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Design and synthesis of trivalent ligands targeting opioid, cholecystokinin, and melanocortin receptors for the treatment of pain

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ABSTRACT

It has been known that co-administration of morphine with either cholecystokinin (CCK) receptor or melanocortin (MC) receptor antagonists enhance morphine's analgesic efficacy by reducing serious side effects such as tolerance and addiction.[1–4] Considering these synergistic effects, we have designed trivalent ligands in which all three different pharmacophores for opioid, CCK, and MC receptors are combined in such a way as to conserve their own topographical pharmacophore structures. These ligands, excluding the cyclic compound, were synthesized by solid phase synthesis using Rink-amide resin under microwave assistance in very high yields. These trivalent ligands bind to their respective receptors well demonstrating that the topographical pharmacophore structures for the three receptors were retained for receptor binding. Ligand **10** was a lead compound to show the best biological activities at all three receptors.

Neuropathic pain which is induced by injury of nervous system and characterized by hyperalgesia and allodynia is particularly difficult to treat because of its complicated developmental mechanism. The most commonly used opioids such as morphine have little effect on such pain and possess a high risk of addiction, constipation, and other toxicities. Repeated administration of morphine results in enhanced levels of substance P, a transmitter of pain signals. This induces increased pain that require a higher dose of pain-relief and thus produces tolerance and intense physical dependence.[5] Cholecystokinin (CCK) acting at CCK receptors is known to give an anti-opioid effect and as a result, causes an increase of pain by inhibiting the opioid response.[6,7] Other studies have shown further that agonists for melanocortin (MC) receptors, mainly subtype MC-4R, produce an increase in response to pain stimuli.[8,9] Therefore, we have postulated that inappropriate interactions of opioid ligands with endogenous anti-opioid receptors such as CCK are one of the major problems to be addressed in treating neuropathic pain states.[10] In previous studies, we have examined a new approach for designing drugs that will be effective in these pain states, and have illustrated the potential for this approach with examples of designing in single molecule ligands that have agonist activities at μ and δ opioid receptors and antagonist activities at CCK-1/-2 receptors (or the Neurokinin 1 receptor).[11–16] In this article, we introduce the design of new trivalent ligands which were set to have biological activities at opioid, CCK, and MC receptors. The design of the trivalent ligands was based on the hypothesis of targeting multiple receptors with overlapping pharmacophores so as to retain each of their appropriate topological pharmacophore structures. Taking into account their individual structure–activity relationships (SAR), different profiles of opioid pharmacophores were linked at their C-terminal to the N-terminal of the MC pharmacophore followed by the CCK pharmacophore, and the CCK and MC pharmacophores were overlapped by a Trp residue (Fig. 1). To explore the SAR of opioid with CCK and MC pharmacophores, modifications were performed mainly in the opioid pharmacophore (Fig. 2).

Results and discussion: The ligands designed were synthesized by microwave assisted coupling with HBTU/HOBt on Rink-amide resin using the N^{α}-Fmoc strategy in high yields (overall yields >40% except for **3**–**5**)[17] and the structures were confirmed by high

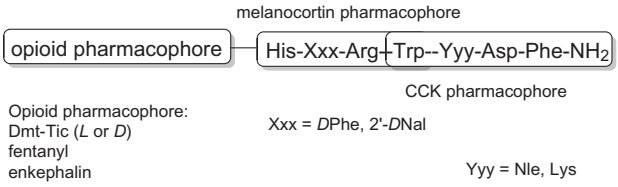

Figure 1. Design of trivalent ligands.

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1 Fen-$COCH_2CH_2CO$-His-*D*Phe-Arg-Trp-Nle-Asp-Phe-NH_2
2 His-*D*Phe(Cl)-Arg-Trp-Lys(Tyr- *D*Ala-Gly-Phe-)-Asp-Phe-NH_2
3 Tyr-c[*D*Glu-Gly-His-*D*Phe(Cl)-Arg-Trp-Lys]Asp-Phe-NH_2
4 Dmt-c[*D*Cys-Gly-His-*D*Phe-Arg-Trp-Cys]-Asp-Phe-NH_2
5 Dmt-c[*D*Cys-Gly-His-2'-*D*Nal-Arg-Trp-Cys]-Asp-Phe-NH_2
6 Dmt-*D*Tic-His-2'-*D*Nal-Arg-Trp-Nle-Asp-Phe-NH_2
7 Dmt-Tic-His-2'-*D*Nal-Arg-Trp-Nle-Asp-Phe-NH_2
8 Tyr-*D*Ala-Gly-Phe-His-*D*Phe-Arg-Trp-Nle-Asp-Phe-NH_2
9 Tyr-*D*Ala-Gly-Phe-His-2'-*D*Nal-Arg-Trp-Nle-Asp-Phe-NH_2
10 Dmt-*D*Ala-Gly-Phe-His-2'-*D*Nal -Arg-Trp-Nle-Asp-Phe-NH_2
11 Dmt-*D*Ala-Phe-His-2'-*D*Nal -Arg-Trp-Nle-Asp-Phe-NH_2
12 Tyr-*D*Ala-Phe-His-2'-*D*Nal -Arg-Trp-Nle-Asp-Phe-NH_2
13 Tyr-*D*Ala-Gly-Phe-NH_2
14 H-Trp-Nle-Asp-Phe-NH_2
15 Phe-His-2'-*D*Nal -Arg-Trp-Nle-NH_2

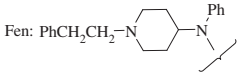

Figure 2. Structure of trivalent ligands synthesized.

resolution mass spectroscopy (Table 1). Each coupling reaction used 3 equiv HBTU/3 equiv HOBt/6 equiv DIPEA for less than 1 min under microwave condition and the syntheses went through very well resulting in high purity of peptides (Fig. 3). The crude linear peptides showed more than 80% purity and could be further purified by preparative RP-HPLC using a gradient system composed of acetonitrile and a 0.1% aqueous TFA solution. No racemized byproducts were detected by HPLC. The formation of the cyclic lactam ring of ligand **3** was accomplished using 3 equiv HBTU/3 equiv HOBt/6 equiv DIPEA for 1 h at room temperature on the solid support after removing Allyl and Alloc groups by cat. tetrakis(triphenylphosphine)palladium(0) and 25 equiv triphenylsilane. For the disulfide bond formation of ligands **4** and **5**, fully protected peptides were cleaved from resin and oxidized by the air in the presence of DMSO. From the synthetic results, it was demonstrated that microwave aided solid phase synthesis using Rink-amide resin is a very efficient method in view of shorten times and easy purification due to the clean coupling reactions.

The biological activities of the synthesized trivalent ligands were tested for their binding affinities for μ and δ opioid receptors, for CCK-1 and CCK-2 receptors, and for the MC-4 receptor. Their opioid binding affinities at the human δ opioid receptor (hDOR) and the rat μ opioid receptor (rMOR) were determined by competition analyses against [^{3}H]DPDPE (δ) and [^{3}H]DAMGO (μ) using membrane preparations from transfected HN9.10 cells that constitutively express the respective receptors.[11] Binding affinities to the CCK-1 and CCK-2 receptors were made by competition analyses against [^{3}H]CCK-8(sulfated) using stably transfected cell lines that

Table 1
Analytical data of the trivalent ligands

No.	Molecular formula	*a* log *P*[18]	LRMS[a]	HRMS[b]		HPLC[c] (t_R, min)
			Observed	Observed	Calculated	
1	$C_{74}H_{92}N_{16}O_{11}$	3.49	1381.6	1381.7310	1381.7209	18.9
2	$C_{74}H_{92}ClN_{19}O_{14}$	0.26	1506.3	1506.6829	1506.6837	13.4
3	$C_{67}H_{83}ClN_{18}O_{14}$	−0.58	1399.5	700.3059[d]	1399.6102	12.6
4	$C_{64}H_{79}N_{17}O_{13}S_2$	−0.49	1358.4	679.7847[d]	1358.5562	13.1
5	$C_{68}H_{81}N_{17}O_{13}S_2$	0.02	704.9[d]	704.7890[d]	1408.5718	13.6
6	$C_{76}H_{90}N_{16}O_{12}$	1.37	1419.5	710.3550[d]	1419.7002	15.9
7	$C_{76}H_{90}N_{16}O_{12}$	1.37	1419.5	1419.7014	1419.7002	16.5
8	$C_{74}H_{92}N_{18}O_{14}$	0.47	1457.5	729.3617[d]	1457.7118	14.9
9	$C_{78}H_{94}N_{18}O_{14}$	0.95	1507.5	754.3677[d]	1507.7274	16.8
10	$C_{80}H_{98}N_{18}O_{14}$	1.08	768.2[d]	768.3822[d]	1535.7587	17.0
11	$C_{78}H_{95}N_7O_{13}$	1.21	739.7[d]	739.8700[d]	1338.7065	17.4
12	$C_{76}H_{91}N_{17}O_{13}$	1.11	725.7[d]	725.8579[d]	1450.7059	17.7
13	$C_{23}H_{29}N_5O_5$	0.32	456.1	456.2239	456.2246	14.0
14	$C_{30}H_{38}N_6O_6$	−0.44	579.1	579.2918	579.2930	15.4
15	$C_{51}H_{63}N_{13}O_6$	0.49	954.5	954.5151	954.5102	15.6

[a] $(M-TFA+H)^+$, ESI method [Finnigan, Thermoelectron, LCQ classic].
[b] $(M-TFA+H)^+$, FAB-MS (JEOL HX110 sector instrument) or MALDI-TOF.
[c] Performed on a Hewlett-Packard 1100 [C-18, Vydac, 4.6 mm × 250 mm, 5 μm, 10–90% of acetonitrile containing 0.1% TFA within 40 min and up to 100% within additional 5 min, 1 mL/min].
[d] $(M+2H)^{2+}$.

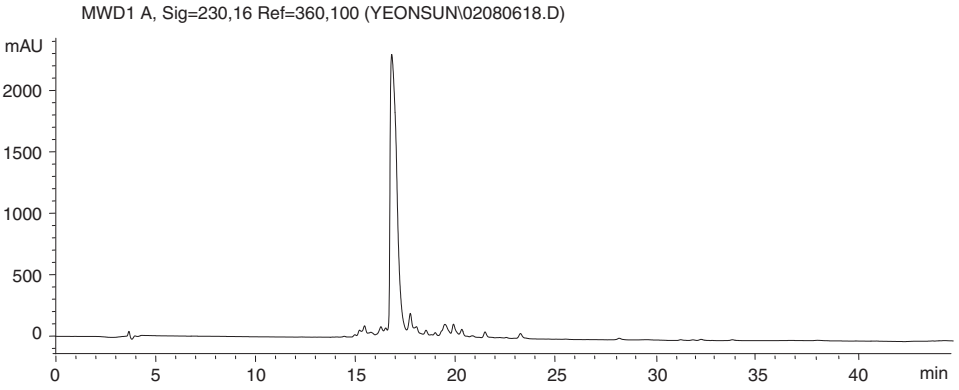

Figure 3. HPLC profile of crude **9** synthesized with microwave assistance.

express the human CCK-1 or CCK-2 receptors.[11] Transfected HEK293 cells were used to assess ligand binding at the human MC-4 receptor (hMC-4R), which was evaluated using a previously described lanthanide (Eu) based competitive binding assay.[19] Functional activities were evaluated using the stimulated isolated mouse vas deferens (MVD, δ) and guinea pig ileum (GPI, μ) bioassays, and the unstimulated GPI/LMMP (CCK receptor, vs the sulfated CCK-8 for antagonist activity) as described previously.[11]

Most ligands showed moderate to high binding affinities at both opioid and CCK receptors related to their respective structures and retained moderate binding affinity at the MC-4 receptor with the conserved pharmacophore structure with the exception of position 6 (Tables 2 and 3). While antagonist activity for the CCK receptor was not shown in the trivalent ligands except for **10**, potent opioid agonist activities for both receptors were observed in several ligands with binding affinities in the high nanomolar range. From the design point of view, a major concern was that the melanocortin receptor pharmacophore, which is located in the middle of the trivalent ligands, could maintain the same topographical structures as they would have as a α-melanotropin analog. For clarity, only two core structures from **SHU9119**,[20] which is a well known MC-4R antagonist, and **MTII**,[21] a potent agonist, were chosen for a MC pharmacophore (Fig. 4). As **SHU9119** resembles **MTII**, **9** differs from **8** only at position 6 where a *D*Phe is replaced by a 2′-*D*Nal.

To explore how such replacement affects the topographical structure, computer modeling experiments were performed on ligands **8** and **9**.[22] From these experiments it was observed that their lowest energy conformations retain a reverse turn structure at positions 5 and 6 in the MC pharmacophore, as was found for **MTII** and **SHU9119** (Fig. 5).[23] Interestingly, the aromatic groups of His^5 and 2′-*D*Nal^6 in **9** have the same orientation which results in a π−π stacking arrangement. On the contrary, ligand **8** shows the opposite orientation of the two aromatic groups of His^5 and *D*Phe^6 without π−π stacking. These results are different than those previously found for the primary structure and conformational relationships of **MTII** and **SHU9119**.[23] In these earlier studies, the two aromatic rings of His^5 and *D*Phe^6 in **MTII** were shown to be located on the same surface, whereas those of 2′-*D*Nal^6 and His^5 in **SHU9119** were on the opposite surface. Based on these observations, it can be suggested that the residue at position 6 (*D*Phe^6 or

Table 2
Binding affinities of trivalent ligands at μ/δ opioid receptors, CCK-1/CCK-2 receptors, and MC-4 receptor

No.	hDOR (δ)		rMOR (μ)		hCCK-1		hCCK-2		hMC-4	
	K_i[a] (nM)	log IC_{50}[a]	K_i[a] (nM)	log IC_{50}[a]	K_i[b] (nM)	log IC_{50}[b]	K_i[b] (nM)	log IC_{50}[b]	IC_{50}[c] (nM)	log IC_{50}[c]
1	2300	−5.3 ± 0.2	1240	−5.6 ± 0.1	>10,000	—	160	−6.9 ± 0.1	340	−6.4 ± 0.1
2	3.5	−8.1 ± 0.1	7.4	−7.5 ± 0.1	nc	—	nc	—	—	—
3	1300	−5.6 ± 0.2	2100	−5.4 ± 0.1	560	−6.9 ± 0.5	930	−6.0 ± 0.4	32	7.5 ± 0.1
4	9.1	−7.7 ± 0.1	8.6	−7.7 ± 0.1	>10,000	—	nc	—	400	5.8 ± 0.6
5	5.6	−7.9 ± 0.2	5.9	−7.9 ± 0.1	nc	—	nc	—	440	6.4 ± 0.2
6	6.2	−7.9 ± 0.1	18	−7.4 ± 0.1	32	−7.5 ± 0.2	100	−6.9 ± 0.1	160	6.8 ± 0.2
7	0.91	−8.7 ± 0.1	47	−7.0 ± 0.1	15	−7.8 ± 0.1	200	−6.7 ± 0.1	230	6.6 ± 0.1
8	6.2	−7.9 ± 0.1	14	−7.5 ± 0.1	1400	−5.6 ± 0.2	18	−7.5 ± 0.2	1300	−5.9 ± 1.5
9	2.9	−8.2 ± 0.1	0.69	−8.9 ± 0.2	9.0	−8.0 ± 0.2	120	−6.9 ± 0.1	260	6.6 ± 0.1
10	0.21	−9.4 ± 0.1	0.13	−9.6 ± 0.1	18	−7.7 ± 0.2	110	−6.9 ± 0.1	160	−6.8 ± 0.1
11	3.7	−8.1 ± 0.1	4.9	−8.0 ± 0.0	0.81	−9.1 ± 1.1	180	−6.7 ± 0.1	500	−6.3 ± 0.2
12	42	−7.1 ± 0.1	250	−6.3 ± 0.1	1.6	−8.8 ± 0.8	190	−6.7 ± 0.1	700	−6.2 ± 0.1
13	300	−6.1 ± 0.2	2.8	−8.2 ± 0.1	—	—	—	—	—	—
14	—	—	—	—	nc	—	61	−7.2 ± 0.1	—	—
15	—	—	—	—	—	—	—	—	200	−6.7 ± 0.1

nc, no competition.
[a] Competition analyses against radiolabeled ligand ([^{3}H]DPDPE for δ, [^{3}H]DAMGO for μ) were carried out using membrane preparations from transfected HN9.10 cells that constitutively express the respective receptor types.
[b] Competition analyses against radiolabeled ligand ([^{125}I]CCK-8(SO_3) were carried out using whole cell lysate preparations from transfected HEK293 cells that constitutively expressed the respective receptor types.
[c] Competetion analyses against lanthanide labeled ligand (Eu-NDP-αMSH) were carried out using whole cell lysate preparations from transfected HEK293 cells that constitutively expressed the hMC-4 receptor.

Table 3
Functional assay result for trivalent ligands at opioid, and CCK receptors

No.	Opioid, IC_{50}[a] (nM)		CCK GPI/LMMP (nM)	
	MVD (δ)	GPI (μ)	Agonist[b] (%)	Antagonist Ke[c] (nM)
1	730 ± 77	4% at 1 μM	0	None at 1 μM
2	240 ± 56	330 ± 160	0	None at 1 μM
3	16% at 1 μM	0% at 1 μM	0	None at 1 μM
4	410 ± 130	190 ± 38	0	None at 1 μM
5	610 ± 190	280 ± 69	0	None at 1 μM
6	130 ± 55	570 ± 180	0	None at 1 μM
7	2.5 ± 0.9[d], antagonist	5% at 1 μM	0	None at 1 μM
8	40 ± 4	130 ± 13	0	None at 1 μM
9	60 ± 16	200 ± 46	0	None at 1 μM
10	11 ± 3	8.3 ± 1.6	0	940 ± 480
11	110 ± 32	66 ± 7	0	None at 1 μM
12	370 ± 99	2300 ± 400	0	None at 1 μM
13	120 ± 13	47 ± 9	—	—

[a] Concentration at 50% inhibition of muscle concentration at electrically stimulated isolated tissues.
[b] Contraction of isolated tissue relative to initial muscle contraction with KCl at 1 μM.
[c] Inhibitory activity against the CCK-8 induced muscle contraction.
[d] Ke, concentration of antagonist needed to inhibit DPDPE to half its activity.

MTII Ac-Nle-c[Asp-His-*D*Phe-Arg-Trp-Lys]-NH_2
SHU9119 Ac-Nle-c[Asp-His-2'-*D*Nal-Arg-Trp-Lys]-NH_2

Figure 4. Structure of MC-4R ligands.

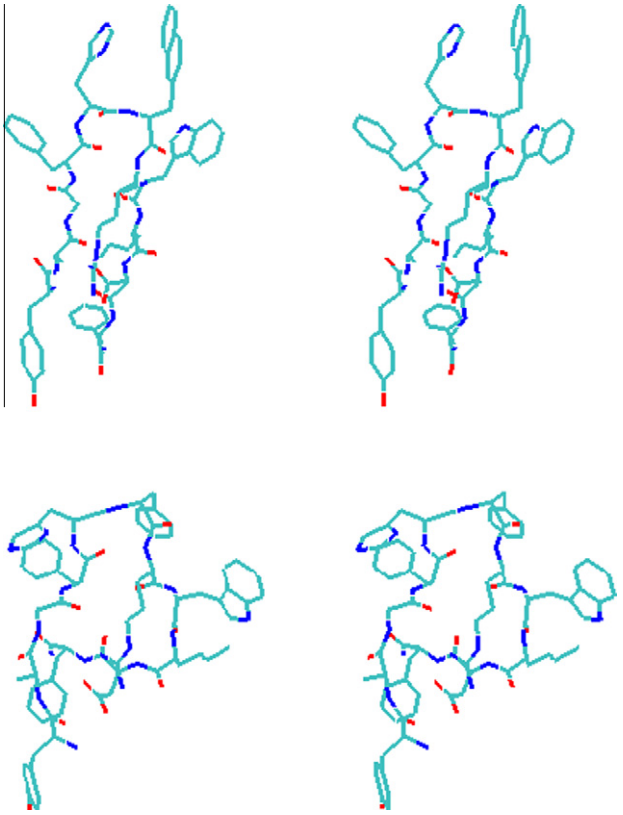

Figure 5. Stereo views of the lowest energy conformations of **8** (lower) and **9** (upper). The figures were produced using VMD.[24] Hydrogens were omitted for the clarity.

2′-DNal) might play an important role in determining the function of these trivalent ligands at the MC-4 receptor, and the *D*Phe^6 in **8** might be involved in antagonist activity due to the opposite surface location of the two aromatic groups, unlike in **MTII**. Interestingly, the 6 position residue in the MC pharmacophore has an effect not only on MC-4 receptor but also on CCK receptors. The CCK-2 selectivity (CCK-1/CCK-2 = 78) of **8** with a *D*Phe^6 residue over the CCK-1 receptor was reversed by a factor of 1000 in CCK-1 selectivity (CCK-1/CCK-2 = 0.075) by replacement of the 2′-*D*Nal^6 in **9** (Table 2). This applied to all of the other trivalent ligands with a 2′-*D*Nal^6 (**6**, **7**, and **9–12**). As evident from Figure 5, the side chain orientation of Trp^8 and Nle^9 of **8** differs from that of **9**. The side chains of Trp^8 and Nle^9 in **8** are oriented on the same surface resulting in hydrophobic interactions, but those in **9** are on the opposite surface. Therefore, the reversal of the selectivity between CCK-1 and CCK-2 receptors can be attributed to the structural differences caused by the replacement at position 6.

In an effort to build up highly opioid active trivalent ligands, major structural modifications were carried out on the N-terminal opioid pharmacophore structure. As seen in Figure 2, three pharmacophore structures, the tetrapeptide from enkephalin analog, fentanyl, and Dmt-Tic, were chosen for the opioid receptors, since each has a different biological activity profile. Occasionally, the residue at position 2 of the pharmacophoric region was used for cyclization by a lactam or a disulfide bridge. Apparently, when the fentanyl structure was connected by a succinic acid linker, lower binding affinities and biological activities were observed for μ and δ opioid receptors and even for CCK-1 and CCK-2 receptors. Ligand **1** with the fentanyl structure showed only micromolar binding affinities and very low agonist activities at both opioid receptors. Interestingly these ligands also lost their binding affinities at the CCK receptors, and this fact suggests that the CCK pharmacophore structure was affected by the modification of opioid area which was fully separated by the MC pharmacophore. The result can be explained by the spatial proximity between the two pharmacophores due to the turn structure of the MC pharmacophore mentioned above.

In order to verify the efficacy of the design of trivalent ligands shown in Figure 1, ligand **2** in which three pharmacophores were connected together in three directions through a Lys^5 residue, was synthesized and tested for their binding affinities and biological activities at opioid and CCK receptors (Tables 2 and 3). The ligand showed potent binding affinities at δ and μ opioid receptors (K_i = 3.5 and 7.4 nM, respectively), but lost its binding affinities at CCK-1 and CCK-2 receptors. This also is the case with the ligands **4** and **5** which have a Cys residue used for a disulfide bridge. However in the case of cyclization using the Lys residue (ligand **3**), the ligand was able to retain modest binding affinities at CCK-1 and CCK-2 receptors (K_i = 560 and 930 nM, respectively). It is clear that the hydrophobic Nle residue is essential for the CCK activities. It is envisioned that ligand **2** will lose its binding affinity at MC-4 receptor due to the difficulty in making a turn structure at the N-terminal. For the opposite reason, ligand **3** showed the best binding affinity (IC_{50} = 32 nM) at MC-4 receptor. This fact indicates that the formation of a lactam ring enhanced the MC pharmacophore by stabilizing the turn structure.

Dmt-Tic, the shortest δ opioid antagonist, also was applied to the trivalent ligands as an opioid pharmacophore. The application resulted in δ opioid receptor selective binding affinity (K_i = 0.91 nM at hDOR, μ/δ = 51) with strong antagonist activity (Ke = 2.5 nM) in **7** and it did not affect the binding and biological activities at the CCK and MC receptors. Interestingly, no agonist activity was observed at the μ opioid receptor despite good binding affinity (K_i = 47 nM). To regain the opioid agonist function, the chirality of Tic residue was reversed to its *D*-form. As a result, ligand **6** recovered opioid agonist activities for both receptors with slightly δ opioid selective binding affinities (K_i = 6.2 and 18 nM for hDOR and rMOR, respectively).

Based on the SAR result, the enkephalin-related tetrapeptide, Tyr-*D*Ala-Gly-Phe-, was chosen as a good opioid pharmacophore structure for the trivalent ligands. Ligands **8** and **9** with this tetrapeptide pharmacophore showed the same range of opioid activities in the binding and functional assays (Tables 2 and 3). In an effort to compact the trivalent ligand structures, the Gly residue in the opioid pharmacophore was truncated. This truncation in ligand **12** decreased binding affinities at the MC-4 receptor and at the μ and δ opioid receptors, especially at the μ receptor. By this modification, opioid agonist activities were decreased up to 12-fold (IC_{50} = 200–2300 nM in GPI assay) in the functional assays. Interestingly, the modification also resulted in a highly CCK-1 selective trivalent ligand (CCK-2/CCK-1 = 119) by increasing the binding affinity from 9 to 1.6 nM at CCK-2 receptor but decreasing it from 120 to 190 nM at CCK-1 receptor. It was observed that Gly truncation decreased binding affinities at the MC-4 receptor, too. The SAR between **10** and **11** correlated well with that between **9** and **12**. Again, it was demonstrated that a slight modification on the opioid pharmacophoric area affects not only the opioid receptor but also the other two receptors, especially the CCK receptor. Based on these SAR results, the Gly residue should be conserved in the opioid pharmacophore to retain high biological activities at δ and μ opioid receptors with balanced activities at CCK-1/-2 receptors.

It has been known that the substitution of Dmt for Tyr in opioid pharmacophore results in increase in opioid activities, especially,

an increase in μ receptor affinity relative to δ receptor.[25,26] To enhance the opioid activities without disrupting the other biological activities, the N-terminal Tyr was replaced by Dmt. The replacement noticeably increased biological activities for both δ and μ opioid receptors, especially the μ opioid agonist activity. In the binding assay, **10** showed a 14-fold increase in affinity at the δ receptor (K_i = 0.21 nM) and a fivefold increase in affinity at the μ receptor (K_i = 0.13 nM) as compared to **9**, thus resulting in balanced affinities at both receptors. In addition, **10** was about fivefold more potent than **9** in the MVD (IC_{50} = 11 nM) assay, and 24-fold more potent in the GPI (IC_{50} = 8.3 nM) assay. It should be noted that the replacement did not greatly affect the other pharmacophores and even resulted in an increase of potencies. More interestingly, ligand **10** showed CCK antagonist activity (Ke = 940 nM) in the GPI/LMMP assay.

In summary, the trivalent ligands for μ-/δ-opioid, CCK-1/-2, and MC-4 receptors were designed and synthesized by SPPS under microwave conditions, and their binding affinities and biological activities were examined at their respective receptors. Of the structural modifications, ligand **10** is the best lead compound having good binding affinities at all three receptors with excellent agonist activities at both opioid receptors, which directly relate to a strong analgesic effect, and moderate antagonist activity at the CCK receptor. These results demonstrated that several different pharmacophores can be combined in one single molecule which possesses complimentary topographical structures for their respective receptors. On the basis of our working hypothesis, the results also showed the potential of these trivalent ligands for the treatment of pain. Further study of the trivalent ligands will include expanded assays such as functional assays for the MC-4 receptor and in vivo tests for anti-allodynic and hyperalgesic effects.

Acknowledgments

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