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Synthesis, biological activity and structure–activity relationship of endomorphin-1/substance P derivatives

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ABSTRACT

Endomorphins have been shown to produce potent analgesia in various rodent models of pain. However, their central administration led to the development of tolerance and physical dependence. Conjugation of C-terminal substance P (SP) fragments to opioids and opioid peptides was previously shown to produce hybrid peptides with strong analgesic activity, with low or no propensity to develop tolerance. In this study, four peptides (**2**–**5**) comprised of endomorphin-1 (**1**) and C-terminal fragments of SP (four or five amino acids, SP_{8-11} (**2**) or SP_{7-11} (**4**), respectively), with an overlapping Phe residue, were synthesized. To overcome low metabolic stability and poor membrane permeability of the peptide, the N-terminus of **2** and **4** was further modified with a C10-carbon lipoamino acid (C10LAA) achieving **3** and **5**, respectively. LAA-modification of the hybrid peptides resulted in a significant increase in metabolic stability and membrane permeability compared to peptides **1**, **2** and **4**. Compound **5** showed potent μ-opioid receptor binding affinity ($K_{i\mu}$ = 3.87 ± 0.51 nM) with dose-dependent agonist activity in the nanomolar range (IC_{50} = 45 ± 13 nM). In silico modeling was used to investigate the binding modes and affinities of compounds **1**–**5** in the active site of μ-opioid receptors. The docking scores were in agreement with the $K_{i\mu}$ values obtained in the receptor binding affinity studies. The more active LAA-modified hybrid peptide showed a lower total interaction energy and higher negative value of MolDock score.

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1. Introduction

Most of the biological effects of endogenous opioid peptides are mediated through activation of three opioid receptors designated μ, δ and κ in the peripheral or central nervous system.[1] They belong to the superfamily of seven transmembrane-spanning G-protein coupled receptors.[2,3] Potent analgesics such as morphine and opioid peptides mainly target μ-opioid (MOP) receptors to mediate their pain-relieving effects.[4] Two important endogenous opioid peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), were shown to exhibit high selectivity and affinity for MOP receptors.[5] Endomorphins elicited potent antinociception in different rodent models of pain after central administration.[6–8] However, similar to other agonist ligands of MOP receptors, tolerance was developed following acute (in high doses) or chronic treatment with endomorphins.[9–13]Apart from analgesia, MOP receptor agonists are responsible for the emergence of both tolerance and physical dependence with high potential for abuse.[14–16] Development of tolerance to opioids occurs through several complex adaptation processes, yet the exact mechanism has remained unclear.[17,18] Generally, it has been shown that desensitization, internalization and down-regulation of the receptors play an important role in development of tolerance by opioids.[19] To prevent this adverse effect, some hybrid molecules, combining an MOP receptor agonist with either a δ-opioid receptor antagonist or substance P, were synthesized and investigated for their in vitro or in vivo biological activities.[20–23] For a long time, it was believed that the undecapeptide substance P (SP: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), is a neurotransmitter associated with mediating painful or nociceptive signalling.[24,25] In contrast to the hyperalgesic effects of SP, Stewart et al. demonstrated that low doses of the peptide produced a modest analgesic response.[26] This effect was characterized to be operated through the opioid system[27] by virtue of being reversible following pretreatment with the opioid antagonist naloxone.[28] SP is also capable of intensifying opioid-mediated analgesia in low doses which was also shown to be naloxone reversible.[29] Different C-terminal SP fragments with antinociceptive activity were conjugated with opioid alkaloids or opioid peptides. The hybrid alkaloid/peptide chimeric compounds produced strong antinociceptive response with little or no development of opioid tolerance or dependence following central (intrathecal) administration to rats.[22,23,30]

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We previously showed that the incorporation of lipoamino acid (LAA) into the structure of peptides such as endomorphin-1 can improve their metabolic stability and membrane permeability,[31,32] rendering these peptides active upon peripheral administration.[32,33] The LAAs combine structural features of lipids and amino acids for facile incorporation into the structure of short peptides. Moreover, various alkyl chain lengths of the LAA can be used to tune the physico-chemical properties of the peptides.[34] Data from our previous studies demonstrated that among different LAA alkyl chain lengths, a 10-carbon (C10) LAA conjugated with endomorphin-1 provided the best results in in vivo analyses, as it elicited potent and prolonged analgesia in a rat-model of neuropathic pain following systemic administration.[33] Therefore, in this study C10LAA was attached to the N-terminus of endomorphin-1/SP hybrid peptides to increase their resistance against enzymatic hydrolysis. Furthermore, lipid-modification can also improve the peptides' passive diffusion across biological membranes such as the blood brain barrier (BBB) due to the increase in lipophilicity.

Three N-terminal amino acids of endomorphin-1 (**1**) were conjugated to the C-terminal fragments of SP, either SP_{8-11} (-Phe-Gly-Leu-Met-NH_2) resulting in compound **2** or SP_{7-11} (Phe-Phe-Gly-Leu-Met-NH_2) resulting in compound **4**, while sharing one Phe residue as the overlapping domain of both peptides. The N-terminus of endomorphin-1/SP hybrid peptides **2** and **4** was modified with C10LAA to achieve compounds **3** and **5**, respectively. These endomorphin-1/SP hybrid peptides were tested for their in vitro membrane permeability, metabolic stability, affinity and selectivity to bind to MOP over δ-opioid (DOP) receptors. The agonist activity of the compounds was also studied in MOP receptor-expressing HEK-293 cells. Moreover, in silico docking was used to predict the binding affinity of compounds **1**–**5** to MOP receptors and the impact of different modifications on the structure of the endomorphin-1 peptide. The possible binding mode of compound **D5**, as the most promising MOP agonist ligand was investigated in the active site of the receptor.

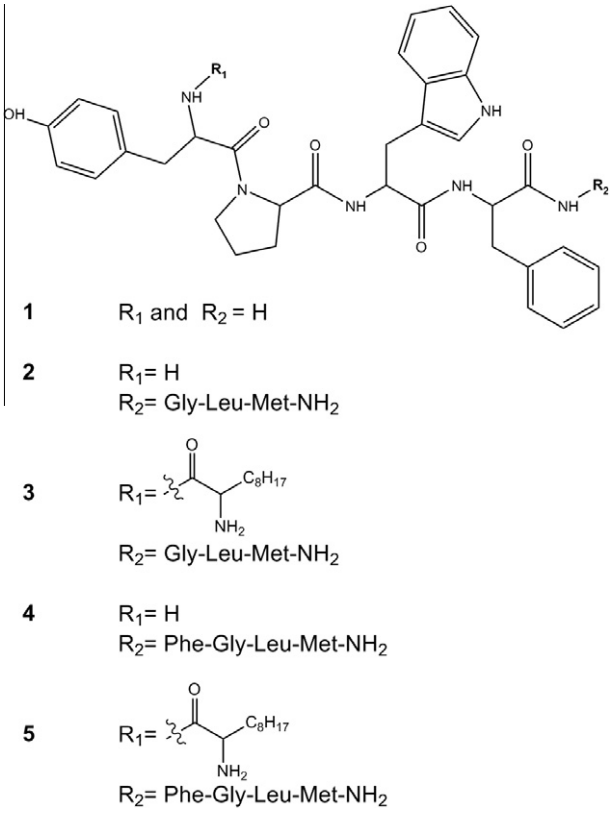

Figure 1. Structure of endomorphin-1 (**1**) and its derivatives (**2**–**5**).

2. Results

2.1. Chemistry

Endomorphin-1 (**1**) and the peptide analogues, **2**–**5** (Fig. 1), were synthesized by solid phase peptide synthesis and purified to a single peak (>95% purity) by analytical RP-HPLC. Peptides were characterized by obtaining the corresponding $[M+H]^+$ peak in the ESI-MS. A summary of compound characterization is shown in Table 1.

Table 1
Summary of the chemical characterization of compounds **1**–**5**

Compound	Yield (%)	ESI-HRMS mass $[M+H]^+$		RT-HPLC (min) t_R [a]
		Calculated	Found	
1	81	611.2976	611.2987	15.9
2	73	912.4437	912.4438	18.1
3	46	1081.5903	1081.5889	21.4, 21.5
4	68	1059.5121	1059.5112	19.8
5	51.7	1228.6587	1228.6568	22.5, 22.8

[a] HPLC elution on a Vydac C18 column (4.6 mm × 250 mm, 5 mm) using the solvent system of 0.1% (v/v) TFA in water (A) and 0.1% (v/v) TFA in acetonitrile (B) and a gradient of 0–100% B over 30 min at a flow rate of 1 mL/min.

2.2. Metabolic stability and membrane permeability

In vitro stability and permeability of the peptide analogues were evaluated using the Caco-2 cell line derived from human colorectal carcinoma cells. Caco-2 cells are widely used as an in vitro model system to predict the enzymatic stability of a compound after systemic or oral administration.[35] The half-life of peptide **1** in the Caco-2 cell homogenates was 5.6 ± 0.9 min. The stability of hybrid peptides **2** and **4** slightly increased, with a half-life of 10.7 ± 2.4 and 15.6 ± 3.3 min, respectively, compared to the parent peptide **1**. In contrast, the half-life of **3** and **5** significantly increased to 52.5 ± 8.4 and 75.8 ± 12.7 min, respectively, relative to peptides **1**, **2** and **4** (Table 2, Fig. 2, $p < 0.05$).

In the Caco-2 cell membrane permeability assay,[35] TEER values after completion of the experiments were within 10% of the original values, indicating that none of the compounds were toxic to the cells. Apparent permeability (P_{app}) of the negative control, ^{14}C mannitol, was proved to be 4.88 (±1.69) × 10^{-7} cm/s, which was consistent with values previously reported for confluent, intact Caco-2 cell monolayers.[36] Peptides **1**, **2** and **4** displayed low permeability across Caco-2 cell monolayers (P_{app} values: 1.01 (±0.52) × 10^{-6}, 1.41 (±0.85) × 10^{-6} and 2.04 (±0.68) × 10^{-6} cm/s, respectively). However, analogues **3** and **5** exhibited improved membrane permeability with P_{app} of 8.08 (±1.79) × 10^{-6} and 10.2 (±1.34) × 10^{-6} cm/s. Propranolol, which is known to be absorbed completely from the GI tract,[37] was used as the positive control and a P_{app} of 3.05 (±0.28) × 10^{-5} was obtained (Table 2).

2.3. Receptor binding and agonist activity

MOP and DOP receptor binding affinity and selectivity of the hybrid analogues were measured by displacement of $[^3H]$DAMGO and $[^3H]$DPDPE from recombinant MOP and DOP receptors expressed in CHO-K1 cells. Receptor binding affinity results demonstrated that hybrid peptides **2** and **4** bound to MOP receptors with high affinity ($K_{i\mu}$ 1.6 ± 0.15 and 0.73 ± 0.20 nM, respectively), similar to that of peptide **1** ($K_{i\mu}$ 0.76 ± 0.19 nM). Although C10LAA modification led to a noticeable drop in the MOP receptor binding

Table 2
Summary of in vitro results

Compound	$t_{1/2}$[a] (min, Caco-2)	P_{app}[b] ($\times 10^{-6}$ cm/s)	$K_{i\mu}$[c] (nM)	$K_{i\delta}$[c] (nM)	Selectivity ($K_{i\delta/\mu}$)	cAMP, IC_{50}[d] (nM)
1	5.6 ± 0.9	1.01 ± 0.52	0.76 ± 0.19	2765 ± 176	9534	3.2 ± 0.48
2	10.7 ± 2.4	1.41 ± 0.85	1.6 ± 0.15	1977 ± 125	1235	ND
3	52.5 ± 8.4 *	8.08 ± 1.79*	132 ± 14.8	2254 ± 199	17	ND
4	15.6 ± 3.3	2.04 ± 0.68	0.73 ± 0.20	1557 ± 156	2132	12 ± 2.1
5	75.8 ± 12.7 *	10.2 ± 1.34*	3.87 ± 0.51	1230 ± 74	318	45 ± 13
Morphine[e]	ND	ND	0.14 ± 0.032	295 ± 19.8	2107	ND

[a] The half-life of compounds **1**–**5** in a homogenate of Caco-2 cells.
[b] Their apparent permeability, P_{app}(cm/s), across Caco-2 cell monolayers. Mannitol was used as the negative control with P_{app} of 4.88 (±1.69) $\times 10^{-7}$ and propranolol was used as the positive control with P_{app} of 3.05 (±0.28) $\times 10^{-5}$. The data were quantified by LC/MS.
[c] Binding affinity values of **1**–**5** and morphine at MOP ($K_{i\mu}$) and DOP ($K_{i\delta}$) receptors were determined by competitive displacement of their selective radioligands, [^{3}H]DAMGO and [^{3}H]DPDPE, respectively, using CHO-K1 cell membranes stably transfected to express the corresponding receptors.
[d] Inhibition of cAMP formation in forskolin-stimulated MOP receptor-expressing cells represented as IC_{50} values (nM) from dose–response curves using non-linear regression.
[e] Data cited from Ref. 59. ND, not determined. All values are mean ± SEM of three independent experiments performed in triplicate.
* p <0.05, compared to compounds **1**, **2** and **4**.

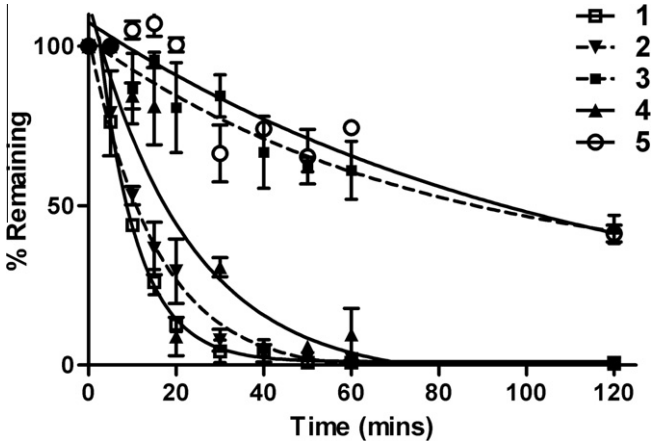

Figure 2. Degradation profile of parent peptide **1** and four hybrid peptide analogues, **2**–**5**, in Caco-2 cell homogenate. Data was quantified by LC/MS and expressed as mean ± SEM (n = 3 from three independent experiments). GraphPad Prism was used to perform the one phase decay analysis to measure $t_{1/2}$.

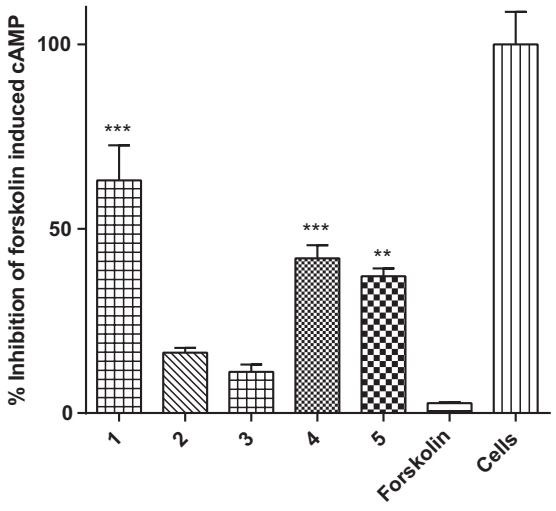

Figure 3. Inhibition level of cAMP production (%) in forskolin-stimulated MOP receptor-expressing HEK-293 cells treated with 1 μM solution of the test compounds. Forskolin-stimulated cells treated with buffer (illustrated as Forskolin) that showed no inhibition (%) in the production of cAMP were used as the positive control. Cells treated with buffer but without stimulation with forskolin (illustrated as cells) represented the minimum level of cAMP and were used as the negative control in this experiment. Each value represents mean ± SEM (n = 3 from three independent experiments). $^{**}p$ <0.01 and $^{***}p$ <0.001, versus the negative control.

affinity of compound **3** ($K_{i\mu}$ 132 ± 14.8 nM), the $K_{i\mu}$ value for compound **5** was close to that of native peptide **1** and in the low nanomolar range (3.87 ± 0.51 nM). Like parent peptide **1**, compounds **2**–**5** exhibited weak receptor binding affinities at DOP receptors, with $K_{i\delta}$ values above 1000 nM. Morphine, the gold-standard analgesic acting at MOP receptors, was also used as the positive control for comparison. The binding affinity of morphine was examined at MOP and DOP receptors with $K_{i\mu}$ and $K_{i\delta}$ values of 0.14 ± 0.032 and 295 ± 19.8 nM, respectively (Table 2).

Additional in vitro experiments were performed using stably transfected MOP receptor-expressing cells to investigate the ability of the hybrid peptides to activate these receptors as agonists. Hybrid peptides **2**–**5** were initially tested at a single concentration (1 μM) to establish their potency to inhibit forskolin-stimulated cAMP accumulation (Fig. 3). Although all hybrid peptide analogues showed some agonist activity in stimulated HEK-293 cells, only analogues **4** and **5** significantly inhibited the accumulation of cAMP in forskolin-stimulated cells (p <0.05). Hence, analogues **4**, **5** and native peptide **1**, were further examined at a range of concentrations to obtain a dose-response curve and calculate their IC_{50} values (Fig. 4,Table 2). Peptide **1** exhibited potent agonist activity with an IC_{50} value of 3.2 ± 0.48 nM. Compounds **4** and **5** showed dose-dependent MOP receptor agonist activity by inhibiting forskolin-stimulated cAMP formation at IC_{50} values of 12 ± 2.1 and 45 ± 13 nM, respectively.

According to several preclinical and clinical studies, spinal excitatory peptides such as SP may play a favourable role by attenuating development of tolerance after opioid use.[38,39] It was previously shown that central administration of SP with morphine significantly inhibited development of tolerance to morphine's analgesia.[29] Moreover, an SP/opioid peptide chimera produced opioid-dependent analgesia after central administration without development of tolerance. Although a reduced binding affinity and agonist activity compared to the opioid peptide, endomorphin-2, was observed at MOP receptors, this hybrid peptide elicited potent in vivo antinociception.[23]

A peptide analogue must confer sufficient enzymatic stability and membrane permeability to elicit analgesic activity following peripheral administration for it to be considered for clinical application. A Caco-2 cell homogenate assay was used to examine the metabolic stability of the hybrid peptide analogues against enzymatic hydrolysis. Caco-2 cells express several drug-metabolizing enzymes and transporters present in the human enterocytes.[40] One of the enzymes they produce is dipeptidyl peptidase IV (DPP IV) which is found to be the main enzyme responsible for degrada-

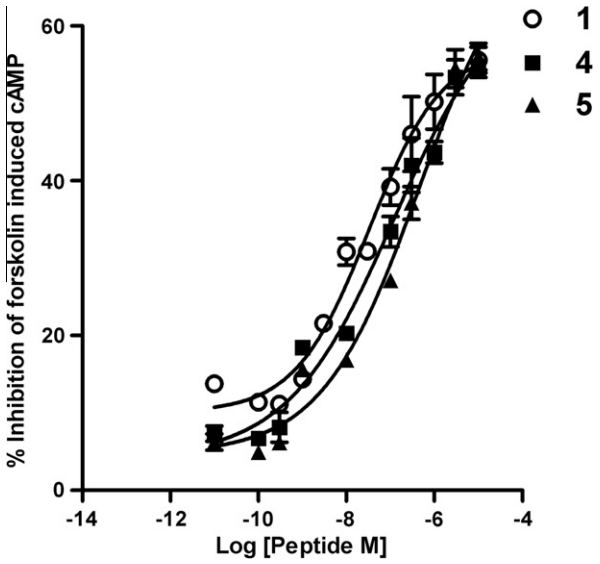

Figure 4. Inhibition of forskolin-stimulated cAMP formation by peptide **1** and analogues **4** and **5** in stably transfected HEK-293 cells. Data presented are means (±SEM) from three separate experiments, each performed in triplicate. Non-linear regression, as implemented in GraphPad Prism™ (v5.0), was used to estimate IC_{50} values.

Table 3
Calculated binding affinities for ligands and MOP receptors

Compound number	MolDock score[a] (kcal/mol)
1	−170
2	−155
D3	−36
L3	−66
4	−140
D5	−130
L5	−91

[a] The binding affinity was predicted by energy minimization and given a MolDock score, which was derived from the PLP scoring function.[59] Larger negative values indicate larger affinities of the compound for the receptor.

tion of endomorphins.[41] N-terminal conjugation of both hybrid peptides **3** and **5** with C10LAA resulted in significant resistance against enzymatic degradation in Caco-2 cell homogenates. More specifically, the half-life of compounds **3** and **5** increased by 9.4- and 13.5-fold compared to compound **1**, and approximately five-fold compared to compounds **2** and **4**.

The hydrophilicity of peptides makes them relatively impermeable across the BBB by transmembrane diffusion.[42] Increasing lipid solubility of peptides is a useful strategy to promote permeation across the BBB[43] via passive diffusion, a nonsaturable mechanism that largely depends on lipid solubility.[44] Both C10LAA-conjugated hybrid peptides **3** and **5** showed improved membrane permeability with an approximately 8- and 10-fold higher P_{app}, respectively, compared to peptide **1**. It has been shown that the Caco-2 cell monolayers are reliable models when studying new chemicals, particularly CNS-targeted compounds, for either passive or active transport across the BBB.[45,46] There has been substantial correlation between Caco-2 cell permeability coefficients (P_{app}) and fractional absorption (Fa) values in humans according to biopharmaceutics classification system (BCS). Based on this classification, the corresponding P_{app} values for compounds with high Fa(%), are in the range of 10^{-5}–10^{-6} cm/s.[47,48] Accordingly, LAA-modified derivatives **3** and **5** are within the range of permeable compounds to cross barriers like the BBB.

It is essential to maintain binding affinity and agonist activity of the hybrid analogues at MOP receptors in order to achieve in vivo analgesia. Analogues **2** and **4** exhibited MOP receptor binding affinities similar to that of the peptide **1**. The $K_{i\mu}$ value for compound **5** was also close to native peptide **1**, and within the nanomolar range. However, C10LAA modification was not well tolerated in compound **3** and therefore, a 170-fold decrease in binding affinity was observed at MOP receptors, compared to peptide **1**. Moreover, the binding affinity of compounds **1-5** was tested at DOP receptors. Similar to peptide **1**, neither hybrid peptides with high MOP receptor binding affinity had appreciable affinities for DOP receptors, indicating maintained MOP receptor selectivity.

Opioid receptor agonists inhibit cAMP production within the cells upon activation of the G_i subunit of GPCRs.[49] Moreover, treatment of cells with forskolin results in the accumulation of cAMP within the cells.[50] Unlike compounds **2** and **3**, analogues **4** and **5** significantly inhibited the production of cAMP in forskolin-stimulated HEK-293 cells at a single concentration. A slight to moderate decrease in the potency of compounds **4** and **5** was observed, respectively, to inhibit cAMP accumulation compared with native peptide **1**; yet, dose-dependent agonist activity was obtained for both compounds with IC_{50} values in the nanomolar range.

Based on the molecular modeling studies, there was an obvious matching correlation between the molecular modeling scores for compounds **1**–**5** docked onto the MOP receptor (Table 3) and the $K_{i\mu}$ values (Table 2) where compounds with higher binding affinities, revealed lower total interaction energies and higher negative MolDock scores. Specifically, for compounds **1**, **2** and **4** with lower $K_{i\mu}$ values, higher negative MolDock scores were obtained in a similar range (−170, −155 and −140, respectively). As C10LAA was incorporated into **3** and **5** as a racemic mixture, these peptides contain equal amounts of each diastereomer. The docking scores demonstrated relatively high binding affinities for both diastereomers **D5** and **L5** to the receptor (−130 and −91) in comparison with **D3** and **L3** (−36 and −66). **D5** and **D3** were selected for further molecular modeling analysis. Since the only difference between the structures of compound **3** (Fig. 5) and **5** (Fig. 6) was an extra Phe in compound **5**, these findings suggested that the Phe residue played an important role in the stretching of the long hydrophobic chain of the LAA in both **D5** (Fig. 6, Fig. 7a) and **L5** (Fig. 7b) into the hydrophobic pocket, in the active site of the receptor. While in compound **D3** the lack of this Phe possibly caused the hydrophobic alkyl chain of LAA to be oriented away from the hydrophobic pocket in the MOP receptor, resulting in lower binding affinity compared to compound **D5** (Fig. 5b).

The investigation of the highest docking score conformation of compound **D5** in the active site of MOP receptor showed a hydrophobic interaction between the two benzene rings of Trp in **D5** and Phe221 in the receptor. Apart from this interaction, there were six hydrogen bonds between **D5** and the receptor (Fig. 7a). Furthermore, the alkyl chain of C10LAA in **D5** bound to a hydrophobic pocket in the active site of the receptor (Fig. 6b). According to these finding, the spatial conformational properties of the alkyl side chain influenced the interaction of **D5** with the receptor within the hydrophobic pocket. Binding of the alkyl chain to the hydrophobic pocket along with the hydrophobic interactions and hydrogen bonding between **D5** and different functional groups can play crucial roles in the high binding affinity of **D5** to the active site of the MOP receptor.

3. Conclusion

Lipid-modification of endomorphin-1/SP hybrid peptides in compounds **3** and **5** resulted in a significant improvement in permeability across Caco-2 cell monolayers and stability in Caco-2 cell

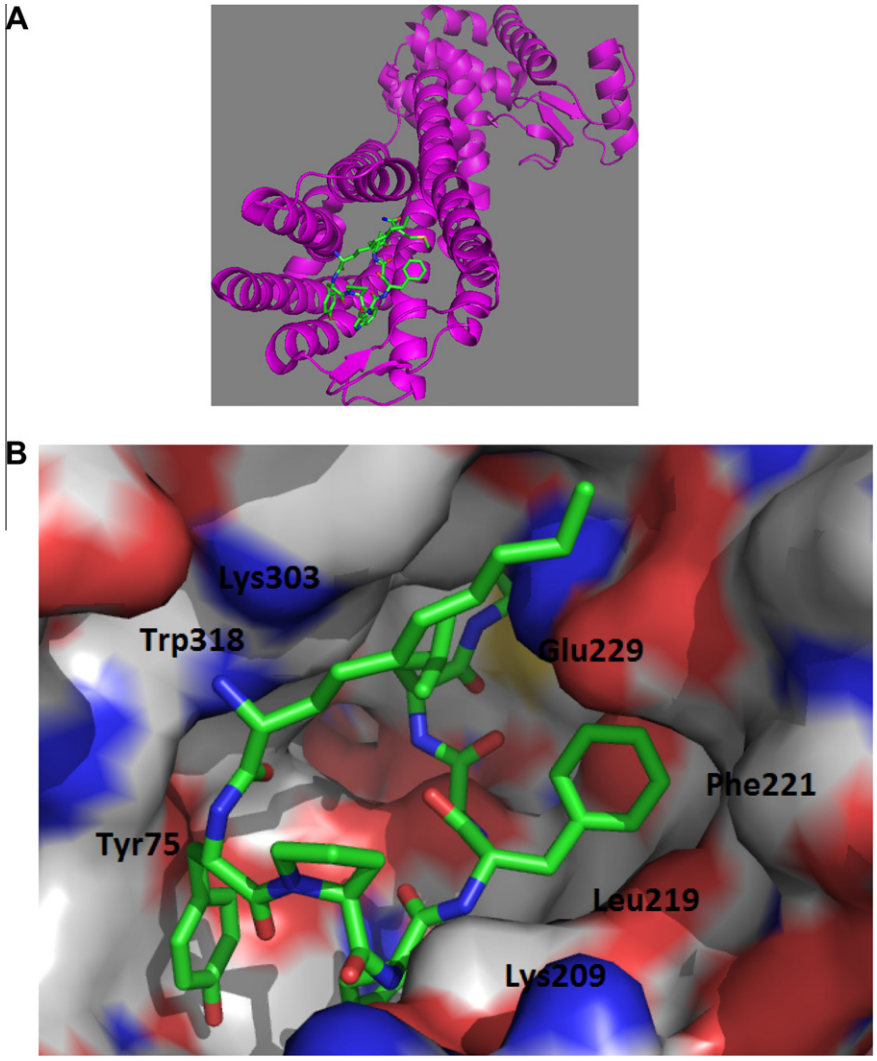

Figure 5. (A) Docking of **D3** in the active site of MOP receptor. (B) Surface view of the active site of the MOP receptor for the highest docking score conformation of MOP receptor with **D3** docked into the active site. For clarity, color of the atoms are as follows: blue—nitrogen, red—oxygen, white—carbon (MOP receptor) and green—carbon (on ligand).

homogenates, compared to their parent peptides, **2** and **4,** and native peptide **1** (p <0.05). Interestingly, among the stable, permeable analogues **3** and **5**, analogue **5** showed a relatively high MOP receptor binding affinity, conserved selectivity, and agonist activity with nanomolar inhibition of forskolin-stimulated cAMP production. Molecular modeling was used to compare the expected binding orientation of **D3** and **D5** in the active site of the MOP receptor. The MolDock scores of peptides (**1**–**5**) were shown to correspond with the values obtained in the receptor binding affinity studies. Our results suggest that hybrid peptide **5** is a promising candidate for the development of peptide-based analgesics with improved bioavailability.

4. Experimentals

4.1. General

Dimethylformamide (DMF), trifluoroacetic acid (TFA) and piperidine (peptide synthesis grade) were obtained from Merck Biosciences (Kilsyth, VIC, Australia), and acetonitrile (HPLC grade) was purchased from RCI Labscan Ltd. (Bangkok, Thailand). Fmoc-protected amino acids and Rink amide MBHA resin (100–200 mesh) with a loading of 0.4–0.8 mmol/g were purchased from Novabiochem Mimotopes (Clayton, VIC, Australia). Cell culture reagents were purchased either from Sigma Aldrich or Gibco (VIC, Australia). Geneticin (G418) was obtained from Life Technologies (VIC, Australia). Frozen membranes providing human MOP receptors (1×400 units/μL), wheatgerm agglutinin coated poly vinyl toluene SPA (scintillation proximity assay) beads, [^{3}H]-[D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin ([^{3}H]DAMGO, 49.6 Ci/mmol, [Tyrosyl-3,5-^{3}H(N)]−), D-Penicillamine(2,5)-enkephalin ([^{3}H]DPDPE, 25.2 Ci/mmol, [Tyrosyl-2,6-^{3}H(N)]−), 384-well OptiPlates and ProxiPlates, and clear adhesive Topseal-A were purchased from Perkin Elmer (Massachusetts, USA). Other chemicals were sourced from Sigma Aldrich (VIC, Australia). Boc-C10-LAA was synthesized according to a published procedure.[51] A Varian Cary 50 Bio UV/vis Spectrophotometer (λ = 595 nm) was used for absorbance measurements. Preparative HPLC was carried out on a Waters system, equipped with a 600 controller and pump connected to a 490E UV/vis detector operating at a wavelength of 230 nm. An Agilent 1100 analytical HPLC equipped with binary pump, autosampler and UV/vis detector set to 214 nm was used. Electrospray ionization mass spectrometry (ESI-MS) analysis was performed on a PE Sciex API3000, with triple quadrupole system, operating with a constant

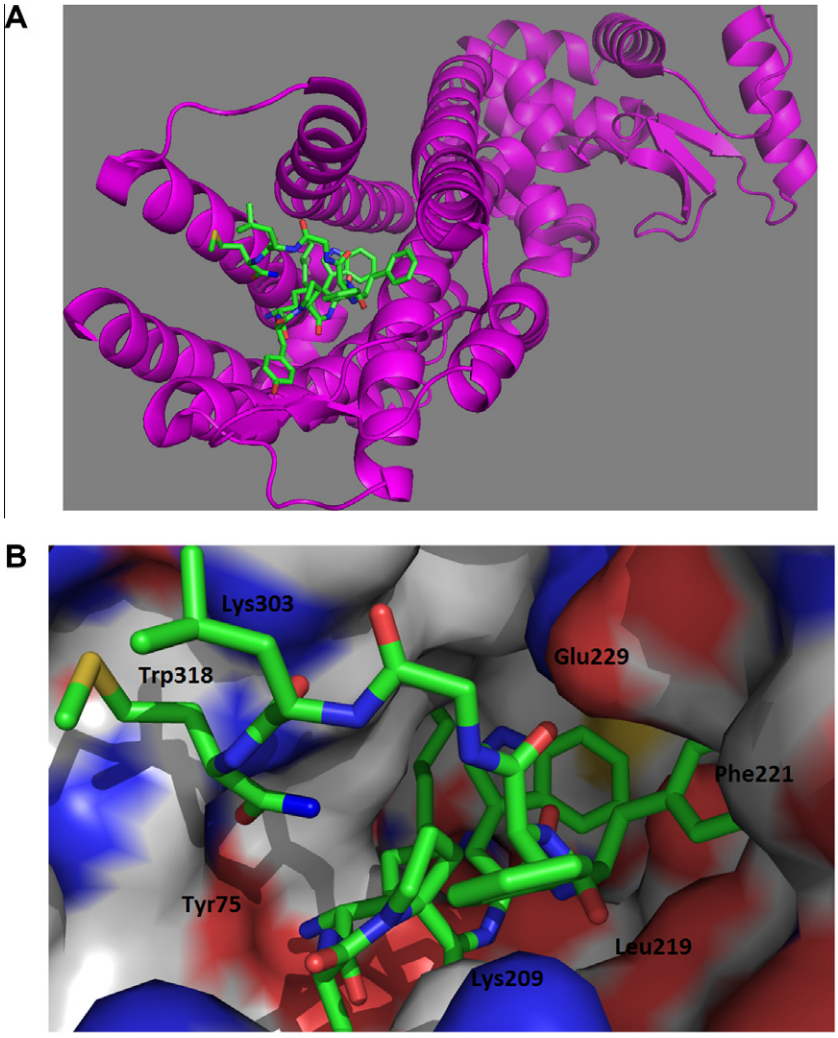

Figure 6. (A) Docking of **D5** in the active site of MOP receptor. (B) Surface view of the active site of the MOP receptor for the highest docking score conformation of MOP receptor with **D5** docked into the active site. For clarity, color of the atoms are as follows: blue—nitrogen, red—oxygen, white—carbon (mu-opioid receptor) and green—carbon (on ligand).

flow of a 1:1 mixture of solvent A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile/water 9:1) at a rate of 0.05 mL/min.

4.2. Chemistry

4.2.1. Lipoamino acid synthesis

2-amino-D,L-decanoic acid was synthesised from diethyl acetamido malonate and the appropriate alkyl bromide using published procedures.[52,53] Lipoamino acids were protected using tertbutoxycarbonyl (Boc). Boc protection was performed according to published procedures.[52,53]

4.2.2. Solid-phase peptide synthesis

Peptides were synthesized using the in situ neutralization protocol for Fmoc chemistry on Rink amide 4-methylbenzhydrylamine (MBHA) resin according to the published procedure.[54] The following protected amino acids (4.2 equiv) were double coupled after activation with HBTU (4 equiv)/DIPEA (5 equiv) prior to coupling: Fmoc-Met, Fmoc-Leu, Fmoc-Gly, Fmoc-Phe, Fmoc-Trp(Boc), Fmoc-Pro, Fmoc-Tyr(*t*Bu), and Boc-C10LAA. One hour coupling time was used for each amino acid. When all amino acids were coupled, the resin was washed with dichloromethane and dried in vacuo overnight. Peptides were cleaved from the resin by treatment with a mixture of TFA (95%), triisopropylsilane (2.5%) and water (2.5%) for 6 hours. The solvent was removed and the peptide was precipitated using cold diethyl ether. The resulting solid was lyophilized from acetonitrile, water and TFA (50:50:0.1).

4.2.3. Purification

A Vydac C18 column (22 × 250 mm) at a flow rate of 10 mL/min with a 70 min gradient of 40–70% solvent B (0.1% TFA in acetonitrile/water 9:1; solvent A: 0.1%TFA in water) was used to purify the crude peptides. ESI mass spectrometry and analytical HPLC were used for characterization of the fractions, and pure fractions were pooled and lyophilized. The purity of the peptides was confirmed by analytical HPLC on a Vydac C18 column (4.6 × 250 mm, 5 μm) at a flow rate of 1 mL/min and a gradient of 0–100% B over 30 min.

4.3. In vitro experiments

4.3.1. Cell culture

Human colorectal adenocarcinoma (Caco-2) cells were obtained from the American Type Culture Collection (Rockville, USA). Human embryonic kidney 293 (HEK-293) cells, stably transfected to express MOP receptors, were a gift from Center for Integrated Preclinical Drug Development at The University of Queensland (Brisbane, Australia). Caco-2 and transfected HEK-293 cells, were

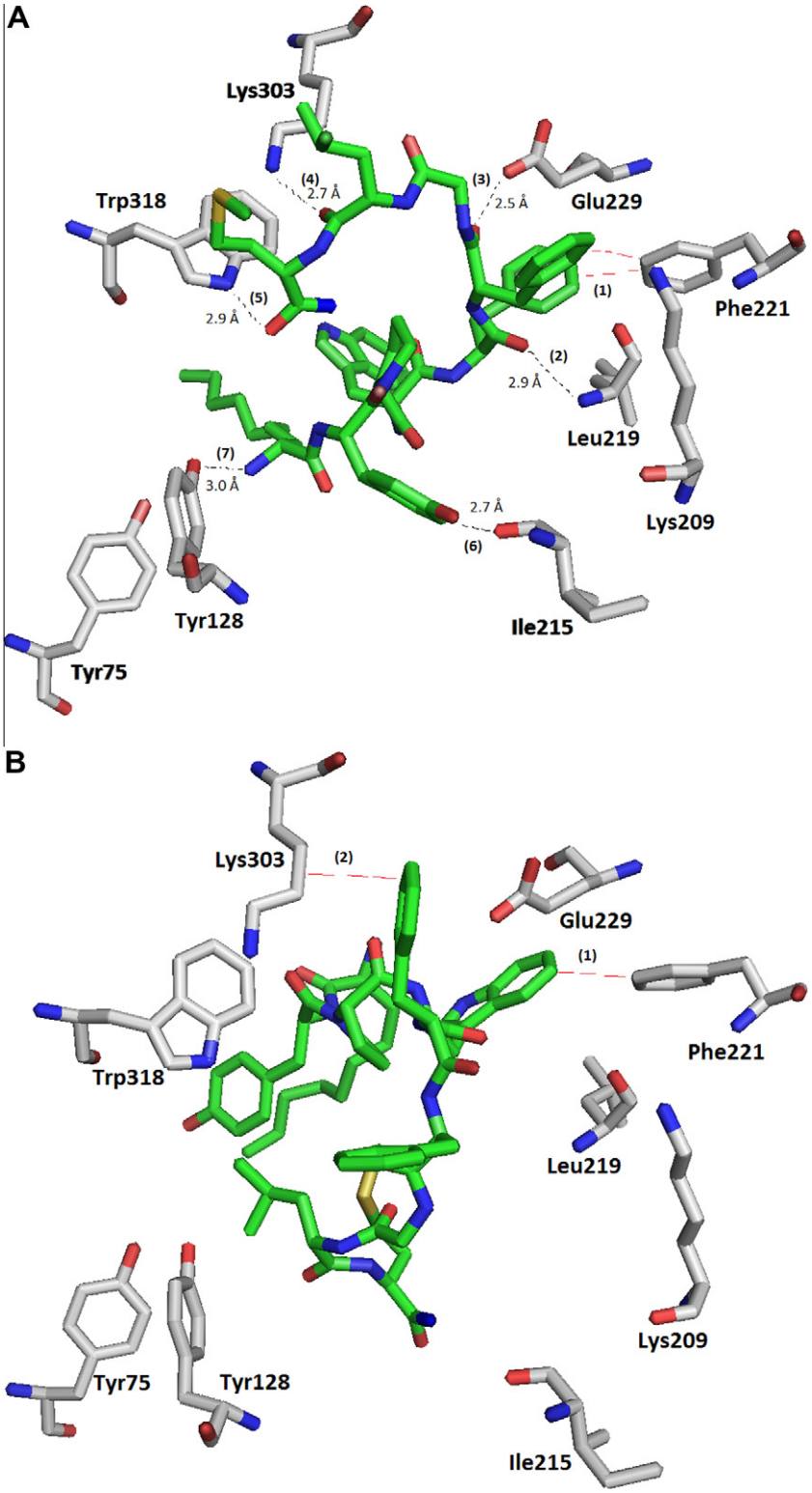

Figure 7. (A)Surface view of the active site of the MOP receptor for the highest docking score conformation of MOP receptor with **D5** docked into the active site shows (1) a hydrophobic interaction between the two benzene rings of both Phe in **D5** and Phe221 in the receptor,(2) a hydrogen bond between the oxygen of the carbonyl group of Phe in **D5** and the NH of Leu219 in the receptor (O–N distance 2.9 Å), (3) a hydrogen bond between the oxygen of the carbonyl group of Phe in **D5** and the OH of carboxylic group of Glu229 in the receptor (O–O distance 2.5 Å), (4) a hydrogen bond between the oxygen of the carbonyl group of Leu in **D5** and the backbone NH of Lys303 in the receptor (O–N distance 2.7 Å), (5) a hydrogen bond between the oxygen of the carbonyl group of Met in **D5** and the NH of the indole moiety of Trp318 in the receptor (O–N distance 2.9 Å), (6) a hydrogen bond between the phenolic hydroxyl group of Tyr in **D5** and the oxygen of the carbonyl group of Ile215 in the receptor (O–O distance 2.7 Å) and (7) a hydrogen bond between the nitrogen atom of the amino group of LAA in **D5** and the phenolic hydroxyl group of Tyr128 in the receptor (N–O distance 3.0 Å);(B) Surface view of the active site of the MOP receptor for the highest docking score conformation of MOP receptor with **L5** docked into the active site shows (1) a hydrophobic interaction between the benzene ring of Trp in **L5** and Phe221 in the receptor and (2) a hydrophobic interaction between the benzene ring of Phe in **L5** and the alkyl chain of Lys303 in the receptor. Atom colors are as follows: blue—nitrogen, red—oxygen, white—carbon (on IMP-1), green—carbon (on inhibitor). The hydrophobic interactions represented by red dots and the hydrogen bonds represented by black dots.

maintained in T-75 flasks with culture medium. The medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% non-essential amino acids. To the medium used to culture transfected HEK-293 cells, 10 μL/mL G418 (Geneticin) was added. Flasks were kept in an incubator at 95% humidity and 37 °C, in an atmosphere of 5% CO_2. When cells were 80% confluent, they were subcultured using 0.25% trypsin–EDTA (ethylenediaminetetraacetic acid). Experiments were performed using Caco-2 cells with passage numbers 55–75 and transfected HEK-293 cells with passage number 49–70.

4.3.2. Caco-2. cell homogenate stability assay

Cells at subconfluence in 75 cm^2 flasks were washed with 0.02% EDTA (3 × 5 mL) and Hanks' balanced salt solution (HBSS) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), with pH adjusted at 7.4 (2 × 5 mL). Using a cell scraper, cells were detached from the flask and suspended in 8 mL HBSS–Hepes buffer. Cells were disrupted by two ten-second pulses with a Sonics Vibracell ultrasonic processor set at 60% amplitude and 130 W. The cell debris were removed by centrifugation at 4 °C (2000 rpm, 10 min) and the protein content of the supernatant was determined using the Bio-Rad protein assay kit,[55] by comparison with a standard curve generated from known concentrations of bovine serum albumin. The protein content of the cell homogenate was adjusted to 0.6–0.8 mg/mL.

Endomorphin-1 and the peptide analogues were dissolved in 5% DMSO (dimethyl sulfoxide) in HBSS–Hepes buffer to achieve a 200 μM solution and then 100 μL was added to four wells in a 96-well plate prefilled with 100 μL cell homogenate. The plate was incubated at 37 °C in a shaker at 400 rpm. Samples (10 μL) were collected at 5, 10, 15, 20, 30, 40, 50, 60, and 120 min and added to a solution containing 5.5% TFA to stop enzymatic digestion. These samples were then analyzed by LC–MS to determine the concentration of peptide remaining in the solution.

LC–MS was carried out on a Luna Phenomenex C18 column (2.0 × 50 mm, 5 μm) with a gradient of 100% A (0.1% acetic acid in water) to 100% B (0.1% acetic acid in acetonitrile/water 9:1) over 5.5 min at a flow rate of 0.5 mL/min. The mass spectrometer was set to selective ion monitoring in the positive ion mode. A standard curve was obtained each time from a standard solution containing known peptide concentrations before running unknown samples, and used to determine the peptide concentration in the samples.

4.3.3. Caco-2. cell permeability assay

Caco-2 cells at a density of 10^5 (100 μL) in culture medium supplemented with 1% penicillin/streptomycin (100 U/mL) were seeded into polycarbonate cell culture inserts (pore size 0.4 μm, 6.5 mm diameter, Transwell®) in a 24-well plate. The same medium was also added to the basolateral chamber at 600 μL. Every second day the medium in both chambers was removed and fresh medium was added.

After 21–28 days, the transepithelial electrical resistance (TEER) was measured using the Millicell-ERS epithelial voltohmmeter system (Millipore Corporation) to determine the integrity of the tight junctions and the monolayers. A TEER value of 1500–4280 (Ωcm^2) was considered acceptable to be used for the experiments. TEER values were also measured after the experiment. After the first measurement of TEER values, the Caco-2 cell monolayers were washed three times with prewarmed HBSS-Hepes (25 mM) buffer and then incubated with the same buffer for 30 min at 37 °C. The buffer was removed from the apical chamber and 100 μL aliquots of peptides, at 200 μM concentration in HBSS-Hepes (25 mM) containing 2.5% DMSO, were added to three wells. The Caco-2 cell monolayer integrity was further examined using radiolabeled [^{14}C]-D-mannitol (1.80 μCi) in HBSS-Hepes (25 mM) buffer in three wells. The plates were incubated in a shaker-incubator set to 400 rpm at 37 °C. At 30, 90, 120 and 150 min, samples (400 μL) were collected from the basolateral chamber and replaced with the same volume of buffer. LC–MS quantification of the peptides was performed as described in Caco-2 cell homogenate stability assay. The radioactivity of the [^{14}C]-D-mannitol samples was quantified by liquid scintillation counting (Liquid Scintillation Systems, BECKMAN LS3801, USA). And the apparent permeability (P_{app}) for each compound was calculated using the following equation:

$$P_{app}(cm/s) = dC/dt \times V_r/(A \times C)$$

dC/dt steady-state rate of change in the chemical concentration (M/s) or radiochemical concentration (dpm mL/s) in the receiver chamber V_r volume of the receiver chamber (mL) A surface area of the cell monolayers C_0 initial concentration in the donor chamber (M or dpm/mL)

4.3.4. Receptor radioligand binding scintillation proximity assay (SPA)

The SPA radioligand binding assay was performed in 384-well clear-bottom OptiPlates in a total volume of 40 μl in Hepes buffer (20 mM Tris–HCl, 1 mM MgCl, pH 7.4). Buffer or peptides (dissolved in 5% DMSO) in a range of concentrations (10^{-12}–10^{-5} M), membranes derived from CHO-K1 cells exclusively expressing either MOP or DOP receptors (1 unit/well), [^{3}H]DAMGO (10 nM) or [^{3}H]DPDPE (10 nM) and SPA beads (5 mg/well) were added in sequential order based on the 'T0' assembly format. Non-specific binding was determined using 10 μM endomorphin-1. The plates were sealed using clear adhesive Topseal-A, incubated at room temperature overnight and read on a MicroBeta[2] Plate Counter (Perkin Elmer). Data were expressed as the mean (±SEM) of three independent experiments, each performed in triplicate. Inhibitory constants (K_i, nM) were calculated from the competition experiments using one site curve fitting and the Cheng-Prusoff equation with the GraphPad Prism (v5.0) software.

4.3.5. MOP receptor agonist activity

HEK-293 cells, stably transfected to express MOP receptors, were seeded at a density of 1000 cells/well in a 384-well ProxiPlate (Perkin Elmer) in stimulation buffer. Stimulation buffer was made fresh before each experiment and contained 5 mM HEPES, 0.5 mM IBMX and 0.1% BSA in HBSS (pH was adjusted to 7.4). Cells were stimulated by 30 μM forskolin and concomitantly treated with endomorphin-1, buffer or peptide analogues (dissolved in 5% DMSO) in a range of concentrations (10^{-11}–10^{-5} M). After 30 min incubation at room temperature, Eu-cAMP tracer and *U*Light-anti-cAMP antibody, which were provided in the kit (LANCE *Ultra* cAMP kit, Perkin Elmer) were added to a final volume of 20 μL. Plates were sealed using Topseal-A and incubated for 1 h at room temperature. EnVision® Multilabel (Perkin Elmer) reader was used to read the plates. Data were expressed as the mean (±SEM) of three separate experiments each performed in triplicate. IC_{50} values were calculated by nonlinear regression curve fit in GraphPad Prism (v5.0) software.

4.3.6. Molecular modelling studies

Molecular modeling was employed to predict the binding affinity based on energy minimization for compounds **1**–**5** at MOP receptors using the software Molegro Virtual Docker.[56] The MOP receptor crystal structure (PDB Code:4DKL)[57] was used as the ligand target in this study. Since analogues **3** and **5** were synthesised as diastereomers, as C10LAA was introduced as a racemate, docking simulations for both D- and L-isomers (**D3**, **L3**, **D5** and **L5**) were performed. MOP receptor binding affinities of compounds **1**–**5** were represented by docking scores (MolDock Scores[58]).

4.4. Statistical analysis

All data are expressed as mean (±SEM). One way ANOVA and Dunnett's post hoc test were used to compare the results, as implemented in the GraphPad PrismTM (v5.0) software package. The statistical significance criterion was $p < 0.05$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.09.003. These data include MOL files and InChiKeys of the most important compounds described in this article.

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