

Dual Alleviation of Acute and Neuropathic Pain by Fused Opioid Agonist-Neurokinin 1 Antagonist Peptidomimetics

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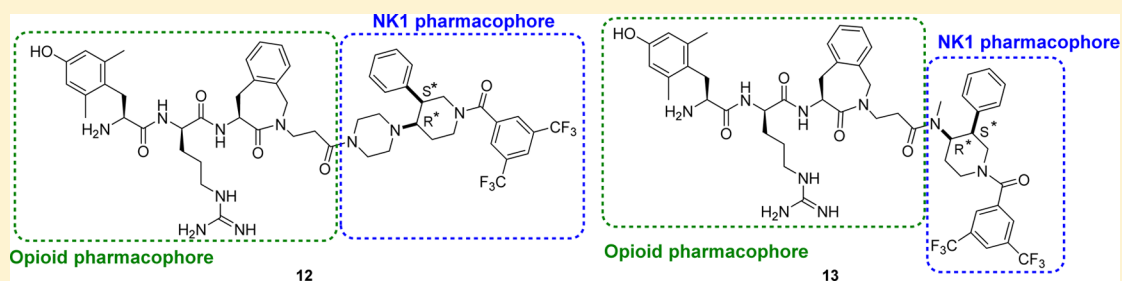
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S Supporting Information



ABSTRACT: Herein, the synthesis and biological evaluation of dual opioid agonists–neurokinin 1 receptor (NK1R) antagonists is described. In these multitarget ligands, the two pharmacophores do not overlap, and this allowed maintaining high NK1R affinity and antagonist potency in compounds **12** and **13**. Although the fusion of the two ligands resulted in slightly diminished opioid agonism at the μ - and δ -opioid receptors (MOR and DOR, respectively), as compared to the opioid parent peptide, balanced MOR/DOR activities were obtained. Compared to morphine, compounds **12** and **13** produced more potent antinociceptive effects in both acute (tail-flick) and neuropathic pain models (von Frey and cold plate). Similarly to morphine, analgesic tolerance developed after repetitive administration of these compounds. To our delight, compound **12** did not produce cross-tolerance with morphine and high antihyperalgesic and antiallodynic effects could be reinstated after chronic administration of each of the two compounds.

KEYWORDS: Designed multiple ligands, opioid agonism, NK1 antagonism, acute pain, neuropathic pain

The adequate treatment of different pain states remains the major medical challenge in pain research. Morphine and other opioids are the most widely used analgesic drugs for the treatment of moderate to severe and chronic pain, despite the serious side effects that emerge upon chronic administration (e.g., nausea, constipation, physical dependence, and tolerance).¹ These drawbacks become increasingly important during prolonged opioid administration, limiting their beneficial effect and rendering these drugs suboptimal for treatment of chronic pain.² Moreover, of all chronic pain states, neuropathic pain (resulting from nerve tissue damage) is especially difficult to treat, as opioids lose their efficacy in attenuating common symptoms of neuropathy, such as allodynia (painful perception

of neutral stimuli) or hypersensitivity to painful stimuli. When opioids are administered for a long period of time, analgesic tolerance develops; higher doses of painkillers are required to maintain the analgesic effect, and simultaneously other deleterious effects become more apparent as well. In addition, the sustained opioid administration results in an increased sensitivity to pain that occurs in undamaged tissues, also termed hyperalgesia.

Received: September 9, 2015

Accepted: October 31, 2015

Published: October 31, 2015

The mechanisms behind opioid tolerance and opioid-induced hyperalgesia are not well understood, but neuroplastic changes that occur in the central nervous system (CNS) are being held responsible.^{2,3} Under neuropathic pain conditions, changes in the activity of endogenous opioid systems, reduced number of receptors or glial interactions, have been reported,^{4–6} and sustained exposure to opioids provokes further changes. These can, for example, lead to an increased release of neurotransmitters like substance P (SP) and the up-regulated expression of the corresponding neurokinin 1 (NK1) receptor.^{3,7} SP, an endogenous neuropeptide, is a known pronociceptive neurotransmitter involved in chronic pain states, and it has been shown to contribute to the development of hyperalgesia.⁸ It activates the NK1 receptors that are expressed both in the CNS and in peripheral tissues. The analgesics currently in use do not counteract these changes in the CNS, rendering them ineffective for chronic pain treatment. The strategy to regulate pain transmission through a simultaneous interaction with the opioid and NK1 receptors, has hence been proposed as an approach for improved/prolonged therapy.^{9–11}

One alternative to the coadministration of different drugs consists of using designed multiple ligands (DMLs), or single molecules exerting multiple pharmacological effects.^{12–14} DMLs specifically targeting both opioid and NK1 receptors were designed in the past.^{3,10,15–18} In these DMLs the opioid agonist pharmacophore activates the μ and δ opioid receptors (MOR and DOR, respectively) and is responsible of the analgesic effect, while the NK1 antagonist ligand blocks pain signaling induced by SP, thus potentially increasing and extending the antinociceptive effect of the drug.

A potent hybrid opioid-NK1 peptidomimetic (Dmt-D-Arg-Aba-Gly-NMe-3',5'-(CF₃)₂-Bn **1**), containing an opioid pharmacophore at the *N*-terminus and a NK1 pharmacophore at the *C*-terminus, was designed by our group,¹⁹ and its structure was recently optimized via the preparation of Dmt-D-Arg-Aba- β Ala-NMe-Bn **2**, which proved to be more active in the neuropathic pain models.²⁰ Both compounds belong to the subclass of “merged” DMLs, in which the two pharmacophores, interacting with two different targets, overlap (Figure 1). The

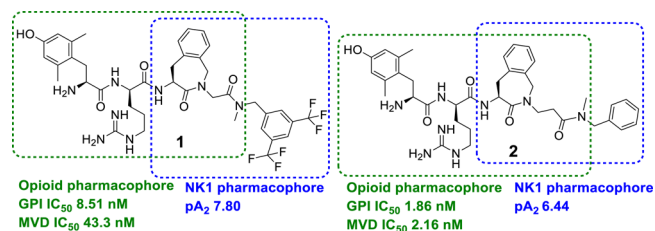


Figure 1. Previously reported opioid agonist-NK1 antagonist hybrid ligands with merged frameworks.

opioid agonist part is derived from the μ opioid receptor agonist Dmt-D-Arg-Phe-Lys-NH₂ ([Dmt]¹-DALDA **3**)²¹ and contains the 4-amino-2-benzazepin-3-one (Aba) scaffold, a conformationally constrained amino acid used in the design of very potent opioid agonists.^{22–25} The Aba scaffold is also part of the NK1 pharmacophore and enabled the design of very compact chimeric compounds.

The *in vitro* evaluation of peptidomimetics **1** and **2** showed that these bifunctional ligands have high (**1**) to subnanomolar (**2**) binding affinity for both the μ and δ opioid receptors and good binding affinity for the NK1 receptor.^{19,20,26} Moreover,

the *in vivo* studies showed activity in acute pain²⁶ as well as in neuropathic pain models.²⁰ The potent analgesic responses after intravenous (i.v.) administration indicated that these hybrid compounds were capable of crossing the blood–brain barrier (BBB), but unfortunately they still produced analgesic tolerance and cross-tolerance with morphine.^{20,26}

The activity profile of DMLs, such as the opioid agonist-NK1 antagonist hybrids, is often dependent on the relative potency at the two target receptors, the modulation of which is a very challenging aspect of DML design.

In this letter we report the synthesis and the biological evaluation of new hybrid opioid-NK1 ligands, in which potent opioid agonist pharmacophores based on the [Dmt]¹-DALDA peptide **3** are conjugated to three different NK1 antagonist pharmacophores with the aim to obtain hybrids producing a more potent NK1 antagonism than **1** or **2**. The 3-aryl-piperidine pharmacophores **4**, **5**,^{27,28} and **6**^{29,30} are known potent NK1 antagonists or fragments thereof (Figure 2).

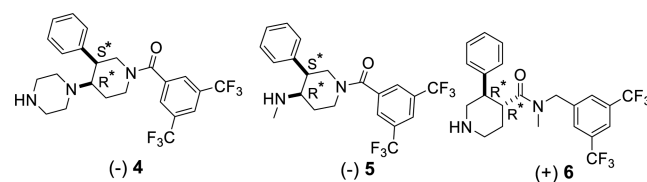
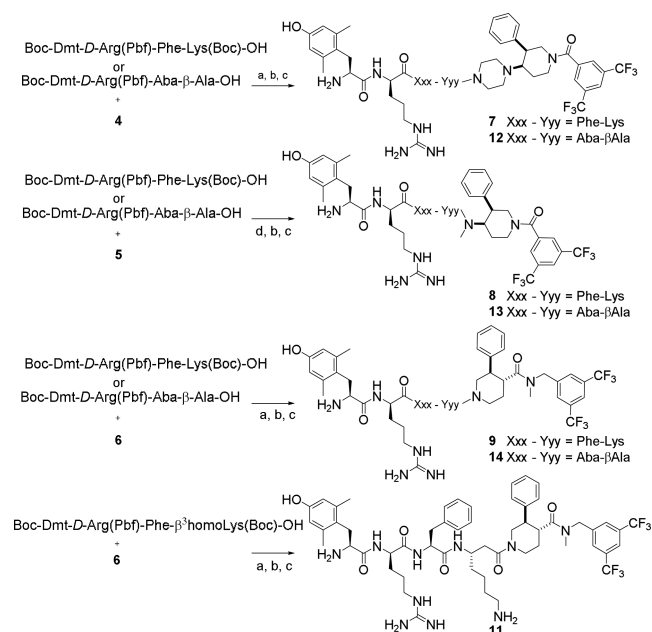


Figure 2. NK1 antagonist pharmacophores used in the hybrids.

The hybrid peptidomimetics **7–9** and **11–14** (Scheme 1) were prepared in two steps: the fully protected tetrapeptide precursors were synthesized via solid phase peptide synthesis (SPPS) using the 2-chlorotrityl chloride resin as solid support, and subsequently coupled to the secondary amines **4–6** in

Scheme 1. Coupling of the Opioid and NK1 Pharmacophores in Solution^a



^aReaction conditions: (a) DIC 1.5 equiv, HOBt 1.5 equiv in CH₂Cl₂, rt, on; (b) flash chromatography purification; (c) TFA/TEA/H₂O, 95:2.5:2.5 v/v, 3 h; (d) DIC 1.5 equiv, HOAt 1.5 equiv in CH₂Cl₂, rt, on.

solution. Boc-Dmt-D-Arg(Pbf)-Phe-Lys(Boc)-OH and Boc-Dmt-D-Arg(Pbf)-Phe- β^3 homoLys(Boc)-OH, precursors of compounds 7–9 and 11, respectively, were prepared via standard SPPS using N^{α} -Fmoc (or N^{α} -Boc for terminal residue Dmt) protected amino acids. The Boc-Dmt-D-Arg(Pbf)-Aba- β Ala-OH peptide, precursor of compounds 12–14, was prepared by direct assembly of the Aba scaffold on the solid support following a protocol recently described.²⁰ The coupling of Boc-Dmt-OH was performed using 3 equiv of DIC/3 equiv of HOBt as the coupling mixture to avoid side reactions that occur when uronium reagents (e.g., TBTU/DIPEA) are used. The fully protected tetrapeptides were cleaved from the resin using a mixture of 1% TFA in DCM for 1 h. The protected peptide acids were coupled to the secondary amines (1.5 equiv of amines 4–6) using 1.5 equiv of DIC/1.5 equiv of HOBt (for 4 and 6) or HOAt (for 5) in DCM at room temperature. After overnight reaction, the reaction mixture was evaporated and the residue was purified by flash chromatography, using a mixture of DCM/MeOH as mobile phase. The removal of the Boc and Pbf protecting groups was achieved using a mixture of TFA/ TES/H₂O 95:2.5:2.5 v/v for 3 h (Scheme 1). After evaporation of the cleavage mixture and purification by preparative RP-HPLC, all compounds were obtained in high purity (>95%). The intermediate purification of the fully protected peptidomimetics was mandatory because of the overlap between the peaks of the desired unprotected hybrids and their corresponding parent amines 4–6, in the HPLC chromatogram.

The ligands 7–14 were evaluated in *in vitro* binding and tissue functional assays (Table 1). [Dmt]¹-DALDA 3 is a highly

Table 1. Opioid and NK1 Receptor Affinity (nM) and Activity (nM) of the Synthesized Compounds

compd	pA ₂ ^a (NK ₁ R)	hNK ₁ R ^b (K _i)	GPI ^c (IC ₅₀)	MVD ^c (IC ₅₀)	MOR ^c (K _i)	DOR ^c (K _i)
1	7.80	0.50	8.51	43.3	0.42	10.4
2	6.44	13.0	1.86	2.16	0.08	0.28
3			1.41	23.1	0.58	877
7	nd	0.99	137	384	9.91	533
8	nd	1.1	70.2	814	54.0	>1000
9	nd	1.2	658	349	39.8	339
10	nd	nd	5.90	7.70	0.187	290
11	8.33	2.2	538	1660	20.2	>1000
12	8.11	1.4	20.3	14.1	1.18	10.6
13	7.54	1.4	13.0	12.6	1.82	11.2
14	6.72	1.9	30.1	10.3	3.07	7.07

^aThe pA₂ values were calculated using Schild's equation. ^bBinding affinities for hNK₁ receptor in CHO cells determined by displacement of [³H]SP. ^cThe GPI functional assay is representative of MOR activation, whereas the MVD is a DOR receptor-representative assay. Binding affinities for MOR and DOR opioid receptors were determined by displacement of [³H]DAMGO ([D-Ala²,NMePhe⁴,Gly-ol⁵]enkephalin) and [³H]DSLET ([D-Ser²,Leu⁵]enkephalin-Thr⁶) from rat brain membrane binding sites, respectively. Values represent means of 3–4 experiments. nd: not determined.

potent MOR agonist that is able to cross the BBB and induce a potent analgesic effect *in vivo*.²¹ When this sequence was conjugated to the NK1 fragments 4–6, the resulting hybrids 7–9 showed high affinity for the NK1 receptor, but disappointingly, their affinity and activity at μ - and δ -opioid receptors was drastically reduced. For this reason these compounds were not considered for further testing, and potential antagonism at the NK1 receptor was not determined.

When a DML is designed, advantages linked to pharmacophore overlap (e.g., lower molecular weight) can be neutralized by mutual steric hindrance and, consequently, this may result in reduced drug–target interaction. This phenomenon was observed for the chimeric compound 1: reduced binding affinity at both μ and δ opioid receptors, compared to the parent opioid sequence, was observed.^{22,26} In the present study, the opioid and NK1 pharmacophores do not overlap, but are “fused” or in close proximity of each other. Nevertheless, for hybrids 7–9 it is apparent that the bulky NK1 antagonist component severely hinders opioid binding. In order to increase the distance between the opioid and the NK1 pharmacophores, an additional methylene group was inserted via use of a β -homo amino acid. The [Dmt]¹-DALDA sequence 3 modified with a β^3 -homo-Lys residue (i.e., peptide 10, Dmt-D-Arg-Phe- β^3 homoLys-NH₂) proved to have a 3-fold better affinity for both the μ - and δ -opioid receptors than the parent peptide 3 (Table 1). Agonist activity was slightly decreased at MOR but significantly increased at DOR, thereby providing an ideal balanced μ -/ δ -agonist profile. Unfortunately, when conjugated to NK1 fragment 6, the high opioid agonist activity of hybrid 11 was, again, severely reduced.

We therefore switched to the opioid sequence Dmt-D-Arg-Aba- β Ala-NH₂, which was known to be tolerant to C-terminal conjugations.^{19,20,26} The functional activities at the μ and δ opioid receptors for these hybrid opioid-NK1 ligands 12–14 are significantly reduced, compared to the opioid parent compound Dmt-D-Arg-Aba- β Ala-NH₂ (GPI IC₅₀ 0.80 nM and MVD IC₅₀ 0.24 nM, data not shown).²⁰ However, the MVD (DOR) activity of compounds 12–14 was improved compared to the previous lead hybrid compound 1, confirming that the replacement of Gly with the β Ala residue is still beneficial to maintain the δ opioid activity. Whereas this replacement seemed to be detrimental for NK1 receptor antagonism in the previous compounds²⁰ [pA₂ from 7.80 (1) to 6.44 (2)], it does not have the same negative effect in this new series of compounds. The NK1 receptor affinity is maintained for compounds 12–14, and associated with potent antagonism for 12 and 13. Even though MOR agonism was slightly decreased in compounds 12 and 13, as compared to hybrid 1, improved DOR activity with concomitantly a potent antagonism at NK1 justified further *in vivo* evaluation of these ligands.

Compound 12 and 13 were selected for further *in vivo* evaluation.

The compounds were administered intrathecally (i.t.) at different doses, and the effect was measured by the tail-flick test (acute pain model). The analgesic effect of compounds 12 and 13 and of a representative NK1 pharmacophore (5) was compared to the effect of morphine in naïve rats (Figure 3) 30 min after the administration. The hybrid compounds 12 and 13 give potent antinociceptive responses (at 8 and 10 nmol doses, respectively) comparable to the effect of morphine used at dose 35 nmol; hence being 3.5 to 4.5-fold more effective than morphine in alleviating acute pain. In contrast, the analgesic effect of the parent NK1 antagonist 5 was very weak compared to its hybrid 13. The antinociceptive effect of compound 12 was recorded in function of time and gave significant antinociception up to 2 h postadministration (data not shown).

Additionally, the hybrid compounds 12 and 13, together with the parent NK1 antagonist 5, were tested in the chronic constriction injury (CCI) model of neuropathic pain after i.t. administration, in order to verify their ability to suppress allodynia or hyperalgesia (Figure 4). Seven days after CCI to

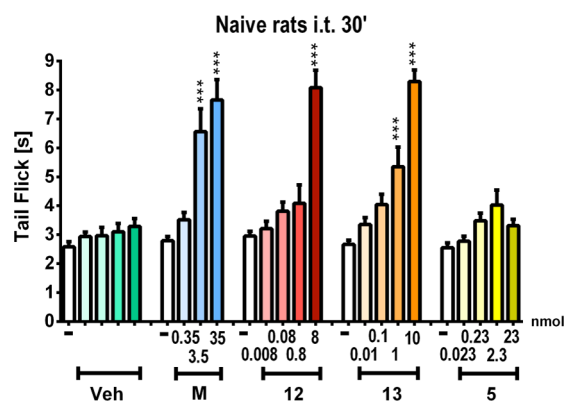


Figure 3. Effect of intrathecal (i.t.) administration of morphine (M), 12, 13, and the parent NK1 antagonist 5 as measured in the tail flick-test 30 min after administration in naïve rats; $***p < 0.001$ vs vehicle (Veh)-treated naïve rats.

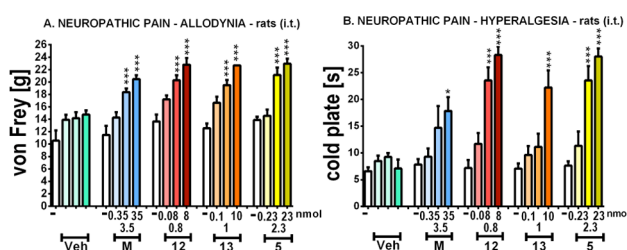


Figure 4. Effect of i.t. administration of morphine (M), hybrids 12 and 13, and the parent NK1 antagonist 5 on (A) allodynia, measured by von Frey test, and (B) hyperalgesia, measured by cold plate test, 30 min after administration in CCI-exposed rats; $*p < 0.05$, $***p < 0.001$ vs Veh-treated CCI-exposed rats.

the sciatic nerve in rats, compounds 12, 13, and 5, as well as morphine, were administered at increasing doses, and their effect on reducing allodynia (von Frey test) or hyperalgesia (cold plate test) was tested 30 min after administration. The antiallodynic effect of the hybrid compounds 12 and 13 was comparable to the one of morphine at a 3.5- to 4.5-fold lower dose, while compounds 12 and 13 were significantly stronger than morphine in the cold plate test, which shows the attenuation of hyperalgesia (Figure 4). From these data, the chimeric compound 12 seems to be the most potent hybrid, efficiently attenuating allodynia and hyperalgesia even at a low dose of 0.8 nmol. The efficiency of the opioid-NK1R hybrids in the applied neuropathic pain models clearly results from the added NK1R component, as the parent NK1 antagonist 5 was very potent in attenuating neuropathic pain symptoms, whereas this NK1R pharmacophore did not give any added value with regard to acute pain thresholds (cf. Figure 3).

The hybrid compounds 12 and 13, parent NK1 antagonist 5, and morphine were injected i.t. for 6 consecutive days (once daily) starting from day 7 after CCI, in order to study the effect of chronic drug administration on allodynia and hyperalgesia. As can be noticed in Figure 5, all compounds lost their initial effectiveness, both in the von Frey and cold plate test, on the sixth day of repeated administrations, and the effect of the investigated compounds was similar to the long-term profile of morphine administration. Particularly in the cold plate, the hybrids 12 and 13 were more potent than morphine in attenuating hyperalgesia on the fourth and the fifth day of the experiment (Figure 5B).

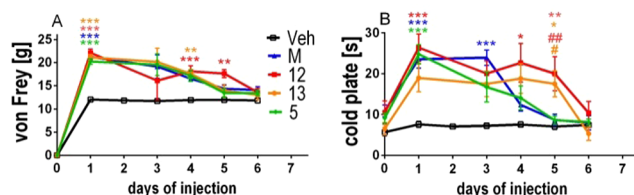


Figure 5. Development of tolerance to antiallodynic effect, measured by the von Frey test (A) and antihyperalgesic effect measured by the cold plate test (B) after repeated once daily (6 days) i.t. injections of morphine (M, 3.5 nmol), hybrid compounds 12 (0.8 nmol) and 13 (1 nmol), and the NK1 parent compound 5 (2.3 nmol); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs Veh-treated CCI-exposed rats; $\#p < 0.05$, $\#\#p < 0.01$ vs morphine-treated CCI-exposed rats.

The hybrid compounds 12, 13 and morphine were examined in a cross tolerance study (Figure 6). Starting from day 7 after

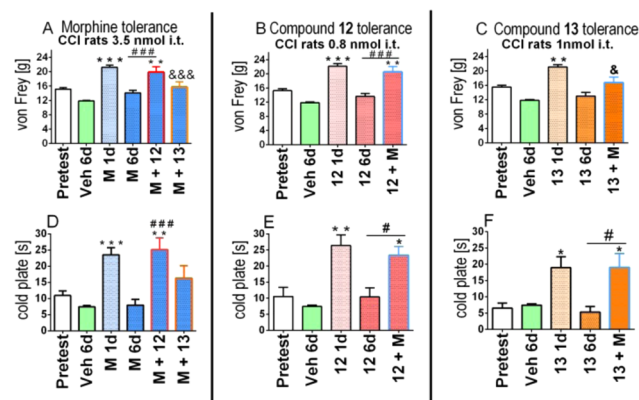


Figure 6. Cross-tolerance of hybrid compounds 12 and 13 with morphine (M) measured in neuropathic pain. The influence of repeated 6 days i.t. administration of morphine (3.5 nmol), 12 (0.8 nmol), and 13 (1 nmol) on the effect of a single dose of 12 (0.8 nmol) or 3.5 nmol of morphine (in 12 or 13 repeatedly treated CCI-exposed rats) was measured on day 7. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicates a significant difference compared with pretest. $\#p < 0.05$, $\#\#\#p < 0.001$ indicates a significant difference compared with the effect of a drug on 6th day of repeated once daily administration. $\&p < 0.05$, $\&\&\&p < 0.001$ indicates a significant difference compared with the effect of a drug on the first day of repeated administration.

CCI compounds 12 and 13 or morphine were daily administered to rats. As tolerance is developed within 6 days of repeated injections for all compounds (Figure 5), on day 7 of the repeated administration (day 14 after CCI) the animals that were tolerant to morphine were injected with a single dose of 12 or 13 (Figure 6A,D), and the animals tolerant to 12 or 13 were administered with a single dose of morphine (Figure 6B,E or C,F, respectively). When morphine is administered to animals tolerant to the hybrid compounds 12 and 13, the antinociceptive effect, especially for hyperalgesia (Figure 6E,F), increases. This indicates that no cross-tolerance occurs between the hybrid compounds and morphine. Similarly, when compound 12 is injected to an animal tolerant of morphine, it is also able to reduce allodynia (Figure 6A). This lack in cross-tolerance was even more pronounced in the cold plate test, a model of hyperalgesia (Figure 6D). In contrast, a single dose of compound 13 was not effective in rats that were rendered tolerant to morphine (Figure 6A,D).

In conclusion, hybrid opioid agonist-neurokinin 1 antagonist ligands were prepared by fusion of peptide opioid pharmacophores and nonpeptide neurokinin 1 receptor antagonists. Hybridization of the pharmacophores gratifyingly maintained the desired dual profile in vitro. Furthermore, this fusion resulted in compounds that manage to alleviate acute pain, an effect mostly induced by the opioid subunit of the chimeric compounds, and neuropathic pain in vivo. From the data it is clear that NK1R antagonism contributes to the observed effect in the neuropathic pain model. In comparison to our earlier reported ligands (Figure 1), these ligands manage to suppress to some extent of tolerance to antihyperalgesic effects (cf. Figure 5), and compounds 12 and 13 diminish cross tolerance with morphine. Therefore, a mixed opioid agonism-neurokinin 1 antagonist profile with highly potent NK1R antagonism represents a viable approach toward analgesics that remain orthogonal to the existing clinically applied painkillers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.5b00359.

Experimental procedures as well as compound characterization data (PDF)

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Author Contributions

C.B., L.F., and A.N. were in charge of the chemical synthesis of all GPCR ligands. B.P., J.S., J.M., and W.M. were in charge of the evaluation of in vivo potency of all ligands. N.N.C. performed the in vitro functional opioid assays. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

The work of C.B., S.B., D.T., and P.W.S. was supported by a collaboration convention between the Ministère du Développement Économique, de l'Innovation et de l'Exportation du Québec, and the Research Foundation—Flanders (FWO Vlaanderen). The work of B.P., J.S., J.M., and W.M. was supported by MAESTRO grant 2012/06/A/NZ4/00028 and statutory funds; J.S. is a Ph.D. student and has a scholarship from the KNOW funds sponsored by Ministry of Science and Higher Education, Poland. The work of P.W.S. was also supported by grants from the NIH (DA004443) and the Canadian Institutes of Health Research (MOP-89716).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Aba, 4-amino-2-benzazepinone scaffold; BBB, blood–brain barrier; CCI, chronic constriction injury; CNS, central nervous system; DIC, *N,N'*-dicyclohexylcarbodiimide; DIPEA, diisopropylethylamine; DML, designed multiple ligand; Dmt, 2',6'-dimethyl tyrosine; DOR, δ opioid receptor; GPI, guinea pig ileum; HOAt, 1-hydroxyazabenzotriazole; HOBt, 1-hydroxybenzotriazole; MOR, μ opioid receptor; MVD, mouse vas

deferens; NK1, neurokinin-1; RP-HPLC, reverse phase high pressure liquid chromatography; SP, substance P; SPPS, solid phase peptide synthesis; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; TES, triethylsilane; TFA, trifluoroacetic acid

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