Bioorganic & Medicinal Chemistry Letters xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

An imidazole based H-Phe-Phe-NH_2 peptidomimetic with anti-allodynic effect in spared nerve injury mice

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ARTICLE INFO

Keywords:
Substance P 1–7
Peptidomimetics
Neuropathic pain
SNI mice
Phenylalanine bioisostere

ABSTRACT

The dipeptide amide H-Phe-Phe-NH_2 (**1**) that previously was identified as a ligand for the substance P 1–7 (SP_{1-7}) binding site exerts intriguing results in animal models of neuropathic pain after central but not after peripheral administration. The dipeptide **1** is derived from stepwise modifications of the anti-nociceptive heptapeptide SP_{1-7} and the tetrapeptide endomorphin-2 that is also binding to the SP_{1-7} site. We herein report a strong anti-allodynic effect of a new H-Phe-Phe-NH_2 peptidomimetic (**4**) comprising an imidazole ring as a bioisosteric element, in the spare nerve injury (SNI) mice model after peripheral administration. Peptidomimetic **4** was stable in plasma, displayed a fair membrane permeability and a favorable neurotoxic profile. Moreover, the effective dose (ED_{50}) of **4** was superior as compared to gabapentin and morphine that are used in clinic.

The neuropeptide substance P (SP, H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) that acts through the G-protein coupled neurokinin-1 receptor, is an important neurotransmitter and neuromodulator of pain signaling in the nervous system.[1] As other tachykinins, the C-terminal part accounts for the signal transduction at the receptor, whereas the *N*-terminal part of the undecapeptide is important for receptor subtype selectivity.[2] SP coexists in neurons with classic transmitters and is after release subjected to enzymatic cleavage, which results in fragments, some with very diverse bioactivities.[3–5] The *N*-terminal fragment, substance P 1–7 (SP_{1-7}, Fig. 1) has attracted most attention owing to its biological effects that oppose those elicited by the parent peptide with regard to nociception as well as opioid tolerance and withdrawal.[6–9] Our interest in this heptapeptide was reinforced owing to its anti-hyperalgesic and anti-allodynic effects in animal models of neuropathic pain,[10–12] and this prompted us to commence a medicinal chemistry program aimed at converting the heptapeptide into drug-like peptidomimetics.

Neuropathic pain affects 7–10% of the general population and is a complex clinical condition that can arise as a result of several underlying etiologies such as diabetes mellitus, *Herpes zoster* infection, multiple sclerosis (MS) or spinal cord injury (SCI).[13,14] Current first-line treatment includes tricyclic antidepressants (TCA), serotonin and noradrenaline reuptake inhibitors (SNRI) and anticonvulsants like gabapentin and pregabalin, all hampered by CNS side effects.[15] Gabapentin is commonly used in clinic and is in comparison relatively safe despite the high doses needed *vide infra* to combat neuropathic pain.[16] Since opioids are addictive and associated with burdensome side effects like constipation, they are considered only as third line treatment. Morphine is a representative example of opioids used in clinic and is administrated for severe nociceptive pain, which also provides effectiveness in short-term neuropathic pain relief.[17,18] In light of this, safe pharmacological treatments that more efficiently target other sites in the neuropathic pain pathways are of interest as future drug candidates.

Several observations suggest that SP_{1-7} binds to a unique binding site, probably G-protein dependent and distinct from the neurokinin receptors or other receptors associated with pain such as opioid receptors.[19,20] In line with this, the binding is specific, saturable, and reversible. However, it is possible that the heptapeptide elicits its action by interfering with allosteric sites present on other neuropeptide receptors.[3] It is notable that the μ-receptor agonist endomorphin-2 (EM-2,

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https://doi.org/10.1016/j.bmcl.2018.06.009
Received 21 May 2018; Accepted 3 June 2018

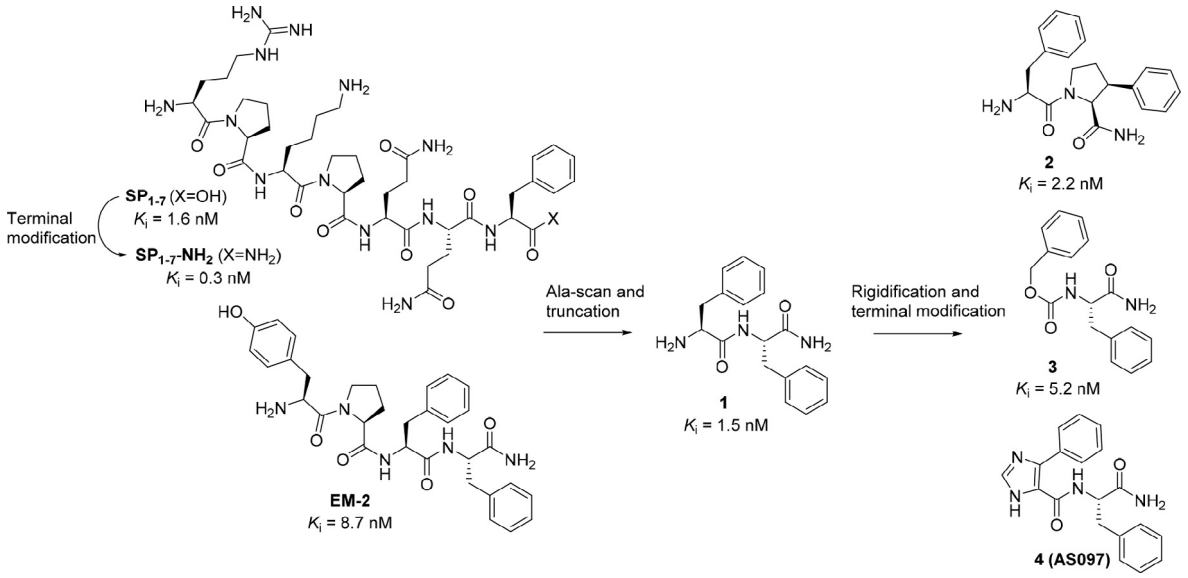


Fig. 1. Lead compound **1** derived from SAR studies of SP_{1-7} and EM-2 along with three rigidified and terminal modified analogues **2**, **3** and **4**. Compounds evaluated in a displacement binding assay using spinal cord membrane from rats and radioactive [^{3}H]SP_{1-7} tracer.

Fig. 1), but not EM-1, exhibited high affinity for the SP_{1-7} binding site.[20,21]

In our ongoing medicinal chemistry program, we aimed at developing pharmacological tools for in depth studies of the SP_{1-7} system but also employing SP_{1-7} as starting point in a design process with the long term goal to discover new chemical entities as potential new therapeutics useful for treatment of neuropathic pain. Important anchor points of SP_{1-7} and EM-2 were identified through a structure activity relationship (SAR) study.[22,23] The extensive SAR study revealed that a C-terminal primary amide is improving the binding affinity and, hence, resulted in the identification of SP_{1-7}-NH_2 with a K_i of 0.3 nM (Fig. 1). *N*-terminal truncation of SP_{1-7}-NH_2 and EM-2 along with an alanine scan led to the remarkable discovery of the dipeptide lead compound **1** (H-Phe-Phe-NH_2) with equipotent affinity (K_i = 1.5 nM) as the SP_{1-7} itself (Fig. 1).

A pharmacophore hypothesis was proposed for **1** where (*S,S*) configuration of the side-chains, primary amine in the N-terminal and primary amide in the C-terminal were essential features for binding.[24] The *in vivo* effect of **1** was further evaluated in a diabetic neuropathy model induced by streptozotocin (STZ). Following central (intrathecal, i.t.) administration **1** produced potent anti-allodynic and anti-hyperalgesic effect at a 0.5–4 pmol dosage range.[25] However, when evaluated in mice suffering from spared nerve injury (SNI) after peripheral (intraperitoneal, i.p.) administration of a dose at 185 nmol/kg, the dipeptide amide **1** failed to reach any distinct anti-allodynic effect.[12] This observation is probably due to low brain exposure since **1** displayed poor drug-like properties such as poor plasma stability, high hepatic metabolism and low permeability and stability over Caco-2 cells.[24]

With the aim to develop drug-like analogues to **1** acting as the neuropeptide SP_{1-7}, compound **2** and **3** were designed (Fig. 1).[24,26] Both compounds exhibited retained binding *in vitro* along with improved metabolic stability and permeability. When evaluated *in vivo* in the SNI model, the rigidified compound **2** displayed a strong anti-allodynic effect. The effect, however, was short-lasting with a peak of effect after 30 min, which may be attributed to low brain exposure due to high blood-brain barrier (BBB) efflux as indicated *in vitro* by Caco-2 cells measurements.[12] The carbamate **3** on the other hand, displayed no *in vitro* efflux over the Caco-2 monolayer and was shown to enter the CNS according to an *in vivo* infusion study but failed to exhibit any anti-allodynic effect in the SNI mice, possibly as a results of fast hydrolysis of the carbamate functionality in plasma.[12,26]

Herein, as an alternative to the primary amine in **2** and the carbamate in **3**, compound **4** was designed and prepared, comprising an C5 phenyl substituted imidazole carboxamide in the *N*-terminal (Fig. 1).[27] This phenylalanine isostere match both the side chain phenyl and contains nitrogen atoms that can act as hydrogen bond donors (HBD), hydrogen bond acceptors (HBA), and sites for positive charges, depending on tautomeric state and pH. The assessment of *in vitro* plasma stability and permeability as well as its *in vitro* cytotoxic profile in primary neuronal cell cultures of the new imidazole based H-Phe-Phe-NH_2 peptidomimetic **4** is presented. Furthermore, the *in vivo* anti-allodynic potential of the compound in an SNI model of neuropathic pain after peripheral administration (i.p.) was assessed. Moreover, to confront its action with the SP_{1-7}-NH_2 peptide and with gabapentin and morphine used in clinic the activity of the four compounds were compared in the SNI model.

The introduction of the *ortho*-substituted imidazole moiety in **4** complies with a suitable atom-to-atom match to the *N*-terminal phenylalanine residue in **1** (Fig. 2). In order to investigate if **1** and **4** can obtain similar spatial arrangement of their potential pharmacophore groups in low energy conformations, a pharmacophore group alignment analysis was performed using Phase.[28] Conformational analysis of **1** and **4** resulted in 69 and 76 conformations, respectively, within 21 kJ mol^{-1} of the lowest energy conformation found, which were included in the pharmacophore group alignment analysis. Requirement of matching all the mutual potential pharmacophore groups identified by Phase (three HBD, two HBA, and two aromatic rings), produced six clusters of pharmacophore group match hypotheses. The match with the lowest energy conformations (ΔE = 3.6 kJ mol^{-1} and 7.8 kJ mol^{-1} from the lowest energy conformation found for **4** and **1**, respectively) is presented in Fig. 2 and shows a very good overlap of the mutual structural features that can be of importance for target-ligand interactions. Taken together, the analysis show that compounds **4** and **1** indeed can obtain a similar spatial arrangement of their potential pharmacophore groups in low energy conformations. Albeit the rigidified **4** should not be able to cover all spatial arrangements of the more flexible **1**, the analysis supports that the *ortho*-substituted imidazole can act as a suitable mimic of the *N*-terminal phenylalanine residue in **1.**

The synthesis of **4** was performed adopting a previously described method[27] and is outlined in Scheme 1. The Pd-catalyzed C5 arylation of 1-benzyl-1*H*-imidazole was conducted under microwave irradiation for 1 h at 160 °C using bromobenzene, $Pd(OAc)_2$, $P(2\text{-furyl})_3$, pivalic acid

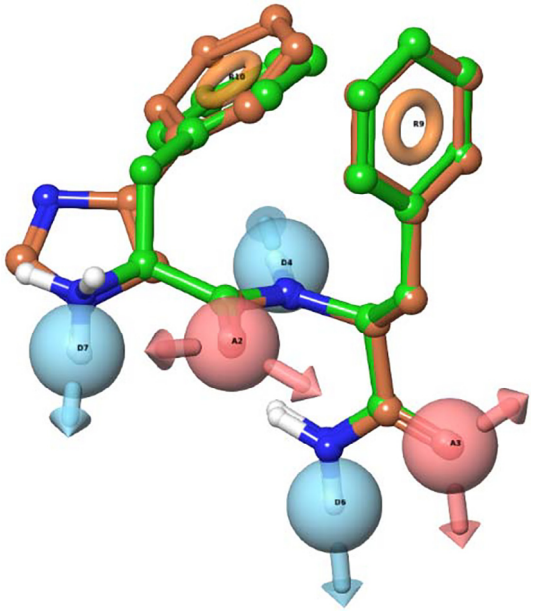
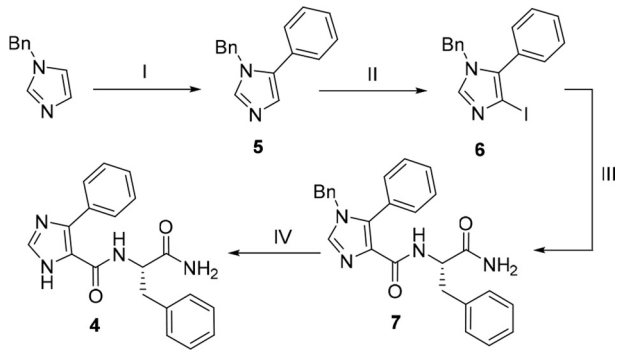


Fig. 2. Potential pharmacophore group alignment of **1** (green carbons) and **4** (orange carbons). Blue sphere = HBD, red sphere = HBA, orange torus = aromatic ring.



Scheme 1. Synthesis of target compound **4**. [a]Reaction conditions: (I) The reaction was performed under microwave irridation in a sealed vial at 160 °C for 1 h: 1-Benzyl-1*H*-imidazole, bromobenzene, $Pd(OAc)_2$, $P(2\text{-furyl})_3$, PivOH and K_2CO_3 in DMF. (II) NIS, TFA in MeCN, 80 °C for 1 h. (III) The reaction was performed in a sealed two-chamber system at 120 °C overnight: Chamber A: $Mo(CO)_6$, DBU in DMF. Chamber B: **6**, H-Phe-NH_2·HCl, $Pd(PPh_3)_4$, K_2CO_3 and DMAP in 1,4-dioxane. (IV) The reaction was performed in an autoclave at 60 °C for 6 h with H_2 (10 bar): 10% Pd/C in MeOH.

(PivOH) and potassium carbonate in DMF, securing **5** in 64% yield. Next, iodination was performed by mixing **5** with *N*-iodosuccinimide (NIS) and catalytic trifluoroacetic acid (TFA) in acetonitrile (MeCN). The reaction was stirred at 80 °C in 1 h, giving **6** in 78% yield. In the Pd-catalyzed aminocarbonylation reaction a two-chamber system set-up was used which enabled *ex situ* generation of CO from $Mo(CO)_6$. The reaction was run at 120 °C overnight and **7** was obtained in a 75% yield. Final reductive Pd-catalyzed *N*-debenzylation provided the target compound **4** (76%), resulting in an overall yield of 28% over four steps.

First, compound **4** was subjected to *in vitro* plasma stability and Caco-2 permeability in both the apical to the basolateral (a – b) and the basolateral to apical (b – a) direction in order to evaluate important pharmacokinetic parameters (Table 1). The introduction of an imidazole phenylalanine mimetic in the *N*-terminal improved the plasma stability and the compound was completely stable for more than 180 min in both human and mouse plasma. Moreover, Caco-2 permeability[29] revealed a moderate permeability of 0.84×10^{-6} cm/s (a – b). In our laboratory's classification, a P_{app} value below 0.2×10^{-6} cm/s indicates a low permeability, a P_{app} value ranging from 0.2 to 1.6×10^{-6} cm/s moderate, and a P_{app} value above 1.6×10^{-6} cm/s indicates a high permeability.[30] The a – b P_{app} value is however affected by a significant efflux as shown by the ratio of 19, most likely due to the efflux transporter multidrug resistance protein 1 (protein name: multidrug resistance protein 1 [MDR1] often referred to as P-glycoprotein [P-gp]; gene name: ABCB1).[31] It is possible that the high concentration (100 μM) used here may, at least partly, saturate the MDR1/P-gp transporter and thereby result in a higher apical to basolateral (a – b) permeability than what would have been seen if a lower concentration was used. According to previous studies (*vide supra*) an N-terminal basic moiety is important for binding to the SP_{1-7} binding site, but also for the efflux. The introduction of the less basic imidazole scaffold in the *N*-terminal did not circumvent the efflux problem.

Compound **4** did not elicit any significant neurotoxic effect *in vitro* in rat primary cortical cell cultures as compared to control. Neither did **4** affect cell viability in the MTT assay that addresses the mitochondrial function, nor in the LDH assays that is associated with membrane integrity and measure lactate dehydrogenase release from the cells (Fig. 3). Thus, a favorable safety profile was demonstrated.

Compound **4** was evaluated for its *in vivo* anti-allodynic efficacy in the SNI model[32] of neuropathic pain (Fig. 4). At 14 days after surgery, **4** was administrated i.p. and mechanical threshold was assessed by using the von Frey test.[33,34] The anti-allodynic effect of **4** was examined at four different dosages (1.85–1850 nmol/kg)[12] and compared with acidified saline as control.

Peripheral (i.p.) administration of **4** produced a time and dose dependent increase of mechanical thresholds in mice with SNI-induced neuropathic pain (Fig. 4A). Mice injected with a dose of 1850 nmol/kg and 185 nmol/kg of **4** showed a significant decrease in mechanical threshold (Fig. 4B). The anti-allodynic effect of **4** was transient and lasted 30 min whereupon it declined rapidly and reached control values at 45 min. Gratifyingly, the results suggest that the *N*-terminal rigidification displayed in compound **4** is allowed for *in vivo* efficacy and that the *ortho*-phenyl substituted imidazole carboxamide serves as an effective phenylalanine bioisostere replacement.

Furthermore, assuming that the observed *in vivo* effect requires BBB (or blood-spinal cord barrier or blood-nerve barrier) transport for a centrally mediated effect, the data (Fig. 4.) suggest that **4** has sufficient exposure, despite the high efflux measured *in vitro* and at fairly low

Table 1
In vitro plasma stability and permeability study of compound **4**.

Compound	Plasma stability $t_{½}$ (min)		Caco-2 permeability P_{app} [a] ($\times 10^{-6}$ cm/s)				
	Mouse	Human	(a – b)[b]	Mass balance (%)	(b – a)[b]	Mass balance (%)	Efflux ratio[d]
4	> 180	> 180	0.84 ± 0.11[c]	65	16.0 ± 1.50[c]	67	19

[a] P_{app} = apparent permeability coefficient.
[b] (a – b) = apical to basolateral.
[c] Average of three filters. Results are expressed as mean ± SD.
[d] Efflux ratio (b – a)/(a – b).

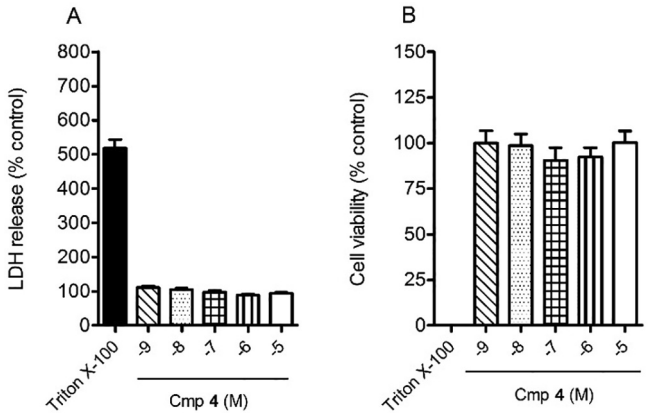


Fig. 3. Primary cortical cell culture cytotoxic death (A) and viability (B) measured in the LDH and MTT assay, respectively, after treatment with **4**. Doses are in 10^{-9}–10^{-5} M. Triton X-100 served as positive control.

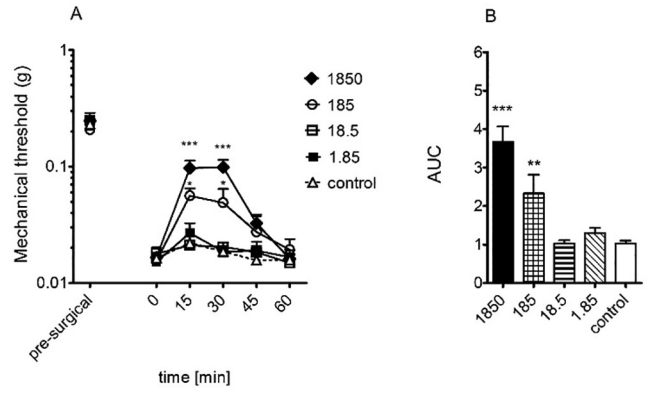


Fig. 4. Time course (A) and total effect (B) of i.p. administration of **4** on mechanical von Frey threshold SNI mice (n = 15–18). Doses are given in nmol/kg. Control mice were injected with 0.1 M acidified saline. Statistical significance was marked as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (vs. control).

doses. Thus, the *in vitro* Caco-2 assay may be a poor predictor of brain exposure in these mice, i.e. **4** is an apparent substrate for MDR1/P-gp, but has sufficient passive permeation and/or aided by active uptake transporters *in vivo*.[31]

However, it cannot be excluded that a peripheral target for full-length SP_{1-7} and corresponding peptidomimetics, such as **4**, is involved in signal transduction of the anti-allodynic effect. Observations strengthening this hypothesis were shown in a previous study indicating that SP_{1-7} modulates neurogenic inflammatory process in peripheral tissue by inhibition of the vascular inflammatory response. It has been speculated that the heptapeptide interacts with a post-terminal binding site located on endothelial cells.[35] In contrast, observations indicating that the SP_{1-7} analogues exert their effect through a centrally located target is that specific binding sites have been observed in the CNS.[19,20]

In line with this, as mentioned before, when **1** is delivered i.t. at a dose in pico molar range it exerts anti-allodynic and anti-hyperalgesic effects in diabetic mice.[25] The effectiveness of low dose administration indicated a centrally located binding site. Additionally, it has been shown that peripheral nerve injury can cause an increase in BBB, as well as blood-spinal cord barrier and blood-nerve barrier permeability.[36,37] Altogether, whether the SP_{1-7} peptidomimetic, such as compound **4** described herein, exerts its effect through a central or peripheral target, or both, needs to be further elucidated.

To further evaluate whether the small peptidomimetic compound **4** really acts and bind as the mother peptide SP_{1-7}, the SNI mice were

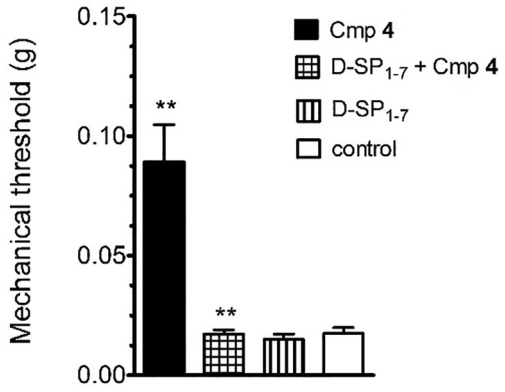


Fig. 5. Effect of D-SP_{1-7} (1850 nmol/kg, i.p.) on the anti-allodynic effect of **4** (185 nmol/kg, i.p.) in SNI mice (n = 6–9). Control mice were injected with 0.1 M acidified saline. Statistical significance was marked as follows: ** $p < 0.01$ (vs. relevant control).

pretreated with an antagonist to the SP_{1-7} binding site, [D-Pro^2, D-Phe^7]SP_{1-7} (D-SP_{1-7}, K_i = 1.8 nM) (Fig. 5).[20] This antagonist have previously been documented to reverse several *in vivo* effects displayed by the SP_{1-7}.[8,35] Here, we could for the first time reverse the analgesic effect produced by a small peptidomimetic compound with D-SP_{1-7}. Mice receiving **4** that were pretreated with D-SP_{1-7} showed lower peak mechanical threshold values than mice injected with injected with **4** alone. D-SP_{1-7} alone had no effect on mechanical thresholds in SNI mice. This observation implies that **4** and the full-length D-SP_{1-7} antagonist interacts with the same molecular target.

As mentioned before, several interesting effects on neuropathic pain have been observed for the full-length SP_{1-7} and its amidated analogue SP_{1-7}-NH_2. More specifically, the peptides have displayed anti-hyperalgesic effect in diabetic mice following i.t. administration as well as anti-allodynic effect in rats suffering from the spinal cord injury (SCI) and in SNI mice following i.p. administration, even though the plasma half-life was measured to 4.4 and 6.4 min, respectively.[10–12,38,39] Throughout the studies, the observed effect for the amidated analogue is higher than for the endogenous SP_{1-7} which corresponds well with binding data (Fig. 1). Here, the potent anti-allodynic effect evoked by **4** was compared to corresponding treatment with the full-length SP_{1-7}-NH_2 peptide and gratifyingly the small peptidomimetic compound **4** displayed equipotent effect as the full-length SP_{1-7}-NH_2 (Fig. 6A). Interestingly, this observation indicates that the prolonged plasma stability (180 min vs. 6.4 min) did neither result in a longer-lasting or increased *in vivo* anti-allodynic effect, which is in agreement with previous observation from *N*-methylated SP_{1-7}-NH_2 peptides.[40]

Finally, compound **4** was compared to gabapentin and morphine and dose-response curves are shown in Fig. 7A. When compared with

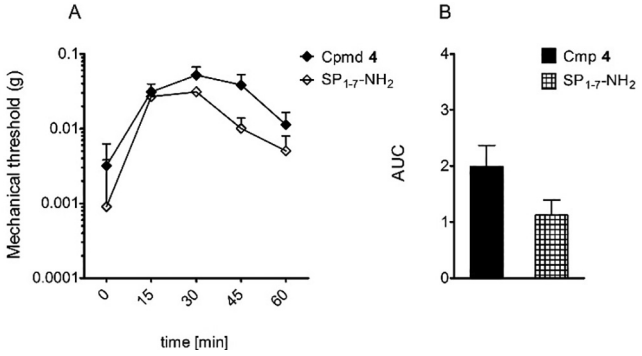


Fig. 6. Time course (A) and total effect (B) of i.p. administration of 185 nmol/kg of SP_{1-7}-NH_2 and **4** on mechanical von Frey threshold in SNI mice (n = 13–18). No statistical significance between the groups.

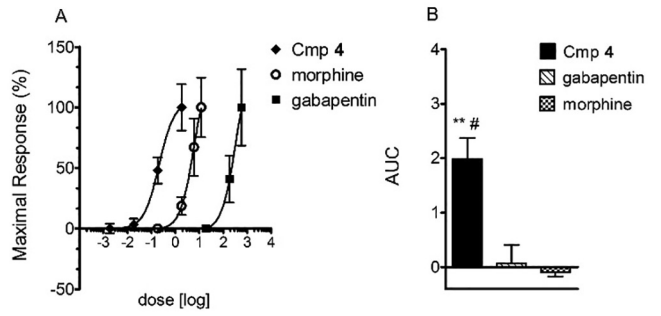


Fig. 7. Dose-response curves of **4** (1.85–1850 nmol/kg, i.p.), gabapentin (18.5–585 μmol/kg, i.p.) and morphine (0.185–12 μmol/kg, i.p.) (A) and the control subtracted total anti-allodynic effect (AUC) (B) on mechanical von Frey threshold in SNI mice (n = 10–18). Statistical significance was marked as follows: ** $p < 0.001$ (vs. gabapentin), # $p < 0.05$ (vs. morphine).

Table 2
ED_{50} values of **4**, gabapentin and morphine.[a]

Compound	ED_{50} (μmol/kg)
4	0.21**#
Gabapentin	336
Morphine	6.5

[a] Statistical significance was marked as follows: ** $p < 0.001$ (vs. gabapentin), # $p < 0.05$ (vs. morphine).

gabapentin, **4** showed effective pain relief at a 1600-times lower effective dose. This observation is in line with clinical data showing that very high doses of gabapentin ranging from 1800 to 3600 mg/day are required to achieve adequate pain suppression.[41] Furthermore, a nearly 30-times superiority in effectiveness was also observed over morphine. Additionally, the total anti-allodynic effect confirmed that it was a significant difference between the groups (Fig. 7B), since mice injected with 0.185 μmol/kg of **4** showed significantly higher AUC values than mice treated with 18.5 μmol/kg gabapentin or 0.185 μmol/kg of morphine.

ED_{50} values for these compounds are displayed in Table 2. The injection of **4** evoked a half-maximum peak anti-allodynic effect at a dose of 0.21 μmol/kg. Thus, **4** was more potent than both gabapentin with an ED_{50} value of 336 μmol/kg and morphine with an ED_{50} value of 6.5 μmol/kg. However, when increasing the dose of gabapentin to 585 μmol/kg a strong anti-allodynic effect was observed with a peak at 30 min, lasting for 60 min and reaching control values after 180 min.[40] Morphine, displayed anti-allodynic effect when mice were injected with 12 μmol/kg. The effect was short-lasting, with a peak at 45 min (see Supplementary data).

In conclusion, evaluation of a conformationally constrained imidazole based H-Phe-Phe-NH_2 analogue **4** revealed improved plasma stability, a good cell permeability but also a relatively high efflux ratio in the *in vitro* experiments. More importantly, compound **4** exhibits a strong anti-allodynic effect in the SNI mice model after peripheral administration. In fact, **4** exhibits superior effects as compared to both gabapentin and morphine that are used in clinic. Moreover, **4** displayed a favorable neurotoxic profile as deduced from cortical cell culture experiments. Design-wise, compound **4** proves that an *ortho*-phenyl substituted imidazole carboxamide serves as a bioisosteric replacement of the *N*-terminal phenylalanine in **1** based on the strong *in vivo* effect.

Altogether, these encouraging data suggest that **4** (called AS097) might serve as a promising starting point for further development into improved analgesics aimed for the management of neuropathic pain.

Acknowledgement

We gratefully acknowledge Kjell and Märta Beijer Foundation, the Berzelii Technology Centre for Neurodiagnostics, the Swedish Medical Research Council (Grant no. 9459) for financial support.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2018.06.009.

References

1. Otsuka M, Yoshioka K. *Physiol Rev.* 1993;73:229–308.
2. Nakanishi S, Nakajima Y, Yokota Y. *Regul Pept.* 1993;46:37–42.
3. Hallberg M. *Med Res Rev.* 2015;35:464–519.
4. Nyberg F, Hallberg M. *Curr Drug Targets.* 2007;8:147–154.
5. Hallberg M, Nyberg F. *Curr Protein Pept Sci.* 2003;4:31–44.
6. Sakurada C, Watanabe C, Sakurada S, Tan-No K, Sakurada T. *J Pharm Sci.* 1999;88:1127–1132.
7. Sakurada C, Watanabe C, Sakurada T. *Methods Find Exp Clin Pharmacol.* 2004;26:171–176.
8. Kreeger JS, Larson AA. *J Pharmacol Exp Ther.* 1996;279:662–667.
9. Zhou Q, Carlsson A, Hallberg M, Nyberg F. *Peptides.* 2011;32:1661–1665.
10. Carlsson A, Ohsawa M, Hallberg M, Nyberg F, Kamei J. *Eur J Pharmacol.* 2010;626:250–255.
11. Carlsson-Jonsson A, Gao T, Hao J-X, et al. *Eur J Pharmacol.* 2014;738:319–325.
12. Jonsson A, Fransson R, Haramaki Y, et al. *Neuroscience.* 2015;298:112–119.
13. Van Hecke O, Austin SK, Khan RA, Smith BH, Torrance N. *Pain.* 2014;155:654–662.
14. Costigan M, Scholz J, Woolf CJ. *Annu Rev Neurosci.* 2009;32:1–32.
15. Finnerup NB, Attal N, Haroutounian S, et al. *Lancet Neurol.* 2015;14:162–173.
16. Kremer M, Salvat E, Muller A, Yalcin I, Barrot M. *Neuroscience.* 2016;3:183–206.
17. Gilron I, Bailey JM, Tu D, Holden RR, Weaver DF, Houlden RL. *N Engl J Med.* 2005;352:1324–1334.
18. McNicol ED, Midbari A, Eisenberg E. *Opioids for Neuropathic Pain.* Rev: Cochrane Database Syst; 2013.
19. Igwe OJ, Kim DC, Seybold VS, Larson AA. *J Neurosci.* 1990;10:3653–3663.
20. Botros M, Hallberg M, Johansson T, et al. *Peptides.* 2006;27:753–759.
21. Botros M, Johansson T, Zhou Q, et al. *Peptides.* 2008;29:1820–1824.
22. Fransson R, Botros M, Nyberg F, Lindeberg G, Sandström A, Hallberg M. *Neuropeptides.* 2008;42:31–37.
23. Fransson R, Botros M, Sköld C, et al. *J Med Chem.* 2010;53:2383–2389.
24. Fransson R, Sköld C, Kratz JM, et al. *J Med Chem.* 2013;56:4953–4965.
25. Ohsawa M, Carlsson A, Asato M, et al. *Mol Pain.* 2011;7:85.
26. Fransson R, Nordvall G, Bylund J, et al. *ACS Med Chem Lett.* 2014;5:1272–1277.
27. Skogh A, Fransson R, Sköld C, Larhed M, Sandström A. *J Org Chem.* 2013;78:12251–12256.
28. Phase, version 4.5; Schrödinger, LLC: New York, NY, 2015.
29. Hubatsch I, Ragnarsson EGE, Artursson P. *Nat Protoc.* 2007;2:2111–2119.
30. Bergström CAS, Strafford M, Lazorova L, Avdeef A, Luthman K, Artursson P. *J Med Chem.* 2003;46:558–570.
31. Giacomini KM, Huang S-M, Tweedie DJ, et al. *Nat Rev Drug Discov.* 2010;9:215–236.
32. Bourquin AF, Süveges M, Pertin M, et al. *Pain.* 2006;122 14.e1-14.e14.
33. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. *J Neurosci Methods.* 1994;53:55–63.
34. Dixon W. *J Am Stat Assoc.* 1965;60:967–978.
35. Wiktelius D, Khalil Z, Nyberg F. *Peptides.* 2006;27:1490–1497.
36. Beggs S, Liu XJ, Kwan C, Salter MW. *Mol Pain.* 2010;6:74.
37. Lim TKY, Shi XQ, Martin HC, et al. *Pain.* 2014;155:954–967.
38. Ohsawa M, Carlsson A, Asato M, et al. *Peptides.* 2011;32:93–98.
39. Skogh A, Lesniak A, Gaugaz FZ, et al. *Eur J Pharm Sci.* 2017;106:345–351.
40. Skogh A, Lesniak A, Gaugaz FZ, et al. *Eur J Pharm Sci.* 2017;109:533–540.
41. Backonja M, Glanzman RL. *Clin Ther.* 2003;25:81–104.