ACS Medicinal Chemistry Letters

Letter

pubs.acs.org/acsmedchemlett

Hydrazone Linker as a Useful Tool for Preparing Chimeric Peptide/Nonpeptide Bifunctional Compounds

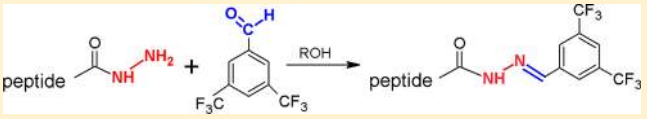
Jolanta Dyniewicz,*[,†] Piotr F. J. Lipiński,[†] Piotr Kosson,[†] Anna Leśniak,[†] Marta Bochyńska-Czyż,[†] Adriana Muchowska,[†] Dirk Tourwé,[§] Steven Ballet,[§] Aleksandra Misicka,*[,†] and Andrzej W. Lipkowski[†]

[†]Department of Neuropeptides, Mossakowski Medical Research Centre Polish Academy of Sciences, 5 Pawińskiego Str., 02-106 Warsaw, Poland

[§]Research Group of Organic Chemistry, Departments of Chemistry and Bio-engineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

Supporting Information

ABSTRACT: The area of multitarget compounds, joining two pharmacophores within one molecule, is a vivid field of research in medicinal chemistry. Not only pharmacophoric elements are essential for the design and activity of such compounds, but the type and length of linkers used to connect them are also crucial. In the present contribution, we describe compound **1** in which a typical opioid peptide sequence is combined with a fragment characteristic for neurokinin-1 receptor (NK1R) antagonists through a hydrazone bridge. The compound has a high affinity for μ- and δ-opioid receptors (IC_{50}= 12.7 and 74.0 nM, respectively) and a weak affinity for the NK1R. Molecular modeling and structural considerations explain the observed activities. In in vivo test, intrathecal and intravenous administrations of **1** exhibited a strong analgesic effect, which indicates potential BBB penetration. This letter brings an exemplary application of the hydrazone linker for fast, facile, and successful preparation of chimeric compounds.



KEYWORDS: *Bifunctional ligands, hydrazone linker, opioid agonists, NK1 antagonists*

The idea of making bivalent active compounds started to emerge as an interesting and fruitful approach in medicinal chemistry about 40 years ago, much earlier than the existence of membrane-bound receptor dimerization was discovered.[1−3] Bifunctional ligands are compounds constructed by joining two pharmacophores with a linker.[4] Essential features for their design and biological activity are not only the pharmacophoric elements but also the type and length of linkers used.[5−7] In case of peptides or chimeric compounds mixing peptide and nonpeptide elements, the hydrazide bridge (as in biphalin: H-Tyr-D-Ala-Gly-Phe-NH-NH ← Phe ← Gly ← D-Ala ← Tyr-H), piperazine, polyamide, or ethylene glycol residues have found most widespread application. The hydrazone moiety had been used in the past by Lipkowski et al. to join oxymorphone and naltrexone fragments to peptides bearing the address portion of enkephalin and dynorphin (1−8).[8] However, this strategy has not been applied in the following years, although hydrazone and *N*-acyl hydrazone (NAH) derivatives are extensively studied in small molecule medicinal chemistry and are often considered to be privileged structures.[9−11] From the standpoint of designing new bivalent ligands (be it of peptidic or chimeric character), the hydrazone function has clear benefits: the ease of synthesis, a variety of commercially available condensation reagents, and their significant contributions in conformation−activity relationship. On the basis of the advantages, we chose the linker for the synthesis of bifunctional compounds. Here we report compound JZ031: H-Tyr-D-Ala-Gly-Phe-NH-N=CH-[3′,5′-$(CF_3)_2$-Ph] (**1**) in which the hydrazone serves to join a typical opioid peptide sequence with a small moiety common to a neurokinin-1 receptor (NK1R) antagonist pharmacophore. The opioid and NK1 receptors play a key role in transmission and modulation of pain signal.[12] It was suggested that simultaneous activation of opioid receptors and blockade of NK1R may prevent side effects induced by opioids administered alone such as nausea, analgesic tolerance, euphoria, or addiction.[13−15] Therefore, a lot of attention was devoted to preparing bifuntional ligands containing opioid agonist and NK1R antagonist pharmacophores.[16−20]

Compound **1** was synthesized by the method published previously (Scheme 1).[21] Briefly, Boc-protected tetrapeptide Boc-Tyr-D-Ala-Gly-Phe-OH (**3**) was made in solution by use of DCC and HOSu as a coupling mixture in DMF. Hydrazide Boc-Tyr-D-Ala-Gly-Phe-NH-NH_2 (**2**) was also obtained by the same coupling method using an excess of hydrazine. Next, intermediate **2** was isolated by preparative RP-HPLC and mixed with 3,5-bis(trifluoromethyl)benzaldehyde in ethanol.

After a few minutes, a protected hydrazone Boc-Tyr-D-Ala-Gly-Phe-NH-N=CH-[3′,5′-$(CF_3)_2$-Ph] (**4**) was obtained in a good yield. Deprotection of **4** was carried out using trifluoroacetic acid, and the final product was purified by RP-HPLC. The purity and structure was confirmed by high-performance liquid chromatography (HPLC), electrospray

Received: September 28, 2016
Accepted: November 1, 2016
Published: November 1, 2016

DOI: 10.1021/acsmedchemlett.6b00381
ACS Med. Chem. Lett. 2017, 8, 73−77

Scheme 1. Synthesis of Compound 1 (JZ031: H-Tyr-D-Ala-Gly-Phe-NH-N=CH-[3′,5′-$(CF_3)_2$-Ph])

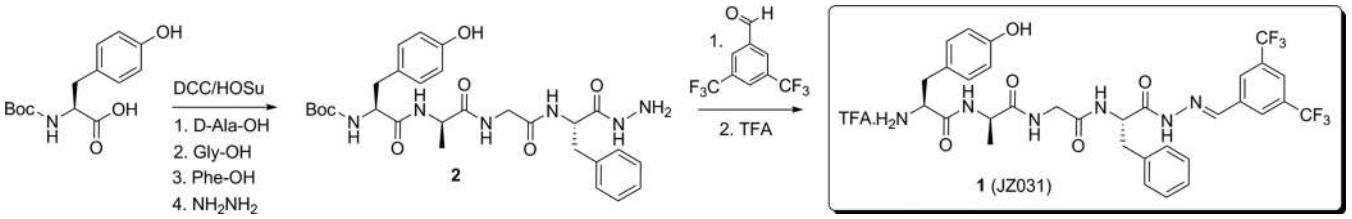


Table 1. Affinity of Bifunctional Ligands at MOR, DOR, and NK1R

compound	sequence	IC_{50}[a] (nM) MOR	DOR	NK1
1 (JZ031)	Tyr-D-Ala-Gly-Phe-NH-N=CH-[3′,5′-$(CF_3)_2$-Ph]	12.7 ± 0.7	74.2 ± 0.4	4.24 μM ± 0.8
5[22]	Tyr-D-Ala-Gly-Phe-NH-NH_2	4.7	230	n/d[b]
6[22]	Tyr-D-Ala-Gly-Phe-NH-NH ← Phe	0.74	15	n/d[b]
biphalin[22]	(Tyr-D-Ala-Gly-Phe-NH-)$_2$	1.4 ± 0.4	2.6 ± 0.3	n/d[b]
substance P	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	n/d[b]	n/d[b]	1.09 ± 0.42

[a]Mean IC_{50} value ± SEM (n = 3). [b]n/d (not determined).

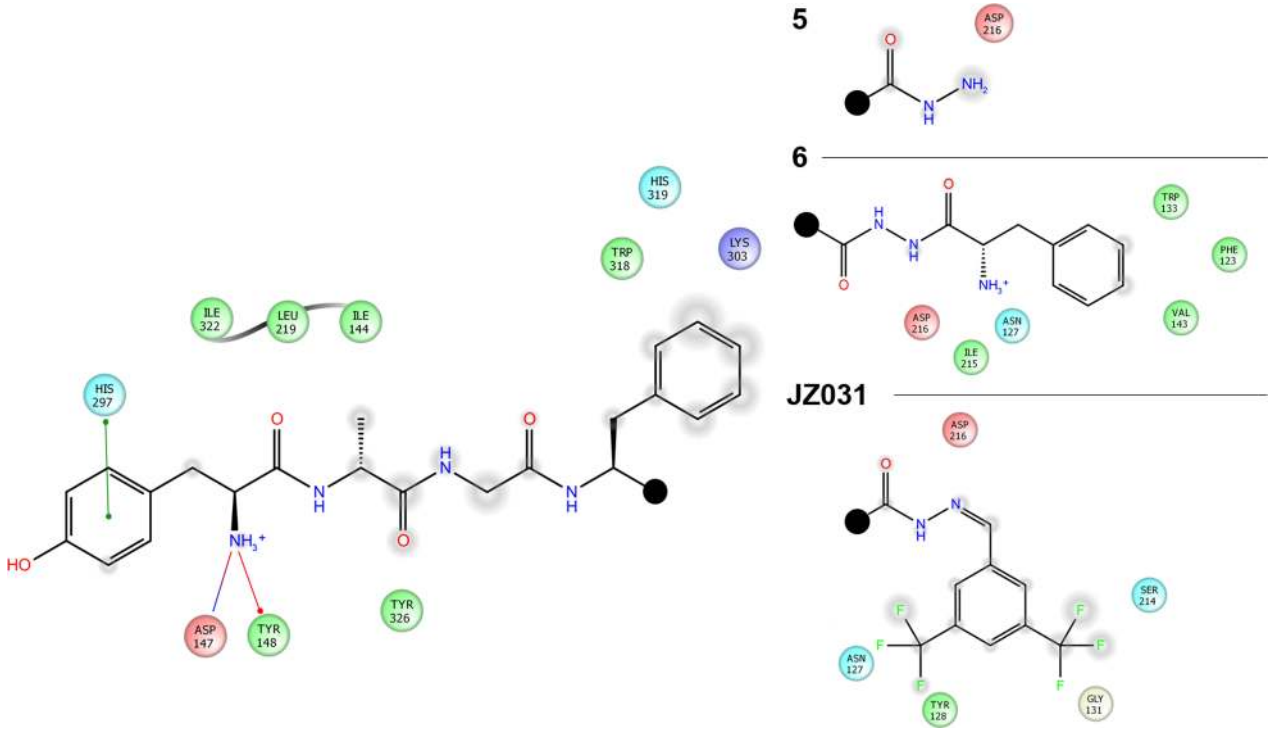


Figure 1. Contacts of compounds **1**, **5**, and **6** with the MOR binding site. On the left interactions of the Tyr-D-Ala-Gly-Phe- part common to 1, 5 and 6 are depicted; on the right, interactions of the C-terminal parts of these molecules. The black dot marks the joint between the common part and the rest of the molecule in each case.

ionization mass spectrometry (ESI-MS), and one and two-dimensional nuclear magnetic resonance methods (1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, HSQC, HMBC) NMR) (details of the experimental and analytical parts are described in the Supporting Information).

The compound JZ031 (**1**) was checked for activity both in vitro and in vivo. First, binding affinity to MOR, DOR, and NK1 receptors was determined (Table 1). JZ031 binds with IC_{50} (half maximal inhibitory concentration) of 12.7 and 74.0 nM to MOR and DOR in whole rat brain preparation, respectively. JZ031's MOR binding is lower than the one of biphalin (Tyr-D-Ala-Gly-Phe-NH-)$_2$ or its truncated variants **5** (Tyr-D-Ala-Gly-Phe-NH-NH_2) and **6** (Tyr-D-Ala-Gly-Phe-NH-NH ← Phe). Regarding DOR binding, compound **1** exhibits significantly higher affinity than hydrazide **5**, but it is far less effective than biphalin and **6**. However, compound **1** possesses only a moderate affinity for NK1R with IC_{50} = 4.24 ± 0.80 μM.

The gradation of affinities for opioid receptors in the considered molecules may be partially rationalized by molecular modeling. When docked in MOR (PDB structure: 4DKL, Figure 1), JZ031 (**1**), **5**, and **6** all have a very similar overall binding mode. The protonated amine of Tyr[1] forms a salt bridge with Asp147 and the side chain is bent so that the hydroxy group is able to interact with His297. The rest of the

DOI: 10.1021/acsmedchemlett.6b00381
ACS Med. Chem. Lett. 2017, 8, 73−77

peptide chain is extended, and the aromatic ring of Phe[4] points toward Trp318 and His319. The hydrazide moiety of **5** and **6** contacts Asp216 by hydrogen bonding. The most potent compound **6** has a second protonated free amine that is able to interact with Ile215 and Asn127. Moreover, this compound assumes a twisted conformation of the diacylhydrazine subunit,[23] which allows the aromatic ring of the terminal Phe to accommodate an additional interaction with Trp133. In contrast, JZ031 **1** is able to interact with neither Asp216 nor to place the aromatic ring in the vicinity of Trp133 (the acylhydrazone bridge is preferentially planar, or close to planarity, and extended as shown by quantum mechanical calculations[24] and a query in PDB database (see Supporting Information). However, the $-CF_3$ groups can potentially contact Asn127, Tyr128, Gly131, or Ser214. Molecular docking to DOR (PDB structure: 4RWA, Figure 3 in Supporting Information) presents similar binding poses. For all compounds, the protonated amine of Tyr[1] forms a salt bridge with Asp128, and the side chain is bent so that the hydroxy group of Tyr[1] is able to contact with His278. The rest of the peptide chain is extended and the aromatic ring of Phe[4] interacts again with Trp284. In case of DOR, however, the hydrazide bridge protons do not seem to have an interaction partner (DOR has Val197 in place of MOR Asp216), and this may explain relatively low affinity of **5** for DOR. The terminal Phe of **6** could pack in a position only slightly different than that of Phe[3] in Dmt-Tic-Phe-Phe-NH_2 (a strong DOR binder cocrystallized in 4RWA structure).[25] In the case of JZ031, the CF_3 groups can reach carbonyl oxygens of Lys108 or Glu112 for interactions.

Regarding the weak binding affinity of JZ031 to NK1R, three remarks can be made. First, based on the above-mentioned preferential planarity of the acylhydrazone linker, it may be speculated that JZ031 **1** is unable to acquire the appropriate pharmacophoric arrangement of two aromatic rings without significant distortion from the energetic minimum.[26−28] Additionally, further deterioration of the binding could arise from a suboptimal distance, as one additional atom was inserted. Finally, in the so far reported potent, bifunctional peptides containing NK1R and opioid pharmacophores, a Trp residue is always present.[29,30] Designing JZ031, we tried to mimic the two phenyl rings as found in numerous small molecule NK1R antagonists.

The high affinity of JZ031 for the opioid receptors prompted us to test it for analgesic activity in vivo. The compound exerts a strong, time- and dose-dependent analgesic effect following both systemic i.v. (intravenous) and central i.th. (intrathecal) administration. The effect was measured in plantar (i.v.) and tail-flick (i.v. and i.th.) tests (see Supporting Information for experimental details). The results are presented in Figure 2. At the supraspinal level, JZ031 evoked the strongest analgesic response 30 min after i.v. administration of 80 μmol/kg dose, reaching 75.41% ± 8.27 MPE (maximal possible effect) (Figure 2a). This effect diminished after 60 min and dropped to baseline values after 120 min. Here, our compound was less potent than morphine. However, at the spinal level (Figure 2b), the analgesic response quickly reached 84.81% ± 15.00 MPE at 5 min after administration of JZ031, whereas in the case of morphine no effect was observed at this particular time point. Thus, **1** produces an impressive rapid onset of analgesia, but the effect decreases faster than the response to morphine does. In the plantar test, only the administration of the highest 80 μmol/kg dose produced most effective and long-lasting analgesia. The peak analgesic response was recorded as early

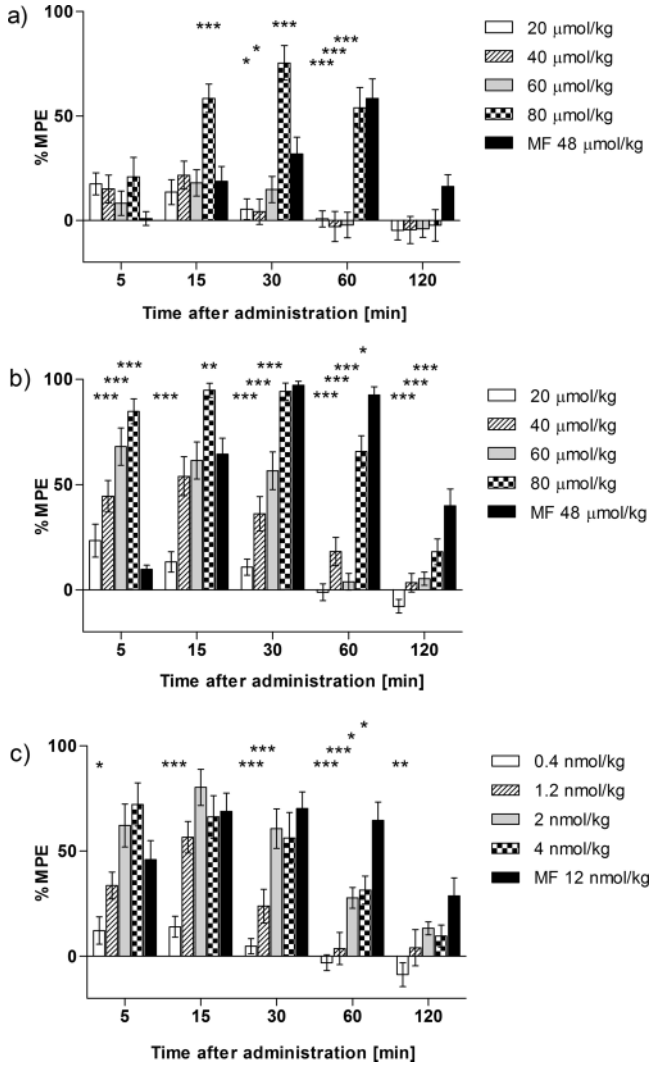


Figure 2. Analgesic responses of **1** (JZ031) in four different doses compared to morphine (MF) expressed as % MPE (means ± SEM); n = 6−8. Significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as compared to morphine. (a) i.v. administration, plantar test; (b) i.v. administration, tail-flick test; (c) i.th. administration, tail-flick test.

as 5 min postinjection and lasted up to 30 min being 94.26% ± 4.00 MPE. A decline in analgesic effect was reported 60 min after administration (69.84% ± 19.5 MPE), and thereafter, a significant loss was observed at 120 min. (16.84% ± 9.7 MPE). Intrathecal administration of JZ031 produced strong analgesic response already after 5 min (Figure 2c). The analgesic curve has a characteristic bell-shape with the peak at 15 min postinjection for all examined doses. Animals injected with **1** exhibited analgesia equipotent to that of morphine-treated animals, but at much lower dose. Here, 2 nmol/kg was enough to produce the same magnitude of peak analgesia as morphine at a dose of 12 nmol/kg, reaching 80.31% ± 8.59 MPE at 15 min postinjection. A further dose increase (4 nmol/kg) did not improve the analgesic response. Ligand **1** retained the peak activity up to 30 min postadministration, whereas morphine sustained peak analgesia up to 60 min. In the case of both compounds, a major decline in the antinociceptive effect was observed 120 min postinjection.

The analgesic effect of JZ031 (**1**) in dose 2 nmol/kg is weaker than that of biphalin, which between 5 and 30 min after

DOI: 10.1021/acsmedchemlett.6b00381
ACS Med. Chem. Lett. 2017, 8, 73−77

injection reaches 90−100% MPE and decreases to 68% MPE at 60 min.[31] This is consistent with lower affinity of compound **1** for MOR and DOR.

Taking into account the fast onset of action obtained in the tail-flick test after i.v. administration, it may be speculated that this effect is caused by fast BBB penetration of our ligand. The similar onset of morphine and JZ031 after i.th. administration and higher lipophilicity of our ligand (compared to morphine) support this explanation.

In conclusion, we presented **1** (JZ031), a chimeric compound in which a typical opioid peptide sequence is fused with a fragment characteristic of the neurokinin-1 receptor antagonist pharmacophore by means of a hydrazone bridge. The compound has high affinity for MOR and DOR, but it is only a weak binder of NK1R. Molecular modeling suggests that **1** (JZ031) preserves interactions with MOR and DOR, typically observed for opioid peptides, and the [3′,5′-$(CF_3)_2$-Ph] fragment is able to find additional contacts in the binding sites. The weak NK1R binding may be explained in terms of a larger distance between pharmacophoric aromatic rings (here, we refer to pharmacophores derived for small molecule antagonists[26−28]) and a reduced flexibility of the hydrazone bridge. Furthermore, our compound lacks a Trp residue, which is found in all so-far published potent bifunctional compounds of this kind (chimeric peptides). Gratifyingly, compound **1** (JZ031) exhibited good analgesic effects in animal models, and a remarkably fast onset of antinociception was noticed, even after i.v. administration. This report is an exemplary application of the hydrazone linker for a fast and facile preparation of bifunctional chimeric compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00381.

Experimental procedures for the synthesis, binding experiments, in vivo tests, and computational analysis, as well as compound characterization data (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: jolantadyniewicz@gmail.com.
*E-mail: misicka@chem.uw.edu.pl.

Author Contributions

D.J. synthesized all compounds, D.J., L.A., and M.A. participated in binding assays, D.J., K.P., and L.A. carried out in vivo tests, and P.F.J.L. carried out modeling study. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

The research was supported by National Science Centre (NCN) Poland, PRELUDIUM grant DEC-2011/03/N/ST5/04725 and a scholarship from the European Social Fund, Human Capital Operational Programme for the execution of the project "Support for bio tech med scientists in technology transfer"; (UDA-POKL.08.02.01-14-041/09). The work was cofinanced with the European Union funds by the European Social Fund.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Thanks to Barbara Nowicka and Zdzisława Kowalska for the help with laboratory work. Molecular modelling was supported by Computational Grant G63-10 from the Interdisciplinary Centre for Mathematical and Computer Modelling (ICM) at the University of Warsaw. Świerk Computing Centre of the National Centre for Nuclear Research in Świerk, Poland is also acknowledged for generous allotment of computer time. Project subject carried out with the use of CePT infrastructure financed by the European Union−the European Regional Development Fund within the Operational Programme "Innovative economy" for 2007-2013. S.B. and D.T. acknowledge the Research Foundation−Flanders (FWO Vlaanderen) for the financial support.

■ ABBREVIATIONS

BBB, blood−brain barrier; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DOR, δ-opioid receptor; HOSu, *N*-hydroxysuccinimide; i.th., intrathecal; i.v., intravenously; NK1R, neurokinin-1 receptor; MF, morphine; MOR, μ-opioid receptor; MPE, maximal possible effect; NAH, *N*-acyl hydrazone; NMR, nuclear magnetic resonance; RP-HPLC, reverse phase high pressure liquid chromatography; SP, substance P; TFA, trifluoroacetic acid

■ REFERENCES

(1) Hebert, T. E.; Moffett, S.; Morello, J. P.; Loisel, T. P.; Bichet, D. G.; Barret, C.; Bouvier, M. A Peptide Derived from a beta2-Adrenergic Receptor Transmembrane Domain Inhibits Both Receptor Dimerization and Activation. *J. Biol. Chem.* **1996**, *271*, 16384−16392.

(2) Cvejic, S.; Devi, L. A. Dimerization of the δ Opioid Receptor: Implication for a Role in Receptor Internalization. *J. Biol. Chem.* **1997**, *272*, 26959−26964.

(3) Ng, G. Y. K.; Coulombe, N.; Ethier, N.; Hebert, T. E.; Sullivan, R.; Kargman, S.; Chateauneuf, A.; Tsukamoto, N.; Mcdonald, T.; Johnson, M. P.; Whiting, P.; Liu, Q.; Kolakowski, L. F.; Evans, J. F.; Bonner, T. I.; O'Neill, G. P. Identification of a GABA B Receptor Subunit, gb2, Required for Functional GABA B Receptor Activity. *J. Biol. Chem.* **1999**, *274*, 7607−7610.

(4) Portoghese, P. S. From Models to Molecules: Opioid Receptor Dimers, Bivalent Ligands, and Selective Opioid Receptor Probes. *J. Med. Chem.* **2001**, *44*, 2259−2269.

(5) Lipkowski, A. W.; Konecka, A. M.; Sroczyńska, I. Double-enkephalins—Synthesis, Activity on Guinea-Pig Ileum, and Analgesic Effect. *Peptides* **1982**, *3*, 697−700.

(6) Mollica, A.; Davis, P.; Ma, S. W.; Lai, J.; Porreca, F.; Hruby, V. J. Synthesis and Biological Evaluation of New Biphalin Analogues with Non-Hydrazine Linkers. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2471−2475.

(7) Shimohigashi, Y.; Costa, T.; Chen, H. C.; Rodbard, D. Dimeric Tetrapeptide Enkephalins Display Extraordinary Selectivity for the Delta Opiate Receptor. *Nature* **1982**, *297*, 333−335.

(8) Lipkowski, A. W.; Tam, S. W.; Portoghese, P. S. Peptides as Receptor Selectivity Modulators of Opiate Pharmacophores. *J. Med. Chem.* **1986**, *29*, 1222−1225.

(9) da Silva, G. S.; Figueiró, M.; Tormena, C. F.; Coelho, F.; Almeida, W. P. Effects of Novel Acylhydrazones Derived from 4-Quinolone on the Acetylcholinesterase Activity and Aβ42 Peptide Fibrils Formation. *J. Enzyme Inhib. Med. Chem.* **2016**, *31*, 1464−1470.

(10) Duarte, C. D.; Barreiro, E. J.; Fraga, C. a M. Privileged Structures: A Useful Concept for the Rational Design of New Lead Drug Candidates. *Mini-Rev. Med. Chem.* **2007**, *7*, 1108−1119.

DOI: 10.1021/acsmedchemlett.6b00381
ACS Med. Chem. Lett. 2017, 8, 73−77

(11) Maia, R. D. C.; Tesch, R.; Fraga, C. A. M. Acylhydrazone Derivatives: A Patent Review. *Expert Opin. Ther. Pat.* **2014**, *24*, 1161−1170.
(12) Trafton, J.; Abbadie, C.; Marchand, S.; Mantyh, P. W.; Basbaum, A. I. Spinal Opioid Analgesia: How Critical Is the Regulation of Substance P Signaling? *J. Neurosci.* **1999**, *19*, 9642−9653.
(13) Powell, K. J.; Quirion, R.; Jhamandas, K. Inhibition of Neurokinin-1-Substance P Receptor and Prostanoid Activity Prevents and Reverses the Development of Morphine Tolerance in Vivo and the Morphine-Induced Increase in CGRP Expression in Cultured Dorsal Root Ganglion Neurons. *Eur. J. Neurosci.* **2003**, *18*, 1572−1583.
(14) Misterek, K.; Maszczynska, I.; Dorociak, A.; Gumulka, S. W.; Carr, D. B.; Szyfelbein, S. K.; Lipkowski, A. W. Spinal Co-Administration of Peptide Substance P Antagonist Increases Antinociceptive Effect of the Opioid Peptide Biphalin. *Life Sci.* **1994**, *54*, 939−944.
(15) Bonney Maszczynska, I.; Foran, S. E.; Marchand, J. E.; Lipkowski, A. W.; Carr, D. B. Spinal Antinociceptive Effects of AA501, a Novel Chimeric Peptide with Opioid Receptor Agonist and Tachykinin Receptor Antagonist Moieties. *Eur. J. Pharmacol.* **2004**, *488*, 91−99.
(16) Dvoracsko, S.; Stefanucci, A.; Novellino, E.; Mollica, A. The Design of Multitarget Ligands for Chronic and Neuropathic Pain. *Future Med. Chem.* **2015**, *7*, 2469−2483.
(17) Largent-Milnes, T. M.; Yamamoto, T.; Nair, P.; Moulton, J. W.; Hruby, V. J.; Lai, J.; Porreca, F.; Vanderah, T. W. Spinal or Systemic TY005, a Peptidic Opioid Agonist/neurokinin 1 Antagonist, Attenuates Pain with Reduced Tolerance. *Br. J. Pharmacol.* **2010**, *161*, 986−1001.
(18) Ballet, S.; Feytens, D.; Buysse, K.; Chung, N. N.; Lemieux, C.; Tumati, S.; Keresztes, A.; Van Duppen, J.; Lai, J.; Varga, E.; Porreca, F.; Schiller, P. W.; Vanden Broeck, J.; Tourwé, D. Design of Novel Neurokinin 1 Receptor Antagonists Based on Conformationally Constrained Aromatic Amino Acids and Discovery of a Potent Chimeric Opioid Agonist-Neurokinin 1 Receptor Antagonist. *J. Med. Chem.* **2011**, *54*, 2467−2476.
(19) Guillemyn, K.; Kleczkowska, P.; Novoa, A.; Vandormael, B.; Van den Eynde, I.; Kosson, P.; Asim, M. F.; Schiller, P. W.; Spetea, M.; Lipkowski, A. W.; Tourwé, D.; Ballet, S. In Vivo Antinociception of Potent Mu Opioid Agonist Tetrapeptide Analogues and Comparison with a Compact Opioid Agonist-Neurokinin 1 Receptor Antagonist Chimera. *Mol. Brain* **2012**, *5*, 4.
(20) Betti, C.; Starnowska, J.; Mika, J.; Dyniewicz, J.; Frankiewicz, L.; Novoa, A.; Bochynska, M.; Keresztes, A.; Kosson, P.; Makuch, W.; Van Duppen, J.; Chung, N. N.; Vanden Broeck, J.; Lipkowski, A. W.; Schiller, P. W.; Janssens, F.; Ceusters, M.; Sommen, F.; Meert, T.; Przewlocka, B.; Tourwé, D.; Ballet, S. Dual Alleviation of Acute and Neuropathic Pain by Fused Opioid Agonist-Neurokinin 1 Antagonist Peptidomimetics. *ACS Med. Chem. Lett.* **2015**, *6*, 1209−1214.
(21) Lipkowski, A. A Method of Producing an N-Blocked Biphalin Intermediate. WO2009093918 A1, July 30, 2009.
(22) Lipkowski, A. W.; Misicka, A.; Davis, P.; Stropova, D.; Janders, J.; Lachwa, M.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. Biological Activity of Fragments and Analogues of the Potent Dimeric Opioid Peptide, Biphalin. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2763−2766.
(23) Reynolds, C. H.; Hormann, R. E. Theoretical Study of the Structure and Rotational Flexibility of Diacylhydrazines: Implications for the Structure of Nonsteroidal Ecdysone Agonists and Azapeptides. *J. Am. Chem. Soc.* **1996**, *118*, 9395−9401.
(24) Pol-Fachin, L.; Fraga, C. A. M.; Barreiro, E. J.; Verli, H. Characterization of the Conformational Ensemble from Bioactive N-Acylhydrazone Derivatives. *J. Mol. Graphics Modell.* **2010**, *28*, 446−454.
(25) Fenalti, G.; Zatsepin, N. A.; Betti, C.; Giguere, P.; Han, G. W.; Ishchenko, A.; Liu, W.; Guillemyn, K.; Zhang, H.; James, D.; Wang, D.; Weierstall, U.; Spence, J. C. H.; Boutet, S.; Messerschmidt, M.; Williams, G. J.; Gati, C.; Yefanov, O. M.; White, T. A.; Oberthuer, D.; Metz, M.; Yoon, C. H.; Barty, A.; Chapman, H. N.; Basu, S.; Coe, J.; Conrad, C. E.; Fromme, R.; Fromme, P.; Tourwé, D.; Schiller, P. W.; Roth, B. L.; Ballet, S.; Katritch, V.; Stevens, R. C.; Cherezov, V. Structural Basis for Bifunctional Peptide Recognition at Human δ-Opioid Receptor. *Nat. Struct. Mol. Biol.* **2015**, *22*, 265−268.
(26) Takeuchi, Y.; Shands, E. F. B.; Beusen, D. D.; Marshall, G. R. Derivation of a Three-Dimensional Pharmacophore Model of Substance P Antagonists Bound to the Neurokinin-1 Receptor. *J. Med. Chem.* **1998**, *41*, 3609−3623.
(27) Young, J. R.; Eid, R.; Turner, C.; DeVita, R. J.; Kurtz, M. M.; Tsao, K. L. C.; Chicchi, G. G.; Wheeldon, A.; Carlson, E.; Mills, S. G. Pyrrolidine-Carboxamides and Oxadiazoles as Potent hNK1 Antagonists. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5310−5315.
(28) Silva, Y. K. C. Da; Augusto, C. V.; Barbosa, M. L. D. C.; Melo, G. M. D. A.; Queiroz, A. C.; Dias, T. D. L. M. F.; Júnior, W. B.; Barreiro, E. J.; Lima, L. M.; Alexandre-Moreira, M. S. Synthesis and Pharmacological Evaluation of Pyrazine N-Acylhydrazone Derivatives Designed as Novel Analgesic and Anti-Inflammatory Drug Candidates. *Bioorg. Med. Chem.* **2010**, *18*, 5007−5015.
(29) Nair, P.; Yamamoto, T.; Cowell, S.; Kulkarni, V.; Moye, S.; Navratilova, E.; Davis, P.; Ma, S.-W.; Vanderah, T. W.; Lai, J.; Porreca, F.; Hruby, V. J. Discovery of Tripeptide-Derived Multifunctional Ligands Possessing Delta/mu Opioid Receptor Agonist and Neurokinin 1 Receptor Antagonist Activities. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 3716−3720.
(30) Yamamoto, T.; Nair, P.; Davis, P.; Ma, S.; Navratilova, E.; Moye, S.; Tumati, S.; Lai, J.; Vanderah, T. W.; Yamamura, H. I.; Porreca, F.; Hruby, V. J. Design, Synthesis, and Biological Evaluation of Novel Bifunctional C-Terminal-Modified Peptides for Delta/mu Opioid Receptor Agonists and Neurokinin-1 Receptor Antagonists. *J. Med. Chem.* **2007**, *50*, 2779−2786.
(31) Kosson, D.; Bonney, I.; Carr, D. B.; Mayzner-Zawadzka, E.; Lipkowski, A. W. Antinociception after Intrathecal Biphalin Application in Rats: A Reevaluation and Novel, Rapid Method to Confirm Correct Catheter Tip Position. *Pharmacol. Reports* **2005**, *57*, 545−549.

DOI: 10.1021/acsmedchemlett.6b00381
ACS Med. Chem. Lett. 2017, 8, 73−77