Letter

The hydrazone linker as a useful tool for preparing chimeric peptide/non-peptide bifunctional compounds

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The hydrazone linker as a useful tool for preparing chimeric peptide/non-peptide bifunctional compounds

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KEYWORDS Bifunctional ligands, hydrazone linker, opioid agonists, NK1 antagonists.

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 nM and 74.0 nM, respectively) and a weak affinity for the NK1R. Molecular modelling 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 paper brings an exemplary application of the hydrazone linker for fast, facile and successful preparation of chimeric compounds.

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 receptors 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 non-peptide elements, the hydrazide bridge (as in biphalin: H-Tyr-D-Ala-Gly-Phe-NH-NH←Phe←Gly←D-Ala←Tyr-H), piperazine, poly-amide 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 molecules medicinal chemistry, and are often considered to be privileged structures.[9–11] From the standpoint of designing new multivalent 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 bivalent ligands containing opioid agonist and NK1R antagonist pharmacophores.[16–20]

Compound **1** was synthesized by the method published previously.[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 the preparative RP-HPLC and mixed with 3,5-bis(trifluoromethyl)benzaldehyde in ethanol.

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

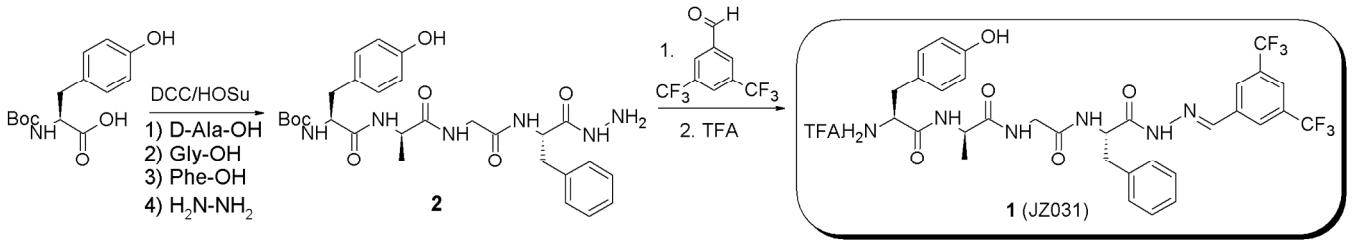

Table 1. Affinity of bifunctional ligands at MOR, DOR, and NK1R.

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 IC50 value ± SEM (n=3), [b] n/d (not determined)

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 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 nM 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**. On the other hand, 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 modelling. 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 peptide chain is extended and the aromatic ring of Phe^4 points towards 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). On the other hand, 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 co-crystallized in 4RWA structure).[25] In the case of JZ031, 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

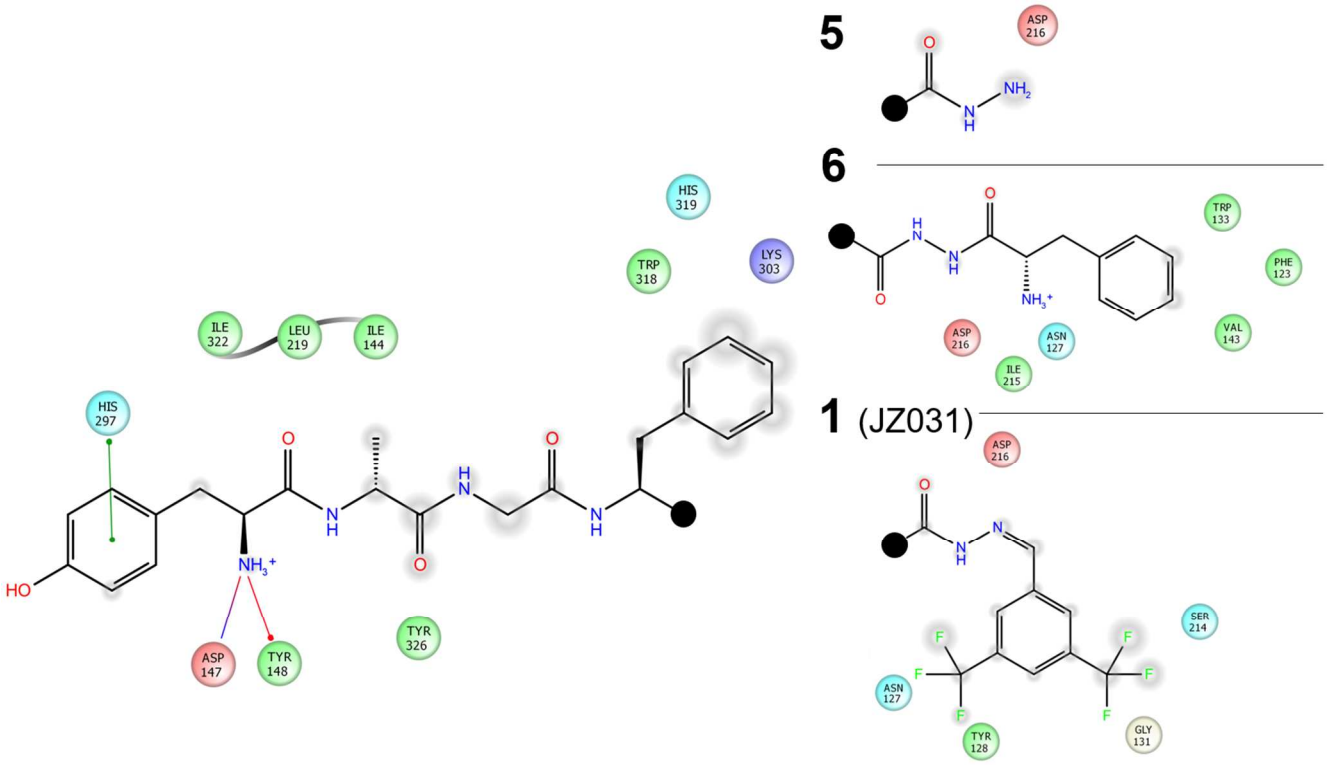

Figure 1. Contacts of compounds 1, 5 and 6 with the MOR binding site. On the left given are interactions of the Tyr-D-Ala-Gly-Phe- part common to 1, 5 and 6; on the right interactions of the C-terminal parts of these molecules. The black dot marks the joint between the common part and the rest of the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

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 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 minutes after *i.v.* administration of 80 µmol/kg dose, reaching 75.41% ± 8.27 MPE (maximal possible effect) (Figure 1a). This effect diminished after 60 minutes and dropped to baseline values after 120 minutes. Here, our compound was less potent than morphine. However, at the spinal level (Figure 1b), the analgesic response quickly reached 84.81% ± 15.00 MPE at 5 minutes 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 80µmol/kg dose produced most effective and long-lasting analgesia. The peak analgesic response was recorded as early as 5 minutes post-injection and lasted up to 30 minutes being 94.26% ± 4.00 MPE. A decline in analgesic effect was reported 60 minutes 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 minutes (Figure 1c). The analgesic curve has a characteristic bell-shape with the peak at 15 minutes post-injection 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 minutes post-injection. A further dose increase (4 nmol/kg) did not improve the analgesic response. Ligand **1** retained the peak activity up to 30 minutes post-administration, 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 minutes post-injection.

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

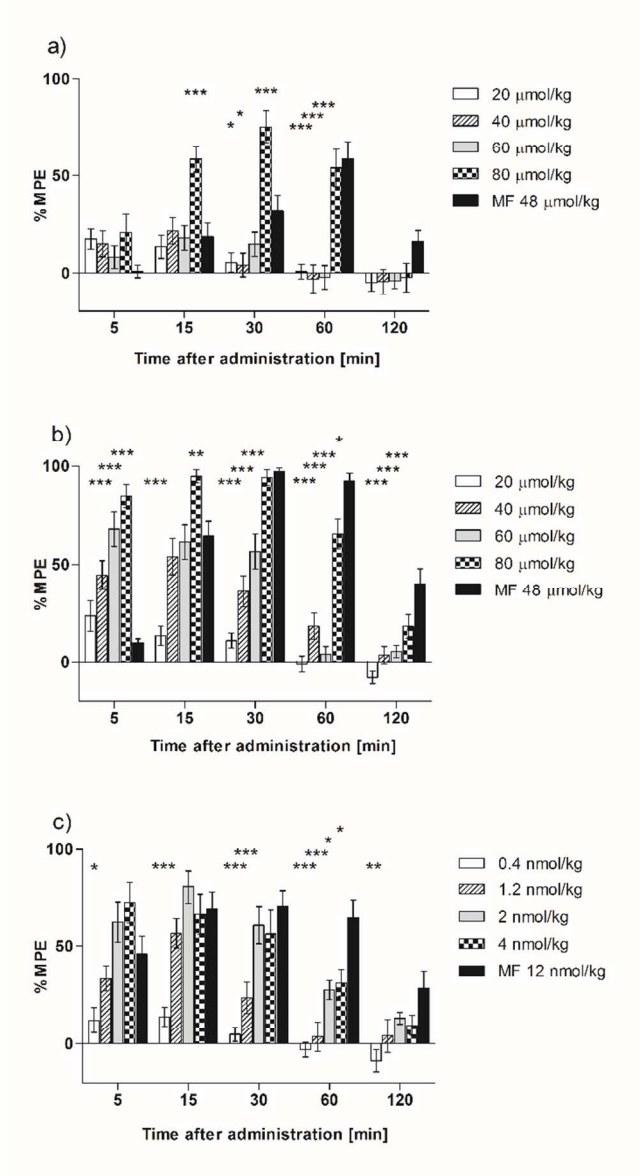

Figure 2. Analgesic responses of **1** (JZ031) in four different doses compared to morphine (MF) expressed as % MPE (means ± SEM); n=6–8. Significance: *p < 0.05; **p < 0.01 ***p < 0.001 as compared to morphine. a) i.v. administration, plantar test b) i.v. administration, tail-flick test. c) i.th. administration,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

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 modelling 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

Experimental procedures for the synthesis, binding experiments, in vivo tests and computational analysis, as well as compound characterization data are available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

DJ synthesized all compounds, DJ, LA, MA participated in binding assays, DJ, KP, LA carried out in vivo tests, PFJL 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.

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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.

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The hydrazone linker as a useful tool for preparing chimeric peptide/non-peptide bifunctional compounds

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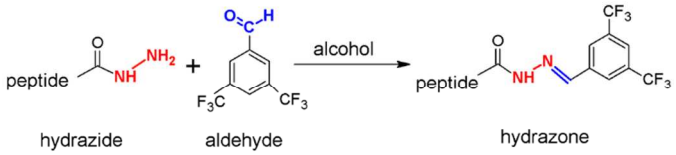

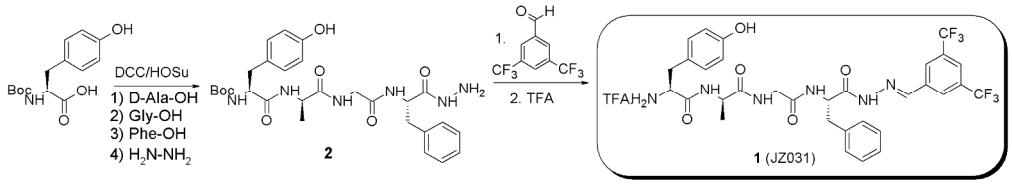

Scheme 1. The synthesis of compound 1 (JZ031: H-Tyr-D-Ala-Gly-Phe-NH-N=CH-[3',5'-(CF3)2-Ph]).

222x39mm (300 x 300 DPI)

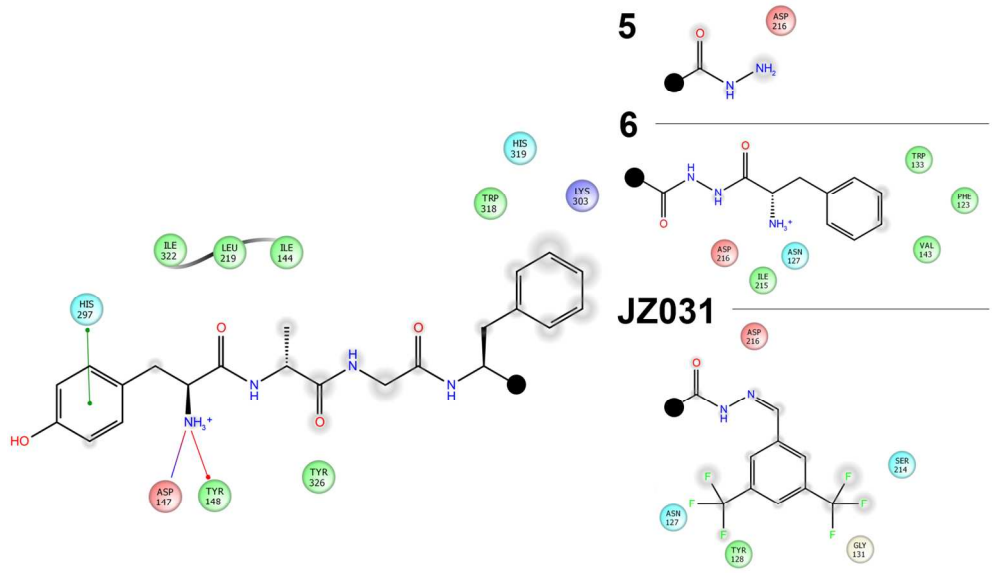

Figure 1. Contacts of compounds 1, 5 and 6 with the MOR binding site. On the left given are interactions of the Tyr-D-Ala-Gly-Phe- part common to 1, 5 and 6; on the right interactions of the C-terminal parts of these molecules. The black dot marks the joint between the common part and the rest of the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

441x272mm (96 x 96 DPI)

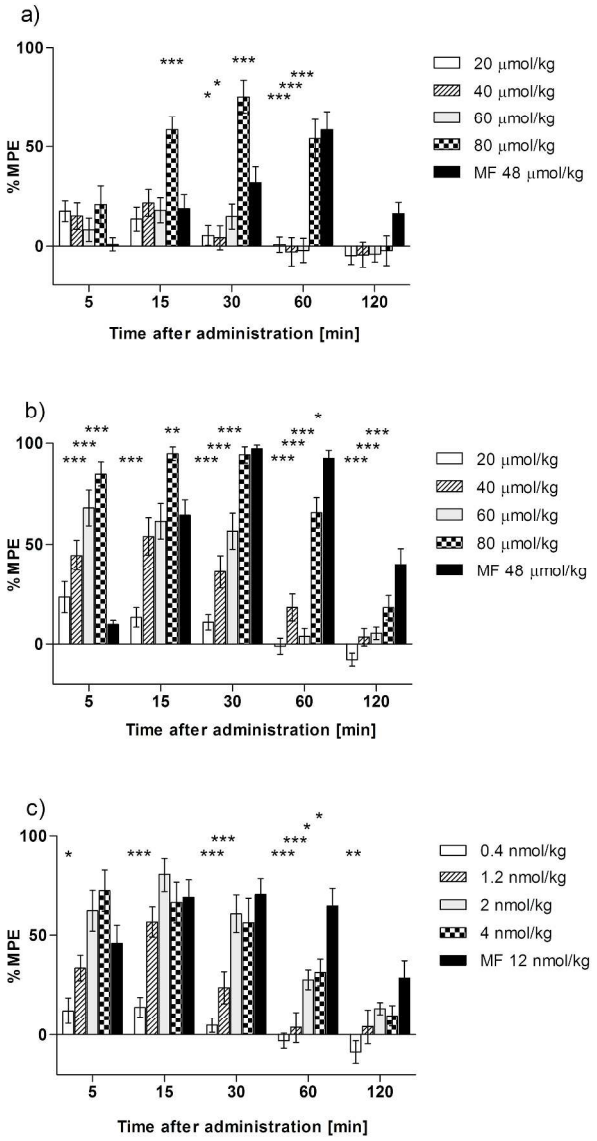

Figure 2. Analgesic responses of 1 (JZ031) in four different doses compared to morphine (MF) expressed as % MPE (means ± SEM); n=6–8. Significance: *p < 0.05; **p < 0.01 ***p < 0.001 as compared to morphine. a) i.v. administration, plantar test b) i.v. administration, tail-flick test. c) i.th. administration,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

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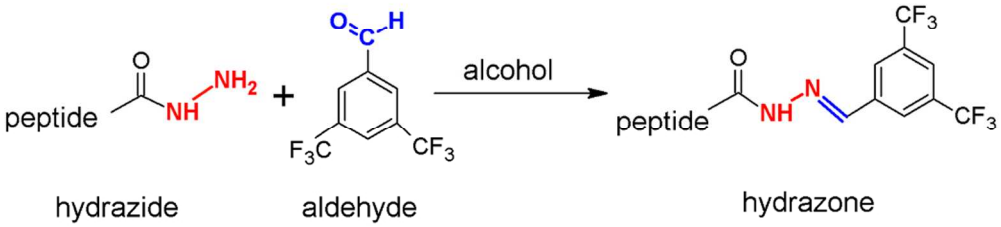

hydrazide aldehyde hydrazone

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88x34mm (299 x 299 DPI)