Structure–activity relationships of opioid ligands

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STRUCTURE-ACTIVITY RELATIONSHIPS OF
OPIOID LIGANDS

by

Iain James McFadyen

A Doctoral thesis

Submitted in partial fulfillment of the requirements for the award of

Doctor of Philosophy of Loughborough University

October 1999

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Title: Structure-activity relationships of opioid ligands

Iain James McFadyen

Key words: Mu opioid receptor, structure-activity relationships, pharmacophore, molecular modelling, radioligand binding, [35S]-GTPγS binding, SH-SY5Y cells, C6μ cells.

Abstract

There are three different types of opioid receptor, namely mu, delta and kappa. Morphine and related clinically useful analgesics exert their actions through the mu-opioid receptor. Such compounds represent a huge structural diversity, including both peptides and alkaloids. Nevertheless, there exists a common pharmacophore comprising two critical features, namely an amine nitrogen and an aromatic ring, usually with a hydroxyl substituent; the spatial relationship between them is also vital.

In the first part of this work the roles of the aromatic ring and hydroxyl substituent in opioid peptides were investigated. Twenty-five cyclic tetrapeptides with variations in the first amino-acid and in the size of the cyclic peptide ring were characterised for receptor affinity and specificity using radioligand binding assays, and for relative efficacy using [35S]-GTPγS binding assays. The data show that neither the hydroxyl nor the aromatic ring are critical for high affinity, potency or efficacy at the mu-opioid receptor, but this does depend upon the ring-size of the tetrapeptide.

In the second part, an aminosteroid (SC17599) which lacks both an aromatic ring and a para-hydroxyl substituent was also shown to have good affinity, selectivity and efficacy at the mu-opioid receptor. Molecular modelling of this compound has been used to investigate the relationship between SC17599 and more traditional opioid ligands in three ways. Firstly, the pharmacophore for mu-opioid ligands has been refined using GASP (Genetic Analysis of Spatial Parameters). Secondly, the docking interaction of SC17599 with the mu opioid receptor has been compared with the docking of morphine using GOLD (Genetic Optimization for Ligand Docking).
Thirdly, QSAR (Quantitative Structure-Activity Relationships) of morphine-like opioid ligands and SC17599 have been investigated using FBSS (Field Based Similarity Search) and CoMFA (Comparative Molecular Field Analysis).

The above findings have profound implications for the future design of mu opioid ligands and for the accepted theories of the binding of such ligands to the mu opioid receptor.
Acknowledgements

First and foremost I must thank my supervisor, Dr John Traynor (JRTB !). An amazing teacher at both undergraduate and graduate level, he always manages to infect others with his boundless scientific curiosity. He was kind enough to bring me across the ocean with him when he changed jobs, which opened the door to opportunities I will never be able to thank him enough for. At Lufbra my ‘substitute’ supervisor Dr Ged Salt helped me out in many ways both before and after the move to America. One day I will e-mail him without asking for a favour.

A great many people have given me technical assistance through the years; at Lufbra, Phil Szekeres helped me get started as a postgrad. At the U of M, Kimon for his help with the glucocorticoid receptor binding, and Dr. Henry Mosberg for his collaboration on the peptide project and for teaching me peptide synthesis. At Parke Davis I was fortunate enough to learn molecular modelling from the very patient and helpful Dan Ortwine, Jack Bikker and David Wild. Thanks also go to the undergrads who worked with me, first Mike and then Swati.

I am convinced that large departments can only function because of the efforts of their support staff; Grahame in Chemistry and everyone in the Postgraduate Office at Lufbra, and Denise and Dennus in Pharmacology and Jim in the International Centre at the U of M have all helped deal with my problems. Finally the Engineering and Physical Sciences Research Council for their financial aid and for allowing me to move out to the U of M.

I want to thank my family for all of the usual things; mine is a small family but full of wonderful people – the Rouths, my grandparents (both sides), my dad, and of course my mother. Thanks for always supporting me.

Finally, my friends. I am lucky enough to know many amazing people in different places around the world; at home (Dave, Rob and especially Andy), at Lufbra (Rich, Tom, Kirsten and especially Barry), and in America (Jordan, Amy, Paul, Dave, Tim, and especially Andy, Mary, and most of all Claire). I could write pages about each of them. As a terminally single guy, the next best thing is to have friends like these.

Right, I think it’s time for a few Long Island Iced Teas ... anyone fancy a quick pint ? :-)

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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>AMI</td>
<td>Austin Method 1</td>
</tr>
<tr>
<td>Boc</td>
<td>t-butyloxycarbonyl</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BW 373,U86</td>
<td>(+)-(1(S*)2α,3α,5β)-4-[[2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-hydroxyphenyl)methyl]-N,N-diethyl-benzamide hydrochloride</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>CI 977</td>
<td>5R-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]-4-benzofuranacetamide</td>
</tr>
<tr>
<td>CoMFA</td>
<td>Comparative Molecular Field Analysis</td>
</tr>
<tr>
<td>CTAP</td>
<td>D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂</td>
</tr>
<tr>
<td>CTOP</td>
<td>D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂</td>
</tr>
<tr>
<td>DADLE</td>
<td>[D-Ala², D-Leu⁵]-enkephalin</td>
</tr>
<tr>
<td>DAMGO</td>
<td>[D-Ala², NMePhe⁴, Gly⁵-ol]-enkephalin</td>
</tr>
<tr>
<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPDPE</td>
<td>[D-Pen², D-Pen⁵]-enkephalin</td>
</tr>
<tr>
<td>DPN</td>
<td>diprenorphine</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>concentration required to exhibit 50% of the observed effect</td>
</tr>
<tr>
<td>ED₅₀</td>
<td>dose required to exhibit 50% of the observed effect</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>EKC</td>
<td>ethylketocyclazocine</td>
</tr>
<tr>
<td>FBSS</td>
<td>Field Based Similarity Search</td>
</tr>
<tr>
<td>G protein</td>
<td>guanosine binding protein</td>
</tr>
<tr>
<td>GASP</td>
<td>Genetic Algorithm Superposition Program</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GOLD</td>
<td>Genetic Optimization for Ligand Docking</td>
</tr>
<tr>
<td>GPBH</td>
<td>guinea pig brain homogenate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine 5'→[γ-thio]triphosphate</td>
</tr>
<tr>
<td>Hat</td>
<td>6-hydroxy-2-aminotetralin-2-carboxylic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HOBT</td>
<td>1-hydroxybenzotriazole</td>
</tr>
<tr>
<td>Hpp</td>
<td>3-(4'-hydroxyphenyl)proline.</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>dose required to inhibit 50% of the observed effect</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>[Leu&lt;sup&gt;5&lt;/sup&gt;]enkephalin</td>
<td>leucine enkephalin (Tyr-Gly-Gly-Phe-Leu)</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>[Met&lt;sup&gt;5&lt;/sup&gt;]enkephalin</td>
<td>methionine enkephalin (Tyr-Gly-Gly-Phe-Met)</td>
</tr>
<tr>
<td>MNDO</td>
<td>Modified Neglect Differential Overlap method</td>
</tr>
<tr>
<td>MPE</td>
<td>maximum possible effect</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>nor-BNI</td>
<td>norbinaltorphimine</td>
</tr>
<tr>
<td>NTB</td>
<td>naltriben</td>
</tr>
<tr>
<td>NTI</td>
<td>naltrindole</td>
</tr>
<tr>
<td>NX</td>
<td>naloxone hydrochloride</td>
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</table>
ORL1  opioid-receptor-like receptor 1
Pen  penicillamine
QSAR  Quantitative Structure-Activity Relationships
RP-HPLC  reverse phase-high performance liquid chromatography
SAR  structure activity relationships
s.c.  subcutaneous
SC17599  17α-acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxy-pregna-3,5-dien-20-one
SNC-80  (±)-[1(S*),2α,5β]-4-[[2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]- (3-methoxyphenyl)methyl]-N,N-diethyl-benzamide hydrochloride
TA  triamcinolone acetonide
TFA  trifluoroacetic acid
TLC  thin-layer chromatography
TMD  transmembrane domain
Tris  tris[hydroxymethyl]aminomethane
U 69,593  5α,7β,8γ-(S)-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide
# Amino acid structures

Structure and coding of amino acids of the general structure: $\text{H}_2\text{N} \rightarrow \text{COOH}$

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Three letter symbol</th>
<th>Single letter symbol</th>
<th>$-\text{R}$</th>
</tr>
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<tr>
<td>Alanine</td>
<td>Ala</td>
<td>A</td>
<td>$-\text{CH}_3$</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
<td>$-(\text{CH}_2)_2\text{NHC}(=\text{NH})\text{NH}_2$</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
<td>$-\text{CH}_2\text{CONH}_2$</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
<td>$-\text{CH}_2\text{CO}_2\text{H}$</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
<td>$-\text{CH}_2\text{SH}$</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
<td>$-\text{CH}_2\text{CONH}_2$</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu</td>
<td>E</td>
<td>$-\text{CH}_2\text{CO}_2\text{H}$</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>G</td>
<td>$-\text{H}$</td>
</tr>
<tr>
<td>Histidine</td>
<td>His</td>
<td>H</td>
<td>$-\text{CH}_2(4\text{-imidazolyl})$</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Ile</td>
<td>I</td>
<td>$-\text{CH}(\text{CH}_3)\text{CH}_3\text{CH}_3$</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
<td>$-\text{CH}_2\text{CH}(\text{CH}_3)_2$</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
<td>$-\text{(CH}_2)_4\text{NH}_2$</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
<td>$-\text{(CH}_2)_2\text{SCH}_3$</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
<td>$-\text{CH}_2\text{Ph}$</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
<td>P</td>
<td>*</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser</td>
<td>S</td>
<td>$-\text{CH}_2\text{OH}$</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
<td>T</td>
<td>$-\text{CH}(\text{CH}_3)\text{OH}$</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>W</td>
<td>$-\text{CH}_2(3\text{-indolyl})$</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
<td>$-\text{CH}_2(4\text{-hydroxyphenyl})$</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
<td>$-\text{CH}(\text{CH}_3)_2$</td>
</tr>
</tbody>
</table>

* Proline is an imino acid of the structure:
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5.5 Overlap of SC17599 and etorphine generated by GASP

5.6 Overlap of SC17599, morphine, etorphine and its ozonolysis product generated by GASP

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5.9 Alignment E1 generated by FBSS

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5.14 SC17599 docked to the mu opioid receptor model

5.15 Comparison of the docked conformations of SC17599 and morphine
What more do you want to know?

The names of all of the stars, and of all living things, and the whole history of the earth and the heavens and the sundering seas. Of course! What less?

On the nature of scientific curiosity, by Peregrine Took

from The Lord of the Rings, by J.R.R. Tolkien.
Chapter 1

GENERAL INTRODUCTION
1.1 History of opioids and opioid receptors

The sensation of pain is integral to human existence and is generally defined as comprising of separate sensory and emotional components. Pain is typically the result of noxious stimuli to which our bodies respond when threatened with actual or potential tissue damage and at least in part serves as a warning. The degree of sensation can range from mildly unpleasant to physically debilitating. Some of the earliest medicines recorded in human history are analgesics, substances which act to control pain by alleviating one or both components. For example, the use of opium, the latex obtained by cutting the unripe seed capsules of the poppy *Papaver somniferum*, predates the written word.

In 1803 the German pharmacist Sertürner isolated the active constituent of opium, which he named Morphine, after Morpheus the god of dreams from the works of Ovid. (Figure 1.1). However, it took 120 years for the chemical structure of morphine to be elucidated [Gulland and Robinson, 1923], and another 30 until the first total synthesis of the molecule was achieved [Gates and Tschudi, 1952].

![Morphine structure](image)

Figure 1.1  Morphine

Morphine and related analgesics, collectively termed opiates, are unique in their ability to alleviate pain without affecting the primary sensory modalities, namely vision, touch, hearing, taste and smell. Opiates are also able to reduce the emotional components of pain, including fear, anxiety and tension. Thus preparations containing morphine rapidly became the treatment of choice in cases of moderate to severe pain.
Chapter 1: Introduction

However, morphine is by no means an ideal analgesic agent. Patients can become tolerant to its effects, such that escalating doses are required to provide the same level of pain relief. In addition, it is liable to produce dependence, whereby the user (typically a recreational user) develops a psychological and/or physical need for the drug. In addition, morphine can cause drowsiness, respiratory depression, reduced gastro-intestinal motility, nausea and vomiting [Rang and Dale, 1995]. Therefore researchers became interested in understanding the mechanisms behind the actions of morphine in order to create alternative analgesics with reduced side effects, particularly abuse potential. As the relatively new science of pharmacology advanced, experiments were carried out first on whole animals, then on isolated tissue preparations, more recently using membranes prepared from cultured cells endogenously expressing opioid receptors, and in the last few years on membranes from cells expressing recombinant opioid receptors.

1.1.1 Endogenous opioid peptides

The German pharmacologist Ehrlich proposed in the late nineteenth century that 'corpora non agent nisi fixata', or 'a substance will not act unless it is bound'. In the late 1960’s Martin suggested that specific binding sites for opiates must exist in the brain [Martin, 1967], and in the early 1970’s Goldstein and co-workers began developing an assay system which used radiolabeled compounds to locate these sites [Goldstein et al., 1971]. In 1973 several groups working independently were able to show that opiates bound to receptors in membranes from central nervous system tissues [Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973; Wong and Hong, 1973]. The only logical explanation for the existence of such receptors is that they act as binding sites for endogenous substances, sparking a race to discover the identity of these substances. In 1975 Hughes and Kosterlitz isolated two pentapeptides from porcine brain which had opiate-like activity in an in vitro bioassay which measures inhibition of the electrically stimulated contractions of smooth muscle [Hughes et al., 1975a; Hughes et al., 1975b]. The two peptides differed only in their carboxy-terminal amino acid and were named the Enkephalins (Figure 1.2).
Chapter 1: Introduction

a) Tyr-Gly-Gly-Phe-Met   b) Tyr-Gly-Gly-Phe-Leu

Figure 1.2  a) [Met$^5$]enkephalin and b) [Leu$^5$]enkephalin.

The [Met$^5$]enkephalin sequence is found at the amino-terminus of the endogenous peptide β-endorphin, which also proved to have high affinity for opiate binding sites [Bradbury et al, 1976] (Figure 1.3). Just as β-endorphin is a carboxy-terminal extension of [Met$^5$]-enkephalin, the dynorphins were discovered as opioid-active extensions of [Leu$^5$]-enkephalin [Goldstein et al, 1979; Goldstein et al, 1981] (Figure 1.3). Other families of endogenous peptides are the dermorphins [Montecucchi et al, 1981], the deltorphins [Kreil et al, 1989; Erspamer et al, 1989] and the endomorphins [Zadina et al, 1997] (Figure 1.3). All retain the Tyr$^1$ residue and a second aromatic amino acid in either the 3 or 4 position. The new term opioids was coined to differentiate these newly discovered endogenous peptides from the morphine-like opiates.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynorphin 1-17</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn</td>
</tr>
<tr>
<td>Dermorphin</td>
<td>Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH$_2$</td>
</tr>
<tr>
<td>Deltorphin 1</td>
<td>Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH$_2$</td>
</tr>
<tr>
<td>Endomorphin 1</td>
<td>Tyr-Pro-Trp-Phe-NH$_2$</td>
</tr>
</tbody>
</table>

Figure 1.3  Structures of some endogenous opioid ligands.

1.1.2 Opioid receptors and ligands

The existence of multiple types of endogenous ligands nicely complemented the proposed existence of multiple types of opioid receptor. The first experimental evidence for multiple receptor types came from in vivo studies by Martin [Martin et al, 1976]. This pioneering group looked for the ability of various compounds to
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prevent the appearance of the withdrawal symptoms which develop after an animal is no longer administered a drug on which it has become dependent. The authors identified three groups of drugs, individual members of which would substitute only for compounds within the same group. Thus they proposed the existence of three types of opioid receptor, named after the prototypical drug in each group — mu (morphine), kappa (ketocyclazocine) and sigma (SKF 10,047 or N-allylnormetazocine). However, opioid receptors are defined by their ability to bind naloxone (Figure 1.4), and since many of the effects mediated by the sigma receptor are not naloxone reversible it is no longer considered an opioid receptor [Walker et al, 1990].

![Figure 1.4 Naloxone](image)

When comparing the effects of morphine and the enkephalins in the inhibition of electrically stimulated contractions in guinea pig ileum and mouse vas deferens, Kosterlitz and colleagues found that the rank order of potency was different in the two tissues. Thus, they proposed the existence of a third opioid receptor, the delta receptor, named after the tissue in which it was discovered (mouse vas deferens) [Lord et al, 1977].

Of the endogenous opioid peptides, β-endorphin and the enkephalins bind to both the mu- and delta-receptors [Paterson et al, 1983], the dynorphin family of peptides bind preferentially to kappa receptors, the dermorphins and the endomorphins are selective for the mu receptor, whilst the delorphins are highly selective for delta receptors, as their name implies. The pioneering work of the 1970’s on multiple opioid receptor...
types was greatly hindered by the lack of ligands with both high selectivity and resistance to enzymatic degradation. In the search for better research tools and analgesic agents, a huge number of peptide and non-peptide opioid ligands were synthesised and older ligands recharacterised, many of which offer high affinity, improved selectivity and increased stability. For example, the phenylpiperidine derivative fentanyl [Janssen et al., 1963] and the synthetic peptide DAMGO ([D-Ala$^2$, N-Me-Phe$^4$, Gly-ol$^5$]enkephalin) [Handa et al., 1981] are both excellent mu ligands. Commonly used delta ligands include DADLE ([D-Ala$^2$, D-Leu$^5$]enkephalin) [Magnan et al., 1982], DPDPE ([D-Pen$^2$, D-Pen$^5$]enkephalin) [Mosberg et al., 1983] and BW 373, U86 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzyl]-N,N-diethylbenzamide) [Chang et al., 1993]. Selective kappa ligands include U69,593 (5R-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-4-benzeneacetamide) [Lahti et al., 1985] and CI 977 (5R-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-4-benzofuranacetamide) [Hunter et al., 1990] (Table 1.1, Figure 1.5). A selection of high affinity compounds displaying lesser selectivity are also widely used, for example the kappa agonists EKC (ethylketocyclazocine) [Harris and Sethy, 1980] and bremazocine [Romer et al., 1980].

Although excellent non-specific opioid antagonists such as naloxone [Takemori et al., 1972] (Figure 1.4) are available, truly selective antagonists have in general proved more elusive than their agonist counterparts. However, the mu selective CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$) [Pelton et al., 1986], the delta antagonist NTT (naltrindole) [Porthogese et al., 1988a] and the kappa antagonist nor-BNI (norbinaltorphimine) [Porthogese et al., 1987] are examples that are all widely used, and there are many others (Table 1.1, Figure 1.5).
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<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Agonists</th>
<th>Antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>mu DAM</td>
<td>DAMGO</td>
<td>CTOP</td>
</tr>
<tr>
<td>fentanyl</td>
<td>CTAP</td>
<td>cyprodime</td>
</tr>
<tr>
<td>morphiceptin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>delta DPDPE</td>
<td>ICI 174,864</td>
<td></td>
</tr>
<tr>
<td>DSLET</td>
<td>naltrindole</td>
<td></td>
</tr>
<tr>
<td>DADLE</td>
<td>naltriben</td>
<td></td>
</tr>
<tr>
<td>kappa U69,593</td>
<td>norbinaltorphimine</td>
<td></td>
</tr>
<tr>
<td>CI 977</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1 Highly selective opioid ligands.

The next logical step in opioid pharmacology was to isolate the three opioid receptors and determine their amino acid sequence. However, this proved difficult due to both the paucity of opioid receptors in most tissues and their lability in detergent [Loh and Smith, 1990] which is the first step in traditional purification schemes. Indeed, it was not until 1992 that two groups published independent descriptions of the expression cloning of cDNA encoding the delta receptor from the neuroblastoma x glioma (NG108-15) cell line [Evans et al, 1992; Kieffer et al, 1992].

In the year following this breakthrough, a multitude of reports appeared detailing the cloning of other opioid receptors: the rat mu-opioid receptor [Chen et al, 1993a; Fukuda et al, 1993; Wang et al, 1993], the rat kappa-opioid receptor [Chen et al, 1993b; Minami et al, 1993; Li et al, 1993; Meng et al, 1993], the mouse kappa-opioid receptor [Yasuda et al, 1993], and the rat delta-opioid receptor [Fukuda, Kato, Mori, Nishi, and Takeshima, 1993]. In the next two years, all three human opioid receptors were cloned: mu [Wang et al, 1994], delta [Knapp et al, 1994; Simonin et al, 1994], and kappa [Simonin et al, 1995].
Figure 1.5 Structure of some of the opioid ligands shown in Table 1.1.
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Based on the differential effects of the antagonist naloxonazine on the various physiological actions of morphine in vivo [Ling et al, 1985], it has been suggested that there may be two subtypes of the mu opioid receptor, termed $\mu_1$ and $\mu_2$ [Pasternak and Wood, 1986]. Similar divisions have been proposed for both the delta and kappa receptors, based on pharmacology (delta) and binding data (kappa). However, there is only a single occurrence of each opioid receptor on the genome [Thompson et al, 1993], and all of the receptors of each type cloned to date from different sources have virtually identical sequences and properties. Thus, the idea of subtypes within the mu, delta and kappa receptor populations remains controversial [Fowler and Fraser, 1994]. The observed discrepancies in the actions of ligands such as naloxonazine may be the result of splice variants or post-translational modifications of a single receptor gene [Rossi et al, 1995].

In 1994 a receptor type was discovered with an amino acid sequence very similar to the opioid receptors, but which did not bind naloxone and is therefore not considered part of the immediate opioid receptor family. Several research groups published details of this ORL1 (opioid-receptor-like) or Orphanin receptor almost simultaneously [Mollereau et al, 1994; Bunzow et al, 1994; Fukuda et al, 1994; Chen et al, 1994; Wang et al, 1994]. The full extent of the pharmacological and physiological effects mediated by the ORL1 receptor are not yet fully understood, but it appears to mediate certain "anti-opioid" actions, such as the inhibition of opioid induced analgesia. The endogenous ligand for the orphanin receptor was quickly isolated by two groups (Figure 1.6), and was named orphanin FQ by one [Reinscheid et al, 1995], and nociceptin by the other [Meuneir et al, 1995] due to its apparent ability to cause hyperalgesia. Nociceptin is unlike the endogenous opioid peptides since it contains Phe rather than Tyr as the N-terminal residue. Despite the similarities in sequence between both the ORL1 and opioid receptors and between their endogenous ligands, all known opioids exhibit low or negligible affinity for the ORL1 receptor. Likewise, all ORL1 ligands show low affinity at mu, delta and kappa opioid receptors. Subsequently, an endogenous antagonist with specificity for the ORL1 receptor has been discovered, named nocistatin [Okuda-Ashitaka et al, 1998].
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Figure 1.6 The endogenous ligand for the ORL1 receptor, Orphanin FQ or Nociceptin.
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1.2 Structure of the opioid receptors

The opioid receptors belong to the superfamily of seven transmembrane domain spanning G-protein coupled receptors [Uhl et al, 1994], currently numbering over 200 members. These receptors share considerable structural homology, despite the huge diversity of ligands with which they interact. This reflects their common mechanism of action, namely an ability to activate intracellular proteins (G proteins) following agonist occupation. To date, although many G protein coupled receptors have been cloned, none has been crystallised. However, much structural detail has been inferred from the known structure of bacteriorhodopsin, which was isolated from bacterial culture and its structure resolved in 1975 [Henderson and Unwin, 1975]. Each G protein coupled receptor is a single polypeptide chain consisting of approximately 350-500 amino acids which spans the cell membrane seven times via α helical segments 20-28 amino acids in length. The amino-terminus resides in the extracellular space whilst the carboxy-terminus lies within the cell. This general structure is shown schematically in Figure 1.8, using the rat mu opioid receptor as an example.

The three opioid receptors are closely related. For example, the mouse delta, mouse kappa and rat mu opioid receptors have approximately 57% sequence homology [Reisine and Bell, 1993] (Figure 1.7). The greatest similarities in sequence are seen in the three intracellular loops, transmembrane domains II, III, V and VII, and the first extracellular loop. The remaining regions exhibit decreasing sequence homology in the order: transmembrane domains VI > I > IV > extracellular loops 2 > 3 (Table 1.2). The amino- and carboxy- terminals exhibit little similarity in sequence or even in size.


<table>
<thead>
<tr>
<th>Transmembrane 1</th>
<th>Transmembrane 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAS - SLALAA ITALYSA VCAVGL LGNVLVMFG IVRT KlKTATNIY IFNLALADALATS</td>
<td>100 δ</td>
</tr>
<tr>
<td>SAHISPAPAIV ITAVSYYVFVVGL VGNSLHMVFIVRTKMTATNIY IFNLALADALVT</td>
<td>110 k</td>
</tr>
<tr>
<td>TGSPSHVTAALTIMLYSIYVCYGFLNVLHIVYVHLRTATNIY IFNLALADALATS</td>
<td>119 μ</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transmembrane 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLFPQSAKYLHETWPPGEL LCKAYLSIDYNNFTSTEPILTLMNSVDPYIAVCHPVKALDFR</td>
</tr>
<tr>
<td>TLFPSAIVALHNWPPGDEVLCKYVSIDYNNFTSTEPILTLMNSVDPYIAVCHPVKALDFR</td>
</tr>
<tr>
<td>TLFPQSVNYLHETWPPGEL LCKYVSIDYNNFTSTEPILTLMNSVDPYIAVCHPVKALDFR</td>
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<tbody>
<tr>
<td>TPAKAKLINCTWVLASGVVP1WVMAVTQFPGDA - VVMLOFSSP - SYWDTVTKIC</td>
</tr>
<tr>
<td>TPLAKAKLINCTWVLASGVISAIVLGKRTREDVVECSLQFDEYSH WDLFHIC</td>
</tr>
<tr>
<td>TPATKINCVNWLSSAIQLGELVHFMATKXQGS - IDCTLSTFSP - TAYWENLHIC</td>
</tr>
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<table>
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<tr>
<th>Transmembrane 5</th>
<th>Transmembrane 6</th>
</tr>
</thead>
<tbody>
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<td>VFLFAFVFVIPITTVCGYLMRLRSLRVLSSGSESKEKDRSLRIRTRMLVLYVCGAFVVCFWAE</td>
<td>276 δ</td>
</tr>
<tr>
<td>VFLFAFVFVIPITTVCGYLMRLRSLRVLSSGSESKEKDRSLRIRTRMLVLYVCFWAE</td>
<td>289 k</td>
</tr>
<tr>
<td>VFLFAFVFVIPITTVCGYLMRLRSLRVLSSGSESKEKDRSLRIRTRMLVLYVCFWAE</td>
<td>295 μ</td>
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</table>

<table>
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<th>Transmembrane 7</th>
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</thead>
<tbody>
<tr>
<td>LHIYVTVVTLDINRDPFVAAALHCIALGYANSSLNPVLYAFIDENFKRCFQULCRTP</td>
</tr>
<tr>
<td>LHIYVTVVTLDINRDPFVAAALHCIALGYANSSLNPVLYAFIDENFKRCFQULCRTP</td>
</tr>
<tr>
<td>LHIYVTVVTLDINRDPFVAAALHCIALGYANSSLNPVLYAFIDENFKRCFQULCRTP</td>
</tr>
</tbody>
</table>

| CGRQEGPSLRPRQATTRVATCPSD - - - - - - GPPGGAAA | 372 δ |
| KNMERQSTNVRN TVQDPA - - - - - - MDVQGMKNKPV | 380 k |
| STIBQONOOSTRONT REHTSANTVDRTNQOLENLEASTAPLP | 398 μ |

Figure 1.7. Sequence alignment of the mouse delta [Evans et al., 1992], mouse kappa [Yasuda et al., 1993] and rat mu [Chen et al., 1993a] opioid receptors. Underlined regions indicate amino acids conserved between all three receptors, adapted from [Reisine and Bell, 1993].
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<table>
<thead>
<tr>
<th>Loop</th>
<th>Sequence homology (%)</th>
<th>Transmembrane domain</th>
<th>Sequence homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular 1</td>
<td>90</td>
<td>I</td>
<td>45</td>
</tr>
<tr>
<td>Intracellular 2</td>
<td>91</td>
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<td>84</td>
</tr>
<tr>
<td>Intracellular 3</td>
<td>78</td>
<td>III</td>
<td>82</td>
</tr>
<tr>
<td>Extracellular 1</td>
<td>67</td>
<td>V</td>
<td>71</td>
</tr>
<tr>
<td>Extracellular 2</td>
<td>24</td>
<td>VI</td>
<td>50</td>
</tr>
<tr>
<td>Extracellular 3</td>
<td>7</td>
<td>VII</td>
<td>71</td>
</tr>
</tbody>
</table>

Table 1.2 Sequence homology amongst the three opioid receptors, by region.

The molecular weight of the opioid receptors as predicted from their amino acid sequences is much lower than that observed using physiochemical techniques such as electrophoresis. The difference in molecular weight comes from the addition of small chemical groups such as palmitoyl, myristoyl or glycosyl during post-translational modification. The three opioid receptors have a variable number of consensus sites for N-linked glycosylation on the extracellular amino-terminus; the mu receptor contains five such sites (asparagine residues 9, 12, 33, 40, 48 in the human mu opioid receptor), whilst the delta and kappa receptors each contain two (Figure 1.8). Whilst there may be tissue-specific differences in the glycosylation patterns of these sites, they do not seem to be implicated in ligand binding or receptor activation [Rands et al, 1990]. The carboxy-terminus of all three opioid receptors contain a highly conserved cysteine residue, as indeed do many other G protein coupled receptors. Palmitoylation of this residue, for example Cys353 in the human mu receptor [O'Dowd et al, 1988], constrains the structure of the receptor by anchoring a part of the carboxy-terminus to the intracellular face of the plasma membrane, in effect forming a fourth intracellular loop (Figure 1.8).
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G protein coupled receptors also contain a number of other conserved cysteine residues which may play an important role in constraining the structure of the receptor through the formation of intramolecular disulphide bridges. One such bridge links cysteine residues in the first and second extracellular loops, Cys140 and Cys217 in the mu receptor, Cys121 and Cys198 in the delta receptor, and Cys131 and Cys210 in the kappa receptor. In β-adrenergic receptors, breaking this bridge by mutagenesis of either cysteine residue dramatically disrupts binding [Dixon et al, 1987]. In addition, opioid receptors lose the ability to bind ligands after treatment with compounds that destroy disulphide bonds [Smith and Simon, 1980]. Interestingly, the receptor is protected against this effect in the presence of pre-bound ligand, implying that this disulphide bridge is close to the ligand binding region.

All G protein coupled receptors possess an invariant aspartate residue in the third transmembrane domain. This residue, for example Asp147 in the human mu opioid receptor, is postulated to act as a counter-ion for the positively charged amino-group present in the majority of ligands for G protein coupled receptors [Dohlman et al, 1991]. Mutation of this residue with neutral alanine or asparagine residues diminishes both agonist and antagonist binding [Surratt et al, 1994a]. The second transmembrane domain also contains a highly conserved aspartate residue (Asp116 in the human mu opioid receptor) which is vital to binding of agonists but not antagonists in both mu and delta opioid receptors [Kong et al, 1993; Surratt et al, 1994b]. This residue forms part of a binding site which can accommodate either a water molecule or a Na\(^+\) ion [Kong et al, 1993]. The presence of Na\(^+\) is predicted to disrupt a network of hydrogen bonds in this region which are vital to activation of the receptor, and hence Na\(^+\) decreases agonist but not antagonist binding. Mutation of Asp116 mimics the presence of Na\(^+\) by disrupting the same hydrogen bonding network [Pogozheva et al, 1998]. A histidine in transmembrane domain VI (His299 in the human mu opioid receptor) is implicated in ligand binding through interaction with the phenolic hydroxyl group moiety present in virtually all opioid peptides and many opiates. This residue can be protected from histidine-specific alkylating agents by the presence of opioid ligands [Spivak et al, 1997].
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A variety of other residues in the transmembrane domains have also been implicated in ligand binding; for example, Tyr148 [Befort et al, 1996] and Trp293 [Pogozheva et al, 1998], as well as Gln and Tyr in TM II, Cys, Lys, Val, Asp, Tyr and Met in TM III, Lys, Ile, and Phe in TM V, Trp, Ile, and His in TM VI and Cys, Ile, and Tyr in TM VII.

The extracellular loops of the opioid receptors have been shown using molecular modelling techniques to lie across the top of the ligand binding cavity [Pogozheva et al, 1998]. Thus, they have been implicated as at least partial determinants of ligand selectivity between the three opioid receptor subtypes [Fukuda et al, 1995; Wang et al, 1995].

The second intracellular loop of most G protein coupled receptors contains an extremely well conserved Asp-Arg-Tyr sequence. Point mutations in this region generally cause a drastic reduction in the ability of receptors to stimulate downstream effectors [Fraser et al, 1988; Zhu et al, 1994], indicating that this triplet of amino acid residues is of general importance in the coupling of receptors to G proteins. A putative α-helical region near the C-terminus of the third intracellular loop is also implicated, since mutations which disrupt the tertiary structure of this region greatly diminish G protein coupling [Duerson et al, 1993]. The amino acid sequence of this region is such that one ‘face’ of the helix is primarily charged whilst the other is hydrophobic. Point mutations which disrupt this arrangement without altering tertiary structure also diminish G protein coupling [Blüml et al, 1994].

All G protein coupled receptors contain sites which may be targets for phosphorylation by protein kinase enzymes. These may be important in the desensitisation of the receptor after occupancy by agonist, resulting in reduced response. In the mu opioid receptor such sites include Ser263 and Thr281 in the third intracellular loop and Ser365 located in the carboxy-terminus [Arden et al, 1995].
Figure 1.8 Cartoon depicting the proposed seven membrane spanning domains of the rat mu opioid receptor. The single letter amino acid code has been used. Shaded amino acid residues are conserved in the majority of G protein coupled receptors (black) or between all three opioid receptors (light grey). Amino acid sequence and proposed topology was obtained from the Center for Opioid Research and Design, Department of Medicinal Chemistry, University of Minnesota.
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1.3 Receptor-effector coupling via G proteins

Activation of an opioid receptor by agonist leads to a cellular response via an extremely intricate signal transduction cascade. The initial steps of this cascade involve interaction with, and activation of, a G protein. These are heterotrimeric proteins consisting of an \( \alpha \) subunit and a tightly associated \( \beta \gamma \) dimer which separate only under denaturing conditions (Figure 1.9). They are called G proteins because the \( \alpha \) subunit contains a site which binds a guanine nucleotide. There are at least 20 different known \( \text{G}\alpha \), 5 \( \text{G}\beta \) and 12 \( \text{G}\gamma \) subunits. Thus the number of possible distinct G protein trimer combinations is very large, and more than 30 have been reported. These are grouped into four major families according to the amino acid sequence of their \( \alpha \) subunits, namely \( \text{G}\alpha_5 \), \( \text{G}\alpha_1 \), \( \text{G}\alpha_2 \), and \( \text{G}\alpha_12 \) [Hepler and Gilman, 1992]. The G protein is anchored to the intracellular surface of the plasma membrane via an isoprenoid group attached to the \( \gamma \)-subunit. The \( \alpha \)-subunits of most G proteins are modified at the amino-terminus glycine residue by either a myristate (\( \text{G}\alpha_1 \) and \( \text{G}\alpha_2 \)) or palmitoyl (\( \text{G}\alpha_5 \) and \( \text{G}\alpha_12 \)) group [Yamane and Fung, 1993]. The G protein is thought to interact with the receptor through two regions of the \( \alpha \) subunit which are in close proximity (Figure 1.9) [Higashijima and Ross, 1991]; an \( \alpha \)-helical area at the carboxy-terminus [Hamm et al, 1988; Weingarten et al, 1990] and another at the amino-terminus [Taylor et al, 1994].

These G proteins, once activated, can couple in turn to a large variety of downstream effectors which typically regulate membrane conductance (\( \text{K}^+ \) and \( \text{Ca}^{2+} \) ion channels) or levels of second messenger molecules (adenylcyclase, guanylyl cyclase, phospholipase C and phospholipase A\(_2\)) [Hille, 1992]. Until recently it was thought that only the \( \alpha \) subunit coupled with downstream effector [Birnbaumer et al, 1990], whilst the \( \beta \gamma \) dimer acted in a regulatory role. However, it has become apparent that the \( \beta \gamma \) dimer can also activate a variety of effector systems [Tang and Gilman, 1991; Taussig et al, 1993].
Figure 1.9  Ribbon model of a heterotrimeric G protein. Gα is green; Gβ is yellow; and Gγ is red.

a), view down the axis of Gβγ. b), view rotated 70 degrees around the horizontal axis compared to (a).

Adapted from [Lambright et al, 1996].
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Opioid receptors have been shown to couple to and inhibit adenylyl cyclase via $G_i/G_o$ proteins [Carter and Medzirhadsky, 1993], leading to decreased levels of the second messenger molecule cAMP (cyclic adenosine monophosphate). Indeed, inhibition of cAMP accumulation is often used as a functional measure of mu opioid agonist action. In addition, opioid receptors inhibit the activity of voltage gated Ca$^{2+}$ channels [Seward et al, 1991] and activate K$^+$ channels [Williams et al, 1988]. How does one particular receptor couple to a small subset of G proteins which in turn activates perhaps only a single effector? Opioid receptors have been shown to activate multiple members of the $G_i/G_o$ families [Prather et al, 1994]. However the interaction between G protein and effector may be much more specific, possibly even to the extent that specific G protein triplets couple different receptors to the same effector. For example, it has been shown that in GH3 cells inhibition of calcium channels by muscarinic m4 receptors is mediated through $\alpha_2 \beta_2 \gamma_4$ whilst somatostatin receptors are coupled via $\alpha_2 \beta_1 \gamma_3$ [Kleuss et al, 1993]. Additionally, G-protein activating proteins and/or targeting or compartmentation of signalling components may play a role in determining specificity of signal transduction [Neubig, 1998].

The exact mechanism by which G proteins mediate the signal from activated receptor to effector is largely unknown, but depends upon the kinetics of guanine nucleotide exchange and hydrolysis, as illustrated in schematic form in Figure 1.10. In the basal state, G proteins exist in the trimeric form, and the $\alpha$ subunit guanine nucleotide binding site contains a molecule of guanosine diphosphate (GDP). The binding of agonist results in an increase in the affinity of the receptor for the $\beta_2 \gamma_4$ complex. Interaction with the receptor causes the $\alpha$ subunit to exhibit reduced affinity for all guanine nucleotides. This is a consequence of an ‘opening’ of the nucleotide binding site [Bourne, 1993], and prompts the release of the bound GDP molecule. A receptor which is complexed to a G protein in which the nucleotide binding site is empty has increased affinity for its ligand. In the absence of guanosine triphosphate (GTP) this ‘high affinity’ state of the receptor forms part of an agonist-receptor-G protein complex which is relatively stable. However, in the presence of relatively high endogenous intracellular concentrations of GTP the nucleotide
Figure 1.10 The G protein cycle, in cartoon form. Adapted from Rang and Dale [1995].
binding site is rapidly filled. Binding of GTP leads to a conformational change in three ‘switch’ regions of G\(\alpha\) that are the primary regions for contact with G\(\beta\gamma\) [Lambright et al, 1996].

This activation of the G protein results in dissociation of the agonist-receptor-G protein complex. The \(\alpha\) and \(\beta\gamma\) subunits show greatly reduced affinity both for each other and for the receptor, and are freed to act separately on downstream targets. This dissociation also causes the receptor to return to the basal state of low affinity for agonist, but before the ligand is released the receptor may interact with another G protein. Therefore a single activated receptor may in turn activate many G proteins, resulting in signal amplification. The dissociated G protein subunits continuously activate their target effector systems until the intrinsic GTPase activity of the \(\alpha\) subunit hydrolyses bound GTP to give bound GDP and a free molecule of phosphate. This prompts the reassociation of the \(\alpha\) and \(\beta\gamma\) subunits to form the basal state \(\beta\gamma\cdot\alpha\cdot\text{GDP}\) complex. Thus the degree of signal amplification at the effector level is regulated by the rate of GTP hydrolysis, which is intrinsically relatively slow. In order to prevent excessive levels of effector activation, GTPase activating proteins or GAPs act to increase the rate of GTP hydrolysis. GAPs include effector proteins themselves, for example phospholipase C\(\beta\) [Biddlecome et al, 1996] and the large family of ‘regulators of G protein signalling’ or RGS proteins. At least 19 genes coding for RGS-like proteins have been found, defined by a 120-amino acid core domain. Some of the characterised RGS proteins are capable of increasing the rate of GTP hydrolysis by G\(\alpha\), including G\(\alpha\), which couple to opioid receptors, by up to 100-fold [Berman and Gilman, 1998]. They may act by stabilising the transition state of G\(\alpha\) [Dohlman and Thorner, 1997].

A variety of biochemical tools are used to probe the individual steps of the G protein cycle at a molecular level. Two of the most commonly used are bacterial toxins which target the \(\alpha\) subunits of specific G proteins. Cholera toxin, isolated from \textit{Vibrio cholera}, catalyses the transfer of an ADP-ribose unit from nicotinamide adenine dinucleotide (NADH) to an arginine residue on G\(\alpha\) and G\(\alpha\). Those \(\alpha\) subunits which have been ADP-ribosylated exhibit greatly
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reduced GTPase activity, thus resulting in constitutive activation of the G protein [Ribeiro-Neto et al, 1985]. Similarly, pertussis toxin from Bordatella pertussis ADP-ribosylates those α subunits which posses a cysteine residue in a specific position close to the carboxy terminus, namely Gtα, Gtα and Gtα. This uncouples the G protein from the receptor, preventing any functional interaction [Katada et al, 1986]. The G proteins which regulate receptor-mediated activation of phospholipase C, namely Gq and G12, are insensitive to both cholera and pertussis toxin.

The G protein cycle is the basis of the [35S]-GTPγS assay [Hilf et al, 1987; Traynor and Nahorski, 1995], which provides a functional measure of receptor activation [Lazareno and Birdsall, 1993]. GTPγS is an analog of GTP which is much less susceptible to hydrolysis by the α subunit. By using small amounts of radiolabelled [35S]-GTPγS (Figure 1.11) in the absence of GTP, the accumulation of α subunits with bound [35S]-GTPγS can be measured. This accumulation will be greatly increased by agonist through activation of G protein.

![Structure of [35S]-GTPγS](image)

Figure 1.11 Structure of [35S]-GTPγS
1.4 Theoretical considerations

There are two major principles involved in ligand-receptor interactions, namely affinity and efficacy.

Affinity is defined as the strength of attraction between ligand and receptor, or more specifically as the reciprocal of the binding association constant:

\[ K_d = \frac{1}{K_a} = \frac{k_1 \times [AR]}{k_2 \times [A] \times [R]} \]

where \( K_d \) is the dissociation constant for the ligand-receptor pair, \( K_a \) is the association constant, \( k_1 \) is the rate constant for the association reaction, \( k_2 \) is the rate constant for the dissociation reaction, \([A]\) is the concentration of ligand, \([R]\) is the concentration of receptor, and \([AR]\) is the concentration of ligand-receptor complex.

Efficacy is the ability of a ligand-receptor complex to elicit a response. For example, full agonists produce maximal response and thus have high efficacy, a pure antagonist has zero efficacy, and a partial agonist has intermediate efficacy. Efficacy is independent of affinity, since compounds with high affinity can exhibit low or zero efficacy, and vice versa.

One of the earliest theories of drug-receptor interactions, Occupancy Theory, proposes that the magnitude of a biological response is linearly proportional to the fraction of receptors occupied by agonist. Thus any agonist that is present in a high enough concentration to occupy all available receptors will produce a maximal response. However, there are many drugs which do not cause a full tissue response even at concentrations much greater than that required for full receptor occupancy. Ariens [1954] proposed that these "partial agonists" could be accounted for in terms of occupancy theory by use of a fractional value which he called 'intrinsic activity'. Thus a partial agonist with an intrinsic activity of 0.25 would only produce a quarter of the response seen with a full agonist, when both occupied all receptors.
Later work recognised that the relationship between receptor occupancy and tissue response was non-linear. Stephenson [1956] introduced efficacy and the transducer function in an attempt to separate those factors involved in receptor activation which are drug dependent from those which are tissue dependent. Implicit in this model is the concept of receptor reserve, where a high efficacy agonist occupying less than 100% of the available receptors nevertheless produces a maximal response. Furchgott [1966] showed that efficacy as defined by Stephenson is still a tissue dependent term, since a compound that acts as a full receptor in one tissue system may appear to be a partial agonist in another due to differences in receptor density [Kenakin, 1993]. He defined the related term ‘intrinsic efficacy’ which is a strictly drug-related property. Thus for any given agonist-receptor interaction, intrinsic efficacy should be constant across all tissues and species [Kenakin, 1983].

The simplest model of receptor activation holds that a receptor can exist in either inactive (R) or active form (R*), Figure 1.12. An agonist has higher affinity for R* and is therefore able to displace the equilibrium in that direction, whereas an antagonist has equal affinity for both states of the receptor. This is the two-state model, originally proposed for the interaction of oxygen and haemoglobin [Monod, 1965]:

\[
\begin{align*}
\text{L} + \text{R} & \rightleftharpoons \text{LR} \\
\text{L} + \text{R}^* & \rightleftharpoons \text{LR}^*
\end{align*}
\]

Figure 1.12  The two state model. L, ligand; R, inactive receptor; R*, active receptor; G, G protein.
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However, this model makes no allowance for the involvement of G proteins. Thus De Lean et al [De Lean et al, 1980] proposed a modified version of the two state model called the ternary complex model, Figure 1.13.

\[
\begin{align*}
L + R + G & \rightleftharpoons LR + G \\
L + R^*G & \rightleftharpoons LR^*G
\end{align*}
\]

Figure 1.13 The ternary complex model. L, ligand; R, inactive receptor; R*, active receptor; G, G protein.

The discovery that certain receptors which spontaneously activate in the absence of agonist, or 'constitutively active' receptors, exhibit affinities for agonists that increase not linearly, but in proportion with their efficacies [Samana et al, 1993], caused Lefkowitz et al [Lefkowitz et al, 1993] to propose the 'allosteric ternary complex model', Figure 1.14. This model allows for several important concepts:

1) receptors exist in equilibrium between inactive (R) and active (R*) states, only the latter of which is capable of binding to G protein;
2) efficacy is a measure both of the ability of a ligand to convert R to R* and to facilitate binding of R* to G protein;
3) constitutive activity depends on both the ability of R to spontaneously convert into R* in the absence of ligand and on the affinity of R* for G protein.

Recently it has become obvious that the same receptor can couple to multiple biochemical response pathways, with the same agonists showing different rank orders of potency at each [Spengler et al, 1993; Eason et al, 1994]. It has been suggested that there may be more than one active confirmation of each receptor, each promoted by different agonists and coupling to a different effector system [Leff et al, 1997].
Opioid ligands include agonists, antagonists, and inverse agonists. The delta receptor has been shown to exhibit constitutive activity [Neilan and Traynor, 1999], although this has yet to be shown for the mu and kappa receptors. Therefore the theoretical models of ligand actions discussed here are useful in the understanding of opioid pharmacology.
1.5 Opioid ligands and structure-activity relationships

Since the discovery that morphine exhibits many undesirable side effects, thousands of analogs have been synthesised in an attempt to discover a potent analgesic without these drawbacks. To date, the huge amount of work dedicated to this search has led to many advances in opioid pharmacology and pharmacology in general, but no ‘perfect’ analgesic.

The concept of ligand as ‘key’ and receptor as ‘lock’ is an old but still valuable one. It is currently impossible to directly observe the binding of opioid ligand to receptor, so designing ‘keys’ which fit the lock is difficult, to say the least. One approach is to synthesise many variations of a promising ligand, hoping to discover an even better one by chance. However, when searching for the perfect key to a particular lock it makes little sense to manufacture key after key based on an imperfect original. Instead, a more rational approach is to analyse the structures of all the available keys, including those which fit the lock well, poorly and not at all. It should then be possible to create a key which includes all the features conducive to fitting the lock, whilst simultaneously excluding the deleterious ones. This is the study of structure-activity relationships (SAR), although in practice the results are rarely ideal.

The number of discrete structural classes which are capable of interacting with the mu opioid receptor is perhaps greater than for any other receptor. The sheer number of opioids and opiates and their structural diversity makes it extremely difficult to derive a comprehensive set of structure-activity relationships which are predictive of binding affinity. An even harder task is the structural separation of agonist from antagonist. When compared to the broad diversity of opioids, the differences between an agonist and an antagonist of the same class, for example naloxone and morphine, are small.

The vast array of mu opioid ligands can be divided into the following five structural classes (Figure 1.15):

1) the rigid polycyclic opioids, which include 4,5-epoxymorphinans, morphinans, and benzomorphans;
2) 4-arylpiperidines, 3-phenylpyrrolidines and 4-anilinopiperidines;
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3) 3,3-diphenylpropylamines;
4) miscellaneous compounds, including benzimidazoles;
5) endogenous and synthetic opioid peptides;

![Opioid structural classes](image)

However, although these divisions are useful they are very simplistic, since within each of these groups there is often a huge degree of structural variation. For example, the endogenous and synthetic opioid peptides can vary in size from 4 amino acids to more than 30, with either a linear or a cyclic tertiary structure, and include a
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staggering number of natural and synthetic amino acids of L- and sometimes D-
stereochemistry.

Despite these difficulties in SAR studies it is commonly acknowledged that there are
three key features which define the vast majority of mu opioid ligands [Casy and
Parfitt, 1986]. All references to atom or ring numbers are taken from the standard
morphinan/epoxymorphinan scheme shown in Figure 1.16.

![Figure 1.16](image)

**Figure 1.16** Numbering of atoms and rings in typical epoxymorphinan structure.

1) A nitrogen atom. This is secondary or tertiary in alkaloid opioids, but can be
primary in peptides. Opioids are largely protonated at physiological pH, and the
positively charged nitrogen is thought to be involved in an ionic interaction with a
complementary anionic centre in the receptor. The extremely well-conserved aspartate
in the third intracellular loop, for example Asp147 in the human mu opioid receptor,
has been implicated as the anion. In addition, the orientation of the nitrogen relative to
the rest of the molecule may be important [Belleau et al, 1974], since this determines
the position of both the nitrogen substituent and the lone pair. In the majority of
opioids the lone pair is anti to the aromatic ring.

There appears to be a causal relationship of sorts between the nature of the substituent
on the nitrogen atom and the agonist or antagonist properties of the ligand. In the
polycyclic opioids a small 3 to 5 carbon substituent such as propyl, allyl,
cyclopropylmethyl or cyclobutylmethyl is traditionally associated with antagonism. It
has been proposed that such groups interfere with activation of the receptor by steric hindrance of the critical Asp147 residue [Pogozheva et al, 1998]. An N-arylalkyl group is necessary for activity in 4-anilinopiperidines such as fentanyl, the majority of which are agonists. This moiety can fit into the ligand binding domain in such a way as to mimic the position of the second aromatic residue of many opioid peptides [Pogozheva et al, 1998]. However, it may be the case that fentanyl and related 4-anilinopiperidines occupy the binding domain of the receptor in a manner unrelated to more traditional, rigid polycyclic opioids [Ferguson et al, 1999] since in the fentanyl series, N-cyclopropylmethyl, N-cyclobutylmethyl and N-allyl substituents do not confer antagonist properties [Casy et al, 1969]. Rather, substitution of the piperidine ring with methyl groups in the 3 and 4 positions results in antagonists [Zimmerman et al, 1993], suggesting that this class of compounds binds to the opioid receptor in a novel manner.

2) An aromatic ring. This is usually phenyl, for example the ‘A’ ring in the rigid polycyclic opioids and the Tyr¹ moiety of opioid peptides. It has been a long-held hypothesis that this aromatic ring forms van der Waals interactions with a complementary feature in the ligand binding domain of the receptor. A molecular modelling study has demonstrated that when a selection of opioid ligands were ‘docked’ in the receptor, their aromatic rings all occupied similar positions which promoted interaction with a tryptophan residue in the sixth transmembrane domain (Trp293 in the human opioid receptor) [Pogozheva et al, 1998]. Many highly potent alkaloid and peptide ligands contain two aromatic rings, suggesting that there may be a second such site for van der Waals bonding. In vivo, the increased lipophilicity conferred upon a molecule by the presence of aromatic features also aids penetration of the blood-brain barrier.

The presence of substituents on the aromatic ring generally causes a decrease in affinity for the mu opioid receptor, with one important exception. In the polycyclic opioids a 3-OH substituent is generally necessary for high affinity, and the equivalent tyrosyl-OH moiety is considered vital in opioid peptides. In both cases the -OH group has been suggested to form a hydrogen bond with the same histidine residue in transmembrane domain VI, for example His 297 in the human opioid receptor [Pogozheva et al, 1998]. Masking the 3-OH substituent of morphine by methylation
gives codeine, which is approximately 10 to 30-fold less active than the parent compound in a variety of assay systems. Acetylation of both the 3 and 6 position hydroxyl groups of morphine affords heroin, which is at least equipotent with the parent compound. The activity of heroin is due to enzymatic hydrolysis of either one or both acetoxy groups, and in both cases the 3-OH group is ‘unmasked’. However, hydroxylated analogs of the fentanyl and pethidine series designed to mimic the 3-OH substituent of morphine actually exhibit a decrease in affinity [Lobezzo et al, 1981], further evidence for distinct binding modes of 4-anilinopiperidines and morphine-like ligands.

3) The spatial arrangement of the nitrogen, aromatic ring, and (if present) the hydroxyl function. The distances and angles between these features comprise the ‘pharmacophore’ for mu opioids, which is at present relatively simple and of limited use in predicting accurate binding affinities. However, it is still useful as a basic screen since a molecule which does not fit the pharmacophore is unlikely to bind to the mu opioid receptor at all.

More generally, the stereochemistry of the molecule is also critical. In the rigid polycyclic opioids the (−) absolute configuration is necessary for maintained affinity. For example, (−)-levorphanol exhibits high affinity for mu opioid receptors, whilst its stereoisomer dextrorphan has the (+) configuration and almost completely abolished affinity. In opioid peptides the amino acids are generally of the L-configuration, especially the first residue which is typically L-Tyr or an analogous residue. However, there are many examples of opioid peptides containing D-configuration amino acids since these are more resistant to enzymatic degradation.

As in most things, there are exceptions to the pharmacophoric ‘rules’ outlined above. Although there are no known opioids which lack an appropriate nitrogen feature, there are a very few which lack an aromatic ring. One set of examples are the ozonolysis products of a series of very similar etorphine-like compounds (Figure 1.17) which have analgesic potency in the rat much reduced when compared to the parent compounds, but still comparable to that of morphine [Bentley et al, 1969]. In view of the traditional opioid SAR theory that an aromatic ring is essential for opioid activity, this was a surprising finding. The authors proposed that the extraordinary
potency afforded to the parent molecules by the inclusion of the additional cyclic ring and the substituent on the 7 position could compensate for the loss of the aromatic ring in the ozonolysis product.

![Chemical Structure](image)

Figure 1.17  Etorphine-like compounds (a) and their ozonolysis products (b), R = n-Pr, n-Bu or n-pentyl.

Another example with an even less typical opioid structure is the steroid SC17599 (Figure 1.18). In 1968 Craig [1968] reported that the steroid 17α-acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxy-pregna-3,5-dien-20-one, or SC17599, possessed marked antinociceptive activity in three *in vivo* procedures, namely the rat tail flick, mouse writhing, and mouse hot plate assays. In addition, it caused respiratory depression in the rabbit, reduced gastrointestinal motility in mice, and the Straub tail response, also in mice. However, the antinociception observed in the rat tail flick assay was not nalorphine reversible, unlike that of morphine. Craig concluded that SC17599 was most likely acting in a similar manner to morphine.
In addition to the nitrogen centre, aromatic ring and sterochemistry described above, there are several other elements of opioid ligand structure that have been proposed to be important in binding to the opioid receptors. For example, in the rigid polycyclic systems a 14-position hydroxyl or ketone group increases ligand binding affinity, possibly through interaction with an asparagine residue which is present in the mu but not delta or kappa opioid receptors, Asn230 [Pogozheva et al, 1998]. In opioid peptides, a second aromatic ring is often present as part of a phenylalanine or analogous residue. This residue may be especially important in delta selective opioids and an analogous aromatic moiety is also present in several high affinity delta selective alkaloid ligands [Lomize et al, 1996]. It has been shown in opioid peptides that the side chain of this 'second' aromatic residue occupies different well-defined orientations in compounds which exhibit selectivity for the mu or delta opioid receptor [Mosberg et al, 1996; McFadyen et al, 1999].

There is a wealth of SAR information available on a huge diversity of opioids, and hence only a small selection has been presented here. An extremely thorough if slightly outdated review is given by Casy and Parfitt [1986]. The field of SAR study has been revolutionized by the increased availability of extremely powerful and relatively affordable computers in the last decade. It is
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now possible to analyze even the largest structures in terms of minimum energy conformations, partial energy charges, electrostatic potential, steric bulk, and other physical characteristics. Custom written software can search for features and conformations common to all molecules in a given group, allowing for pharmacophore definition. It is possible to build mathematical models which attempt to predict the properties of novel compounds, for example ligand binding affinities. This last is increasingly used as a tool to help direct ligand synthesis programs and testing studies, and there are a variety of appropriate software packages available.
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1.6 Aims

The exact nature of the ligand binding domain of the receptor, the nature of the interaction of a ligand with this domain, the determinants of specificity of a ligand for its receptor, the mechanism by which binding of agonist causes activation of receptor, and the determinants of agonist/antagonist properties are some of the most fundamental questions concerning G protein coupled receptor pharmacology.

These questions are still largely unanswered. In particular, elucidation of the relationships between the structure of a ligand and its affinity, specificity and extent of agonist action is still in its infancy.

The aim of the work reported here is to test the following hypotheses:
1) the aromatic ring and para-hydroxyl groups of the typical Tyr\(^1\) residue are critical to the binding of opioid peptides;
2) the steroid SC 17599 exerts its antinociceptive actions through the mu opioid receptor;
3) SC 17599 binds to the mu opioid receptor in a similar manner to morphine and other traditional opiates despite lacking both the aromatic and hydroxyl moieties;
4) molecular modelling techniques can be applied to the redefinition of the mu opioid pharmacophore in light of the activity of several ligands with unusual structures.
Chapter 2

MATERIALS AND METHODS
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2.1 Materials

2.1.1 Radiochemicals

$[^3]H$-DAMGO ([D-Ala$^2$, N-MePhe$^4$, Gly(ol)$^5$]enkephalin) (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, USA.

$[^3]H$-CI977 (5R-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-4-benzofuranacetamide) (21.1 Ci/mmol; 0.78 T bq/mmol) was a kind gift from Dr. J.C. Hunter, Parke-Davis Neuroscience Research Centre, Cambridge, UK.

$[^3]H$-Nociceptin (60 Ci mmol; 2.22 TBq/mmol), $[^3]H$-triamcinolone acetonide (38 Ci/mm mol; 1.41 TBq/mm mol), and $[^35]S$-GTP$\gamma$S (guanosine-5'-O-(3-thio)triphosphate) (1250 Ci/mm mol; 46.25 TBq/mm mol) were all purchased from DuPont NEN, Hounslow, UK or Boston, MA, USA.

Structures of the radioligands used are given in Figure 2.1.

2.1.2 Chemicals

EDTA (ethylenediaminetetraacetic acid), Folin & Ciocalteu’s phenol reagent, GDP (guanosine diphosphate), GTP (guanosine triphosphate), HEPES (N-[2-hydroxyethyl]-piperazine-N’-[2-ethanesulfonic acid], DL-dithiothreitol, sodium hydroxide, sodium potassium tartrate, magnesium chloride, magnesium sulfate, copper sulfate, sodium hydrogen carbonate, hydrochloric acid (sp. gr. 1.16), sodium molybdate, phenylmethylsulfonyl flouride, dextran, ammonia solution (specific gravity 0.88) and Trizma base (tris[hydroxymethyl]-aminomethane) were purchased from Sigma Chemical Co., Poole, UK or St. Louis, MO, USA.
Calcium chloride, potassium chloride, potassium phosphate monobasic, and sodium chloride were purchased from Mallinckrodt, St. Louis, MO, USA.
Activated charcoal and DMSO (dimethylsulfoxide) were from Fisher Scientific, Loughborough, UK or Pittsburgh, PA, USA.
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Sodium bicarbonate was from Columbus Chemical Industries, Inc., Columbus, WI, USA.
UltimaGold liquid scintillation fluid was from Packard Bioscience, Groningen, Holland.

a) $[3,5,3^\text{H}]\text{Tyr-\text{d-Ala-Gly-N-MePhe-NH(CH}_2)_2\text{-OH}}$

\[
\begin{array}{c}
\text{b)}\\
\text{c)}
\end{array}
\]

\[
\begin{array}{c}
\text{d)} \text{Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-[3,5,3^\text{H}]Tyr-Ala-Asn-Gln}
\end{array}
\]

\[
\begin{array}{c}
\text{e)}\\
\text{f)}
\end{array}
\]

Figure 2.1  a) $[^3\text{H}]$-DAMGO, b) $[^3\text{H}]$-diprenorphine, c) $[^3\text{H}]$-CI 977, d) $[^3\text{H}]$-nociceptin, e) $[^3\text{H}]$-triamcinolone, f) $[^{35}\text{S}]$-GTP$\gamma$S.
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2.1.3 Drugs and related compounds

The following drugs were generous gifts from the National Institute on Drug Abuse, Rockville, MD, USA: fentanyl HCl, alfentanil HCl, α-prodine, profadol, SNC 80 ((+)-(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethyl[benzamide), naloxone HCl, and naltrexone HCl.

BW 373, U86 ((+)-(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxy-benzyl]N,N-diethyl[benzamide) was from Burroughs Wellcome, Research Triangle Park, NC, USA.

Morphine sulfate was purchased from Mallinckrodt, St. Louis, MO, USA.

SC 17599 (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, USA.

Nociceptin, 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, USA.

Codeine sulfate, U69,593 (5α,7β,8γ(-)-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide) and etonitazene HCl were from RBI, Natick, MA, USA.

2.1.4 Peptides

DAMGO was from Tocris Cookson, Bristol, UK or Ballwin, MO, USA.

All of the peptides described in Chapter 3 were supplied by Dr. Henry Mosberg, School of Pharmacy, University of Michigan.

2.1.5 Cell culture media

Dulbecco’s Modified Eagle Medium (without sodium pyruvate; with 4,500 mg L⁻¹ glucose), Minimum Essential Medium (with Earle’s salts), foetal calf serum, penicillin/streptomycin, fungizone, trypsin, EDTA, and geneticin were all from Gibco Life Sciences, Grand Island, NY, USA.

Sterile DMSO was from Sigma Chemical Co., St. Louis, MO, USA.
2.1.6 Buffers

The composition of Buffer A used in all $^{35}$S\textsc{GTP}$\gamma$S assays was (mM): HEPES (20) MgCl$_2$.6H$_2$O (10) and NaCl (100). The pH was adjusted to 7.4 with NaOH. The Tris buffer used in radioligand binding studies was Trizma base (50 mM) acidified with HCl to pH 7.4. Buffer B used in the glucocorticoid receptor binding assay was composed of (mM): HEPES (10), EDTA (0.1), sodium molybdate (20) and phenylmethylsulphonyl fluoride (3). The pH was adjusted to 7.5 with NaOH.

2.1.7 Equipment

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2.2 Methods

2.2.1 Cell culture

The SH-SYSY human neuroblastoma cell line was kindly donated by Dr. D. Lambert, Department of Anaesthesia, Leicester University, UK. Cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% foetal calf serum, 2.5 μg mL⁻¹ amphotericin B (fungizone), 50 μg mL⁻¹ penicillin/streptomycin, and 250 μg mL⁻¹ L-glutamine at 37 °C in a humidified 5% CO₂ atmosphere.

C6 glioma cells transfected with either the cloned rat mu- or delta-receptor were a kind donation from Dr. Huda Akil, Mental Health Research Institute, University of Michigan, MI, USA. Cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% foetal calf serum at 37 °C under a 5% CO₂ atmosphere. For subculture one flask from each passage was grown in the presence of 1 mg mL⁻¹ 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 delta-opioid receptor were a kind gift from Dr. C.J. Evans, Department of Psychiatry, UCLA, Los Angeles, California. Cells were cultured in Dulbecco’s Modified Eagle Medium supplemented with 5% foetal calf serum, 2.5 μg mL⁻¹ fungizone, 50U mL⁻¹ penicillin, 50μg mL⁻¹ streptomycin and 258 μg mL⁻¹ L-glutamine at 37°C in a humidified 5% CO₂ atmosphere.

All cell lines were subcultured at confluency by splitting one flask per passage, as follows: the medium was aspirated, and trypsin (5 mL) added. After approximately 2 mins the cells were lifted from the flask by gentle agitation, removed, and pelleted by centrifugation for 3 mins at 1,600 rpm. Supernatant was removed, the pellet was resuspended in fresh medium, and finally the cell suspension was added to fresh medium in new flasks.
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2.2 Membrane preparation

Once cells had reached confluency they were harvested in HEPES (20 mM pH 7.4) buffered saline containing 1 mM EDTA, dispersed by agitation and collected by centrifugation at 1,600 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 final pellet was resuspended in 50 mM Tris-HCl buffer pH 7.4; separated into 0.5 mL aliquots (0.75 - 1.0 mg protein) and frozen at -80 °C.

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, USA.

2.2.3 Preparation of brain homogenates

Male Dunkin-Hartley guinea-pigs (250-500 g) from David Hall, Newchurch, Burton-on-Trent, were decapitated and the brain removed, weighed and homogenised in Tris-HCl buffer for 15 sec using a Polytron homogeniser. After centrifugation (35,000 × g, 15 mins, 4 °C) the pellet was resuspended in 10 times the volume of Tris buffer and incubated at 37 °C for 30 min to enable dissociation of endogenous ligands. The homogenate was re-centrifuged as above and the pellet was finally resuspended in Tris buffer to give a known concentration of approximately 1:60 w/w (original weight of wet tissue).

2.2.4 Determination of protein

Protein concentration for both cell membrane and guinea-pig brain membrane preparations was determined by the method of Lowry et al. [1951], using a bovine serum albumin standard.
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2.2.5 Radioligand binding assays

2.2.5.1 Competition assays

C6 glioma cell membranes (30 – 60 μg protein), SH-SY5Y cell membranes (100 – 150 μg protein), or guinea-pig brain homogenates (400 μg protein) were incubated at 25°C in 50 mM Tris-HCl buffer, pH 7.4 for 1 hour with radiolabelled ligand at the concentrations indicated in the text, and varying concentrations of unlabelled ligand to give a final volume of 1 mL. Non-specific binding was defined with 10 μM naloxone. The reaction was terminated by filtering the samples through glass fiber filters mounted in a Brandel 24 well harvester. The filters were subsequently washed 3 times with ice-cold Tris-HCl, pH 7.4 and radioactivity determined by scintillation counting after addition of 3 mL of liquid scintillation fluid.

The displacement of [3H]-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]. Briefly, cytosol fractions (20-30 μg protein) were incubated at 4 °C in buffer B, pH 7.5 for 18 h with [3H]-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, and non-specific binding was defined by dexamethasone (10 μM). Free [3H]-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 x 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).

2.2.5.2 Saturation assays

Membrane homogenates (as described above) were incubated at 25°C for 1 hour with varying concentrations of tritiated ligand (10 – 0.005 nM) in the presence of either water (control) or 10 μM naloxone (non-specific binding) to determine total specific binding. The reaction was terminated by rapid filtration and filters subjected to liquid scintillation counting as above.
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2.2.6 \(^{35}\text{S}\)-GTP\(_{\gamma}\)S binding assays

Membrane homogenates (as described above) were incubated for 1 hour at 30 °C in the presence of \(^{35}\text{S}\)GTP\(_{\gamma}\)S (100 pM), GDP (10 μM for SH-SY5Y cell homogenates, 30 μM for C6 glioma cell homogenates) and various concentrations of unlabeled ligand. Maximal stimulation was determined using fentanyl (10 μM) for mu-receptor studies and BW 373,U86 (10 μM) for delta-receptor studies. After 1 hour samples were rapidly filtered and filters subjected to liquid scintillation counting as before. The amount of stimulated \(^{35}\text{S}\)GTP\(_{\gamma}\)S binding is given as a percentage of the maximal stimulation evoked by either fentanyl (10 μM) or BW 373,U86 (10 μM).

2.2.7 Data analysis

All data were analysed using Microsoft Excel 97 (Microsoft, Seattle, WA) and GraphPad Prism 2.01 (GraphPad, San Diego, CA). From saturation binding assays, binding capacities (B\(_{\text{max}}\)) and equilibrium dissociation constants (K\(_{D}\)) were calculated from non-linear regression using GraphPad Prism. For radioligand binding assays mean IC\(_{50}\) values were calculated from individual IC\(_{50}\) values from at least three separate experiments, which were in turn determined using GraphPad Prism.

\(K_i\) values were determined using the Cheng-Prusoff equation [Cheng and Prusoff, 1973]:

\[K_i = \frac{IC_{50} \times K_D}{K_D + [L]}\]

where \(K_i\) is the equilibrium dissociation constant for the competing ligand; IC\(_{50}\) is the concentration of the competing ligand required for half-maximal inhibition of the binding of the radioligand; \(K_D\) is the equilibrium dissociation constant for the radioligand; and \([L]\) is the free radioligand concentration, assumed to be equal to the total radioligand concentration when present in large excess.

K\(_D\) values used were determined by saturation assay and were: 0.2 nM for \(^{3}\text{H}\)diprenorphine at mu receptors in either SH-SY5Y or C\(_{6}\) cell membranes, 1.0 nM for \(^{3}\text{H}\)DAMGO at mu receptors in SH-SY5Y cell membranes, 0.1 nM for \(^{3}\text{H}\)CI977 at kappa receptors in guinea-pig brain homogenates, and 1.0 nM for \(^{3}\text{H}\)triamcinolone at glucocorticoid receptors in Sf9 cytosol fractions.
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For $[^{35}\text{S}]-\text{GTPyS}$ binding assays, $EC_{50}$ values (effective concentration producing a 50% maximal response) were calculated from individual $EC_{50}$ values from at least three separate experiments, which were in turn determined using GraphPad Prism.

2.2.8 Antinociceptive assays

Male NIH mice (20-30 g) were used (Harlan Sprague Dawley Inc., Indianapolis, IN, USA). Subjects were housed in groups in a colony maintained at 20 °C, 40-50% humidity with a 12 hour light/dark cycle. Food and water were available ad libitum until the time of the experiments. Each subject was tested only once and all experiments were performed between 8 a.m. and 6 p.m.

2.2.8.1 Acetic acid induced writhing assay

The procedure of Koster et al. was used [Koster et al., 1959]. Briefly, mice received subcutaneous injections of drug prior to intraperitoneal injection of 0.6% acetic acid (0.4 mL). Five minutes after the injection of acetic acid, the number of writhes occurring during a 5 min period was recorded, with a ‘writhe’ defined as an extension of the animal’s abdomen and hind legs. Inhibition of writhing was expressed as the percentage of writhes observed in control mice, which was determined for each new batch of mice:

\[
\% \text{ control writhes} = \frac{\text{writhes with sterile water} - \text{writhes with drug}}{\text{writhes with sterile water}} \times 100
\]

2.2.8.2 Warm water tail withdrawal assay

The procedure of Janssen et al. was used [Janssen et al., 1963]. Briefly, the lower third of the tail was immersed into a 50 °C water bath and the latency to tail withdrawal was measured, with a cut-off latency of 20 sec. Drugs were injected i.p. at 30 min intervals and tail withdrawal latencies measured after 25 mins. Antinociception was measured as percent of maximum possible effect (% MPE):

\[
\% \text{ MPE} = \frac{(\text{mean latency following drug} - \text{control latency}) \times 100}{(\text{cutoff time} - \text{control latency})}
\]
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2.2.9 Molecular modelling

All computations were carried out on either an SGI Oxygen R10,000 workstation or an SGI Octane workstation. The majority of the work was done using SYBYL 6.4.3 form 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, except where noted. 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 minimised using the Tripos force field engine incorporating the use of charges (from above), and with termination by gradient at 0.001 kcal/mol. All minimisations were allowed to run until converged (usually < 1,000 iterations).

2.2.9.1 Pharmacocophore generation

The GASP (Genetic Algorithm Superposition Program) module of SYBYL was used to align from 2 to 4 energy minimised 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 due to limitations on computation time. The ligands used were: morphine, etorphine, the ozonolysis product of etorphine, and SC17599. Four alignments were generated in each run, and distance constraints were imposed such that all amine nitrogens had to be located within a 0.5 Å radius sphere. The alignments were scored as a weighted average of measures describing steric overlap, hydrogen bonding contribution, and increased internal energy in the molecules. 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.
2.2.9.2 Structure alignment

The FBSS (Field Based Similarity Search) module developed by David Wild at the University of Sheffield, Sheffield, UK [Wild and Willett, 1996] uses a genetic algorithm (GA) to align a set of molecules one at a time to a target. Alignments are determined by the GA based not on common atomic features as is the case in GASP, but on the similarity between the target and the test molecule calculated as a function of one or more of the following fields; electrostatic, hydrophobic, and shape.

Here, FBSS was used to align a relatively small but structurally diverse database of 17 opioid ligands as a precursor to analysis of quantitative structure-activity relationships (QSAR). The database comprised: etorphine, buprenorphine and diprenorphine (thebaine analogues), morphine, codeine, nalbuphine and naloxone (4,5-epoxymorphinans), butorphanol (a morphinan), pentazocine (a benzomorphan), pethidine and α-prodine (4-phenylpiperidines), profadol (a 3-phenylpyrrolidine), fentanyl and alfentanil (4-anilinopiperidines), methadone (a diphenylpropylamine), etonitazene (a benzimidazole), and SC17599 (a steroid). For structures, see Figure 5.7.

All of the ligands had AM1 (Austin Model 1) charges attached via the MOPAC module [Dewar et al, 1985] rather than the MNDO charges used elsewhere, due to the requirements of the FBSS genetic algorithm. Two target molecules were used to align the set in separate runs, namely etorphine (most active) and morphine (most typical structure). The genetic algorithm uses a scoring function to test the ‘goodness’ of its alignment of the target and database molecule in order to determine when the analysis has converged. Part of the scoring function analyses the steric overlap between the target and database molecule, and two variations were used. In the first, full steric scoring (FSS), overlap between the two molecules generates a positive scoring contribution whilst regions of excess volume in the database molecule generate a negative scoring contribution. In the second, partial steric scoring (PSS), there is no negative contribution from excess volume in the database molecule.
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In each case (morphine or etorphine target, full steric scoring or partial steric scoring), four separate analyses used the following combinations of field(s): electrostatic, electrostatic plus hydrophobic, electrostatic plus shape, or electrostatic, hydrophobic and shape. For each analysis 20 iterations were performed, each consisting of 20,000 operations at a population size of 125 with selection pressure set to 1.10. The target was held rigid whilst the database molecules were allowed to flex.

Five sets of four alignments each were generated, and were assessed on the individual ‘similarity score’ given for each molecule relative to the target and the average ‘similarity score’ for all ligands in the database. The similarity score is an unweighted average of measures describing the similarity of the steric, electrostatic, and hydrophobic fields. The alignments were also assessed visually, using the position of the amine nitrogen atom in each molecule.

2.2.9.3 Quantitative structure-activity relationships

The CoMFA (Comparative Molecular Field Analysis) module of SYBYL was used, based on an alignment of a database of ligands by FBSS (Field Based Structural Similarity). The FBSS module is particularly useful in preparation for a QSAR (Quantitative Structure-Activity Relationships) study since it aligns molecules based not on their structural features but on their steric, electrostatic, and hydrophobic fields, which is also the basis of the CoMFA analysis. The chosen alignments from the FBSS analysis were used to construct separate spreadsheets which also contained log \((1/K_c)\), log \((1/EC_{50})\) and % maximal stimulation data for each molecule as measures of affinity, potency and efficacy at the mu opioid receptor respectively. Ligands which were mis-aligned in a particular FBSS analysis were omitted from the corresponding spreadsheet.

The CoMFA field for each ligand was generated using the default parameters, and used to derive a model describing the variance in one of the three provided measures of biological activity. In each case, an initial cross-validated partial-least squares (PLS) analysis was used to identify the optimal number of components for further analysis, where additional components increase the complexity of the equation which describes the model. Default parameters were used in all cases. The cross-validation
calculation is a sequential process in which one ligand at a time is removed and the remainder used to generate a QSAR model. The predicted biological activity of the 'odd-man-out' is then compared to its actual activity. In addition to identifying the optimal number of components, this analysis also provides a measure of the predictive ability of the QSAR model, and ensures that it is not reliant on any one single data point.

A further PLS analysis was performed using the recommended optimal number of components, with no validation and column filtering turned off. The predicted regions of favourable and unfavourable steric and electrostatic contributions were visualised using the default parameters.

2.2.9.4 Ligand docking

Genetic Optimization for Ligand Docking (GOLD) is a joint collaboration between the CCDC, Cambridge, UK (Cambridge Crystallographic Data Center) and Dr. Gareth Jones, Sheffield University, Sheffield, UK [Jones et al, 1995; Jones et al, 1997]. GOLD uses a genetic algorithm to search out possible docking modes of a given ligand with a given receptor. Here, energy minimised structures (with charges) were docked with a model of the mu opioid receptor kindly provided by Dr. Henry Mosberg [Pogozheva et al, 1998] (http://www-personal.umich.edu/~him). Ten dockings were generated in each run, with 'set atoms types' enabled for both ligand and protein in order to validate atom type assignments, and 'early termination' disabled to ensure that the full number of dockings were produced. The active site was defined by a 15.0 Å radius around the α carbon (atom number 644) of the Asp147 residue of the receptor, since this residue has been consistently implicated in forming an ionic interaction with the positively charged amine nitrogen present in all opioid ligands. Individual dockings were scored as a weighted average of measures describing internal and external hydrogen bonding contributions, van der Waals interactions, and torsion energies. Those docking models with the best 'overall' scores were selected.
Chapter 3

CYCLIC TETRAPEPTIDES
Chapter 3: Cyclic Tetrapeptides

3.1 Introduction

As discussed in Chapter 1, extensive study of the interactions of hundreds if not thousands of structurally diverse ligands with the opioid receptors has led to the identification of pharmacophore models which detail the spatial arrangement of structural features common to all ligands of a particular opioid receptor. The main focus of these efforts has been the mu receptor, for which the largest number of ligands are available and which has the greatest clinical relevance. These models are constantly being refined, commonly through the design and study of conformationally restricted ligands, many of which display selectivity in binding to one or other opioid receptor. This study examines the mu opioid pharmacophore by characterising the binding affinity, potency and relative efficacy of a series of tetrapeptides in which such restriction is achieved via cyclisation between the second and fourth amino acids.

The endogenous opioid pentapeptides lend themselves well to this approach. For example, DPDPE, [D-Pen², D-Pen⁵]-enkephalin, is a cyclic disulfide containing analogue of Leu-enkephalin, Figure 3.1. This compound displays such high affinity (4.0 nM) and selectivity (200-fold) for the delta opioid receptor that it is considered the standard delta ligand [Mosberg et al, 1983; Mosberg et al, 1988].

![Figure 3.1: Structure of DPDPE, [D-Pen², D-Pen⁵]-enkephalin (cyclised via a disulfide bond between D-Pen² and D-Pen⁵).](image)

A large number of conformationally restricted disulfide- or dithioether-containing tetrapeptides related to DPDPE have been studied, with the focus on refining the delta opioid receptor pharmacophore [Mosberg et al, 1994a; Mosberg et al, 1994b; Mosberg et al, 1996; Lomize et al, 1996]. These compounds can be thought of as des-Gly³ analogues of DPDPE and have the general structure depicted in Figure 3.2. When cyclization is via a disulfide bond (i.e. n = 0), giving a ring size of 11 atoms and the C-terminal group is carboxylic acid, these peptides typically show selectivity...
for the delta receptor. For example, H-Tyr-c[D-Cys-Phe-d-Pen]OH (JOM-13), shows higher affinity for the delta receptor than DPDPE at 0.75 nM, and at the same time displays a similar selectivity ratio as DPDPE (70-fold) over the mu receptor [Mosberg et al, 1988]. However, when the C-terminus of JOM-13 is modified to carboxamide, giving H-Tyr-c[D-Cys-Phe-d-Pen]NH₂ (JOM-5), delta receptor affinity is diminished by 80-fold whilst mu receptor affinity is concurrently enhanced by 7.5-fold to give an affinity of 7.0 nM, such that JOM-5 actually shows moderate, 8-fold, mu receptor selectivity [Mosberg et al, 1988]. Increasing the ring size of JOM-5 from 11 to 13 atoms by incorporating a dithioethane linkage (i.e. -S(CH₂)₂S-) gives H-Tyr-c[D-Cys-Phe-d-Pen]NH₂ (Et) (JOM-6), which exhibits greatly increased mu receptor affinity (0.3 nM) when compared to JOM-5, resulting in a correspondingly higher selectivity (80-fold) for the mu opioid receptor [Mosberg et al, 1988].

![Figure 3.2](image)

General structure of cyclic tetrapeptides, using JOM-6 as an example. Analogues reported include variations in the first and third residues and alterations to the bridging group.
Chapter 3: Cyclic Tetrapeptides

The extensive work carried out on these and related peptides has determined that three of the key pharmacophore elements that govern binding to both the mu and delta opioid receptor are contained within the Tyr$^1$ residue, namely the amino function, and the phenolic aromatic and hydroxyl groups [Casy and Parfitt, 1986; Mosberg et al, 1987]. In opioid peptides, replacement of the Tyr$^1$ residue with an aromatic amino acid lacking a para-hydroxyl group almost invariably results in a drastic, if not complete, loss of affinity for both the mu and delta opioid receptors [Morgan et al, 1976; Chang et al, 1976; Chang et al, 1976]. Two exceptions are the cyclic octapeptides CTAP and CTOP, which are related to a short stretch of the cyclic tetradecapeptide Somatostatin rather than the enkephalins [Pelton et al, 1985; Pelton et al, 1986]. The general structure of these compounds is D-Phe-c[Cys-Tyr-D-Trp-x-Thr-Pen]-Thr-NH$_2$, where x = Ornithine (CTOP) or Arginine (CTAP). Both exhibit high affinity for the mu opioid receptor (approximately 2.8 nM and 3.5 nM respectively) with extremely low affinity for both delta opioid and somatostatin receptors, but both are antagonists at the mu opioid receptor. The ability of these peptides to bind to the mu opioid receptor is especially surprising, since not only does the D-Phe$^1$ residue lack the tyrosyl hydroxyl group, it also has D- stereochemistry, which traditionally abolishes mu receptor binding [Coy et al, 1976; Casy and Parfitt, 1986].

In addition, the aromatic ring of the Phe$^3$ residue, cyclic peptide ring size and C-terminal substitution play important roles particularly as determinants of mu/delta selectivity, as seen above with the conversion from JOM-13 (C-terminal -COOH) to JOM-5 (C-terminal -CONH$_2$) [Heyl et al, 1991; Heyl and Mosberg, 1992; Ho et al, 1999]. The relative positions of the aromatic rings of the first and third amino acids has been shown to be crucial for both binding and selectivity. One of the main determinants of this relative position is the orientation of the Phe$^3$ side chain phenyl group, which must be gauche for high delta receptor affinity [Mosberg et al, 1994b] and trans for optimal mu opioid binding [Mosberg et al, 1996]. Changes to the cyclic peptide ring size also directly affect the relative positions of the first and third aromatic side chains [Wang et al, 1998].
Chapter 3: Cyclic Tetrapeptides

In the first part of this chapter the influence of amino acids substitutions and changes to the cyclic ring system have been examined whilst retaining the para-hydroxyl group considered to be crucial in binding to both mu and delta opioid receptors. In the second section, characterisation of a larger series of peptides lacking any para-substituent highlights a possible reduced role for this group in certain mu opioid receptor selective peptides. Finally, several compounds are considered in which the Tyr¹ residue is replaced by aromatic amino acids containing nitrogen. These experiments were designed to examine the mu opioid receptor pharmacophore for peptide ligands but also to determine if the structural features required for binding affinity are the same as those necessary for agonist potency and efficacy. The general structure of these peptides is shown in Figure 3.2, and the amino acid substitutions and numbering of the peptides is shown in Table 3.1.
## Table 3.1 Structures of cyclic tetrapeptides studied

<table>
<thead>
<tr>
<th>Peptide structure</th>
<th>Bridge</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>JOM-6</td>
</tr>
<tr>
<td>t-Hpp-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>1</td>
</tr>
<tr>
<td>Hat-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>2</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Δ⁸-Phe-D-Pen]NH₂</td>
<td>S–S</td>
<td>3</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–cis-Ey–S</td>
<td>4</td>
</tr>
<tr>
<td>p-F-Phe-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>5</td>
</tr>
<tr>
<td>Phe-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>6</td>
</tr>
<tr>
<td>Phe-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–S</td>
<td>7</td>
</tr>
<tr>
<td>Phe-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–cis-Ey–S</td>
<td>8</td>
</tr>
<tr>
<td>phenylGly-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>9</td>
</tr>
<tr>
<td>homoPhe-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>10</td>
</tr>
<tr>
<td>diphenylAla-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>11</td>
</tr>
<tr>
<td>1-naphthy1Ala-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>12</td>
</tr>
<tr>
<td>2-naphthy1Ala-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>13</td>
</tr>
<tr>
<td>trans-phenylPro-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>14</td>
</tr>
<tr>
<td>cis-phenylPro-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>15</td>
</tr>
<tr>
<td>isoquinoline-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>16</td>
</tr>
<tr>
<td>tetralin-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>17</td>
</tr>
<tr>
<td>cyclohexy1Ala-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
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</tr>
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<td>pyridyl-c[D-Cys-Phe-D-Pen]NH₂</td>
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<td>His-c[D-Cys-Phe-D-Pen]NH₂</td>
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</tr>
<tr>
<td>Trp-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>21</td>
</tr>
<tr>
<td>indan-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S–Et–S</td>
<td>22</td>
</tr>
</tbody>
</table>
Chapter 3: Cyclic Tetrapeptides

3.2 Peptides retaining a para-hydroxyl substituent

Five analogues of JOM-6 (Tyr-c[β-Cys-Phe-d-Pen]NH₂ (Et)), were characterised to examine the effects of conformational restriction of Tyr¹ or Phe³ or alterations in peptide ring size, in addition to one para-fluoro analogue. The residue 1 substituted analogues contain: L-trans-hydroxyphenylproline (L-t-Hpp¹) (1a), d-trans-hydroxyphenylproline (d-t-Hpp¹) (1b), L-hydroxyaminotetralin (L-Hat¹) (2a), or d-hydroxyaminotetralin (d-Hat¹) (2b). The peptide 3 retains tyrosine in position 1 but contains the Phe³ analogue dehydro(E)phenylalanine (Δ⁸-Phe³) and a disulfide bridge, whilst 4 differs from JOM-6 only in its altered ring closure, namely cis-ethene (S(CH₂)S–). Finally, 5 contains para-fluorophenylalanine (p-F-Phe³). The structures of the amino acids and bridging groups used are shown in Figures 3.3, 3.4, and 3.5.

\[ \text{tyrosine} \quad \text{trans-hydroxyphenylproline} \]

peptides: JOM-6, 3, and 4

\[ \text{6-hydroxy-2-amino-2-} \]
\[ \text{para-fluorophenylalanine} \]

peptides: 2a (L) and 2b (D)

Figure 3.3 Structure of the N-terminal portion of the cyclic tetrapeptides. The general structure is as shown in Figure 3.1.
Chapter 3: Cyclic Tetrapeptides

peptides: 3 JOM-6, 1a, 1b, 2a, 2b and 5 4

Figure 3.4 Structure of bridging groups used to close cyclic peptide system. The general structure is as shown in Figure 3.1.

peptides: JOM-6, 1a, 1b, 2a, 2b, 0 0 0 4 and 5

Figure 3.5 Structure of the third residues of the cyclic tetrapeptides. The general structure is as shown in Figure 3.1.

3.2.1 Results

3.2.1.1 Radioligand binding assays

Radioligand binding data for the peptides at mu, delta and kappa opioid receptors in guinea-pig brain were provided by Dr. Henry Mosberg of the School of Pharmacy and are shown in Table 3.2, along with values for the standard ligands for the delta (DPDPE) and mu (DAMGO) receptors.
<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th>Peptide structure</th>
<th>Analogue Name</th>
<th>Bridge ¹</th>
<th>Binding Kᵢ (nM)</th>
<th>Selectivity Kᵢ(δ) / Kᵢ(μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-d-Ala-Gly-N-MePhe-Gly-ol</td>
<td>None</td>
<td>DAMGO ²</td>
<td></td>
<td>4.13 ± 0.84</td>
<td>615</td>
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<tr>
<td>Tyr-c[D-Pen-Gly-Phe-d-Pen]OH</td>
<td>S–S</td>
<td>DPDPE</td>
<td></td>
<td>810 ± 66</td>
<td>&gt; 10,000</td>
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<tr>
<td>Tyr-c[D-Cys-Phe-d-Pen]NH₂</td>
<td>S–S</td>
<td>JOM-5 ³</td>
<td></td>
<td>7.0 ± 0.5</td>
<td>8.23</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-d-Pen]NH₂</td>
<td>S–Et–S</td>
<td>JOM-6 ³</td>
<td></td>
<td>0.29 ± 0.04</td>
<td>83.3</td>
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<tr>
<td>L-t-Hpp-c[D-Cys-Phe-d-Pen]NH₂</td>
<td>S–Et–S</td>
<td>1a</td>
<td></td>
<td>0.32 ± 0.03</td>
<td>85.6</td>
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<tr>
<td>D-t-Hpp-c[D-Cys-Phe-d-Pen]NH₂</td>
<td>S–Et–S</td>
<td>1b</td>
<td></td>
<td>31.3 ± 4.2</td>
<td>133</td>
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<tr>
<td>L-Hat-c[D-Cys-Phe-d-Pen]NH₂</td>
<td>S–Et–S</td>
<td>2a</td>
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<td>D-Hat-c[D-Cys-Phe-d-Pen]NH₂</td>
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<td></td>
<td>0.39±0.04</td>
<td>148</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Δ-Phe-d-Pen]NH₂</td>
<td>S–S</td>
<td>3</td>
<td></td>
<td>8.7±1.2</td>
<td>18.4</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-d-Pen]NH₂</td>
<td>S–cis-Ey–S</td>
<td>4</td>
<td></td>
<td>4.1±0.7</td>
<td>14.9</td>
</tr>
<tr>
<td>p-F-Phe-c[D-Cys-Phe-d-Pen]NH₂</td>
<td>S–Et–S</td>
<td>5</td>
<td></td>
<td>12.3±1.8</td>
<td>≈ 100</td>
</tr>
</tbody>
</table>

Table 3.2 Opioid receptor binding profiles of cyclic tetrapeptides. Displacement of $[^{3}H]$-DAMGO (0.6 nM, mu), $[^{3}H]$-DPDPE (1.8 nM, delta), or $[^{3}H]$-U69,593 (0.9 nM, kappa) from guinea pig brain homogenates. Data represent mean ± standard error of the mean from 3 or more separate experiments. Data provided by Dr. Henry Mosberg of the School of Pharmacy, University of Michigan.

¹ Bridge between the second and fourth amino acids (second and fifth amino acids in DPDPE).

² Determined in rat brain membranes.

³ Taken from Mosberg et al [1988].
Chapter 3: Cyclic Tetrapeptides

As described above, JOM-5 exhibits good affinity for the mu opioid receptor and moderate selectivity for the mu over delta receptor. This selectivity is increased 10-fold when the disulfide bond of JOM-5 is altered to a dithioethane bond, giving JOM-6, as a consequence of greatly increased mu receptor affinity (Table 3.2).

The L-trans-hydroxyphenylproline (L-t-Hpp) derivative 1a exhibited affinity for both mu and delta receptors equivalent to that of the parent compound JOM-6. However, its diastereomer 1b (D-t-Hpp) had 108- and 168-fold reduced affinity at mu and delta receptors respectively. In contrast, both of the diastereomeric Hat containing analogues 2a (L) and 2b (D) showed affinities at the mu opioid receptor identical to that of the parent peptide JOM-6. As a consequence of its slightly reduced affinity for the delta receptor, 2b had improved selectivity for the mu opioid receptor when compared to JOM-6, and at approximately 150-fold was the most selective of the peptides tested here. The peptide 3 (Tyr-c[D-Cys-D-Phe-D-Pen]NH2 (Et)), is the dehydro(E)-phenylalanine analogue of the disulfide-bridged JOM-5. This peptide exhibited affinity for the mu opioid receptor similar to that of JOM-5, but with almost 3-fold decreased delta receptor affinity. The cis-ethene linked analogue of JOM-6 is the peptide 4, which exhibited 10-fold reduced affinity for the mu opioid receptor but unchanged affinity at delta. Finally, the p-F-Phe containing peptide 5 showed 40-fold reduced affinities at both the mu and delta opioid receptors.

All of the cyclic tetrapeptides tested showed very low affinity for the kappa opioid receptor as measured by displacement of [3H]U69,593, with the exception of the peptide 2b which exhibited moderate to low affinity at approximately 280 nM. Consequently, all of the peptides possessed selectivity for mu and delta over kappa opioid receptors. For example, JOM-6 exhibited almost 7,000-fold selectivity for the mu over the kappa opioid receptor, and 80-fold selectivity for delta over kappa.
<table>
<thead>
<tr>
<th>Peptide structure</th>
<th>Analogue</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>max (%)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Max (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-D-Ala-Gly-N-MePhe-Gly-ol</td>
<td>DAMGO</td>
<td>6.4 ± 1.45</td>
<td>110.0 ± 4.6</td>
<td>&gt; 10,000</td>
<td></td>
</tr>
<tr>
<td>Tyr-c[D-Pen-Gly-Phe-D-Pen]OH</td>
<td>DPDPE</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.87 ± 0.52</td>
<td>99.7 ± 0.58</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-D-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>JOM-5</td>
<td>17.8 ± 3.56</td>
<td>98.2 ± 2.3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-D-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>JOM-6</td>
<td>2.93 ± 0.83</td>
<td>125.8 ± 6.0</td>
<td>337.7 ± 72.6</td>
<td>72.6 ± 1.9</td>
</tr>
<tr>
<td>L-t-Hpp-c[D-Cys-Phe-D-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1a</td>
<td>8.76 ± 1.99</td>
<td>125.7 ± 5.1</td>
<td>57.5 ± 3.9</td>
<td>40.9 ± 1.0</td>
</tr>
<tr>
<td>L-Hat-c[D-Cys-Phe-D-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2a</td>
<td>0.40 ± 0.21</td>
<td>102.1 ± 5.5</td>
<td>247.7 ± 37.8</td>
<td>69.6 ± 5.1</td>
</tr>
<tr>
<td>D-Hat-c[D-Cys-Phe-D-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2b</td>
<td>1.44 ± 0.36</td>
<td>109.6 ± 5.4</td>
<td>1,550 ± 117.2</td>
<td>73.9 ± 3.2</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Δ&lt;sup&gt;8&lt;/sup&gt;-Phe-D-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3</td>
<td>1,060 ± 69.1</td>
<td>117.4 ± 5.6</td>
<td>567.6 ± 61.7</td>
<td>35.4 ± 8.8</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-D-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>cis-Ey</td>
<td>4.41 ± 0.44</td>
<td>105.1 ± 7.0</td>
<td>56.9 ± 19.2</td>
<td>65.8 ± 11.8</td>
</tr>
<tr>
<td>p-F-Phe-c[D-Cys-Phe-D-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>S-Et-S</td>
<td>5.13 ± 2.73</td>
<td>105.6 ± 4.4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 3.3 Potency and relative efficacy of cyclic tetrapeptides. Stimulation of [<sup>35</sup>S]-GTPγS binding in membranes of either C<sub>6</sub>μ (mu) or C<sub>6</sub>δ (delta) cells, as described in Methods. Data represent mean ± standard error of the mean from 3 or more separate experiments.

1 Bridge between the second and fourth amino acids (second and fifth amino acids in DPDPE).


3 n.d., not determined

2 Values represent percentage of the maximal response to 10 μM fentanyl (mu) or BW 373, U86 (delta)

4 Significantly different to the maximal response to 10 μM fentanyl (mu) or BW 373, U86 (delta), p < 0.05 (Student's t test)
Chapter 3: Cyclic Tetrapeptides

3.2.1.2 GTP\gamma S binding assays

The $[^{35}\mathrm{S}]-\text{GTP}\gamma \text{S}$ assay provides a functional measure of the efficacy of a ligand interacting with a G protein-coupled receptor [Hilf et al, 1987; Traynor and Nahorski, 1995]. Potencies and relative efficacies of the cyclic peptides for the mu opioid receptors are shown in Table 3.3 and compared to values for DPDPE and DAMGO.

The mu opioid full agonist fentanyl was used as a control in the $[^{35}\mathrm{S}]-\text{GTP}\gamma \text{S}$ assay, and it displayed a potency of $10.3 \pm 0.9 \text{ nM}$. The mu opioid receptor selective DAMGO exhibits high potency, $6.4 \pm 1.5 \text{ nM}$, and stimulation equal to that produced by a maximal concentration of fentanyl. Of the cyclic tetrapeptides reported here, 1a, 2a, 2b and 3 showed potencies comparable to or greater than DAMGO, and all showed maximal response at least equal to those of DAMGO and fentanyl. Indeed, as shown in Table 3.3, the peptides JOM-6, 1a, 3 produced significantly greater stimulation than fentanyl, suggesting very high efficacy. The analogues 2a, 2b and 4 exhibited potencies approximately 7-, 2- and 2-fold higher than JOM-6, respectively. The peptides 1a and 5 showed 3- and 14-fold lower potencies than JOM-6, whilst 3 exhibited very low potency (Figure 3.6).

![Graph](image)

**Figure 3.6** Stimulation of $[^{35}\mathrm{S}]-\text{GTP}\gamma \text{S}$ binding in C6\mu cell membranes by DAMGO (♦), JOM-6 (□), 3 (▲) or 5 (▼). Data represent percentage of the maximal response to $10 \mu\text{M}$ fentanyl, means ± standard error of the mean from three or more separate experiments, as described in Methods.
Chapter 3: Cyclic Tetrapeptides

At the delta receptor, maximal stimulation was defined using the non-peptide delta receptor full agonist BW 373,U86. DPDPE had high potency, 1.87 ± 0.52 nM and produced maximal stimulation equal to that seen with BW 373,U86. None of the peptides tested here were able to produce stimulation of $[^{35}\text{S}]-\text{GTPyS}$ binding equivalent to that of BW 373,U86, instead ranging from 35.4% for 3 to 73.9% for 2b. Potencies were also more varied, and lower, than found in the same assay at the mu receptor, ranging from 56.9 nM (4) to 1,550 nM (2b) (Figure 3.7). All of the peptides but 3 exhibited greater potency and efficacy in the $[^{35}\text{S}]-\text{GTPyS}$ assay at the mu receptor than at the delta receptor, with functional selectivity ranging from 7-fold (1a) to more than 1,000-fold (2b).

![Figure 3.7](image.png)

**Figure 3.7** Stimulation of $[^{35}\text{S}]-\text{GTPyS}$ binding in C6δ cell membranes by DPDPE (●), JOM-6 (□), 2b (▼) or 4 (▲). Data represent percentage of the maximal response to 10 μM BW 373,U86, means ± standard error of the mean from three or more separate experiments, as described in Methods.
Chapter 3: Cyclic Tetrapeptides

3.2.2 Discussion

3.2.2.1 Radioligand binding assays

Conformational restriction of the N-terminal residue was achieved in two ways. Firstly, when compared with the Tyr$^1$ residue in JOM-6, L-trans-hydroxyphenylproline (L-t-Hpp$^1$) restricts rotation about the Cα-Cβ but not Cβ-Cγ bonds, thus reducing the conformational space accessible to the phenolic ring (Figure 3.3). However, the fact that 1a maintained the binding profile of JOM-6 despite Cα-Cβ rotational restriction implies that the required conformation(s) for binding to the opioid receptors lies within the limited accessible conformational space of the L-t-Hpp$^1$ residue. This has been seen previously for delta selective cyclic tetrapeptides containing the L-t-Hpp$^1$ residue [Mosberg and Kroona, 1992]. In contrast, the D-t-Hpp$^1$ containing peptide 1b had approximately 100-fold reduced affinity, indicating that this residue is much less compatible with the required binding conformation at the mu opioid receptor. Delta receptor affinity was likewise affected.

Compared to hydroxyphenylproline, the hydroxyaminotetralin residue (Hat$^1$) restricts rotation about both the Cα-Cβ and Cβ-Cγ bonds through formation of a bicyclic structure spiro to the α-carbon, greatly restricting the number of possible conformers (Figure 3.3). Delta selective D- and L-Hat analogues of the peptide JOM-13 have previously been shown to exhibit similar binding affinities which are reduced, but not abolished, when compared to the parent compound [Mosberg et al, 1994a], and this is also the case at the mu receptor. This ability of both the D- and L-Hat$^1$ containing peptides 2a and 2b to bind with high affinity to the mu, and indeed the delta receptor is intriguing, since they can never be configured in such a way as to allow superimposition of the phenolic rings and primary amine groups of the L- and D-Hat$^1$ residues, two of the essential pharmacophoric elements for binding to both the mu and delta receptors. However, the stereoisomeric amino acids can be configured such that both the primary amine and backbone amide groups, and hence the cyclic peptide system of both molecules, are superimposed (Figure 3.8). The cyclohexyl rings of both are also overlapped, although in slightly different conformations, and there is a difference in the position of the tyrosyl phenyl rings. These are in the same plane but approximately 2.5 Å apart, and as a consequence there is also a difference in the
position of the hydroxyl groups. One model for the binding of ligands to the mu opioid receptor [Pogozheva et al, 1998] proposes that the quaternary nitrogen of the primary amine group of the peptide forms an ion pair with the carboxylic acid group of the Asp147 residue in the third transmembrane domain, whilst the oxygen atom of the tyramine hydroxyl group forms a hydrogen bond interaction with the hydrogen attached to the secondary nitrogen of the imidazole moiety of the His297 residue in the sixth transmembrane domain. Assuming that both peptides occupy the binding site in broadly the same orientation, this difference in position of the tyramine phenolic rings of 2a and 2b requires that the hydroxyl groups of both can form hydrogen bonds with His297. This is certainly feasible, as shown in Figure 3.8.

Figure 3.8 Overlap of L- and D-Hat isomers and their hypothetical interaction with the His297 residue. Nitrogens are shown in blue; oxygens, red; hydrogens, cyan; and carbons in either orange (L-Hat), magenta (D-Hat) or white (His). Dashed yellow lines indicate hydrogen bonds.
In addition, the hydrophobic residues comprising the binding pocket, namely well conserved hydrophobic residues in the fifth (Ile234), sixth (Ile296 and Val300) and seventh (Cys321) transmembrane domains, must be flexible enough to be able to accommodate both L- and D-Hat in position 1 of these tetrapeptides. Thus, the phenolic ring of the first residue of opioid peptides may have a degree of positional flexibility without detriment to binding at the opioid receptors.

The ΔE-Phe3 residue as in peptide 3 introduces a double bond between the α and β carbons, restricting rotation of the Cα-Cβ but not Cβ-Cγ bonds (Figure 3.5). The reduced affinity of 3 for the delta but not mu receptor suggests that the required binding conformation is energetically unfavorable in the former case but not the latter, showing that the side chain of the Phe3 residue must be in the gauche conformation in order to bind with high affinity to the mu receptor. This also implies that binding to the delta receptor requires the trans conformation. Indeed, it has previously been shown in a tetrapeptide series based on JOM-13 (Tyr-c[D-Cys-Phe-D-Pen]OH) that binding to the delta opioid receptor requires a gauche orientation of the Phe3 sidechain, as in the ΔZ-Phe3 analogue [Ho et al, 1999] and in a group of four β-methylphenylalanine isomers [Mosberg et al, 1994b]. In contrast, binding to the mu receptor requires a trans orientation of the Phe3 sidechain, as seen in the ΔE-Phe3 analogue [Mosberg et al, 1996].

Alteration of the bridging group of JOM-6 from −S(CH2)2S− to −S(CH)2S−, as in 4 (Tyr-c[D-Cys-Phe-D-Pen]NH2 (cis-Ey)) resulted in a 10-fold reduction in affinity for the mu opioid receptor but no change in delta affinity. Thus, the reduction in flexibility of the cyclic peptide system upon introduction of a double bond affects the ability of the peptide to assume the binding conformation necessary at the mu, but not delta, receptor. However, it should be noticed that affinity for the delta receptor was extremely low, and this finding may not translate to higher affinity analogues.

Substitution of a para-fluorophenylalanine residue in position 1 of JOM-6 gives 5, which exhibited parallel 40-fold reductions in affinity at the mu and delta receptors. However, affinity for the mu receptor was still 12.3 nM, implying that the para-
hydroxyl substituent of tyrosine is not critical for binding of these peptides to the mu opioid receptor, but does contribute to binding.

Peptidergic opioids related to enkephalin traditionally exhibit very low affinity for the kappa opioid receptor, and this is the case in this series. It is apparent that those peptides containing the \(-\text{S(CH}_2\text{)}_2\text{S}-\) ring closure exhibit higher affinity for the kappa opioid receptor than those with the disulfide linkage, implying that conformational freedom of the peptide ring system is important in allowing for correct orientation within the binding pocket of the kappa opioid receptor.

3.2.2.2 GTP\(_\gamma\)S binding assays

The cyclic tetrapeptide JOM-6 compares favourably with fentanyl and DAMGO in the \([^{35}\text{S}]\)-GTP\(_\gamma\)S binding assay, exhibiting maximal response even greater than these highly efficacious mu opioid agonists. Likewise, the L-\(\tau\)-Hpp\(^1\) analogue 1a exhibited relative efficacy equal to that of JOM-6, whilst the remaining peptides exhibited maximal response equal to fentanyl, suggesting very high efficacy. Thus in the L-\(\tau\)-Hpp residue the tyrosyl residue may be constrained in the correct position for optimal efficacy.

The conformational restrictions imposed on the aromatic moiety of residue 1 in the peptides 1a, 2a and 2b had a greater effect on relative potency in the \([^{35}\text{S}]\)-GTP\(_\gamma\)S assay than on affinity as measured by the ligand binding assay. For example, the L-Hat analogue 2a exhibited a 7-fold higher potency than JOM-6 and the L-\(\tau\)-Hpp\(^1\) peptide 1a showed a 3-fold lower potency in the \([^{35}\text{S}]\)-GTP\(_\gamma\)S assay, whilst both had equal affinity to JOM-6 in the ligand binding assay. The disulfide linked peptide 3 with a \(\Delta^E\)-Phe\(^3\) residue was 60-fold less potent at the mu opioid receptor than JOM-5, but still gave a full maximal response. However, 3 displayed almost equivalent affinity to JOM-5, suggesting that although all of the peptides tested produced maximal stimulation in this assay, their true efficacies at the mu opioid receptor are different. The L- and D-Hat isomers 2a and 2b produced a similar maximal effect and only 3-fold differing potency in the \([^{35}\text{S}]\)-GTP\(_\gamma\)S assay, confirming that the features within the opioid binding site have the degree of conformational freedom necessary to bind both L- and D-isomers of this conformationally restricted amino acid.
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As with the mu receptor, the relationship between affinity and potency at the delta receptor is not parallel for all of the compounds in this series. For example, the peptide 1a exhibited higher potency than JOM-6 at the delta receptor, despite showing equivalent affinity. Also, 2a and 2b had similar affinities at the delta opioid receptor, but 6-fold different potencies in the $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ assay. This differentiation resulted in the D-Hat$^1$ containing peptide 2b becoming functionally very selective for the mu over delta opioid receptors, with a greater than 1,000-fold preference for mu as measured by the $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ assay.

In conclusion, the peptide 2b (D-Hat-c[D-Cys-Phe-D-Pen]NH$_2$(Et)), has high potency and more than 1,000-fold functional selectivity for the mu over delta opioid receptor, comparable to the greater than 1,500-fold selectivity of DAMGO. The structural requirements for binding to the mu or delta opioid receptor are not necessarily the same as the structural requirements for activation of the receptor. Thus, variations in affinity are not matched by parallel changes in potency. This is perhaps most graphically illustrated by the L-trans-hydroxyphenylproline derivative 1a and the L-Hat analogue 2a, which displayed comparable affinities at the mu receptor but 20-fold different potencies. This highlights the need to measure relative efficacy in SAR studies. Finally, the retained high affinity of the p-F-Phe$^1$ analogue 5 for the mu opioid receptor suggests a reduced role for the tyrosyl para-hydroxyl substituent common to opioid peptides in this series of cyclic tetrapeptides.
3.3 Peptides lacking para-hydroxy! subsituents

As shown above, the cyclic tetrapeptide containing para-fluorophenylalanine in position 1 maintains high affinity for the mu opioid receptor despite lacking a para-hydroxy! substituent, implicating a reduced role for this moiety in the binding of this series of cyclic tetrapeptides to the mu opioid receptor. Indeed, a recent publication has reported that a phenylalanine containing peptide is also capable of high affinity mu receptor binding [Mosberg et al., 1998]. To test this hypothesis, thirteen analogues of JOM-6 (Tyr-c[\(\text{D-Cys-Phe-D-Pen}\)]NH\(_2\) (Et)) in which the tyrosine in position 1 was replaced by various aromatic residues lacking a para-hydroxy! substituent, as well as one peptide containing cyclohexylalanine, were characterised for their ligand binding affinities and potencies and relative efficacies in the \([^{35}\text{S}]\text{-GTP}\gamma\text{S}\) binding assay.

Replacement of the Tyr\(^1\) residue of JOM-6 with phenylalanine gave Phe-c[\(\text{D-Cys-Phe-D-Pen}\)]NH\(_2\) (Et) (6), whilst the same substitution in JOM-5 gave Phe-c[\(\text{D-Cys-Phe-D-Pen}\)]NH\(_2\) (7). Retaining the Phe\(^1\) residue of 6 and 7 but altering the ring closure to a cis-ethene bridge gave Phe-c[\(\text{D-Cys-Phe-D-Pen}\)]NH\(_2\) (cis-Ey) (8). The bridging groups used are the same as in the previous section, but are shown again here for convenience (Figure 3.9).

![Structure of bridging groups used to close cyclic peptide system. The general structure is as shown in Figure 3.2.](image)

Figure 3.9
Chapter 3: Cyclic Tetrapeptides

Figure 3.10 Structure of the N-terminal residues of the cyclic tetrapeptides. The general structure is as shown in Figure 3.2.
Chapter 3: Cyclic Tetrapeptides

The following modified amino acids were also incorporated in JOM-6 as substitutions in place of Tyr; phenylglycine (phenylGly) (9), homophenylalanine (homoPhe) (10), diphenylalanine (diphenylAla) (11), 3-(1-naphthylalanine) (1-naphthylAla) (12), 3-(2-naphthylalanine) (2-naphthylAla) (13), trans-phenylproline (trans-phenylPro) (14), cis-phenylproline (cis-phenylPro) (15), 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (isoquinoline) (16), 2-amino-2-carboxytetralin (tetralin) (17), and cyclohexylalanine (cyclohexylAla) (18). The structures of the amino acids used are shown in Figure 3.10.

3.3.1 Results

3.3.1.1 Radioligand binding assays

The Phe analogue of the dithioethane containing JOM-6, 6, retained affinity at the mu receptor only 5-fold less than the parent peptide but exhibited 40-fold reduced affinity for the delta receptor, resulting in a 750-fold selectivity for mu over delta receptors. However, the Phe analogue of JOM-5 (cyclized via a disulfide bond), 7, had 50-fold reduced affinity for the mu receptor and extremely low affinity for the delta receptor. Peptide 8, cyclized via a cis-ethene bond, exhibited intermediate affinity for the mu opioid receptor and very low affinity for the delta receptor.

Varying the α carbon to phenyl ring chain length as in the phenylglycine analogues 9a and 9b and the homophenylalanine peptide 10 resulted in greatly reduced affinity for the mu opioid receptor, by a minimum of 50-fold. Additional steric bulk in the N-terminal residue was however tolerated in the 3-(1-naphthylalanine) (12) and 3-(2-naphthylalanine) (13) analogues which displayed only 10-fold reduced affinities when compared to the Phe compound 6, but not in the diphenylalanine containing peptide (11) which had 100-fold reduced affinity (Table 3.4).
<table>
<thead>
<tr>
<th>Peptide structure</th>
<th>Amino acid sequence</th>
<th>Bridge</th>
<th>Analogue Name</th>
<th>Binding $K_i$ (nM)</th>
<th>Selectivity $K_i(\delta)/K_i(\mu)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-d-Ala-Gly-N-McPhe-Gly-ol</td>
<td>None</td>
<td>DAMGO$^2$</td>
<td>4.13 ± 0.84</td>
<td>2,540 ± 155</td>
<td>615</td>
</tr>
<tr>
<td>Tyr-[d-Pen-Gly-Phe-d-Pen]OH</td>
<td>S–S</td>
<td>DPDPE</td>
<td>810 ± 66</td>
<td>3.98 ± 0.46</td>
<td>0.005</td>
</tr>
<tr>
<td>Tyr-[d-Cys-Phe-d-Pen]NH$_2$</td>
<td>S–S</td>
<td>JOM-5</td>
<td>7.01 ± 0.45</td>
<td>57.7 ± 4.7</td>
<td>8.23</td>
</tr>
<tr>
<td>Tyr-[d-Cys-Phe-d-Pen]NH$_2$</td>
<td>S–Et–S</td>
<td>JOM-6</td>
<td>0.29 ± 0.04</td>
<td>24.8 ± 1.46</td>
<td>83.3</td>
</tr>
<tr>
<td>Phe-[d-Cys-Phe-d-Pen]NH$_2$</td>
<td>S–Et–S</td>
<td>6</td>
<td>1.36 ± 0.4</td>
<td>1,021 ± 91</td>
<td>750</td>
</tr>
<tr>
<td>Phe-[d-Cys-Phe-d-Pen]NH$_2$</td>
<td>S–S</td>
<td>7</td>
<td>352 ± 37</td>
<td>&gt; 10,000</td>
<td>&gt; 28</td>
</tr>
<tr>
<td>Phe-[d-Cys-Phe-d-Pen]NH$_2$</td>
<td>S–cis-Ey–S</td>
<td>8</td>
<td>16.4 ± 4.2</td>
<td>&gt; 10,000</td>
<td>&gt; 610</td>
</tr>
<tr>
<td>phenylGly-[d-Cys-Phe-d-Pen]NH$_2$</td>
<td>S–Et–S</td>
<td>9a</td>
<td>361 ± 47.2</td>
<td>&gt; 10,000</td>
<td>&gt; 27</td>
</tr>
<tr>
<td>phenylGly-[d-Cys-Phe-d-Pen]NH$_2$</td>
<td>S–Et–S</td>
<td>9b</td>
<td>754 ± 53.9</td>
<td>&gt; 10,000</td>
<td>&gt; 13</td>
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<tr>
<td>homoPhe-[d-Cys-Phe-d-Pen]NH$_2$</td>
<td>S–Et–S</td>
<td>10</td>
<td>1,956 ± 249</td>
<td>&gt; 10,000</td>
<td>&gt; 5.1</td>
</tr>
</tbody>
</table>

Table 3.4  Opioid receptor binding profiles of cyclic tetrapeptides. Displacement of [³H]-DAMGO (0.6 nM, mu), [³H]-DPDPE (1.8 nM, delta), or [³H]-U69,593 (0.9 nM, kappa) from guinea pig brain homogenates. Data represent mean ± standard error of the mean from three or more separate experiments. Data provided by Dr. Henry Mosberg of the School of Pharmacy, University of Michigan. Continued on next page.
<table>
<thead>
<tr>
<th>Peptide structure</th>
<th>Bridge (^1)</th>
<th>Analogue Name</th>
<th>Binding (K_i) (nM)</th>
<th>Selectivity (K_i(\delta) / K_i(\mu))</th>
</tr>
</thead>
<tbody>
<tr>
<td>diphenylAla-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>11</td>
<td>146 ± 18.8</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>1-naphthylAla-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>12</td>
<td>15.5 ± 1.6</td>
<td>4,268 ± 446</td>
</tr>
<tr>
<td>2-naphthylAla-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>13</td>
<td>12.2 ± 3.5</td>
<td>4,243 ± 526</td>
</tr>
<tr>
<td>trans-phenylPro-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>14a</td>
<td>1,079 ± 51.1</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>trans-phenylPro-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>14b</td>
<td>14.3 ± 2.1</td>
<td>1,369 ± 39.2</td>
</tr>
<tr>
<td>cis-phenylPro-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>15a</td>
<td>2,645 ± 574</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>cis-phenylPro-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>15b</td>
<td>11.8 ± 2.8</td>
<td>2,260</td>
</tr>
<tr>
<td>isoquinoline-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>16</td>
<td>979 ± 117</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>tetrahn-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>17a</td>
<td>7.2 ± 1.0</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>tetrahn-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>17b</td>
<td>44.5 ± 12.5</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>cyclohexylAla-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>18</td>
<td>32.5 ± 7.4</td>
<td>&gt; 10,000</td>
</tr>
</tbody>
</table>

Table 3.4 Opioid receptor binding profiles of cyclic tetrapeptides. Continued from previous page.

1 Bridge between the second and fourth amino acids (second and fifth amino acids in DPDPE)

   S-Et-S denotes \(\text{--S--CH}_2\text{--CH}_2\text{--S--}\), and S-cis-Et-S denotes \(\text{--S--CH=CH--S--}\)

2 Determined in rat brain membranes

3 Taken from Mosberg et al [1988]
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The peptides 14 to 17 contain analogues of phenylalanine which are conformationally restricted about the Ca-Cβ bond. The peptides 14, 15 and 17 were each prepared using a racemic mixture of the starting material for the N-terminal residue, resulting in a pair of stereoisomers in each case. One of the pair of trans-phenylproline stereoisomers (14b), one of the cis-phenylproline peptides (15b) and both of the 2-amino-2-carboxytetralin stereoisomers (17a and 17b) exhibited high affinity for the mu opioid receptor (Table 3.4). The remainder, namely the phenylproline stereoisomers 14a and 15a and the 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid peptide (16), each showed low affinity.

Surprisingly, when the phenylalanine of 6 is replaced by cyclohexylalanine as in the analogue 18 (cyclohexylAla-c[D-Cys-Phe-d-Pen]NH₂ (Et)), moderate affinity for the mu opioid receptor of 58.8 nM is retained.

The majority of the peptides examined showed low affinity for the delta receptor with Kᵢ values >10,000 nM, but the peptides 7, 12, 13, 14b and 15b showed affinity for the delta receptor of 1,000 nM to 4,500 nM. Selectivities of the individual compounds for the mu over delta receptors were difficult to determine in those cases where delta receptor affinities were greater than 10,000 nM, but in some cases were extremely high. For example, the tetralin analogue 17a exhibited a mu over delta opioid receptor selectivity of almost 1,400-fold.

3.3.1.2 [³⁵S]-GTPγS binding assays

Analogues with affinity for the mu opioid receptor of < 500 nM were examined in the [³⁵S]-GTPγS binding assay, in order to determine their efficacy relative to the full mu agonists fentanyl and DAMGO (Table 3.5 and Figures 3.11 and 3.12). All of the analogues tested gave EC₅₀ values between 1.0 and 4.2 times higher than their affinities (Kᵢ) measured in the ligand binding assay, with the exception of JOM-6, 6, 7 and 15b, which were approximately 9 to 13-fold higher.
<table>
<thead>
<tr>
<th>Peptide structure</th>
<th>Amino acid sequence</th>
<th>Bridge (^1)</th>
<th>Analogue</th>
<th>EC(_{50}) (nM)</th>
<th>max (%) (^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-d-Ala-Gly-N-MePhe-Gly-ol</td>
<td>None</td>
<td>DAMGO</td>
<td>6.4 ± 1.45</td>
<td>108.9 ± 12.0</td>
<td></td>
</tr>
<tr>
<td>Tyr-c[D-Pen-Gly-Phe-D-Pen]OH</td>
<td>S-S</td>
<td>n.d. (^4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-S</td>
<td>JOM-5</td>
<td>17.8 ± 3.56</td>
<td>98.2 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>JOM-6</td>
<td>2.93 ± 0.83</td>
<td>125.8 ± 6.0 (^3)</td>
<td></td>
</tr>
<tr>
<td>Phe-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>6</td>
<td>18.1 ± 4.72</td>
<td>98.1 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Phe-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-S</td>
<td>7</td>
<td>4,465 ± 210</td>
<td>45.1 ± 1.8 (^3)</td>
<td></td>
</tr>
<tr>
<td>Phe-[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-cis-Ey-S</td>
<td>8</td>
<td>52.0 ± 6.19</td>
<td>84.5 ± 1.8 (^3)</td>
<td></td>
</tr>
<tr>
<td>diphenylAla-c[D-Cys-Phe-D-Pen]NH(_2)</td>
<td>S-Et-S</td>
<td>9</td>
<td>286.5 ± 19.4</td>
<td>101.7 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5  Potency and relative efficacy of cyclic tetrapeptides. Stimulation of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding in membranes of \(\text{C}_6\text{μ}\) cells, as described in Methods. Data represent mean ± standard error of the mean from 3 or more separate experiments. Only those peptides exhibiting affinity at the mu opioid receptor < 500 nM were tested. Continued on next page.
<table>
<thead>
<tr>
<th>Peptide structure</th>
<th>Analogue</th>
<th>EC$_{50}$ (nM)</th>
<th>max (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-naphthylAla-c[D-Cys-Phe-D-Pen]NH$_2$</td>
<td>S–Et–S 12</td>
<td>36.9 ± 1.75</td>
<td>93.9 ± 5.1</td>
</tr>
<tr>
<td>2-naphthylAla-c[D-Cys-Phe-D-Pen]NH$_2$</td>
<td>S–Et–S 13</td>
<td>12.7 ± 2.71</td>
<td>114.7 ± 3.3 $^3$</td>
</tr>
<tr>
<td><em>trans</em>-phenylPro-c[D-Cys-Phe-D-Pen]NH$_2$</td>
<td>S–Et–S 14b</td>
<td>44.2 ± 3.56</td>
<td>98.43 ± 5.6</td>
</tr>
<tr>
<td><em>cis</em>-phenylPro-c[D-Cys-Phe-D-Pen]NH$_2$</td>
<td>S–Et–S 15b</td>
<td>105.8 ± 6.26</td>
<td>105.6 ± 1.6</td>
</tr>
<tr>
<td>tetralin-c[D-Cys-Phe-D-Pen]NH$_2$</td>
<td>S–Et–S 17a</td>
<td>23.3 ± 2.00</td>
<td>85.6 ± 3.8 $^3$</td>
</tr>
<tr>
<td>tetralin-c[D-Cys-Phe-D-Pen]NH$_2$</td>
<td>S–Et–S 17b</td>
<td>73.5 ± 11.3</td>
<td>102.7 ± 4.2</td>
</tr>
<tr>
<td>cyclohexylAla-c[D-Cys-Phe-D-Pen]NH$_2$</td>
<td>S–Et–S 18</td>
<td>58.8 ± 11.1</td>
<td>95.8 ± 3.3</td>
</tr>
</tbody>
</table>

Table 3.5  Potency and relative efficacy of cyclic tetrapeptides. Continued from previous page.

1 Bridge between the second and fourth amino acids (second and fifth amino acids in DPDPE)


2 Values represent percentage of the maximal response to 10 µM fentanyl

3 Significantly different to the response to 10 µM fentanyl, $p < 0.05$ (Student's t test)

4 n.d., not determined
Of the Phe^1 peptides containing dithioethane bridging groups, only 13 was capable of stimulating[^{35}S]-GTPγS binding comparable to that produced by a maximal concentration of JOM-6, approximately 115 % and 126 % respectively of that produced the mu full agonist fentanyl. The remainder, namely 7, 11, 12, 14b, 15b, 17b, and including the cyclohexylAla^1 derivative 18, all exhibited maximal stimulation equivalent to that of fentanyl. However, the cis-dithioethene analogue 8 and the dithioethane analogue 17a produced maximal stimulation significantly less than that of fentanyl, approximately 85 % and 86 % respectively, while the disulfide analogue 7 produced only 45 % stimulation at 10 μM, the highest concentration tested.

![Graph showing stimulation of[^{35}S]-GTPγS binding](image)

**Figure 3.11** Stimulation of[^{35}S]-GTPγS binding in C₆μ cell membranes by DAMGO (◆), JOM-6 (■), 6 (▲) or 18 (▼). Data represent percentage of the maximal response to 10 μM fentanyl, means ± standard error of the mean from three or more separate experiments, as described in Methods.
Figure 3.12 Stimulation of $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in C6μ cell membranes by DAMGO (◆), 6 (■), 14b (▲) or 15b (▼). Data represent percentage of the maximal response to 10 μM fentanyl, means ± standard error of the mean from three or more separate experiments, as described in Methods.

3.3.2 Discussion

The results reported here indicate that the tyrosyl hydroxyl moiety is not an absolute requirement for the binding of opioid peptides with agonist properties to the mu opioid receptor, as has previously been believed [Morgan et al, 1976; Chang et al, 1976], since the Phe$^1$ analogue of JOM-6, 6, showed only 4-fold and 6-fold reduced affinity and potency respectively when compared to the parent peptide. This modest decrease in affinity and potency is presumably due to the loss of the favorable contribution from the hydrogen bond which is believed to exist between the tyrosyl hydroxyl group and the His297 residue in transmembrane domain VI of the human mu opioid receptor [Pogozheva et al, 1998]. However, 6 did exhibit affinity and potency 10- and 5.8-fold higher respectively than the para-fluorophenylalanine analog 5, indicating that the electron withdrawing characteristics of the para-fluoro substituent are undesirable for mu opioid receptor binding. The ring present in the first residue, traditionally tyrosine, need not even be aromatic in nature since the
peptide 18 exhibited reasonable affinity and potency and was a full agonist in the 
\[^{35}\text{S}]\text{GTPyS}\) assay despite possessing a cyclohexyl ring.

As discussed earlier, the relative position of the aromatic rings of the first and third residues has been shown to play a crucial role in the binding of peptides to the opioid receptors [Mosberg et al., 1996; Wang et al., 1998]. In this series, the Tyr\(^1\) containing and dithioethane bridged peptide JOM-6 showed approximately 25-fold higher affinity than the corresponding disulfide analogue JOM-5. Replacement of the Tyr\(^1\) residue of JOM-6 with Phe\(^1\), giving 6, resulted in only a 5-fold reduction in affinity and potency at the mu opioid receptor. In contrast, loss of the tyrosyl hydroxyl group of JOM-5, giving 7, caused a drastic reduction in mu receptor binding affinity and potency. Thus, only when cyclization was via a dithioether bridging group as in JOM-6, and not when cyclization was via a disulfide bond as in JOM-5, can the Tyr\(^1\) residue be replaced with Phe\(^1\) without abolishing affinity.

The cis-ethene containing analogue of 6 and 7 is the peptide 8, which exhibits intermediate affinity and potency at the mu opioid receptor. The cis-ethene bridging group of 8 limits flexibility of the ring compared to the ethane group of 6 since the double bond is rotationally restricted, and yet still allows greater flexibility than the direct disulfide bond of 7. Thus the pharmacophoric elements of the analogue 8 may be able to assume the required conformation for binding to the mu opioid receptor, less readily than in 6 due to unfavorable energy barriers imposed by the rotational restriction of the double bond, but more readily than in 7. This confirms similar findings with the related Tyr\(^1\) analogues.

At the delta receptor, inclusion of a Phe\(^1\) residue is extremely deleterious to binding regardless of the nature of the bridging group joining the D-Cys\(^2\) and D-Pen\(^2\) residues. Both 7 and 8 exhibited affinities > 10,000 nM whilst 7 had an affinity of 1,000 nM, 40-fold lower than its Tyr\(^1\) analogue JOM-6. Consequently, 6 showed greatly increased selectivity over JOM-6. Thus the tyrosyl hydroxyl group appears to be vital to interactions of peptides with the delta receptor.
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In the homologous series of analogues 6, 9, and 10, optimal affinity for the mu opioid receptor occurs when the chain length linking the α carbon and the aromatic ring of the first residue is 1 carbon atom, as in phenylalanine. When the chain length is zero, as in phenylglycine (9a and 9b), or two, as in homophenylalanine (10), affinity is reduced at least 250-fold. Affinity at the delta receptor is similarly affected. Thus in the analogues 9 and 10, the conformational space accessible to the N-terminal residue phenyl ring does not allow it to assume a favorable position relative to that of the third residue, and therefore to fit into an appropriate hydrophobic binding pocket in the receptor.

Additional bulk in the aromatic portion of the N-terminal amino acid can be accommodated without adverse steric interactions. When naphthalene is incorporated into the first residue via attachment at either the 1 or 2 position giving increased steric bulk fused to the aromatic ring, as in 12 and 13, mu receptor binding is reduced only 10-fold relative to the analogue 6. Potency at the mu opioid receptor is either unaffected, in 13, or reduced 2-fold, in 12. However, when the first residue incorporates an additional phenyl ring as a substitution at the β carbon, as in 11, mu receptor binding affinity is reduced 100-fold, indicating that the tolerance of additional steric bulk depends upon its position.

The cis and trans isomers of phenylproline (14 and 15) were used to examine the effects of conformational restriction of the first amino acid about the Cα-Cβ but not Cβ-Cγ bonds. Since a racemic mixture of each phenylproline isomer was used in the synthesis, four analogues resulted - a pair of trans-phenylprolines (L and D) and a pair of cis-phenylprolines (L and D). Within each pair, one isomer showed high affinity for the mu opioid receptor (14b and 15b), but the complementary pair exhibited low affinity (14a and 15a). The difference in affinities between diastereomers was approximately 75-fold for the trans-phenylprolines and almost 225-fold for the cis-phenylprolines. The high affinity analogues also exhibited moderate potencies in the [35S]-GTPγS assay. This suggests that the first residues of both high affinity analogues are able to assume a common arrangement of the first residue which is suitable for binding to and activation of the mu opioid receptor, but which is outside the conformational space accessible to the peptides which exhibit low affinity. Since the
L-trans and L-cis-phenylprolines are stereoisomers, they can never assume a conformation in which the primary nitrogen and aromatic rings are overlapped. However, the L-trans and D-cis phenylprolines can assume a common conformation, as can the D-trans and L-cis isomers (Figure 3.13). Generally, in a pair of peptides containing the same N-terminal amino acid in both L and D forms, the L-analogue exhibits far greater affinity for the mu opioid receptor. However, since in each pair of phenylprolines which can assume a common conformation there is an L- and a D-isomer, it is impossible without further stereochemical characterization to predict which pair corresponds to the high affinity analogues 14b and 15b and which to the low affinity 14a and 15a.

Restriction of rotation about the Cα-Cβ and Cβ-Cγ bonds has been examined in two ways. Firstly, formation of a bicyclic structure via cyclization to the amide nitrogen gives the N-terminal 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid analogue 16, which had drastically reduced affinity for both the mu and delta opioid receptors. In contrast, formation of a bicyclic structure spiro to the α carbon gives the 2-amino-2-carboxytetralin analogue 17. Synthesis of 17 used a racemic mixture of the 2-aminotetralin-2-carboxylic acid hydrobromide starting material, giving rise to the two stereoisomers 17a and 17b. Both bound to the mu opioid receptor with affinities of 5.6 nM and 44.5 nM respectively, although this represents almost 20-fold and 150-fold decrease when compared to JOM-6. Potency at the mu opioid receptor as determined in the [35S]-GTPγS binding assay was only reduced approximately 10-fold and 20-fold respectively when compared to JOM-6, but the higher potency analogue 17a showed significantly lower efficacy than either JOM-6. Binding to the delta opioid receptor was completely abolished. The analogue 17a is tentatively assigned as L-2-amino-2-carboxytetralin-D-Cys-Phe-D-PenNH₂ (Et) and therefore 17b is assigned as D-2-amino-2-carboxytetralin-D-Cys-Phe-D-PenNH₂ (Et) on the basis that the higher affinity analogue of a pair of stereoisomers is generally the L-isomer. As seen above, the related L- and D-2-amino-2-carboxy-6-hydroxytetralin analogues exhibited affinity, potency and efficacy at both mu and delta opioid receptors identical to that of their parent compound JOM-6. In contrast, conformational restriction does cause a reduction of affinity in the tetralin-containing analogues 17a and 17b when compared to the Phe⁴ peptide 6.
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Figure 3.13  Overlap of four phenylproline isomers: \textit{L-trans} (green); \textit{L-cis} (magenta); \textit{D-trans} (white); and \textit{D-cis} (orange). Nitrogen is shown in blue; oxygens, red; and hydrogens, cyan.

Perhaps the most surprising finding in this group of peptides was that the cyclohexylAla\textsuperscript{1} containing analogue 18 exhibited approximately 100-fold reduced, but still good, affinity for the mu opioid receptor when compared to JOM-6. Potency in the \(^{[35}S\)-GTP\textsubscript{\gamma}S assay was decreased almost 20-fold, but 18 still produced maximal stimulation, indicating good efficacy. Loss of aromaticity in the first residue would be expected to cause a complete loss of affinity and potency for both the mu and delta opioid receptor, since an aromatic ring is traditionally considered a crucial pharmacophoric element [Casy and Parfitt, 1986]. However, binding to the mu receptor was decreased but not abolished. In contrast, aromaticity in the N-terminal residue was critical for binding to the delta receptor in this series.
In conclusion, it has been shown here that the Tyr\textsuperscript{1} residue in this series of cyclic tetrapeptides can be replaced with Phe\textsuperscript{1} and a variety of related residues lacking a hydroxyl group without drastic reductions in affinity, potency and relative efficacy at the mu opioid receptor, suggesting that although the tyrosyl hydroxyl group is important in interactions with the mu opioid receptor it is not critical. Indeed, even aromaticity of the N-terminal residue is not vital, since the cyclohexylalanine analogue 18 exhibited moderate affinity and potency and full maximal response in the \textsuperscript{[3S]}GTP\gammaS assay. Consequently, it is possible that at the N-terminus any relatively planar, hydrophobic group may be able partially to substitute for the tyrosyl aromatic ring in these cyclic tetrapeptides by forming suitable van der Waals interaction(s) with the appropriate region of the mu receptor, although it must be located in the correct position relative to the \(\alpha\)-carbon and is subject to steric restrictions in fitting into the binding pocket, which comprises at least in part of hydrophobic residues in the fifth (Ile234), sixth (Ile296 and Val300) and seventh (Cys321) transmembrane domains.

As discussed earlier, there are several peptides related to somatostatin which are high affinity mu opioid ligands despite lacking a para-hydroxyl substituent in the initial residue. However, CTAP and CTOP are antagonists, whilst the cyclic tetrapeptides are fully efficacious agonists, many with high potency at the mu opioid receptor. In transmembrane domain VII of the mu receptor there is a tryptophan residue that is substituted for a leucine found in the same position in the delta receptor. It has been proposed [Mosberg et al, 1998] that the interaction of the tryptophan with the cyclic peptide system of a related series of peptides causes a shift in the docking mode such that the oxygen of the Tyr\textsuperscript{1} residue is shifted approximately 0.7 Å away from its proposed hydrogen bonding partner, the His of transmembrane domain VI. This increased separation may explain the reduced role of the tyrosine para-hydroxyl substituent in the binding of these peptides to the mu opioid receptor. Note however that the role of the hydroxyl group is not totally abolished, since JOM-6 is more efficacious and has higher affinity than the phenylalanine analogue 6.
3.4 Peptides containing nitrogen in the N-terminal residue

As a consequence of the discovery that affinity for the mu opioid receptor is retained in certain cyclic tetrapeptides incorporating initial residues lacking a para-hydroxyl substituent, four analogues of JOM-6 (Tyr-c[D-Cys-Phe-D-Pen]NH₂ (Et)) were characterised to examine the influence of aromatic rings containing nitrogen in the first residue. Effects were measured on ligand binding affinities, as well as potencies and relative efficacies in the [³⁵S]-GTPγS binding assay.

The following natural and modified amino acids were incorporated in JOM-6 as residue 1 substitutions; 3-(3-pyridyl)alanine (pyridylAla) (19), histidine (His) (20), tryptophan (Trp) (21), and 2-amino-2-carboxyindan (indan) (22). These peptides were compared with the phenylalanine containing 6 and the tyrosine containing JOM-6. The structures of the amino acids used are shown in Figure 3.14.

![Figure 3.14](image)

Figure 3.14 Structure of the N-terminal residues of the cyclic tetrapeptides. The general structure is as shown in Figure 3.2.
<table>
<thead>
<tr>
<th>Peptide structure</th>
<th>Amino acid sequence</th>
<th>Bridge&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Analogue Name</th>
<th>Binding $K_i$ (nM)</th>
<th>Selectivity $K_i(\delta)/K_i(\mu)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-d-Ala-Gly-N-MePhe-Gly-ol</td>
<td>None</td>
<td>DAMGO&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.13 ± 0.84</td>
<td>2,540 ± 155</td>
<td>615</td>
</tr>
<tr>
<td>Tyr-c[D-Pen-Gly-Phe-d-Pen]OH</td>
<td>S–S</td>
<td>DPDPE</td>
<td>810 ± 66</td>
<td>3.98 ± 0.46</td>
<td>0.005</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-d-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>S–Et–S</td>
<td>JOM-6&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.29 ± 0.04</td>
<td>24.8 ± 1.46</td>
<td>83.3</td>
</tr>
<tr>
<td>Phe-c[D-Cys-Phe-d-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>S–Et–S</td>
<td>6</td>
<td>1.36 ± 0.38</td>
<td>1,021 ± 91</td>
<td>750</td>
</tr>
<tr>
<td>pyrrolylAla-c[D-Cys-Phe-d-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>S–Et–S</td>
<td>19</td>
<td>8.7 ± 0.3</td>
<td>&gt; 10,000</td>
<td>&gt; 1,150</td>
</tr>
<tr>
<td>His-c[D-Cys-Phe-d-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>S–Et–S</td>
<td>20</td>
<td>1,535 ± 246</td>
<td>&gt; 10,000</td>
<td>&gt; 6.5</td>
</tr>
<tr>
<td>Trp-c[D-Cys-Phe-d-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>S–Et–S</td>
<td>21</td>
<td>93.4 ± 13.3</td>
<td>&gt; 10,000</td>
<td>&gt; 107</td>
</tr>
<tr>
<td>indan-c[D-Cys-Phe-d-Pen]NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>S–Et–S</td>
<td>22</td>
<td>137 ± 20.5</td>
<td>&gt; 10,000</td>
<td>&gt; 73.0</td>
</tr>
</tbody>
</table>

Table 3.6  Opioid receptor binding profiles of cyclic tetrapeptides. Displacement of $[^3]H$-DAMGO (0.6 nM, mu), $[^3]H$-DPDPE (1.8 nM, delta), or $[^3]H$-U69,593 (0.9 nM, kappa) from guinea pig brain homogenates. Data represent mean ± standard error of the mean from 3 or more separate experiments. Data provided by Dr. Henry Mosberg of the School of Pharmacy, University of Michigan.

<sup>1</sup> Bridge between the second and fourth amino acids (second and fifth amino acids in DPDPE)

<sup>2</sup> S–Et–S denotes $-S-CH_2-CH_2-S-$, and S–cis-Ey–S denotes $-S-CH=CH-S-$

<sup>3</sup> Taken from Mosberg <i>et al</i> [1988]
Chapter 3: Cyclic Tetrapeptides

3.4.1 Results

3.4.1.1 Radioligand binding assays

The pyridy1Ala\(^1\) analogue of the dithioethane containing JOM-6, 19, retained good affinity (K\(_d\) = 8.7 nM) at the mu opioid receptor, only 6-fold reduced compared to the phenylalanine\(^1\) containing peptide 6. In addition, 19 exhibited a complete loss of affinity for the delta receptor, resulting in an extremely high selectivity (greater than 1,150-fold) for mu over delta opioid receptors. Replacement of Phe\(^1\) with His\(^1\), as in the analogue 20, was greatly detrimental to binding at both mu and delta opioid receptors. Indeed, at the mu receptor affinity was reduced more than 1,000-fold compared to the Phe\(^1\) containing 6. The Trp\(^1\) and indan\(^1\) containing peptides 21 and 22 showed very similar moderate to poor affinities at the mu opioid receptor, approximately 100-fold reduced when compared to 6, and very poor affinities at the delta receptor.

3.4.1.2 \[^{35}\text{S}\]-GTP\(_\gamma\)S binding assay

Analogues with affinity for the mu opioid receptor of < 500 nM were examined in the \[^{35}\text{S}\]-GTP\(_\gamma\)S binding assay, in order to determine their efficacy relative to the full mu agonists fentanyl and DAMGO. These results are shown in Table 3.7 and Figure 3.15.

The pyridy1Ala\(^1\) analogue 19 exhibited maximal stimulation greater than fentanyl and comparable to JOM-6, even though potency was much lower at approximately 588 nM. The difference between affinity and potency in 19 was very large, approximately 67-fold. The tryptophan peptide 21 displayed maximal stimulation of 87 %, significantly less than seen for fentanyl, but potency only about 3-fold lower than its affinity. The indan analogue 22 gave maximal stimulation equivalent to that of fentanyl and 4-fold reduced potency relative to its binding affinity.
<table>
<thead>
<tr>
<th>Peptide structure</th>
<th>Analogue</th>
<th>EC₅₀ (nM)</th>
<th>max (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-D-Ala-Gly-N-MePhe-Gly-ol</td>
<td>None</td>
<td>DAMGO</td>
<td>6.4 ± 1.45</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S-S</td>
<td>JOM-5</td>
<td>17.8 ± 3.56</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S-Et-S</td>
<td>JOM-6</td>
<td>2.93 ± 0.83</td>
</tr>
<tr>
<td>Phe-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S-Et-S</td>
<td>6</td>
<td>18.1 ± 4.72</td>
</tr>
<tr>
<td>pyridylAla-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S-Et-S</td>
<td>19</td>
<td>587.8 ± 34.7</td>
</tr>
<tr>
<td>Trp-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S-Et-S</td>
<td>21</td>
<td>248.7 ± 22.1</td>
</tr>
<tr>
<td>mdan-c[D-Cys-Phe-D-Pen]NH₂</td>
<td>S-Et-S</td>
<td>22</td>
<td>529.7 ± 43.8</td>
</tr>
</tbody>
</table>

Table 3.7  Potency and relative efficacy of cyclic tetrapeptides. Stimulation of [³⁵S]-GTPγS binding in membranes of C6 μ cells, as described in Methods. Data represent mean ± standard error of the mean from 3 or more separate experiments. Only those peptides exhibiting affinity at the mu opioid receptor < 500 nM were tested (see Table 3.6).

¹ Bridge between the second and fourth amino acids (second and fifth amino acids in DPDPE)

S-Et-S denotes -S-CH₂-CH₂-S-, and S-cis-Ey-S denotes -S-CH=CH-S-

² Values represent percentage of the maximal response to 10 μM fentanyl

³ Significantly different to the response to 10 μM fentanyl, p < 0.05 (Student's t test)
3.4.2 Discussion

The introduction of a nitrogen atom into the aromatic moiety of the first residue reduced mu opioid receptor binding in the analogues tested. However, the close analogue of the Phe¹ peptide 6, the 3-(3-pyridyl)alanine containing 19, retained good affinity and exhibited good relative efficacy at the mu receptor. However, 19 displayed much reduced potency, with a much larger difference between potency and affinity than was seen with other cyclic tetrapeptides. The EC₅₀/Kᵲ ratio for 19 was 67.5 compared to 3.9 for 22, 13.3 for 6, or 10.1 for JOM-6. This may indicate that the structural requirements for binding to the mu opioid receptor are different to those for activation.

The indan analogue 22 displayed moderate affinity and low potency, although it produced a full maximal response. As with the tetralin and hydroxytetralin analogues discussed above, in the 2-amino-2-carboxyindan residue rotation about the Cα-Cβ and Cβ-Cγ bonds is restricted by the formation of a bicyclic structure spiro to the α
carbon. The reduction in potency of the indan analogue 22 compared to the pyridylAla-containing 19 (15-fold) is much greater than was seen with the hydroxyaminotetralin peptides 2a and 2b compared to the tyrosine containing JOM-6 (only 1.4-fold), or the tetralin analogue 17a compared to the phenylalanine peptide 6 (5.3-fold). Thus, the low affinity of 22 is not the result of conformational restriction, since similarly constrained peptides have retained high affinity. Neither is it a result of the inclusion of a nitrogen atom, since the pyridylAla peptide exhibited good affinity. Therefore it must be a result of the difference in the nature of the ring spiro to the \( \alpha \)-carbon, which is 6-membered in the case of the hydroxyaminotetralin and aminotetralin analogues 2a, 2b, and 17a but 5-membered in the indan peptide 22. This change forces the pyridyl ring of 22 to assume a different position in space than the phenyl ring of 17a and the pyridyl ring of 19, which can overlap, by approximately half the diameter of the ring (Figure 3.16).

Figure 3.16  Overlap of the initial residues of the peptides 19 (pyridylalanine, white), 22 (2-amino-2-carboxy-indan, orange) and 17a (2-amino-2-carboxy-tetralin, magenta). Nitrogen is shown in blue; oxygens, red; and hydrogens, cyan.
Chapter 3: Cyclic Tetrapeptides

This shift in position of the aromatic ring also moves the nitrogen of the pyridyl ring of 22 relative to that of 19, by approximately 1.7 Å within the plane of the ring. It is unclear whether the cause of the large decrease in affinity seen with 22 is the shift in position of the aromatic ring, the nitrogen, or both.

The His¹ analogue 20 displayed a 5,000 and 1,125-fold decrease in affinity at the mu opioid receptor as compared to the peptides JOM-6 and 7 respectively. The side chain imidazole moiety of histidine has a pKₐ value of 6.2, and therefore will only be 6.3 % protonated at physiological pH [Tandford, 1962]. Thus the decrease in affinity is likely to be due to either the altered size electronic characteristics of the aromatic ring, rather than protonation of the histidine.

There are three possibilities that explain the moderate binding affinity and potency, and reduced maximal response of the tryptophan containing peptide 21 at the mu opioid receptor. In the first, the presence of the indole group causes the phenyl ring to be incorrectly positioned within the binding domain of the receptor and thus unable to interact efficiently with its complementary feature. Secondly, the additional steric bulk of tryptophan compared to the simple phenyl ring of more traditional peptides may cause unfavourable steric interactions with the residues comprising the binding pocket. Lastly, the electronic characteristics of the tryptophan group compared to those of phenylalanine or tyrosine may be incompatible with mu opioid receptor binding.
In conclusion, the results reported here confirm that binding to the mu opioid receptor can be maintained in the absence of a tyrosyl hydroxyl substituent, and furthermore that the aromatic ring may be heterocyclic. However, affinity for the delta receptor is reduced, as a consequence of which a peptide 19 has been identified with greater than 1,150-fold selectivity for mu over delta opioid receptors. As seen previously, changes in affinity are not matched by parallel changes in potency, in particular with the analogues 19 and 22 which exhibited EC$_{50}$/K$_i$ ratios of 67.5 and 3.9 respectively. This highlights the possibility that structural requirements for binding to the mu opioid receptor are not necessarily the same as those for activation.
Chapter 4

THE STEROID SC17599
4.1 Introduction

The traditional pharmacophore for mu opioid ligands highlights the importance of the amine nitrogen, aromatic ring, and hydroxyl group, as discussed in the introduction [Casy and Parfitt, 1986]. In the previous chapter, members of a series of cyclic opioid tetrapeptides lacking any suitable para-hydroxyl feature have been shown to retain high affinity for the mu opioid receptor. Likewise, a single example of a tetrapeptide lacking both the hydroxyl group and the aromatic feature was shown to exhibit good affinity and potency and to be a full agonist. Thus, the stress placed on the importance of the aromatic and hydroxyl moieties in the traditional mu opioid pharmacophore is called into question, at least in this series of opioid tetrapeptides. In this chapter, the role of the same features in the binding of non-peptide ligands to the opioid receptors is examined through the characterisation of a steroid, 17α-acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxy-pregna-3,5-dien-20-one (SC17599) which has been reported to possess opioid actions in vivo [Craig, 1968].

It has been shown that the majority of steroids are incapable of interacting with high affinity with the three opioid receptor subtypes, namely mu, delta and kappa. For example, steroids from the androgen, glucocorticoid, mineralocorticoid, and gestagen families were ineffective at displacing bound radioligand from rat brain homogenates at concentrations up to 100 μM [LaBella et al, 1978; Schwarz and Pohl, 1994]. Of the hundreds of endogenous and synthetic steroids tested, the highest affinity for the mu opioid receptor has consistently been shown to belong to members of the oestrogen family, including diethylstilbestrol, 17α-estradiol, and 17α-dihydroequilinene, presumably due to the presence of an aromatic A ring and suitably placed hydroxyl group. However, even these steroids only display affinities in the micromolar range, 200-fold or more lower than that shown by morphine [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 pharmacophore detailed above. Although the role of the aromatic ring has been questioned in the previous chapter, the importance of the amine nitrogen is still undisputed [Casy and Parfitt, 1986]. There are no known
examples of ligands with high affinity for any of the three opioid receptors that lack such a feature, which is almost invariably tertiary in alkaloid opioid ligands, and either primary or less commonly secondary in peptide ligands. All 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.

There is one exception; the steroid 17α-acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxy-pregna-3,5-dien-20-one (SC17599) contains a tertiary nitrogen (Figure 4.1) and has been shown to possess marked antinociceptive potency *in vivo* as measured by the mouse writhing test, the mouse hot plate, and the rat tail flick assay [Craig, 1968]. In addition, SC17599 markedly depressed the respiratory rate and increased pCO₂ in rabbits, and caused a reduction in gastrointestinal motility and afforded the Straub tail response in mice [Craig, 1968]. Potency in all cases was less than that of morphine. Although the respiratory depressive effects of SC17599 were reversed by pretreatment with nalorphine, the antinociceptive effects of SC17599 in the rat tail flick assay were unaffected [Craig, 1968].

![Figure 4.1](image)

Figure 4.1  The steroid 17α-acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxy-pregna-3,5-dien-20-one, or SC17599.
Thus, with this one exception SC17599 presents the typical in vivo action profile of a mu opioid agonist. However, it is possible that SC17599 could be exerting its in vivo effects indirectly through stimulation of the release of endogenous peptides, rather than by acting directly at mu opioid receptors, although a closely related analogue of SC17599 lacking a methyl substituent in the 10-position, SC22000, has been reported to bind to opioid receptors in mouse brain with affinity 30-fold less than morphine [LaBella et al, 1978].

If SC17599 is indeed exerting its antinociceptive actions through a direct action at the mu opioid receptor, it represents a highly novel structure for a mu opioid ligand. Although SC17599 possesses a tertiary nitrogen, it lacks both the aromatic and hydroxyl features stressed by the traditional mu opioid pharmacophore (Figure 4.1) [Morgan et al, 1976; Casy and Parfitt, 1986; Lomize et al, 1996]. The characterisation of the direct interaction of SC17599 with the mu receptor directly addresses the importance of these moieties.
4.2 Results

4.2.1 Antinociceptive assays

In order to confirm the finding that SC17599 exhibits antinociceptive actions \textit{in vivo} [Craig, 1968], the effects of the steroid in the mouse acetic acid induced writhing and mouse warm-water tail withdrawal procedures were evaluated (see Methods) and compared to those of morphine.

In the warm water tail (50 °C) withdrawal assay, SC17599 and morphine both produced dose-dependent antinociception (Figure 4.2). SC17599 was approximately 2.5-fold less potent than morphine, \( ED_{50} \) values of 25.1 mg/kg and 10.5 mg/kg respectively.

![Figure 4.2](image)

Figure 4.2 Antinociceptive effects of cumulative doses of either morphine (■) or SC17599 (▲) in the warm water (50 °C) tail withdrawal assay, as described in Materials and Methods. Points, mean \( \pm \) standard error of the mean from five separate experiments performed in duplicate.
In the acetic acid induced writhing assay, SC17599 produced a significant and dose-dependent suppression of writhing (Figure 4.3a) with potency approximately 10-fold less than that shown by morphine (Figure 4.3b). The actions of both ligands were reversed by pretreatment with both the non-selective opioid antagonist naltrexone (1.0 mg/kg, 15 min) and the mu selective antagonist M-CAM (1.8 mg/kg, 1 hour).

Figure 4.3  Antagonism of the antinociceptive effects of SC17599 (a) and morphine (b) by naltrexone (NTX) (1.0 mg/kg, 15 min) and methocinnamox (M-CAM) (1.8 mg/kg, 1 hour) in the mouse acetic acid induced writhing assay, as described in Materials and Methods. Points, mean ± standard error of the mean from 6-12 separate experiments performed in duplicate. *, significantly different to control (100 %), p < 0.05 (Student's t test) **, significantly different to agonist treatment (10 mg/kg), p < 0.05 (Student's t test)
4.2.2 Radioligand binding studies

Having confirmed the naltrexone reversible antinociceptive effects of SC17599 in vivo, the direct interaction of the steroid with the opioid receptors was examined. In membranes from SH-SY5Y cells endogenously expressing mu opioid receptors SC17599 displaced both the selective mu agonist $[^{3}\text{H}]$-DAMGO and the non-selective antagonist $[^{3}\text{H}]$-diprenorphine (DPN) in a concentration-dependent manner, giving affinities of 16.3 and 19.1 nM respectively (Figure 4.4 and Table 4.1).

![Graph depicting displacement by SC17599 of the binding of either $[^{3}\text{H}]$-DPN (0.2 nM) (▲) or $[^{3}\text{H}]$-DAMGO (1.0 nM) (▼) from membranes of SH-SY5Y cells in Tris buffer, as described in Materials and Methods. Points, mean ± standard error of the mean from three or more separate experiments performed in duplicate.](image-url)
<table>
<thead>
<tr>
<th>[³H]-Ligand</th>
<th>Membranes</th>
<th>Conditions</th>
<th>Receptor</th>
<th>Kᵢ (nM)</th>
<th>Selectivity over:</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMGO</td>
<td>SH-SY5Y</td>
<td>in Tris</td>
<td>μ</td>
<td>16.3 ± 2.6</td>
<td>143.7</td>
</tr>
<tr>
<td>DPN</td>
<td>SH-SY5Y</td>
<td>in Tris</td>
<td>μ</td>
<td>19.1 ± 3.9</td>
<td>122.9</td>
</tr>
<tr>
<td>DPN</td>
<td>SH-SY5Y</td>
<td>in Buffer A</td>
<td>μ</td>
<td>146.3 ± 20.9</td>
<td></td>
</tr>
<tr>
<td>DPN</td>
<td>C₆μ</td>
<td>in Tris</td>
<td>μ</td>
<td>62.3 ± 5.4</td>
<td>37.7</td>
</tr>
<tr>
<td>DPN</td>
<td>C₆μ</td>
<td>in Buffer A</td>
<td>μ</td>
<td>434.0 ± 43.5</td>
<td></td>
</tr>
<tr>
<td>DPN</td>
<td>CHOδ</td>
<td>in Tris</td>
<td>δ</td>
<td>2,348 ± 509.5</td>
<td></td>
</tr>
<tr>
<td>CI977</td>
<td>GPBH ²</td>
<td>in Tris</td>
<td>κ</td>
<td>1,959 ± 927.5</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Table 4.1 Opioid receptor binding profile of SC17599. Displacement of radioligands from membranes as described in Methods; [³H]-DPN (0.2 nM), [³H]-DAMGO (1.0 nM), [³H]-CI977 (1.0 nM). Data represent mean ± standard error of the mean from three or more separate experiments. Buffer composition as described in Methods; briefly, Buffer A contains 100 mM Na⁺ and 10 mM Mg²⁺, whilst Tris buffer is Na⁺ and Mg²⁺ free.

¹ Morphine displaced [³H]-DPN (0.2 nM) from membranes of C₆μ cells in Tris buffer with a Kᵢ value of 1.90 ± 0.11 nM

² Guinea pig brain homogenate, see Methods.
Similarly, SC17599 displaced $[^3]$H-diprenorphine from membranes of \(C_6\mu\) cells with a \(K_i\) of 62.3 nM, approximately 5-fold lower than that shown by morphine in the same cell line (\(K_i\) 11.7 nM) (Figure 4.5 and Table 4.1).

![Figure 4.5](image.png)

**Figure 4.5** Displacement of the binding of $[^3]$H-DPN (0.2 nM) from membranes of \(C_6\mu\) cells by morphine (■) or SC17599 (▲) in Tris buffer, as described in Materials and Methods. Points, mean ± standard error of the mean from three or more separate experiments performed in duplicate.

When the buffer system was changed from Tris buffer to buffer A which contains 100 mM Na\(^+\) and 10mM Mg\(^{2+}\) ions (see Methods), the dose response curve for the displacement of $[^3]$H-diprenorphine in \(C_6\mu\) cell membranes was shifted to the right in parallel fashion by approximately 7.7-fold, affording a \(K_i\) of 434.0 nM (Figure 4.6 and Table 4.1). Similarly, a 7.0-fold rightward parallel shift was seen in the displacement of $[^3]$H-diprenorphine in SH-SY5Y cell membranes, such that the \(K_i\) in buffer A was 146.3 nM.
Chapter 4: The Steroid SC17599

![Graph showing displacement of binding](image)

Figure 4.6 Displacement of the binding of $[^3H]$-DPN (0.2 nM) from membranes of C6μ cells by SC17599 in the absence (▲) (Tris-HCl buffer) or presence (♦) (buffer A) of 100 mM Na$^+$ and 10 mM Mg$^{2+}$, as described in Materials and Methods. Points, mean ± standard error of the mean from three or more separate experiments performed in duplicate.

A small selection of other steroids were tested for their ability to displace $[^3H]$-DAMGO in SH-SY5Y cell membranes. Three estrogens (17α-estradiol, 17β-estradiol and estrone) were chosen since 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 a relatively high concentration (10 μM) only approximately 40% of the bound $[^3H]$-DAMGO was displaced (Figure 4.7 and Table 4.2). In contrast, SC17599 displaced virtually all bound radioligand at the same concentration.
Figure 4.7 Displacement by 10 μM of the specified steroids of bound [³H]-DAMGO (1.0 nM) from membranes of SH-SY5Y cells. Assays were performed as described in Materials and Methods. Points, mean ± standard error of the mean from three or more separate experiments performed in duplicate. *, significantly different to control (100%), p < 0.05 (Student’s t test)

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Specific binding remaining (%)</th>
<th>Steroid</th>
<th>Specific binding remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-pregnanolone</td>
<td>102.5 ± 5.5</td>
<td>Triamcinolone</td>
<td>95.1 ± 3.3</td>
</tr>
<tr>
<td>β-pregnanolone</td>
<td>99.6 ± 2.9</td>
<td>Dexamethasone</td>
<td>94.8 ± 3.7</td>
</tr>
<tr>
<td>17α-estradiol</td>
<td>59.2 ± 3.8 *</td>
<td>Hydrocortisone</td>
<td>99.6 ± 4.3</td>
</tr>
<tr>
<td>17β-estradiol</td>
<td>94.0 ± 2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>84.4 ± 3.6</td>
<td>SC17599</td>
<td>2.1 ± 3.7 *</td>
</tr>
</tbody>
</table>

Table 4.2 Binding of steroids to the mu opioid receptor. Displacement of [³H]-DAMGO (1.0 nM) from SH-SY5Y cell membranes by 10 μM each of the various steroids, as described in Methods. Data represent mean ± standard error of the mean from three or more separate experiments. * Significantly different to 100 %, p < 0.05 (Student’s t test)
SC17599 also produced dose-dependent displacement of both \[^{3}\text{H}\text{-diprenorphine}\] from CHO\(\delta\) cell membranes and of the kappa selective agonist \[^{3}\text{H}\text{-CI977}\] from guinea pig brain homogenates but with very low affinity, giving \(K_\text{i}\) values of 2,348 nM and 1,950 nM respectively (Figure 4.8 and Table 4.1).

![Graph](image)

**Figure 4.8** Displacement by SC17599 of either bound \[^{3}\text{H}\text{-DPN}\] (0.2 nM) from membranes of CHO\(\delta\) cells (\(\Delta\)) or bound \[^{3}\text{H}\text{-CI977}\] (0.2 nM) from guinea pig brain homogenates (\(\blacktriangledown\)). Assays were performed as described in Materials and Methods. Points, mean ± standard error of the mean from three or more separate experiments performed in duplicate.

In cytosolic fractions from Sf9 cells infected with a mouse glucocorticoid receptor baculovirus, SC17599 was unable to displace \[^{3}\text{H}\text{-triamcinolone}\] (TA) at concentrations up to 100 \(\mu\text{M}\). In contrast, dexamethasone exhibited a \(K_\text{i}\) of 0.17 ± 0.04 nM under the same conditions (Figure 4.9).
Figure 4.9 Displacement of the binding of [³H]-TA (1.0 nM) from cytosolic fractions of Sf9 cells by SC17599 (■) or dexamethasone (▲). Assays were performed as described in Materials and Methods. Points, mean ± standard error of the mean from three or more separate experiments performed in duplicate.

4.2.3 [³S]-GTPγS binding studies

The shift in the affinity of SC17599 for the mu opioid receptor induced by the presence of 100 mM Na⁺ and 10 mM Mg²⁺ ions indicates agonist properties, which was confirmed by the results of [³S]-GTPγS binding assays. In C₆ cell membranes the mu selective agonist fentanyl stimulated the binding of [³S]-GTPγS with a potency (EC₅₀) of 10.3 ± 0.9 nM. A high concentration of fentanyl (10 μM) was used to define maximal stimulation in all [³S]-GTPγS binding studies. In the same cell line morphine stimulated binding of [³S]-GTPγS cells with potency of 21.1 ± 1.8 nM and gave an equivalent maximal response.
In C₆ cell membranes SC17599 stimulated [³⁵S]-GTPγS binding with an EC₅₀ of 248.3 ± 63.2 nM and maximal stimulation equivalent to that of both morphine and fentanyl (Figure 4.10).

Figure 4.10 Stimulation of [³⁵S]-GTPγS (100 pM) binding to membranes of C₆ cells by morphine (□) or SC17599 (▲). Assays were performed as described in Materials and Methods. Points, mean ± standard error of the mean from three or more separate experiments performed in duplicate.

In membranes from SH-SY5Y cells SC17599 stimulated the binding of [³⁵S]-GTPγS with an EC₅₀ of 282.3 ± 42.4 nM and maximal response equivalent to that of fentanyl. This effect was antagonized by naloxone (10 nM), which shifted the dose-response curve to the right by approximately 5.6 fold, allowing an apparent affinity for naloxone of 2.2 nM (Figure 4.11).

In wild type C₆ cells, SC17599 like fentanyl, the delta agonist BW 373,U86 and the kappa agonist U69,593 did not significantly stimulate [³⁵S]-GTPγS binding at concentrations up to 10 µM (data not shown).
Figure 4.11 Stimulation of \([^{35}S]-\text{GTPyS}\) (100 pM) binding to membranes of SH-SY5Y cells by SC17599 in the absence (▲) and presence (▼) of naloxone (10 nM). Assays were performed as described in Materials and Methods. Points, mean ± standard error of the mean from three or more separate experiments performed in duplicate.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Membranes</th>
<th>EC(_{50}) (nM)</th>
<th>Maximal (%) ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>C6μ</td>
<td>23.4 ± 3.7</td>
<td>95.6 ± 2.11</td>
</tr>
<tr>
<td>Morphine</td>
<td>SH-SY5Y</td>
<td>31.8 ± 3.2</td>
<td>98.7 ± 3.5</td>
</tr>
<tr>
<td>SC17599</td>
<td>C6μ</td>
<td>248.3 ± 64.2</td>
<td>93.9 ± 2.3</td>
</tr>
<tr>
<td>SC17599</td>
<td>SH-SY5Y</td>
<td>282.3 ± 42.4</td>
<td>100.5 ± 2.4</td>
</tr>
<tr>
<td>SC17599</td>
<td>SH-SY5Y (10 nM NX)</td>
<td>1,570 ± 175.7</td>
<td>96.5 ± 5.0</td>
</tr>
</tbody>
</table>

Table 4.3 Potency and relative efficacy of SC17599 and morphine. Stimulation of \([^{35}S]-\text{GTPyS}\) binding in membranes of either C6μ or SH-SY5Y cells, as described in Methods. Data represent mean ± standard error of the mean from 3 or more separate experiments.

¹ maximal stimulation (100 %) defined by 10 μM fentanyl
Chapter 4: The Steroid SC17599

4.3 Discussion

4.3.1 Antinociceptive assays

The results reported here confirm the previous finding that SC17599 exhibits antinociceptive actions \textit{in vivo} [Craig, 1968]. In both the acetic acid induced writhing and warm water tail withdrawal procedures in mice, SC17599 was fully effective at dose-dependently producing antinociception. In both assays the steroid was less potent than morphine; 2.5-fold in the tail withdrawal assay, and 10-fold in the writhing assay.

The antinociceptive effects of SC17599 in the acetic acid induced writhing assay were reversed by both the non-selective opioid antagonist naltrexone and the mu selective antagonist M-CAM. Taken together, these data suggest that SC17599 is acting \textit{via} mu opioid receptors to produce its antinociceptive effects. Further work on the \textit{in vivo} actions of SC17599 in this laboratory by Hani Houshyar has shown that pretreatment with naltrexone shifts the dose-response curve for both SC17599 and morphine in the mouse warm water tail withdrawal assay to the right in parallel fashion, whilst pretreatment with M-CAM completely abolishes the ability of SC17599 to exert its antinociceptive effects. Additionally, both morphine and SC17599 produced the Straub tail response in mice [Houshyar \textit{et al}, 1999]. However, some interesting differences between the actions of SC17599 and morphine were highlighted in this study. In a mouse tail withdrawal time course assay, SC17599 was shown to possess a significantly longer duration of action than morphine, with full antinociceptive effects lasting up to 4 hours after injection, compared to 2 1/2 hours for morphine. In a locomotor activity assay in mice, morphine significantly enhanced activity whereas SC17599 did not.

Thus, SC17599 appears to be a mu opioid agonist in several \textit{in vivo} preparations, longer acting than morphine but less potent.
SC17599 displaced two radioligands, namely $[^3]$H-diprenorphine and $[^3]$H-DAMGO, from mu opioid receptors in SH-SY5Y cell membranes with almost identical affinities, approximately 18 nM. In C66 cell membranes affinity was lower by about 3.5-fold at 62.3 nM as measured by displacement of $[^3]$H-diprenorphine, but still good. In this cell line SC17599 exhibited approximately 5-fold lower affinity for the mu opioid receptor than morphine ($K_i, 11.7$ nM). SC17599 bound with greatly reduced affinity to the delta and kappa opioid receptors, exhibiting between 38- and 145-fold and 32- and 120-fold selectivity respectively. In cytosolic fractions from Sf9 cells, dexamethasone displayed high affinity for the glucocorticoid receptor ($K_i, 0.17 \pm 0.04$ nM), whereas SC17599 did not bind significantly at concentrations up to 10 $\mu$M suggesting that the steroid is not a glucocorticoid receptor ligand.

The ability of SC17599 to bind with good affinity to the mu opioid receptor is not shared by any of the other steroids tested. Dexamethasone, $\alpha$-pregnanolone, $\beta$-pregnanolone, triamcinolone, hydrocortisone, 17$\beta$-estradiol, and estrone were all unable to displace $[^3]$H-DAMGO from SH-SY5Y cell membranes. 17$\alpha$-estradiol was able to bind to the mu opioid receptor, but with very low affinity. This is consistent with previous data [LaBella et al, 1978; LaBella, 1985; Schwarz and Pohl, 1994]. The ability of 17$\alpha$-estradiol but not 17$\beta$-estradiol to bind to the mu opioid receptor indicates a role for the 17-hydroxyl group in the interaction of these steroids with the mu receptor. This substituent is unlikely to correspond to the para-hydroxyl group of traditional opioids, a role which would be more logically assigned to the hydroxyl substituent of the phenolic A ring of the steroid. Rather, the 17-hydroxyl moiety provides a favourable interaction which does not correspond to any feature in the more traditional opioid structures.

Displacement of $[^3]$H-diprenorphine from the mu opioid receptor in both SH-SY5Y and C66 cell membranes was shifted to the right in the presence of 100 mM Na$^+$ and 10 mM Mg$^{2+}$. The presence of sodium ions causes a shift in the equilibrium of the high and low affinity states of the receptor in favor of the low affinity state. The 'Allosteric Ternary Complex Model' (Figure 1.14 in the Introduction) [De Lean et al,
Chapter 4: The Steroid SC17599

1980] predicts that agonists bind preferentially to the high affinity state of the receptor, whilst antagonists exhibit no preference. Therefore, the rightward shift in the concentration-effect curve for displacement of bound radioligand by SC17599 in the presence of Na$^+$ ions implies that SC17599 possesses agonist properties.

4.3.3 $[^{35}S]$-GTPγS binding studies

The $[^{35}S]$-GTPγS binding assay provides a functional measure of agonist occupation of mu opioid receptors, allowing for determination of the potency and relative efficacy of a ligand [Hilf et al., 1987; Traynor and Nahorski, 1995]. For example, morphine stimulates binding of $[^{35}S]$-GTPγS in membranes from SH-SY5Y cells with high potency, only 2-fold lower than fentanyl at approximately 20 nM, and equivalent maximal response.

The agonist properties of SC17599 suggested by the rightward shifts in the binding curves in the presence of Na$^+$ and Mg$^{2+}$ ions were confirmed by the stimulation of $[^{35}S]$-GTPγS binding in membranes from two cell lines. In both cases EC$_{50}$ values were approximately 12-fold lower than shown by morphine, with a maximal stimulation equivalent to that afforded by fentanyl. In SH-SY5Y cell membranes and in the presence of naloxone (10 nM), the concentration-effect curve for SC17599 in the $[^{35}S]$-GTPγS binding assay was shifted to the right by 5.6-fold. The apparent pA$_2$ value afforded was 8.66, indicating that SC17599 stimulates binding of $[^{35}S]$-GTPγS under these conditions via mu opioid receptor activation [Traynor and Nahorski, 1995]. This naloxone-induced rightward shift in the concentration effect curve did not cause any change in maximal stimulation, showing that SC17599 interacts with the mu opioid receptor. The low potency of SC17599 relative to its binding affinity is due to the use in the $[^{35}S]$-GTPγS binding assay of buffer A, which contains 100 mM Na$^+$ and 10 mM Mg$^{2+}$ ions, causing a shift in the equilibrium state of the receptor as described above. The K$_s$ value from the ligand binding assay (434 nM) and the EC$_{50}$ value from the $[^{35}S]$-GTPγS binding assay (248 nM) were comparable when the same sodium containing buffer system was used.

In C6 wild type cell membranes lacking any opioid receptors, high concentrations of SC17599 and the traditional opioid ligands fentanyl (mu), BW373, U86 (delta) and
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U69,593 (kappa) were all unable to stimulate $[^{35}S]$-GTPγS binding above baseline, indicating that the agonist activity of SC17599 described above is indeed due to an opioid receptor mediated mechanism of action.

Thus, SC17599 is a full agonist at the mu opioid receptor, and exhibits good affinity and potency both in vivo and in vitro. It is selective for the mu over the delta and kappa receptors, and is unable to bind to the glucocorticoid receptor. The activity of the steroid brings into question traditional structure-activity findings for mu opioid agonists. It lacks both the aromatic and phenolic hydroxyl moieties which are thought to be crucial to the activity of morphine-like and peptide opioid ligands. SC17599 is extremely unusual in its ability to bind to mu opioid receptors whilst lacking any aromatic feature. Aside from the close analog SC22000 [LaBella et al, 1978], the only other reported example known to us is a set of ozonolysis products of etorphine-like compounds which exhibited antinociceptive actions with potencies similar to morphine [Bentley et al, 1969] (Figure 1.17 in the Introduction). In these molecules it has been suggested that the loss of the aromatic ring is compensated for by the contributions of features in other areas of the molecule. A simple manual overlap of SC17599 with morphine and etorphine based on the position of the quaternary nitrogen and the orientation of the N⁰–H bond suggests that this may be possible for the steroid (Figure 4.11). The delocalised electron system of the A and B rings may be able to substitute for the aromatic A ring of more traditional opioids. A redefined model of the mu opioid pharmacophore based on this simple alignment is shown in Figure 4.12.

These results, taken together with those reported in the previous chapter, indicate that the aromatic and hydroxyl groups play a less important role in the binding of both peptides and small ligands to the mu opioid receptor than was previously believed. Therefore a more extensive structure-activity relationship study of mu opioid ligands forms the basis of the next chapter.
Figure 4.12  Manual overlap of morphine (orange), etorphine (magenta) and SC17599 (white). Nitrogens are shown in blue; oxygens, red; hydrogens, cyan and fluorine in green.

Figure 4.13  Modified pharmacophore for the mu opioid receptor based on the manual overlap shown in Figure 4.12.
Chapter 5

MOLECULAR MODELLING
Chapter 5: Molecular Modelling

5.1 Introduction

The use of computers to visualise the three dimensional shape of molecules, their properties and their interactions has only become possible through the revolutionary advances in processing power in the late 1980's and throughout the 1990's. As available computing power increases, modelling software can approach the level of complexity necessary to accurately reproduce experimental results. In pharmacology, this has allowed molecular modelling techniques to be applied to the generation of pharmacophores, the quantitative investigation of structure-activity relationships, the docking of ligands to receptors and even the tracking of the motion of entire receptor proteins in simulated membrane environments.

5.1.1 Pharmacophore modelling

The number of known ligands which bind to opioid receptors is huge, and they represent perhaps more structural classes than ligands for any other type of receptor, making opioids particularly well suited to pharmacophore generation. However, although it is logical to assume that all ligands of similar structure bind to the opioid receptors in the same manner, it is possible that this is not the case for largely unrelated structures. For example, the sheer difference in size between the morphine-like ligands and large endogenous peptides such as \( \beta \)-endorphin suggests at the very least that the latter accesses parts of the binding pocket untouched by the former. This leads to the concept of ‘message’ and ‘address’, whereby certain structural features form a core element common to all opioid ligands (the message) whilst other molecule-specific moieties determine selectivity and potency (the address) [Porthogese et al, 1988b; Metzger et al, 1996]. Similarly, it has has been noted that the structure-activity relationships in fentanyl and related analogues do not follow the same patterns seen in morphine-like ligands [Casy et al, 1969; Casy and Parfitt, 1986], raising the possibility that the 4-anilino-piperidines bind to the mu opioid receptor in a different manner than the tyramine containing peptides and alkaloids [Ferguson et al, 1999].
There have been many attempts to identify opioid pharmacophores using various sets of candidate ligands and different modelling packages [Cometta-Morini et al, 1991; Froimowitz, 1993; Huang et al, 1997]. To date, the majority have identified a single binding mode common to all opioid ligands, stressing the importance of the amine nitrogen, aromatic feature, and hydroxyl substituent. However, as seen in the previous chapters, these moieties may play a lesser role in the binding of ligands to the mu opioid receptor than has previously been believed. Therefore in this work the mu opioid pharmacophore has been re-examined, using a genetic algorithm (GA) based package.

5.1.2 Quantitative structure-activity relationships

A parallel approach to pharmacophore generation is the study of quantitative structure-activity relationships (QSAR), whereby a ‘training set’ of ligand structures together with corresponding biological activity data is used to predict the steric and electronic structural characteristics which enhance or decrease that activity. This approach has several advantages; firstly, it can pinpoint small contributing factors which are missed in more gross analyses such as pharmacophore generation. Secondly, the accuracy of the model can be validated by generating predictions of activity for well characterised ligands. Finally, the predictions of a QSAR study can be used to identify new candidate ligands and hence drive design and synthesis initiatives.

The greatest limitations of the technique lie in the selection of the training set and the manner in which the chemical features of each molecule are aligned. Since all QSAR programs use the spatial co-ordinates of the molecules to compare and contrast their structures and activities, the starting alignment is crucial. Also, the inclusion of an excessive number of ligands from one structural class or the exclusion of more structurally varied compounds can bias the results by effectively ‘weighting’ the calculations. In this work a genetic algorithm has been used to align a diverse set of mu selective opioid ligands with no preset constraints. The package used generates alignments based not on individual atoms or functional groups, but by considering the similarities between the electrostatic, steric, and/or hydrophobic fields of the entire molecule. The resulting alignment has been used to quantify the structure-activity
relationships of opioid ligands using COrparitive Molecular Field Analysis (CoMFA).

5.1.3 Receptor models and ligand docking

A particularly powerful application of molecular modelling is the docking of ligands to receptors, which can highlight important residues in the binding pocket and the manner in which they interact with the ligand. The predicted docking modes can be validated by previously known structure-activity relationship and mutagenesis data. However, the construction of the receptor model is obviously critical [Bikker et al, 1998] and in this case extremely difficult, since high resolution experimental structures of the three opioid receptors are currently unavailable. When this is the case, 'homology modelling' techniques are often applied, whereby a model is generated based upon the crystal structures of closely related proteins. In the case of the opioid receptors this is again impossible since no G-protein coupled receptor structure is available at sufficiently high resolution.

However, structural data is available for bacteriorhodopsin. This is a light sensing protein found in bacteria which contains seven transmembrane domains and a covalently bound ligand. Bacteriorhodopsin does not couple to G-proteins; instead, it acts as a proton pump. One common approach to modelling the opioid receptors is to 'thread' the amino acid sequence of the target receptor onto a scaffold based on the structure of bacteriorhodopsin, taken from either the original x-ray resolution [Henderson and Unwin, 1975] or one of the more recent images [Grigorieff et al, 1996; Pebay-Peyroula et al, 1997].

Mammalian rhodopsin is a closer analogue of G-protein coupled receptors than bacteriorhodopsin. It is the light sensing protein found in the retina, and although it consists of seven transmembrane domains and couples to a G-protein it is not truly a receptor since, like bacteriorhodopsin, it contains its own ligand. An alternative to the approach described above using bacteriorhodopsin is to construct a model by using the low resolution electron cryo-microscopic structure of mammalian rhodopsin [Schertler et al, 1993; Schertler and Hargrave, 1995; Unger et al, 1997] as a guide to the orientation of helices built de novo, using hydrophobicity data, sequence analysis
of many similar receptors, or distance constraints based upon hydrogen bonding networks. Both methods benefit from the large quantities of mutagenesis data available for G protein coupled receptors, either as initial design constraints or as independent validation.

Within the transmembrane helices are invariant residues common to all G protein coupled receptors which serve as common anchor points when modelling these regions [Bikker et al, 1998]. However, the extracellular and intracellular loops vary greatly between receptors, not only in terms of amino acid sequence but also in length. In addition, the loops have much greater conformational flexibility than the highly structured helices. Due to these difficulties, these regions are omitted entirely from many models as being inherently speculative. However, the loops are thought to play important roles in both ligand recognition (extracellular) and G-protein coupling (intracellular) and therefore more recent models have tended to include the smaller loops. Modelling of the N- and C-terminus portions of receptors, which are often very large, is still impossible with any degree of certainty.

It is important to note that all receptor models provide only a single, static conformation, whereas these proteins are predicted to possess a great deal of flexibility in vivo. Indeed, both ligand docking and receptor activation are predicted to involve the movement of individual residues, small regions, or whole helices. However, molecular dynamics of G-protein coupled receptors is unfortunately still unrealistic, since the starting models are of uncertain accuracy. Therefore, for the moment at least, static models are the norm, although there is a great deal of work being done in this area [Fanelli et al, 1995; Ferguson et al, 1999].

Only a few attempts have been made to model the opioid receptors. One such mapped the mu opioid receptor onto a consensus model generated from the sequence of almost 500 G-protein coupled receptors [Ferguson et al, 1999], and examined the docking of a number of fentanyl analogues. Another model was derived from distance geometry calculations using hydrogen bonding constraints [Pogozheva et al, 1998]. In previous work this latter group used the sequence of over 400 G-protein coupled receptors to generate a set of distance constraints between pairs of residues which participate in H-
bonds within the α-helical bundle. Application of these distance constraints in iterative calculations gave a template for the position and orientation of the seven α-helical transmembrane domains in an ‘average’ G-protein coupled receptor. This was initially used as the basis for a model of bovine rhodopsin [Pogozheva et al, 1997], and later extended to the opioid receptors [Pogozheva et al, 1998] and used to examine the docking of a selection of peptide and non-peptide opioid ligands.

In this study, the latter model was kindly provided by Dr. Henry Mosberg of the School of Pharmacy, University of Michigan and used to dock a small selection of mu opioid ligands, including the steroid SC17599. A ribbon representation of the model is shown in Figure 5.1. The first intracellular loops as well as the first, second and third extracellular loops are included in the model. However, the second and third intracellular loops are omitted, as are both terminii. The binding pocket as identified by the authors [Pogozheva et al, 1998] occurs towards the extracellular surface, near to transmembrane domains III, IV, V, VI and VII, and directly underneath the large second extracellular loop.
Figure 5.1  Ribbon representation of the mu opioid receptor model kindly provided by Dr. Henry Mosberg of the School of Pharmacy, University of Michigan. Morphine is shown docked.
5.2 Results

5.2.1 Automated pharmacophore generation

The mu opioid pharmacophore has been re-examined in light of the activity of the unusual opioid ligands discussed in previous chapters, namely the steroid SC17599, the cyclic tetrapeptides and the ozonolysis product of etorphine. SC17599 does not fit the traditional mu opioid pharmacophore since it lacks a suitable hydroxyl substituent (Figure 1.18). There are very few examples of peptide (with the exception of the cyclic tetrapeptides discussed earlier) or morphine-like opioid ligands which lack such a group, although it is more common amongst less traditional ligands such as the 4-arylpiperidines (e.g. fentanyl) and 3,3-diphenylpropylamines (e.g. methadone). In addition, SC17599 lacks an aromatic ring, and in this regard is almost unique amongst opioid ligands. The only other examples known to us are the cyclohexylalanine containing cyclic tetrapeptide 18 described in this thesis, and several ozonolysis products of etorphine-like compounds (see Figure 1.17). Due to the complexity and flexibility of the cyclic tetrapeptides this work has focused on SC17599 and the ozonolysis product of etorphine.

Automated pharmacophore generation using the GASP (Genetic Algorithm Superposition Program) module of SYBYL gave poor steric overlap between the quaternary forms of morphine and SC17599 (Figure 5.2). The overlap shown is a result of the emphasis placed by the algorithm on the creation of shared ‘site points’, hypothetical features with which both molecules can interact. For example, the nitrogen groups of both morphine and SC17599 can act as hydrogen bond donors interacting with the same theoretical feature within the receptor. Both molecules have several oxygen functionalities which biases the algorithm in favour of any alignment which allows the inclusion of multiple shared site points created by oxygen atoms acting as hydrogen acceptors. For example, the highest ranked solution to the overlap of morphine and SC17599 (of the four generated by GASP) contains 4 such site points (Figure 5.2). The positive contribution to the alignment score from these points far outweighs the negative contribution from poor steric overlap.
This analysis seems unrealistic in light of available structure-activity relationship data which indicates that the 6-hydroxyl and 4,5-epoxy moieties of morphine-like ligands are not required in order to maintain high binding affinity at the mu opioid receptor. For example, levorphanol is a high affinity mu opioid ligand which lacks these moieties [Randall and Lehmann, 1950]. It is possible to adjust the weighting factors assigned to the site points and steric factors in order to calculate an overlap score. However, satisfactory steric overlap of morphine and SC17599 is impossible since the steroid skeleton is much larger, with van der Waals volume of 439.1 compared to 242.8, as measured by SYBYL (arbitrary units).

Figure 5.2 Overlap of morphine (orange) and SC17599 (magenta) generated by GASP. Nitrogens are shown in blue; oxygens, red; hydrogens, cyan; fluorine, green, and hypothetical site points in green-blue.
Consequently, etorphine was included in the study since it is a high affinity opioid ligand with a volume comparable to that of SC17599 (368.0 and 439.1 respectively). An initial analysis of morphine and etorphine gave excellent overlap of the morphinan skeletons and close spatial superposition of the quarternary nitrogen and the interacting site point (Figure 5.3) in all four solutions. In all cases GASP also identified six additional site points, interacting with the 3-hydroxyl substituents (three site points), the 6-hydroxyl groups (two site points), and the aromatic A rings (one point) common to both molecules (Figure 5.3). Comparison of etorphine and its ozonolysis product gave the logical result, with the morphinan skeletons and 7-substituents being closely overlapped (Figure 5.4). There are a total of seven site points which interact with the quarternary nitrogen (one), the 6-methoxy group (two), the 19-hydroxyl substituent (three), and the 3-hydroxy group of etorphine and the ketone groups of the ozonolysis product (two).

Figure 5.3 Overlap of morphine (orange) and etorphine (magenta) generated by GASP. Nitrogens are shown in blue; oxygens, red; hydrogens, cyan, and hypothetical site points in green-blue.
Figure 5.4  Overlap of etorphine (orange) and its ozonolysis product (magenta) generated by GASP. Nitrogens are shown in blue; oxygens, red; hydrogens, cyan; and hypothetical site points in green-blue.

The four analyses of the alignment of SC17599 and etorphine were remarkably similar, with the top ranked solution being shown in Figure 5.5. The A, B and C rings of the steroid coincide with, and are broadly co-planar with, the etorphine rings A, B and E. The overlap of the A ring of the steroid with the aromatic A ring of etorphine suggests that the electron rich, relatively planar feature of the former may be able to substitute for the aromatic moiety of the latter in binding to the mu opioid receptor. The quaternary nitrogens are in close proximity and interact with the same site point. The steroid D ring and its subsituents project beyond the volume occupied by the morphinan skeleton, into the space corresponding to the etorphine 7-substituents. Here there is another site point which interacts with the oxygens of the 19-hydroxyl substituent of etorphine and the carbonyl of the 17α-acetoxyl group of SC17599. The increased steric bulk of etorphine allows for a more complete steric overlap. Only a few substituents lie outside the shared volume, namely the 19-methyl substituent of etorphine and the 17β-fluoroacetone and 3-ethoxy groups of SC17599.
Figure 5.5  Overlap of SC17599 (orange) and etorphine (magenta) generated by GASP. Nitrogens are shown in blue; oxygens, red; hydrogens, cyan; fluorine, green, and hypothetical site points in green-blue.

Finally, morphine, etorphine, the ozonolysis product of etorphine and SC17599 were analysed together (Figure 5.6). As shown, the four molecules aligned in manner consistent with the results of the previous three analyses (i.e. morphine/etorphine, etorphine/ozonolysis product and etorphine/SC17599). The net result is that the overlap of morphine and SC17599 in this case is similar to that of etorphine and SC17599, and in complete contrast to that seen when SC17599 and morphine were analysed alone. When all four molecules are present there is only one identified site point, which interacts with the amine nitrogen in all cases.
The amine nitrogen has always been considered the single most crucial pharmacophoric element, and of the many thousands of opioid ligands reported to date, none has lacked such a feature. As the single pharmacophoric element whose role has remained unchallenged to date, the amine nitrogen must be assumed to play a central role in any new opioid pharmacophore or docking mode. This has been confirmed by the results presented here, which show the amine nitrogen as the single pharmacophoric point common to all of the ligands analysed. The ability of SC17599 to bind with high affinity at the mu opioid receptor can be explained by its unexpected ability to mimic more traditional mu ligands such as morphine and etorphine, despite lacking any aromatic ring or para-hydroxyl substituent. However, as seen in previous chapters, these moieties may play reduced roles in the binding of ligands to the mu opioid receptor. The A ring of SC17599 is relatively planar and electron rich, which may enable it to substitute for an aromatic ring in interactions with the mu opioid receptor.

Figure 5.6 Overlap of SC17599 (white), morphine (orange), etorphine (magenta) and its ozonolysis product (purple) generated by GASP. Nitrogens are shown in blue; oxygens, red; hydrogens, cyan; fluorine, green, and hypothetical site points in green-blue.
5.2.2  Structure alignment

As described previously, GASP performs automated pharmacophore generation using atomic features to align molecules. In order to confirm the results seen with GASP, a set of 17 opioid ligands was aligned using a genetic algorithm (FBSS) which searches for similarity in the steric, hydrophobic and/or electrostatic fields surrounding the molecules. The ligands were chosen to represent a wide range of structural classes, and comprised:

etorphine, buprenorphine, diprenorphine (thebaine analogues); morphine, codeine, nalbuphine, naloxone (4,5-epoxymorphinans); butorphanol (a morphinan); pentazocine (a benzomorphan); pethidine and \( \alpha \)-prodine (4-phenylpiperidines); profadol (a 3-phenylpyrrolidine); fentanyl and alfentanil (4-anilinopiperidines); methadone (a diphenylpropylamine); etonitazene (a bezimidazole); and SC17599 (a steroid). See Figure 5.7 for structures.

No constraints were imposed on the nature of the overlap. Either morphine or etorphine were used as the target, the molecule to which the remainder of the ligands were aligned. Five runs were performed, as follows:

A) protonated form of ligands, full steric scoring, etorphine target
B) tertiary form of ligands, full steric scoring, etorphine target
C) tertiary form of ligands, full steric scoring, morphine target
D) tertiary form of ligands, partial steric scoring, morphine target
E) tertiary form of ligands, partial steric scoring, etorphine target

Within each run, the following analyses were used:

1) electrostatics + shape
2) electrostatics + hydrophobics + shape
3) electrostatics
4) electrostatics + hydrophobics

Thus, 20 alignments and associated scores were generated, as shown in Table 5.1. Note that the average similarity scores for the 17 ligands in the database cannot be used for comparison purposes between runs, since in each case either the database ligands, the target molecule, or the scoring function used was different. The scores can however be used to compare analyses within a run where the parameters are
consistent and the only variable is the combination of fields used to generate the alignment. The spatial position of the amine nitrogens of each of the 17 ligands was used as an additional measure of the goodness of the fit, since this feature has been highlighted in every examination of the mu opioid receptor pharmacophore to date.

Figure 5.7 Structures of mu opioid ligands used in the QSAR study. From left to right: Top row; etorphine, buprenorphine, and diprenorphine; 2nd row; morphine, codeine, nalbuphine, and naloxone; 3rd row; butorphanol, pentazocine, pethidine, α-prodine and profadol; and 4th row; fentanyl, alfentanil, methadone, and etonitazene.
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For the structure of SC17599 see Figure 1.18.

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Table 5.1 Scoring of the twenty FBSS alignments.

1 For the parameters used in each run see text.
2 A 'mis-aligned' ligand is defined as one whose amine nitrogen is not within 1 Å of the corresponding feature in the target molecule.
3 Qualitative score based on average similarity score, number of misaligned ligands and a visual assessment.
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The initial run using the quaternary, protonated form of each of the 17 ligands (run A) did not give good alignment in any of the four analyses due to the presence of the large charge on the amine nitrogen. Any alignment of a database molecule to the etorphine target which included close overlap of the amine nitrogens generated such a large positive electrostatic scoring contribution that any negative scores based on poor steric overlap were almost negligible in comparison. Although coincidence of the crucial amine nitrogens is the one remaining unequivocal criteria available for the generation of a new opioid pharmacophore, the alignment of other charge concentrations and of steric bulk is nonetheless important. Inclusion of the formal positive charge on the amine nitrogen inevitably biases the genetic algorithms calculations.

Using a database of the same ligands in uncharged, tertiary form (run B) produced much more balanced alignments. Within the run, analysis B1 scored highest on the qualitative scale, containing only 4 mismatches, i.e. molecules in which the amine nitrogens are not within 1 Å of the corresponding feature of the target. The lowest scoring analyses were those which did not include the steric field, namely B3 and B4. Inclusion of the hydropobicity field as in B2 and B4 actually decreases the ‘goodness’ of the alignment.

This pattern holds true for the remaining runs C, D and E also. Thus, within each run the same rank order of goodness of fit is seen:

- electrostatic + shape > electrostatic + shape + hydrophobicity ≈ electrostatics alone > electrostatic + hydrophobicity

i.e. \(1 > 2 \approx 3 > 4\)

Use of the full steric scoring (FSS) function, as in runs A, B and C, had a tendency to produce overlaps in which ligands with large nitrogen substituents were mis-aligned, for example pentazocine, nalbuphine and butorphanol. This was due to the negative scoring contribution generated by poor steric overlap of the large N-substituent of the database molecule with the much smaller N-methyl of the target (morphine or etorphine). The partial steric scoring (PSS) function corrects this problem by removing the negative scoring contribution that results from excess steric bulk of the database molecule compared to the target. One alternative would have been to choose
a target molecule with a large N-substituent with high affinity, such as buprenorphine or diprenorphine. However, the alignments generated by FBSS were used to create a quantitative-structure activity model not just of affinity but also of potency and efficacy, which necessitates using a full agonist as the target.

Figure 5.8  Alignment D1 generated by FBSS. Nitrogens are shown in blue; oxygens, red; fluorine, green and carbons in either magenta (SC17599) or white (etorphine, buprenorphine, diprenorphine, morphine, codeine, nalbuphine, naloxone, pethidine, butorphanol, α-prodine, pentazocine and profadol).
In addition to ligands with large N-substituents, some other molecules were also commonly mis-aligned. For example, FBSS was unable to correctly align the 4-anilinopiperidines, namely fentanyl and alfentanil, in any of the twenty analyses. This might be considered as indirect evidence that these ligands may interact with the mu opioid receptor in a different manner than the more traditional ligands, and indeed there is evidence in the literature for a separate binding mode for 4-anilinopiperidines [Ferguson et al, 1999]. This may also be the case for other commonly mis-aligned ligands, including etonitazene (incorrect in 14 of the 20 alignments), SC17599 (12), pentazocine (11), and methadone (10). However, in the case of the steroid the mis-alignment may simply be the result of the extreme difference in its structure from those of the more traditional opioid ligands. More traditional morpine-like ligands were rarely mis-aligned; for example, morphine and etorphine were correct in all 20 alignments.

There was little difference between the four high scoring analyses B1, C1, D1, and E1, since all have between 3 and 5 mismatched ligands. There is some variance in which ligands these are; for example, only runs D1 and E1 aligned SC17599 in a manner similar to that found above in the automated pharmacophore generation, although both involved mis-alignment of fentanyl, alfentanil and methadone. Other commonly mis-aligned ligands within this sub-set were etonitazene and methadone. Selected ligands within the 17 strong data set are shown for alignments D1 (Figure 5.8) and E1 (Figure 5.9).

The best alignment as measured on both quantitative and qualitative scores was E1 (Figure 5.9), which used an etorphine target with the PSS function. It is notable that in this alignment SC17599 had almost exactly the same orientation relative to morphine and etorphine as was seen with analysis by GASP, confirming the probable role of the A ring of SC17599 in compensating for the lack of an aromatic ring.
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Figure 5.9 Alignment E1 generated by FBSS. Nitrogens are shown in blue; oxygens, red; fluorine, green; and carbons in either magenta (SC17599) or white (etorphine, buprenorphine, diprenorphine, morphine, codeine, nalbuphine, naloxone, pethidine, butorphanol, α-prodine, pentazocine and profadol).

5.2.2 Quantitative structure-activity relationships

The FBSS-generated alignments D1 and E1 were used as the basis for a CoMFA study on the structural basis of affinity, potency and efficacy. The mis-aligned ligands were removed from each data set, namely fentanyl, alfentanil, etonitazene and methadone in E1 and fentanyl, alfentanil, and methadone in D1. Since the aim of the study was to validate the FBSS alignment by generating a prediction of the activity of
SC17599, the steroid was also omitted. The remaining ligands constituted two separate data sets for the QSAR analysis. Both D1 and E1 therefore included α-prodine, buprenorphine, butorphanol, codeine, diprenorphine, etorphine, pethidine, morphine, nalbuphine, naloxone, pentazocine and profadol, and D1 also included etonitazene. The biological activity data used included ligand binding affinity (log (1/K)), potency (log (1/EC50)) and efficacy (% maximal stimulation), as shown in Table 5.2.

These data were generated as described in the Methods. Affinity values of the ligands in this study were determined by the displacement of [3H]diprenorphine from membranes of C6μ cells, as described in Methods. The ligands used were chosen to represent a variety of structural classes, and they possessed a wide range of affinities for the mu opioid receptor, for example 0.15 nM for the thebaine analogue diprenorphine, 21 nM for the benzomorphan pentazocine, approximately 400 nM for the 4-phenylpiperidines α-prodine and pethidine, and 3,439 nM for codeine. In all cases displacement curves with slope of unity indicated simple competitive binding.

In the [35S]GTPγS assay in membranes of C6μ cells, a similarly wide range of potencies and relative efficacies were seen. Maximal stimulations as compared to fentanyl ranged from 0 % for the pure antagonists diprenorphine and naloxone, to approximately 8-12 % for the weak partial agonists pentazocine, butorphanol and nalbuphine, to 64 % and 71 % for the strong partial agonists pethidine and profadol respectively, to roughly 100 % for the full agonists etorphine and SC17599. In those cases where maximal stimulation was less than 10 % the potency (EC50) could not be accurately determined. In the remaining ligands, potency ranged from 0.21 nM for the thebaine analogue buprenorphine to 5,476 nM for the 4-phenylpiperidine pethidine.
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<table>
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<tr>
<th>Ligand</th>
<th>$K_t$ (nM)</th>
<th>log ($1/K_t$)</th>
<th>$EC_{50}$ (nM)</th>
<th>log ($1/EC_{50}$)</th>
<th>Max. Stim. (%)</th>
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<td>NA $^3$</td>
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<td>83</td>
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<tr>
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<td>n.d. $^2$</td>
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<tr>
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<td>NA $^3$</td>
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<tr>
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Table 5.2  Affinity ($K_t$), potency ($EC_{50}$) and relative efficacy (% maximal stimulation) in C6J1 cell membranes of the mu opioid ligands used in the CoMFA analysis. Affinities were determined by displacement of $[^3H]$DPN from membranes of C6J1 cells whilst potency and efficacy were determined by $[^35S]$-GTPyS binding assays, as described in Methods. All data represent the mean of three or more individual experiments, and s.e.m. were within 10% of the given value in all cases.

1 maximal stimulation, defined as the percentage of the stimulation of $[^35S]$-GTPyS binding by 10 μM fentanyl

2 not determined

3 NA - $EC_{50}$ values could not be accurately determined for those ligands with maximal stimulation less than 10%
Generally, the thebaine analogues possessed the highest affinity and potency, whilst those ligands with less traditional structures typically displayed lower affinity and potency. The steroid SC17599 had affinity and potency approximately in the middle of the observed range, although it was one of only two full mu agonists in the dataset, the other being etorphine (fentanyl and alfentanil were removed from the dataset due to mis-alignment).

A total of six analyses were performed, using affinity, potency and efficacy measures in conjunction with two starting alignments, namely D1 and E1. Analysis of variance of relative efficacy (maximal stimulation) and potency (EC\textsubscript{50}) failed to produce a useful QSAR model with either starting alignments. In all cases, cross-validated $r^2$ were below 0.5, and non-validated $r^2$ were less than 0.85. Predicted potencies of 2.2 nM and 1.6 nM for the steroid SC17599 were inaccurate by at least 100-fold when compared to the experimentally observed value of 248 nM. Similarly, relative efficacy predictions of 46.7 % and 34.8 %, i.e. partial agonism, were distant from the observed full agonist (94 %) value. Thus, it would appear that use of alignments generated by FBSS in conjunction with QSAR analysis by CoMFA is incapable of accurately modelling the structural basis of potency and efficacy, at least with this selection of ligands. Indeed, this is a problem that has thwarted opioid pharmacology for decades, and one that seems to be no closer to resolution at the current time.

The model of affinity based on the D1 alignment was also inaccurate, with a predicted $K_i$ for SC17599 of 0.32 nM, 200-fold higher than the observed value of 62.4 nM. However, a QSAR model based on the FBSS alignment E1 was more promising. Initial cross-validated partial least squares (PLS) analysis gave an $r^2$ of 0.514, implying that this initial model was able to predict slightly more than half of the variance in affinity for the mu opioid receptor based on the differences in the steric and electrostatic fields of the molecules. It should be noted that this is an inherently conservative estimate since the cross-validation analysis is based on the omission of data points. This initial analysis also indicated that the optimal number of components to be included in the algorithm was 3, since the inclusion of further components did not significantly increase the cross-validated $r^2$. 
Further analysis using three components without validation gave $r^2 = 0.957$, explaining over 95% of the variance in affinities. The correlation between predicted and actual affinity for the compounds within the data-set is shown in Figure 5.10.

![Graph showing predicted versus actual affinities](image)

Figure 5.10  Actual versus predicted binding affinity for the dataset of 12 ligands used in the CoMFA study (▲) and SC17599 (●).

Using this model to predict the binding affinity of the steroid SC17599 gave a $K_i$ value of 7.19 nM which compared reasonably well with the experimentally-observed 62.3 ± 5.4 nM. The ability to predict the binding affinity of such an unusual opioid ligand to within 10-fold of the actual value suggests that quantitative prediction of affinity as a function of structure is possible. It is also a testament to the advantages of field-based alignment (FBSS) and QSAR (CoMFA) modules over more traditional atom-based methods. The predicted regions of favourable and unfavourable steric and electrostatic influences are shown in Figure 5.11. The fact that the structure-affinity model reported here predicts higher affinity for SC17599 than the observed value suggests that there may be unfavourable steric interactions between the steroid and the mu opioid receptor binding pocket which have not been recognised by the CoMFA algorithm. Indeed there are features of SC17599 which project beyond the shared volume occupied by the remainder of the dataset ligands, for example the 3-ethoxy and 17-substituents.
Figure 5.11  Quantitative structure analysis of a database of opioid ligands using the CoMFA module of SYBYL. Nitrogens are shown in blue; oxygens, red; fluorine, green; and carbons in either white (SC17599), magenta (etorphine), or orange (morphine). CoMFA regions - positive steric influence, green; negative steric influence, yellow; positive electrostatic influence, blue and negative electrostatic influence, red.
5.2.4 Ligand docking

The GOLD program (Genetic Optimisation of Ligand Docking) was used in order to explore the docking of morphine and SC17599 to the mu opioid receptor model. The Asp147 residue in transmembrane domain III has been implicated in forming an ionic interaction with the quaternary nitrogen of mu opioid ligands [Dohlman et al, 1991]. Analysis of the area within a 15 Å radius of the α carbon of Asp147 revealed only two possible cavity regions (Figure 5.12). The larger cavity extends diagonally from 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 where it is closest to TMD III and lies directly beneath the large second extracellular loop. The second cavity is small, and lies between the upper end of the first cavity and TMD V. The size of the two cavities suggests that only the larger is likely to be involved in ligand docking.

Figure 5.12 Ribbon representation of the mu opioid receptor model (green) showing the cavity regions identified by GOLD (magenta).
Figure 5.13 Morphine (magenta) docked to the mu opioid receptor model. Only selected residues in the binding pocket are shown for clarity (white). Nitrogens are shown in blue; oxygens, red; hydrogens, cyan. The dashed yellow line indicates a hydrogen bond between the quaternary nitrogen of morphine and Asp147 in TMD III.
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When docked to the mu opioid receptor, morphine lies close to TMD III, IV, V, VI and VII with the A and B rings parallel to the membrane and the C and D rings parallel to the helix bundle (Figure 5.13). The bulk of the morphine molecule is surrounded by largely hydrophobic residues, including Ile234, Trp293, Ile296, Val 300, Cys321 (ring A of morphine), Tyr148, Met151, Ile322 (ring D), Lys233, Tyr 148, Asn230, and Trp318 (ring C). The quaternary nitrogen lies close to and is involved in hydrogen bonding to Asp147, whilst the tyrosyl hydroxyl group lies close to His297. This alignment (generated using GOLD) is almost identical to that reported earlier using the same receptor model and performed using QUANTA, a different molecular modelling package [Pogozheva et al, 1998].

Docking of SC17599 to the mu opioid receptor shows that the quaternary nitrogen is also involved in a hydrogen bond with Asp147 (Figure 5.14). The remainder of the molecule extends diagonally upward towards the extracellular end of TM III, with the 17-substituents located at the extracellular surface of the membrane directly beneath the second extracellular loop. The binding pocket for SC17599 is therefore larger than that for morphine, and includes residues Gln124, Cys 140, Lys141, Ile144, Ala206, Thr207, Gln212, Ile 215, Gln229.

Both morphine and the steroid lie almost entirely within the larger cavity region identified by GOLD (Figure 5.15). The only projecting groups are the D ring and 6-hydroxyl group of morphine, and the 17α-acetoxy and 6-dimethylamino substituents of SC17599. Therefore the quaternary nitrogens of both molecules lie outside the identified binding cavity in order to form the hydrogen bond to Asp147.

Comparison of the docked alignments of the two molecules shows only partial steric overlap. In the analysis of morphine and SC17599 by GASP and FBSS the A ring of the steroid was broadly co-planar and overlapped with the A ring of morphine. Here, 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. This shift of the relative positions of the two molecules when compared to earlier alignments by GASP and FBSS may explain the results of the CoMFA analysis. The binding affinity of SC17599 as predicted by CoMFA based on an alignment by FBSS was 10-fold too
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high, possibly as a result of a more complete and advantageous overlap of the A rings of the two molecules than is seen here. Otherwise the alignments were very similar.

Figure 5.14 SC17599 (orange) docked to the mu opioid receptor model. Only selected residues in the binding pocket are shown for clarity (white). Nitrogens are shown in blue; oxygens, red; hydrogens, cyan. The dashed yellow line indicates a hydrogen bond between the quaternary nitrogen of SC17599 and Asp147 in TMD III.
Figure 5.15  Comparison of the docked conformations of SC17599 (white) and morphine (orange). The mu opioid receptor model is represented in ribbon form (green). The binding cavities identified by GOLD are shown in magenta. Nitrogens are shown in blue; oxygens, red; hydrogens, cyan.
In conclusion, the ability of SC17599 to bind to the mu opioid receptor despite its unusual structure has been explained by the results of two structural analyses and one docking study. In a pharmacophore analysis by GASP, SC17599 gave excellent overlap with morphine, etorphine and its ozonolysis product. The lack of an aromatic ring in both the steroid and the ozonolysis product are compensated for by relatively planar areas of electron density. The GASP module compares atomic features, whereas FBSS compares various combinations of whole molecule fields. Use of FBSS analysis with no imposed constraints was successful in reproducing the alignment seen in GASP with a much larger number of opioid ligands of various structural classes. A quantitative structure-activity relationship study based on the FBSS alignment was able to predict the ligand binding affinity of SC17599 to within 10-fold, but was unable to accurately predict either potency or efficacy. Finally, docking of morphine to a model of the mu opioid receptor reproduced the previously reported binding mode. SC17599 was also successfully docked to the model. Both molecules fit within an identified binding cavity located towards the extracellular surface of the membrane and between helices III, IV, V, VI and VII. Comparison of the binding modes of morphine and the steroid was in close but not complete agreement with the alignments produced earlier, possibly explaining the 10-fold error in the affinity estimate produced by CoMFA for SC17599.

The tentative pharmacophore shown in Figure 4.13 has been broadly validated by the results presented here. In all cases show the critical amine nitrogens were very closely overlapped, whilst the aromatic A rings or appropriate relatively planar and electron rich region are also close, although there may be some freedom in the exact position of this last moiety. The para-hydroxyl substituent has again been shown to play a reduced but not abolished role in the binding of ligands to the mu opioid receptor.
Chapter 6

OVERVIEW
Chapter 6: Overview

One of the fundamental questions in pharmacology is the nature of the recognition process that allows a receptor to recognise and bind its ligands. In opioid pharmacology a pharmacophore detailing the elements crucial to recognition by the mu opioid receptor has been available and has remained essentially unchanged for many years. In the work presented here, this traditional mu opioid pharmacophore has been challenged, with important implications for the future design of mu opioid ligands.

Initially, a series of cyclic tetrapeptides related to DPDPE were investigated, several of which exhibited exceptional selectivity for the mu over the delta and kappa opioid receptors in either radioligand or $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding assays. Moreover, characterisation of peptides containing conformationally restricted amino acids has indirectly revealed details of the mu opioid receptor binding pocket. Importantly, it has been shown that high affinity for the mu opioid receptor can be maintained despite the loss of the tyrosyl hydroxyl group, in direct contradiction of the traditional pharmacophore. However, it must be emphasised that the role of this substituent is only reduced, not abolished. Even more surprisingly, affinity for the mu opioid receptor is maintained despite a loss of aromaticity in the initial residue. Again, this is traditionally considered to be a critical pharmacophoric element, which is shown here to play a reduced, but not abolished, role.

Thus, two of the critical features of the traditional mu opioid pharmacophore are shown to play reduced roles in the binding of certain cyclic tetrapeptides to the mu opioid receptor. Characterisation of the recognition by the opioid receptors of the steroid SC17599 (17α-acetoxy-6-dimethylaminomethyl-21-fluoro-3-ethoxy-pregna-3,5-dien-20-one) has shown this to also be the case in at least one non-peptide ligand. The ability of SC17599 to bind with high affinity and selectivity to mu opioid receptors, upon which it acts as an agonist with good potency and high relative efficacy, confirms that in certain mu ligands the para-hydroxyl and aromatic functionalities play a reduced role.

Automated pharmacophore generation using a small selection of mu opioid ligands has revealed that the traditional pharmacophore is largely correct, but must be
modified to account for the activity of the unusual opioid ligands detailed here. The spatial position and lone pair orientation of the amine nitrogen are indeed as critical as has previously been believed. Although an aromatic ring is not strictly necessary, it can only be replaced by relatively planar, electron rich moieties and some loss of affinity is still involved. The presence of a para-hydroxyl group promotes affinity, but is not critical. These results have been confirmed by field-based similarity searches in a larger set of mu opioid ligands, which produced very similar alignments and allowed accurate prediction of the binding affinity of SC17599. Data presented here suggest that whilst the steroid may bind to the mu opioid receptor in a manner similar to morphine, fentanyl does not.

However, accurate prediction of the potency or efficacy of SC17599 was not possible with this model. In the study of cyclic tetrapeptides shifts in affinity were not always matched by parallel shifts in potency; that is, the EC50/Ki ratio differed greatly within the series. Thus, the structural requirements for high affinity at the mu opioid receptor are not necessarily the same as for potency. This provides some evidence that affinity and potency are separable characteristics from a ligand design stand-point, with different structure-activity relationships.

SC17599 was docked successfully to a model of the mu opioid receptor, and comparison of the binding modes of morphine and SC17599 was largely compatible with the previous alignments produced by either atomic- or field-based analysis. Both molecules docked inside a binding pocket located towards the extracellular surface of the membrane and in contact with transmembrane domains III, IV, V, VI and VII.

The lack of an aromatic ring in the steroid and other mu opioid ligands, including the ozonolysis product of etorphine and certain cyclic tetrapeptides reported here, can be compensated for by the presence of a relatively planar, electron rich region in the appropriate position. Thus, the work reported here represents a significant advance upon the previous understanding of structure-activity relationships in mu opioid ligands, both peptide and alkaloid. Future ligand design initiatives will be able to further characterise the nature of the structural requirements for binding to the mu opioid receptor, although understanding the structural basis of efficacy is still problematic.
Chapter 7

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