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Structure–activity relationships of modified C-terminal endomorphin-2 analogues by molecular dynamics simulations

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ABSTRACT

Motivated by recent experimental works on the modifications of endomorphin-2 (EM2, H-Tyr-Pro-Phe-Phe-NH_2) to develop better painkiller, we performed structure–activity-relationship (SAR) studies to investigate modified C-terminal ligands by using molecular dynamics (MD) simulations. Specifically, instead of the $CONH_2$ for the unmodified EM2's C-terminus, the analogue 2 with its C-terminus being $CONHNH_2$ and analogue 3 with its C-terminus being COOMe are studied. First, a systematic conformer search was performed via the quantum chemical AM1 calculations. The *cis*/*trans* isomers of the lowest energy were hence selected as MD initial structures. We further showed that EM2s in water exhibited similar dihedral angles to those in DMSO, obtained from the NMR experiment. This similarity indicates the reliability of our MD simulations, and enables us to discuss related bioactivity. Our results showed that the interactions of the Tyr^1-Phe^3 pair for *cis*-/*trans*-EM2s played a considerable role for structural stability. Furthermore, we utilized the χ^1 rotamers of individual aromatic side chains to examine the structural bioactivity. It is shown that this criterion to determine the conformational bioactivity toward μ-opioid receptor (MOR) is insufficient. Thus, we have further employed rotamer-combination approaches to examine the characteristics of SAR for *cis*-/*trans*-EM2s. Our results suggested that the bioactive χ^1 rotamers for Tyr^1-Phe^3 pair remained to favor the [*trans*–*trans*] status for MOR selectivity. Therefore, based on the analysis of the χ^1 rotamers, it is suggested that the analogue 2 exhibit greater structural bioactivity for MOR than the analogue 3, and both of them be greater than unmodified EM2 for *trans* isomers.

1. Introduction

Improving the anti-nociceptive effect for acute pain treatment with minimal side effects has long been an important research topic in studying opioid systems. Recently, it has been known that the structure–activity relationships (SARs) of opioid ligands are an important parameter to measure bioactivities, and hence have attracted much attention. There are three well-defined types of opioid receptors, namely the δ, μ, and κ receptors, all of which belong to the G-protein-coupled receptor (GPCR) family [1]. Two novel endogenous tetrapeptide ligands, endomorphin-1 (EM1, H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM2, H-Tyr-Pro-Phe-Phe-NH_2) have been demonstrated to exhibit high affinity and selectivity for μ-opioid receptor (MOR) [2]. They are more effective than other major opioid peptides against neuropathic pain even at low doses. EM1 is widely distributed throughout the brain whereas EM2 is mostly present in the terminal regions of primary afferent neurons in the dorsal horn and medulla of spinal cord [3]. Thus, EM2 can modulate pain at an earlier stage of pain perception than EM1.

The endomorphins mainly consist of two biologically important segments, the 'message' and 'address' sequences [4]. The "message" sequence is defined as the first three N-terminal residues of EMs (Tyr^1-Pro^2-Trp^3 or Tyr^1-Pro^2-Phe^3), and usually influences the molecular recognition function of MOR. As for the "address" sequence, the remaining C-terminal fragment (Phe^4-NH_2), it affects the conformational stability and selectivity to MOR [5]. Based on the "address" sequence concept, many studies have indicated that the C-terminal modified ligands do significantly influence the bioactivity of opioid receptors [6–11]. These include

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aromatic substitution at Phe[4], C-terminal amide group to alcohol conversion, aliphatic or hydrophobic groups. Most of these analogues were determined by opioid receptor binding assays or *in vitro* pharmacological activity, these results revealed detrimental for the μ-opioid affinity. Lazarus et al. have modified the C-terminus of deltorphin by converting $CONH_2$ into COOMe and $CONHNH_2$ [6]. Their results indicate that the binding frequency of the $CONH_2$, COOMe and $CONHNH_2$ terminuses toward MOR increased 6.6-, 67- and 149-folds, respectively. Similar approaches were applied to study EM2 by Gao et al. and their results also confirmed that modifying C-terminal $CONH_2$ to COOMe and $CONHNH_2$ increased the selectivity and conserved binding affinity toward MOR [9]. As for conformational properties on modifications at C-terminus, a series of hydrophobic non-aromatic residues for Phe[4] by substitution of the C-terminal amide group has been studied [10]. Furthermore, it has been found that the (R) chiral center of the fourth amino acid may induce a proper spatial arrangement of the third aromatic ring of endomorphin analogues [12]. Hence, not only pharmacophoric residues, but also the C-terminal residue plays a critical role in the biological activity of the endomorphins. Moreover, the amino acid, Phe[4], is free to adopt a bioactive conformation that is independent of the other amino acids [11]. These results further point out the importance of the orientation of the C-terminal aromatic side chain for binding affinity to the MOR, indicating different structural features for each residues would influence the SARs and further affect the affinity and selectivity to MOR strongly [12].

On the other hand, it is known that *cis*/*trans* isomers of Tyr[1]-Pro[2] peptide bond play a critical role in relation to bioactive conformations of EMs and their C-terminal modified analogues. Various theoretical and experimental approaches have been conducted on conformational analysis in different environments, but those results have led to diverse conclusions. Podlogar et al. conducted a combination of ^{1}HNMR measurements in aqueous/DMSO solutions and molecular dynamics (MD) simulation for EM1 [13]. The authors concluded that the extended structure is the bioactive conformation, and the percentages of the *cis* and *trans* conformations in Tyr[1]-Pro[2] peptide bonds are 25% and 75%, respectively. Yet, Fiori et al. studied the EM1 in amphipathic environments, such as reverse micelles of bis(2-ethylhexyl)sulfosuccinate sodium salt (AOT), and found the bent structure is the bioactive conformation [14]. Their results show that the percentages of *cis* and *trans* conformations in Tyr[1]-Pro[2] peptide bonds are 45% and 55%, respectively. Moreover, In et al. employed the NMR experiment to study EM2 in DMSO solution and dynamical simulated annealing (SA) computation [15]. They concluded that extended conformations are more suitable for MOR, and a population ratio of *ca.* 1:2 was observed between *cis* and *trans* isomers around Tyr[1]-Pro[2] peptide bond. Furthermore, experiments using NMR spectral analysis in pseudoproline-containing analogues of morphiceptin and EM2 show that the majority of bioactive conformations (>98%) are in *cis* conformation and therefore bent structure should be the bioactive conformation [16]. However, by studying the conformational features of EM2 and EM2OH through experiments in different solutions, i.e. TFE, DPC, H_2O, and DMSO, and various pH environments, i.e. 2.7, 3.5, 5.2, In et al. showed that these factors cause various mixtures and ratios of *cis*/*trans* isomers [17].

Aromatic–aromatic interactions play a relevant role in stabilizing the structure of EMs. These interactions between each aromatic side chain can be a stabilizing factor in the various conformations of EMs in different environment. In AOT micelles, Fiori et al. reported the tight packing of the aromatic side chain of Tyr[1] and Trp[3] in *cis*-EM1 [14], and the close contact between Tyr[1] and Phe[4] for *trans*-EM1 in aqueous solution. On the other hand, for the EM2OH, In et al. observed close contact between the aromatic side chain of (Tyr[1], Phe[3]) and (Tyr[1], Phe[4]) in the conformation with folded backbone in DMSO-*d*6 solution [15]. In addition, compact sandwich conformations were experimentally observed for both *cis*-EM1 and *trans*-[D-Pro[2]]EM1. In both cases, the aromatic side chains of Tyr[1] and Phe[3] were packed against Pro[2] [11,13,24]. Moreover, this compact structure was also found in *cis*-EM2 [15,17,24].

Additionally, the basic structural characteristics of EMs were determined in detail by using SA and MD theoretical works from Leitgeb et al. [18,19]. The results revealed these EMs are a highly flexible conformation on their backbone and side chain rings. Although the secondary structures of β- and γ-turns were ever occurred during their MD simulation, these turns were formed for only a few picoseconds. In the present review, Leitgeb summarized numerous researches with respect to hunting for the possibly bioactive conformation of EMs [20].

Despite many studies have been performed to investigate preferred bioactive conformations of endomorphins in the past, to the best of authors' knowledge, none of the structure–activity relationships of modified EM2s with C-terminal modifications are explored for their benefits in increasing bioactivity *via* MD simulations. Since the pharmacophoric elements and orientations of aromatic side chains are suggested as the two essential indicators for the analysis of bioactive conformations of endomorphins [15,24], the present study is focused on explaining how the EM2s with the C-terminal modification are more bioactive through analyzing the conformations and bioactive χ^1 rotamers for Tyr[1], Phe[3], and Phe[4] residues with MD simulations.

2. Methodology

2.1. Systematic conformer search

Fig. 1 illustrates the geometric structures of EM2s. These structures contain the parent peptide EM2 (also called the analogue 1), and analogues 2 and 3 with $CONHNH_2$ and COOMe modifications, respectively, at the EM2's C-terminus. The dihedral angles of the backbone and side chain are labelled. The initial structures of the EM2s were constructed using the SPARTAN software package [21]. Since the NMR measurements in DMSO have revealed that EM2 existed in an equilibrium mixture of *cis* and *trans* isomers of Tyr[1]-Pro[2] peptide bonds, and the dihedral angles between these two residues of backbone stayed at a stable conformation [15,17], the conformer searches for EM2s were performed by systematically restraining the dihedral angle Ψ_1 and peptide bond ω_1 to find global energy minimum. Initially, these angles were assigned to 0°, and then by separately tuning the

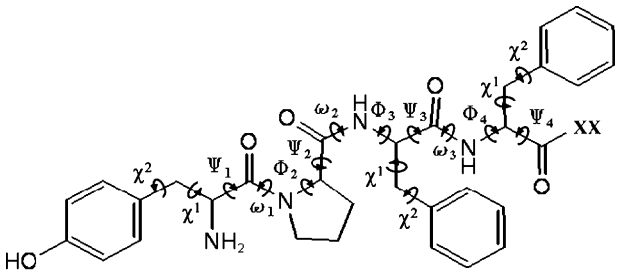

Fig. 1. Illustration of the structural model of the EM2's containing the parent peptide EM2 (known as analogue 1), analogue 2, and analogue 3.

angles we calculated the lowest-energy conformers of EM2s using the SPATRAN/AM1 approach. Subsequently, the dihedral angles of Ψ_1 and ω_1 were mutually rotated by increments of 30° and repeat above steps. Finally, we obtained the 12 by 12 (totally 144) conformers of EM2 with minimized energy. The *cis* and *trans* conformers of the lowest energy derived from EM2, analogues 2 and 3, were selected as the initial conformers for the MD simulations.

2.2. Molecular dynamics simulation

The MD simulations were performed using GROMACS computer program (version 3.2.1) with the GROMOS96 (ffG43a1) force field [22,23]. During the simulations, the initial structures of EM2 and its analogues were specified in accordance with the lowest-energy conformers obtained from the conformers found by AM1 calculations. Then, all simulations assumed that the EM2s were surrounded by a total of 2900 water molecules (simple point charge with SPC216 model), and the systems, including EM2s and waters, were placed within a cubic box with dimensions 44 Å × 44 Å × 44 Å, subjected to periodic boundary conditions. An energy minimization technique was then used to refine the initial structure of EM2s in aqueous solution. The MD simulations assumed the atoms have a random velocity which is consistent with a Maxwellian distribution. The positions and velocities of the atoms were integrated using the standard Verlet algorithm with a time step of dt = 2 fs and LINCS to constrain all bond length. The system was initially equilibrated to a temperature of 300 K and a constant pressure of 1 atm (NPT ensemble) using the Berendsen coupling scheme with a time constant of 0.1 ps for each case. Following the equilibration, the time evolutions of the quantities of interest were recorded over the duration of 20 ns. The data were sampled every 2 ps, and in total 10,000 structures were collected. The Particle–Mesh–Ewald (PME) method was then applied to calculate the Coulomb-interactions and the non-bonded potentials were truncated using a cut-off radius of 12 Å.

3. Results and discussion

The characteristics of conformations for EM2 and its C-terminal modified analogues 2 and 3 are to be discussed here through analysis of the relationships among structure and stability, aromatic–aromatic interactions, aromatic side chain characteristics and the rotamer-combination analysis of aromatic side chains using MD simulations. During the entire MD simulations, peptide energies have not changed significantly.

3.1. Structure and stability

To explore the structural features of analogues 2 and 3, and compare them with the parent peptide (i.e. analogue 1), the root-mean-square-deviations (RMSD) of the backbone, the end-to-end distance (r_{ee}) between N-terminal N atom and C-terminal C atom and the probability distributions of backbone dihedral angles (Φ, Ψ) are specifically examined. The RMSD and r_{ee} variations of EM2s are shown in Fig. 2. They are grouped into *cis* and *trans* isomers, as shown in Fig. 2a and b, respectively. It can be seen from the RMSD results that all the simulation cases have reached to an equilibrated state at *ca.* 4000 ps of simulation time. Furthermore, the results show that the *trans*-EM2's r_{ee} values are higher than the *cis*-EM2s. This indicates that the occurrences of open structures with extended backbone in *trans*-EM2s during the MD simulations, and the hydrogen bond (HB) interactions between backbone-to-backbone are relatively lower (~3%). In contrast, for *cis*-EM2s, the HB interactions (~17%) mainly take place between the carboxylic

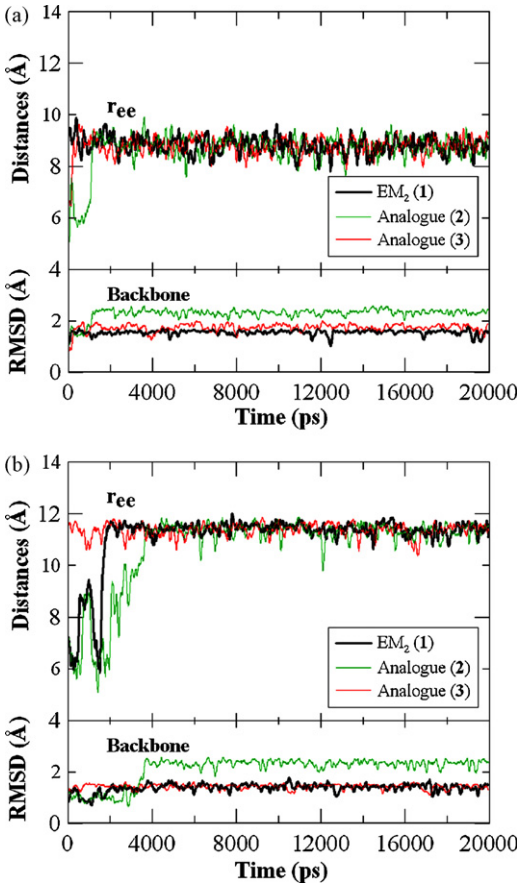

Fig. 2. The RMSD values of backbone and r_{ee} distances between two termini of EM2s for the 20 ns MD simulations: (a) *cis* isomers and (b) *trans* isomers.

oxygen of Pro^2 and the amide nitrogen of Tyr^1. Hence, it is suggested that the HB interactions for *cis*-EM2s cause its r_{ee} value less than that of *trans*-EM2s.

The probability distributions of dihedral angles (Φ, Ψ) in backbone for *cis*/*trans* isomers of the EM2s are shown in Fig. 3. The solid and dotted lines indicate the Φ and Ψ dihedral angles, respectively. In addition, parent peptide, analogues 2 and 3 are decorated with the black, green and red colour, respectively, for easy identification. It can be seen that the Ψ angle for Tyr^1 and the dihedral angles (Φ, Ψ) for Pro^2 reveal the unique peak of the probability distribution for both *cis*-EM2s and *trans*-EM2s. This feature indicates the Tyr^1-Pro^2 moiety of EM2s is more stable than other residue segments. In contrast, for both *cis*-/*trans*-EM2s, the dihedral angles Φ of Phe^3 and Phe^4 are more flexible, due to the local dynamic conformations that can exist at peak A or B for Phe^3, and peak C or D for Phe^4. The peaks correspond to the degrees of dihedral angles *ca.* −60° and −120°. These regular variations of dihedral angles of backbone were referred that the spatial conformational properties contain key factors for binding to the MOR [25]. Compared with the MD calculation from Leitgeb et al. [19], except for Ψ_1 and Φ_2, most of the dihedral angles of backbone were more narrow range in our analytic data. Furthermore, we examine the backbone conformations, containing the parent peptide, analogues 2 and 3, and it is found that the EM2s with modified C-terminus affected the variations of backbone slightly, especially on phenylalanine residues. From the aforementioned analysis, by and large, we conclude that the conformations of N-terminal fragments, i.e. Tyr^1 and Pro^2, are more stable than C-terminal segments, i.e. Phe^3 and Phe^4. These theoretical findings are in agreement with previous study from In et al. [15,17].

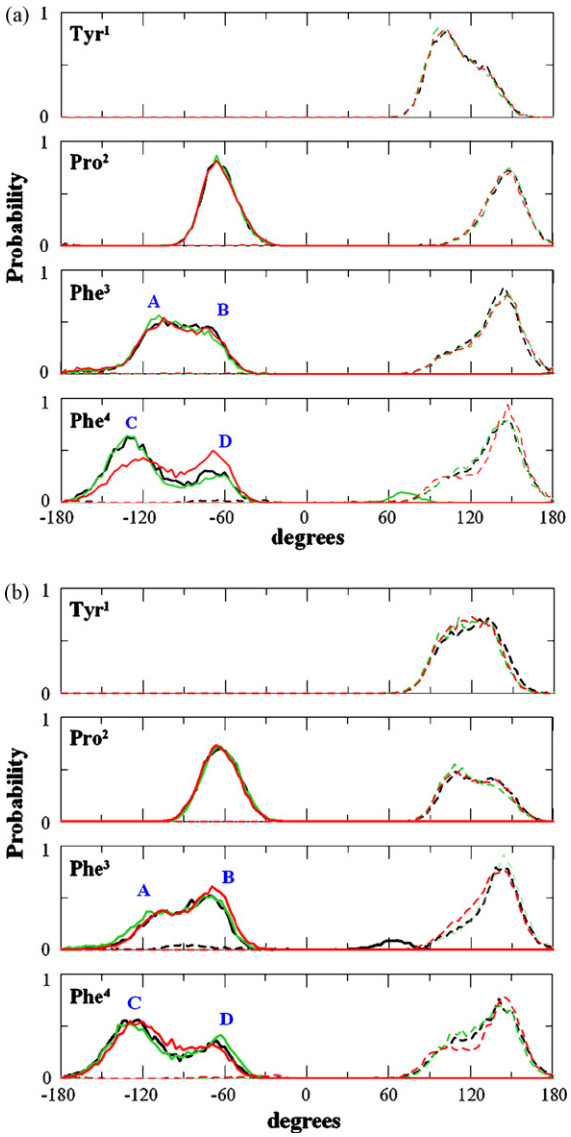

Fig. 3. The probability distributions of dihedral angles (Φ, Ψ) in backbone for the 20 ns MD simulations: (a) *cis* isomers and (b) *trans* isomers. The real and dot lines indicate the Φ and Ψ dihedral angle, respectively.

3.2. Aromatic–aromatic interactions

The aromatic–aromatic interactions are an important factor to infer the structural information of EM2s. During the course of our MD simulations, the proportions of the aromatic–aromatic interactions between different aromatic side chain residues in *cis*-/*trans*-EM2s are sampled from the stable conformations, based on the data shown in Fig. 2. The definition of existence of the aromatic–aromatic interactions with the distance between two aromatic centroids, that is less than 5.5 Å, was adopted herein [24]. In our works, we classified the interactions into three interacting aromatic pairs (Tyr^1-Phe^3, Tyr^1-Phe^4 and Phe^3-Phe^4) for all *cis*/*trans* isomers. The proportions of aromatic–aromatic interactions between these pairs for all three kinds of the two types (*cis* and *trans*) EM2s are given in Table 1. The interacting percentage is defined as the ratio of the number of the events, whose distance between the two aromatic centroids is less than 5.5 Å, to the number of total sampling in the MD simulations. It can be seen that the highest ratio of aromatic–aromatic interactions belongs to the Tyr^1-Phe^3 interacting pair for both *cis*-/*trans*-EM2s. Furthermore, the interactions between the Tyr^1 and Phe^3 pair, or Phe^3 and Phe^4 interacting pair are higher than the Tyr^1-Phe^4 interacting pair for both of the types, due to higher interacting percentages found in our simulation, as shown in the table. The results indicate the interactions of the Tyr^1-Phe^3 pair play a considerable role for the structural stability. We conclude that the aromatic–aromatic interactions may replace the conventional H-bonds (~17% for *cis*-EM2s and ~3% for *trans*-EM2s) and become the major force in stabilizing all the structures analyzed here. This finding is in agreement with the experimental work by Shao et al. [25].

Table 1
Proportions (in %) of aromatic–aromatic interactions between the pairs of Tyr^1-Phe^3, Tyr^1-Phe^4 and Phe^3-Phe^4 for *cis* and *trans* isomers during the MD trajectories[a].

	cis-EM2-$CONH_2$	*cis*-EM2-$CONHNH_2$	*cis*-EM2-COOMe
Tyr^1-Phe^3	50.28	56.43	54.30
Tyr^1-Phe^4	0.02	0.32	0.02
Phe^3-Phe^4	2.79	2.20	4.56
	trans-EM2-$CONH_2$	*trans*-EM2-$CONHNH_2$	*trans*-EM2-COOMe
Tyr^1-Phe^3	12.25	12.04	14.34
Tyr^1-Phe^4	0.15	0.32	0.15
Phe^3-Phe^4	4.99	4.53	0.54

[a] The data were sampled from the stable structure of backbone for *cis*-/*trans*-EM2s.

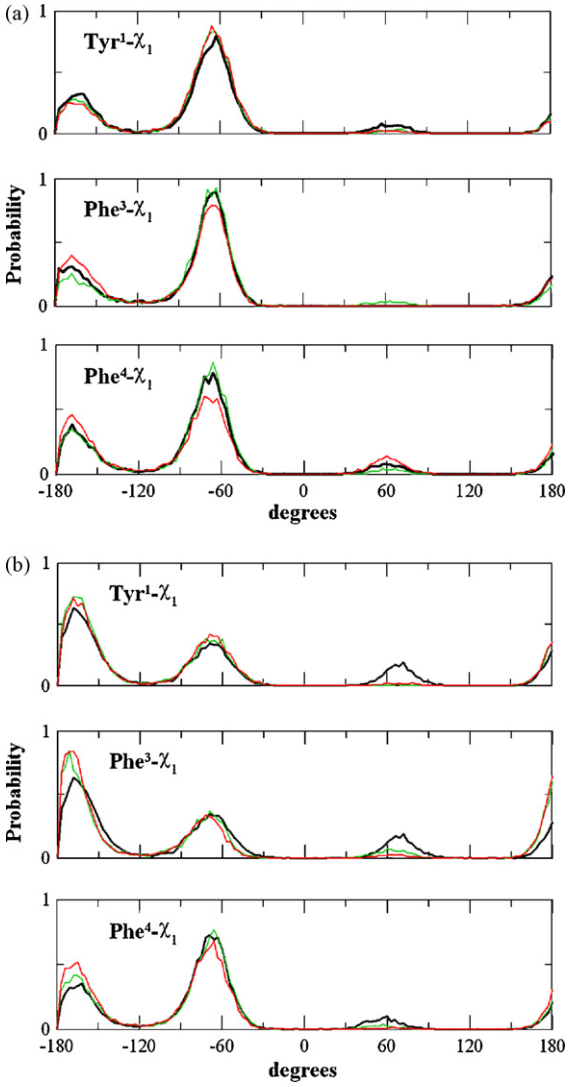

Fig. 4. The probability distributions of aromatic χ^1 rotamers for the 20 ns MD simulations. (a) *cis* isomers and (b) *trans* isomers.

Table 2
Proportions (in %) of bioactive di-rotamer-combination for *cis*-EM2s.

Aromatics	Di-rotamer-combinations	*cis*-EM2-$CONH_2$	*cis*-EM2-$CONHNH_2$	*cis*-EM2-COOMe
Tyr^1-Phe^3	[g(-)–*trans*]	12.16	12.70	17.60
	[*trans*–*trans*]	7.43	2.74	8.69
	Sum	19.59	15.44	26.29
Tyr^1-Phe^4	[g(−)–g(−)]	35.55	43.01	33.01
	[*trans*–g(−)]	15.77	13.85	9.39
	Sum	51.32	56.86	42.40
Phe^3-Phe^4	[*trans*–g(−)]	12.76	10.99	12.95

Table 3
Proportions (in %) of bioactive di-rotamer-combination for *trans*-EM2s.

Aromatics	Di-rotamer-combinations	*trans*-EM2-$CONH_2$	*trans*-EM2-$CONHNH_2$	*trans*-EM2-COOMe
Tyr^1-Phe^3	[g(−)–*trans*]	17.19	22.89	26.18
	[*trans*–*trans*]	26.19	35.93	30.14
	Sum	43.38	58.82	56.32
Tyr^1-Phe^4	[g(−)–g(−)]	16.43	16.10	16.64
	[*trans*–g(−)]	24.41	32.73	27.11
	Sum	40.84	48.83	43.75
Phe^3-Phe^4	[*trans*–g(−)]	31.18	38.54	30.74

3.3. Preferred χ^1 rotamers of individual aromatic side chain

It is well known that the spatial arrangement of side chains for peptides and proteins plays a critical role in biological activities, particularly for the opioid peptides and their synthetic analogues. To date, several considerable studies and many efforts have been devoted into the research of bioactivity between ligands and their relevant opioid receptors, including modified C-terminus [6–10]. Furthermore, prior studies show that the spatial disposition of aromatic side chains is important when ligands bind with the opioid receptors [26,27]. The probability distributions of χ^1 rotamers of *cis*-/*trans*-EM2s, including the Tyr^1, Phe^3 and Phe^4 are shown in Fig. 4. The topographical space of χ^1 rotamers are divided into three angle groups, i.e. g(−) (∼−60°), g(+) (∼+60°), and *trans* (∼±180°). The colour scheme is the same as that in Fig. 3.

In Fig. 4, the preferred orientation of the side chain conformers for both *cis*-/*trans*-EM2s lies in the g(−) and *trans* rotamers. For the *trans*-EM2s, Tyr^1 and Phe^3, however, prefer the *trans* orientation, and Phe^4 favors the g(−) rotamer, whereas the *cis*-EM2s favor the g(−) more than the *trans* orientation in all aromatic χ^1 rotamers. From our results, it is found that the g(−) and *trans* conformations have the characteristics of a high degree of tolerance in structural

Table 4
Proportions (in %) of bioactive tri-rotamer-combination for *cis*/*trans* isomers.

Bioactive tri-rotamer-combination conformers (Tyr^1-Phe^3-Phe^4)			
Peptides	[g(−)–*trans*–g(−)]	[*trans*–*trans*–g(−)]	Sum
cis-EM2-$CONH_2$	5.47	4.92	10.39
cis-EM2-$CONHNH_2$	7.23	1.45	8.68
cis-EM2-COOMe	6.55	4.96	11.51
trans-EM2-$CONH_2$	9.19	12.38	21.57
trans-EM2-$CONHNH_2$	12.95	20.97	33.92
trans-EM2-COOMe	12.40	14.27	26.67

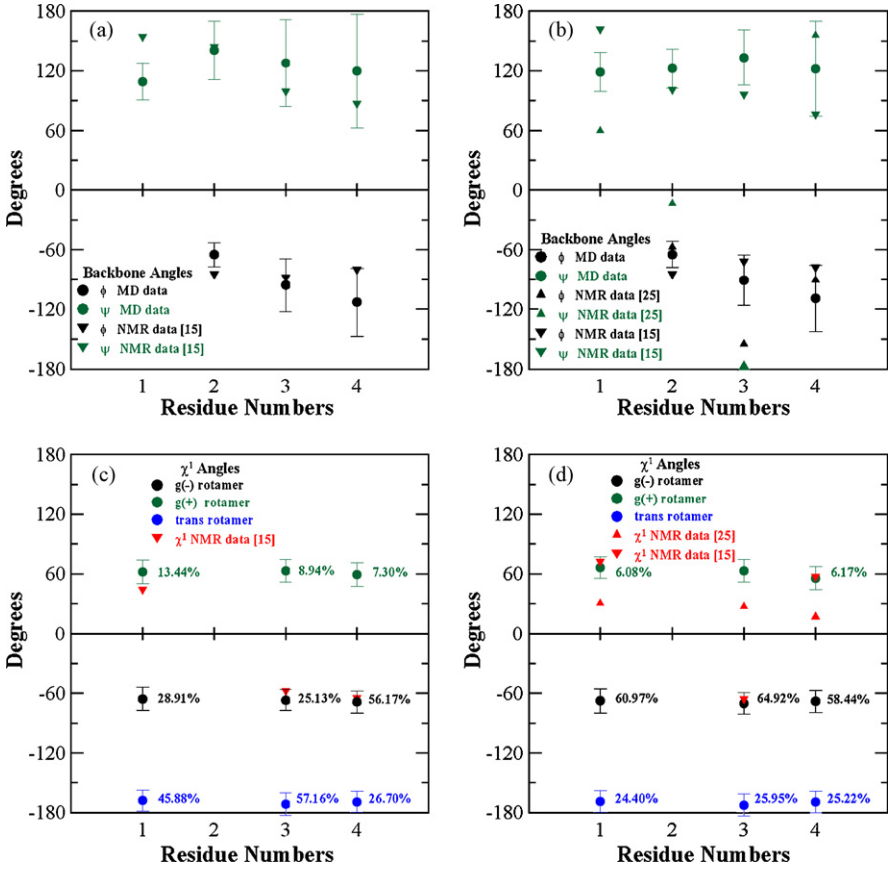

Fig. 5. Dihedral angles of backbone and χ^1 rotamer of the EM2. (a) Backbone angles for the *cis* isomers, (b) backbone angles for the *trans* isomers, (c) χ^1 angles for the *cis* isomers, and (d) χ^1 angles for the *trans* isomers.

differences in Tyr^1. This results is in agreement with the finding of Grieco et al. that the Tyr^1 prefers to accommodate both the g(−) and *trans* side chain conformations [28]. Thus, this conformational feature indicates that the g(−) and *trans* are the main rotamers for the receptor binding site. In addition, it can be seen in Fig. 4 that the probability of the *trans* rotamers of Tyr^1 is increased more for analogues 2 and 3, when compared with the parent peptide. However, this situation is not obvious in the *cis* isomers. Regarding the Phe^3 residues, two cyclic tetrapeptides were studied by Mosberg et al. [29–31], i.e. JOM-6 (Tyr-c(S-Et-S)[D-Cys-Phe-D-Pen] NH_2) and JOM-13 (Tyr-c[D-Cys-Phe-D-Pen]OH). The Phe^3 side chain of JOM-13 assumes a g(−) orientation for optimal δ binding, while the Phe^3 side chain of JOM-6 must be in the *trans* orientation for high-affinity μ binding. Additionally, based on the investigation by Yamazaki et al. [32], the bioactive structure of morphiceptin (Tyr^1-Pro^2-Phe^3-Pro^4-NH_2), known as the ligand moderated select to MOR, requires the χ^1 rotamers of Tyr^1 and Phe^3 to be in the *trans* orientation. From these results, it can be envisaged that possessing a *trans* rotamer in Phe^3 can be highly selective for the MOR. Indeed, our results have clearly shown that the χ^1 rotamer of Phe^3 is more predilection to have the *trans* orientation for modified peptides, i.e. analogues 2 and 3. In contrast, analogue 3 increases such probability more than analogue 2 for the *cis* isomers. This indicates that analogue 3 has most potency for the MOR. Tömböly et al. replaced the Phe^4 of endomorphins with β-$MePhe^4$ to explore the binding affinities for the MOR [33]. They found that the (2S,3S)-β-$MePhe^4$ configuration can enhance enzymatic stability, and their μ opioid affinities are four times higher than the parent peptides. Furthermore, from their NMR experiments, it is shown that the substituted C-terminal (2S,3S)-β-$MePhe^4$ in EM2 strongly favors the g(−) spatial orientation, which implies g(−) is one of the bioactive rotamers for the MOR. Our simulation results show, among the three EM2s, only analogue 3 decreases the probability of Phe^4 χ^1 rotamer for the *cis* isomers. This indicates that the analogue 3 may possess less bioactivity for the MOR. From the viewpoint of using individual χ^1 rotamer to determine the bioactivity of EM2s toward to MOR, we demonstrate that the *trans* isomers have increased bioactivity than the *cis* isomers. However, according to individual χ^1 rotamers, it is clearly insufficient to determine the conformational bioactivity toward relevant opioid receptors. Because of conflicts between the χ^1 rotamers of Phe^3 and Phe^4 residues regarding the bioactivity of *cis* isomers for analogue 3, we further utilize the di-rotamer-combination (Tyr^1-Phe^3, Tyr^1-Phe^4 and Phe^3-Phe^4) and tri-rotamer-combination approaches to examine the SAR of C-terminal modified analogues of EM2s.

3.4. Rotamer-combination analysis

According to individual χ^1 rotamers, the determination of the conformational bioactivity toward relevant opioid receptors is insufficient, and hence we further employ the di-rotamer-combination approach to examine the characteristics of SAR for *cis*-/*trans*-EM2s. In our MD simulations, the pairs of di-rotamer-combination contain [g(−)–*trans*] and [*trans*–*trans*] for Tyr^1-Phe^3, [g(−)–g(−)] and [*trans*–g(−)] for Tyr^1-Phe^4, and [*trans*–g(−)] for Phe^3-Phe^4. The proportions of bioactive di-rotamer-combination for *cis*-EM2s and *trans*-EM2s have been shown in Tables 2 and 3, respectively. It is generally accepted that the most significant pharmacophoric parameters in opioid peptides are: (1) the distance from the protonated amine to the Tyr aromatic ring,

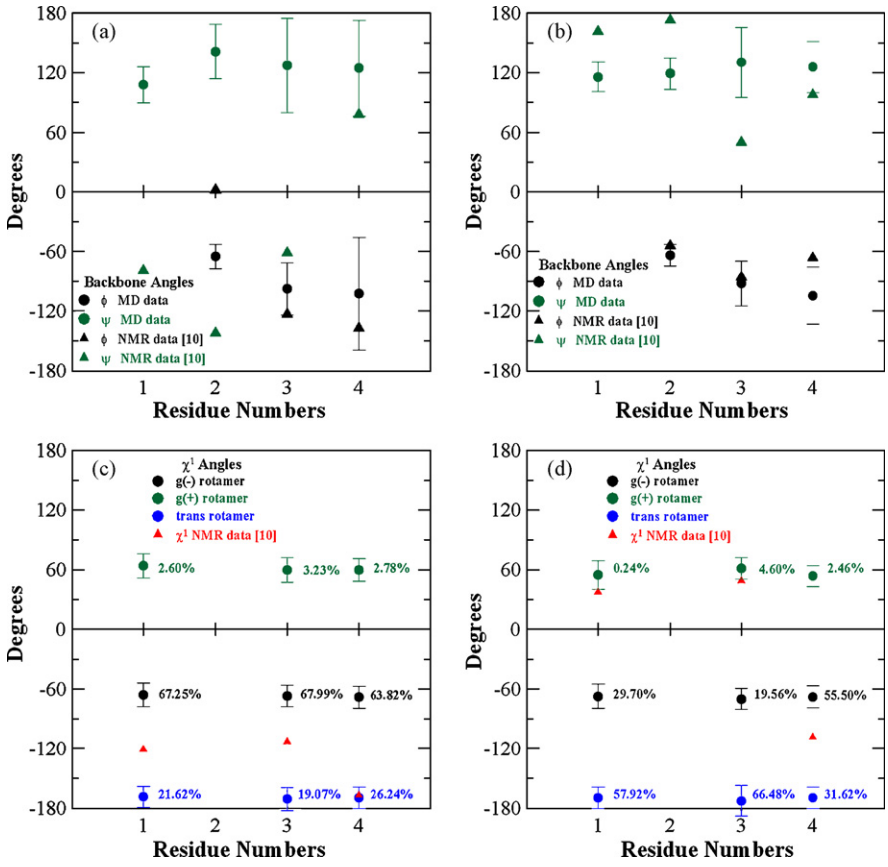

Fig. 6. Dihedral angles of backbone and χ^1 rotamer of the $CONHNH_2$-modified EM2. (a) Backbone angles for the *cis* isomers, (b) backbone angles for the *trans* isomers, (c) χ^1 angles for the *cis* isomers, and (d) χ^1 angles for the *trans* isomers.

(2) the distance from the protonated amine to a second hydrophobic center, and (3) the distance between the Tyr ring and the hydrophobic center [14,28,32]. In fact, these significant distances are also strongly related to aromatic χ^1 rotamers, due to the dependence between the angles and distances. Thus, here, we only discuss the most important factor, i.e. di-rotamer-combination for the Tyr^1-Phe^3 pair, that influences the bioactivity of EM2s. For *trans*-EM2s (Table 3), it can be seen that the χ^1 rotamers of the [*trans*–*trans*] pair for Tyr^1-Phe^3 exhibit a higher ratio than the [g(−)–*trans*] pair, whereas the [g(−)–*trans*] pair bears the higher ratio for *cis*-EM2s (Table 2). Moreover, the total proportions of χ^1 rotamers of the Tyr^1-Phe^3 for *trans*-EM2 are higher than either of the *cis*-EM2 or analogues 2 and 3. Meanwhile, the *trans* isomers of the analogues 2 and 3 display a tendency to increase the proportions when compared with *trans*-EM2 (parent peptide). These results provide the evidence that the *trans*-EM2s may possess higher selectivity and possibly higher binding affinity to MOR than *cis*-EM2s, and hence analogues 2 and 3 may bear the conformational bioactivity more than *cis*-EM2. In particular, the analogue 2 shows larger increase in bioactivity than analogue 3. Although Grieco et al. report that the Tyr^1 prefers to accommodate both the g(−) and *trans* χ^1 rotamers [28], our results suggest that the bioactive χ^1 rotamers for the Tyr^1-Phe^3 pair remain to favor the [*trans*–*trans*] configuration for MOR selectivity.

Furthermore, a tri-rotamer-combination approach was utilized to analyze the conformational bioactivity of *cis*-/*trans*-EM2s. Leitgeb and Szekeres have studied the major rotamer combinations from 1000 possible conformations of endomorphins that are generated by the SA method [34]. Our MD simulation shows that the preferred tri-rotamer-conformation can generally be classified into two tri-conformer-combination groups that are [*trans*–*trans*–g(−)] and [g(−)–trans–g(−)] for Tyr^1-Phe^3-Phe^4. Based on the data in Table 4, the percentages of combined two preferred tri-conformer-combinations in *cis*-EM2s are 10.39%, 8.68%, and 11.51% for parent peptide, analogue 2, and analogue 3, respectively. Moreover, the percentages of combined preferred tri-conformer-combinations in *trans*-EM2s are 21.57%, 33.92%, and 26.67% for parent peptide, analogue 2, and analogue 3, respectively. These results indicate that the probability of the preferred tri-conformer-combinations for *trans*-EM2s is much higher than *cis*-EM2s. Furthermore, the analogues 2 and 3 would enhance the presence of these two rotamer-combinations, indicating the C-terminal modified analogues have a higher selectivity to the MOR, and analogue 2 exhibits even higher selectivity than analogue 3. Our results conclude that *trans*-EM2s possess the selectivity and bioactivity to MOR better than *cis*-EM2s, and, hence, the C-terminal modified analogues are better fitted for the MOR binding site as suggested by many other studies [27–30,32,33].

In Figs. 5–7, we show the comparison of the dihedral angles of the backbone and χ^1 rotamers from MD simulations in water and ^{1}HNMR experimental data in DMSO for the *cis* and *trans* isomers. The experimental data are obtained from Refs. [10,15,25]. Residues 1, 2, 3 and 4, labelled on the horizontal axis, are Tyr^1, Pro^2, Phe^3 and Phe^4, respectively. The backbone angles are shown in (a) and (b) in Figs. 5–7. Symbols on the figures are explained as follows: black circles indicate our MD results for angle ϕ and green circles for Ψ. Triangles are data from the experiment in the references. They are colour-coded in the same way as our MD data. For the χ^1 angles (in (c) and (d) in Figs. 5–7), black circles represent the g(−) rotamer, green circles the g(+) rotamer, and blue circles the *trans* rotamer. The red triangles show the experimental ^{1}HNMR data from the literatures. For *cis*-/*trans*-EM2 (Fig. 5), it can be seen that the dihedral angles (Φ, Ψ, χ) are in relatively good agreement between

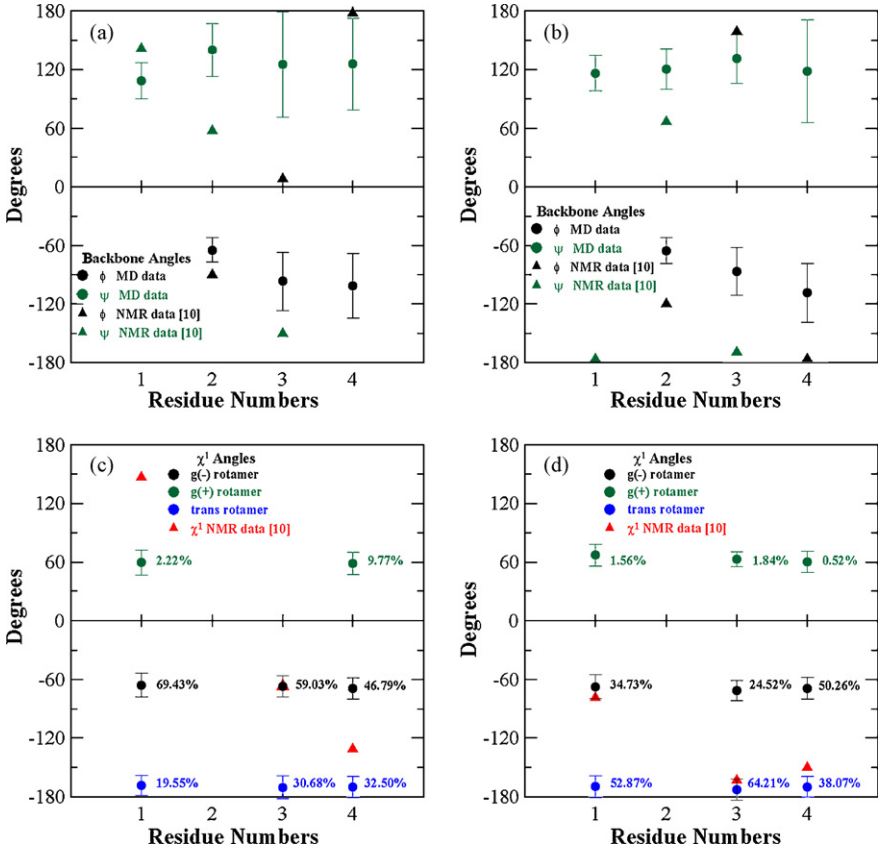

Fig. 7. Dihedral angles of backbone and χ^1 rotamer of the COOMe-modified EM2. (a) Backbone angles for the *cis* isomers, (b) backbone angles for the *trans* isomers, (c) χ^1 angles for the *cis* isomers, and (d) χ^1 angles for the *trans* isomers.

MD and experimental data. Furthermore, the results of *trans*-EM2-$CONH_2$ reveal most of the dihedral angles are different from unmodified *trans*-EM2. It can be seen that there are only Φ_2, Φ_4 and Ψ_4 consist with the ^{1}HNMR data for the *trans* isomer. For *cis*/*trans* isomers of EM2 analogues, as shown in Figs. 6 and 7, the MD data are compared with the recent investigation concerning the SAR of EM2 analogues with C-terminal modifications [10]. Our simulation data are partially in agreement with ^{1}HNMR experimental data for the backbone angles. As for χ^1 rotamers, it can be seen that only *trans* isomers of analogues 2 and 3 have a relatively good agreement between the MD and experimental data. The data points that do not show good agreement between the MD and ^{1}HNMR may be due to different solvent or experimental conditions. For the χ^1 angle of residue 2, due to the lack of the definition in the literature, our MD results are not shown. Error bars are determined from the standard deviation of our MD simulation data in the time period with stabilized conformations. Overall, our MD results predict similar trend as experimental findings. Further MD simulation work may be required to answer the inconsistency between MD and experiment that are reported here.

4. Conclusions

Through the molecular dynamics simulations, we have examined the structure–activity-relationships (SAR) of the EM2's parent peptide ($CONH_2$), and its analogues 2 ($CONHNH_2$) and 3 (COOMe) with modified C-terminal groups. Our results reveal that after these modifications, noticeable enhancement in its pharmacological activity to μ-opioid receptor (MOR) can be achieved both in terms of binding affinity and bioactivity. Better understandings of the structural characteristics of these μ-selective opioid ligands have been obtained through the rotamer-combination analyses. Furthermore, it is identified that the interaction of the Tyr^1-Phe^3 pair for *cis*-/*trans*-EM2s plays a considerable role for the structural stability. From the analysis of di-rotamer-combination for Tyr^1-Phe^3 pair, our results provide significant evidence to show that the *trans*-EM2s possess higher percentage of bioactive di- and tri-rotamer-combination to MOR than *cis*-EM2s, and hence analogues 2 and 3 bear the conformational bioactivity more than *trans*-EM2. In particular, the analogue 2 shows the highest bioactivity. Moreover, our MD simulations show the *trans*-EM2s possess higher selectivity and possibly higher binding affinity to MOR than *cis*-EM2s. The order of structural bioactivity is analogue 2 greater than analogue 3, and the analogue 3 greater than EM2 parent peptide for the *trans* isomers. We remark that consideration of the structure of MOR is necessary in the future molecular dynamics simulations for binding analysis.

Acknowledgements

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