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'Carba'-carfentanil (*trans* isomer): a μ opioid receptor (MOR) partial agonist with a distinct binding mode

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Abstract

There is strong evidence to indicate that a positively charged nitrogen of endogenous and exogenous opioid ligands forms a salt bridge with the Asp residue in the third transmembrane helix of opioid receptors. To further examine the role of this electrostatic interaction in opioid receptor binding and activation, we synthesized 'carba'-analogues of the highly potent μ opioid analgesic carfentanil (**3**), in which the piperidine nitrogen was replaced with a carbon. The resulting *trans* isomer (**8b**) showed reduced, but still significant MOR binding affinity ($K_i^\mu = 95.2$ nM) with no MOR versus DOR binding selectivity and was a MOR partial agonist. The *cis* isomer (**8a**) was essentially inactive. A MOR docking study indicated that **8b** bound to the same binding pocket as parent **3**, but its binding mode was somewhat different. A reevaluation of the uncharged morphine derivative *N*-formylnormorphine (**9**) indicated that it was a weak MOR antagonist showing no preference for MOR over KOR. Taken together, the results indicate that deletion of the positively charged nitrogen in μ opioid analgesics reduces MOR binding affinity by 2–3 orders of magnitude and may have pronounced effects on the intrinsic efficacy and on the opioid receptor selectivity profile.

Keywords

carfentanil; 'carba'-carfentanil; opioid activity profiles; μ opioid receptor binding mode

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1. Introduction

There is strong evidence to indicate that formation of a salt bridge between the Asp residue in the third transmembrane helix ((TMH3) of opioid receptors and a positively charged nitrogen present in most non-peptide opiates and most opioid peptides plays an important role in receptor binding and activation. The recent determinations of the crystal structures of the μ (MOR), δ (DOR) and κ (KOR) opioid receptors in complex with an antagonist confirmed the existence of a salt bridge between one or two charged nitrogens of the ligand with the TMH3 Asp residue in each case.¹⁻³ Two studies on mutations of the TMH3 Asp residue of the DOR^{4,5} and a subsequent re-interpretation of the results⁶ indicated that the protonated amine of opioid ligands indeed interacts with Asp¹²⁸ in the wild-type receptor. An elegant two-dimensional mutagenesis study revealed that the carboxylate group of Asp¹⁴⁷ (TMH3) in the MOR also engages in an electrostatic interaction with the protonated nitrogens of naltrexone and morphine.⁷

In opioid peptides the positively charged amino group of the N-terminal tyrosine residue is thought to play the same role as the protonated nitrogen of opiates in the interaction with the TMH3 Asp residue of opioid receptors. In cyclic opioid peptide analogues containing a 2', 6,-dimethyltyrosine (Dmt) residue in place of Tyr¹, elimination of the positive charge through removal of the N-terminal amino group or its replacement with a methyl group resulted in selective antagonists with very high binding affinity^{8,9} and in a DOR agonist with moderate potency.¹⁰

The positively charged nitrogen of the piperidine ring in fentanyl (**1**) (Fig. 1) is thought to form a salt bridge with the MOR TMH3 Asp¹⁴⁷ residue. In a fentanyl analogue containing a 3-(guanidinomethyl)-benzyl group in place of the phenyl moiety attached to the ethylamide group, the piperidine nitrogen was replaced with a carbon (**2**).¹¹ The resulting *cis* and *trans* isomers (**2a,2b**) showed decreased MOR binding affinities but retained full MOR agonist activity, indicating that an electrostatic interaction of the protonated nitrogen in the piperidine ring of fentanyl analogues with the THM3 Asp¹⁴⁷ residue is not a requirement for receptor activation. However, these 'carba'-analogues still carry a positive charge on the guanidine group which, according to a performed receptor docking study, interacts with Asp²¹⁶ of the MOR.¹¹ This interaction compensates to some extent for the loss of the salt bridge with Asp¹⁴⁷.

Carfentanil (**3**) is one of the most potent μ opioid analgesics known.¹² In the present study we describe the synthesis and in vitro opioid activity profiles of the two isomers (*trans* and *cis*) of carfentanil in which the piperidine nitrogen was replaced with a carbon. The resulting 'carba'-carfentanils (c-carfentanils) **8a** (*cis*) and **8b** (*trans*) lack a positive charge and are unable to engage in salt bridge formation with the receptor. Because of the extraordinary potency of carfentanil it was thought that a 'carba'-analogue might still retain significant opioid receptor binding affinity despite the loss of an electrostatic interaction with the receptor. Furthermore, it was of interest to determine the effect of the carbon substitution on the intrinsic efficacy and on opioid receptor selectivity.

2. Results and discussion

2.1. Chemistry

The 'carba'-analogues of carfentanil were synthesized according to Scheme 1. Phenylethylcyclohexanone **4** was prepared as described.¹¹ An anhydrous modification of the Strecker reaction using **4**, aniline and trimethylsilyl cyanide (TMSCN) in glacial acetic acid¹³ yielded isomers **5a** and **5b** (isomer ratio 1:3) in 90% yield. The conversion of nitriles **5a** and **5b** to amides **6a** and **6b** was performed with basic H₂O₂ in DMSO.¹⁴ Methyl esters **7a** and **7b** were obtained by direct methanolysis of the amides in the presence of *p*-toluenesulfonic acid in a sealed tube.¹⁵ Acylation of **7a** and **7b** with propionic anhydride gave target compounds **8a** and **8b**. NMR resonance assignments based on 1H-1H and 1H-13C correlations permitted the stereochemical assignment. **8a** and **8b** were identified as the *cis* and *trans* isomer, respectively.

2.2. In vitro opioid activity determinations

The *trans* isomer of *c*-carfentanil (**8b**) showed marked binding affinity for the MOR in the nanomolar range ($K_i^\mu = 95.2$ nM) (Table 1). In comparison with the carfentanil parent (**3**), its MOR binding affinity is about 4000-fold decreased. Interestingly, the DOR binding affinity of **8b** ($K_i^\delta = 110$ nM) is comparable to that at the MOR. This lack of selectivity is in contrast to the preference of the carfentanil parent for MOR over DOR ($K_i^\delta/K_i^\mu = 138$). The compound showed no significant KOR binding affinity ($K_i^k > 5$ μ M). In the guinea pig ileum (GPI) assay, **8b** turned out to be a MOR partial agonist (maximal inhibition of electrically evoked contractions = 50%) with an IC₂₅ of 116 nM. The effect was completely reversible with naloxone (50 nM), indicating that it was mediated by opioid receptors. In the DOR-representative mouse vas deferens (MVD) assay, **8b** was a weak partial agonist (70% inhibition of contractions) with an IC₃₅ of 3890 nM. The relatively weak DOR partial agonist activity of **8b** as compared to its DOR binding affinity may be due to differences between central and peripheral DORs or to somewhat impeded DOR access in the vas preparation.

The *cis* isomer of *c*-carfentanil (**8a**) showed no significant MOR-, DOR- or KOR-binding affinity at concentrations up to 5 μ M. In the GPI assay, it was a very weak MOR and KOR antagonist ($K_e^\mu = 4050$ nM; $K_e^k = 2980$ nM) and it was inactive in the MVD assay. The lack of significant opioid activity of **8a** can be explained with the different orientation of its phenylethyl substituent (axial position) as compared to **3** and **8b** (equatorial position) (see section 2.3).

For comparative purposes, the in vitro opioid activity profile of *N*-formylnormorphine (**9**), a derivative of morphine (**10**) lacking a positive charge, was determined. This compound had previously been synthesized and characterized in a MOR binding assay and in a MOR [³⁵S]GTP γ S binding assay.⁷ It had been found to be unable to activate the MOR, but antagonist activity had not been determined. In the opioid receptor binding assays, *N*-formylnormorphine (**9**) showed MOR affinity ($K_i^\mu = 420$ nM) 290-fold lower as compared to morphine (**10**) ($K_i^\mu = 1.45$ nM) and MOR versus DOR selectivity ($K_i^\delta/K_i^\mu = 49$) similar to that of its parent (**10**) ($K_i^\delta/K_i^\mu = 57$) (Table 1). Its binding affinity for the KOR ($K_i^k = 363$

nM) was similar to that to that for the MOR. Thus, unlike morphine ($K_i^K/K_i^M = 19$), it showed no preference for the MOR over the KOR. In the GPI assay, *N*-formylnormorphine (**9**) was a weak antagonist at the MOR ($K_e^M = 1240$ nM) and at the KOR ($K_i^K = 1890$ nM). In agreement with its very low DOR binding affinity, it was inactive in the MVD assay.

2.3. Molecular modeling studies

A molecular mechanics study was performed to determine the lowest energy conformations of carfentanil and its 'carba'-analogues. The lowest-energy conformers of all compounds have the piperidine or cyclohexane ring in the chair conformation. In the lowest-energy conformers of carfentanil (**3**) and the *trans*-'carba'-analogue (**8b**) the phenylethyl- and *N*-phenylpropionamido substituents are equatorial and the methyl carboxylate is axial. The lowest-energy conformer of the *cis* 'carba' analogue (**8a**) has the *N*-phenylpropionamido substituent in the equatorial position and the phenylethyl and methyl carboxylate groups in the axial positions.

A study of flexible docking of carfentanil (**3**) and of the *trans* isomer of *c*-carfentanil (**8b**) in their lowest-energy conformations was performed using Mosberg's model of the MOR in the activated form¹⁶ (Fig. 2). Carfentanil (**3**) fits into a binding pocket formed by helices 2, 3, 5, 6 and 7. Its positively charged piperidine nitrogen forms a salt bridge with Asp¹⁴⁷ in TMH3 with a distance of 2.8 Å between the nitrogen and the Asp¹⁴⁷ carboxylate group. The phenylethyl substituent is in an extended conformation with the phenyl ring interacting with Tyr¹⁴⁸, Val³⁰⁰, Phe²³⁷, Met¹⁵¹ and Phe¹⁵². The phenyl ring of the *N*-phenylpropionamido substituent interacts with Tyr¹²⁸ and Thr²¹⁸, and the methyl carboxylate group is in close proximity to Trp³¹⁸ and Leu²¹⁹.

The *trans* 'carba'-carfentanil analogue (**8b**) is located in the same binding pocket as carfentanil, but is rotated by about 180° relative to the orientation of its parent. The centroid of the cyclohexane ring of **8b** is shifted by 1.8 Å relative to the centroid of the piperidine ring of **3**. Obviously, there is no salt bridge formation due to the replacement of the piperidine nitrogen with a carbon which is located at an increased distance (6.7 Å) from the Asp¹⁴⁷-COOH group as compared to the distance between that residue and the piperidine nitrogen in carfentanil (2.8 Å). The phenylethyl substituent is in an extended conformation and its aromatic ring interacts with the same five residues with which the phenyl moiety of the phenylethyl substituent of carfentanil interacts. Due to the rotated orientation of the of MOR-bound **8b** relative to **3**, the two other substituents on the cyclohexane ring have different orientations as compared to the corresponding substituents in **3**. The phenyl group of the *N*-phenylpropionamido substituent interacts with Trp³¹⁸ and Phe³¹³, and the methylcarboxylate group is located close to Thr³¹⁵ and Tyr¹²⁸.

3. Conclusions

The *trans* isomer of 'carba'-carfentanil (**8b**) has about 4000-fold lower MOR binding affinity than the carfentanil parent (**3**), corresponding to a loss in binding energy (ΔG) of 5 kcal/mol. This reduction in binding energy is due to the inability of **8b** to engage in an electrostatic interaction with Asp¹⁴⁷. Such electrostatic interactions typically contribute about 3 – 5 kcal/mol to the binding energy in ligand-protein interactions.¹⁷ The receptor

docking study revealed that while the phenylethyl substituent in **3** and **8b** interacts with the same receptor residues, the MOR binding mode of **8b** differs somewhat from that of **3**, with its cyclohexane ring rotated and shifted relative to the position of the piperidine ring of **8b** and with the *N*-phenylpropionamido- and methylcarboxylate substituent assuming a different orientation and interacting with different receptor residues. The different binding mode of **8b** may induce a distinct receptor conformation, resulting in the MOR partial agonist behaviour of this compound. Interestingly, the carbon substitution also had an effect on receptor selectivity, as **8b** showed no preference for MOR over DOR, in contrast to the high MOR versus DOR selectivity of **3**.

In contrast to the MOR partial agonist behaviour of **8b**, the 'carba' analogues containing a guanidinomethyl group in the *meta* position of the phenyl moiety attached to the ethylamido group (**2a**, **2b**) were full agonists. In these compounds the guanidino group forms a salt bridge with Asp²¹⁶. *N*-formylnormorphine (**9**) showed 290-fold lower MOR binding affinity than morphine (**10**), corresponding to a ΔG reduction of ~ 3.5 kcal/mol due to the loss of salt bridge formation. As described above, **9** turned out to be an antagonist with an altered MOR versus KOR binding selectivity profile. Taken together, the results obtained with **2a**, **2b**, **8b** and **9** indicate that deletion of the positively charged nitrogen can have diverse effects on the intrinsic efficacy (agonism, partial agonism, antagonism). As formation of a salt bridge with the TMH3 Asp¹⁴⁷ residue is no longer possible, ligand moieties other than the positively charged nitrogen present in the parent compounds may play an important role in inducing or recognizing distinct receptor conformations, leading to altered opioid activity profiles.

A number of 'non-classical' opioid receptor ligands lacking a positively charged nitrogen have been described in the literature. The neoclerodane diterpene salvinorin A (KOR agonist)¹⁸ and its analogue herkinorin (MOR agonist)¹⁹ are nonnitrogenous opioid receptor ligands with high binding affinities. However, there is evidence to indicate that these compounds have receptor binding modes totally different from the binding modes of all 'classical' nitrogen-containing agonists.²⁰ A neutral cyclic pentapeptide, c[YpwFG] (MOR agonist)²¹ and an uncharged cyclic tetrapeptide, c[FpFW] (CJ-15,208) (KOR agonist),^{22,23} are opioid receptor ligands with quite high binding affinities that likely have receptor binding modes quite different from that of 'classical' opioid peptides.

4. Experimental section

4.1. Chemistry: general

Melting points were recorded on an Electrothermal melting point apparatus and are uncorrected. Reactions were monitored by ascending TLC using precoated plates (silica gel 60F₂₅₄, 250 μ m, Merck Darmstadt). Flash chromatography was performed with silica gel columns (particles size 230–400 μ m). Preparative reversed-phase HPLC was performed using a Vydac 218-TP1022 column (22 \times 250mm) with a linear gradient of 55–85% MeOH in 0.1% TFA/H₂O over 30 min at a flow rate of 12 ml/min. Purity of compounds was determined using an analytical Vydac 218-TP54 column (5 \times 250mm) with a linear gradient of 45–75% MeOH in 0.1% TFA/H₂O over 30 min at a flow rate of 1 ml/min. High-resolution mass spectra were obtained by electrospray mass spectrometry on a hybrid Q-ToF mass spectrometer interfaced to a Mass Lynx 4.0 data system. ¹H and ¹³C NMR spectra

were recorded on Varian INOVA 500 MHz and Bruker 700 MHz AVANCEII spectrometers, and are referenced with respect to the residual signals of the solvent. Singlet, broad singlet, doublet, triplet and multiplet are abbreviated as s, br s, d, t, and m, respectively. *J* stands for the coupling constant measured in hertz (Hz). IR spectra were recorded on an ABB-Bomem MR Series spectrophotometer.

4.1.1. Phenethyl-1-(phenylamino)cyclohexanecarbonitrile 5a, 5b—To a stirred solution of 4-phenethylcyclohexanone **4** (3.5 g, 17.3 mmol) and aniline (1.88 g, 21 mmol) in 30 ml of glacial acetic acid, trimethylsilyl cyanide (2.48 ml, 17.3 mmol) was added dropwise over 5 min. The solution was stirred at room temperature overnight and then poured into 50 ml of NH₄OH conc. and 50 ml of ice. For adjustment of the pH to 10 an additional portion of NH₄OH was added. The reaction mixture was extracted with CHCl₃ (3 × 50ml) and the combined organic phase was washed with water and dried over MgSO₄. Evaporation of the organic layer afforded **5** (4.8 g, 90%) as a white solid of the mixture of *cis* and *trans* isomers. The isomer ratio (**5a/5b** = 1:3) was determined from the ¹H NMR spectrum by integration of the peaks at 2.33 and 2.47 ppm, respectively. Purification by flash chromatography on silica gel with hexane/ethyl acetate (9:1) afforded **5a** (0.5 g) and **5b** (2.8 g).

5a (isomer 1): yield 10%; mp 102–103° C; IR (KBr) 2234 cm⁻¹ ∪(CN); ¹H NMR (500 MHz, CDCl₃) δ 7.17–7.35 (m, 7H), 6.91–6.96 (m, 3H), 3.60 (br s, 1H), 2.62 (t, 2H, *J* = 8 Hz), 2.33 (d, 2H, *J* = 14 Hz), 1.94 (t, 2H, *J* = 14 Hz), 1.74–1.78 (m, 2H), 1.49–1.62 (m, 1H), 1.35–1.43 (m, 2H); ¹³C NMR (125MHz, CDCl₃) δ 143.6, 142.5, 129.5, 128.6, 128.5, 126.0, 122.4, 120.7, 117.4, 45.0, 37.7, 35.5, 34.2, 33.4, 26.6; HRMS (EI) *m/e* calcd for C₂₁H₂₅N₂ [M+H]⁺ 305.2096, obsd 305.2019.

5b (isomer 2): yield 50%; mp 136–138° C; IR (KBr) 2237 cm⁻¹ ∪(CN); ¹H NMR (500 MHz, CDCl₃) δ 7.19–7.32 (m, 7H), 6.92–6.95 (m, 3H), 3.82 (br s, 1H), 2.66 (t, 2H, *J* = 8 Hz), 2.48 (d, 2H, *J* = 12 Hz), 1.92 (d, 2H, *J* = 15 Hz), 1.63 (dd, 2H, *J* = 6 Hz, *J* = 10 Hz), 1.43–1.57 (m, 4H), 1.32–1.45 (m, 1H); ¹³C NMR (125MHz, CDCl₃) δ 143.8, 142.5, 129.5, 128.6, 125.5, 126.0, 121.2, 120.9, 118.2, 45.0, 38.2, 37.0, 36.3, 33.4, 29.3; HRMS (EI) *m/e* calcd for C₂₁H₂₅N₂ [M+H]⁺ 305.2096, obsd 305.2016.

4.1.2. 4-Phenethyl-1-(phenylamino)cyclohexanecarboxamide (6a, 6b)—To a solution of nitrile **5a** (0.3 g, 1 mmol) or **5b** (0.9 g, 3 mmol) in DMSO (10 or 20 ml) at room temperature was added K₂CO₃ × 1.5 H₂O (0.2 g or 0.6 g, respectively) and dropwise 30% H₂O₂ (1 or 2 ml). After 10 h for isomer **5a** and 40 h for **5b** no more substrate was observed (monitoring on TLC [hexane/ AcOEt = 1:1]). The reaction mixture was then poured into cool water and the obtained solid precipitate was filtered and washed with cool water to give **6a** (304 mg, 94%) or **6b** (870 mg, 90%) respectively.

6a (isomer 1): yield 94%; mp 230–230° C; IR (KBr) 1669 cm⁻¹ ∪(CO); ¹H NMR (500 MHz, CDCl₃) δ 6.67–7.35 (m, 12H), 4.04 (br s, 1H), 2.61 (t, 2H, *J* = 8Hz), 1.98–2.12 (m, 4H), 1.72 (d, 2H, *J* = 12 Hz), 1.53 (dd, 2H, *J* = 7 Hz, *J* = 9 Hz), 1.39–1.42 (m, 1H), 0.99–1.08 (m, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 179.5, 144.1, 142.7, 129.3, 128.5, 128.3,

125.9, 119.3, 116.4, 60.3, 39.1, 36.3, 33.3, 31.0, 27.8; HRMS (EI) m/e calcd for $C_{21}H_{27}N_2O$ $[M+H]^+$ 323.2118, obsd 323.2123.

6b (isomer 2): yield 90%; mp 142–144°C; IR (KBr) 1668 cm^{-1} $\nu(CO)$; 1H NMR (500 MHz, $CDCl_3$) δ 7.30–7.77 (m, 9H), 6.91 (t, 1H, $J = 14$ Hz), 6.77 (d, 2H, $J = 7.8$ Hz), 4.0 (br s, 1H), 2.61 (t, 2H, $J = 8$ Hz), 2.34 (m, 2H) 1.59–1.69 (m, 6H), 1.50 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.0, 144.5, 142.8, 129.3, 128.6, 128.5, 125.3, 119.3, 116.2, 60.0, 35.8, 34.3, 33.9, 31.9, 27.3; HRMS (EI) m/e calcd for $C_{21}H_{27}N_2O$ $[M+H]^+$ 323.2118, obsd 323.2119.

4.1.3. Methyl 4-phenethyl-1-(phenylamino)cyclohexanecarboxylate (**7a**, **7b**)—

Amide **6a** (250 mg, 0.77 mmol) or **6b** (337 mg, 1 mmol), p-toluenesulfonic acid monohydrate (670 mg, 3.5 mmol) and 10 ml MeOH was sealed in a glass pressure vessel. The reaction vessel was kept in an oil bath at 105–110°C for 4 days. After cooling the vessel was opened and was vented. The mixture was filtered and the solvent evaporated. A concentrated aqueous solution of NH_4OH was added to the residue until a pH of 8 was reached and the solution was then extracted with AcOEt (3 \times 25ml). The combined organic extracts were dried ($MgSO_4$) and evaporated. Purification by flash chromatography on silica gel with hexane/AcOEt (7:3) afforded **7a** (80 mg) or **7b** (100 mg) in the form of a colorless oil in both cases.

7a (isomer 1): yield 48%; 1H NMR (500 MHz, $CDCl_3$) δ 7.15–7.3 (m, 7H), 6.75 (t, 1H, $J = 7$ Hz), 6.57 (d, 2H, $J = 8$ Hz), 4.03 (br s, 1H), 3.70 (s, 3H), 2.62 (t, 2H, $J = 8$ Hz), 2.22 (d, 2H, $J = 13$ Hz), 1.82 (t, 2H, $J = 13$ Hz), 1.70 (d, 2H, $J = 13$ Hz), 1.56 (dd, 2H, $J = 6$ Hz, $J = 10$ Hz), 1.26–1.40 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.1, 144.9, 142.8, 129.3, 128.5, 125.9, 118.7, 115.3, 60.0, 52.5, 38.7, 36.4, 33.3, 32.3, 27.5; HRMS (EI) m/e calcd for $C_{22}H_{28}NO_2$ $[M+H]^+$ 338.2115, obsd 338.2112.

7b (isomer 2): yield 30%; 1H NMR (500 MHz, $CDCl_3$) δ 7.12–7.35 (m, 10H), 4.0 (br s, 1H), 3.77 (s, 3H), 2.59 (t, 2H, $J = 8$ Hz), 2.40 (d, 2H, $J = 12$ Hz), 1.78–1.85 (m, 4H), 1.50 (dd, 2H, $J = 7$ Hz, $J = 9$ Hz), 1.37 (m, 1H), 1.09–1.17 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.5, 142.8, 129.4, 128.5, 128.4, 125.9, 122.1, 118.8, 63.6, 52.4, 38.1, 35.9, 33.8, 33.6, 29.1; HRMS (EI) m/e calcd for $C_{22}H_{28}NO_2$ $[M+H]^+$ 338.2115 obsd 338.2116.

4.1.4. Methyl 4-phenethyl-1-(N-phenylpropionamido)cyclohexanecarboxylate (**8a**, **8b**)—

A solution of **7a** or **7b** (60 mg, 0.2 nmol) in propionic anhydride (2 ml) was stirred and refluxed for 72 h or 3.5 h, respectively (completion of the reaction was monitored by TLC [hexane/AcOEt = 4:1]). After cooling the mixture was poured into ice and basified with conc. NH_4OH . The separated oil was extracted into $CHCl_3$. The organic layer was washed with H_2O , dried ($MgSO_4$) and the solvent was evaporated. Purification of the crude products by preparative HPLC afforded **8a** (20 mg) or **8b** (55 mg). Compound **8a** was identified as the *cis* isomer and **8b** as the *trans* isomer (see Supplementary data).

8a (cis isomer): yield 34%; 1H NMR (500 MHz, $CDCl_3$) δ 7.12–7.50 (m, 10H), 3.79 (s, 3H), 2.52 (t, 2H, $J = 8$ Hz), 2.17 (d, 2H, $J = 4$ Hz), 1.86–1.92 (m, 4H), 1.56–1.59 (m, 2H), 1.43–1.47 (m, 2H), 1.36 (m, 1H), 0.93–1.0 (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.8,

175.0, 142.8, 140.4, 131.4, 129.2, 128.6, 128.5, 128.4, 125.9, 62.2, 52.5, 38.0, 35.0, 33.4, 31.5, 29.2, 27.5, 9.4; HRMS (EI) m/e calcd for $C_{25}H_{32}NO_3$ $[M+H]^+$ 394.2377 obsd 394.2385.

8b (trans isomer): yield 93%; 1H NMR (500 MHz, $CDCl_3$) δ 7.13–7.45 (m, 10H), 3.80 (s, 3H), 2.55 (t, 2H, $J = 8$ Hz), 2.34 (d, 2H, $J = 13$ Hz), 1.86 (dd, 2H, $J = 7$ Hz, $J = 8$ Hz), 1.64 (br d, 2H), 1.39–1.53 (m, 4H), 1.12–1.22 (m, 3H), 0.96 (t, 3H, $J = 7$ Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.3, 174.2, 142.9, 139.7, 130.6, 129.5, 128.8, 128.5, 125.8, 64.9, 52.2, 38.6, 36.4, 34.2, 33.4, 29.3, 9.5; HRMS (EI) m/e calcd for $C_{25}H_{32}NO_3$ $[M+H]^+$ 394.2377 obsd 394.2385.

4.2. Theoretical conformational analyses and receptor docking studies

All calculations were performed using the SYBYL software version 7.0 (Tripos Associates, St. Louis, MO). The Tripos force field was used for energy calculations. A dielectric constant of 78 was used for the conformational analysis of carfentanil and 'carba'-carfentanil. A stepwise approach was used to determine the low-energy conformations of carfentanil (**3**) and analogues **8a** and **8b**.²⁴ First, the piperidine or cyclohexane ring was retrieved from the fragment library. The nitrogen of the piperidine ring was in the charged (protonated) form. Functional groups were attached to the six-membered ring in each of the four different arrangements: equatorial-equatorial, equatorial-axial, axial-equatorial and axial-axial, resulting in four different starting structures for each compound studied. For each structure, a systematic conformational grid search was performed to identify low-energy structures. Each exocyclic rotatable bond was rotated in 30° increments over all space. Energies were calculated and the obtained conformers were grouped into low-energy families. The lowest-energy member of each family was minimized and the resulting conformations were ranked according to energy.

The software program GLIDE (Schrödinger LLC) was used in the studies of flexible docking of compounds **3** and **8b** to a model of the MOR in the activated state.¹⁶ A dielectric constant of one was used in the docking studies and in all subsequent calculations involving ligand-receptor complexes. Each of the resulting ligand-receptor complexes was minimized using the conjugate gradient approach.²⁵ Molecular dynamics simulations of 100 ps at 300 K were performed to assess the stability of each complex. In each case, no significant change in the complex structure was observed during the simulation.

4.3. In vitro bioassays and receptor binding assays

Opioid receptor binding studies were performed as described in detail elsewhere.²⁶ Binding affinities for the MOR and DOR were determined by displacing, respectively, [3H]DAMGO (Multiple Peptide Systems, San Diego, CA) and [3H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites and KOR binding affinities were measured by displacement of [3H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0° C with [3H]DAMGO, [3H]DSLET and [3H]U69,593 at respective concentrations of 0.72, 0.78 and 0.80 nM. IC_{50} values were determined from log-dose response curves, and K_i values were calculated from the IC_{50}

values by means of the equation of Cheng and Prusoff,²⁷ using values of 1.3, 2.6 and 2.9 nM for the dissociation constants of [³H]DAMGO, [³H]DSLET and [³H]U69,593, respectively.

The GPI²⁸ and MVD²⁹ bioassays were carried out as described elsewhere.^{26,30} A dose-response curve was determined with [Leu⁵]enkephalin as standard for each ileum or vas preparation, and IC₅₀ values of the compounds being tested were normalized according to a published procedure.³¹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

c-carfentanil	'carba'-carfentanil
DAMGO	H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol
Dmt	2',6'-dimethyltyrosine
DSLET	H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH
GPI	guinea pig ileum
HPLC	high performance liquid chromatography
MVD	mouse vas deferens
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TMH3	transmembrane helix 3
TMSCN	trimethylsilyl cyanide
U69,593	(5 α ,7 α ,8 β)-(—)- <i>N</i> -methyl- <i>N</i> -[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide

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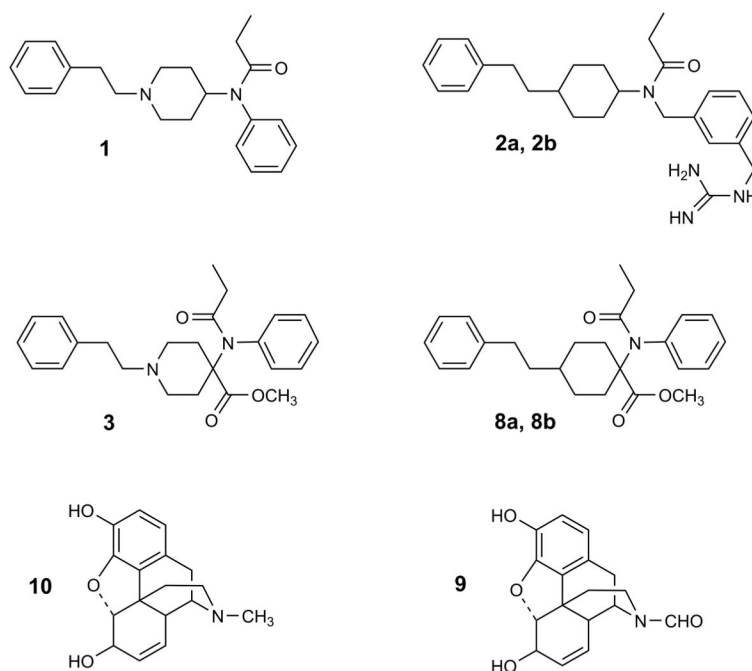


Figure 1. Structural formulas of fentanyl (**1**), fentanyl analogues (**2a**, **2b**), carfentanil (**3**), 'carba'-carfentanils (**8a**, **8b**), *N*-formylnormorphine (**9**) and morphine (**10**).

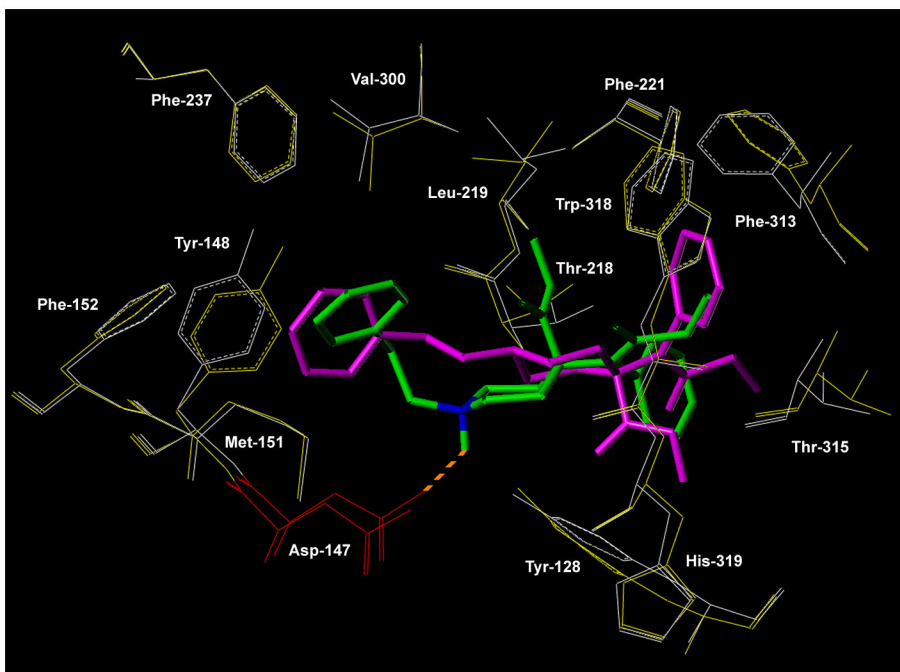
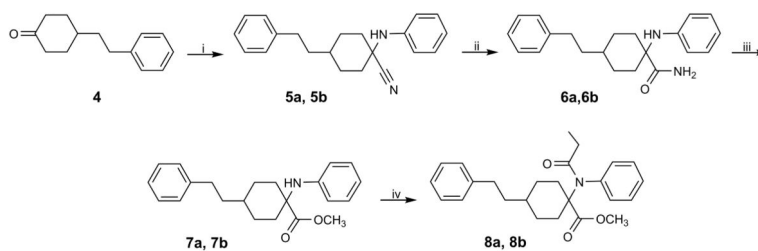


Figure 2. Docking study. Carfentanil (**3**) in green bound to the MOR in the activated state (key residues depicted in white) and 'carba'-carfentanil (*trans*) (**8b**) in magenta bound to the MOR in the activated state (key residues depicted in yellow). Asp¹⁴⁷ depicted in red.

**Scheme 1.**

Synthetic route to compounds **8a** and **8b**. Reagents and conditions: (i) aniline, TMSCN, AcOH, rt, 16 h, 90%; (ii) 30% H₂O₂, DMSO, K₂CO₃ × H₂O, rt, 10 – 40 h, 90 – 94%; (iii) *p*-TSOH, MeOH, 105 – 110° C, 4 days, 30 – 48%; (iv) (CH₃CH₂CO)₂O, reflux, 3.5 – 72 h, 34 – 93%.

Table 1
Opioid receptor binding affinities of 'carba'-carfentanil and N-formylnormorphine

No.	Compound	K_i^{μ} (nM) ^a	K_i^{δ} (nM) ^a	K_i^{κ} (nM) ^a	potency ratio $\mu/\delta/\kappa$
8a	c-carfentanil (<i>cis</i>)	> 5000	> 5000	> 5000	-
8b	c-carfentanil (<i>trans</i>)	95.2 ± 16.3	110 ± 5	> 5000	1/1/> 53
3	carfentanil ^b	0.024 ± 0.04	3.30 ± 0.04	43 ± 4	1/138/1790
9	N-formylnormorphine	420 ± 30	17700 ± 1200	363 ± 32	1/49/1
10	morphine	1.45 ± 0.16	82.5 ± 9.2	28.1 ± 2.8	1/57/19

^aMean of 3–4 determinations ± SEM.

^bData taken from ref. 32.

Table 2

GPI and MVD assay of 'carba'-carfentanil and N-formylmorphine

No.	Compound	GPI ^a			MVD ^a	
		IC ₅₀ [nM]	K _e ^d [nM]	K _e ^e [nM]	IC ₅₀ [nM]	
8a	c-carfentanil (<i>cis</i>)		4050 ± 1050	2980 ± 560	inactive	
8b	c-carfentanil (<i>trans</i>)	116 ± 7 (IC ₂₅) ^b			3890 ± 350 (IC ₃₅) ^b	
3	carfentanil ^c	0.019 ± 0.002			17 ± 8	
9	N-formylmorphine		1240 ± 10	1890 ± 300	> 10,000	
10	morphine		29.3 ± 2.2		155 ± 31	

^aMean of 3–4 determinations ± SEM.^bPartial agonist.⁵^cData taken from ref.³².