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DESIGN, SYNTHESIS AND BIOLOGICAL PROFILE OF MIXED OPIOID AGONIST/N-VGCC BLOCKER PEPTIDES

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In this paper we reported the synthesis, the *in vitro* and the *in vivo* biological evaluation of linear pseudo-peptides incorporating the N-VGCC blocker tripeptide Phe-*N*Me-Leu-Tyr(OBz)-N*t*Bu and the biphalin pharmacophore Tyr-D-Ala-Gly-Phe. The novel sequences have been designed by using amino acids of different length to join the two pharmacophores and explore the structure activity relationships of the novel compounds.

The clinical management of chronic pain still represents a serious worldwide health problem. The currently available analgesic drugs are not always efficacious and pain control remains a large unmet therapeutic need. Opioid agonists are widely used for the treatment of moderate and severe pain; however, they have limited efficacy for neuropathic pain at tolerable doses. Recent findings in biological systems and clinical care showed that the "single-target" drugs can block or stimulate a specific target though not always produce the desired effect, because many compensatory paths are involved in their activity.[1-3]

Opioid drugs exert their activities through the stimulation of the μ-opioid receptors (MOR), which are responsible not only for the antinociceptive effect but also for a number of serious drawbacks, including the development of tolerance, physical dependence, addiction liability, urinary retention, constipation and respiratory depression, many of which are due to the up-regulation/signal inactivation of the opioid receptors.[4] Therefore, there is a vivid interest in the development of bi- or multi-functional compounds to address the complex nature of pain associated to the hyperactivation of several pro-nociceptive systems. Hybrid analgesic compounds may stimulate the opioid system in neuropathic pain and minimize the activation of compensatory pro-nociceptive systems in response to consecutive opioid administration.[5]

In the past 30 years the N-type Voltage Gated Calcium Channels (VGCC) have been elected as strength targets for analgesic development since they are involved in both ascending and descending pain pathways. Among the VGCC located in the central nervous system (CNS), Cav2.1 and Cav2.2. are abundant in presynaptic nerve terminals and are principally involved in the neurotransmitter release.[6] Cav2.2 channels are highly concentrated in dorsal root ganglia cell bodies, in the spinal cord dorsal horn and play an essential role in the perception of pain. The nociceptive signal is propagated to spino-talamic tract neurons into the spinal cord dorsal horn, where Cav2.2 mediates calcium influx and promotes the release of substance P and glutamate neurotransmitters.[6]

Dorsal horn expression of the Cav2.2 is upregulated contemporary to mechanical and thermal allodynia in rat chronic sciatic nerve constriction injury model of neuropathic pain, and its activity is modulated by G-protein coupled receptors (GPCR) activation, many of which are also targets for opioids, cannabinoids, neuropeptide Y and substance P.[7]

Interestingly, MOR and VGCC are co-localized on overlapping population of neurons in pain modulating regions of the CNS, thus molecular hybrids targeting both of them are expected to produce synergism in terms of improved potency, while decreasing the unwanted side effects.[5,8]

A multi-target molecule designed by the hybridization of Cav2.2 blocker and μ-opioid agonist, would be an ideal therapeutic candidate for chronic and neuropathic pain treatment. Recently, the venom peptide Ziconotide derived from marine cone snails *Conus magus*, has been approved by the US Food and Drug Administration (FDA) and the European

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Medicines Agency (EMA) for treatment of patients with severe chronic pain refractory to other treatments.[9] Ziconotide selectively inhibits Cav2.2 VGCC subtype resulting in analgesia in chronic pain states.[10] Successful attempts by Dr. Hruby and Dr. Ballet to link the agonist opioid activity and CCK inhibitors in one bivalent ligand,[11,12] stimulate us to design a series of analogues in which the opioid agonist enkephalin-like fragment is joined at the *C*-terminus to the *N*-terminus of a novel short ω-conotoxin pharmacophore *H*-Phe*N*(Me)-Leu-Tyr(*O*Bn)-*NHt*Bu to form a single peptide. We have selected the opioid pharmacophore Tyr-D-Ala-Gly-Phe basing on our previous papers on the super potent opioid peptide Biphalin.[13] Biphalin has two pharmacophores derived from enkephalin's sequence held together by a hydrazine spacer. This synthetic peptide is able to bind simultaneously μ- and δ-opioid receptors with good affinity due to their anatomical superimposition. Such approach has allowed to synthesize very potent opioid peptides, particularly suitable for the treatment of severe pain.[7,13]

Polt *et al.* described synergism between μ- and δ-opioid receptors by the glycopeptide MMP-2200,[14] Hruby *et al.* also reported the discovery of novel multifunctional ligands with μ/δ opioid agonist/neurokinin-1 (NK1) antagonist activities for the treatment of pain.[15] Our group previously reported a multi target analgesic drug joining together a portion of the ω-conotoxin pharmacophore and an opioid peptide, in order to obtain peptide **10** (Figure 1). The opioid agonist's pharmacophore has been attached to the N-terminal fragment of the ω-conotoxin loop 2. However, the design was not entirely satisfactory since the potency of the pharmacophore blocking the VGCC channels was significantly lower than that of the opioid portion.[16] In this study we have designed our novel bi-functional molecules by following different approaches showed schematically in Figure 2. The pharmacophores have been joined together following the *i*) overlapping pharmacophores approach to give compounds **5a-b**; *ii*) adjacent pharmacophores approach to give the compounds **6a-b** and *iii*) linked pharmacophores approach to give the compounds **7-9**. The novel N-VGCC blocker pharmacophore, namely *H*-Phe-*N*(Me)-Leu-Tyr(*O*Bn)-*NHt*Bu (IC50 8.369 nM Cav2.2; IC50 10.299 nM Cav3.2, unpublished data), was obtained by studying a series of modifications of the potent *N*-methyl-*N*-aralkyl-peptidylamine N-VGCC blocker, described by Hu et *al.*[17] aimed to introduce an *N*-terminus group suitable for coupling to the opioid pharmacophore. It is worth noting that this modified fragment is 1000 times more active than the pharmacophore used in our previous design (Ser-Arg-Leu-Met-Tyr-NH_2; EC_{50} = 80μM) [16] (Figure 1).

Opioid Pharmacophore - ω-conotoxin pharmacophore
Tyr-D-Ala-Gly-Phe-Ser-Arg-Leu-Met-Tyr-NH_2
Compound **10**

Figure 1. Bivalent peptide (**10**) previously described by Mollica *et al.*[16]

RESULTS AND DISCUSSION

The final products **5-9** have been synthetized by using combined solid phase peptide synthesis (SPPS) and solution phase peptide approach. The two approaches have been used in order to avoid the amidation of long peptides by *t*-butyl amine, which is sterically hindered. The intermediate tripeptide **3** was prepared following the Fmoc-SPPS procedure reported by Mollica *et al*. by growing the peptide on 2-Chlorotrityl chloride resin (Cl-CTC) (loading 0.60 mMol/g).[18,25] *t*-Butylamide formation required *N*-Boc protection of the last amino acid, a mild acid cleavage with 1% of TFA was performed to cleave the peptide from the resin. Then tripeptide **3** was treated with *t*-butylamine in presence of EDC·HCl, HOBt anhydrous as coupling reagents and NMM as base in DCM for 24h, to give the corresponding *t*-butylamide in 60% yield. The so obtained *t*-butylamide was deprotected at the *N* terminus, with a mixture of TFA:DCM = 1:1 at room temperature to obtain the corresponding peptide **4** as TFA salt (Figure 3). Peptide elongation was performed following the procedure previously described until to reach the complete sequence.[16] Isolation of the intermediates was performed on silica gel chromatography. Final products **5-9** were obtained as TFA salts and purified in RP-HPLC following the procedure described in Methods. Products **5** and **6** have been isolated as two distinct diastereoisomers, namely **5a,b** and **6a,b** while products **7-9** were obtained as inseparable mixture. It has been previously reported that *N*-methylated residues are prone to give from none up to 40% of racemization during the coupling. In literature is reported that during the activation of protected *N*-methyl residues, racemization can take place through tautomerization of the oxazolonium ion leading to pseudo-aromatic structures.[19]

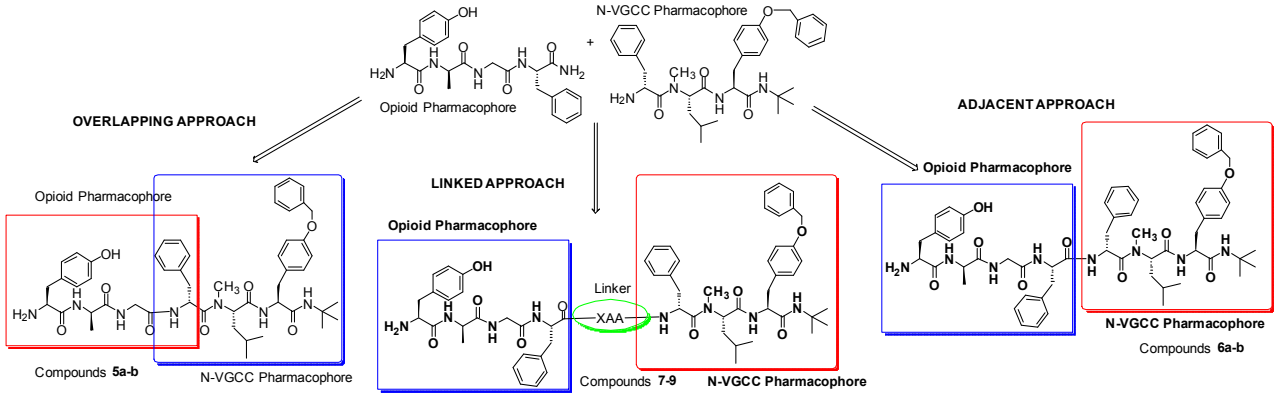

Figure 2. Different approaches used to design the bivalent peptides reported in this paper.

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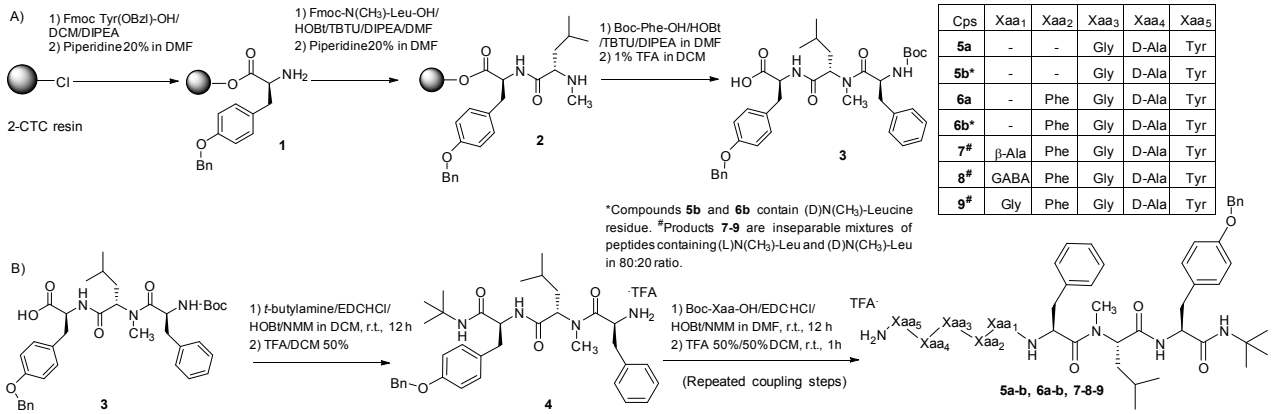
Several racemization-free procedures were tested, such as the use of HATU and Oxyme/*O*suc combination as coupling reagents,[20] however, their effects were found to be negligible to reduce the racemization to less than 20%, for the intermediate compound **3**. On the other hands, several other procedures may be applied in future to improve this synthetic step.[21] All the final peptides have been identified by MS and ^{1}H-NMR in DMSO-d6 (see SI). All peptides displayed affinity to MOR within the nanomolar range (Table 1) whereas compound **8** showed weak affinity for MOR and negligible affinity for DOR. Compounds **5a**, **5b** and **9** showed higher MOR selectivity whereas **6a** and **6b** were twice as selective towards DOR than MOR. Compound **7** displayed slightly higher selectivity for the MOR. The G-protein activity of the novel compounds was investigated in 10 μM concentration. Overall the **5-9** peptides showed week, but significant inverse agonist activity, indicated by lower [^{35}S]GTPγS specific binding compared to basal activity (100 % → ~50-80 %, Figure 2S, SI). In case of **6a**, **7** and **8** this activity was mediated by MOR, since DAMGO was able to significantly reduce the inverse agonist activity of these compounds (Figure 2S). For comparison DAMGO and IleDelt II selective ligands significantly increased the specific binding of the [^{35}S]GTPγS compared to basal level, which demonstrates their well-documented agonist activity. Compounds **5a** and **5b**, which have the best binding affinity for MOR receptor, were selected for evaluation of the blocking activity on Cav2.2. **5a** and **5b** possess activity on Cav2.2 at low micromolar range (0.453 μM and 0.710 μM respectively), thus **5a** was selected for the in vivo tests. The *in vivo* formalin (FT) and tail flick (TF) tests were performed (Figure 4). Both the *in vivo* tests confirm that **5a** is able to induce analgesia after i.t. administration. A close comparison of the analgesic effect can be done with the previously synthetized multi-target compound **10** reported by *Mollica et al.*[16] (Figure 1 and 4). Both these compounds suffer of reduced potency and efficacy if compared to a full opioid agonist such as biphalin or DAMGO. The same pattern was found for the blocking activity toward Cav2.2 when compared to ziconotide.[22-25] However, it is worth noting that the improvement of the antinociceptive effect recorded for compound **5a** compared with that previously reported for compound **10** in the tail flick test, (Figure 4), may be either related to a higher potency to the Cav2.2 and/or to better tissue penetration and pharmacokinetic. Indeed, the calcium channel blocker activity of **5a** is 200 times higher than that of compound **10.**

CONCLUSION

The aim of this work was to develop a set of new molecules to prove the hypothesis that combining an opioid pharmacophore and a N-type calcium channel ($Ca_v2.2$) blocker, would enhance the overall analgesic activity of the resulting molecules by additive or synergistic effects. The design and development of these novel hybrids peptides fall in modern research of safe and potent analgesics based on a multi-target approach that may increase their efficacy, bioavailability, and could simplify the route of administration avoiding the well-known side effects of the opiates. *In vitro* biological data showed that the pseudo tripeptide **4** containing natural phenylalanine at position 1, was active in the nanomolar range as blocking agent of the N-type calcium channels ($Ca_v2.2$), thus it was selected as Cav2.2 pharmacophore, in the preparation of bivalent compounds **5-9**. Among them, the multi target compound **5a** showed the best binding affinity for MOR, and a considerable activity as Cav2.2 blocker, about 200 folds than the activity of the parent peptide **10**,[16] but around 50 times less potent than the pharmacophore (**4**) alone.



Cps	Xaa_1	Xaa_2	Xaa_3	Xaa_4	Xaa_5
5a	-	-	Gly	D-Ala	Tyr
5b*	-	-	Gly	D-Ala	Tyr
6a	-	Phe	Gly	D-Ala	Tyr
6b*	-	Phe	Gly	D-Ala	Tyr
7#	β-Ala	Phe	Gly	D-Ala	Tyr
8#	GABA	Phe	Gly	D-Ala	Tyr
9#	Gly	Phe	Gly	D-Ala	Tyr

*Compounds **5b** and **6b** contain (D)N(CH_3)-Leucine residue. #Products **7-9** are inseparable mixtures of peptides containing (L)N(CH_3)-Leu and (D)N(CH_3)-Leu in 80:20 ratio.

Figure 3. Part A: SPPS. Part B: solution phase synthesis of the pseudo peptides **5a-b,6a-b,7-8-9**.

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Compound **5a** was tested *in vivo* following i.t. administration. The analgesic potency of the novel compounds resulted moderate, demonstrating that these hybrid molecules deserve further investigation and design efforts.

Table 1. Affinity values for DOR and MOR, Cav2.2 of **5-9** peptides in competition binding experiments.

Compound	$(K_i)^{\mu}$	$(Ki)^{\delta}$	Cav2.2 (IC_{50})
5a	12.0 nM	66.3 nM	453.0 nM
5b	12.8 nM	79.5 nM	0.71 μM
6a	140.9 nM	77.8 nM	nd
6b	95 nM	55.9 nM	nd
7	129.8 nM	209.8 nM	nd
8	218.4 nM	not relevant[3]	nd
9	61.6 nM	221.3 nM	nd
4	--	--	8.37 nM
YaGF-NH_2	2.8 nM	300 nM	--
Ziconotide	--	--	0.055 nM
Homologous lig[1]	1.5 nM	3.8 nM	--

[1]: MOR: DAMGO; DOR: IleDelt II; [2]: calculated with the K_i values; [3]: The compound did not displace [^{3}H]IleDelt II total specific binding to non-specific binding level (0 %), the maximum inhibition was ~50% at highest applied concentrations. The values are reported as means of three independent experiments. nd = not determined.

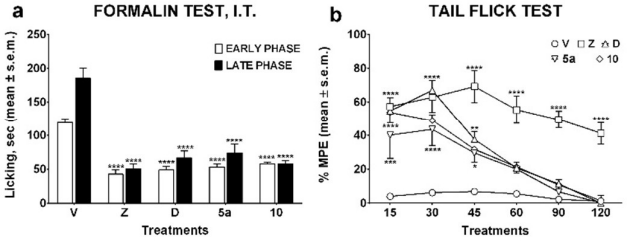

Figure 4. (a) Antinociceptive activity in the formalin test of ziconotide (Z, 0.1 nmol), DAMGO (D, 0.1 nmol) **5a** (5 nmol), **10** (5 nmol) and vehicle (V) after i.t. administration in mice (n = 10). The analgesic effects are evidenced by decreased licking (seconds, mean ± SEM) quantified from 0 to 10 min (early phase) and from 15 to 40 min (late phase) following formalin injection. Statistical significance was defined as $p < 0.05$ (****$p < 0.0001$ versus vehicle). (b) Time-response of the analgesic activity in the tail flick test of ziconotide (0.1 nmol), DAMGO (0.1 nmol), **5a** (5 nmol) and **10** (5 nmol) after i.t. administration in mice (n = 8). The activity is reported as percentage of the maximum possible effect (% M.P.E.) ± SEM. Statistical significance was assumed at $p<0.05$ (*$p<0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p<0.0001$).

DECLARATION OF INTEREST

Declared none.

Notes and references

ESI available: Materials and methods, compounds characterization, *in vivo* and *in vitro* biological assays.

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