

“Carba”-Analogues of Fentanyl are Opioid Receptor Agonists[‡]

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There is evidence to indicate that the Asp residue in the third transmembrane helix (TMH) of opioid receptors forms a salt bridge with the positively charged nitrogen of endogenous and exogenous opioid ligands. To further examine the role of this electrostatic interaction in receptor binding and activation, we synthesized “carba”-analogues of a published fentanyl analogue containing a 3-(guanidinomethyl)-benzyl group in place of the phenyl moiety attached to the ethylamido group (C. Dardonville et al., *Bioorg. Med. Chem.* **2006**, *14*, 6570–6580 (**1**)), in which the piperidine ring nitrogen was replaced with a carbon. As expected, the resulting *cis* and *trans* isomers (**8a** and **8b**) showed reduced μ and κ opioid receptor binding affinities as compared to **1** but, surprisingly, retained opioid full agonist activity with about half the potency of leucine-enkephalin in the guinea pig ileum assay. In conjunction with performed receptor docking studies, these results indicate that the electrostatic interaction of the protonated nitrogen in the piperidine ring of fentanyl analogues with the Asp residue in the third TMH is not a *conditio sine qua non* for opioid receptor activation.

Introduction

The positively charged nitrogen in nonpeptide opiates and in opioid peptides with agonist or antagonist activity has been thought until recently to be an absolute requirement for binding to opioid receptors, presumably through formation of a salt bridge with the side chain of the Asp residue in the third transmembrane helix (TMH)^a of opioid receptors.¹ A cyclic β -casomorphin-derived opioid peptide, CHO-Dmt-c[D-Orn-2-Nal-Pro-Gly], lacking a positively charged nitrogen, was the first example of a neutral opioid compound with significant μ and δ opioid receptor binding affinity and μ and δ antagonist activity.² Subsequently, it was reported that replacement of the Tyr¹ residue in cyclic opioid peptide analogues with 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (Dhp), (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)-propanoic acid [(2*S*)-Mdp], or (3*S*)-3-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid [(3*S*)-Mdp] resulted in opioid antagonists lacking a positively charged N-terminal amino group and showing very high opioid receptor binding

affinity.^{3–5} Recently, a cyclic enkephalin analogue containing Dhp in the place of Tyr obtained via ring closing metathesis has been described.⁶ This peptide also lacks a positively charged nitrogen and represents the first example of a neutral compound with δ opioid agonist activity.

N-Formylnormorphine is an example of a nonpeptide opioid compound lacking a positive charge. This compound retained significant μ opioid receptor binding affinity, but was unable to activate the μ opioid receptor, as demonstrated using the [³⁵S]GTP γ S binding assay.⁷ More recently, *N*-formylnormorphine was shown to be a μ and κ opioid antagonist with similar binding affinity for the two receptors (K_i^μ = 420 nM; K_i^κ = 363 nM) (G. Weltrowska et al., manuscript in preparation).

In the μ opioid agonist fentanyl, the positively charged nitrogen is contained in the piperidine ring of this compound. In an effort to develop bifunctional μ opioid receptor/I₂-imidazoline binding site ligands, a fentanyl analogue containing a 3-(guanidinomethyl)-benzyl group in place of the phenyl moiety attached to the ethylamido group was prepared (Figure 1, compound **1**).⁸ This compound showed no affinity for the I₂-imidazoline binding site but very high μ opioid receptor binding affinity (K_i^μ = 0.0098 nM), about 300-fold more potent than fentanyl under the assay conditions used. This compound was not examined for binding affinity at δ and κ receptors and was not characterized in a functional opioid activity assay. To examine the role of the positively charged nitrogen of fentanyl compounds in the interaction with opioid receptors, we prepared “carba”-analogues of compound **1**, in which the piperidine nitrogen is replaced with a carbon atom. Both the *cis* and the *trans* isomer were obtained and pharmacologically characterized (Figure 1,

[‡]Dedicated to the memory of Ralph F. Hirschmann.

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^a Abbreviations: DAMGO, H-Tyr-D-Ala-Gly-N^{Me}Phe-Gly-ol; Dhp, 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid; DMAP, 4-(dimethylamino)-pyridine; Dmt, 2',6'-dimethyltyrosine; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; GPI, guinea pig ileum; LENK, [Leu⁵]enkephalin; (2*S*)-Mdp, (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid; (3*S*)-Mdp, (3*S*)-3-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid; MVD, mouse vas deferens; 2-Nal, 3-(2-naphthyl)-L-alanine; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TMH, transmembrane helix; U69,593, (5 α ,7 α ,8 β -(—)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

compounds **8a** and **8b**). Compound **1** was chosen as parent structure because the presence of the guanidino group increases water solubility as compared to a carba analogue of fentanyl itself which likely would be poorly soluble in water. To assess the possible contribution of the guanidino group to receptor binding, an analogue of **1** lacking the guanidino group (compound **10**) was also synthesized and pharmacologically characterized.

Chemistry

The “carba”-analogues **8a** and **8b** were synthesized according to Scheme 1. 4-Phenylethylcyclohexanone **5** was prepared by following a synthetic route described in the literature.⁹ The Wittig reagent was obtained from phosphonium salt **2** with NaH/DMSO and was condensed with 4,4-(ethylenedioxy)-cyclohexanone to form a mixture of benzylmethylene **3a** and phenylmethylene **3b**. Catalytic hydrogenation of the mixture of **3a** and **3b** yielded phenylethyl derivative **4**. Removal of the ketal blocking group from **4** with HCl/THF afforded 4-phenylethylcyclohexanone **5**. Cyclohexanone **5** was then used to prepare the target compounds **8a** and **8b** by following synthetic steps described for the preparation of fentanyl analogue **1**.⁸ Reductive amination of **5** with mono-Boc-protected *m*-xylenediamine provided compound **6**. Acylation of **6** with propionic anhydride followed by Boc-deprotection

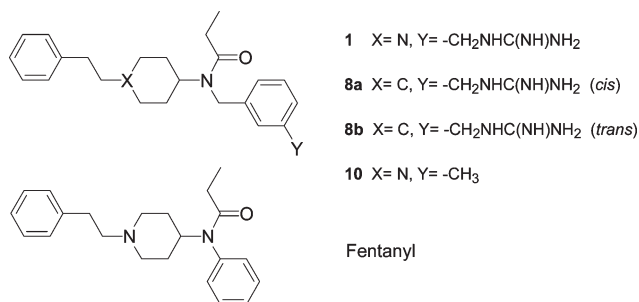
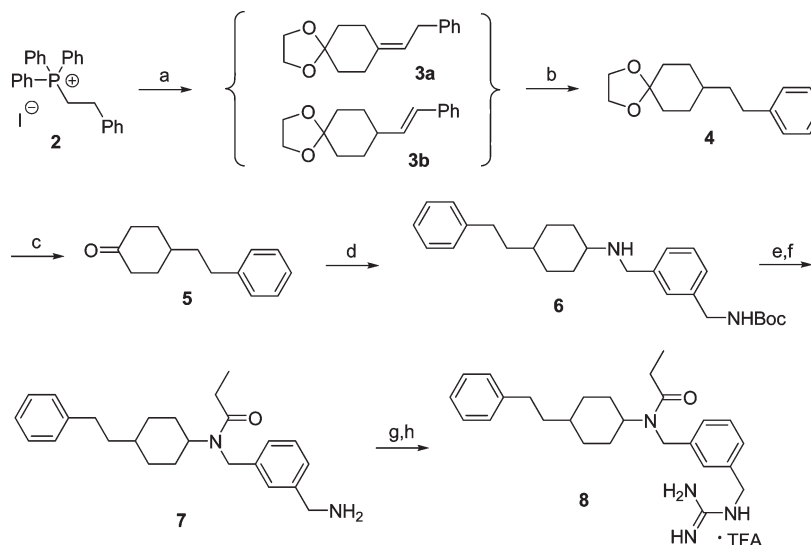


Figure 1. Structural formulas of fentanyl and fentanyl analogues **1**, **8a**, **8b**, and **10**.

Scheme 1^a



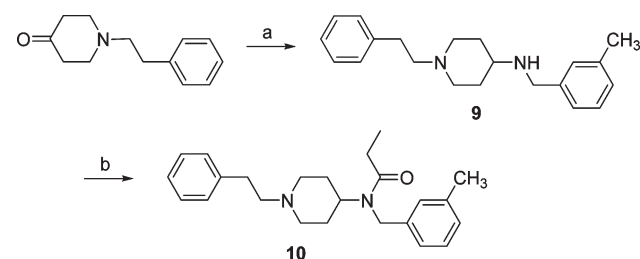
^a Reagents and conditions: (a) NaH, dry DMSO, 4,4-(ethylenedioxy)-cyclohexanone, rt, 20 h, 70%; (b) Pd/C, H₂ (g), EtOAc/AcOH, 60 psi, rt, 2 h, 100%; (c) 2 N HCl, THF, rt, 48 h, 82%; (d) *tert*-Butyl 3-(aminomethyl)benzylcarbamate, NaBH₃CN, 3 Å molecular sieves, 1% AcOH/MeOH, rt, 3 h, 71%; (e) (CH₃CH₂CO)₂O, DMAP, pyridine, dry CH₂Cl₂, rt, 20 h, 95%; (f) 50% TFA/CH₂Cl₂, rt, 45 min, 63%; (g) *N,N'*-Boc-thiourea, HgCl₂, Et₃N, dry CH₂Cl₂, rt, 3 h, 96%; (h) 50% TFA/CH₂Cl₂, rt, 1 h, 95%.

gave amine **7**. Reaction of **7** with the Boc-protected thiourea reagent and subsequent Boc-deprotection afforded the target compounds **8a** and **8b** as the trifluoroacetate salts. Compounds **6**, **7**, and **8** were obtained as mixtures of *cis* and *trans* isomers, which could be separated by preparative HPLC. In the case of **8**, the stereochemical assignment was made on the basis of the results of homonuclear decoupling NMR experiments. The faster-eluting component was identified as the *cis* isomer (**8a**) and the slower-eluting one was the *trans* isomer (**8b**). Compound **10** was prepared by reductive amination of 1-phenylethyl-4-piperidone with 3-methylbenzylamine, yielding **9**, followed by acylation with propionic anhydride (Scheme 2).

Results and Discussion

In the guinea pig ileum (GPI) assay, the carba analogues **8a** and **8b** both showed full agonist activity with about half the potency of [Leu⁵]enkephalin (LENK) (Table 1). Compounds **8a** and **8b** were about 25-fold less potent agonists than the piperidine nitrogen-containing parent **1**, which was characterized as a potent agonist in this assay system (IC₅₀ = 22.4 nM). The agonist effects of **8a**, **8b**, and **1** in the GPI assay were naloxone-reversible with K_e (naloxone) values ranging from 2 to 13 nM, indicating that they were mediated by opioid

Scheme 2^a



^a Reagents and conditions: (a) 3-methylbenzylamine, NaBH₃CN, 3 Å molecular sieves, 1% AcOH/MeOH, rt, 5 h, 50%; (b) (CH₃CH₂CO)₂O, DMAP, pyridine, dry CH₂Cl₂, rt, 20 h, 73%.

Table 1. In Vitro Opioid Activity Profiles of Fentanyl Analogues^a

cpd	GPI	MVD	receptor binding			<i>K_i</i> ratio
	IC ₅₀ (nM)	IC ₅₀ (nM)	<i>K_i</i> ^μ (nM)	<i>K_i</i> ^δ (nM)	<i>K_i</i> ^κ (nM)	μ/δ/κ
1	22.4 ± 3.7	114 ± 9	0.448 ± 0.079	43.5 ± 4.1	0.536 ± 0.043	1/97/1.2
8a	521 ± 67	5820 ± 1040	300 ± 8	4110 ± 80	238 ± 41	1/14/0.8
8b	590 ± 85	4750 ± 790	224 ± 13	7800 ± 280	162 ± 8	1/35/0.7
10	647 ± 77	610 ± 78	96.0 ± 5.9	6820 ± 670	1850 ± 160	1/71/19
Fentanyl	3.45 ± 0.45 ^b	9.45 ± 4.05 ^b	5.9 ± 1.4 ^c	568 ± 159 ^c	298 ± 40 ^c	1/96/51
LENK	246 ± 39	11.4 ± 1.1	9.43 ± 2.07	2.53 ± 0.35	4570 ± 550	1/0.3/485

^a Values represent means of 3–6 determinations ± SEM. ^b ref 14. ^c ref 15.

receptors. In the δ receptor-representative MVD assay, the carba analogues **8a** and **8b** displayed about 10-fold lower potency as compared to the potencies determined in the GPI assay, similar to the 5-fold weaker potency seen with compound **1** in this assay system.

In the opioid receptor binding assays, compounds **8a** and **8b** showed marked binding affinities for μ opioid receptors (*K_i*^μ = 220–300 nM) and κ opioid receptors (160–230 nM) (Table 1). Subnanomolar binding affinities were determined for **1** at both μ and κ receptors. The discrepancy between the μ receptor binding affinity of **1** (*K_i*^μ = 0.448 nM) determined in this study and the *K_i*^μ of 0.0098 nM reported for this compound in the literature⁸ is likely due to the different μ receptor binding assays used in the two laboratories. The δ opioid receptor binding affinities of **8a**, **8b**, and **1** were more than 10-fold lower than their respective μ and κ receptor binding affinities, and these three compounds showed similar μ/δ/κ *K_i* ratios (Table 1). This result indicates that the carbon replacement of the piperidine nitrogen in **1** did not have much of an effect on the opioid receptor selectivity profile. Furthermore, the opioid receptor binding affinities obtained for **8a**, **8b**, and **1** indicate that the agonist effects produced by these three compounds in the GPI assay are due to activation of both μ and κ receptors which are both present in the ileum. It is also of interest to point out that the configuration at the cyclohexane ring in the carba analogues does not have much of an effect on the in vitro opioid activity profile, as both the *cis* isomer (**8a**) and the *trans* isomer (**8b**) show very similar agonist potencies and opioid receptor binding affinities and similar opioid receptor selectivity profiles. Compound **10**, an analogue of **1** lacking the guanidino group, showed about 30-fold lower agonist potency than **1** in the GPI assay as well as lower potency in the MVD assay. In the receptor binding assays, compound **10** displayed about 150–200-fold lower μ and δ receptor binding affinities in comparison with **1** and 3500-fold lower κ receptor affinity. These results indicate that the guanidino group of **1** significantly contributes to the receptor binding energy of this compound at all three receptors and to the largest extent at the κ receptor. In comparison with carba analogues **8a** and **8b**, compound **10** has similar μ and δ receptor binding affinities but about 10-fold lower κ receptor binding affinity. Interestingly, compound **10** has about 15-fold lower μ receptor binding affinity than fentanyl and about 180-fold lower μ agonist potency in the GPI assay (Table 1).

Flexible docking studies of the fentanyl analogues were performed using Mosberg's model of the μ opioid receptor in the activated state.¹⁰ The results indicated that in all cases the phenylethyl group is accommodated in a binding pocket formed by transmembrane helices (TMHs) 3, 5, and 6, but significant differences in the detailed binding modes among the different ligands are observed (Figure 2). In the case of the docked fentanyl analogue containing the

3-(guanidinomethyl)-benzyl group (**1**), the phenylethyl group assumes an almost fully extended conformation with the phenyl ring interacting with the Tyr¹⁴⁸, Val³⁰⁰, Phe²³⁷, and Met¹⁵¹ side chain residues of the receptor. The piperidine nitrogen forms a salt bridge with Asp¹⁴⁷ in the third TMH (nitrogen–oxygen distance = 2.8 Å), as is typically observed with nitrogen-containing opioid receptor ligands. A second salt bridge between the guanidino moiety of the ligand and the side chain of Asp²¹⁶ in the second extracellular loop close to the interface between the cell membrane and the extracellular domain is also observed. The aromatic ring of the 3-(guanidinomethyl)-benzyl moiety interacts with the side chains of Phe³¹³, Trp³¹⁸, Thr²¹⁸, and Leu²¹⁹.

The lowest-energy conformer of compound **8b**, the *trans* isomer of the “carba”-analogue of **1**, with the phenylethyl and *N*-(3-guanidinomethyl)benzylpropanamide moieties equatorial, shows a receptor binding mode which overall is quite similar to that of its parent (**1**) (Figure 2). Again, the phenylethyl substituent is in an extended conformation and interacts with the same receptor residues as the phenylethyl group of **1**. However, the distance between the carbon replacing the nitrogen in the piperidine ring of **1** and the Asp¹⁴⁷ oxygen is larger (5.2 Å as compared to the corresponding distance of 2.8 Å in the case of **1**) and the cyclohexane ring is displaced by about 1.2 Å as compared to the piperidine ring in **1**. The guanidino group of **8b** forms a salt bridge with the side chain of Asp²¹⁶ but from the opposite side as compared to the salt bridge formation of the guanidino group of **1** with that same residue. The phenyl ring of the 3-(guanidinomethyl)benzyl moiety of **8b** interacts with the same receptor moieties as in the case of compound **1** (Phe³¹³, Thr²¹⁸, Trp³¹⁸).

In the lowest-energy conformer of compound **8a**, the *cis* isomer of the “carba”-analogue of **1**, the phenylethyl moiety is axial and the *N*-(3-guanidinomethyl)benzylpropanamide moiety is equatorial. The binding mode of compound **8a** differs somewhat from the binding modes of **8b** and **1**. The phenylethyl group assumes a bent conformation and its aromatic ring is displaced by about 4 Å relative to the corresponding aromatic ring of docked **1**, interacting with Tyr¹⁴⁸, Phe²³⁷, Val³⁰⁰, and Lys³⁰⁸. The cyclohexane ring is displaced by 3.6 Å relative to the cyclohexane ring of the *trans* isomer **8b**, and the carbon replacing the nitrogen in the piperidine moiety of **1** is located 8.5 Å away from the Asp¹⁴⁷ side chain oxygen. The guanidino group of **8a** again forms a salt bridge with the side chain of Asp²¹⁶ and the aromatic ring of its 3-(guanidinomethyl)-benzyl moiety interacts with the side chains of the Tyr¹²⁸, Thr³¹⁵, and His³¹⁹ residues of the receptor.

Compound **10**, the analogue of **1** lacking the guanidino group, has a binding mode very similar to that of **1**, with the phenylethyl moiety in an extended conformation. To compare the μ receptor binding mode of **10** with that of fentanyl, a docking study with fentanyl was also performed (data not

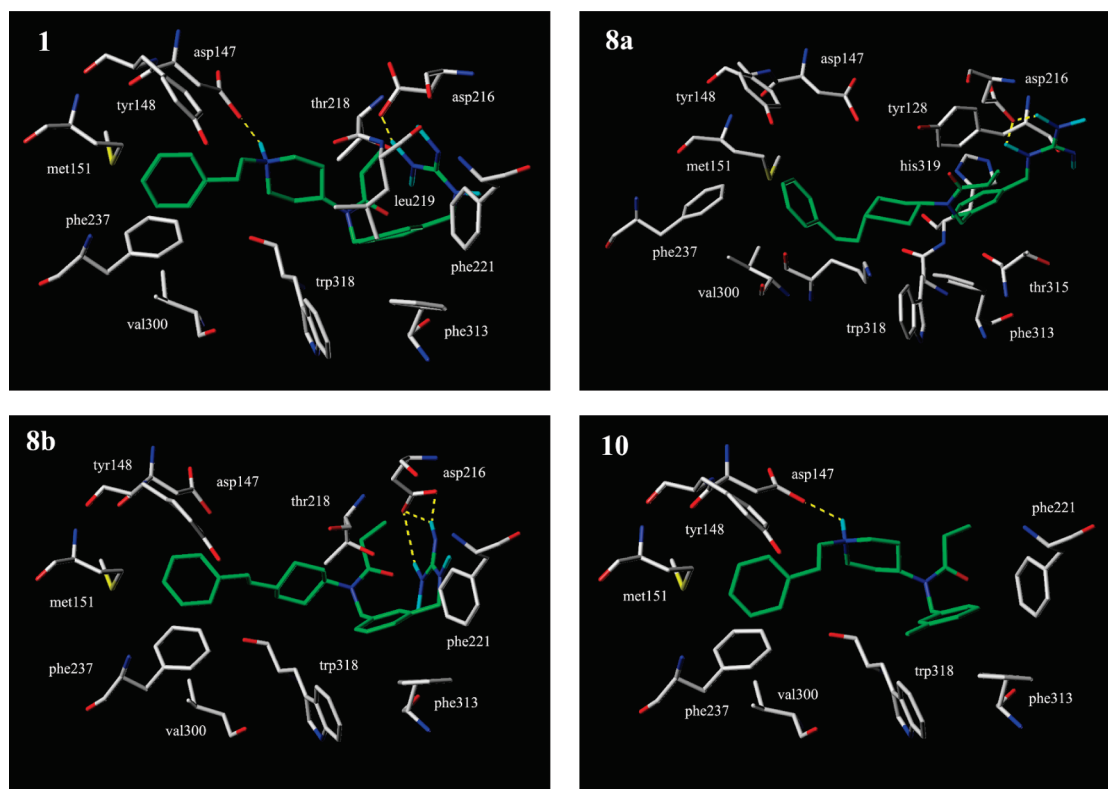


Figure 2. Receptor docking studies. Fentanyl analogues bound to the μ opioid receptor in the activated state: **1**, **8a**, **8b**, **10**.

shown). Earlier studies on the docking of fentanyl to the μ receptor, using receptor models developed prior to the rhodopsin crystal structure determination have been reviewed.¹¹ In agreement with two of these studies,^{12,13} the results of the present docking study indicated an interaction of the positively charged piperidine nitrogen with Asp¹⁴⁷ in the third TMH and an orientation of the phenylethyl moiety downward into a cavity formed by TMHs 3, 5, and 6, as described in one of these studies,¹² whereas in the other study, it was located in a cavity formed by TMHs 2, 3, and 7.¹³ Furthermore, as in the case of one of these studies,¹³ the phenethyl moiety assumed an extended conformation. The aromatic rings of the phenylethyl group and of the 3-methylbenzyl group in **10** interact with the same receptor residues as the corresponding aromatic rings of **1**, and the nitrogen in the piperidine ring forms a salt bridge with Asp¹⁴⁷. In a comparison of docked fentanyl and docked compound **10**, the *N*-phenylethyl piperidine moiety of the two compounds shows the same receptor binding mode. However, the aromatic rings of the *N*-phenyl group in fentanyl and of the *N*-3-methylbenzyl group in compound **10** occupy different receptor sites. This is in part due to the presence of the methylene group between the aromatic ring and the amide nitrogen in **10**. Furthermore, the torsion angle at the bond between the amide nitrogen and the piperidine ring is quite different for fentanyl ($\Phi = 172^\circ$) and compound **10** ($\Phi = 11^\circ$). Consequently, the propanamide moiety also has a different orientation in the two docked compounds. The different receptor interactions of the *N*-propanamide substituents may explain the observed decrease in μ opioid agonist potency of **10** as compared to fentanyl (Table 1).^{14,15}

The observation that the “carba”-analogues of **1**, compounds **8a** and **8b**, behave as full opioid agonists in the GPI assay with about half the potency of leucine-enkephalin represents a novel and important finding. Compounds **8a**

and **8b** have 670- and 500-fold lower μ receptor binding affinity than **1**, corresponding to a loss in binding energy (ΔG) of 3.95 and 3.71 kcal/mol, respectively. This reduction in receptor binding energy is due to the inability of these “carba”-analogues to engage in an electrostatic interaction with Asp¹⁴⁷. Such electrostatic interactions typically contribute about 4 kcal/mol to the binding energy in ligand–protein interactions.¹⁶ The analogous analysis of the κ receptor binding data of **8a** and **8b** as compared to **1** indicates a similar loss in binding energy of the two “carba”-analogues (3.71 and 3.47 kcal/mol, respectively). This decrease in binding energy is likely due to the elimination of the electrostatic interaction with the Asp residue (Asp¹³⁸) in the third TMH of the κ receptor, which was observed in a study on the docking of **1** to this receptor in the activated state (data not shown). The performed flexible docking studies reveal overall similar μ receptor binding modes for **8a**, **8b**, and **1**. In all cases, an electrostatic interaction between the guanidino group of these three compounds and the Asp²¹⁶ side chain of the receptor is observed. The analogue of compound **1** lacking the guanidino group (compound **10**) shows a 214-fold decrease in μ receptor binding affinity. This corresponds to a reduction in binding energy of 3.26 kcal/mol, in agreement with the observation that the guanidino group of **1** engages in an electrostatic bond with Asp²¹⁶.¹⁶ The agonist activity of “carba”-analogues **8a** and **8b** can be explained with their overall similar binding modes as compared to **1** and **10**, and with their guanidino group interacting with Asp²¹⁶. The latter electrostatic interaction contributes to binding the active conformation of the receptor and, thereby, compensates to some extent for the loss of the salt bridge with Asp¹⁴⁷ in the third TMH.

Conclusions

Compound **1** was confirmed as a ligand with subnanomolar μ receptor binding affinity and, furthermore, it was

determined that this fentanyl analogue also has high κ receptor binding affinity as well as high agonist potency in the GPI assay. The most important finding of this study is the novel observation that an electrostatic interaction between the positively charged nitrogen of fentanyl compounds and the Asp¹⁴⁷ residue of the μ opioid receptor is not a *conditio sine qua non* for μ opioid receptor activation. To the best of our knowledge, this has not been demonstrated before for opioid receptor ligands or for other GPCRs having a conserved Asp residue in the third TMH and interacting with endogenous ligands containing an amine moiety. Previously, it has been shown that elimination of the N-terminal amino group or its replacement with a methyl group in opioid peptides containing an N-terminal 2',6'-dimethyltyrosine (Dmt) residue led to neutral compounds showing high opioid antagonist activity.^{3–5} Similarly, *N*-formylnormorphine, which also lacks a positively charged nitrogen, also turned out to be an opioid antagonist (G. Weltrowska et al., manuscript in preparation). Taken together, these results indicate that the elimination of the positively charged nitrogen in opioid compounds may have divergent effects on the efficacy, depending on the receptor binding interactions of other moieties present in the molecule. The nonnitrogenous salvinorin A analogue herkinorin, which also lacks a positive charge, has been reported to be a μ opioid agonist with an EC₅₀ of 500 nM in a functional in vitro assay.¹⁷ However, there is evidence to indicate that salvinorin A and its analogues have a receptor binding mode totally different from the binding modes of all classical, nitrogen-containing opioid agonists.¹⁸

Experimental Section

General Methods. Reactions were monitored by ascending TLC using precoated plates (silica gel 60 F₂₅₄, 250 μ m, Merck Darmstadt, Germany). Flash chromatography was performed with silica gel 60 columns (particle size 230–400 μ m). ¹H and ¹³C NMR spectra were recorded on a Varian INOVA 500 MHz spectrometer and referenced with respect to the residual signals of the solvent. The following abbreviations were used in reporting spectra: s = singlet, d = doublet, t = triplet, m = multiplet. Preparative reversed-phase HPLC was performed on a Vydac 218-TP1022 column (22 \times 250 mm) with a linear gradient of 50–70% MeOH in 0.1% TFA/H₂O over 40 min at a flow rate of 12 mL/min. For analytical reversed-phase HPLC a Vydac 218-TP54 column (5 \times 250 mm) was used with a linear gradient of 50–70% MeOH in 0.1% TFA/H₂O over 30 min at a flow rate of 1 mL/min. All final compounds were obtained in >95% purity, as established by analytical HPLC. Molecular masses of compounds were determined by electrospray mass spectrometry on a hybrid Q-ToF mass spectrometer interfaced to a Mass Lynx 4.0 data system.

Syntheses of Compounds 8a, 8b, and 10. Phenylethyltriphenylphosphonium Iodide (2). A solution of (2-iodoethyl)benzene (10.4 mL, 72 mmol, 1.2 equiv) and 15.64 g (60 mmol) triphenylphosphine in 80 mL xylene was heated at 90 °C under argon for 24 h. After cooling in an ice bath, the formed precipitate was collected, washed with Et₂O, and dried in vacuo. Crystallization from acetone–ethyl ether yielded **2** as white crystals (25.0 g, 83%); mp 130–132 °C. ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.77–7.95 (m, 15H), 7.24–7.35 (m, 5H), 3.92–3.98 (m, 2H), 2.86–2.91 (m, 2H). HRMS (EI) *m/e* calcd for C₂₆H₂₄P [M]⁺ 367.1610, obsd 367.1611.

8-(2-Phenylethylidene)-1,4-dioxaspiro[4.5]decane (3). 1.2 g (30 mmol) NaH (60% oil dispersion) was stirred in 75 mL DMSO (dry) at 60 °C under argon for 1 h. After cooling of the solution to room temperature, 12.8 g (26 mmol) of phosphonium salt **2** was added under stirring. 4,4-(Ethylenedioxy)-cyclohexanone (4.06 g, 26 mmol) was then added and the reaction mixture was

stirred for 20 h. The mixture was then poured over ice and extracted with petroleum ether. The organic layers were combined, dried over MgSO₄, and evaporated. Flash chromatography on silica gel with hexane–EtOAc (6:1, v/v) afforded **3** as a mixture of 8-(2-phenylethylidene)-1,4-dioxaspiro[4.5]decane (**3a**) and 8-styryl-1,4-dioxaspiro[4.5]decane (**3b**) in the form of a colorless oil (4.5 g, 70%). The mixture of **3a** and **3b** was used without separation in the subsequent hydrogenation step.

8-Phenylethyl-1,4-dioxaspiro[4.5]decane (4). An argon-purged reaction vessel was charged with **3a** and **3b** (4.5 g, 18 mmol), 10% Pd/C (0.5 g) in 75 mL EtOAc and AcOH (10 μ L). The reaction vessel was then pressurized to 60 psi with hydrogen and the mixture was stirred for 2 h at room temperature. After filtration, the solvent was evaporated to yield **4** as a colorless oil (4.5 g, 100%). ¹H NMR (500 MHz, CDCl₃) δ 7.18–7.35 (m, 5H), 3.95 (s, 4H), 2.63–2.66 (t, 2H, *J* = 8.0 Hz), 1.28–1.82 (m, 11H). ¹³C NMR (125 MHz, CDCl₃) δ 142.7, 128.5, 125.7, 108.5, 64.4, 38.2, 36.5, 33.1, 29.5, 28.9. HRMS (EI) *m/e* calcd for C₁₆H₂₃O₂ [M+H]⁺ 247.1698, obsd 247.1691.

4-Phenylethylcyclohexanone (5). 4.5 g (18 mmol) of **4** in a mixture of 50 mL THF and 25 mL 2 N HCl was stirred for 48 h at room temperature. After evaporation of the solvent, the product was partitioned between EtOAc and H₂O. Evaporation of the organic layer afforded **5** (3.0 g, 82%) as a colorless oil. ¹H NMR (500 MHz, CDCl₃) δ 7.18–7.38 (m, 5H), 2.68–2.71 (t, 2H, *J* = 8.0 Hz) 1.42–2.45 (m, 11H). ¹³C NMR (125 MHz, CDCl₃) δ 211.8, 142.7, 128.5, 128.2, 125.7, 41.0, 38.3, 36.5, 31.2, 28.1. HRMS (EI) *m/e* calcd for C₁₄H₁₉O [M+H]⁺ 203.1436, obsd 203.1431.

tert-Butyl-3-((4-phenylethylcyclohexylamino)methyl) Benzylcarbamate (6). NaBH₃CN (942 mg, 15 mmol) was added to a solution of mono-Boc-protected *m*-xylenediamine⁸ (2.8 g, 12 mmol), 4-phenylethylcyclohexanone (**5**) (2.02 g, 10 mmol) and 3 Å molecular sieves in 1% AcOH/MeOH dry, and the mixture was stirred for 3 h at room temperature. After filtration on a pad of Celite, the organic solvent was evaporated and **6** was obtained as a 50%/50% mixture of the *cis* and *trans* isomers, as determined by integration of the isomeric proton peaks at 2.94 and 3.08 ppm in the ¹H NMR spectrum. Purification by flash chromatography on silica gel with CH₂Cl₂/7N NH₃ in MeOH (97:3) afforded **6** (3.080 g, 71% [3 g of *cis/trans* mixture, 40 mg of isomer 1 and 40 mg of isomer 2]). Isomer 1: ¹H NMR (500 MHz, CDCl₃) δ 8.45 (s, 1H), 7.08–7.25 (m, 9H), 5.12 (s, 1H), 4.12 (s, 2H), 4.08 (s, 2H), 3.08 (br s, 1H), 2.60 (t, 2H, *J* = 8.0 Hz), 1.42–1.9 (m, 11H), 1.4 (s, 9H). ¹³C NMR (125 MHz, CDCl₃) δ 156.2, 142.6, 137.4, 136.2, 128.5, 128.3, 128.1, 127.7, 125.9, 80.0, 56.3, 52.5, 45.4, 38.3, 36.7, 32.1, 28.4. HRMS (EI) *m/e* calcd for C₂₇H₃₉N₂O₂ [M+H]⁺ 423.3012, obsd 423.3013. Isomer 2: ¹H NMR (500 MHz, CDCl₃) δ 8.45 (s, 1H), 7.08–7.22 (m, 9H), 5.12 (br s, 1H), 4.12 (s, 1H), 4.05 (br s, 1H), 2.94 (br s, 1H), 2.60 (t, 2H, *J* = 8.0 Hz), 2.10 (br, 2H), 1.98 (br, 2H), 1.25–1.4 (br m, 14H), 0.81 (br m, 2H). ¹³C NMR (125 MHz, CDCl₃) δ 156.2, 142.6, 137.4, 136.2, 128.5, 128.3, 128.1, 127.7, 125.9, 80.0, 56.3, 52.5, 45.5, 38.3, 36.7, 32.1, 28.4. HRMS (EI) *m/e* calcd for C₂₇H₃₉N₂O₂ [M+H]⁺ 423.3012, obsd 423.3018.

***N*-(3-(Aminomethyl)benzyl)-*N*-(4-phenylethylcyclohexyl)propionamide (7).** Propionic anhydride (1 mL, 7.8 mmol) was added at room temperature to a solution of amine **6** (mixture of *cis/trans* isomers), DMAP (0.1 equiv), and pyridine (2.11 mL, 26 mmol) in dry CH₂Cl₂, and the mixture was stirred for 20 h at room temperature. After evaporation of the organic solvent, the oily residue was dissolved in EtOAc, extracted with a saturated NH₄Cl aqueous solution, brine, and water, and dried under MgSO₄ and concentrated. The crude product (2.5 g, 95%) was then taken up in 50% TFA/CH₂Cl₂, and the solution was stirred for 45 min at room temperature. The volatiles were evaporated, and the resulting crude oil was triturated with dry Et₂O. Purification of the oily residue by flash chromatography on silica gel with CH₂Cl₂/7N NH₃ in MeOH (97:3) afforded **7** as a mixture of *cis* and *trans* isomers (1.2 g, 63%). The mixture was

used in the next synthetic step without separation of the isomers. For the analytical characterization, the isomers were separated by preparative HPLC of a 100 mg sample of the mixture. Isomer 1: ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.26 (s, 2H), 7.25–7.49 (m, 9H), 4.58 (d, 2H, $J = 23$ Hz), 4.36 (br t, 0.5H), 4.05–4.11 (dd, 2H, $J_1 = 5$ Hz, $J_2 = 17$ Hz) 3.82 (br t, 0.5H), 3.16 (m, 0.5H), 2.82 (d, 0.5H, $J = 5$ Hz), 2.52 (m, 1H), 2.21 (m, 1H), 1.44–1.71 (m, 11H), 1.12 (t, 1.5H, $J = 7$ Hz), 1.0 (t, 1.5H, $J = 7$ Hz). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 171.7, 142.4, 141.3, 136.2, 128.5, 128.3, 128.1, 127.7, 125.9, 125.2, 46.7, 46.2, 38.3, 36.7, 29.4, 28.8, 26.6, 10.3. HRMS (EI) m/e calcd for $\text{C}_{25}\text{H}_{35}\text{N}_2\text{O}$ $[\text{M}+\text{H}]^+$ 379.2749, obsd 379.2735. Isomer 2: ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.22 (br s, 2H), 7.15–7.41 (m, 9H), 4.46 (d, 2H, $J = 24$ Hz), 4.31 (t, 0.5H, $J = 12$ Hz), 3.99 (m, 2H), 3.72 (t, 0.5H, $J = 12$ Hz), 3.05 (m, 0.5H), 2.75 (d + m, 1.5H, $J = 5$ Hz), 2.47 (m, 3H), 2.13 (m, 1H), 1.75 (d, 2H, $J = 12$ Hz), 1.56 (t, 2H, $J = 18$ Hz), 0.92–1.48 (m, 8H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 171.7, 142.4, 141.3, 136.2, 128.5, 128.3, 128.1, 127.7, 125.4, 125.2, 46.7, 46.2, 38.3, 36.7, 29.4, 28.8, 26.6, 10.3. HRMS (EI) m/e calcd for $\text{C}_{25}\text{H}_{35}\text{N}_2\text{O}$ $[\text{M}+\text{H}]^+$ 379.2749, obsd 379.2738.

***N*-(3-(Guanidinomethyl)benzyl)-*N*-(4-phenylethylcyclohexyl)-propionamide TFA Salt (8).** To a solution of **7** (500 mg, 1 mmol), *N,N'*-di-(*tert*-butoxycarbonyl)-thiourea (327 mg, 1.2 mmol) and TEA (0.68 mL) in dry CH_2Cl_2 (20 mL), HgCl_2 (325 mg, 1.2 mmol) was added. After stirring under argon for 3 h at room temperature, the reaction mixture was filtered and the organic phase was washed with water, dried over MgSO_4 , and concentrated. The resulting crude oil (600 mg, 96%) was dissolved in 50% TFA/ CH_2Cl_2 and the solution was stirred for 1 h at room temperature. The volatiles were then evaporated and the crude oily residue was triturated with dry Et_2O . After evaporation of Et_2O , the TFA salt of **8** (mixture of *cis* and *trans* isomers) was obtained as a very hygroscopic solid (400 mg, 95%). The *cis* and *trans* isomers were separated by preparative HPLC and were identified by homonuclear decoupling NMR experiments (see Supporting Information). Isomer 1 (*cis*, **8a**): ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 7.94 (d, 1H, $J = 27$ Hz), 7.09–7.37 (m, 9H), 4.50 (d, 2H, $J = 23$ Hz), 4.32 (dd, 2H, $J = 6$ Hz), 4.26 (br t, 0.5H), 3.73 (br 0.5H), 2.75 (d, 1H, $J = 5$ Hz), 2.46 (m, 3H), 2.12 (m, 1H), 1.17–1.63 (m, 12H), 1.04 (t, 1.5H, $J = 7$ Hz), 0.92 (t, 1.5H, $J = 7$ Hz). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 171.7, 158.0, 142.6, 137.4, 128.5, 128.1, 127.7, 125.9, 53.3, 46.7, 44.9, 38.3, 36.7, 29.4, 29.1, 28.8, 26.6, 10.2. HRMS (EI) m/e calcd for $\text{C}_{26}\text{H}_{37}\text{N}_4\text{O}$ $[\text{M}+\text{H}]^+$ 421.2967, obsd 421.2955. Isomer 2 (*trans*, **8b**): ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 7.94 (d, 1H, $J = 27$ Hz), 7.08–7.37 (m, 9H), 4.46 (d, 2H, $J = 26$ Hz), 4.33–4.37 (dd, 2.5H, $J = 6$ Hz), 3.73 (br t, 0.5H), 2.74 (d, 1H, $J = 5$ Hz), 2.53 (t, 2H, $J = 8$ Hz), 2.40 (m, 1H), 2.12 (m, 1H), 1.75 (d, 2H, $J = 12$ Hz), 1.54 (m, 2H), 0.98–1.46 (m, 12H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 171.7, 158.0, 142.6, 137.4, 128.5, 128.1, 127.7, 125.0, 53.3, 46.7, 44.9, 38.3, 36.7, 29.4, 29.1, 28.8, 26.6, 10.2. HRMS (EI) m/e calcd for $\text{C}_{26}\text{H}_{37}\text{N}_4\text{O}$ $[\text{M}+\text{H}]^+$ 421.2967, obsd 421.2955.

***N*-(3-Methylbenzyl)-1-phenylethylpiperidine-4-amine (9).** NaBH_3CN (0.55 g, 8.9 mmol) was added to a solution of 3-methylbenzylamine (0.88 mL, 7 mmol), 1-phenylethyl-4-piperidinone (1.2 g, 5.9 mmol) and 3 Å sieves in 1% AcOH/MeOH (dry). The mixture was stirred at room temperature for 5 h, filtered over Celite, and washed with dry MeOH. The combined organic filtrates were evaporated and the crude product was purified by flash chromatography on silica gel ($\text{CHCl}_3/\text{MeOH}$, 9:1), affording **9** as a colorless oil (0.9 g, 50%). ^1H NMR (500 MHz, CDCl_3) δ 7.08–7.32 (m, 9H), 3.81 (s, 2H), 3.0 (d, 2H, $J = 14$ Hz), 2.83 (m, 2H), 2.58 (m, 4H), 2.37 (s, 3H), 2.15 (t, 2H, $J = 11$ Hz), 1.97 (d, 2H, $J = 12$ Hz), 1.50 (dd, 2H, $J = 4$ and 11 Hz). ^{13}C NMR (125 MHz, CDCl_3) δ 140.5, 140.4, 129.1, 129.0, 128.9, 128.6, 128.5, 127.9, 126.3, 125.3, 60.7, 54.0, 51.0, 45.0, 38.9, 33.8, 32.5, 24.0, 21.6. HRMS (EI) m/e calcd for $\text{C}_{21}\text{H}_{29}\text{N}_2$ $[\text{M}+\text{H}]^+$ 309.2325, obsd 309.2319.

***N*-(3-Methylbenzyl)-*N*-(1-phenylethylpiperidine-4-yl)propionamide (10).** Propionic anhydride (0.55 mL, 4.35 mmol) was

added at room temperature to a solution of amine **9** (0.9 g, 2.9 mmol), DMAP (30 mg, 0.1 equiv) and pyridine (1.17 mL, 14.5 mmol) in dry CH_2Cl_2 . After stirring the reaction mixture for 20 h, the organic solvent was evaporated and the crude product was dissolved in AcOEt . The solution was washed with sat. NH_4Cl aqueous solution, brine, and water, dried over MgSO_4 , and concentrated. The resulting crude oil was purified by flash chromatography on silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{TEA}$, 95:5:0.2), affording **10** as a colorless oil (0.8 g, 73%). ^1H NMR (500 MHz, CDCl_3) δ 6.93–7.34 (m, 9H), 4.88 (m, 1H), 4.52 (s, 2H), 3.66 (d, 2H, $J = 12$ Hz), 3.16 (br, 2H), 3.00 (m, 2H), 2.77 (br, 2H), 2.37 (d, 2H, $J = 8$ Hz), 2.35 (s, 3H), 2.11 (dd, 2H, $J = 11$ and 36 Hz), 1.82 (d, 2H, $J = 12$ Hz), 1.14 (t, 3H, $J = 8$ Hz). ^{13}C NMR (125 MHz, CDCl_3) δ 175.8, 139.1, 137.6, 135.8, 129.3, 128.8, 128.5, 127.7, 126.4, 122.7, 58.6, 52.8, 49.0, 46.5, 45.0, 30.7, 27.2, 26.7, 21.6, 9.7. HRMS (EI) m/e calcd for $\text{C}_{24}\text{H}_{33}\text{N}_2\text{O}$ $[\text{M}+\text{H}]^+$ 365.2593, obsd 365.2580.

In Vitro Bioassays and Receptor Binding Assays. The GPI¹⁹ and MVD²⁰ bioassays were carried out as reported in detail elsewhere.^{21,22} A dose–response curve was determined with [Leu⁵]enkephalin as standard for each ileum and vas preparation, and IC₅₀ values of the compounds being tested were normalized according to a published procedure.²³ K_e -values for naloxone as antagonist were determined from the ratio of IC₅₀ values obtained with an agonist in the presence and absence of a fixed naloxone concentration (20 nM).²⁴ Opioid receptor binding studies were performed as described in detail elsewhere.²¹ Binding affinities for μ and δ opioid receptors 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 κ opioid receptor 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. IC₅₀ values were determined from log–dose displacement curves, and K_i values were calculated from the IC₅₀ values by means of the equation of Cheng and Prusoff,²⁵ 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 free energy change, ΔG , associated with ligand–receptor interactions was calculated using the equation $\Delta G = -2.303 \text{ RT log } K_i$, which under physiological conditions ($T = 310^\circ \text{ K}$) is approximated (in kcal/mol) by $\Delta G = -1.4 \text{ log } K_i$.

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 the isolated ligands in vacuo. A stepwise approach was used to determine the low-energy conformations of the fentanyl analogues.²⁶ First, the cyclohexane ring or piperidine ring was retrieved from the fragment library. The piperidine ring was in the charged (protonated) form. Functional groups were attached to the six-membered ring in each of four different arrangements: axial–axial, axial–equatorial, equatorial–axial, and equatorial–equatorial, 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 lowest-energy conformations of all compounds have the cyclohexane or piperidine ring in the chair conformation. In the lowest-energy conformation of the *cis* isomer of the “carba”-analogue (**8a**), the larger substituent on the cyclohexane ring is equatorial and the smaller substituent is

axial. The lowest-energy conformation of the *trans* isomer of the "carba"-analogue (**8b**) has both substituents on the cyclohexane ring in the equatorial position. In the lowest-energy conformations of compounds **1** and **10**, both substituents on the piperidine ring are equatorial.

The model of the μ opioid receptor in the activated state, constructed by Mosberg et al. by homology modeling based on the crystal structure of rhodopsin¹⁰ (<http://mosberglab.phar.umich.edu/resources/>), was used in the docking studies. A dielectric constant of 1 was used for the receptor docking studies as well as in all subsequent calculations involving receptor–ligand complexes. Flexible docking of the lowest-energy conformation of each compound was performed using the software program *GLIDE* (Schrödinger LLC). Each of the resulting ligand–receptor complexes was minimized using the conjugate gradient approach.²⁷ Molecular dynamics simulations of 100 ps at 300 K were performed in order to assess the stability of each complex. In each case, no significant change in the complex structure was observed during the simulation.

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Supporting Information Available: NMR data for stereochemical assignment. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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