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Design, Synthesis and Biological evaluation of two opioid agonist and $Ca_v2.2$ blocker multi-target ligands

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Abstract: *N*-type voltage-dependent Ca^{2+} channels ($Ca_V2.2$) are located at nerve endings in the central and peripheral nervous systems and are strongly associated with the pathological processes of cerebral ischemia and neuropathic pain. $Ca_V2.2$ blockers such as the ω-conotoxin MVIIA (Prialt) are analgesic and have opioid-sparing effects. With the aim to develop new multi-target analgesic compounds, we designed the first ω-conotoxin/opioid peptidomimetics based on the enkephalin-like sequence Tyr-D-Ala-Gly-Phe (for the opioid portion) and two fragments derived from the loop-2 pharmacophore of ω-conotoxin MVIIA. Antinociceptive activity evaluated *in vitro* and *in vivo* revealed differential affinity for $Ca_V2.2$ and opioid receptors and no significant synergistic activity.

Keywords: Conotoxin, Opioid, $Ca_V2.2$, pain, analgesic, multi-target ligands.

Pharmacological management of severe and chronic pain is still a challenging task. Currently available analgesic drugs are not always efficacious, and pain control remains a large unmet therapeutic need.(1) Opioids are considered the standard of analgesic care for severe pain, but they have significant limitations in the management of neuropathic and chronic pain at

tolerable doses. Chronic opioid therapy is often connected to undesired side-effects like dyschezia, vomiting, drowsiness and hypoventilation, resulting in a poor quality life for patients.(2)

Recently, regulatory agencies in the Unites States of America and Europe approved ziconotide (Prialt) for treatment of severe neuropathic pain. This complex peptide, the synthetic analogue of ω-conotoxin MVIIA which is produced by the cone snail *Conus magus*, acts by blocking the Ca^{2+} flow mediated by *N*-type Ca^{2+} channels or $Ca_V2.2$ at the level of the spinal cord. This inhibition results in the suppression of neurotransmitter release in ascending key pain pathway, to mediate analgesia.(3)

Importantly, ziconotide has opioid-sparing effects and provides superior pain relief compared to opioids alone,(4) although serious side-effects restrict its use to a subset of chronic neuropathic pain sufferers that are refractory to opioids.(5)

Despite the high potency and selectivity for $Ca_V2.2$, and its well established role as pain regulator in humans, ziconotide is not an ideal drug due to its peptidic nature, large size, 3D structural complexity (three disulphide bridges), toxicity and administration route.

To overcome these limitations, over the last decade, several research groups have focused on developing small molecules with $Ca_V2.2$ blocking activity.(6) The ω-conotoxin pharmacophore incorporates a two-point binding model including the hydroxyl group of residue Tyr^{13} and the amino group of residue Lys^{2},(7) and was later extended to include additional residues including Arg^{10} and Leu^{11}.(8) Development of novel small molecules that mimic the functional amino acids present in loop 2 of ω-conotoxin includes dendroids by Menzler ad co-workers,(6b) linear ligands developed by Parke-Davis and cyclic peptides developed at the University of Queensland by the Lewis Group (Figure 1).(9)

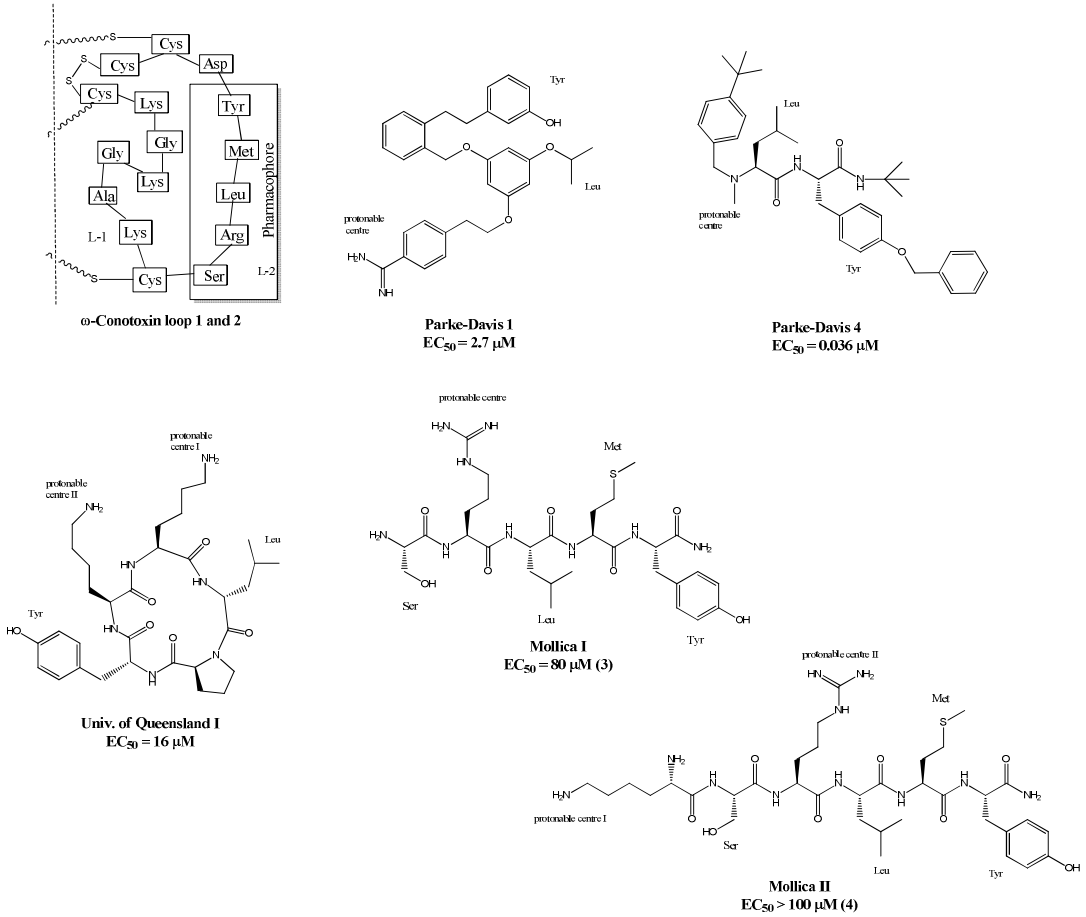

Figure 1: Relevant structural analogies between native ω-conotoxin loop-two (MVIIA), other ω-conotoxin analogues (Parke-Davis 1,4, Univ. of Queensland I) and our novel mono-functional ω-conotoxin analogues **3** and **4** (namely: Mollica I and Mollica II).(6b,9)

Interestingly, a synergistic analgesic effect is achieved by intrathecal (*i.th.*) administration of ziconotide with opioids showing a cooperative analgesic effect, accompanied by reduced development of dependence and tolerance. Pain control achieved by co-administration of morphine and Ziconotide is higher than that obtained by using one of these drugs alone.(10) This suggests that compounds with multi-target pharmacology, in particular molecules combining opioid agonist and a $Ca_V2.2$ inhibitor pharmacophores may provide superior pain relief while reducing the incidence of side effects (Table 1).

Thus, following the recent literature on bivalent ligands (11) and on the adjacent pharmacophores concept recently developed by Hruby,(12) and Ballet,(13) we report two novel multi-target analogues **1** and **2** (Figure 2) as starting point to design a new class of bivalent compounds with potent antinociceptive effects in acute and chronic pain.

For the design of multi-target peptides **1** and **2**, the well known opioid portion represented by the enkephalin-like opioid motif H-Tyr-D-Ala-Gly-Phe (YaGF) (14) was used. This sequence was chosen because it has been previously used in the design of dimeric enkephalins (H-Tyr-

D-Ala-Gly-Phe-NH-)$_2$,(15) DADLE (YaGPl) (16) and other bivalent ligands.(17) YaGF was linked through its *C*-terminus to the *N*-terminus of the ω-conotoxin pharmacophores **3** and **4** (Table 1).

Conversely, development of a short and effective ω-conotoxin pharmacophore was challenging. In the design of the conotoxin portion of peptides **1-4**, we took into account the numerous and diverse ω-conotoxin SAR published in the last two decades. Initially we selected two small linear peptides derived directly from the native sequence of ω-conotoxin loop-2: H-Ser-Arg-Leu-Met-Tyr-NH_2 (**3**) and H-Lys-Ser-Arg-Leu-Met-Tyr-NH_2 (**4**) namely Mollica I and Mollica II respectively, depicted in Figure 1.

3 is the natural linear amino acid sequence loop-2 of the ω-conotoxin MVIIA,(18) which has been further simplified to obtain the short sequence Arg-Leu-Tyr. Both sequences were used in the design of the bivalent peptides **1** and **2**. As depicted in Figure 1, the Arg-Leu-Tyr motif is frequently present in other highly active ω-conotoxin-analogues.

It was also hypothesized that an additional cationic centre, as in the analogue 'Queensland I' (Figure 1), could further enhance the binding affinity of the linear residue. Thus, the sequence H-Lys-Ser-Arg-Leu-Met-Tyr-NH_2 (**4**) was also tested for $Ca_V2.2$ blocking activity.(8,19) All results of the *in vitro* tests for the bivalent peptides **1** and **2** and the mono-functional compounds YaGF, **3** and **4** are shown in Tables 2 and 3. Compounds **1** and **2** preserved a good opioid activity but showed only a micromolar range functionality for VGGC blocking activity. Peptides **3** and **4** showed the same range of activity to *N*-Type-VGCC. All compounds where also tested by *in vivo* nociception tests (Figures 3 and 4).

Opioid portion Conotoxin portion

1: Tyr-D-Ala-Gly-Phe-Ser^{9}-Arg^{10}-Leu^{11}-Met^{12}-Tyr^{13}-NH_2

2: Tyr-D-Ala-Gly-Phe-Arg^{10}-Leu^{11}-Tyr^{13}-NH_2

Figure 2: Design of bivalent compounds **1** and **2**.

Table 1; Structures, data analysis of peptides **1**-**4**.

Name	Products	MW	ESI-MS	HPLC-RT (min)[a]
1	H-Tyr-D-Ala-Gly-Phe-Ser-Arg-Leu-Met-Tyr-NH_2	1105.5	1106.3	7.68
2	H-Tyr-D-Ala-Gly-Phe-Arg-Leu-Tyr-NH_2	888.0	889.2	5.35
Mollica I (3)	H-Ser-Arg-Leu-Met-Tyr-NH_2	667.8	668.9	7.66
Mollica II (4)	H-Lys-Ser-Arg-Leu-Met-Tyr-NH_2	795.9	796.8	7.47

[a] HPLC chromatograms were recorded by a Kromasil 100-5C18 column (4.5 mm x 250 mm / 5 µm); flow rate = 0.8 mL/min; eluent: linear gradient of H_2O/CH_3CN, 0.1% trifluoroacetic acid, starting from 10% to 60% CH_3CN in 15 min. The MS data were recorded by a LCQ (Thermo Finnigan) ion trap mass spectrometer equipped with an electrospray ionization source (ESI). The fluid was nebulized by using N_2 sheath and auxiliary gas. Capillary temperature: 300 °C, spray voltage: 4.25 kV.

Methods and Material

Chemistry

The bifunctional derivatives **1**-**2** and mono-functional conotoxin fragments **3-4** were synthesized on solid phase (SPPS), following standard Fmoc-strategy by using TBTU/HOBt for coupling reactions and piperidine 20% solution in DMF for Fmoc group deprotection as previously described.(20) Boc group for Lysine, Pbf for Arginine and tBu for Tyrosine and Serine were used as orthogonal protection groups. For detailed experimental procedures see Supporting Information.

Biological evaluation

The biological activity of compounds **1**-**4** was assessed in a range of *in vitro* assays, including µ, δ and κ receptor binding assays, G-protein stimulation assay in rat brain membranes and functional $Ca_v2.2$ assay. Mouse *in vivo* analgesic efficacy testing was also performed on peptides **1** and **2**.(6c,21) For detailed experimental procedures see Supporting Information.

Table 2: $Ca_v2.2$ and opioid receptors affinity of peptides **1-4** and reference compounds.

Compounds	Receptor binding K_i[a] (nM)			$Ca_v2.2$-mediated responses
	µ	δ	κ	EC_{50}[a,c] (µM)
Control[b]	0.36 ± 0.1	1.5 ± 0.3	5.9 ± 1.6	--
1	14.8 ± 4.8	22 ± 4.4	5.5 ± 1.5	93 ± 30
2	9.64 ± 2.4	37.4 ± 7.4	226.5 ± 57.3	72 ± 37
3	>1000	>1000	>1000	80 ± 6
4	>1000	>1000	>1000	> 100
Ziconotide[d]	--	--	--	0.055 nM
YaGF-NH_2[e]	2.8	300	--	--

[a] ± S.E.M.; [b] the control was the appropriate opioid receptor specific ligand (µ, DAMGO; δ, $Ile^{5,6}$ deltorphin II; κ, U69593); [c] EC_{50} values were calculated from the dose–response graphics; each data point was done in triplicate; [d] Ref. (22) and Ref. (17a).

Results and Discussion

In vitro binding affinity

In vitro assessment of bivalent peptides **1** and **2** and the mono-functional compounds YaGF, **3** and **4** are shown in Tables 2 and 3. Compounds **1** and **2** maintained affinity at δ and μ opioid receptors compared with the native opioid tetrapeptide YaGP-NH_2, (20a) with only a moderate decrease of the μ opiate receptor affinity and improved affinity (~ 10-fold) at the δ affinity.

Table 3: GTP binding assays of compounds **1** and **2**.

Cpd	1		2	
	E_{max}[a] (%)	$LogEC_{50}$[a] (EC_{50})[b]	E_{max}[a] (%)	$LogEC_{50}$[a] (EC_{50})[b]
Control[c]	186 ± 4	−5.97 ± 0.07 (1.06)	179 ± 5	-6.26 ± 0.13 (0.54)
+Cyp	146 ± 6	−5.62 ± 0.18 (2.38)	140 ± 4	-6.19 ± 0.21 (0.65)
+nor-BNI	158 ± 9	−5.52 ± 0.17 (3.05)	122± 4	-5.91 ± 0.34 (1.23)
+NTI	117 ± 8	−5.89 ± 0.67 (1.29)	117 ± 2	-6.53 ± 0.29 (0.29)

[a] ± S.E.M.; [b] μM; [c] The control were peptides **1-2** in absence of specific antagonist opioids receptor, (Cyp, Cyprodime, μ selective; nor-BNI, Norbinaltorphimine, κ selective; NTI, Naltrindole, δ selective).

Assessment of G-protein activation by peptides **1** and **2** towards opioid receptors was also performed. We applied the functional [^{35}S]GTPγS binding assays on rat brain membrane preparations using the specific opioid receptor antagonists for comparison. Compounds **1** and **2** stimulated G-protein activation, indicating that these two hybrid peptides were agonists at opioid receptors (Table 3), consistent with results from opioid receptor binding studies (Table 2).

All molecules **1-4** were tested for the $Ca_V2.2$ assay. In contrast to native ω-conotoxins, compounds **1**–**4** exhibited weak or no functional activity at $Ca_V2.2$, with EC_{50} values in the micromolar range (Table 2). Peptide **4** showed the weakest blocking activity for $Ca_V2.2$, indicating that the addition of a Lys in position one did not enhance activity.

These results suggest that conservation of the three-dimensional structure of the pharmacophore residues is critical for activity at $Ca_V2.2$, although a peptidomimetic with similar structure (Parke-Davis 4) was recently reported to possess nanomolar activity at $Ca_V2.2$ (Fig. 1).

In *vivo* assays

To investigate how the different pharmacological properties of compounds **1**-**4** affected their analgesic profile, the *in vivo* formalin and tail flick tests were performed on all compounds. Results are shown in Figure 3 and Figure 4.

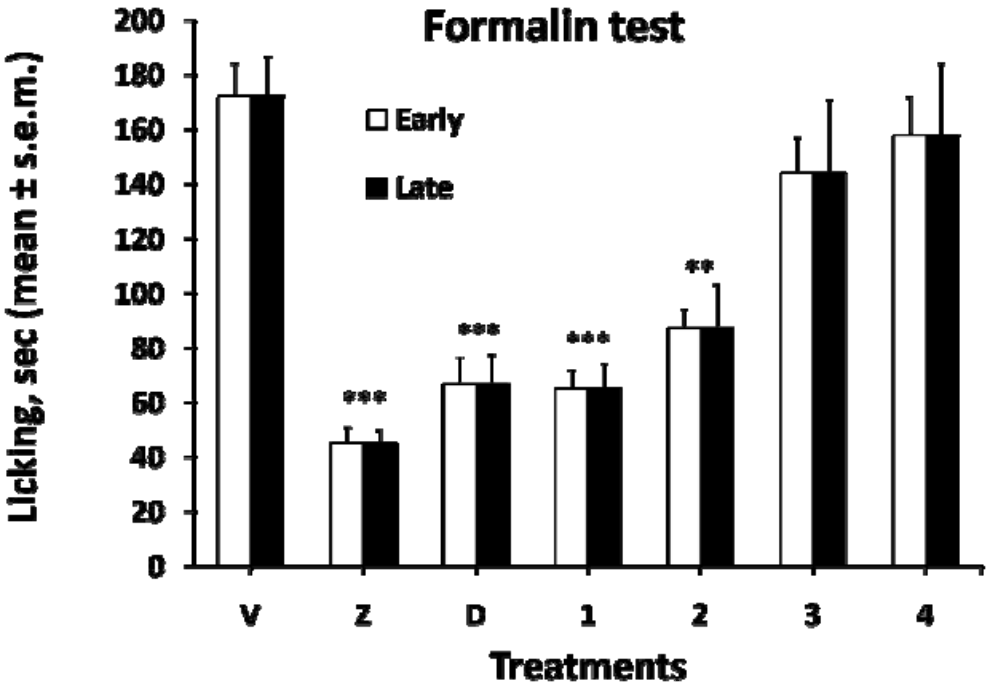

Figure 3: Antinociceptive activity (formalin test) of ziconotide (Z, 0.1 nmol), DAMGO (D, 0.1 nmol) peptides **1**-**4** (5 nmol) and Vehicle (V) after *i.th.* administration in mice (n = 10). The analgesic effects are evidenced by decreased licking (seconds, mean ± S.E.M.) quantified from 0 to 10 min (early phase) and from 15 to 40 min (late phase) following formalin injection. Statistical significance was defined as $P < 0.05$ (**$P < 0.05$, *** $P < 0.001$ vs vehicle).

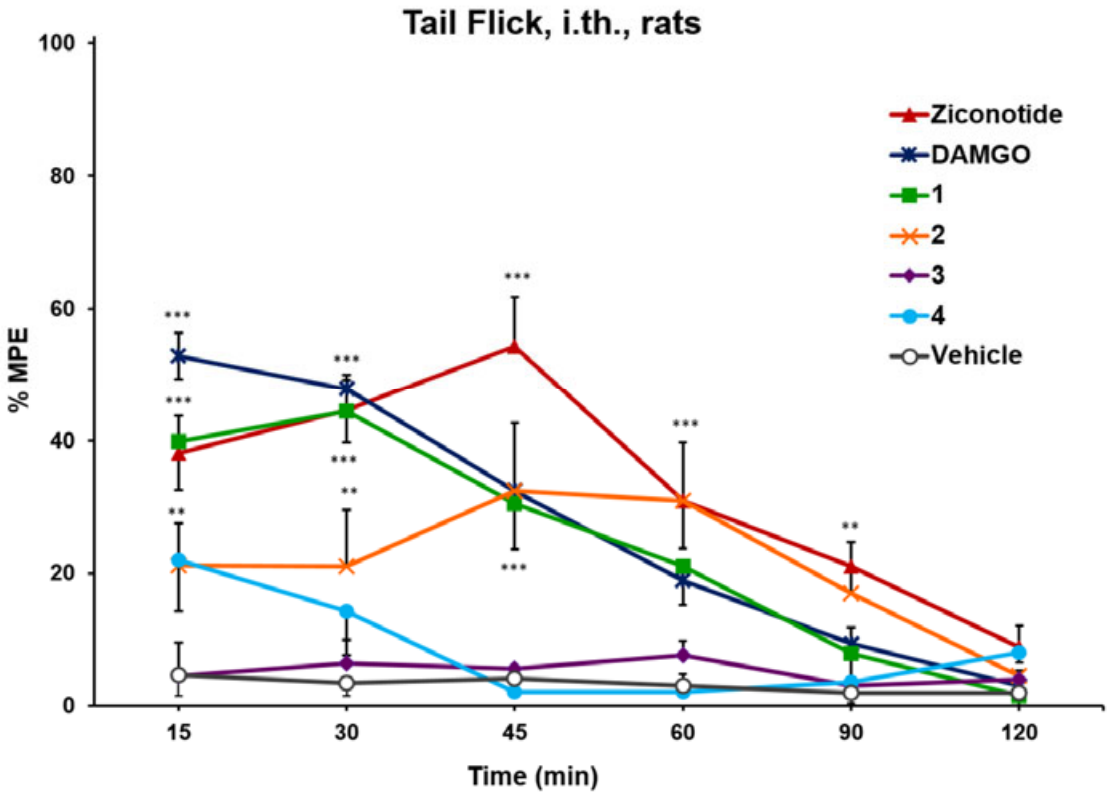

Figure 4: Time-response of the analgesic activity (Tail Flick test) of ziconotide (0.1 nmol), DAMGO (0.1 nmol) and compounds **1**-**4** (5 nmol) after *i.th.* administration in rats (n = 8). The activity is reported as percentage of the maximum possible effect (% M.P.E.) ± S.E.M. Statistical significance was assumed at P<0.05 (**P<0.05; ***P<0.001).

Both the *in vivo* formalin and tail flick data confirm that bivalent compounds **1**-**2** have a higher analgesic activity than monovalent ziconotide fragments **3** and **4**, which are almost inactive at the concentration range of the tests. Overall, the activity of peptides **1-4** in terms of potency and efficacy was modest to weak and correlated well with activity at opioid receptors.

Conclusion

Previous studies indicate that bi-functional analgesic compounds can reverse neuropathic pain in a pain models and demonstrated some ability to cross the blood-brain barrier (BBB), in specific cases even with suppression of tolerance or addiction (*i.e.* mixed opioid-NK1 antagonist Tyr-D-Ala-Gly-Phe-Met-Pro-Leu-Trp-NH-3',5'-Bn-$(CF_3)_2$] and opioid-CCK Tyr-DNle-Gly-Trp-Nle-Asp-Phe-NH_2) (12,23) validating our hypothesis that a single drug with opioid pharmacophore/$Ca_V2.2$ blocker activity could be very effective for pain treatment. This work represents the first attempt to investigate the potential of multi-target compounds combining opioid agonist and $Ca_V2.2$ inhibitor pharmacophores (Table 1). The pharmacological profile of such compounds would be expected to combine potent analgesic efficacy in acute and neuropathic pain states and reduce the extent of tolerance.

Additional advantages over a multi-drug combinations might also include: (*i*) simpler administration; (*ii*) improved pharmacokinetic properties; (*iii*) reduced drug-drug interactions and (*iv*) better tissue targeting (because of significant anatomical overlap between VGCCs and opioid receptors in the CNS).(12,13)

The data obtained in this study show that our bivalent molecules (**1** and **2**) bind and activate μ and δ opioid receptors in nanomolar range, but bind $Ca_V2.2$ with μM affinity. Compounds **3** and **4**, which were designed as small ω-conotoxin fragments and had no activity at opioid receptors, had little or no analgesic efficacy. In contrast, the bivalent derivatives **1** and **2**, which maintained good activity at opioid receptors effectively reversed pain behaviours in both the formalin and tail flick nociceptive assays despite relatively weak activity at $Ca_V2.2$. We conclude that the antinociceptive activity observed in the *in vivo* tests are likely explained by the opioid receptor activation evidence for a synergistic effect was not obtained. Although it has been previously demonstrated that properties of opioid peptides could be readily transposed to small multi-target ligands,(12,16,23) incorporation of more potent $Ca_V2.2$ activity is more challenging. Further investigations are presently underway in our laboratory to develop bivalent molecules with more potent activity at $Ca_v2.2$.

Supporting Information

Synthetic procedures, characterization data for novel compounds and the biological methods used for *in vitro*/*in vivo* bio-assays are provided in the Supporting Information File.

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References

1. Woodcock J., Witter J., Dionne R.A. (2007) Stimulating the development of mechanism-based, individualized pain therapies. Nat Rev Drug Discov;6:703-710.
2. Reisine T., Pasternak G. (1996) In Goodman and Gilman's the Pharmacological Basis of Therapeutics 9th edn. Hardman J.G., Limbird L.E., Molinoff P.B., Ruddon R.W. Eds McGraw-Hill: New York;521-577.
3. Jain K.K. (2000) An evaluation of intrathecal ziconotide for the treatment of chronic pain. Expert Opin Investig Drugs;9:2403-2410.
4. Williams J.A., Day M., Heavner J.E. (2008) Ziconotide: an update and review. Expert Opin Pharmacother;9:1575-1583.
5. (a) Lynch S.S., Cheng C.M., Yee J.L. (2006) Intrathecal ziconotide for refractory chronic pain. Ann Pharmacother;40:1293-1300. (b) Wermeling D., Drass M., Ellis D., Mayo M., McGuire D., O'Connell D., Hale V., Chao S. (2003) Pharmacokinetics and pharmacodynamics of intrathecal ziconotide in chronic pain patients. J Clin Pharmacol;43:624-636. (c) Staats P.S., Yearwood T., Charapata S.G., Presley R.W., Wallace M.S., Byas-Smith M., Fisher R., Bryce D.A., Mangieri E.A., Luther R.R., Mayo M., McGuire D., Ellis D. (2004) Intrathecal ziconotide in the treatment of

refractory pain in patients with cancer or AIDS: a randomized controlled trial. JAMA;291:63-70. (d) Rauck R.L., Wallace M.S., Leong M.S., Minehart M., Webster L.R., Charapata S.G., Abraham J.E., Buffington D.E., Ellis D., Kartzinel R. (2006) A randomized, double-blind, placebo-controlled study of intrathecal ziconotide in adults with severe chronic pain. J Pain Symptom Manage;31:393-406.

6. (a) Yamamoto T., Niwa S., Tokumasu M., Onishi T., Ohno S., Hagihara M., Koganei H., Fujita S., Takeda T., Saitou Y., Iwayama S., Takahara A., Iwata S., Shoji M. (2012) Discovery and evaluation of selective *N*-type calcium channel blockers: 6-unsubstituted-1,4-dihydropyridine-5-carboxylic acid derivatives. Bioorg Med Chem Lett;22:3639-3642.l (b) Menzler S., Bikker J.A., Suman-Chauhan N., Horwell D. C. (2000) Design and biological evaluation of non-peptide analogues of omega-conotoxin MVIIA. Bioorg Med Chem Lett;10:345-347. (c) Duggan P.J., Lewis R.J., Phei Lok Y., Lumsden N.G., Tuck K.L., Yang A. (2009) Low molecular weight non-peptide mimics of ω-conotoxin GVIA. Bioorg Med Chem Lett;19:2763-2765. (d). Baell J.B, Duggan P.J., Forsyth S.A., Lewis R. J., Lok Y.P., Schroeder C.I. (2004) Synthesis and biological evaluation of nonpeptide mimetics of ω-conotoxin GVIA. Bioorg Med Chem;12:4025-4037.
7. Kim J. I., Takahashi M., Ogura A., Kohno T., Kudo Y., Sato K. (1994) Hydroxyl group of Tyr^{13} is essential for the activity of ω-conotoxin GVIA, a peptide toxin for *N*-type calcium channel. J Biol Chem;269:23876-23878.
8. (a) Lew M.J., Flinn J.P., Pallaghy P.K., Murphy R., Whorlow S.L., Wright C.E., Norton R. S., Angus J.A. (1997) Structure-function relationships of ω-conotoxin GVIA. Synthesis, structure, calcium channel binding, and functional assay of alanine-substituted analogues. Biol Chem;272:12014-12023. (b) Schroeder C.I., Lewis R.J. (2006) ω-Conotoxins GVIA, MVIIA and CVID: SAR and clinical potential. Mar Drugs;4:193-214.
9. (a) Hu L.Y., Ryder T.R., Rafferty M.F., Dooley D.J., Geer J.J., Lotarski S.M., Miljanich G. P., Millerman E., Rock D.M., Stoehr S.J., Szoke B.G., Taylor C.P., Vartanian M.G. (1999) Structure-activity relationship of *N*-methyl-*N*-aralkyl-peptidylamines as novel *N*-type calcium channel blockers. Bioorg Med Chem Lett;9:2151-2156. (b) Ryder T.R., Hu L.Y., Rafferty M.F., Millerman E., Szoke B.G., Tarczy-Hornoch K. (1999) Multiple parallel synthesis of *N,N*-dialkyldipeptidylamines as *N*-type calcium channel blockers. Bioorg Med Chem Lett;9:1813-1818. (c) Schroeder C.I., Smythe M.L., Lewis R.J. (2004) Development of small molecules that mimic the binding of ω-conotoxins at the *N*-type voltage-gated calcium channel Mol Divers;8:127-134.
10. Alicino I., Giglio M., Manca F., Bruno F., Puntillo F. (2012) Intrathecal combination of ziconotide and morphine for refractory cancerpain: a rapidly acting and effective choice. Pain;153:245-249.
11. (a) Portoghese P.S. (2001) From models to molecules: opioid receptor dimers, bivalent ligands, and selective opioid receptor probes. J Med Chem;44:2259-2269. b) Morphy R., Rankovic Z. (2009) Designing multiple ligands – medicinal chemistry strategies and challenges. Curr Pharm Des;15:587-600. (c) Kleczkowska P.,

Lipkowski A.W., Tourwé D., Ballet S., (2011) Hybrid opioid/non-opioid ligands in pain research. Top Curr Chem;299:239-275. (d) Fujii H. (2011) Twin and triplet drugs in opioid research. Top Curr Chem;299:239-275.

12. (a) Agnes R.S., Lee Y.S., Davis P., Ma S.W., Badghisi H., Porreca F., Lai J., Hruby V.J. (2006) Structure-activity relationships of bifunctional peptides based on overlapping pharmacophores at opioid and cholecystokinin receptors. J Med Chem;49:2868-2875. (b) Hanlon K.E., Herman D.S., Agnes R.S., Largent-Milnes T.M., Kumarasinghe I.R., Ma S.W., Guo W., Lee Y.S., Ossipov M.H., Hruby V.J., Lai J., Porreca F., Vanderah T.W., (2011) Novel peptide ligands with dual acting pharmacophores designed for the pathophysiology of neuropathic pain. Brain Res;1395:1-11. (c) Agnes R.S., Ying J., Kövér K.E., Lee Y.S., Davis P., Ma S.W., Badghisi H., Porreca F., Lai J., Hruby V.J. (2008) Structure–activity relationships of bifunctional cyclic disulfide peptides based on overlapping pharmacophores at opioid and cholecystokinin receptors. Peptides;29:1413-1423. (d) Largent-Milnes T.M., Yamamoto T., Nair P., Moulton J.W., Hruby V.J., Lai J., Porreca F., Vanderah T. W. (2010) Spinal or systemic TY005, a peptidic opioidagonist/neurokinin 1 antagonist, attenuates pain with reduced tolerance. Br J Pharmacol;161:986-1001.
13. 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. (2011) 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;54:2467-2476.
14. McGregor W.H., Stein L., Belluzzi J.D. (1978) Potent analgesic activity of the enkephalin-like tetrapeptide H-Tyr-D-Ala-Gly-Phe-NH_2. Life Sci;23:1371-1376.
15. Lipkowski A.W., Konecka A.M., Sroczyńska I. (1982) Double-enkephalins - synthesis, activity on guinea-pig ileum, and analgesic effect. Peptides;3:697-700.
16. Eriksson P.S., Hansson E., Rönnbäck L., (1990) Delta and kappa opiate receptors in primary astroglial cultures from rat cerebral cortex. Neurochem Res;15:1123-1126.
17. (a) Lee Y. S., Petrov R., Park C. K., Ma S. W., Davis P., Lai J., Porreca F., Vardanyan R., Hruby V.J. (2007) Development of novel enkephalin analogues that have enhanced opioid activities at both μ and δ opioid receptors. J Med Chem;50:5028-5032. (b) Lee Y.S., Agnes R.S., Badghisi H., Davis P., Ma S.W., Lai J., Porreca F., Hruby V.J. (2006) Design and synthesis of novel hydrazide-linked bifunctional peptides as δ/μ opioid receptor agonists and cck-1/cck-2 receptor antagonists. J Med Chem;49:1773-1780.
18. Yamamoto, T. Takahara, A. (2009) Recent updates of *N*-type calcium channel blockers with therapeutic potential for neuropathic pain and stroke. Curr Top Med Chem;9:377-395.
19. (a) Kohno T., Kim J.I., Kobayashi K., Kodera Y., Maeda T., Sato K. (1995) Three-Dimensional structure in solution of the calcium channel blocker ω-conotoxin MVII. Biochemistry;34:10256-10265. (b) Nadasdi L., Yamashiro D., Chung D., Tarczy-Hornoch K., Adriaenssens P., Ramachandran J. (1995) Structure- activity analysis of

a *Conus* peptide blocker of *N*-type neuronal calcium channels. Biochemistry;34:8076-8081.

20. Mollica A., Pinnen F., Stefanucci A., Costante R. (2014) The evolution of peptide synthesis: from early days to small molecular machines. Curr Bioact Compd;9:184-202.
21. (a) Mollica A., Pinnen F., Costante R., Locatelli M., Stefanucci A., Pieretti S., Davis P., Lai J., Rankin D., Porreca F., Hruby V.J. (2013) Biological active analogues of the opioid peptide biphalin: mixed α/β^3−peptides. J Med Chem;56:3419-3423. (b) Mollica A., Costante R., Stefanucci A., Pinnen F., Lucente G., Fidanza S., Pieretti S. (2013) Antinociceptive profile of potent opioidpeptide AM94, a fluorinated analogue of biphalin with non-hydrazine linker. J Pept Sci;19:233-239. (c) Mollica A., Costante R., Stefanucci A., Pinnen F., Luisi G., Pieretti S., Borsodi A., Bojnik E., Benyhe S. (2013) Hybrid peptides endomorphin-2/DAMGO: design, synthesis and biological evaluation. Eur J Med Chem;68:167-177.
22. Lewis R. J., Nielsen K. J., Craik D. J., Loughnan M. L., Adams D. A., Sharpe I. A., Luchian T., Adams D. J., Bond T., Thomas L., Jones A., Matheson J. L., Drinkwater R., Andrews P. R., Alewood P. F. (2000) Novel ω-conotoxins from *Conus catus* discriminate among neuronal calcium channel subtypes. J Biol Chem;275:35335-35344.
23. Bonney I. M., Foran S. E., Marchand J. E., Lipkowski A. W., Carr D. B., (2004) Spinal antinociceptive effects of AA501, a novel chimeric peptide with opioid receptor agonist and tachykinin receptor antagonist moieties. Eur J Pharmacol;488:91-99.