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Article

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The Pharmacological Heterogeneity of Nepenthone Analogs in Conferring Highly Selective and Potent κ-Opioid Agonistic Activities

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ABSTRACT

To develop novel analgesics with no or less side effects than that of traditional opioids is highly demanded to treat opioid receptors mediated pain and addiction issues. Recently, κ-opioid receptor (KOR) is established as an attractive target, although its selective agonists could bear heterogeneous pharmacological activities. In this study, we designed and synthesized two new series of nepenthone derivatives by inserting a spacer (carbonyl) between 6α,14α-endo-ethenyl-thebaine and the 7α-phenyl substitution of the skeleton, and by substituting the 17-N-methyl group with a cyclopropylmethyl group. We performed *in vitro* tests (binding and functional assays) and molecular docking operations on our newly designed compounds. The results of wet-experimental measures and modeled binding structures demonstrate that these new compounds are selective KOR agonists with nM level affinities. Compound **4** from these new derivatives, showed highest affinity (K_i= 0.4 ± 0.1) and the highest selectivity (μ/κ = 339, δ/κ = 2034) toward KOR. The *in vivo* tests turned out that compound **4** is able to induce stronger (ED_{50} = 2.1 mg/kg) and much longer antinociceptive effect than that of the typical KOR agonist U50488H (ED_{50} = 4.4 mg/kg). Therefore, compound **4** can be used as a perfect lead compound for future design of potent analgesics acting through KOR.

KEYWORDS

κ-opioid receptor, μ-opioid receptor, nepenthones, 4,5-epoxymorphinans, pharmacological heterogeneity, structure-activity relationship, binding structure

Introduction

Opioid receptors are composed of three major subtypes (μ, δ, and κ), they modulate various physiological and pathophysiological activities in the central nervous system (CNS), including pain, psychotomimetic effects and neuroprotection[1, 2]. When bound with opioids and opioid analogs, these receptors demonstrated heterogeneous pharmacological profiles[1]. Typical narcotics as morphine, fentanyl and mepridine, which have been used as analgesics, are the agonists of μ-opioid receptor (MOR),[2] while some other opioids like **butorphanol**[3] and **nalbuphine**[4] **(Chart 1)** are also agonists of κ-opioid receptor (KOR) with lower affinities than that with the MOR. In order to avoid the opioid-like adverse effects (e.g. addiction, respiratory depression and constipation) mainly from the binding of MOR with opioids[1, 2], recent research efforts are focused on the design of potent and selective KOR agonists[5, 6]. Early efforts led to the discovery of KOR-selective agonists as **spiradoline**[7] and **enadoline**[8, 9] **(Chart 1)**. However, these two KOR-selective agonists have failed to survive clinical trials of analgesia due to their negative benefit-risk ratios. A successful example is discovery of the KOR-selective agonist **nalfurafine** (**Chart 1**).[10, 11] Chemically, this compound is a 4,5-epoxymorphinan derivative. It has been approved and marketed in Japan, as it is able to produce a long-term suppression of pruritus, but can only be used for hemodialysis patients showing resistance to conventional treatments[12]. On the other hand, a lot of studies are continuously performed to develop dynorphin A analogs as potential treatment of chronic neuropathic pain,[6] as the KOR-dynorphin system has been found to have counter-modulatory effects on the

reward from narcotics exposure, and exhibits plasticity in addictive-like states.[13] These discoveries[5, 6] indicate that KOR-selective agonists could bear heterogeneous pharmacological activities. So far, it is still a hard work to design and develop novel KOR agonists as potent therapeutics, but with no or much less adverse effects than that of MOR agonists.

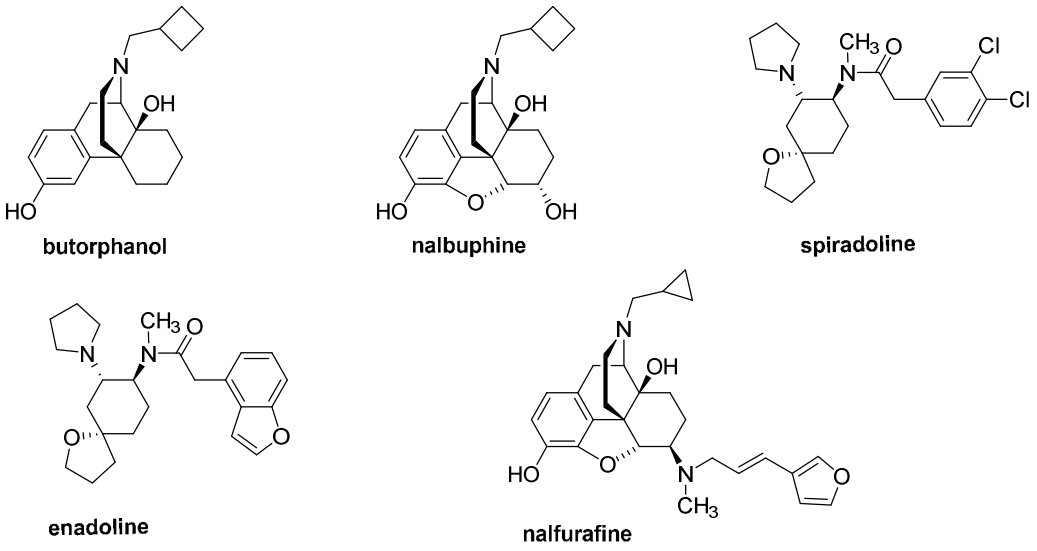
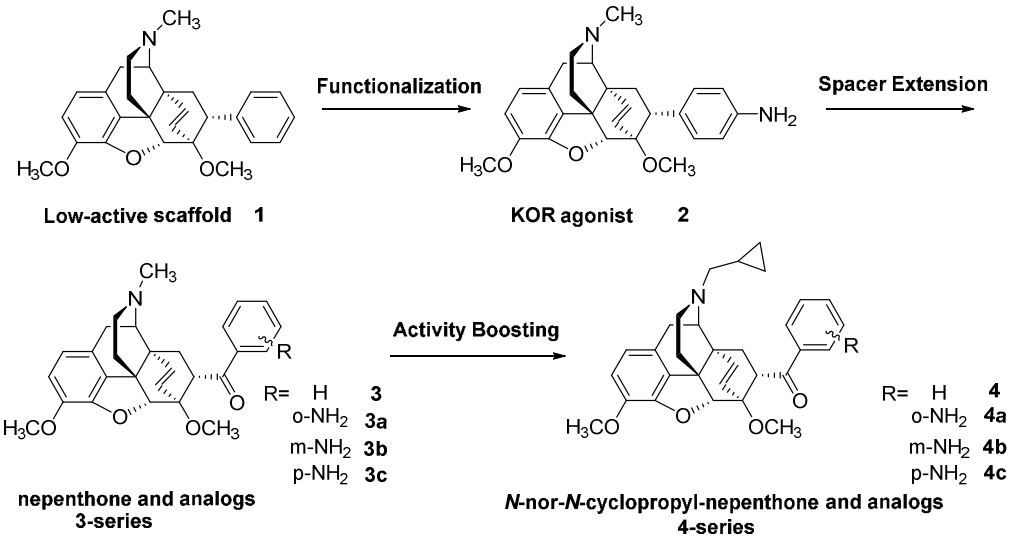


Chart 1. Chemical structures of representative kappa opioid agonists

In our previous work[14-16], we spent much efforts to design and chemically synthesize novel derivatives of thebaine based on its morphine-like structure, and obtained MOR and KOR agonists.[15, 16] Inspired by the structures and transformed activities of GNTI and 6'-GNTI in the literature,[17,18] we introduced a 7α-aminophenyl substitution to 6α,14α-endo-ethenyl-7α-phenylthebaine (compound **1** in **Scheme 1**) analogs, to see whether this substitution could help to increase KOR activity and selectivity. Our initial pharmacological results suggested that, compound **2 (Scheme 1**) with 7α-*p*-aminophenyl substitution has *ex vivo* KOR agonistic activities by the test

as rabbit vas deferens (RVD) preparations, and such *ex vivo* activity can be reversed by the KOR-selective antagonist nor-BNI[16]. These initial results encouraged us to finely tune the structure of compound **2**, in order to design better KOR-selective agonists. For this purpose, we inserted a carbonyl group as a spacer extension between the substructures of 6α,14α-endo-ethenyl-thebaine and the 7α-phenyl substitution. Such spacer should help us to test the role of *p*-amino substitution in conferring KOR agonistic activity of compound **2**. We also wanted to test the effect on KOR agonistic activity by substituting the 17-*N*-methyl group with a cyclopropylmethyl group, as the cyclopropymethyl group appeared in some opioid analogs[19, 20]. Following these strategies, we chemically synthesized, pharmacologically tested (*in vitro* and *in vivo*) the two series of compounds (**3-4 series**, **Scheme 1**). Furthermore, we performed molecular modeling of KOR structure, and molecular docking our newly designed KOR-selective agonists into the agonist-binding site of KOR, in order to elucidate the structural determinates for their selective affinities toward KOR.

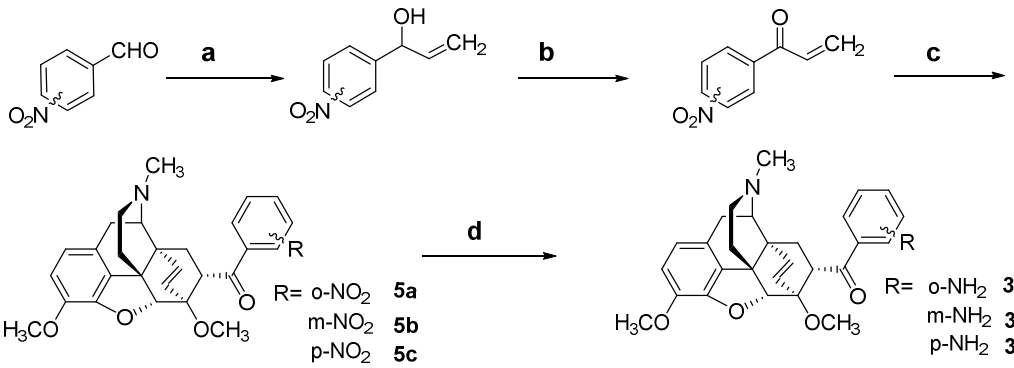


Scheme 1 Initial design rationale for KOR agonists from nepenthone and *N*-nor-*N*-cyclopropylmethyl-nepenthone derivatives

RESULTS AND DISCUSSION

Chemical Designs.

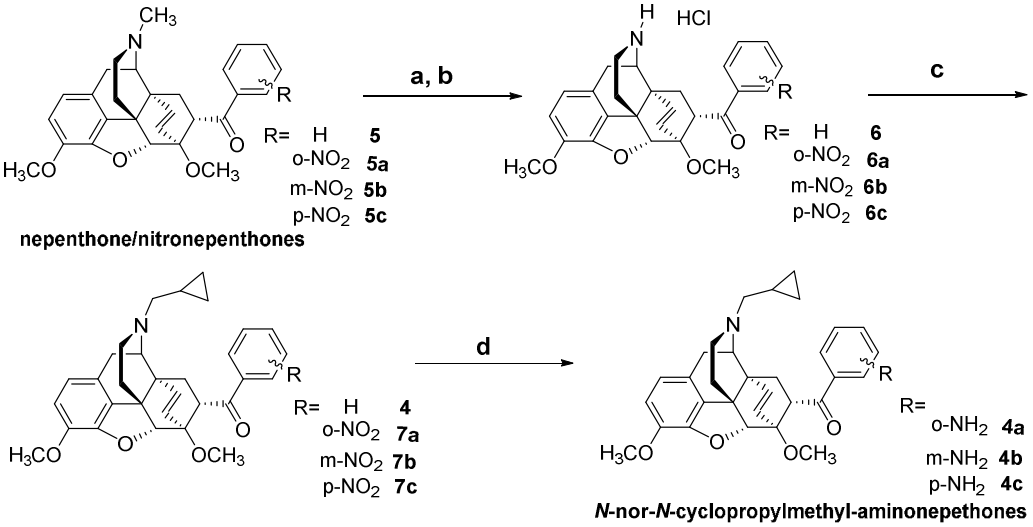
4,5-Epoxymorphinan has been recognized to be a privileged structural component for ligands of opioid receptors [19]. However, the Diels-Alder adducts (*e.g.*, nepenthone and thevinone) between thebaine and alkyl or aryl vinyl ketones would be much of interest if the adducts were converted into orvinols first through Grignard reaction and then the optimization strategies like *N*-cyclopropylmethyl substitution, 3-*O*-demethylation or saturation of the 17,18-ethenyl group[21-23]. These synthetic approaches led to the discovery of some important semi-synthetic opioids, including etorphine and buprenorphine[19]. Structurally, nepenthone and its analogs were not fully explored and optimized, possibly due to their low pharmacological activities toward opioid receptors. As outlined in **Scheme 1**, we started from synthesizing nepenthones by Diels-Alder reactions, using thebaine and appropriate phenyl vinyl ketones as the reactant materials. The reported approach[24] for preparing phenyl vinyl ketone did not work for nitrophenyl vinyl ketones, so we designed an alternative synthetic route (**Scheme 2**). In our synthetic route, nitrobenzaldehydes reacted first with vinyl magnesium bromide to afford the Grignard products, and then the products were oxidized by Jones reagent to yield the expected nitrophenyl vinyl ketones. The ketones underwent Diels-Alder reactions with thebaine to afford **5a-c**. These intermediates were further chemically reduced to generate compounds **3a-c**.



Reagents and conditions: **(a)** vinyl magnesium bromide, THF, -65 °C; **(b)** CrO_3/H_2SO_4, acetone, <10 °C; **(c)** thebaine, toluene, reflux; **(d)** Raney Ni/85% Hydrazine hydrate, ethanol, 60 °C.

Scheme 2 Synthesis route of compounds **3a-c**

In order to synthesize *N*-nor-*N*-cylcopropylmethyl-nepenthones, we designed another route as shown in **Scheme 3**, starting from compound **5** and **5a-c**. These intermediates **5, 5a-c** were demethylated with diethyl azodicarboxylate (DEAD) and hydrolyzed to yield **6** and **6a-c**, which were further alkylated with propyl methyl bromide to afford **7a-c**, and subsequently reduced to generate the expected compounds **4a-c**.



Reagents and conditions: **(a)** diethyl azodicarboxylate, benzene, reflux; **(b)** 1N HCl, 70-80 °C; **(c)** propylmethyl bromide, potassium carbonate, DMF, 100 °C ; **(d)**. Raney

Ni/85% Hydrazine hydrate, ethanol, 60 °C.

Scheme 3 Synthesis route of compounds **4a-c**

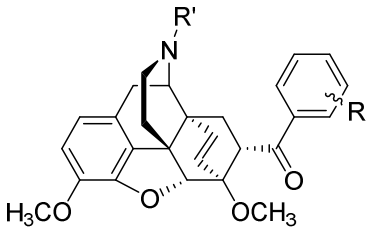
In Vitro Activities.

We expected the newly synthesized compounds (**3-4 series**, **Scheme 1**) to be KOR agonists. This was based on the reports[23] that nepenthones are active ligands of opioid receptors. The only difference between the nepenthones and the 6α,14α-endo-ethenyl-7α-phenylthebaines (**Scheme 1**) is the inserted carbonyl group in the structural scaffold of nepenthones. Would these newly designed compounds share similar pharmacological profiles as that of nepenthones?

To test the agonistic activities of these new compounds toward KOR, we performed both the competitive binding assays and functional assays. The competitive binding for each of the new compounds was measured in a similar way as that was used in our previous studies[15], that was, each compound was tested to competitively inhibit the binding of [^{3}H]DAMGO for MOR, [^{3}H]DPDPE for δ-opioid receptor (DOR) and [^{3}H]U69,593 for KOR [25]. The functional assays for these new compounds were performed through classic [^{35}S]GTPγS binding. The results of these assays are summarized and listed in **Table 1** for the binding affinities, and **Table 2** for their abilities to stimulate [^{35}S]GTPγS binding. As shown in Table 1, nepenthone, **3** has higher affinity with MOR than that with KOR. Adding the amino-substituent to the 7α-phenylcarbonyl group of compound **3** does not obviously change the receptor affinity for the derivatives **3a-c**, no matter the position of the amino-substituent (*o*- for **3a**, *m*- for **3b** and *p*- for **3c**). However, substituting the 17-*N*-methyl group with a

cyclopropylmethyl group dramatically increased KOR affinity for the derived compound **4** (K_i = 0.4 ± 0.1 nM for KOR**)**, and also increased KOR selectivity (μ/κ = 339, and δ/κ of 2034). Such increase in both affinity and selectivity for KOR were counteracted by adding the amino-substitutent to the 7α-phenylcarbonyl group, as seen the effect on compounds **4a-c** (**Table 1**). The effect of increasing KOR affinity for the 17-N-cyclopropylmethyl substitution was repeated when comparing the difference in KOR affinity between the intermediate compounds **7a-c** and compounds **5a-c** (Table 1). The extent of affinity increase from 17-*N*-cyclopropylmethyl group can be roughly estimated to be the affinity ratio of 100/1 for compound **4** over compound **6**. Based on these KOR affinity data, the 17-*N*-cyclopropylmethyl group can be viewed as the KOR affinity enhancer for nepenthone derivatives.

Table 1 Binding affinities of nepenthone and *N*-nor-*N*-cyclopropylmethyl-nepenthone analogs for μ, δ and κ opioid receptors



Compd	R'	R	K_i±SEM (nM)			Selectivity ratio	
			μ[a]	δ[b]	κ[c]	μ/κ	δ/κ
3	CH_3	H	21.9±5.1	ND	170.0±10.0	0.13	NA
3a	CH_3	*o*-NH_2	9.6±1.6	ND	80.0± 10.0	0.12	NA
3b	CH_3	*m*-NH_2	38.9±1.7	ND	370.0±50.0	0.11	NA

3c	CH_3	*p*-NH_2	30.1±0.8	ND	410.0±60.0	0.07	NA
4	CPM[d]	H	129.5±3.1	773.7±29.8	0.4±0.1	339	2034
4a	CPM[d]	*o*-NH_2	59.3±1.4	253.1±13.1	2.0±0.1	30.1	128
4b	CPM[d]	*m*-NH_2	13.6±0.6	329.7±20.7	2.3±0.13	5.99	145
4c	CPM[d]	*p*-NH_2	40.4±0.6	>5000	14.6±1.8	2.76	>342
5a	CH_3	*o*-NO_2	195.6±30.2	>5000	>5000	NA	NA
5b	CH_3	*m*-NO_2	13.5±0.7	617.9±6.7	918.4±62.5	0.02	0.67
5c	CH_3	*p*-NO_2	21.1±1.1	1547.0±65.5	1264.0±61.5	0.02	1.22
6	H	H	55.8±0.8	962.9±11.4	39.8±0.9	1.40	24.2
7a	CPM[d]	*o*-NO_2	350.7±24.9	500.8±22.6	349.7±1.6	1.00	1.43
7b	CPM[d]	*m*-NO_2	30.0±1.6	219.3±18.5	6.5±1.3	4.62	33.7
7c	CPM[d]	*p*-NO_2	195.6±30.2	1865.0±31.0	22.3±0.6	8.77	83.6
morphine			6.9±1.3	116.8±15.4	76.9 ±16.3	0.089	1.5

[a]Displacement of [^{3}H]DAMGO from CHO cell membrane expressing MOR; [b]Displacement of [^{3}H]DPDPE from CHO cell membrane expressing DOR; [c]Displacement of [^{3}H]U69,593 from CHO cell membrane expressing KOR. [d] CPM denotes cyclopropylmethyl; Values are expressed as the Mean ± S.E.M. for three independent experiments performed in triplicate. ND: Not determined. NA: Not applicable.

As shown in Table 2 for the results of functional assays, compound **3** showed poor agonistic activity toward KOR, although it has better MOR agonistic activity. Surprisingly, the addition of the amino-substituent to the 7α-phenylcarbonyl group at compound **3a** brought in great agonistic activity toward KOR, i.e. ~ 600-fold (EC_{50} = 1368 ± 248.9 nM of compound **3** *vs*. 2.3 ± 0.2 nM of compound **3a**) more active. The *m*-NH_2 substitution (**3b**) and *p*-NH_2 substitution (**3c**) showed similar increase of the agonistic activity toward KOR. Could this positive functional effect from the

additional NH_2-substituent at the 7α-phenylcarbonyl group be repeated? The answer is yes, as seen from the increased agonistic activity toward KOR for compounds **4a-c** (**Table 2**). Thus, the amino-substituent at the 7α-phenylcarbonyl group of nepenthone derivatives is a real KOR functionalizer. We also wondered how strong of the functional effect from 17-*N*-cyclopropylmethyl group of nepenthone derivatives toward KOR. As shown in **Table 2**, this substitution can only enhance the MOR agonistic activity, but not that toward KOR for compounds **4a-c**. Very interestingly, compound **4** showed highest binding affinity with the KOR, and also the highest agonistic activity among all the new compounds.

Table 2 Functional activity in stimulating [^{35}S]GTPγS binding of nepenthone and *N*-nor-*N*-cyclopropylmethyl-nepenthone analogs for μ, δ and κ opioid receptors

Compd	μ		δ		κ	
	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}	EC_{50} (nM)	E_{max}
3	NA[a]	122.5±1.6 stimulation at 10 μM	NA[a]	NA[a]	1368.0±248.9	180.2±1.1
3a	NA[a]	136.5±1.1 stimulation at 10 μM	NA[a]	NA[a]	2.3±0.2	204.5±2.7
3b	NA[a]	NA[a]	NA[a]	NA[a]	30.2 ± 2.3	205.6±10.7
3c	NA[a]	NA[a]	NA[a]	NA[a]	62.2 ± 7.4	180.5 ± 9.0
4	130.2±6.6	202.1±2.7	678.2±20.3	224.6±10.8	1.0±0.2	211.9±1.3
4a	18.7±2.0	206.2±3.2	186.0±15.5	273.1±4.1	9.1±1.4	190.9±0.2
4b	75.1±7.3	208.8±0.3	442.3±59.0	263.4±8.4	7.6±0.2	197.7±0.9

4c	29.7±5.9	176.9±0.5	1567.0±35.1	231.9±11.2	28.7±2.1	217.2±2.5
morphine	123.5±30.5	209±15.9	NA[a]	NA[a]	NA[a]	NA[a]
U50488H	NA[a]	122.1±1.1	NA[a]	112.6±2.0	1.8±0.6	262.0±0.6

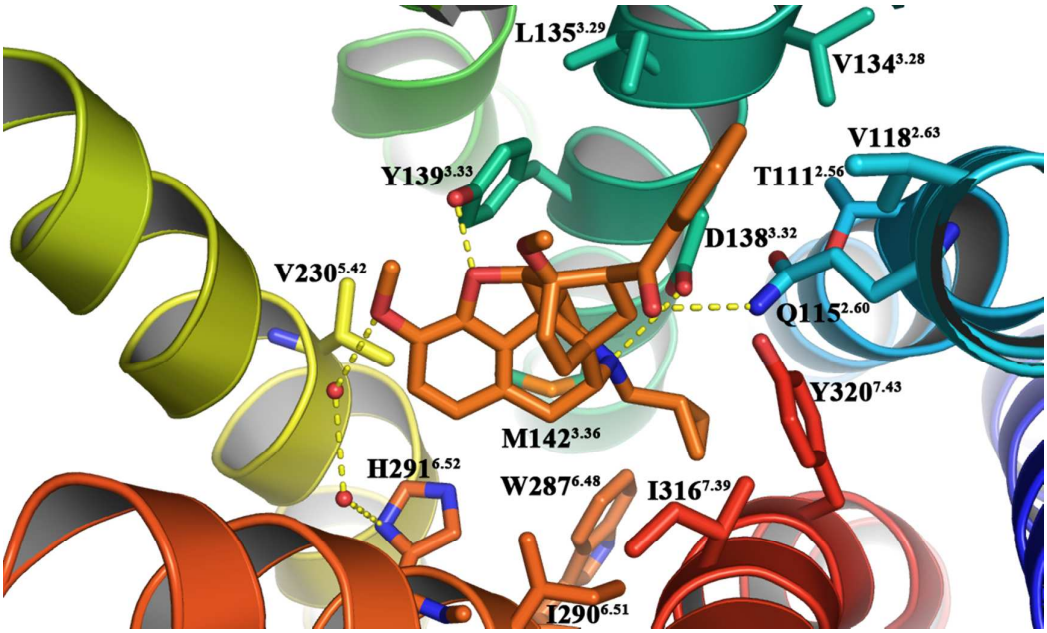
Potency and efficacy in stimulating [^{35}S]GTPγS binding to membranes of CHO cells stably expressing opioid receptors. Data are expressed as the Mean ± S.E.M. of independent experiments performed in triplicate. [a] NA: Not applicable.

Binding Structures

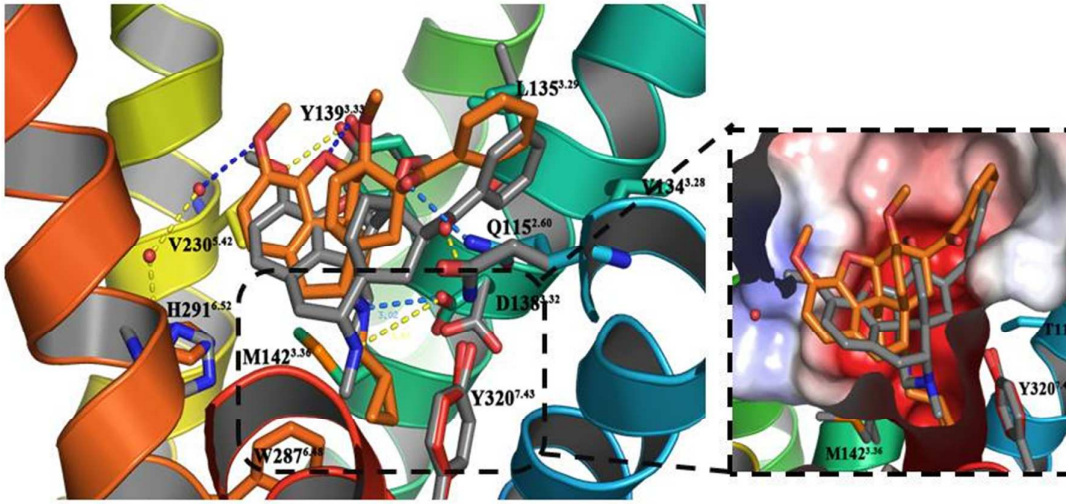
Based on the structure-activity relationship (SAR) for our newly designed nepenthone derivatives as described above, we wanted to explore how these compounds bind with KOR, and what are the structural determinants for the measured affinity differences. For this purpose, we performed molecular docking operations to dock these new compounds into the agonist-binding site of KOR. The active KOR structure was homology modeled based on the X-ray structure of the active murine MOR[26]. As shown in **Figure 1**, the docked structure of KOR binding with compound **4** indicates that compound **4** takes a similar binding mode as that for other opioid ligands, which have been revealed in the published X-ray structures of opioid receptors.[27-30] In particular, compound **4** interacts with KOR residues Asp138$^{3.32}$, Ile290$^{6.51}$, Met142$^{3.36}$, Tyr320$^{7.43}$, Trp287$^{6.48}$ and Tyr139$^{3.33}$ (**Figure 1a**). In addition, it forms the water bridge with His291$^{6.52}$ through two water molecules, and the oxygen atom of the 7α-phenylcarbonyl group of compound **4** could provide a hydrogen bond acceptor to residue Gln115$^{2.60}$ of KOR. This additional hydrogen bonding interaction makes the 7α-phenylcarbonyl group orientated very well inside the hydrophobic subpocket composed of residues Trp124^{ECL2}, Leu135$^{3.29}$, Val118$^{2.63}$,

Val134$^{3.28}$ and Gln115$^{2.60}$ of KOR (**Figure 2(a)**). Compounds **4a-c** take similar binding mode at the agonist-binding site of KOR. The amino-substituent at the 7α-phenylcarbonyl group of compounds **4a-c** is elongated into the hydrophobic subpocket between TM2 and TM3 as well. Due to the local steric hindrance, the amino-substituent is obviously not favorable to this subpocket, resulting in the reduced KOR affinities for these compounds, especially for the KOR affinity of compound **4c**. On the contrary, compounds **4a-c** can fit better to the agonist-binding site of MOR than that of compound **4** with MOR, as the amino-substituent at the 7α-phenylcarbonyl group could form additional hydrogen bonds with the non-conserved residue Asn127$^{2.63}$ around the top of this hydrophobic subpocket (**Figure 2(b)**). So, the compounds **4a-c** have higher affinities with MOR than that of compound **4** (as listed in **Table 1**), this leads to higher KOR selectivity of compound **4** than that of compounds **4a**-**4c**. As measured from affinity assays, the KOR affinity of compound **4** is ~400 fold stronger than that of compound **3** (**Table 1**). The superimposed structures of **4** and **3** at the active site of KOR (**Figure 1b**) show that compound **4** binds more tightly, oriented deeper to the binding pocket, and its *N*-cyclopropylmethyl group fits better inside the hydrophobic subpocket formed by residues Tyr320$^{7.43}$, Ile316$^{7.39}$, W287$^{6.48}$ and M142$^{3.36}$ of KOR. Based on the binding structure, compound **4** forms stronger electrostatic interaction and hydrogen bonding interaction with the negative charged side chain of residue Asp138$^{3.32}$, with a hydrogen bond distance of 3.0 Å, while the distance for similar interation between

compound **3** and residue Asp138$^{3.32}$ is much longer (4.0 Å). These differencs in the inter-molecular interactions significantly make compound **4** a better KOR agonist.



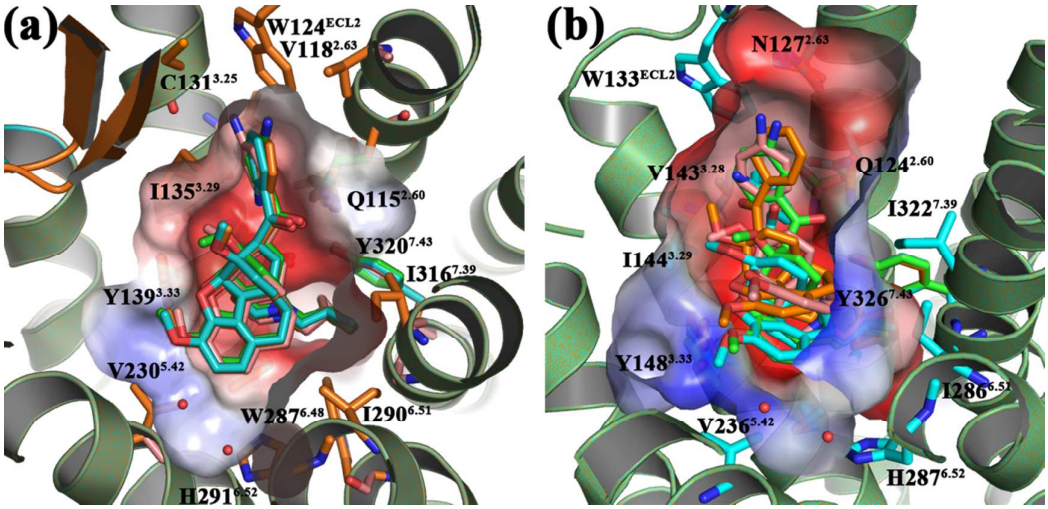
a



b

Figure 1 a) The binding mode for *17*--nor-*N*-cyclopropylmethyl-nepenthone (compound **4**, colored in orange) at the agonist-binding site of KOR; b). The superimposition of the binding structure of compound **3** (colored in grey; and its

inter-molecular interactions are colored yellow) with that of compound **4** (colored in orange; and the inter-molecular interactions are colored blue).

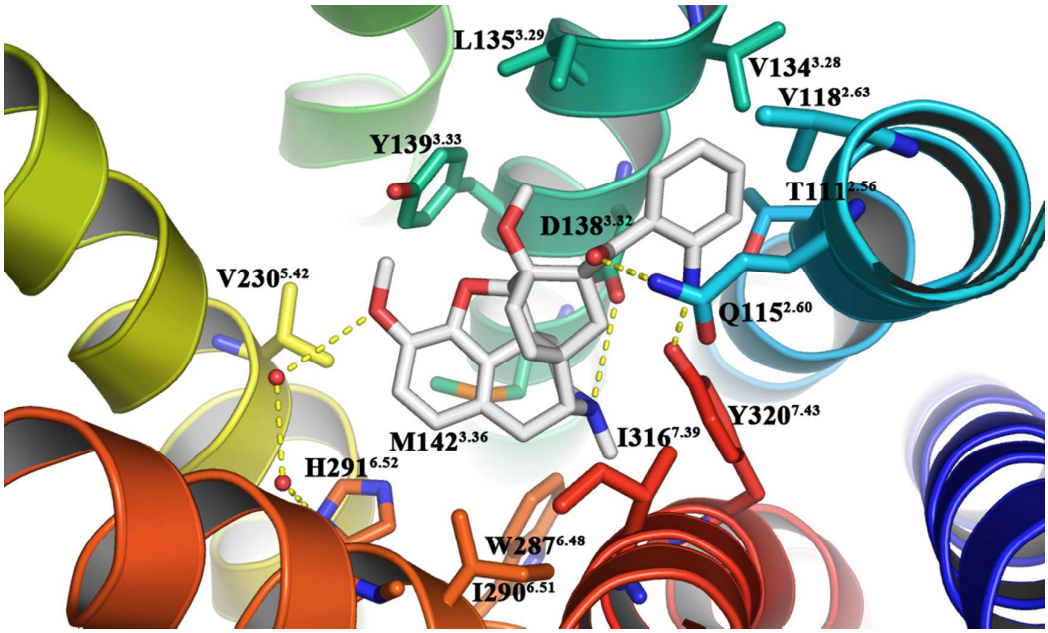


(a) **(b)**

Figure 2 (a) Superimposition for binding structures of compounds **4-4c** at the agonist-binding site of KOR; **(b)** superimposed structures for compounds **4a-c** at the agonist-binding site of MOR (color patterns: **4** as orange; **4a** as cyan; **4b** as green; and pink for **4c**)

We also docked the compounds **3** and **3a** into the agonist-binding site of KOR. As shown in **Figure 3**, compound **3a** shares the conserved interactions with KOR as that of compound **3** binding with KOR, for example, compound **3a** interacts with Asp138$^{3.32}$, Ile290$^{6.51}$, Met142$^{3.36}$, Tyr320$^{7.43}$, Trp287$^{6.48}$, Tyr139$^{3.33}$ and H291$^{6.52}$ of KOR. The difference is that the *o*-amino substitution at the 7α-phenylcarbonyl group

forms hydrogen bond with Tyr320$^{7.43}$ of KOR, which makes compound **3a** show stronger affinity toward KOR than that of compound **3**.



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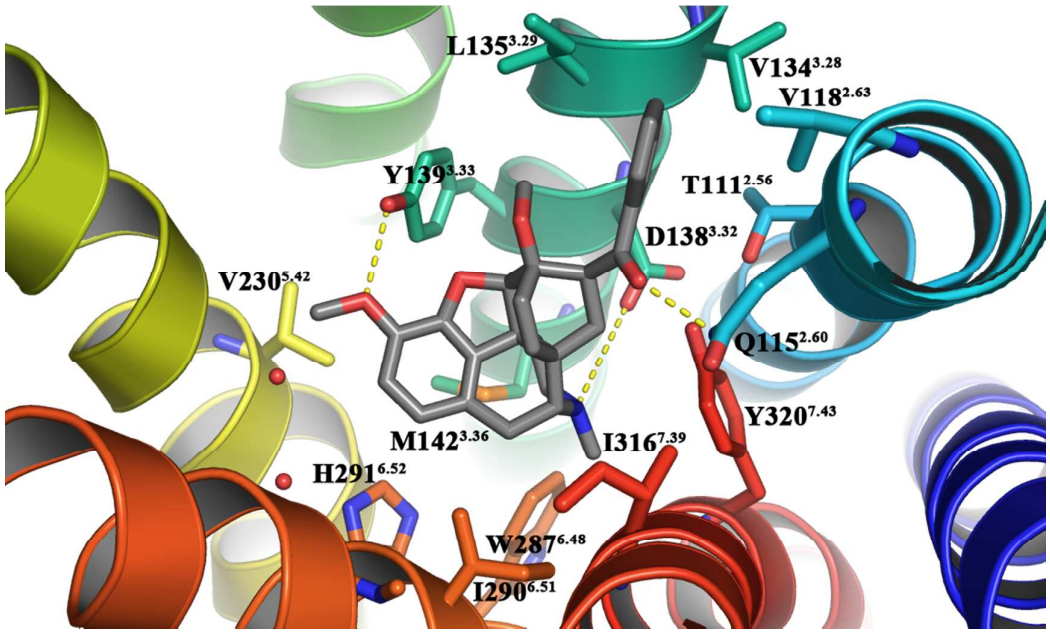

b

Figure 3 a). The binding mode of **3a** with KOR; b). The binding mode of **3** with KOR

Based on the docked structures of our compounds binding with either KOR or MOR, we performed binding energy calculations by means of X-Score 1.2, and explored the correlation between the calculated binding energies and the wet-experimentally measured binding affinities using the two-dimensional correlation analyses. The calculated results are shown in Figure S2 and listed in Table S1, which demonstrated favorable R^2 (coefficient of determination) values (for MOR complex, $R^2 = 0.7196$; for KOR complex, $R^2 = 0.8484$). The good correlation between the calculated binding affinities and the measured binding affinities support that our docked binding structures are reasonable.

In Vivo Activities

The encouraging results of *in vitro* tests and the modeled binding structures of our newly designed nepenthone derivatives made us want to know whether or not these compounds are able to produce analgesic effect *in vivo*. We selected compound **4**, compound **3**, and compound **U50488H** as a control, for further animal tests using hot plate approach. The results of ED_{50} values are listed in **Table 3**. Both compound **3a** and **4** showed strong antinocieceptive effect in hot plate test, and compound **4** showed a dose-dependent ED_{50} value of 2.1 (1.5-3.1) mg/kg, which is approximately 2-fold more potent than that of **U50488H**[31].

Table 3 ED_{50} values of the antinociception induced by **Compounds 3a, 4** and **U50488H** in the hot plate test. The ED_{50} value was calculated from data obtained at 15 min (**3a**) or 2h (**4**) after administration. In parentheses are 95% confidence limits.

Compd	Antinociception ED_{50} (mg/kg)
3a	6.8 (6.5-7.1)
4	2.1 (1.5-3.1)
U50488H	4.4 (2.5-7.8)[a]

[a]The data of **(-)U50,488H** were cited from our group's results in previously published paper.[31]

More interestingly as shown in **Figure 4**, the antinocieceptive effect of compound **4** peaked from 2h to 8h after injection in the hot plate test and then gradually declined. After 24 h, it still produced almost 35% of antinociception. In comparison, **U50488H**-induced antinociception returned to the pre-injection level after 2h. These results suggest that compound **4** is able to produce much longer active antinociception than that of the classical agonist **U50488H**. Further *in vivo* tests showed that, the compound **4**-induced antinociception can be significantly inhibited by the KOR-selective antagonist nor-BNI (as shown in **Figure 5**), we concluded that the antinociceptive effect of compound **4** showed in the hot plate test should work through its selective binding with KOR.

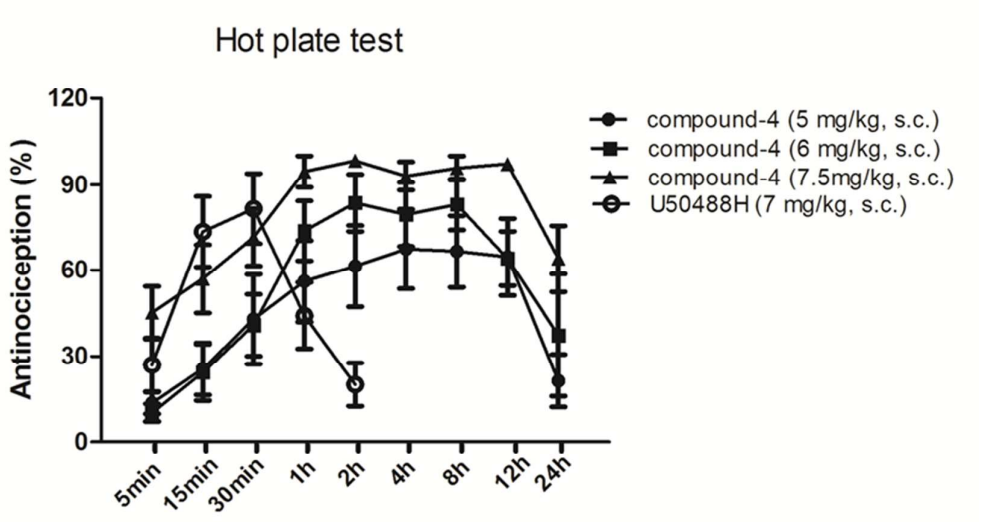

Figure 4 Time courses for the effects of Compound **4** (5, 6, 7.5 mg/kg, s.c.) and **U50488H** (7 mg/kg, s.c.) induced antinociception in the hot plate test.

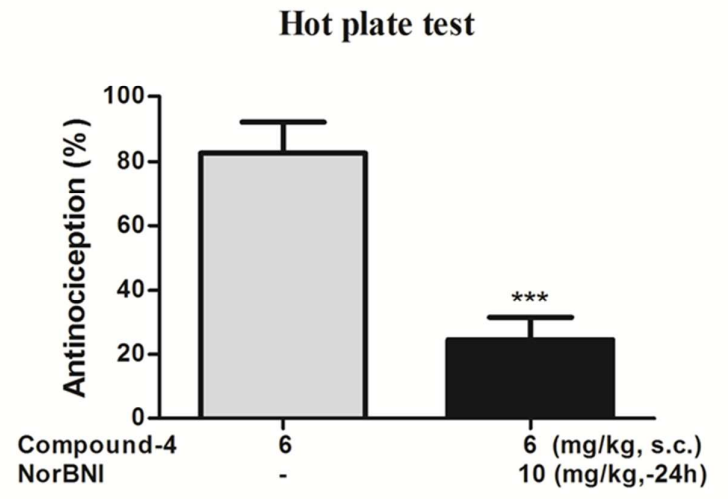

Figure 5 Compoud **4**-induced antinociception was mediated by kappa opioid receptor. Mice were pretreated with nor-BNI (10 mg/kg, s.c.) for 24 h, then injected with **compound 4** (6 mg/kg, s.c.). 2 h later, the antinociceptive effect was assessed in the hot plate test.

CONCLUSION

Starting from the morphine-like structure of thebaine derivatives in our previous work[15, 16], we explored possibilities to make them as selective KOR agonists, by designing novel nepenthone derivatives. We applied two strategies on the structure of nepenthone, i.e., inserting a spacer (carbonyl group) between the substructure of 6α,14α-endo-ethenyl-thebaine and the 7α-phenylcarbonyl substitution; and replacing the 17-*N*-methyl group with a cyclopropylmethyl group. We synthesized two series of new compounds (**3**, **3a-c**; and **4**, **4a-c**) and subjected them to both the *in vitro* tests (binding assays and functional assays), and molecular docking operations to obtain their binding structures with KOR. The measured data and binding structures showed that compounds **4**, **4a-c** are potent selective KOR agonists at nM level of binding affinities. We performed *in vivo* tests for antinociception on compounds **4**, **3a** with **U50488H** as the control, and found that compound **4** is able to induce stronger and much longer antinociceptive effect than that of **U50488H**, and that antinociception can be reversed by the KOR antagonist nor-BNI. All these results support that compound **4** is a very good lead compound for future design of potential chemical entitles acting through KOR.

METHODS

Chemistry

Reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd, Acros and other suppliers, which were used as received. Tetrahydrofuran was boiled

with sodium and distilled before use. Melting points were determined in glass capillary tubes and were uncorrected. IR data were taken on an AVATAR 360 FT-IR spectrometer (KBr). NMR data were recorded with Mercury *plus* 400 MHz or Bruker ASCEND 600 MHz NMR system. Chemical shifts (δ) are expressed in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard. Signals of active hydrogen were examined by D_2O exchange. Mass spectra were measured on an Agilent 1100 series LC/MSD 1946D spectrometer. High-resolution mass spectra (HRMS) were conducted on an AB SCIEX TripleTOF 5600+ mass spectrometer. The content of compounds for biological evaluation was examined by WATERS Breeze ^{TM} 2 HPLC system with methanol: water (80:20 or 85:15) as the eluent. Unless specified, the purity of target compounds was >95% which was considered to be pure enough for biological assays.

Synthesis of nepenthone, 3

Nepenthone was prepared according to the procedure described in the literature[24].

Nepenthone, 5.92 g (83.4%), mp: 153-154°C (lit. mp: 155°C).

General Procedure for the synthesis of amino-nepenthones, 3a-c

1 mL (17.3 mol) of 85% Hydrazine hydrate /Raney Ni (*cat.*) was added fractionally to a solution of 1 g (2.05 mmol) of nitronepenthone in 50 mL ethanol at 60°C. The reaction was completely confirmed by TLC. Raney Ni was removed by filtration and the product was recrystallized from appropriate solvents to yield aminonepenthones.

***o*-amino-nepenthone, 3a**

Colorless needle (from ethanol/water) 0.75g, 79.9%, mp: 182-183°C; **IR** (KBr) υ 3447.21, 3335.72, 1647.29 cm^{-1}; **^{1}H NMR** (DMSO-d^6) δ 7.98 (1H, d, J=7.44 Hz), 7.21 (1H, m, J_1=7.82 Hz, J_2=7.64 Hz), 7.11 (2H, s, N-H, this peak is diminished when treated with D_2O), 6.70 (1H, brd, J_1=1.17 Hz, J_2=8.60 Hz), 6.59 (1H, d, J=8.22 Hz), 6.55 (1H, m, J_1=7.63 Hz, J_2=7.43 Hz), 6.48 (1H, d, J=8.21 Hz), 5.79 (1H, d, J=8.99 Hz), 5.35 (1H, d, J=8.61 Hz), 4.78 (1H, s), 3.68 (3H, s), 3.24 (3H, s), 3.12-3.06 (2H, m), 2.98 (1H, dd, J_1=10.12 Hz, J_2=12.13 Hz), 2.46-2.35 (2H, m), 2.25 (3H, s), 2.25-2.23 (2H, m), 1.66 (1H, brd, J=9.78 Hz), 1.08 (1H, dd, J_1=6.65 Hz, J_2=12.33 Hz) ppm; **^{13}CNMR** (DMSO-d^6) δ 200.71, 151.23, 147.54, 141.04, 134.14, 133.68, 132.86, 131.45, 128.17, 127.79, 119.01, 117.21, 116.66, 114.17, 113.23, 92.21, 81.21, 59.09, 55.92, 51.70, 46.40, 44.94, 42.94, 42.62, 42.54, 32.63, 32.34, 21.67 ppm; **MS** (LCMS) *m/z* 459.1 $(M+H)^+$; **HRMS** (m/z): $[M+H]^+$ Calculated for $C_{28}H_{30}N_2O_4$: 459.2283; found: 459.2258.

***m*-amino-nepenthone, 3b**

Light yellow powder, 6.36g, 84.6%, mp: 172-173°C. **IR** (KBr) υ 3463.37, 3320.72, 1674.88 cm^{-1}; **^{1}H NMR** (DMSO-d^6) δ 7.19-7.09 (3H, m), 6.76 (1H, brd, J=8.99 Hz), 6.60 (1H, d, J=8.21 Hz), 6.49 (1H, d, J=8.22 Hz), 5.75 (1H, d, J=9.00 Hz), 5.39 (1H, d, J=8.99 Hz), 5.30 (2H, s, this peak is diminished when treated with D2O), 4.76 (1H, d, J=0.78 Hz), 3.93 (1H, dd, J_1=7.04 Hz, J_2=9.39 Hz), 3.68 (3H, s), 3.21 (3H, s), 3.14-3.07 (2H, m), 2.99 (1H, dd, J_1=9.78 Hz, J_2=11.74 Hz), 2.46-2.36 (2H, m), 2.25 (3H, s), 2.22-2.17 (2H, m), 1.69 (1H, d, J=11.73 Hz), 1.09 (1H, dd, J_1=6.65 Hz, J_2=12.14 Hz) ppm; **^{13}CNMR** (DMSO-d^6) δ 209.94, 159.35, 158.10, 151.65, 149.11,

144.59, 144.11, 139.41, 138.77, 137.96, 129.66, 128.73, 128.64, 126.38, 123.93, 123.23, 123.13, 102.81, 91.83, 69.64, 66.54, 62.28, 57.03, 55.53, 53.51, 53.23, 43.06, 42.77, 32.27 ppm; **MS** (LCMS) *m/z* 459.2 $(M+H)^+$; **HRMS** (m/z): $[M+H]^+$ Calculated for $C_{28}H_{30}N_2O_4$: 459.2283; found: 459.2257.

***p*-amino-nepenthone, 3c**

Off-white solid, 10.25g (76.2%), mp: 238-240°C; **IR** (KBr) υ 3482.74, 3347.15, 1595.69 cm^{-1}; **^{1}H NMR** (DMSO-d^6) δ 7.74 (2H, d, J=8.61 Hz), 6.59 (1H, d, J=8.21 Hz), 6.54 (2H, d, J=8.60 Hz), 6.48 (1H, d, J=8.22 Hz), 6.00 (2H, s, N-H, this peak is diminished when treated with D_2O), 5.73 (1H, d, J=9.00 Hz), 5.35 (1H, d, J=9.00 Hz), 4.76 (1H, s, H-5), 3.89 (1H, dd, J_1=7.43 Hz, J_2=8.30 Hz), 3.67 (3H, s), 3.21 (3H, s), 3.11-3.06 (2H, m), 2.92 (1H, dd, J_1=10.17 Hz, J_2=11.74 Hz), 2.50-2.35 (2H, m), 2.25 (3H, s), 2.23-2.20 (2H, m), 1.68-1.65 (1H, m), 1.05 (1H, dd, J_1=6.65 Hz, J_2=11.94 Hz) ppm; **^{13}CNMR** (DMSO-d^6) δ 195.95, 153.30, 147.54, 141.02, 134.14, 133.04, 130.52, 128.19, 127.60, 125.30, 118.99, 113.21, 112.31, 92.30, 81.19, 59.10, 55.91, 51.64, 46.40, 44.96, 42.96, 42.62, 41.36, 32.42, 32.27, 21.66 ppm; **MS** (LCMS) *m/z* 459.1$(M+H)^+$; **HPLC** purity**:** 92.6%, T_R=11.58min (MeOH:water= 80:20); **HRMS (m/z)**: $[M+H]^+$ Calculated for $C_{28}H_{30}N_2O_4$: 459.2283; found: 459.2256.

Synthesis of 17-*N*-nor-*N*-cyclopropylmethyl nepenthones, 4

10 g (21.5 mmol) of *N*-nor-nepenthone hydrochloride was dissolved in 100 mL anhydrous DMF. Then anhydrous K_2CO_3 9.0 g (65.1 mmol) and 2.6 mL (26.8 mmol) cyclopropylmethyl bromide were added. The mixture was heated to 80-90 °C and

reacted for 10 h. The K_2CO_3 was removed by filtration and 90 mL of 10% Na_2CO_3 and 150 mL ethyl acetate were added to the filtrate. The organic phase was separated and washed with of appropriate amount of water and brine. After being dried with anhydrous Na_2SO_4, it was filtered and evaporated to dryness. The residue was further purified by flash column chromatography to afford the title compound.

White foam, 3.6 g (34.6%), m.p. 57.5-58.9°C, **IR** (KBr) υ3435.62, 2925.92, 1683.13, 1212.49, 1102.81, 652.05 cm^{-1}, **^{1}H NMR** ($CDCl_3$) δ 8.02(2H,d,J=7.04Hz), 7.61(1H,t,J_1=7.73Hz, J_2=7.44Hz), 7.51 (2H, t, J_1=7.44Hz, J_2=7.43Hz), 6.59(1H, d, J=8.02Hz), 6.47(1H, d, J=8.22Hz), 5.75(1H, d, J=8.61Hz), 5.45(1H, d, J=8.61Hz), 4.81(1H,s), 4.16-4.13(1H,m), 3.68(3H,s), 3.47(1H, d, J=6.26Hz), 3.21(3H,s), 3.05-2.98(2H,m), 2.68(1H, d, J=6.26Hz), 2.45-2.39(1H, m), 2.31-2.25(4H, m), 1.69(1H, d, J=9.00Hz), 1.21-1.13(2H, m), 0.76(1H, brs), 0.45-0.39(2H,m), 0.09-0.06(2H,m) ppm. **^{13}CNMR** (DMSO-d^6) δ 200.18, 147.679, 141.352, 138.089, 134.674, 134.445, 133.15, 128.842, 128.53, 128.393, 127.211, 119.512, 113.527, 92.716, 81.937, 59.227, 56.780, 56.201, 52.222, 47.518, 43.806, 42.899, 42.693, 32.752, 32.074, 22.773, 9.463, 4.066, 3.494 ppm. **MS** (LCMS) *m/z* 484.3 $(M+H)^+$. **HRMS (m/z):** $[M+H]^+$ Calculated for $C_{31}H_{33}N_1O_4$: 484.2482; found: 484.2486.

General Procedure for the synthesis of 17-*N*-nor-*N*-cyclopropylmethyl–aminonepenthones, 4a-c

1 mL (17.3 mol) of 85% Hydrazine hydrate /Raney Ni (*cat.*) was added fractionally to a solution of 500 mg (1.89mmol) of *N-CPM-N-nor*-nitronepenthone in 50 mL ethanol at 60 °C. The reaction was complete as shown by TLC analysis. Raney Ni

was removed by filtration. The crude product was further purified by flash column to yield the appropriate *N*-cyclopropylmethyl-*N*- nor-aminonepenthone.

***N-cyclopropylmethyl-N-nor*methyl *-o-*aminonepenthone, 4a**

Light yellow foam, 940 mg (89.1%), mp: 88-90°C, **IR** (KBr) υ 3453.41, 2915.07, 1614.78, 1210.36, 1102.48 cm^{-1}; **^{1}H NMR** (DMSO-d^6) δ 8.02(1H, d, J=7.83 Hz), 7.21 (1H, t, J_1=7.83 Hz, J_2=7.43 Hz), 7.12 (2H, s), 6.70 (1H, d, J=7.83Hz), 6.60-6.54 (2H, m), 6.47 (1H, d, J=8.20 Hz), 5.80 (1H, d, J=9.00 Hz), 5.37 (1H, d, J=8.61 Hz), 4.05-4.00(1H, m), 4.80 (1H, s), 3.68 (3H, s), 3.46 (1H, d, J=6.26 Hz), 3.25 (3H, s), 3.05-2.97 (2H, m), 2.67 (1H, brs), 2.44-2.38 (1H, m), 2.33-2.22 (4H, m), 1.68 (1H, d, J=10.56 Hz), 1.14-1.07 (1H, m), 0.76 (1H, brs), 0.42 (2H, brs), 0.076 (2H, brs) ppm; **^{13}CNMR** (DMSO-d^6) δ 201.331, 151.331, 147.824, 141.344, 134.567, 134.194, 133.500, 131.785, 128.576, 128.050, 119.428, 117.591, 116.935, 114.709, 113.474, 92.510, 81.678, 59.212, 56.735, 56.209, 52.008, 47.457, 43.859, 42.868, 42.685, 32.973, 32.821, 22.781, 9.471, 4.119, 3.441 ppm; **MS** (LCMS) *m/z* 499.3$(M+H)^+$. **HRMS** (m/z): $[M+H]^+$ Calculated for $C_{31}H_{34}N_2O_4$: 499.2597; found: 499.2566.

***N-cyclopropylmethyl -N-nor*methyl *-m-* aminonepenthone, 4b**

Light yellow foam, 450 mg (92.4%), mp: 93-96°C; **IR** (KBr) υ3445.65, 3372.95, 2925.69, 1681.38, 1627.18, 1600.10, 1451.95, 1101.86 cm^{-1}; **^{1}H NMR** (DMSO- d^6) δ 7.21(1H, d, J=7.83 Hz), 7.16-7.10(2H, m), 6.77(1H, dd, J1=1.56 Hz, J2=7.82 Hz), 6.59(1H, d, J=8.22 Hz), 6.47(1H, d, J=8.21 Hz), 5.74(1H, d, J=8.61 Hz), 5.41(1H, d, J=8.61Hz), 5.29 (2H, s, this peak is diminished when treated with D_2O), 4.76 (1H, s), 3.97 (1H, dd, J_1=7.04 Hz, J_2=9.19 Hz), 3.68 (3H, s), 3.47 (1H, d, J=6.26 Hz), 3.22

(3H, s), 3.05-2.98 (2H, m), 2.68(1H, brd, J=9.00 Hz), 2.42 (1H, dd, J_1=6.65 Hz, J_2=18.78 Hz), 2.26-2.18 (4H, m), 1.70 (1H, brd, J=12.13 Hz), 1.12-1.10 (1H, m), 0.77 (1H, s), 0.46-0.39(2H, m), 0.079(2H, brs) ppm; **^{13}CNMR** (DMSO-d^6) δ 200.165, 148.922, 147.740, 141.344, 138.875, 134.415, 134.186, 129.307, 128.568, 127.630, 119.496, 118.604, 116.325, 113.489, 112.933, 92.434, 81.723, 59.220, 56.712, 56.193, 51.970, 47.472, 43.844, 42.868, 42.670, 32.897, 32.485, 22.758, 9.456, 4.104, 3.471 ppm; **MS** (LCMS) *m/z* 499.3 $(M+H)^+$. **HRMS** (m/z): $[M+H]^+$ Calculated for $C_{31}H_{34}N_2O_4$: 499.2597; found: 499.2565.

***N-cyclopropylmethyl -N-nor*methyl *-p*-amino-nepenthone, 4c**

White powder, 550 mg (99.3%), mp 214-217°C; **IR** (KBr) υ3454.36, 3367.90, 1660.69, 1596.93, 1178.60 cm^{-1}; **^{1}H NMR** (DMSO-d^6) δ 7.74 (2H, d, J=8.61 Hz), 6.58 (1H, d, J=8.22 Hz), 6.56 (2H, d, J=9.00 Hz), 6.46 (1H, d, J=7.82 Hz), 6.00(2H, s, this peak is diminished when treated with D_2O), 5.72 (1H, d, J=9.00 Hz), 5.37 (1H, d, J=8.61 Hz), 4.77 (1H, s), 3.94-3.90 (1H, m), 3.68 (3H, s), 3.44 (1H, d, J=6.26), 3.22 (3H, s), 3.02-2.94(2H, m), 2.67(1H, brd, J=7.04 Hz), 2.41 (1H, dd, J_1=6.26 Hz, J_2=18.58 Hz), 2.29-2.20 (4H, m), 1.68 (1H, brd, J=9.78), 1.07 (1H, dd, J_1=6.65 Hz, J_2=12.12 Hz), 0.76 (1H, brs), 0.45-0.39 (2H, m), 0.051 (2H, brs) ppm; **^{13}CNMR** (DMSO-d^6) δ196.727,153.534, 147.809, 141.322, 134.575, 133.683, 130.931, 128.583, 127.882, 125.717, 119.405, 113.436, 112.681, 103.678, 92.571, 81.655, 59.220, 56.780, 56.193, 51.940, 47.442, 43.867, 42.860, 41.526, 32.874, 32.781, 9.478, 4.089, 3.471 ppm; **MS** (LCMS) *m/z* 499.3 $(M+H)^+$. **HRMS** (m/z): $[M+H]^+$ Calculated for $C_{31}H_{34}N_2O_4$: 499.2597; found: 499.2569.

Computational Approaches

Homology Modeling and Structure Validation:

The active murine MOR was structurally determined at a resolution of 2.1 Å ((Protein Data Bank entry: 5C1M [26]), it provides a solid template for modeling the active 3D structures of MOR and KOR. The active human 3D MOR model and the active human 3D KOR model were constructed using a homology-modeling approach based on the x-ray crystal structure of murine active MOR. The "canonical" sequences of human MOR (P35372-1) and human KOR (P41145-1) were downloaded from UniProt database (www.uniprot.org). The residues of human MOR sequence before P65 at the N terminus, as well as the residues after I349 at the C terminus were truncated. The Blast program encoded in Discovery Studio 3.5 (Discovery Studio 3.5 (San Diego, CA)) was used to align the sequences of murine active MOR and human MOR and KOR, the Modeller program encoded in Discovery Studio 3.5 was used to build the models of human MOR and KOR. 50 homology models were built for each receptor. The one with the lowest DOPE scores was submitted to energy minimization (1000 steps steepest descent with backbone constrained in CHARMm force field). The resulting models were then validated by PROCHECK[32] Ramachandran plots for their stereochemical qualities (See **Figure S1**). Energy minimizations of the models were carried out using the Discover module encoded in Discovery Studio 3.5 with the same parameters as that of our previous studies [33].

Molecular Docking: The structures of compound **3a, 4**, **4a**, **4b** and **4c** were sketched in SYBYL (Tripos, St Louis, MO, USA) and energy-minimized using the Tripos

Force Field (Gasteiger-Hückel charges, distance-dependent dielectric constant is 4.0; non-bonded interaction cutoff is 8 Å; termination criterion: the energy gradient <0.05 kcal/(mol×Å) for 10,000 iterations).

The docking program GOLD Suite 5.1 (Cambridge Crystallographic Data Centre, Cambridge, UK) with the genetic algorithm was employed to dock the ligands into the active sites of human MOR and KOR. The binding site was defined as a sphere with the radius of 15 Å around the atom C^{β} of Asp138 $^{3.32}$. Side chains of residues $Glu^{2.60}$, $Asp^{3.32}$, $Tyr^{3.33}$ and $Tyr^{7.43}$ were set to be flexible during the docking run, referring to the rotamer library in GOLD. Full flexibility was allowed for all the rotatable bonds of ligands. The number of genetic algorithm (GA) runs was set to 10 and the GoldScore was used as the scoring function. The top-ranked poses were visually inspected by considering favorable interactions with the key residues demonstrated by available mutagenesis studies. The Binding affinities were predicted by X-Score 1.2 with empirical scoring functions. The predicted affinities and all of available experimental data were used for the selection of the final complex. The resulting ligand-receptor complexes were plotted using the PyMOL (The PyMOL molecular graphics system, version 0.98, DeLano Scientific, Palo Alto, CA) software.

Pharmacological assays

U50488H, nor-BNI (nor-binaltorphimine) were purchased from Sigma-Aldrich. Morphine hydrochloride was supplied by Qinghai Pharmaceutical General Factory (Qinghai province, China).

Cell culture and membrane preparation

CHO cells stably expressing human κ-, rat μ- or rat δ- opioid receptors were maintained in F12 medium with 10% fetal calf serum and 0.25 mg/ml G418. Cells were incubated in a humidified atmosphere consisting of 5% CO_2, 95% air at 37˚C. When cell growth reached 90% confluence, cells were washed with phosphate-buffered saline (PBS) and detached by incubation with PBS containing 1 mM EDTA and centrifuged at 1000 g for 10 min. The cell pellet was suspended in ice-cold homogenization buffer composed of 50 mM HEPES, pH7.4, 1 mM $MgCl_2$, and 1 mM EGTA. Cells were homogenized with a glass Dounce homogenizer, followed by centrifugation at 40,000 *g* for 10 min at 4°C. The membrane pellets were resuspended in homogenization buffer, and the homogenization, and the centrifugation steps were repeated. The final pellets were resuspended in a 50 mM Tris-HCl buffer, pH 7.4. Protein concentration was determined and aliquots were stored at -80°C.

Receptor Binding Assay.

Ligand binding experiments were carried out with [^{3}H]U69,593 for κ-opioid receptor, [^{3}H]DAMGO for μ-opioid receptor and [^{3}H]DPDPE for δ-opioid receptor[25]. Competition inhibition by compounds or morphine of [^{3}H]ligands binding to opioid receptors was performed in the absence or presence of various concentrations of each drug. Binding was carried out in 50 mM Tris.HCl buffer (pH7.4) at 37°C for 30 min in triple in a final volume of 0.5 ml with 30 μg of membrane protein prepared from CHO cell expressing human κ-, rat μ- or rat δ- opioid receptors. Naloxone (10 μM) was used to define nonspecific binding. Bound and free [^{3}H]-labeled ligands were

separated by filtration under reduced pressure with GF/B filters. Radioactivity on filters was determined by liquid scintillation counting.

[^{35}S]GTPγS Binding Assay.

[^{35}S]GTPγS binding was performed as described previously[34]. Briefly membranes (15 μg/sample) prepared from CHO cell expressing human κ-, rat μ- or rat δ- opioid receptors were incubated with 0.1 nM [^{35}S]GTPγS (1,030 Ci/mmol, Perkin Elmer) in a binding buffer composed of 50 mM Tris.HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, and 40 μM GDP at 30°C for 1h in the presence of increasing concentrations of compounds, morphine or U50,488H. Nonspecific binding was determined in the presence of nonradioactive GTPγS (10 μM). Reactions were terminated by rapid filtration and bound radioactivity was determined by liquid scintillation counting as described above. The percentage of stimulated [^{35}S]GTPγS binding was calculated as $100\times (cpm_{sample}-cpm_{nonspecific}) /(cpm_{basal}-cpm_{nonspecific})$.

Animals.

Males Kunming mice (18-22 g) were obtained from the Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Animals were housed in a temperature- and humidty-controlled room with food and water freely available under a 12 h light/dark cycle.

Antinociceptive effects of Compounds 3a and 4

Antinociception was assessed using mouse hot-plate test. In brief, mice were placing on a 55°C heated surface, and the time to licking of the back paws or to an escape jump was recorded. Before drug administration, the nociceptive response of

each mouse was measured three times. Mice not responding within 20 s were excluded from further studies. The cut-off time of 60 s for the temperature 55℃ hot-plate test was used to minimize tissue damage. For drawing the dose-response curves, percentage analgesia was expressed as: 100 ×[(test latency-baseline latency)/(cut-off time –baseline latency)].

Drug Treatment.

Mice were injected with U50488H (7 mg/kg, s.c.), varying doses of compound **3a** or **4**. Antinociception was assessed at varying times after compounds injections.

To further determine the *in vivo* opioid receptor profile of compound **4**, mice were pretreated with a-selective kappa antagonist (nor-BNI, 10 mg/kg s.c., -24 h). Then, mice received compound **4** administration (6 mg/kg, s.c.). After 2 h, the antinociceptive effects were assessed in the hot plate test.

ASSOCIATED CONTENT

Supporting Information

The following Supporting Information is available free of charge on the ACS Publications website at DOI:

1. **Methods for the synthesis of key intermediates**
2. **Figure S1.** Ramachandran plots of the active human MOR model and active human KOR model.
3. **Figure S2.** Validation of the docking results
4. **Table S1.** Data for correlation analysis.

5. **Figure S3.** Overlay of **3** (grey) and **4a** (cyan) in the binding pocket of kappa opioid receptor.
6. **Figure S4.** Sequence alignment of human mu opioid receptor and human kappa opioid receptor. Residues interacted with ligands were labeled.
7. **Figure S5.** ^{1}H and ^{13}C NMR spectra for compounds **3a-3c**, **4a-4c**

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Author Contributions

# W.L., J.D.L. and Y.Y.Q. contributed equally to this work.

* W.F. and J.G.L equally supervised the work.

Z.B.Q. and W.F. conceived the research. W.L., Z.N.W., and X.C.Y. conducted chemical synthesis and structural characterization. *In vitro* and *in vivo* assays were completed by J.D.L., Y.L., X.J.X., Y.J.W. and Y.Y.Q under the supervision of J.G.L. Molecular modeling works were conducted by Y.Y.Q, Q.S and W.L. under the supervision of W.F.. Y.Y.Q., X.C.Y., L.X., H.P.S., Y.L.X., Y.Y.C., Q.X., Y.H.W. and L.M.S. were involved in data analysis and discussion. The manuscript was drafted by W.L., Y.Y.Q and Y.J.W., and revised by J.G.L., W.F., and Y.H.W. All authors approved the final version of manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATION USED

brd, broad doublet; CHO, Chinese hamster ovary; CPM, cyclopropylmethyl; DOR, delta opioid receptor; EGTA, ethylene glycol tetraacetic acid; GTPγS, guanosine 5'-O-[gamma-thio]triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; KOR, kappa opioid receptor; MOR, mu opioid receptor; nor-BNI, Norbinaltorphimine

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