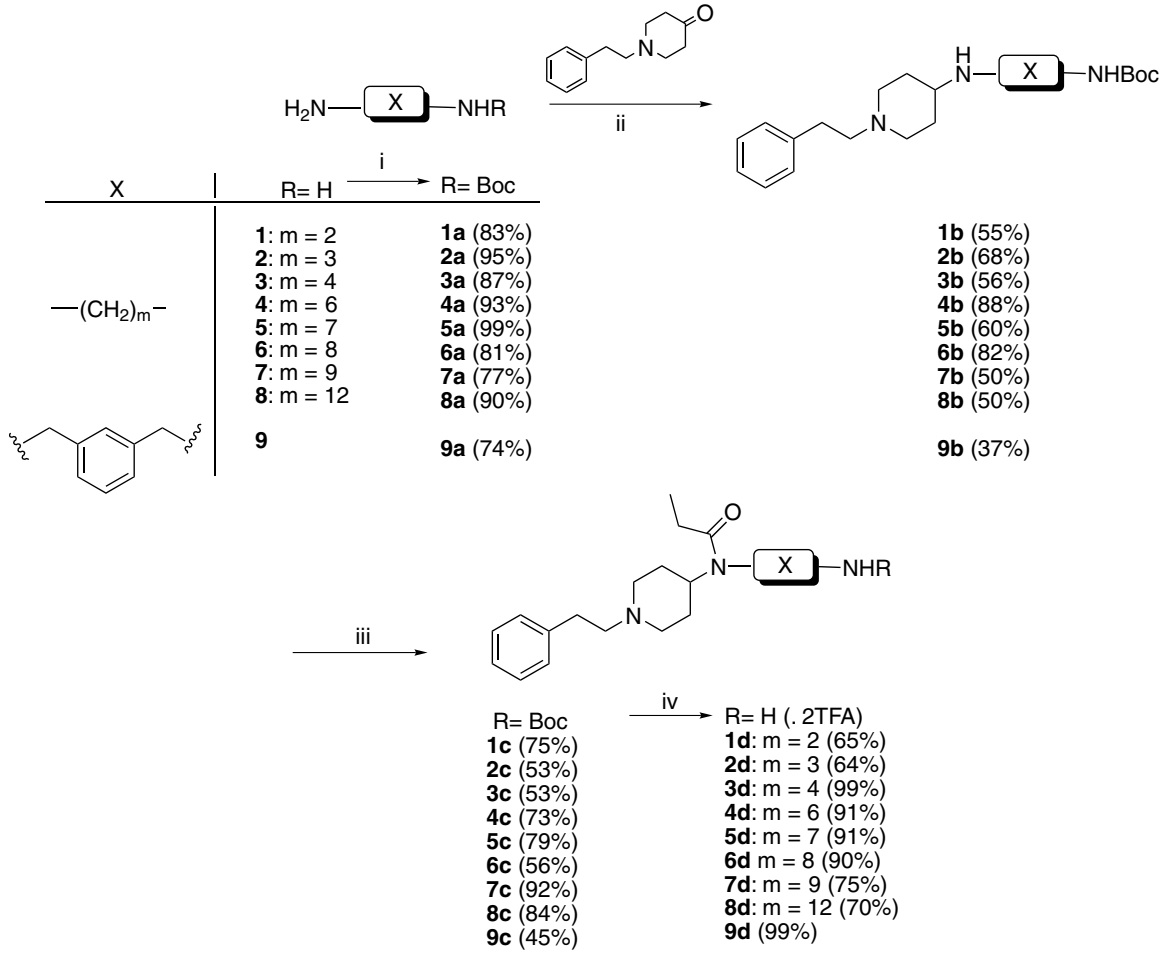


Chart 1.

had high affinity for I_2–IBS.[11] Of special interest was the structure–activity relationship for a series of agmatine-like hybrid molecules, the methylene linker length of which seemed to correlate well with I_2–IBS affinity. To probe further these findings, three new derivatives with an alkyl spacer (**3f**, **5f** and **7f**)] were synthesized and evaluated. For these series, an extensive modulation of the chain length of the alkyl spacer between the two pharmacophoric groups, fentanyl and guanidine, is presented (Scheme 1, Table 1). In addition, a more rigid analogue with a *m*-xylene spacer (**9f**) was also prepared and evaluated in order to explore possible structural needs for these hybrid molecules.

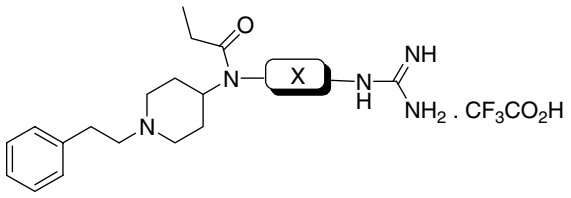
Besides, we also wish to report the synthesis of new compounds (**2g**, **4g**, **6g** and **8g**) that display the combination of the potent and selective I_2–IBS ligand BU224 and the μ-opioid ligand fentanyl. BU224[1] (Chart 1) is a selective I_2–IBS ligand which has also demonstrated interesting spinal antinociceptive properties in vitro.[12]

The affinity of the two series of compounds presented here was evaluated at the I_2–IBS and at the μ-opioid receptor. In addition, the functional activity of two hybrid molecules was also investigated through [^{35}S]GTPγS assays on postmortem human frontal cortex membranes. We chose to use membranes from human brain which could be more relevant from a therapeutically vantage point. The analgesic potential of the most promising dual ligand (**4f**) was evaluated in mice by hot plate and writhing tests.



Scheme 1. Reagents and conditions: (i) diamine (5 equiv), Boc_2O (1 equiv), $CHCl_3$, rt; (ii) $NaBH_3CN$, molecular sieves (3 Å), MeOH, rt; (iii) $(EtCO)_2O$, DMAP, pyridine, CH_2Cl_2, rt; (iv) CF_3CO_2H, CH_2Cl_2, 30 min.

Table 1. Guanidine hybrid molecules. Affinity data [K_i (nM)] for I_2–IBS and μ-opioid receptors



Compound	Spacer (X)	[^{3}H]-2BFI I_2	[^{3}H]-DAMGO μ	n[a]
Idazoxan	—	28 ± 11	nd[b]	5
Fentanyl	—	8593 ± 738	2.9 ± 1.5	4
1f[c]	$-(CH_2)_2-$	437 ± 228	433 ± 83	3
2f[c]	$-(CH_2)_3-$	1920 ± 996	23 ± 4.5	3
3f	$-(CH_2)_4-$	>10,000	0.59 ± 0.18	3
4f[c]	$-(CH_2)_6-$	409 ± 238	1.04 ± 0.28	3
5f	$-(CH_2)_7-$	6627 ± 3106	0.37 ± 0.19	3
6f[c]	$-(CH_2)_8-$	126 ± 72	37 ± 9.7	3
7f	$-(CH_2)_9-$	58 ± 46	26 ± 6	3
8f[c]	$-(CH_2)_{12}-$	6.5 ± 3.0	477 ± 75	3
9f	*m*-Xylene	>10,000	0.0098 ± 0.0033	6

[a] Values expressed as means ± standard error mean of n experiments.
[b] Not determined.
[c] From Ref. 11.

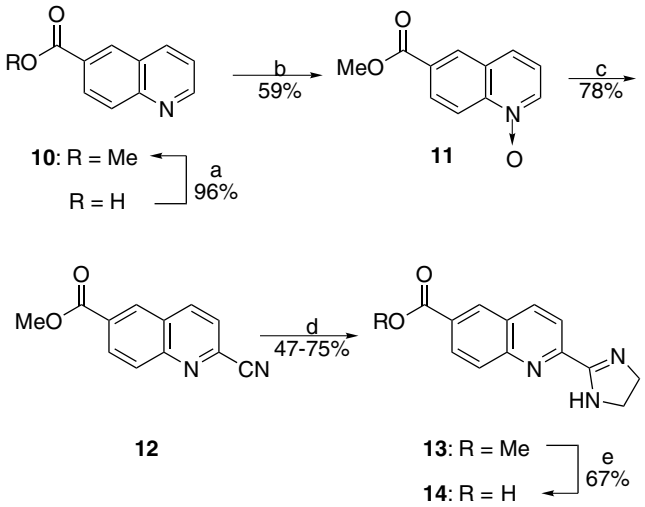
2. Results

2.1. Chemistry

The amines **1d**–**9d** were synthesized in four steps starting from the corresponding diamines **1**–**9** and following a procedure previously described by us.[11] In summary, the reductive amination ($NaBH_3CN$, pH 7–8) of 1-phenethyl-4-piperidone with the mono-protected diamines **1a**–**9a** afforded amines **1b**–**9b** which were subsequently acylated with propionic anhydride to afford the corresponding propanamides **1c**–**9c**. Treatment with CF_3CO_2H afforded the amines **1d**–**9d** as their trifluoroacetate salts (Scheme 1).

The BU224 moiety (**14**) was prepared in five steps from the commercially available quinoline precursor (Scheme 2). Esterification of methylquinoline-6-carboxylate with $SOCl_2$/MeOH yielded **10** which was converted to the cyano-derivative **12** in two steps applying the procedure that Kaneko et al. used for the synthesis of methyl 2-cyanoquinoline 5-carboxylate.[13] Formation of the imidazoline nucleus was accomplished by heating the cyanide **12** in the presence of ethylenediamine and a catalytic amount of P_2S_5 in refluxing toluene to afford **13**. Mild hydrolysis of the methyl ester **13** by treatment with LiOH afforded **14**. This reaction has to be carefully monitored since hydrolysis of the imidazoline moiety is a possible competitive reaction.

Two series of hybrid molecules were prepared starting from the diamines **1d**–**9d** (Scheme 3). The guanidines were introduced with the classical Boc-protected thiourea reagent[14] which allows the purification of the Boc-protected guanidines by SiO_2 chromatography. Removal of the Boc-protecting group with CF_3CO_2H afforded the guanidine hybrids **1f**–**9f**. Coupling of the



Scheme 2. Reagents and conditions: (a) $SOCl_2$, MeOH; (b) 30% H_2O_2, AcOH, 80 °C; (c) KCN, BzCl, H_2O, 0 °C; (d) $H_2NCH_2CH_2NH_2$, P_2S_5 cat, PhMe, reflux; (e) LiOH, H_2O–THF, rt.

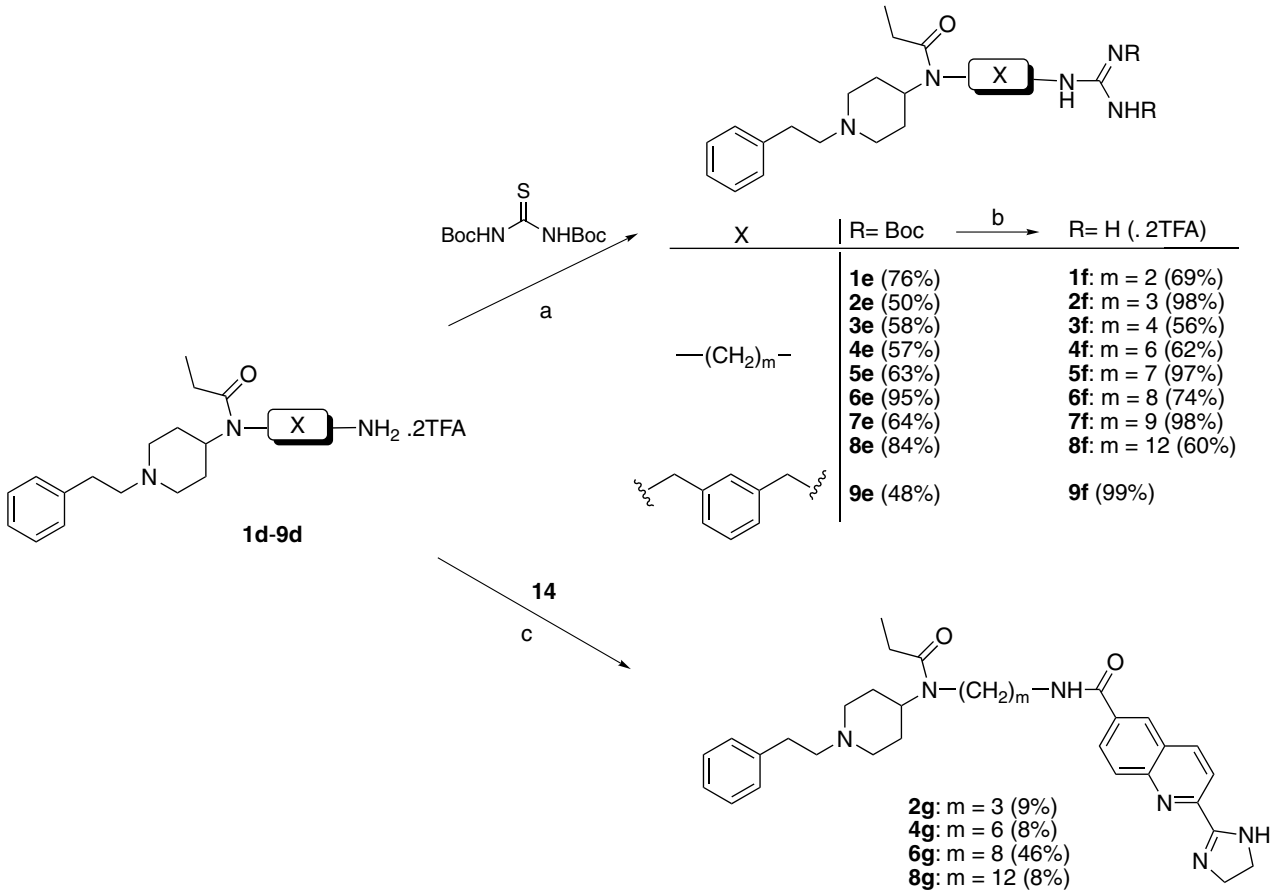
acid **14** with amines **2d**, **4d**, **6d** and **8d** in the presence of Mukayama's reagent afforded the corresponding hybrid molecules **2g**, **4g**, **6g** and **8g**, respectively. The difficulty of the silica purification was evidenced by the presence of multiple by-products. Isolation of a significant quantity of 1-methyl-pyridinium adducts of the amines (**2d**, **4d** and **8d**) and of further substitution products of the imidazoline nitrogen might account for the low yield of these couplings (8–46%).

The NMR spectra of the propanamide compounds showed characteristic duplicated signals corresponding to the observation of the amide *cis*–*trans* isomerism around the N–CO bond as previously reported.[10]

2.2. Pharmacology

2.2.1. Binding affinity. The binding affinity of the new compounds (**3f**, **5f**, **7f**, **9f**, **2g**, **4g**, **6g** and **8g**) and **1f**, **2f**, **4f**, **6f** and **8f**[11] was evaluated through competition binding assays against the selective I_2–IBS radioligand [^{3}H]-2-BFI and the selective μ-opioid ligand [^{3}H]DAMGO following the procedure previously described.[10,15–17] The assays were performed in membranes from post-mortem human frontal cortex, a brain area that shows an important density of I_2–IBS and μ-opioid receptors. Drug competition studies were performed with either [^{3}H]-2-BFI (1 nM) or [^{3}H]-DAMGO (2 nM) in the absence or presence of various concentrations of competing drugs (10^{-11}–10^{-3} M, nine concentrations). Non-specific binding was estimated in the presence of 10^{-3} M idazoxan in experiments with [^{3}H]-2-BFI, or with 10^{-4} M naloxone in [^{3}H]-DAMGO assays. The opioid agonist fentanyl and the I_2–IBS-selective ligand idazoxan were used as references (Tables 1 and 2).

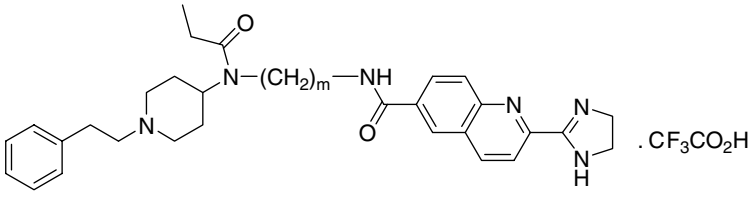
Guanidine hybrid molecules. Table 1 shows the results of binding of the guanidine hybrid series of the newly synthesized compounds (**3f**, **5f**, **7f** and **9f**) and previously reported molecules (**1f**, **2f**, **4f**, **6f** and **8f**). Regarding



Scheme 3. Reagents and conditions: (a) $HgCl_2$, Et_3N, CH_2Cl_2, rt; (b) TFA, CH_2Cl_2; (c) 2-chloro-1-methylpyridinium iodide, Et_3N, CH_2Cl_2, rt.

μ-opioid receptor, all the molecules bound with affinities in the nanomolar range; the best μ-opioid ligands being obtained with spacers of four (**3f**), six (**4f**) and seven (**5f**) methylene units with affinity of 0.59, 1.04 and 0.37 nM, respectively. Either increasing (m = 8, 9, 12) or decreasing (m = 2, 3) the length of the spacer afforded less potent ligands. On the contrary, increasing the length of the methylene spacer improved I_2–IBS binding affinity.[11]

Table 2. BU224 hybrid molecules. Affinity data [K_i (nM)] for I_2–IBS and μ-opioid receptors



Compound	–$(CH_2)_m$–[a]	[^{3}H]-2BFI I_2	[^{3}H]-DAMGO μ	n[b]
Idazoxan	—	28 ± 11	nd[c]	5
Fentanyl	—	8593 ± 738	2.9 ± 1.5	4
BU224	—	9.8 ± 0.3[d]	nd	2
13	—	18 ± 11	>10,000	3
14	—	588 ± 285	>10,000	3
2g	3	875 ± 713	6142 ± 2123	3
4g	6	323 ± 270	2168 ± 66	3
6g	8	>10,000	339 ± 35	3
8g	12	547 ± 316	545 ± 179	3

[a] m, number of methylene units in the spacer.
[b] Values expressed as means ± standard error mean of n experiments.
[c] Not determined.
[d] From Ref. 18.

Noteworthy is the excellent μ-opioid affinity observed for the hybrid compound **9f** with the *m*-xylene spacer ($K_i = 0.0098 \pm 0.0033$ nM). However, this compound was not a μ/I_2 dual ligand since it lacked I_2–IBS binding affinity.

BU224 hybrid molecules. The affinity for the μ-opioid receptor was lower in this series, affording only low micromolar binding for hybrid molecules with linkers of 8 and 12 methylene units (**6g** and **8g**, Table 2). Shorter spacers (3 or 6 methylene units, **2g** and **4g**, respectively) afforded low affinity μ-opioid ligands. In this series, the best affinities for I_2–IBS were also observed for the longest linkers **4g** and **8g** ($m = 6$ and 12) with the exception of **6g** ($m = 8$) which did not bind to I_2–IBS. The most balanced I_2/μ binding affinity of this series was obtained with the 12 methylene spacer hybrid molecule **8g** (Table 2).

Besides the binding studies performed on the BU224 hybrid molecules, two new analogues of BU224 (**13** and **14**) were evaluated as I_2–IBS ligands. Noteworthy was the excellent I_2–IBS affinity displayed by **13**, a new 6-methyl ester analogue of BU224 ($K_i = 18 \pm 11$ nM vs 9.8 ± 0.3 nM for BU224[18]). Interestingly, hydrolysis of the ester group of **13** to give the 6-carboxylic acid analogue (**14**) led to a 30-fold loss in binding affinity for I_2–IBS.

2.2.2. [^{35}S]GTPγS functional assay. The therapeutic potential of these new families of I_2–IBS/μ-opioid hybrid compounds as possible μ-opioid modulators depends on their functional activity (i.e., agonist/antagonist) because μ-opioid agonists would have analgesic properties, whereas antagonists would not. Hence, we decided to assess the functional activity of two of these new hybrid molecules using the [^{35}S]GTPγS assay in postmortem human brain samples.[19] This assay constitutes a functional measure of the interaction of the receptor and the G-protein, the first step in activation of G-protein coupled receptors (GPCRs). It is a useful tool to distinguish between agonists (increasing the nucleotide binding), inverse agonists (decreasing the nucleotide binding), and neutral antagonists (not affecting the nucleotide binding) of GPCRs.

In order to confirm the nature of the functional activity of both families of dual compounds, we selected in each family one molecule that presented the 'best dual affinity.' Hence, the intrinsic activity of **4f** and **8g**, which showed the highest μ-opioid receptor along with an acceptable I_2–IBS affinity, was investigated with [^{35}S]GTPγS binding experiments on membranes of postmortem human frontal cortex.

The guanidine derivative **4f** induced an increase in the [^{35}S]GTPγS binding of 25% compared to basal level (Fig. 1a) revealing agonist properties. This effect was reverted in the presence of naloxone 10^{-5}M indicating agonist properties at the human μ-opioid receptor. The apparent low potency of this compound ($EC_{50} = 4.21 \pm 0.57$ μM) compares favourably with that of the μ-opioid agonist DAMGO ($EC_{50} = 77.1 \pm 17.1$ μM) in this assay. It should be noted that 'affinities of tritiated agonists for receptors in postmortem human brain are usually several orders of magnitude higher than the potencies of these agonists stimulating [^{35}S]GTPγS binding both in rat and in postmortem human brain membranes.'[20]

Compound **8g** showed a marked increase in nucleotide binding compared to basal level (+125%). However, this effect was not reverted in the presence of naloxone 10^{-5}M (Fig. 1b). Several classes of GPCRs present in membranes from postmortem human frontal cortex (e.g., α_2–adrenoceptors, CB_1–cannabinoid, or $5HT_1$) could potentially be activated by this compound and explain the observed [^{35}S]GTPγS binding activity.

2.2.3. In vivo assays. Because **4f** proved to be a μ-opioid agonist in the binding experiments, we decided to evaluate its analgesic potential in vivo in the hot plate and writhing tests in mice (Table 3). Unfortunately, this compound did not produce significant analgesia in both assays up to 40 mg/kg ip. The experiments were discontinued because of toxicity problems (27.3% death at 40 mg/kg). The lack of analgesic activity of **4f** in the hot plate and writhing tests may possibly relate to its low efficacy in the functional assay. Alternatively, a poor blood–brain barrier (BBB) penetration of this dicationic compound and the consequent lack of activation of the opioid receptors in the CNS might also explain the in vivo results.

3. Discussion

Our approach towards the management of opioid-induced tolerance developed during treatment with opioid drugs like fentanyl led us to design and synthesize several hybrid molecules that are able to interact with both I_2–IBS and μ-opioid receptors. The first goal of this study was the development of new agmatine-like hybrid analogues modulating the chain length of the methylene spacer (**1f**–**8f**, Table 1) in order to find the optimum distance between the pharmacophoric units for a balanced affinity on I_2–IBS and μ-opioid receptors. In this series, we clearly found that the best spacer for μ-opioid affinity should have between four and seven methylene units (**3f**, **4f** and **5f**). Longer (**6f**–**8f**) or shorter (**1f**–**2f**) spacers lead to a decrease in binding affinity for these receptors. On the other hand, the best binding affinity for I_2–IBS was observed with the longest spacers (**7f** and **9f**, 9 and 12 methylene units, respectively), confirming our previous findings on this kind of alkaneguanidine derivatives.[11,15] We previously observed[11] that the presence of the phenethylpiperidyl propanamide pharmacophore of the fentanyl structure could lead to a synergistic effect for the binding of alkaneguanidinium moieties to I_2–IBS since alkaneguanidines alone (i.e., monoguanidinium) show very poor I_2–IBS affinity.[15] This synergistic effect seems to exist also for the binding to μ-opioid receptors. Accordingly, we observed that amines **2d** and **3d** (as their TFA salts) showed no binding to these receptors ($K_i\mu = 4689 \pm 2232$ and >10,000 nM, respectively), whereas their guanidinium derivatives **2f** and **3f** had K_i

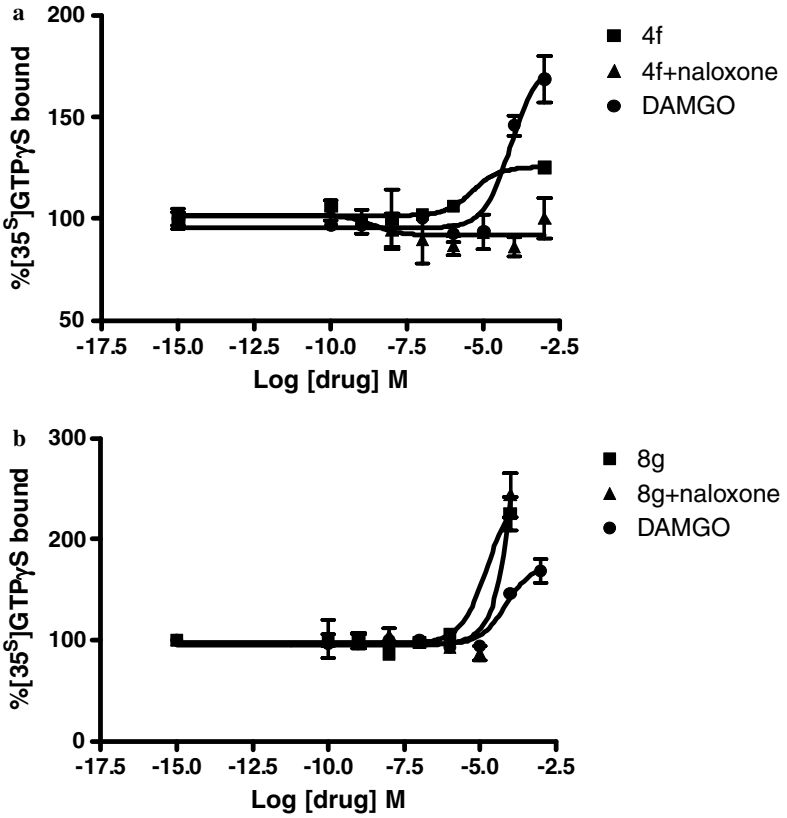


Figure 1. Concentration–response curves of the stimulation of [^{35}S]GTPγS-specific binding to postmortem human brain membranes by **4f** (1a) or **8g** (1b) in the absence (■) or in presence (▲) of the μ-opioid antagonist naloxone, and by the selective μ-opioid agonist DAMGO (●).

Table 3. Antinociceptive effect of compound **4f** and morphine on hot plate and acetic acid writhing tests in mice

Compound	Dose (mg/kg, ip)	Hot plate		Writhing	
		(% MPE)[a]	*n*	(% inhibition)	*n*
4f	1	−5.6 ± 3.8	6		
	10	−1.2 ± 9.7	6		
	20	2.8 ± 5.7	6	7.6 ± 22.2	10
	40	8.9 ± 12.1	5	28.0 ± 23.6	11
Morphine	2.5	25.5 ± 18.0	6		
	5	51.1 ± 12.6**	6		
	10	92.3 ± 7.7***	6	100.0 ± 0.0**	9

Values are expressed as means ± SEM from n mice per group.

[a] Values correspond to data obtained 30 min after compound **4f** or morphine administration.

**$p < 0.01$; ***$p < 0.001$ versus saline (ANOVA followed by the Student-Newman–Keuls test).

of 23 and 0.59 nM, respectively. These results altogether indicate that in this series of hybrid molecules the presence of the guanidinium cation is crucial for the binding to μ-opioid receptors, while the fentanyl skeleton confers I_2–IBS affinity to the alkylguanidinium moiety.

The remarkable μ-opioid affinity of the *m*-xylene derivative **9f** ($K_i = 0.0098 \pm 0.0033$ nM), which represents a 1000-fold increase compared to its *m*-phenyl analogue ($K_i = 7.8 \pm 2.5$ nM),[10] seems to indicate the needs of more flexibility for this ligand to fit into the μ-opioid receptor active site. The *m*-xylene spacer may alter the spatial positioning of the guanidinium group to bind to the μ-opioid receptor. On the contrary, **9f** was devoid of I_2–IBS affinity ($K_i > 10{,}000$ nM) compared to its *m*-phenyl analogue ($K_i = 1890 \pm 499$),[10] indicating that more conformational flexibility of the guanidine moiety around the phenyl ring was detrimental for the binding of these aromatic derivatives to I_2–IBS.[1] Compound **9f** is not a dual ligand but has impressive picomolar affinity for the μ-opioid receptor. Further in vitro and in vivo studies of this compound are warranted even though this goes beyond the present work focusing on dual acting ligands.

The second family of hybrid molecules synthesized, the BU224 derivatives **2g**, **4g**, **6g** and **8g**, only afforded micromolar ligands for both receptors, the longest spacers giving the best affinities for both receptors. These trends may reveal the importance of steric factors for the binding of these hybrid molecules to μ-opioid receptors and I_2–IBS.

The binding affinity observed for the new 6-substituted analogues of BU224 (**13** and **14**) is especially interesting since they demonstrate that the incorporation of a methyl ester group in this position of the quinoline ring of BU224 hardly affects its binding to I_2–IBS (Table 2). In contrast, a carboxylic acid moiety in that position leads to a 60-fold decrease in binding affinity. Since the carboxylate group is likely to be deprotonated under physiological conditions, these differences may indicate

that the presence of a negatively charged substituent in the 6-position of the quinoline ring has a detrimental effect on the binding to I_2–IBS.

4. Conclusion

The synthesis of new hybrid molecules incorporating the fentanyl skeleton and a guanidine moiety linked with an alkyl or aromatic spacer allowed to gain insights into the SAR of this family of compounds. Hence, we verified that the presence of the guanidine and the *N*-phenethyl piperidyl propanamide core was necessary for these hybrid compounds to bind to both I_2–IBS and μ-opioid receptors. The new family of BU224-derived dual compounds only produced moderate affinity ligands for these receptors, possibly due to a bigger steric hindrance of the BU224 core.

To conclude, the guanidinoalkyl fentanyl derivatives represent an interesting family of μ-opioid/I_2–IBS hybrid ligands that showed agonist properties (i.e., **4f**) at the human brain μ-opioid receptor. Even though **4f** was devoid of analgesic action in vivo, possibly due to its low efficacy and/or to unfavourable pharmacokinetic properties, these hybrid molecules represent attractive lead compounds.

5. Experimental

5.1. Chemistry

Reactions were monitored by TLC using pre-coated silica gel 60 F254 plates. Chromatography was performed either with silica gel 60 PF_{254} (particle size 40–63 μm) or with 50 g SI Isolute prepacked columns. All reactions requiring anhydrous conditions or an inert atmosphere were performed under a positive pressure of N_2. Dry CH_2Cl_2 was obtained by distillation over $CaCl_2$. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 200, Bruker Advance 300 or Varian Inova 400 spectrometer. Chemical shifts of the ^1H NMR spectra were internally referenced to the residual proton resonance of the deuterated solvents: $CDCl_3$ (7.26 ppm), D_2O (δ 4.6 ppm), CD_3OD (3.49 ppm) and DMSO (δ 2.49 ppm). Melting points were determined with a Reichert-Jung Thermovar apparatus and are uncorrected. Elemental analysis was performed on a Heraeus CHN–O Rapid analyser. Analytical results were within ± 0.4% of the theoretical values unless otherwise noted. Electrospray mass spectra were recorded on an MSD–Serie 1100 Hewlett Packard apparatus.

Synthetic and spectroscopic data for **1a**, **3a**, **5a**–**8a**, **1b**, **3b**, **5b**–**8b**, **1c**–**3c**, **5c**–**8c**, **1d**–**3d**, **5d**–**8d**, **1e**, **3e**, **5e**–**8e**, **1f**, **3f**, **5f**–**8f**, **2g**, **6g** and **8g** are available online as Supplementary material.

5.2. General synthetic procedures

A. Mono-protection of diamines with Boc. A 0.5 M solution of Boc_2O (1 equiv) in $CHCl_3$ was added dropwise over a 2 h period to a 0.25 M solution of diamine **1**–**9** (5 equiv; typical scale: 250–500 mmol) in $CHCl_3$ cooled with an ice-bath. The reaction mixture was stirred overnight at room temperature and filtered. The filtrate was concentrated under vacuum and the resulting oil dissolved in EtOAc (400 mL) was washed with half-saturated brine (3× 150 mL), dried ($MgSO_4$) and concentrated under vacuum to afford pure mono-Boc-protected diamine **1a**–**9a**. Further purification by silica chromatography was necessary for the diamine with more than six methylene units and **1a**.

***N*-(*tert*-Butoxycarbonyl)-1,6-hexanediamine (4a).** Colourless oil (93%). $R_f = 0.59$ (50% MeOH–$NH_{3(satd)}$ in CH_2Cl_2); ^1H NMR ($CDCl_3$, 200 MHz) δ 4.70 (br, 1H, NH), 3.03 (m, 2H), 2.62 (t, 2H, $J = 6.6$ Hz), 1.38 (s, 9H), 1.50–1.20 (m, 10H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 155.7, 78.3, 41.6, 40.0, 33.2, 29.6, 28.0, 26.2, 26.1; LRMS (ES^+) *m*/*z* 217 [(M+H), 100].

***N*-(*tert*-Butoxycarbonyl)-1,3-phenylenedimethanamine (9a).** Purification by chromatography on silica with CH_2Cl_2/MeOH–NH_3 (90:10) yielded **9a** as a colourless oil (96%); ^1H NMR ($CDCl_3$): δ 7.34–7.13 (m, 4H), 5.34 (br s, 1H, NH), 4.24 (m, 2H), 3.74 (s, 2H), 2.73 (br s, 2H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 161.9, 142.4, 139.3, 128.5 (2× CH), 126.0, 125.9, 79.1, 45.7, 44.3, 28.2; LRMS (ES^+) *m*/*z* 237 [(M+H), 80%].

B. Reductive amination. $NaBH_3CN$ (1.5 equiv) was added to a 0.2 M solution of 1-phenethyl-4-piperidone (1 equiv; typical scale: 15–20 mmol), mono-Boc-protected diamine **1a**–**8a** (1.2 equiv) and 3 Å molecular sieves in dry MeOH. The pH of the reaction was adjusted to 6–7 with AcOH during the course of the reaction. When the reaction was complete (typically 2–5 h), the mixture was filtered on a pad of Celite. The filtrate was concentrated under vacuum and the crude product was purified by chromatography.

***tert*-Butyl [6-(1-phenethylpiperidin-4-ylamino)hexyl]carbamate (4b).** Purification by chromatography with CH_2Cl_2–Et_3N (1%)/MeOH–$NH_{3(satd)}$ (100:0 → 90:10) afforded the product as an orange solid (64–88%). ^1H NMR ($CDCl_3$, 200 MHz) δ 7.00 (m, 5H), 4.85 (br, 1H), 2.90 (m, 2H), 2.75 (m, 2H), 2.66–2.52 (m, 2H), 2.50–2.20 (m, 5H), 2.11 (br, 1H), 1.96–1.60 (m, 4H), 1.40–1.04 (br m, 17H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 155.7, 140.1, 128.3, 127.9, 125.5, 78.4, 60.2, 54.6, 52.1, 46.2, 40.1, 33.4, 32.1, 29.72, 29.66, 28.1, 26.3, 26.1; LRMS (ES^+) *m*/*z* 404 [(M+H), 100%]. Anal. ($C_{27}H_{45}N_3O_3$) C, H, N.

***tert*-Butyl {3-[(1-phenethylpiperidin-4-ylamino)methyl]benzyl}carbamate (9b).** $NaBH_3CN$ (1.4 g, 3.6 mmol); 1-phenethyl-4-piperidone (331 mg, 2.4 mmol); mono-Boc-protected amine **9a** (681 mg, 2.9 mmol). Purification by flash chromatography on silica with CH_2Cl_2/MeOH–NH_3 (100:0 → 95:5)] yielded **9b** as a colourless oil (49%); ^1H NMR ($CDCl_3$): δ 7.29–7.16 (m, 9H), 5.12 (br s, 1H, NH), 4.26 (m, 2H), 3.80 (br s, 2H), 3.06 (m, 2H), 2.84 (m, 2H), 2.70 (m, 2H), 2.28 (br t, 2H, $J = 10.7$ Hz), 1.99 (br d, 2H, $J = 11.5$ Hz), 1.61

(dq, 2H, $J = 2.9$ Hz, $J = 10.2$ Hz), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 156.4, 140.3, 140.0, 139.7, 129.3, 129.1, 128.9, 128.8, 127.7, 127.6, 126.6, 80.0, 60.5, 52.3, 50.9, 46.9, 33.6, 31.9, 28.8, 9.41; LRMS (ES^+) *m/z* 424 [(M+H), 100%].

C. Acylation with propionic anhydride. Propionic anhydride (1.5 equiv) was added at room temperature to a 0.2 M solution of amine **1b**–**9b** (1 equiv; typical scale: 5–15 mmol), DMAP catalytic (0.1 equiv) and pyridine (5 equiv) in dry CH_2Cl_2. The reaction mixture was stirred at room temperature until all the starting material was consumed. The volatiles were removed under vacuum and the crude oil dissolved in EtOAc was extracted successively with satd NH_4Cl aqueous solution and brine, dried (Na_2SO_4) and concentrated. The purification was carried out as outlined for each compound.

***tert*-Butyl {6-[(1-phenethylpiperidin-4-yl)propionylamino]hexyl}carbamate (4c).** Following method C. Purification by chromatography with CH_2Cl_2–Et_3N (0.5%)/MeOH–$NH_{3(satd)}$ (100:0 → 98:2) afforded **4c** as a colourless oil (73%). $R_f = 0.34$ (4% MeOH in CH_2Cl_2); Mixture of two conformers as observed by NMR. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30–7.10 (m, 5H), 4.70–4.40 (m, 1.5H), 3.57 (br, 0.5H), 3.32 (br d, 1.5H), 3.20–3.00 (m, 4H), 3.00–2.85 (m, 2.5H), 2.85–2.60 (m, 2H), 2.53 (br t, 1H), 2.30 (m, 2H), 2.25–1.65 (m, 4H), 1.60–1.20 (m, 17H), 1.10 (t, 3H, $J = 7.3$ Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.9, 172.9, 156.0, 137.6, 128.7, 128.6, 128.4, 126.7, 126.2, 77.9, 60.0, 59.1, 54.8, 53.0, 52.8, 50.1, 43.7, 41.9, 40.3, 33.3, 31.9, 31.3, 30.3, 29.9, 29.5, 28.3, 27.8, 26.8, 26.6, 26.3, 26.2, 9.6, 9.5; LRMS (ES^+) *m/z* 460 [(M+H), 100%].

***tert*-Butyl {3-[(*N*-(1-phenethylpiperidin-4-yl)propionamido)methyl]benzyl} carbamate (9c).** Method C. The crude product was pre-purified on silica gel by flash chromatography eluting with CH_2Cl_2/MeOH–NH_3 (100:0 → 98:2), then it was purified on silica gel eluting with CH_2Cl_2/ Et_3N (100:1) to give **9c** as a colourless oil (63%); ^{1}H NMR ($CDCl_3$): δ 8.5 (br, 2H), 7.32–7.08 (m, 9H), 4.90–4.0 (m, 5H), 4.12 (br, 2H), 3.80–3.50 (m, 2H), 3.25–2.65 (m, 5H), 2.47 (m, 2H), 2.10–1.70 (m, 3H), 1.30–1.10 (m, 3H). ^{13}C NMR ($CDCl_3$): δ 174.7, 155.8, 140.2, 139.6, 139.1, 128.9, 128.6, 128.4, 126.1, 126.0, 125.8, 124.5, 79.6, 60.4, 55.9, 53.1, 51.7, 46.3. 33.8, 29.7, 28.4, 27.1, 9.2; LRMS (ES^+) *m/z* 480 [(M+H), 100%].

D. Removal of the Boc-protecting groups with TFA. A solution of the Boc-protected amine **1c**–**9c** in 50% TFA/CH_2Cl_2 was stirred for 30 min at room temperature. The volatiles were removed under vacuum. Et_2O was added to the crude residue and rotatory evaporation was repeated. The crude oil was dissolved in a little quantity of EtOH (ca 5 mL) and Et_2O was added until a precipitate formed. The product was allowed to stand in the freezer for 2 h. The mother liquor was discarded and the oily residue that settled at the bottom of the flask was collected and dried under high-vacuum, affording the TFA salt of the product as a highly hygroscopic solid.

***N*-(6-Aminohexyl)-*N*-(1-phenethylpiperidin-4-yl)propionamide (4d).** Trifluoroacetate salt of **4d**: brownish hygroscopic solid (91%); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.40–7.20 (m, 5H), 4.34 (t, 0.5H, $J = 11.9$ Hz), 4.14 (t, 1H, $J = 11.1$ Hz), 3.73 (br d, 2H, $J = 11.1$ Hz), 3.40–2.80 (m, 10H), 2.60–2.10 (m, 5H), 1.96 (m, 2H), 1.80–1.26 (m, 10H), 1.13 (br t, 3H, $J = 7.7$ Hz); ^{13}C NMR (CD_3OD, 75 MHz) δ 176.6, 175.97, 137.6, 130.0, 129.8, 128.3, 59.0, 53.6, 53.1, 52.2, 46.2, 42.8, 40.7, 40.6, 31.7, 31.5, 30.1, 28.9, 28.45, 28.37, 27.9, 27.6, 27.5, 27.3, 27 .0, 26.9, 10.0; LRMS (ES^+) *m/z* 360 [(M+H), 100%]; Anal. ($C_{22}H_{37}N_3O$ 1.6 $C_2F_3HO_2$) Calcd: C, 55.87; H, 7.11; N, 7.76. Found: C, 55.68; H, 6.99; N, 7.20.

***N*-(3-(Aminomethyl)benzyl)-*N*-(1-phenethylpiperidin-4-yl)propionamide (9d).** Trifluoroacetate salt of **9d**: yellowish oil (99%); ^{1}H NMR ($CDCl_3$): δ 7.68–7.40 (m, 9H), 4.85 (s, 0.8H), 4.81 (s, 1.2H), 4.49 (m, 0.6H), 4.32 (s, 1.2H), 4.27 (s, 0.8H), 3.84 (br d, 2H, $J = 12.0$ Hz), 3.50 (m, 2H), 3.47–3.28 (m, 4H), 2.86 (q, 0.8H, $J = 7.3$ Hz), 2.52 (q, 1.2H, $J = 7.3$ Hz), 2.32 (q, 2H, $J = 12.5$ Hz), 2.12 (br d, 2H, $J = 12.9$ Hz), 1.40 (t, 1.2H, $J = 7.3$ Hz), 1.28 (t, 1.8H, $J = 7.3$ Hz); ^{13}C NMR ($CDCl_3$): δ 177.3, 176.8, 141.5, 140.5, 135.3, 134.7, 130.8, 130.3, 129.9, 129.8, 129.1, 128.2, 127.7, 58.9, 53.6, 53.4, 53.1, 51.9, 45.3, 31.4, 29.0, 27.7, 27.5, 9.9, 9.8; LRMS (ES^+) *m/z* 380 [(M+H), 100%]; Anal. ($C_{28}H_{35}F_6N_3O_5{\cdot}4H_2O$) Calcd: C, 52.56; H, 6.13; N, 6.99. Found: C, 52.09; H, 6.40; N, 6.51.

E. Synthesis of the Boc-protected guanidines. $HgCl_2$ (190 mg, 0.7 mmol, 1.2 equiv) was added to a solution of amine **1d**–**8d** (0.58 mmol, 1 equiv), *N*,*N*′-di(*tert*-butoxycarbonyl)thiourea (191 mg, 0.7 mmol, 1.2 equiv) and Et_3N (0.4 mL, 2.9 mmol, 5 equiv) in dry CH_2Cl_2, under a N_2 atmosphere. After 3 h stirring at room temperature, the dark grey reaction mixture was diluted with CH_2Cl_2 and filtered on a pad of Celite. The filter cake was rinsed with CH_2Cl_2 (2× 15 mL). The combined organic phases were washed with water, dried (Na_2SO_4) and concentrated under vacuum. The crude oil was chromatographed on a short plug of silica (2.5 × 2.5 cm) eluting with cyclohexane/EtOAc (50:50).

***N*-[N^2,N^3-(Bis-*tert*-butoxycarbonyl)guanidinohexyl]-*N*-[1-phenethyl-4-piperidyl]propanamide (4e).** Yellowish oil (57%); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 11.43 (br, 1H), 8.22 (br, 1H), 7.30–7.05 (m, 5H), 4.40 (m, 0.5H), 3.50 (m, 0.5H), 3.34 (m, 2H), 3.20–2.95 (m, 4H), 2.74 (m, 2H, 1/2 A_2B_2), 2.54 (m, 2H, 1/2 A_2B_2), 2.27 (quint, 2H), 2.06 (m, 2H), 1.93–1.57 (m, 4H), 1.55–1.40 (m, 22H), 1.27 (br m, 4H), 1.08 (m, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.6 (s), 172.9 (s), 164.4 (s), 155.9 (s), 153.16 (s), 153.10 (s), 139.8 (s), 128.5 (d), 128.2 (d), 125.9 (d), 82.88 (s), 82.76 (s), 79.06 (s), 78.95 (s), 60.2 (t), 55.0 (d), 53.05 (t), 52.98 (t), 51.1 (d), 43.2 (t), 41.9 (t), 40.7 (t), 40.5 (t), 33.5 (t), 33.4 (t), 31.3 (t), 30.5 (t), 29.5 (t), 29.4 (t), 28.8 (t), 28.4 (t), 28.1 (q), 27.9 (q), 26.72 (t), 26.67 (t), 26.56 (t), 26.49 (t), 26.37 (t), 9.62 (q), 9.46 (q); LRMS (ES^+) *m/z* 602.5 [(M+H)]; Anal ($C_{33}H_{55}N_5O_5$) C, H, N.

***N*-[Guanidinohexyl]-*N*-[1-phenethyl-4-piperidyl]propanamide (4f).**[11] Following method D (1 h at room

temperature). Trifluoroacetate salt of **4f**: brownish hygroscopic solid (62%); Anal. ($C_{27}H_{39}F_6N_5O_5$) C, H, N.

***N*-[N^2,N^3-((Bis-*tert*-butoxycarbonyl)guanidinomethyl)-benzyl]-*N*-[1-phenethyl-4-piperidyl]propanamide (9e).** Pale yellow solid; ^{1}H NMR ($CDCl_3$) δ 11.55 (br, 1H), 8.56 (br, 1H), 7.34–7.09 (m, 9H), 4.80–4.40 (m, 4H), 3.03 (m, 2H), 2.74 (m, 2H), 2.56–2.45 (m, 2H), 2.22 (m, 2H), 1.80–1.50 (br m, 5H), 1.48 (m, 18H), 1.26–1.21 (m, 2H), 1.11 (t, 3H, J = 7.3 Hz); LRMS (ES^+) m/z 622 [(M+H), 100%].

***N*-[(3-Guanidinomethyl)benzyl]-*N*-[1-phenylethyl-4-piperidyl]propanamide (9f).** Following method D (2 h at room temperature). Trifluoroacetate salt of **9f**: yellow hygroscopic solid (100%); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.70–7.33 (m, 9H), 5.25 (br, overlap with solvent), 5.00–4.40 (m, 6H), 3.86 (br d, 2H), 3.50 (m, overlap with solvent), 3.42–3.15 (m, 4H), 2.84 (m, 1H), 2.56 (q, 2H, J = 7.3 Hz), 2.50–2.05 (m, 5H), 1.40 (t, 1.5H, J = 7.3 Hz), 1.29 (t, 1.5H, J = 7.4 Hz), 1.1 (br, 1H); ^{13}C NMR ($CDCl_3$) δ 160.7, 160.3, 157.2, 137.4, 136.2, 134.7, 129.9, 129.2, 128.4, 127.8, 127.2, 125.5, 124.5, 58.7, 53.0, 49.8, 47.2, 44.9, 30.4, 27.2, 26.7, 9.5; LRMS (ES^+) m/z 422 [(M+H), 100%].

F. Synthesis of the BU224 moiety

Methyl quinoline-6-carboxylate (10). Thionyl chloride (4 mL, 55 mmol) was added dropwise at 0 °C to a suspension of commercial 6-quinoline carboxylic acid (3.17 g, 18.3 mmol) in MeOH (100 mL). A thick precipitate formed after completion of the addition. The ice-bath was removed and the reaction mixture was stirred at room temperature for 1 h. The stopper of the flask was replaced by a reflux condenser and the reaction mixture was refluxed for 1 h. After stirring overnight at room temperature, the solvent was removed under reduced pressure. The solid was taken up in a 1 M K_2CO_3 solution (50 mL) and the aqueous phase was extracted with CH_2Cl_2 (2× 100 mL). The organic extracts were washed with brine, dried ($MgSO_4$) and concentrated to dryness, affording **10** as an off-white solid (3.28 g, 96%). R_f = 0.22 (33% EtOAc in hexane); mp = 86–88 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.96 (dd, 1H, J = 1.8 and 4.2 Hz), 8.54 (d, 1H, J = 1.8 Hz), 8.27 (dd, 1H, J = 1.8 and 9 Hz), 8.21 (dt, 1H, J = 8.7 Hz), 7.42 (dd, 1H, J = 4.2 and 8.1 Hz), 3.96 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.9, 152.9, 150.4, 137.7, 131.4, 130.2, 129.3, 128.5, 127.8, 122.2, 52.8; LRMS (ES^+) m/z 188 [(M+H), 100%]; Anal. ($C_{11}H_9NO_2$ 0.1 H_2O) Calcd: C, 69.95; H, 4.86; N, 7.42. Found: C, 69.93; H, 4.90; N, 7.33.

Methyl quinoline-1-oxide-6-carboxylate (11). To the ester **10** (2.04 g, 10.9 mmol) dissolved in AcOH (50 mL) was added 30% aqueous H_2O_2 solution (7 mL, 55 mmol). The reaction mixture was heated at ca. 80 °C for 2 h and stirred at room temperature overnight. After the repeated addition of water (50 mL) followed by concentration to the original volume under reduced pressure (3×), the residue was basified with aqueous K_2CO_3 solution and extracted with CH_2Cl_2 (3× 40 mL). The organic extracts were dried ($MgSO_4$) and concentrated to dryness under reduced pressure to yield **11** as a crude off-white solid (1.3 g, 59%). R_f = 0.11 (5% MeOH in Et_2O); mp = 155–156 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.76 (d, 1H, J = 9.2 Hz), 8.54 (m, 2H), 8.27 (dd, 1H, J = 1.5 and 9 Hz), 7.78 (d, 1H, J = 8.5 Hz), 7.32 (dd, 1H, J = 6.2 and 8.3 Hz), 3.96 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.1, 143.6, 137.5, 131.4, 130.8, 130.3, 130.2, 126.9, 122.3, 120.8, 53.1; LRMS (ES^+) m/z 204 [(M+H), 100%]. Anal. ($C_9H_{11}NO_3$) C, H, N.

Methyl 2-cyanoquinoline-6-carboxylate (12). The *N*-oxide **11** (1.25 g, 6.2 mmol) was dissolved in an aqueous solution (25 mL) containing KCN (720 mg, 11 mmol). The solution was cooled with an ice-bath and benzoyl chloride (1.5 mL, 12.9 mmo.l) was added in small portions. The reaction mixture was stirred [note: an efficient stirring is needed due to formation of a pasty precipitate] at 0 °C for 1 h. The resulting precipitate was collected by filtration and washed thoroughly with 1 M aqueous K_2CO_3 and finally with water. The crude product was crystallized from MeOH/CH_2Cl_2 (2:1), rinsed with Et_2O and dried in vacuo, yielding **12** as a pale pink solid (1.02 g, 78%). R_f = 0.66 (100% in Et_2O); mp = 195–196 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.64 (s, 1H), 8.41 (m, 2H), 8.22 (br d, 1H, J = 8.8 Hz), 7.76 (br d, 1H, J = 8.4 Hz), 4.0 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.9, 149.8, 139.0, 135.7, 130.8, 130.7, 130.4, 127.9, 124.1, 117.3, 52.9; LRMS (ES^+) m/z 213 [(M+H), 100%]. Anal. ($C_{12}H_8N_2O_2 \cdot$ 0.1H_2O) Calcd: C, 67.39; H, 3.82; N, 13.10. Found: C, 67.12; H, 3.86; N, 12.91.

Methyl 2-(4,5-dihydro-1*H*-imidazol-2-yl)quinoline-6-carboxylate (13). A mixture of cyanide **12** (706 mg, 3.3 mmol), ethylenediamine (2.2 mL, 33 mmol) and P_2S_5 (73 mg, 0.3 mmol) in dry toluene (10 mL) was stirred at room temperature for 2 days under a N_2 atmosphere. The mixture was then refluxed for 3 h to lead the reaction to completion. The solvent was removed under reduced pressure and the crude residue was partitioned between water and CH_2Cl_2. The aqueous phase was extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried and concentrated to yield **13** as an off-white powder (398 mg, 47%). R_f = 0.08 (10% MeOH in Et_2O); mp = 242–244 °C (180–220 °C sublimation); Free base of **13**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.55 (s, 1H), 8.27 (m, 3H), 8.06 (d, 1H, J = 8.8 Hz), 6.20 (br, 1H, NH), 4.10 (br, 2H), 3.99 (s, 3H), 3.69 (br, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.4 (s), 166.8 (s), 150.9 (s), 149.3 (s), 138.0 (d), 137.6 (d), 131.0 (d), 130.7 (d), 129.6 (d), 129.1 (s), 128.1 (s), 120.6 (d), 57.0 (br), 52.9 (q), 45.4 (br), 43.0 (t, weak), 41.7 (t, weak); LRMS (ES^+) m/z 256 [(M+H), 100%]. TFA salt of **13**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 10.60 (br, 1H), 8.50 (s, 1H), 8.50–8.20 (m, 3H), 8.09 (d, 1H, J = 8.8 Hz), 4.24 (s, 4H), 3.99 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.3, 164.8, 149.3, 141.2, 131.6, 131.4, 131.3, 130.7, 129.6, 120.2, 53.7, 45.8.

2-(4,5-Dihydro-1*H*-imidazol-2-yl)quinoline-6-carboxylic acid (14). Lithium hydroxide monohydrate (55 mg, 1.37 mmol) was added to a stirred solution of **13** (140 mg, 0.55 mmol) in a mixture of THF (5 mL) and

water (2 mL). The ester deprotection was complete after 45 min at room temperature. The solution was acidified to pH ~3 with 1 M HCl and diluted with acetone (100 mL). The flask was allowed to stand for 1 h at room temperature. The white precipitate was collected by filtration and dried in vacuo. Hydrochloride salt of **14**: colourless solid (102 mg, 67%). mp = 310–312 °C; ^{1}H NMR (D_2O, 200 MHz) δ 8.00 (m, 2H), 7.86 (d, 1H, J = 10 Hz), 7.49 (d, 1H, J = 8.8 Hz), 7.38 (d, 1H, J = 8.4 Hz), 4.10 (s, 4H); ^{13}C NMR (D_2O, 50 MHz) δ 168.9, 163.3, 147.7, 141.5, 140.2, 130.9, 130.3, 130.2, 130.1, 127.9, 119.4, 45.2; LRMS (ES^+) m/z 242 [(M+H), 100%]. Anal. ($C_{13}H_{12}ClN_3O_2$) C, H, N.

G. Synthesis of the BU224 hybrid molecules. To a mixture of the trifluoroacetate salt of the amine **1d–8d** (0.15 mmol, 1 equiv) and **14** (0.15 mmol, 1 equiv) in dry CH_2Cl_2 (3 mL) were added Et_3N (0.1 mL, 5 equiv) and 2-chloro-1-methylpyridinium iodide (0.17 mmol, 1.2 equiv). The reaction mixture was stirred at room temperature and quenched when all the acid starting material had been consumed (1–4 days). *Workup A*: the crude slurry was poured into diluted HCl (10 mL) and the aqueous phase was extracted with EtOAc (3× 10 mL). The aqueous phase was basified to pH ~9 with 10% aqueous NaOH solution and extracted with EtOAc (3× 10 mL). The combined organic phases were washed with brine, dried (Na_2SO_4) and concentrated to give a crude yellow oil. *Workup B*: the crude reaction mixture was partitioned between EtOAc and water. The organic phase was collected and the aqueous phase was basified with 10% aqueous NaOH solution. The aqueous phase was extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried and concentrated.

2-(4,5-Dihydro-1*H*-imidazol-2-yl)-*N*-(6-(*N*-(1-phenethylpiperidin-4-yl)propionamido)hexyl)quinoline-6-carboxamide (4g). Following workup B. Chromatography (2 g SI) with CH_2Cl_2/MeOH–$NH_{3(satd)}$ (100:0 → 85:15) yielded the hybrid molecule **4g** as a yellowish oil (8%). R_f = 0.2 (10% MeOH–$NH_{3(satd)}$ in CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.40–8.25 (m, 3H), 8.12 (m, 2H), 7.35–7.15 (m, 5H), 6.79 (br m, 0.5H), 6.52 (br m, 0.5H), 3.92 (br s, 4H), 3.52 (m, 2H), 3.30–3.05 (m, 4H), 2.83 (m, 2H, 1/2 A_2B_2), 2.67 (m, 2H, 1/2 A_2B_2), 2.45–1.20 (m, 18H), 1.15 (t, 1.5H, J = 7.3 Hz), 1.12 (t, 1.5H, J = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.8, 173.2, 166.7, 164.3 (2× C), 149.7, 149.5, 148.2, 140.0, 139.7, 137.4 (2× C), 133.6, 130.1, 130.0, 128.6, 128.4 (br), 128.0, 127.9, 127.7, 127.2, 126.2 (2× C), 120.3, 120.2, 60.4, 60.3, 55.4, 53.2, 51.1, 43.3, 41.4, 40.2, 39.9, 33.8, 33.5, 31.5, 30.9, 30.3, 29.7 (m), 29.4, 29.2, 27.3 (m), 26.9 (m), 26.7 (m), 26.4, 26.0, 9.8 (2× C); LRMS (ES^+) m/z 583.5 [(M+H)], 292.3 [(M+2H), 100]; Anal. ($C_{35}H_{46}N_6O_2$) C, H, N.

5.3. Pharmacology, materials and methods

[^{35}S]GTPγS (1250 Ci/mmol) was purchased from DuPont NEN (Brussels, Belgium), [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO), DL-dithiothreitol (DTT), GDP, GTP, GTPγS and naloxone were purchased from Sigma (St. Louis, USA). Morphine HCl was obtained from Alcaliber S.A. (Madrid, Spain). All other chemicals were of the highest purity commercially available.

5.3.1. Preparation of membranes. Postmortem human frontal cortex samples of each subject (~1 g) were homogenized using a Teflon–glass grinder (10 up-and-down strokes at 1500 rpm) in 30 volumes of homogenization buffer (1 mM EGTA, mM $MgCl_2$, 1 mM DTT and 50 mM Tris–HCl, pH 7.4) supplemented with 0.25 M sucrose. The crude homogenate was centrifuged for 5 min at 1000g (4 °C) and the supernatant was centrifuged again for 10 min at 40,000g (4 °C). The resultant pellet was washed twice in 20 volumes of homogenization buffer and recentrifuged in similar conditions. Aliquots of 1 mg protein were stored at −70 °C until assay. Protein content was measured according to the method of Bradford using BSA as standard, and was similar in the different brain samples.

5.3.2. [^{35}S]GTPγS binding assays. The incubation buffer for measuring [^{35}S]GTPγS binding to brain membranes contained, in a total volume of 500 μL, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 50 mM GDP, 50 mM Tris–HCl at pH 7.4 and 0.5 nM [^{35}S]GTPγS. Protein aliquots were thawed and re-suspended in the same buffer. The incubation was started by addition of the membrane suspension (40 μg of membrane proteins) to the previous mixture and was performed at 30 °C for 120 min with shaking. In order to evaluate the influence of the compounds on [^{35}S]GTPγS binding, eight concentrations (10^{-10}–10^{-3} M) of the different drugs were added to the assay. Incubations were terminated by adding 3 mL of ice-cold re-suspension buffer followed by rapid filtration through Whatman GF/C filters pre-soaked in the same buffer. The filters were rinsed twice with 3 mL of ice-cold re-suspension buffer, transferred to vials containing 5 mL of OptiPhase HiSafe II cocktail (Wallac, UK) and the radioactivity trapped was determined by liquid scintillation spectrometry (Packard 2200CA). The [^{35}S]GTPγS bound was about 7–14% of the total [^{35}S]GTPγS added. Non-specific binding of the radioligand was defined as the remaining [^{35}S]GTPγS binding in the presence of 10 μM unlabelled GTPγS.

5.3.3. In vivo analgesia

Animals. Male Swiss mice (final weight 26–32 g) were housed 6 per cage on a 12 h light–dark cycle at 22 °C with free access to food and water. The animals were allowed to habituate to the laboratory environment for at least 2 h before any experiment was initiated; they were used only once and killed by cervical dislocation after testing.

Writhing test. The abdominal constrictor test was performed by the intraperitoneal (ip) injection of acetic acid (2%; 10 mL/kg) to produce the typical writhing reaction that mimics acute visceral pain (arching of back, development of tension in the abdominal muscles, elongation of the body and extension of the hind limbs). Immediately after the acetic acid administration, the animals were placed in individual transparent cages and the number of writhes was counted during a

20 min period. The test was carried out twenty min after the ip injection of compound **4f** (20 and 40 mg/kg, $n = 10$–11) or the same volume of saline (5 mL/kg, $n = 12$). Morphine (10 mg/kg, ip, $n = 9$) was used as a positive control. The analgesic activity was expressed as the percent inhibition of the number of writhes observed in saline control animals (16.7 ± 3.5, $n = 12$).

Hot-plate test. Mice were placed in a hot plate (LE7406, Panlab S.L.; Spain) at 55 ± 0.1 °C as a nociceptive stimulus and the time of latency until jumping was taken as the end point, with a cut-off time of 120 s. The time of latency was determined 30 min before and at different times (5–120 min) after the ip injection of separated doses of compound **4f** (1, 10, 20 and 40 mg/kg, $n = 6$) or the same volume of saline (5 mL/kg, $n = 10$). Morphine (2.5, 5 and 10 mg/kg, ip) was used as a positive control. Antinociceptive activity was expressed as the percentage of the maximal possible effect (% MPE), which was calculated as follows:

% MPE = 100 × (latency of treated − latency of saline)/(cut-off time − latency of saline). The latency of saline was 25 ± 2s ($n = 10$).

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Supplementary data

Synthetic and spectroscopic data for **1a**, **3a**, **5a**–**8a**, **1b**, **3b**, **5b**–**8b**, **1c**–**3c**, **5c**–**8c**, **1d**–**3d**, **5d**–**8d**, **1e**, **3e**, **5e**–**8e**, **1f**, **3f**, **5f**–**8f**, **2g**, **6g** and **8g**. Combustion analysis data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.06.007.

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