0026-895X/00/040669-08$3.00/0
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 58:669–676, 2000

Vol. 58, No. 4
13069/857332
Printed in U.S.A.

The Steroid 17α-Acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxy-pregna-3,5-dien-20-one (SC17599) Is a Selective μ-Opioid Agonist: Implications for the μ-Opioid Pharmacophore

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Received November 3, 1999; accepted July 27, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The steroid SC17599 (17α-acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxypregna-3,5-dien-20-one) has μ-opioid actions in vivo. The ability of SC17599 to interact with opioid receptors has been studied using radioligand and [^{35}S]guanosine-5′-*O*-(3-thio-)triphosphate (GTPγS) binding assays. SC17599 bound to μ-opioid receptors in SH-SY5Y neuroblastoma cells and to recombinant receptors expressed in rat C6 glioma cells and Chinese hamster ovary cells with good affinity and with greater than 100-fold selectivity for μ- over both δ- and κ-opioid receptors. Binding was much reduced when aspartate 147 in the wild-type μ-opioid receptor was replaced with asparagine. The affinity of SC17599 for the μ-opioid receptor was decreased in the presence of sodium ions, indicating agonist activity. SC17599 stimulated the binding of [^{35}S]GTPγS in a naloxone-reversible manner with good potency and maximal effect equivalent to that of the μ-opioid agonists fentanyl and [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin. In rat brain membranes, SC17599-mediated stimulation of [^{35}S]GTPγS binding was reversed by the antagonist naltrexone. SC17599 lacks an aromatic ring and *para*-hydroxyl substituent considered critical in the pharmacophore for μ-opioids. The structural relationship between SC17599 and more traditional opioid ligands was investigated through genetic algorithm-based modeling techniques for pharmacophore generation (GASP) and ligand-receptor docking (GOLD). The relatively planar and electron-rich A ring of the steroid compensated for the lack of aromaticity. Modeling of ligand-receptor docking showed that both morphine and SC17599 occupy the same binding pocket within the transmembrane helix bundle of the μ-opioid receptor and that the relationship between their binding modes largely mimicked the pharmacophore alignment.

Steroids are considered to be incapable of interacting with high affinity with the three opioid receptors. Steroids from the androgen, glucocorticoid, mineralocorticoid, and gestagen families are ineffective at displacing bound radioligand at concentrations up to 100 μM (LaBella et al., 1978; Schwarz and Pohl, 1994). At best, certain estrogens (for example diethylstilbestrol, 17α-estradiol, and 17α-dihydroequilenin) are capable of binding to the μ-opioid receptor with very low affinity (LaBella et al., 1978; LaBella, 1985; Schwarz and Pohl, 1994).

That steroids should exhibit such uniformly low affinity for the opioid receptors is only to be expected from the known structure-activity relationships of opioid ligands (Casy and Parfitt, 1986). Crucially, most of the steroids that have been investigated to date with regard to opioid receptor binding have lacked an amine moiety, thus explaining their low affinity. In contrast, the steroid SC17599 (17α-acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxy-pregna-3,5-dien-20-one), which contains a tertiary nitrogen (Fig. 1a), possesses marked antinociceptive potency in vivo as measured by the mouse abdominal constriction, the mouse hot-plate, and the rat tail-flick assay (Craig, 1968; Houshyar et al., 2000). Potency in all cases was less than that of morphine. In addition, SC17599 markedly depressed the respiratory rate and increased pCO_2 in rabbits, caused a reduction in gastrointestinal motility and afforded the Straub tail response in mice. The antinociceptive actions of SC17599 are reversed by the μ-selective antagonist methocinnamox (Houshyar et al., 2000).

SC17599 could be producing these in vivo effects by acting directly at μ-opioid receptors or indirectly through stimulation of the release of endogenous peptides. However, a closely related analog of SC17599 lacking a methyl substituent in the 10-position, SC22000, has been reported to bind to opioid receptors defined with [^{3}H]naloxone in mouse brain, albeit with affinity 30-fold less than morphine (LaBella et al., 1978). If SC17599 is indeed exerting its antinociceptive and

This work was supported by National Institute of Health Grants DA03910 and DA00254. We also thank the EPSRC (UK) for the studentship award to I.J.M.

ABBREVIATIONS: GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; TA, triamcinolone acetonide; CHO, Chinese hamster ovary; TMD, transmembrane domain.

other opioid actions through a direct interaction with the μ-opioid receptor, it represents a highly novel structure for a μ-opioid ligand. Although SC17599 possesses a tertiary nitrogen, it lacks both the critical aromatic feature and a phenolic hydroxyl substituent corresponding to the one typically found in opioid peptides and morphine-like opioids (Fig. 1, b and c; Morgan et al., 1976; Casy and Parfitt, 1986; Lomize et al., 1996).

In the current study, we show the selective interaction of SC17599 with the μ-opioid receptor using radioligand binding and confirm the agonist activity of the compound using [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate (GTPγS) binding assays in a variety of cell lines and in rat brain membranes. Molecular modeling techniques explain the relationship between SC17599 and more traditional opioid ligands and a possible binding mode of the steroid within the μ-opioid receptor binding pocket.

Materials and Methods

Chemicals and Drugs. [^{3}H][D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-Enkephalin (DAMGO; 54.5 Ci/mmol; 2.02 TBq/mmol) and [^{3}H]diprenorphine (45 Ci/mmol; 1.66 TBq/mmol or 58 Ci/mmol; 2.14 TBq/mmol) were from Amersham International (Aylesbury, UK or Piscataway, NJ). [^{3}H]CI 977 [5*R*-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-4-benzofuranacetamide] (21.1 Ci/mmol; 0.78 Tbq/mmol) was a kind gift from Dr. J. C. Hunter (Parke-Davis Neuroscience Research Center, Cambridge, UK). [^{35}S]GTPγS (1250 Ci/mmol; 46.25 TBq/mmol) and [^{3}H]triamcinolone (TA; 38 Ci/mmol; 1.41 TBq/mmol) were purchased from DuPont NEN (Hounslow, UK or Boston, MA). The following drugs were generous gifts from the National Institute on Drug Abuse (Rockville, MD): fentanyl HCl, naloxone HCl, and naltrexone HCl. BW 373,U86 [(±)-[1(*S**),2α,5β]-4-{[2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-hydroxyphenyl)methyl}-*N*,*N*-diethyl-benzamide hydrochloride] was from Burroughs Wellcome (Research Triangle Park, NC). Morphine sulfate was purchased from Mallinckrodt (St. Louis, MO). SC17599 free base (17α-acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxy-pregna-3,5-dien-20-one) was a kind gift from G. D. Searle and Co. (Chicago, IL). 5α-Pregnan-3α-ol-20-one, 5α-pregnan-3β-ol-20-one, 17α-estradiol, 17β-estradiol, estrone, hydrocortisone, and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). U69,593 [5α,7β,8γ-(−)-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide] was from RBI (Natick, MA). Dulbecco's modified Eagle's medium (without sodium pyruvate; with 4500 mg/l glucose), minimum essential medium (with Earle's salts), fetal calf serum, penicillin/streptomycin, Fungizone, trypsin, EDTA, and Geneticin were all from GIBCO Life Sciences (Grand Island, NY). All other chemicals were of analytical grade and were purchased from Sigma Chemical Co.

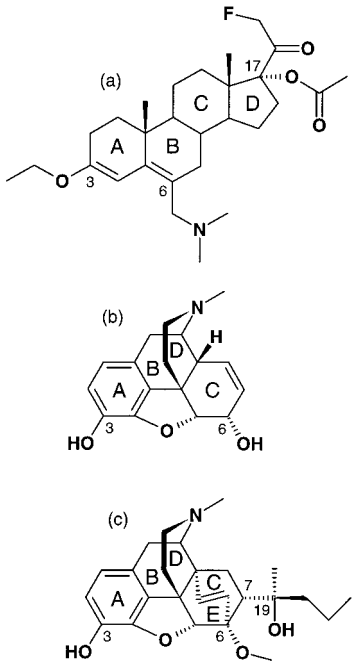

Fig. 1. Structure and atom/ring numbering of SC17599 (a), morphine (b), and etorphine (c).

Cell Membrane Preparation. Undifferentiated human neuroblastoma SH-SY5Y cells were a kind donation from Dr. D. Lambert (Department of Anesthesia, Leicester University, UK). Cells (passages 75–90) were cultured in minimum essential medium supplemented with 10% fetal calf serum, 2.5 μg/ml amphotericin B (Fungizone), 50 μg/ml penicillin/streptomycin, and 250 μg/ml L-glutamine at 37°C in a humidified 5% CO_2 atmosphere. C6 glioma cells transfected with the cloned rat μ-opioid receptor ($C_6\mu$ cells) were a kind donation from Dr. Huda Akil (Mental Health Research Institute, University of Michigan, MI). Cells (passages 15–25) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C under a 5% CO_2 atmosphere. For subculture, one flask from each passage was grown in the presence of 1 mg/ml Geneticin. Cells used for experiments were grown in the absence of Geneticin with no significant reduction in receptor number. Chinese hamster ovary (CHO) cells transfected with the mouse δ-opioid receptor were a kind gift from Dr. C. J. Evans (Department of Psychiatry, UCLA, Los Angeles, CA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 2.5 μg/ml Fungizone, 50 U/ml penicillin, 50 μg/ml streptomycin, and 258 μg/ml L-glutamine at 37°C in a humidified 5% CO_2 atmosphere. CHO cells expressing the rat μ-opioid receptor and its D147N mutant (Li et al., 1999) were established as previously described. Cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 medium supplemented with 10% fetal calf serum and 0.2 mg/ml Geneticin at 37°C in a humidified 5% CO_2 atmosphere. In all cases, cells were grown to confluence and then harvested in HEPES (20 mM, pH 7.4)-buffered saline containing 1 mM EDTA, dispersed by agitation and collected by centrifugation at 1600 rpm. The cell pellet was suspended in 50 mM Tris-HCl buffer, pH 7.4, and homogenized using a Tissue Tearor. The resultant homogenate was centrifuged for 15 min at 18,000 rpm at 4°C and the pellet collected, washed, resuspended, and recentrifuged. The pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4, at a final protein concentration of 100 to 200 μg/ml (Lowry et al., 1951). Cytosol fractions from Sf9 cells stably transfected with the glucocorticoid receptor were kindly supplied by Dr. W. Pratt (Department of Pharmacology, University of Michigan, MI).

Brain Membrane Preparation. Male Sprague-Dawley rats and Duncan-Hartley guinea pigs, (300 g; Harlan, Indianapolis, IN) were housed in standard laboratory cages (three per cage) in a temperature-controlled colony room maintained on a 12-h light/dark cycle. Food (Purina Rodent Chow; Purina Mills, St. Louis, MO) and water were available ad libitum until testing. Studies were carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Brains (minus cerebella) were suspended in ice-cold 0.32 M sucrose, 1 mM Tris-HCl, pH 7.4, disrupted using a Teflon-glass Dounce homogenizer rotating at 1000 rpm as previously described (Emmerson et al., 1996) and centrifuged at 1000*g*. This process was repeated three times and the combined supernatants centrifuged at 15,000*g* for 20 min. The resultant pellet was diluted with 50 mM Tris-HCl (pH 7.4) and centrifuged for 20 min at 20,000*g*. The final pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4, and stored in 1-ml aliquots containing 1 mg/ml protein (Lowry et al., 1951) at −80°C. All procedures were performed at 4°C.

Radioligand Binding Assays. Displacement of bound radioligand from cell membranes or guinea pig brain homogenates was performed as follows. $C_6\mu$ cell membranes (30–60 μg of protein), SH-SY5Y cell membranes (100–150 μg of protein), CHOδ cell membranes (50–80 μg of protein), or guinea pig brain homogenates (400 μg of protein) were incubated at 25°C in 50 mM Tris-HCl buffer, pH 7.4, for 1 h with radiolabeled ligand and varying concentrations of competing ligand to give a final volume of 1 ml. Radiolabeled ligand concentrations used were: [^{3}H]DAMGO, 1.0 nM; [^{3}H]diprenorphine, 0.2 nM; [^{3}H]CI 977, 0.5 nM. For CHO cells expressing the wild-type μ-opioid receptor or the D147N mutant, binding was determined as above using 35 to 55 μg of protein and either 0.2 nM [^{3}H]diprenorphine (wild-type) or 2 nM [^{3}H]diprenorphine (D147N mutant) approximating to the affinity of [^{3}H]diprenorphine for the two receptors (K_d wild-type = 0.24 nM, K_d mutant = 2.4 nM). Wild-type and mutant receptors were expressed at a similar level (approximately 1.5 pmol/mg of protein; Li et al., 1999). In all cases, total binding was determined in the absence of unlabeled ligand, and nonspecific binding was defined by naloxone (10 μM). Bound and free radioligand were separated by vacuum filtration through glass fiber filters and quantified by liquid scintillation counting.

The displacement of [^{3}H]triamcinolone in cytosol fractions from Sf9 worm ovary cells infected with a mouse glucocorticoid receptor baculovirus was performed as described recently (Kanelakis et al., 1999). Cytosol fractions (20–30 μg of protein) were incubated at 4°C in buffer B, pH 7.5 for 18 h with [^{3}H]TA (1.0 nM) and varying concentrations of ligand to give a final volume of 200 μl. Total binding was determined in the absence of unlabeled ligand; nonspecific binding was defined by dexamethasone (10 μM). Free [^{3}H]TA was separated from bound by incubation with a suspension containing charcoal (1% w/v) and dextran (0.2% w/v) for 10 min followed by centrifugation at 12,000*g* for 2 min, and quantified by liquid scintillation counting of the supernatant. Buffer B comprised HEPES (10 mM), EDTA (100 μM), sodium molybdate (20 mM), and phenylmethylsulfonyl fluoride (3 mM).

[^{35}S]GTPγS Binding Assay. Agonist stimulation of [^{35}S]GTPγS binding in cell lines containing cloned receptors was measured as previously described (Traynor and Nahorski, 1995). Briefly, $C_6\mu$ cell membranes (30–60 μg of protein) were incubated at 30°C in buffer A, pH 7.4, for 1 h with [^{35}S]GTPγS (100 pM), GDP (10 μM), and varying concentrations of ligand to give a final volume of 1 ml. Basal binding of [^{35}S]GTPγS was determined in the absence of unlabeled ligand, and maximal stimulation was defined by fentanyl (10 μM). Bound and free [^{35}S]GTPγS were separated by vacuum filtration through glass fiber filters and quantified by liquid scintillation counting. Buffer A comprised HEPES (20 mM), $MgCl_2{\cdot}6H_2O$ (10 mM), and NaCl (100 mM).

[^{35}S]GTPγS binding in rat brain membranes (21–25 μg of protein) was determined as above for 30 min at 25°C using 50 pM [^{35}S]GTPγS and 30 μM GDP. [^{35}S]GTPγS binding was stimulated with 1 μM DAMGO or 1 μM SC17599 in the presence or absence of the opioid antagonists naltrexone or naloxone (30 μM).

Molecular Modeling. All computations were carried out on either an SGI Oxygen R10,000 workstation or an SGI Octane workstation (Silicon Graphics, Mountain View, CA) using SYBYL 6.4.3 from Tripos Inc. (St. Louis, MO). In all calculations, parameters were as default except where noted.

All molecules were constructed within SYBYL in the pharmacologically relevant protonated form. Charges were added using the MOPAC (Molecular Orbital PACkage) module with the following parameters: MNDO (Modified Neglect Differential Overlap) method (Dewar and Thiel, 1977), ESP (ElectroStatic Potential) option, slope = 1.2, convergence = "precise". Structures were then minimized using the Tripos force field engine incorporating the use of charges with termination by gradient at 0.001 kcal/mol. All minimizations were allowed to run until converged (usually <1000 iterations).

The GASP (Genetic Algorithm Superposition Program) module of SYBYL was used to align from two to four energy-minimized opioid ligands and to identify common site points, hypothetical features with which those molecules may interact when bound to a receptor. More than four molecules could not be aligned simultaneously because of limitations on computation time. The number of alignments was set to four, and intramolecular distance constraints of <0.5 Å were imposed between all amine nitrogens. Those pharmacophore models with the best "overall" scores were selected, except when their "internal energy" scores were significantly (>10%) higher than the model with the next best overall score. This resulted in unfavorable distortion of the molecule, which was confirmed by a visual assessment.

The GOLD package (Genetic Optimization for Ligand Docking) is a joint collaboration between the Cambridge Crystallographic Data Center (Cambridge, UK) and Dr. Gareth Jones (Sheffield University, Sheffield, UK; Jones et al., 1995, 1997). GOLD uses a genetic algorithm to search out possible docking modes of a given ligand with a given receptor. Here, energy minimized structures for either morphine or SC17599 (with charges) were docked with a model of the μ-opioid receptor kindly provided by Dr. Henry Mosberg (University of Michigan, Ann Arbor, MI; http://www-personal.umich.edu/~him) (Pogozheva et al., 1998). "Set atoms types" was enabled for both ligand and receptor, "early termination" was disabled, the number of dockings was set to 10, and the active site was defined by a 15.0-Å radius around the α carbon (atom number 644) of the Asp-147 residue of the receptor. Those docking models with the best overall scores were selected.

Results

Radioligand Binding Studies. SC17599 displaced [^{3}H]diprenorphine from membranes of $C_6\mu$ cells with a K_i value of 62.3 ± 5.4 nM (Fig. 2a), approximately 5.5-fold lower than that shown by morphine (K_i = 11.7 ± 2.6 nM). In membranes from SH-SY5Y cells, SC17599 displaced both the selective μ-agonist [^{3}H]DAMGO and the nonselective antagonist [^{3}H]diprenorphine in a concentration-dependent manner, giving higher affinity values than using $C_6\mu$ cells (Table 1). When the binding buffer was changed from Tris buffer to buffer A, which contains 100 mM Na^+ ions, the concentration-response curve for the displacement of [^{3}H]diprenorphine from $C_6\mu$ cell membranes was shifted to the right in parallel fashion by approximately 7.7-fold (Fig. 2b). Similarly, a 7.0-fold rightward parallel shift was seen in the displacement of [^{3}H]diprenorphine in SH-SY5Y cell membranes (Table 1). SC17599 produced a concentration-dependent displacement of both [^{3}H]diprenorphine from CHOδ cell membranes and of [^{3}H]CI 977 from guinea pig brain homogenate but with very low affinity, affording K_i values of 2348 ± 509 nM and 1950 ± 928 nM at δ- and κ-receptors, respectively (Fig. 2c). This gives greater than 100-fold selectivity for the μ-opioid receptor over the δ- and κ-opioid receptors (Table 1).

The aspartic acid residue in transmembrane domain (TMD) III of the μ-opioid receptor is considered important for the binding of opiate alkaloids and opioid peptides (Surratt et al., 1994; Mansour et al., 1997). Consequently, the binding of SC17599 to wild-type opioid μ-receptors and to receptors in which this aspartate had been replaced with asparagine to give a D147N mutant (Li et al., 1999) was studied. Both receptors were expressed in CHO cells. The K_i value for morphine was markedly reduced in the mutant compared with the wild-type with a shift in the K_i value of 78-fold (Table 2). The ability of SC17599 to bind to the μ-opioid receptor expressed in CHO cells was reduced by 17-fold in the

D147N mutant compared with the wild-type μ-receptor (Table 2).

A selection of other steroids was tested for their ability to displace [^{3}H]DAMGO in SH-SY5Y cell membranes. Three estrogens (17α-estradiol, 17β-estradiol, and estrone) were chosen because this class of steroids has been reported to exhibit some affinity for the μ-opioid receptor (LaBella et al., 1978; LaBella, 1985; Schwarz and Pohl, 1994). In addition, three glucocorticoids (hydrocortisone, dexamethasone and triamcinolone) and two pregnanolones (α-pregnanalone and β-pregnanalone) were tested. Of these, only 17α-estradiol was able to bind significantly to the μ-opioid receptor, but even at the high concentration of 10 μM, only approximately 40% of the specifically bound [^{3}H]DAMGO was displaced (Table 3). In contrast, SC17599 displaced virtually all of the μ-selective radioligand at the same concentration.

In cytosolic fractions from Sf9 cells infected with a mouse glucocorticoid receptor baculovirus, SC17599 was unable to displace [^{3}H]triamcinolone at concentrations up to 100 μM. In contrast, dexamethasone showed a K_i value of 0.17 ± 0.04 nM under the same conditions (data not shown).

[^{35}S]GTPγS Binding Studies. In SH-SY5Y cell membranes SC17599 stimulated the binding of [^{35}S]GTPγS with an EC_{50} of 282.3 ± 42.4 nM and maximal response equivalent to that of the μ-agonist fentanyl (10 μM) (Fig. 3a). This effect was antagonized by naloxone (10 nM), which shifted the concentration-response curve to the right by approximately 5.6 fold, affording an apparent K_e value for naloxone of 2.2 nM (Fig. 3a). In membranes from $C_6\mu$ cells, SC17599 stimulated [^{35}S]GTPγS binding with an EC_{50} value of 110 ± 6.2 nM, compared with DAMGO (EC_{50} = 38.3 ± 3.4 nM) and morphine (EC_{50} = 23.9 ± 2.2 nM). The maximal degree of stimulation afforded by SC17599 was 95.9% (95% confidence limits, 91.9–99.9). This was not significantly different from the 95.5% (95% confidence limits, 91.9–99.9) stimulation afforded by morphine or the 104.8% (95% confidence limits, 92.8–117.0) stimulation evoked by the highly efficacious μ-peptide DAMGO (Fig. 3b).

In wild-type C_6 cells, SC17599, like the μ-agonist fentanyl, the δ-agonist BW373,U86, and the κ-agonist U69,593, was unable to significantly stimulate [^{35}S]GTPγS binding at a concentration of 10 μM (data not shown).

In rat brain membranes, both DAMGO (1 μM) and SC17599 (1 μM) stimulated the binding of [^{35}S]GTPγS. This stimulation was fully reversed by naltrexone (30 μM; Fig. 4). A similar reversal was seen with naloxone (data not shown).

Molecular Modeling. Various combinations of morphine, the potent opioid etorphine, and SC17599 (structures shown in Fig. 1) were analyzed using the GASP (Genetic Algorithm Superposition Program) module of SYBYL. Automated pharmacophore generation using morphine and SC17599 alone gave very poor steric overlap (data not shown), whereas overlap between the more complex etorphine and SC17599 was excellent (Fig. 5). The A, B, and C rings of the steroid coincide with, and are broadly coplanar with, the A, B, and E rings of etorphine. The quaternary nitrogens of each molecule are in close proximity and interact with the same hypothetical site

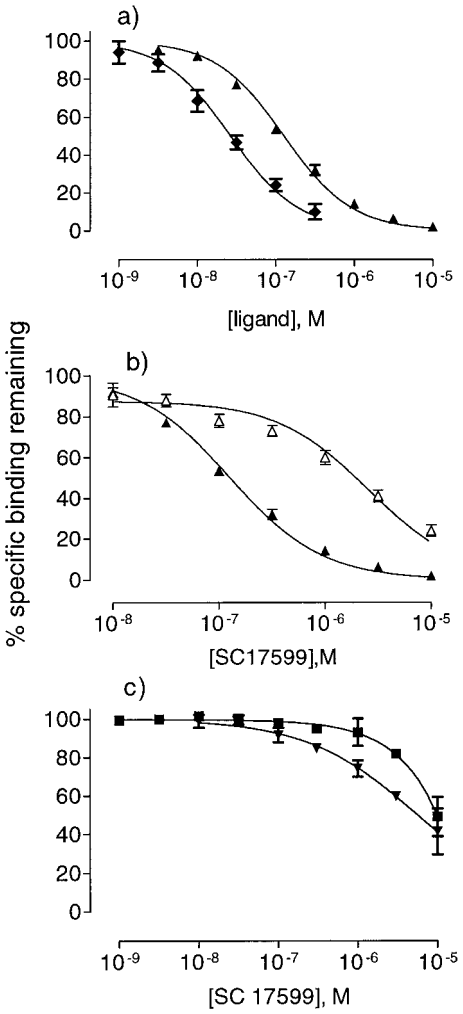

Fig. 2. Radioligand binding of SC17599. a, displacement of the binding of [^{3}H]diprenorphine (0.2 nM) from membranes of $C_6\mu$ cells by morphine (♦) and SC17599 (▲) in Tris buffer. Data shown for morphine were taken from previous work in this laboratory (Lee et al., 1999). b, displacement of the binding of [^{3}H]diprenorphine (0.2 nM) from membranes of $C_6\mu$ cells by SC17599 in the absence (▲) (Tris-HCl buffer) and presence of 100 mM Na^+ (△) (buffer A). For buffer composition, see *Materials and Methods*. c, displacement by SC17599 of either bound [^{3}H]diprenorphine from membranes of CHOδ cells (■) or bound [^{3}H]CI 977 from guinea pig brain homogenates (▲). All assays were performed as described under *Materials and Methods*. Data represents the mean ± S.E.M. from three or more separate experiments performed in duplicate.

TABLE 1

Displacement of bound radioligands by SC17599 in SH-SY5Y cell membranes

Membranes from SH-SY5Y cells were incubated with radioligand ([^{3}H]diprenorphine 0.2 nM or [^{3}H]DAMGO 1.0 nM) and varying concentrations of SC17599 in either 50 mM Tris-HCl buffer or buffer A containing 100 mM Na^+ ions (see *Materials and Methods*). Values are means ± S.E.M. from three or more separate experiments performed in duplicate. Selectivities were calculated using affinities for the δ- and κ-receptors, determined as described in Fig. 2c.

^{3}H-Ligand	Buffer	Receptor	K_i (nM)	Selectivity Over	
				δ	κ
DAMGO	Tris	μ	16.3 ± 2.6	143.7	120.0
Diprenorphine	Tris	μ	19.1 ± 3.9	122.9	102.5
Diprenorphine	Buffer A	μ	146.3 ± 20.9		

point. The steroid D ring and its substituents project beyond the volume occupied by the morphinan skeleton, into the space corresponding to the etorphine 7-substituent. Here there is another theoretical site point that interacts with the oxygen atoms present in the 19-hydroxyl substituent of etorphine and the 17α-acetoxyl group of SC17599. Overall steric overlap is excellent; the only moieties that project beyond the shared volume of the two molecules are the 19-methyl substituent of etorphine and the 17β-fluoroacetone and 3-ethoxy groups of SC17599. Pharmacophore generation using morphine, etorphine, and SC17599 together gave an overlap very similar to that produced by the analysis of etorphine and SC17599 alone (data not shown).

Analysis of the seven-TMD domain model of the μ-opioid receptor provided by Dr. Henry Mosberg (Pogozheva et al., 1998) was carried out using GOLD (Genetic Optimization for Ligand Docking). This identified two cavity regions within a 15-Å radius of the α carbon of Asp-147 in TMD III. This residue is strongly implicated in the binding of opioid ligands to the receptor through the formation of an ionic interaction with the positively charged nitrogen (Surratt et al., 1994; Mansour et al., 1997). The larger cavity extends diagonally with its lower end buried approximately 12 Å within the membrane close to TMD VI, passes between TMDs V and VII, and has its upper end at the extracellular surface of the membrane. Here it is closest to TMD III and lies directly beneath the large second extracellular loop. The second cavity is smaller, and lies between the upper end of the first cavity and TMD V.

When docked to the μ-opioid receptor morphine lies largely within the larger cavity region. The bulk of the morphine molecule is surrounded by largely hydrophobic residues, including Ile-234, Trp-293, Ile-296, Val-300, Cys-321 (ring A of morphine), Tyr-148, Met-151, Ile-322 (ring D), Lys-233, Tyr-148, Asn-230, and Trp-318 (ring C). The A and B rings of morphine lie parallel to the membrane and hence the C and D rings are parallel to the helix bundle (Fig. 6). Parts of the D ring lie just outside the binding cavity, placing the quaternary nitrogen close enough to Asp-147 to allow for interaction. The 6-hydroxyl group also lies outside the binding pocket, whereas the tyrosyl hydroxyl group lies close to His-297. This alignment generated using GOLD is very similar to that reported earlier using the same receptor model but performed using the QUANTA molecular modeling package (Molecular Simulations Inc., San Diego, CA), as reported by Pogozheva et al. (1998).

Despite its larger size, SC17599 is also able to dock to the μ-opioid receptor mostly within the larger binding cavity

TABLE 2

Affinities of morphine and SC17599 for the wild-type μ-opioid receptor and the D147N mutant of the μ-opioid receptor expressed in CHO cells

Affinities (K_i values) were determined from IC_{50} values measured as the displacement of [^{3}H]diprenorphine binding using concentrations of ^{3}H-ligand approximating to the K_d value for [^{3}H]diprenorphine at the two receptors, either 0.2 nM (wild-type) or 2.0 nM (D147N mutant, see *Materials and Methods*). Shown are mean ± S.E.M. of three experiments, each performed in duplicate.

	K_i (nM)	
	Wild-type	D147N Mutant
Morphine	12.9 ± 1.7	1002 ± 111
SC17599	107 ± 5.8	1824 ± 132

TABLE 3

Displacement of bound [^{3}H]DAMGO by various steroids in SH-SY5Y cell membranes

Membranes from SH-SY5Y cells were incubated with [^{3}H]DAMGO (1.0 nM) and 10 μM of various steroids in 50 mM Tris-HCl buffer (see *Materials and Methods*). Values are means ± S.E.M. from three or more separate experiments performed in duplicate.

Steroid	Specific Binding Remaining (%)	Steroid	Specific Binding Remaining (%)
Hydrocortisone	99.6 ± 4.3	17β-Estradiol	94.0 ± 2.2
Triamcinolone	95.1 ± 3.3	Estrone	84.4 ± 3.6
Dexamethasone	94.8 ± 3.7	17α-Estradiol	59.2 ± 3.8*
α-Pregnanolone	102.5 ± 5.5	SC17599	2.1 ± 3.7**
β-Pregnanolone	99.6 ± 2.9		

* Significantly different from control binding ($P < .05$).
** Significantly different from control binding ($P < .001$).

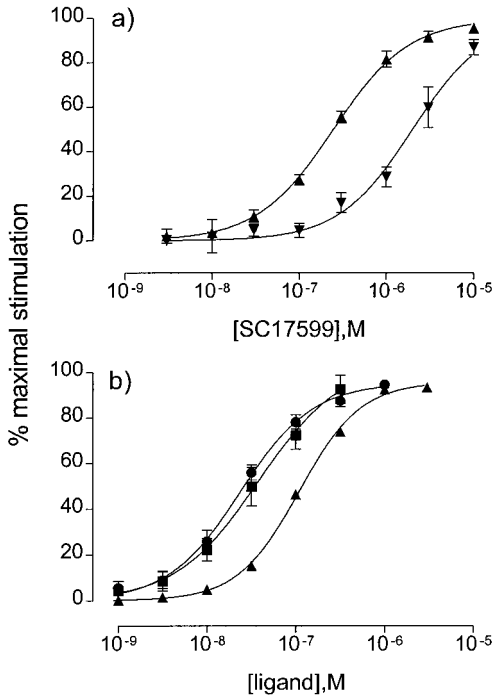

Fig. 3. a, stimulation of [^{35}S]GTPγS (100 pM) binding to membranes of SH-SY5Y cells by SC17599 in the absence (▲) and presence (▼) of naloxone (10 nM). b, stimulation of [^{35}S]GTPγS (100 pM) binding to membranes of C6μ cells by SC17599 (▲), morphine (●), and DAMGO (■). Assays were performed as described under *Materials and Methods*. Data represent the mean ± S.E.M. from three or more separate experiments each performed in duplicate.

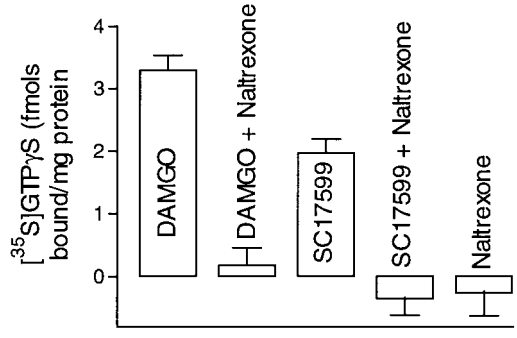

Fig. 4. Stimulation of [^{35}S]GTPγS binding in rat brain membranes by DAMGO (1 μM) and SC17599 (1 μM) and reversal by naltrexone (30 μM). Assays were performed as described under *Materials and Methods*. Values are means ± S.E.M. of three experiments each performed in triplicate.

identified by GOLD (Fig. 6). The only features that extend outside this pocket are the 6-dimethylamino substituent, which allows the quaternary nitrogen of the steroid to interact with Asp-147, and the 17α-acetoxy moiety. From the A ring located close to Asp-147, the molecule extends diagonally upward toward the extracellular end of TM III, with the 17-subsituents located at the extracellular surface of the membrane directly beneath the second extracellular loop. SC17599 contacts more residues than does morphine, including Gln-124, Cys-140, Lys-141, Ile-144, Ala-206, Thr-207, Gln-212, Ile-215, and Gln-229.

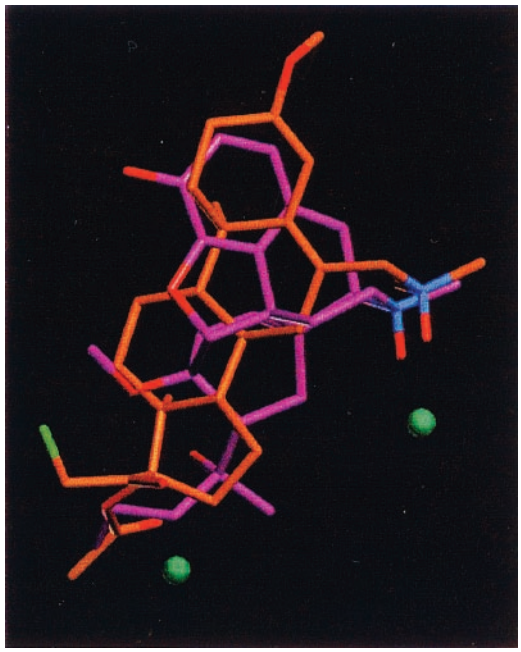

Fig. 5. Overlap of SC17599 (orange) and etorphine (magenta) generated by GASP. Nitrogen is shown in blue, and hypothetical site points in green.

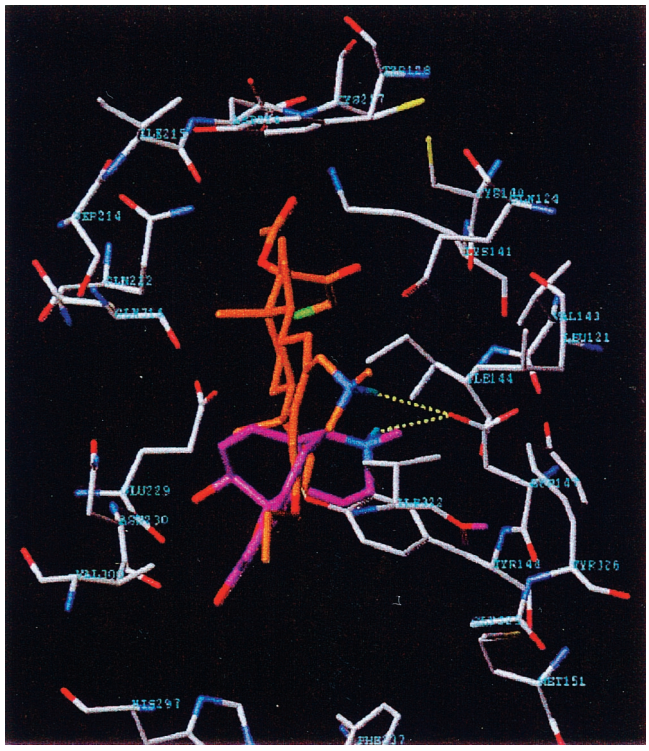

Fig. 6. Docking of morphine (magenta) and SC17599 (orange) to the μ-opioid receptor model. Only selected residues in the binding pocket are shown for clarity (white). Nitrogen is shown in blue, oxygen in red, and hydrogen in cyan. Shown in yellow is possible hydrogen bonding between the N^+ of both molecules and Asp-147 in TMD III.

Discussion

Radioligand Binding Studies. SC17599 bound with good affinity and marked preference for the μ-opioid receptor over the δ- and κ-opioid receptors. In membranes from $C_6\mu$ cells and CHO cells expressing the μ-opioid receptor, affinity remained good but was somewhat reduced. The reasons underlying this discrepancy are unclear, but may relate to the use of the oripavine [^{3}H]diprenorphine in the radioligand binding assays (Lee et al., 1999). The binding of morphine and SC17599 to the μ-opioid receptor in CHO cells was highly dependent on the presence of Asp-147 in TMD III, because mutation of this residue to Asn considerably reduced binding affinity.

Displacement of [^{3}H]diprenorphine from the μ-opioid receptor in both SH-SY5Y and $C_6\mu$ cell membranes was shifted to the right in the presence of 100 mM Na^+. This causes a shift in the equilibrium between high and low agonist affinity states of the receptor in favor of the low affinity state. The ternary complex model (De Lean et al., 1980) predicts that agonists preferentially bind to the high affinity state of the receptor, whereas antagonists exhibit no preference. The rightward shift in the concentration-effect curve for displacement of bound radioligand by SC17599 is consistent with its agonist properties (Houshyar et al., 2000).

The ability of SC17599 to bind with good affinity to the μ-opioid receptor was not shared by any of the other steroids tested. 17α-Estradiol bound with very low affinity to the μ-opioid receptor, consistent with previous data (LaBella et al., 1978; LaBella, 1985; Schwarz and Pohl, 1994), and SC17599 displayed no affinity for the glucocorticoid receptor.

[^{35}S]GTPγS Binding Studies. The [^{35}S]GTPγS binding assay provides a functional measure of agonist occupation of μ-opioid receptors, allowing for determination of potency and relative efficacy (Traynor and Nahorski, 1995). The μ-agonist properties of SC17599 were confirmed by its ability to stimulate [^{35}S]GTPγS binding in membranes from both SH-SY5Y and $C_6\mu$ cell lines. The potency of SC17599 was 3- to 4-fold lower than morphine or DAMGO but maximal stimulation was equivalent to that afforded by the full μ-agonist DAMGO.

In SH-SY5Y cell membranes in the presence of naloxone, the concentration-effect curve for SC17599 was shifted to the right in parallel fashion. The apparent pK_b (8.66) indicated that SC17599 stimulated [^{35}S]GTPγS binding via a reversible interaction with the μ-opioid receptor (Traynor and Nahorski, 1995). The inability of SC17599 to produce an agonist effect in membranes from C6 wild-type cells, plus the fact that the action of SC17599 in rat brain membranes was fully reversed by the opioid antagonist naltrexone, confirmed selectivity for the μ-opioid receptor.

Molecular Modeling. SC17599 is able to bind to μ-opioid receptors despite lacking any aromatic or hydroxyl feature, making it a highly unusual opioid agonist. An aromatic feature is considered critical in traditional opioid pharmacoph-

ores, whereas a phenolic hydroxyl group is thought to be crucial to the activity of morphine-like and peptide opioid ligands (Casy and Parfitt, 1986). On the other hand, SC17599 does have a tertiary nitrogen, several oxygen functionalities, and a relatively planar electron-rich area that may be able to substitute for the pharmacophoric elements of more traditional opioid ligands.

Comparison of morphine and SC17599 by GASP gave very poor steric overlap because of an overemphasis on hypothetical site points interacting with the oxygen features of both molecules. Because it is known that the 4,5-epoxy and 6-hydroxy groups of morphine and related opioids are not necessary for maintained high affinity (Casy and Parfitt, 1986), assigning a crucial pharmacophore role to these moieties is inappropriate. In contrast, alignment of the larger and more complex etorphine molecule and SC17599 was excellent. The A ring of the steroid situated close to and broadly coplanar with the A ring of etorphine and the quaternary nitrogens of both molecules interacted with the same hypothetical site point, in accordance with the importance of the quaternary nitrogen. The alignment of morphine, etorphine, and SC17599 was very similar to that of etorphine and SC17599 alone. This was caused by a decreased emphasis on hypothetical site points identified using morphine alone.

In addition to SC17599, there are several classes of well-characterized μ-opioid ligands that lack any *para*-hydroxyl substituent. Such compounds include the 4-phenylpiperidines (meperidine), 3-phenylpyrrolidines (profadol), 4-anilinopiperidines (fentanyl), and diphenylpropylamines (methadone), although it is possible that these ligands interact with the μ-opioid receptor in a different manner (Subramanian et al., 2000). The hydroxyl substituent is traditionally considered important in morphine-like ligands and opioid peptides, although a series of cyclic tetrapeptides have recently been reported in which some analogues retain high affinity for the μ-opioid receptor despite lacking *para*-hydroxyl substituents in the first residue (Mosberg et al., 1998). These data suggest that the *para*-hydroxyl substituent is not critical to maintained high affinity at the μ-opioid receptor.

The role of an aromatic ring may not be so critical and the relatively planar and electron-rich A ring region of SC17599 is capable of substituting for the aromatic A ring of the more traditional opioid ligands. Although the majority of μ-opioid ligands possess an aromatic ring there are a few exceptions, including SC17599, the closely related steroid SC22000 (LaBella et al., 1978), and a set of ozonolysis products of etorphine-like compounds (Bentley et al., 1969).

Thus, we present a revised pharmacophore for the binding of both peptide and alkaloid ligands to the μ-opioid receptor (Fig. 7). The only required moieties are a quaternary amine nitrogen and a relatively planar, electron-rich region, which is commonly, but not necessarily, a phenyl ring. A *para*-hydroxyl substituent contributes to high-affinity binding but is not critical.

Ligand Docking. To examine whether this pharmacophore was useful in predicting the binding mode of SC17599, the docking of this unusual ligand to the μ-opioid receptor was examined. Using a genetic algorithm for ligand docking, two cavities were identified within the μ-opioid receptor. Only the larger pocket within the TMD helix bundle is likely to be involved in ligand binding because the other lies in the extracellular loops and is very small. Morphine docks almost entirely within the identified binding pocket in an orientation that gives very close agreement to one binding mode for morphine to the same model (Pogozheva et al., 1998). In this position the quaternary nitrogen of morphine is involved in a hydrogen-bonding interaction with Asp-147 in TMD III. Despite its larger steric bulk, SC17599 is also able to bind within the same pocket in an orientation that allows interaction between the quaternary nitrogen and Asp-147. Comparison of the docked alignments of the two ligands shows that the A ring of SC17599 is shifted approximately 3.25 Å within the plane of both ligands such that it now overlaps the B ring of morphine. Despite this shift in the position of the steroid skeleton, the quaternary nitrogen is still appropriately positioned to form a hydrogen bond with Asp-147 with an H-O distance of 2.85 Å and an N-H–O angle of 162.6°. Alternatively, alteration of the configuration of the side chain of Asp-147 allows for an ionic interaction between the steroid N^+ and the Asp carboxyl, with a $N^+ \ldots O$ distance of 5.1 Å. The importance of Asp-147 in the binding of SC17599 to the μ-opioid receptor was confirmed by the large loss in affinity seen at the D147N mutant compared with the wild-type receptor. The finding that a larger loss of affinity was seen with morphine than with SC17599 probably relates to the fact that SC17599 contacts more residues than morphine and so the contribution of the interaction with Asp-147 to the overall binding affinity is reduced.

The steroid as a whole occupies almost the entire binding pocket, from the portion buried within the TMD helix, which is also occupied by the smaller morphine, to the extracellular surface. As discussed above, features of the steroid interact with areas of the binding pocket not interacting with morphine, thereby providing binding contributions that can compensate for the lack of an aromatic ring. Indeed, alignment of the potent opioid etorphine onto the docked morphine molecule shows almost complete steric overlap of the 17-substituents of etorphine with the C and D rings of the steroid.

In conclusion, the results demonstrate that the steroid SC17599 is a selective, full agonist at the μ-opioid receptor with good affinity and potency. It also possesses a highly novel structure, bringing into question traditional structure-activity findings for μ-opioid agonists. The widely held view that an aromatic ring, typically with *para*-hydroxyl moiety in morphine-like and peptide ligands, is an essential part of the

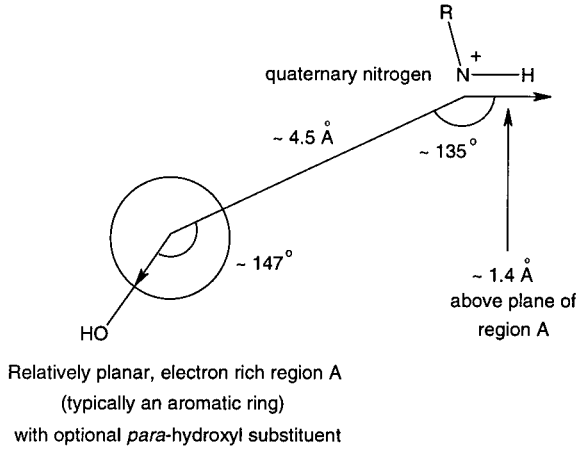

Fig. 7. Revised pharmacophore for μ-opioid ligands, based on comparisons by GASP (see *Materials and Methods*) of the structures of SC17599, morphine, and etorphine.

μ-opioid ligand pharmacophore has to be revised in light of the activity of the steroid SC17599. Any hydrophobic, electron-rich, and relatively planar feature may be able to substitute successfully for an aromatic ring and facilitate binding of the ligand to the μ-opioid receptor.

Acknowledgments

We thank Mary Clark and Hui-Fang Song for help with the binding assays and Kimon Kanelakis and Dr. W. B. Pratt for expert assistance with the glucocorticoid receptor binding assay. Dr. D. Ortwine at Warner Lambert- Parke Davis Pharmaceutical Research Division (Ann Arbor, MI) gave invaluable assistance with the modeling work.

References

Bentley KW, Hardy DG and Mayor PA (1969) Novel analgesics and molecular rearrangements in the morphine-thebaine group. Part XV. Ozonolysis of derivatives of 6,14-*endo*-ethenotetrahydrothebaine. *J Chem Soc* **18:**2385–2389.

Casy AF and Parfitt RT (1986) *Opioid Analgesics: Chemistry and Receptors.* Plenum Press, New York.

Craig CR (1968) Analgetic activity and other pharmacological properties of a steroid, 17α-acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxy-pregna-3,5-dien-20-one hydrochloride (SC 17599). *J Pharmacol Exp Ther* **164:**371–379.

De Lean A, Stadel JM and Lefkowitz RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J Biol Chem* **255:**7108–7117.

Dewar MJS and Thiel W (1977) Ground states of molecules. 38. The MNDO method. Approximations and parameters. *J Am Chem Soc* **99:**4899–4905.

Emmerson PJ, Clark MJ, Mansour M, Akil H, Woods JH and Medzihradsky F (1996) Characterization of opioid agonist efficacy in a C_6 Glioma cell line expressing the μ opioid receptor. *J Pharmacol Exp Ther* **278:**1121–1127.

Houshyar H, McFadyen IJ, Woods JH and Traynor JR (2000) Antinociceptive and other behavioral effects of the steroid SC17599 are mediated by the μ-opioid receptor. *Eur J Pharmacol* **395:**121–128.

Jones G, Willett P and Glen RC (1995) Molecular recognition of receptor sites using a genetic algorithm with a description of desolvation. *J Biol Chem* **245:**43–45.

Jones G, Willett P, Glen RC, Leach AR and Taylor R (1997) Development and validation of a genetic algorithm for flexible docking. *J Mol Biol* **267:**728–747.

Kanelakis KC, Morishima Y, Dittmar KD, Galigniana MD, Takayama S, Reed JC and Pratt WB (1999) Differential effects of the hsp70 binding protein BAG-1 on glucocorticoid receptor folding by the hsp90-based chaperone machinery. *J Biol Chem* **274:**34134–34140.

LaBella FS (1985) Opiate Receptor activity of 17α-estradiol and related steroids. *Prog Clin Biol Res* **192:**323–328.

LaBella FS, Kim RS and Templeton J (1978) Opiate receptor binding activity of 17-alpha estrogenic steroids. *Life Sci* **23:**1797–1804.

Lee KO, Akil H, Woods JH and Traynor JR (1999) Differential binding properties of oripavines at cloned mu- and delta-opioid receptors. *Eur J Pharmacol* **378:**323–330.

Li J-G, Chen C, Yin J, Rice K, Zhang Y, Matecka D, de Riel JK, DesJarlias RL and Lui-Chen L-Y (1999) Asp147 in the third transmembrane helix of the rat mu opioid receptor forms ion-pairing with morphine and naltrexone. *Life Sci* **65:**175–185.

Lomize AL, Pogozheva ID and Mosberg HI (1996) Development of a model for the δ-opioid receptor pharmacophore: 3. Comparison of the cyclic tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]OH with other conformationally constrained δ-receptor selective ligands. *Biopolymers* **38:**221–234.

Lowry OH, Rosebrough NA, Faff AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Mansour A, Taylor LP, Fine JL, Thompson RC, Hoversten MT, Mosberg HI, Watson SJ and Akil H (1997) Key residues defining the mu opioid receptor binding pocket: a site-directed mutagenesis study. *J Neurochem* **68:**344–353.

Morgan BA, Smith CF, Waterfield AA, Hughes J and Kosterlitz HW (1976) Structure-activity relationships of methionine-enkephalin. *J Pharm Pharmacol* **28:**660–661.

Mosberg HI, Ho JC and Sobczyk-Kojiro K (1998) A high affinity, mu-opioid receptor-selective enkephalin analogue lacking an N-terminal Tyrsosine. *Bioorg Med Chem Lett* **8:**2681–2684.

Pogozheva ID, Lomize AL and Mosberg HI (1998) Opioid receptor three-dimensional structures from distance geometry calculations with hydrogen bonding constraints. *Biophys J* **75:**612–634.

Schwarz S and Pohl P (1994) Steroids and Opioid Receptors. *J Steroid Biochem Mol Biol* **48:**391–402.

Subramanian G, Paterlini MG, Porthogese PS and Ferguson DM (2000) Molecular docking reveals a novel binding site model for fentanyl at the mu opioid receptor. *J Med Chem* **43:**381–391.

Surratt CK, Johnson PS, Moriwaki A, Seidlack BK, Blaschak CJ, Wang JB and Uhl GR (1994) μ-Opiate receptor: Charged transmembrane domain amino-acids are critical for agonist recognition and intrinsic activity. *J Biol Chem* **269:**20548–20553.

Traynor JR and Nahorski SR (1995) Modulation by μ-opioid agonists of guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol Pharmacol* **47:**848–854.

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