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Opioid Bifunctional Ligands from Morphine and the Opioid Pharmacophore Dmt-Tic

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Abstract

Bifunctional ligands containing an ester linkage between morphine and the δ -selective pharmacophore Dmt-Tic were synthesized, and their binding affinity and functional bioactivity at the μ , δ and κ opioid receptors determined. Bifunctional ligands containing or not a spacer of β -alanine between the two pharmacophores lose the μ agonism deriving from morphine becoming partial μ agonists **4** or μ antagonists **5**. Partial κ agonism is evidenced only for compound **4**. Finally, both compounds showed potent δ antagonism.

1. Introduction

It is now widely accepted that biological activity at a single receptor is often insufficient and recent research has focused on ligands that have multiple activities.¹ A large body of evidence indicates that δ opioid receptor antagonists suppress tolerance, physical dependence, and related side effects of μ agonists without affecting their analgesic activity.² The simultaneous targeting of both receptors can be accomplished by: (i) co-administering two selective drugs, (ii) administering one non-selective drug, or (iii) designing a single drug that specifically targets both receptors; a bifunctional ligand.^{3, 4} Bifunctional ligands can be classified on the basis of the link between pharmacophores as: conjugates, fused and merged. Furthermore, conjugates and fused bifunctional ligands can be subdivided into cleavable (typically esters which provide a prodrug approach) and non cleavable ones.⁴ On the basis of the chemical structure of the linked pharmacophores, opioid bifunctional ligands are classified as: (i) peptide opioid/opioid, (ii) mixed peptide/non-peptide opioid/opioid, (iii)

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non-peptide opioid/opioid, and (iv) opioid/non-opioid.⁴ Our previous studies in this field reported the synthesis of bifunctional ligands belonging to each of the first three classes mentioned above.^{5–9} Many other opioid bifunctional ligands have been reported by other authors and recently reviewed.^{1,3,4} On the assumption that morphine is the most widely used opioid analgesic, but endowed with a series of side effects, a large number of bifunctional ligands were synthesized with the aim to overcome such drawbacks, but very few analogues incorporating morphine as one of the two pharmacophores have been reported.^{9a,b} In the light of these considerations, here we report the synthesis and pharmacological characterization of two bifunctional ligands (Table 1) made up by the μ agonist (morphine) and the δ antagonist *N*(Me)₂-Dmt-Tic pharmacophore.¹⁴ Although, the length and the type of spacer between the two pharmacophores is considered important,^{7, 8, 15} such variability was not examined in the present study. In fact, we did not use a spacer or used only spacers made of four atoms, ethylenediamine or β -alanine.^{5, 6}

2. Chemistry

Compounds (**4**, **5**) were synthesized by condensation (WSC/HOBt) of morphine with *N*(Me)₂-Dmt-Tic-OH or *N*(Me)₂-Dmt-Tic- β -Ala-OH; where β -Ala can be considered as a spacer or as a part of the pharmacophore in view of its similarity to the selective δ antagonist tripeptides H-Dmt-Tic- β -Ala-OH⁶ and *N*(Me)₂-Dmt-Tic-Ala-OH.¹⁴ As an example, in Scheme 1 the synthesis of compound **4** is reported. *N,N*-dimethylation of the tripeptide H-Dmt-Tic- β -Ala-OH⁶ was accomplished with 37% aqueous formaldehyde and NaBH₃CN according to the reported procedure.¹⁶ Final compounds were purified by preparative reverse phase HPLC.

3. Results and Discussion

3.1. Receptor Affinity Analysis

The new bifunctional ligands **4** and **5** and the single pharmacophores (**2**, **3** and morphine) were evaluated for their affinity and selectivity for μ , δ , κ opioid receptors using Chinese hamster ovary (CHO) cell membranes stably expressing the opioid receptors. The data are summarized in Table 1. The new compounds **4** and **5** containing morphine as a μ agonist pharmacophore, had nanomolar affinity for both μ (K_i = 1.1–3.5 nM) and δ (K_i = 1.4–1.6 nM) receptors of the same order of magnitude when compared with the affinities of the corresponding selective pharmacophores [morphine, K_i (μ) = 0.88 nM; **2**, (δ) = 5.7 nM; **3**, (δ) = 2.7 nM]. κ receptor affinity increased (2–17 fold) in comparison to morphine, especially for the bifunctional ligands **4** containing the tripeptide δ antagonist **2**. An increase in κ affinity was unexpected and difficult to explain in the light of the fact that the Dmt-Tic pharmacophore never showed affinity for this receptor.

3.2. Functional Bioactivity

Tables 2 and 3 indicate agonist and antagonist properties of the new compounds **4** and **5** in stimulating [³⁵S]GTP γ S binding mediated by the μ , δ and κ opioid receptors. Unlike reference bifunctional ligand **1** which retained the pharmacological characteristics of the two constituent pharmacophores, the new derivatives unexpectedly changed their pharmacological profiles. In particular, the mono-ester of morphine **4** with the *N*-dimethylated δ antagonist tripeptide **2** showed weak μ partial agonism and weak δ inverse agonism. It is useful to remember that usually δ antagonists derived from the Dmt-Tic pharmacophore are also endowed with various degree of inverse agonist activity.^{17, 18} Unlike morphine, **4** has partial κ agonist activity. Analogue **5**, containing the *N*-dimethylated δ antagonist dipeptide **3** exhibited only weak δ inverse agonism (no agonism for μ and κ receptors). Surprisingly, analogue **5**, derived from a morphine mono-ester with the dipeptide

δ antagonist **3**, showed μ antagonist activity in the same order of magnitude as naloxone. The corresponding analogue **4**, derived from the δ antagonist tripeptide **2** was endowed with a μ antagonism 35 fold less active than **5**. Both morphine bifunctional ligands **4** and **5** were characterized by a potent δ antagonism activity comparable to the reference **1**. Compound **5** exhibited κ antagonism at micromolar concentration. For the first time here we reported the synthesis of bifunctional ligands of the Dmt-Tic pharmacophore in which their pharmacological profile is changed with respect to the single constituents; until now we haven't hypothesis to explain this different behaviour.

4. Conclusion

The conclusions that can be drawn from the present work are as follows: (i) Morphine, when incorporated in bifunctional ligands with δ antagonist peptides containing the Dmt-Tic pharmacophore, do not conserve its μ agonism. It maintains partial μ agonism when linked to the tripeptide in compound **4**, or becomes a μ antagonist when linked to the dipeptide in compound **5**. (ii) Morphine bifunctional ligands **4** and **5** are endowed with δ inverse agonism and potent δ antagonist activity attributable to the Dmt-Tic sequence. It is useful to remember that the best δ inverse agonist reported to date is $N(\text{Me})_2\text{-Dmt-Tic-NH}_2$ [$\text{EC}_{50} = 2.66 \text{ nM}$; $E_{\text{max}} = -35.95\%$].^{17, 18} Furthermore unlike morphine, **4** shows partial κ agonism, and **5** shows κ antagonism at micromolar concentration. (iii) Finally, compounds **4** and **5** are made up of an ester linkage between pharmacophores (cleavable bivalent ligands), that after administration can be metabolized to the single pharmacophores[morphine and Dmt-Tic-(β -Ala)] characterized by a different pharmacological profile in comparison to the bifunctional ligands **4** and **5**. For this reason, such ligands could be of potential utility in the opioid receptor trafficking and related pharmacological studies.^{19–21}

5. Experimental Section

5.1. Chemistry

5.1.1. General Methods—Crude peptides and pseudopeptides were purified by preparative reversed-phase HPLC [Waters Delta Prep 4000 system with Waters Prep LC 40 mm Assembly column C18 (30 cm $\times$ 4 cm, 15 μm particle)] and eluted at a flow rate of 20 mL/min with mobile phase solvent A (10% acetonitrile + 0.1% TFA in H_2O , v/v), and a linear gradient from 10 to 60% B (60%, acetonitrile + 0.1% TFA in H_2O , v/v) in 25 min. Analytical HPLC analyses were performed with a Beckman System Gold (Beckman ultrasphere ODS column, 250 mm $\times$ 4.6 mm, 5 μm particle). Analytical determinations and capacity factor (K') of the products used HPLC in solvents A and B programmed at flow rate of 1 mL/min with linear gradients from 0 to 100% B in 25 min. Analogues had less than 5% impurities at 220 and 254 nm. TLC was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany): (A) 1-butanol/AcOH/ H_2O (3:1:1, v/v/v); (B) CH_2Cl_2 /toluene/methanol (17:1:2). Ninhydrin (1% ethanol, Merck), fluorescamine (Hoffman-La Roche) and chlorine spray reagents. Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations were assessed at 10 mg/mL in methanol with a Perkin-Elmer 241 polarimeter in a 10 cm water-jacketed cell. Molecular weights of the compounds were determined by a MALDI-TOF analysis (Hewlett Packard G2025A LD-TOF system mass spectrometer) and α -cyano-4-hydroxycinnamic acid as a matrix. ^1H NMR (δ) spectra were measured, when not specified, in $\text{DMSO}-d_6$ solution using a Bruker AC-200 spectrometer, and peak positions are given in parts per million downfield from tetramethylsilane as internal standard. The purity of tested compounds was determined by combustion elemental analyses conducted by the Microanalytical Laboratory of the Chemistry Department, University of Ferrara, with a Yanagimoto MT-5 CHN recorder elemental analyzer. All tested compounds possess a purity of at least 95% of the theoretical values.

5.2. Synthesis

5.2.1. TFA·N(Me)₂-Dmt-Tic-β-Ala-OH (2)—To a stirred solution of TFA·H-Dmt-Tic-β-Ala-OH⁶ (0.23 g, 0.42 mmol) in acetonitrile/H₂O (1:1, v/v, 10 mL); NMM (0.1 mL, 0.84 mmol), 37% aqueous formaldehyde (0.32 mL, 4.2 mmol) and sodium cyanoborohydride (0.08 g, 1.26 mmol) were added. Glacial acetic acid (0.06 mL) was added over 10 min and the reaction was stirred at room temperature for 15 min. The reaction mixture was acidified with TFA (0.1 mL) and directly purified by preparative reverse phase HPLC: yield 0.23 g (95%); *R_f*(A) 0.50; HPLC *K'* 2.97; mp 151–153 °C; [α]_D²⁰ +10.8; *m/z* 469 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 2.27–2.49 (m, 14H), 2.77–3.47 (m, 6H), 3.95–4.51 (m, 3H), 4.90–4.94 (m, 1H), 6.29 (s, 2H), 6.96–7.02 (m, 4H). Anal. C₂₈H₃₄F₃N₃O₇: C; H; N.

5.2.2. 2TFA·3-[N(Me)₂-Dmt-Tic-β-Ala]-O-Morphine Ester (4)—To a solution of TFA·N(Me)₂-Dmt-Tic-β-Ala-OH (0.1 g, 0.17 mmol) and HCl·Morphine (0.06 g, 0.17 mmol) in DMF (10 mL) at 0 °C, NMM (0.06 mL, 0.51 mmol), HOBt (0.03 g, 0.19 mmol), and WSC (0.04 g, 0.19 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in AcOEt and washed with NaHCO₃ (5% in H₂O), and brine. The organic phase was dried (Na₂SO₄) and evaporated to dryness. The residue was precipitated from Et₂O/Pe (1:9, v/v): yield 0.14 g (84%); *R_f*(A) 0.62; HPLC *K'* 3.68; mp 148–150 °C; [α]_D²⁰ −51.1; *m/z* 736 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 1.63–1.88 (m, 2H), 2.19–2.49 (m, 20H), 2.76–3.47 (m, 9H), 3.95–4.51 (m, 5H), 4.92–5.59 (m, 3H), 6.29 (s, 2H), 6.54–6.63 (m, 2H), 6.96–7.02 (m, 4H). Anal. C₄₇H₅₂F₆N₄O₁₁: C; H; N.

5.2.3. 2TFA·3-[N(Me)₂-Dmt-Tic]-O-Morphine Ester (5)—This compound was obtained by condensation of TFA·N(Me)₂-Dmt-Tic-OH¹⁴ with HCl·Morphine via WSC/HOBt as reported for 2TFA·3-[N(Me)₂-Dmt-Tic-β-Ala]-O-Morphine Ester: yield 0.11 g (78%); *R_f*(A) 0.68; HPLC *K'* 4.04; mp 157–159 °C; [α]_D²⁰ −41.6; *m/z* 665 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 1.63–1.88 (m, 2H), 2.19–2.49 (m, 18H), 2.76–3.17 (m, 7H), 3.95–4.51 (m, 5H), 4.81–5.59 (m, 3H), 6.29 (s, 2H), 6.54–6.63 (m, 2H), 6.96–7.02 (m, 4H). Anal. C₄₄H₄₇F₆N₃O₁₀: C; H; N.

5.3 Pharmacology

5.3.1. Radiolabeled Ligand Binding Assays—Binding assays used to screen compounds are similar to those previously reported.²² Membrane protein from CHO cells that stably expressed one type of the human opioid receptor were incubated with 12 different concentrations of the compound in the presence of either 1 nM [³H]U69,593²³ (κ), 0.25 nM [³H]DAMGO²⁴ (μ) or 0.2 nM [³H]naltrindole²⁵ (δ) in a final volume of 1 mL of 50 mM Tris-HCl, pH 7.5 at 25 °C. Incubation times of 60 min were used for [³H]U69,593 and [³H]DAMGO. Because of a slower association of [³H]naltrindole with the receptor, a 3 h incubation was used with this radioligand. Samples incubated with [³H]naltrindole also contained 10 mM MgCl₂ and 0.5 mM phenylmethylsulfonyl fluoride. The binding was terminated by filtering the samples through Schleicher & Schuell no. 32 glass fiber filters using a Brandel 48-well cell harvester. The filters were subsequently washed three times with 3 mL of cold 50 mM Tris-HCl, pH 7.5, and were counted in 2 mL of ScintiSafe 30% scintillation fluid. For [³H]naltrindole and [³H]U69,593 binding, the filters were soaked in 0.1% polyethylenimine for at least 60 min before use. IC₅₀ values were calculated by least squares fit to a logarithm-probit analysis. *K_i* values of unlabeled compounds were calculated from the equation $K_i = (IC_{50})/(1+S)$ where $S = (\text{concentration of radioligand})/(K_d \text{ of radioligand})$.²⁶ Data are the mean $\pm$ SEM from at least three experiments performed in triplicate.

5.3.2. [³⁵S]GTPγS Binding Assays—In a final volume of 0.5 mL, 12 different concentrations of each test compound were incubated with 10 μg (δ), 7.5 μg (μ) or 15 μg (κ) of CHO cell membranes that stably expressed either the human δ, μ or κ opioid receptor. The assay buffer consisted of 50 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 0.2 mM EGTA, 3 μM GDP, and 100 mM NaCl. The final concentration of [³⁵S]GTPγS was 0.080 nM. Non specific binding was measured by inclusion of 10 μM GTPγS. Binding was initiated by the addition of the membranes. After an incubation of 60 min at 30 °C, the samples were filtered through Schleicher & Schuell No. 32 glass fiber filters. The filters were washed three times with cold 50 mM Tris-HCl, pH 7.5, and were counted in 2 mL of Ecoscint scintillation fluid. Data are the mean E_{max} and EC₅₀ values ± S.E.M. from at least three separate experiments, performed in triplicate. For calculation of the E_{max} values, the basal [³⁵S]GTPγS binding was set at 0%. To determine antagonist activity of a compound at the μ opioid receptors, CHO membranes expressing the μ opioid receptor, were incubated with 12 different concentrations of the compound in the presence of 200 nM of the μ agonist DAMGO. To determine if a compound was an antagonist at δ receptors, CHO membranes expressing the δ receptor were incubated with 12 different concentrations of the test compound in the presence of 10 nM of the δ-selective agonist SNC 80. To determine if a compound was an antagonist at κ receptors, CHO membranes expressing the κ receptor were incubated with 12 different concentrations of the test compound in the presence of 100 nM of the κ-selective agonist U50,488.

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Abbreviations

In addition to the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1985**, *260*, 14-42), this paper uses the following additional symbols and abbreviations:

AcOEt	ethyl acetate
DAMGO	[D-Ala ² ,N-Me-Phe ⁴ ,Gly-ol ⁵]enkephalin
DMF	<i>N,N</i> -dimethylformamide
DMSO-<i>d</i>₆	hexadeuteriodimethyl sulfoxide
Dmt	2',6'-dimethyl-L-tyrosine
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
NMM	4-methylmorpholine
OBzl	benzyl ester
Pe	petroleum ether
SNC 80	(+)-4-[(<i>αR</i>)- <i>α</i> -((2 <i>S</i> ,5 <i>R</i>)-4-Allyl-2,5-dimethyl-1-piperaziny)-3-methoxybenzyl]- <i>N,N</i> -diethylbenzamide
TFA	trifluoroacetic acid
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
TLC	thin-layer chromatography

U50	488, <i>trans</i> -(α)-3,4-Dichloro- <i>N</i> -methyl- <i>N</i> -[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride
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
WSC	1-ethyl-3-[3'-dimethyl)aminopropyl]-carbodiimide hydrochloride

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Scheme 1.
Synthetic Methods for Compounds 4 and 5.

Table 1

K_i Values of the Inhibition of μ , δ and κ Opioid Binding to CHO Membranes.

Compound	Structure	K_i (nM) $\pm$ SEM ^d			Selectivity
		[³ H]DAMGO (μ)	[³ H]Naltrindole (δ)	[³ H]U69,593 (κ)	
Morphine ^c		0.88 $\pm$ 0.14	140 $\pm$ 18	24 $\pm$ 2.0	K_i^{μ}/K_i^{δ} ; K_i^{μ}/K_i^{κ} ; $K_i^{\delta}/K_i^{\kappa}$
Naloxone ^d		0.79 $\pm$ 0.020	76 $\pm$ 2.0	1.1 $\pm$ 0.030	159*/5.8***/27
Butorphan ^b MCL-101		0.23 $\pm$ 0.011	5.9 $\pm$ 0.55	0.079 $\pm$ 0.0030	96*/69***/1.4
1 ^b MCL-450		0.69 $\pm$ 0.040	1.5 $\pm$ 0.032	0.28 $\pm$ 0.031	26*/75***/2.9**
2 MCL-612		4500 $\pm$ 715	5.7 $\pm$ 0.80	>10 μ M	2.2*/5.4***/2.5**
3 MCL-613		2300 $\pm$ 115	2.7 $\pm$ 0.13	>10 μ M	789/>1754/>2.2
4 MCL-480		3.5 $\pm$ 0.69	1.4 $\pm$ 0.19	1.4 $\pm$ 0.11	861/>3745/>4.3
5 MCL-481		1.1 $\pm$ 0.09	1.6 $\pm$ 0.065	12 $\pm$ 0.33	2.5/1.0/2.5**
					1.5*/7.5/11

^a See experimental section. The K_d values for [³H]DAMGO, [³H]U69,593, and [³H]naltrindole were 0.56 nM, 0.34 nM, and 0.10 nM, respectively. These values were used to calculate the K_i values.

Selectivity = * K_i^{δ}/K_i^{μ} ; ** K_i^{μ}/K_i^{κ} ; *** $K_i^{\delta}/K_i^{\kappa}$;

^b Data taken from Neumeyer et al.⁶

^c Data were taken from Peng et al.^{9a}

^d Data were taken from Peng et al.^{9c}

Table 2

EC₅₀ and E_{max} Values for the Inhibition of Agonist-Stimulated [³⁵S]GTPγS Binding to the Human μ, δ, and κ Receptors.^a

Comp	[³⁵ S]GTPγS binding (Agonism)					
	μ		δ		κ	
	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)
Butorphan MCL-101 ^b	1.6±0.20	50±2.5	NT	NT	1.3±0.44	80±6.8
1 MCL-450	8.9±0.76	79±2.3	2.8±0.75	18±1.7	6.0±1.0	130±5.1
2 MCL-612	NT	NT	NT	NT	NT	NT
3 MCL-613	NT	NT	NT	NT	NT	NT
4 MCL-480	29±10	14±1.4	NA	-12±2.1	20±1.2	41±8.1
5 MCL-481	NA	0.43±0.062	NA	-4.9±0.15	NA	2.6±1.5

^aSee experimental section. Data are the mean E_{max} and EC₅₀ values (SEM from at least three separate experiments, performed in triplicate. For calculation of the E_{max} values, the basal [³⁵S]GTPγS binding was set at 0%. NT, Not Tested; NA = Not Applicable (no value could be determined).

^bData were taken from Peng et al.^{9a}

Table 3

IC₅₀ and I_{max} Values for the Inhibition of Agonist-Stimulated [³⁵S]GTPγS Binding to the Human μ, δ, and κ Receptors.^a

Comp	[³⁵ S]GTP- γ-S binding (Antagonism)						κ	
	μ			δ			IC ₅₀ (nM)	I _{max} (%)
Butorphan MCL-101	IC ₅₀ (nM)	I _{max} (%)	IC ₅₀ (nM)	IC ₅₀ (nM)	I _{max} (%)	IC ₅₀ (nM)	I _{max} (%)	I _{max} (%)
Butorphan MCL-101	20	50	NT	NT	NT	NT	NT	NT
Naloxone	23	92	NT	NT	NT	10	10	55
1 MCL-450	97±21	47±1.7	3.9±0.34	84±5.2	No Inhibition	No Inhibition	No Inhibition	No Inhibition
2 MCL-612	NT	NT	NT	NT	NT	NT	NT	NT
3 MCL-613	NT	NT	NT	NT	NT	NT	NT	NT
4 MCL-480	450±17	69±2.8	0.81±0.043	120±0.85	No Inhibition	No Inhibition	No Inhibition	No Inhibition
5 MCL-481	13±1.8	97±3.3	1.7±0.63	120±1.7	3100±120	99±0.58	99±0.58	99±0.58

^aSee experimental section. Data are the mean I_{max} and IC₅₀ values (SEM from at least three separate experiments, performed in triplicate. NT, Not Tested; A concentration of 200 nM DAMGO, which gave 96 ± 3.1% stimulation, was used to measure inhibition of [³⁵S]GTPγS binding. A concentration of 100 nM U50,488, which gave 64 ± 1.9% stimulation, was used to measure inhibition of U50,488-stimulated [³⁵S]GTPγS binding. and 10 nM SNC 80, which gave 66 ± 4.3% stimulation, was used to measure inhibition of [³⁵S]GTPγS binding, mediated by the δ opioid receptor.

^bData were taken from Peng et al.^{9a}