Affinity and efficacy studies of buprenorphine analogues at opioid receptors

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AFFINITY AND EFFICACY STUDIES OF BUPRENORPHINE ANALOGUES AT OPIOID RECEPTORS

by

L. Guo

A thesis submitted in partial fulfilment of the requirements for

the award of

Master of Philosophy of the Loughborough University of Technology

April 1996

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Finally, I also wish to thank my husband and my family for their love and support.
Title: Affinity and efficacy studies of buprenorphine analogues at opioid receptors

Li Guo

Key words: Opioid receptor, affinity, efficacy, buprenorphine, mouse vas deferens, guinea-pig ileum, mouse brain, SH-SY5Y cells.

Abstract

Buprenorphine is a widely used analgesic. Its in vivo properties are partial agonism at μ receptors and antagonism at κ receptors. For the investigation of the role of the tertiary alcohol function in the pharmacology of buprenorphine, a series of ring constrained analogues of buprenorphine, in which the tertiary hydroxyl substituent on C19 is fixed in different positions either above (BU46 and BU95) or below (BU47, BU96, BU48 and BU61) the plane of the ring, have been studied in several biological test systems, include binding assays, isolated tissue bioassays and intracellular assays.

In binding assays, all the buprenorphine analogues tested showed similar high affinity (nanomolar) for μ, δ and κ opioid receptors in mouse brain homogenates and μ receptors in SH-SY5Y human neuroblastoma cells. This is consistent with the values obtained for buprenorphine. The results demonstrate that the position of the tertiary alcohol function does not play an important role in controlling receptor-binding affinity.

In the mouse vas deferens preparation, all compounds showed full agonist activity with the IC50 values in the low nanomolar range (0.2nM-7.2nM) and no obvious difference in efficacy and potency between the isomeric pairs (e.g. IC50 values for the isomeric pair BU46 and BU47 were 1.3nM and 3.6nM respectively). Antagonism by the δ selective ligand naltrindole indicated that all compounds exerted their effects via δ receptors. However, the least strained ring derivative BU48, in which the position of the OH is constrained in a 6-membered ring, was the most potent compound with an IC50 value being 10-30 times lower than that of other compounds tested. Hence the position of the OH may affect the efficacy and potency at δ receptors, although the
higher potency of BU48 may relate to the 6-membered ring itself, rather than the slightly different position of the OH function.

In the guinea-pig ileum preparation, all compounds acted as potent and full agonists. For most of compounds IC₅₀ values were in the range 1.4nM-3.7nM. However, compound BU46 (IC₅₀ 0.3nM) was more potent by 5-10 fold than the other compounds. The κ selective antagonist nor-BNI prevented the agonist action affording Ke values in line with κ-mediated responses. The isomers BU46 and BU47 showed a 10-fold different in potency, which suggests that the position of tertiary OH function has a influence in the determination of efficacy and potency at κ sites. However, this was not the case for the isomeric pair BU95 and BU96. These compounds contain two extra methyl substituents which could mask the effects of the OH function.

The efficacy of the compounds at μ opioid receptors was determined as the ability to stimulate [³⁵S]-GTPγS binding in SH-SY5Y cell membranes. All compounds were agonists with slightly lower efficacy than buprenorphine which itself gave 70% of the response seen with the full agonist DAMGO. The EC₅₀ values matched the affinity of the compounds for the μ opioid receptors under similar buffer conditions. The partial agonist profile of some of the buprenorphine analogues was also confirmed by the cyclic AMP assays. It is concluded that the position of tertiary OH function does not alter the potency and efficacy of the compounds at μ opioid receptors.
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Abbreviations

amino acids see overleaf

cAMP cyclic adenosine monophosphate

cDNA complementary deoxyribonucleic acid

CNS central nervous system

DADLE [D-Ala\textsuperscript{2},Leu\textsuperscript{5}]enkephalin

DAMGO [D-Ala\textsuperscript{2},MePhe\textsuperscript{4}Gly(\textit{ol})\textsuperscript{5}]enkephalin

DPDPE [D-Pen\textsuperscript{2},D-Pen\textsuperscript{5}]enkephalin

DSLET [D-Ser\textsuperscript{2}, Leu\textsuperscript{5},Thr\textsuperscript{6}]enkephalin

EDTA ethylenediaminetetraacetic acid

GDP guanosine diphosphate

GTP guanosine triphosphate

G protein guanosine triphosphate binding protein

GTP\textgamma S guanosine 5\textsuperscript{\prime}-[\gamma-thio]triphosphate

HEPES N-[2-hydroxyethyl]piperazine-N\textsuperscript{\prime}-[2-ethanesulfonic acid]

[Leu\textsuperscript{5}]enkephalin leucine enkephalin (Tyr-Gly-Gly-Phe-Leu)

[Met\textsuperscript{5}]enkephalin methionine enkephalin (Tyr-Gly-Gly-Phe-Met)

PLC phospholipase C

Tris tris-[hydroxymethyl]-aminomethane

IP\textsubscript{3} inositol (1,4,5) triphosphate

Nor-BNI norbinaltorphimine
Structure and coding of amino acids of the general structure: 
H₂N-CH(R)-CO₂H.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Three letter symbol</th>
<th>Single letter symbol</th>
<th>-R</th>
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<td>V</td>
<td>-CH(CH₃)₂</td>
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*proline has the structure: 

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*Since this thesis was written it appears that there has been a change in the numbering applied to the bridged oripavine series: the piperidine N atom is now numbered 17 so that the C₇ α side chain starts with C₂₀ and not C₁₉ as used throughout this thesis. Thus C₂₀ becomes C₂₁.
CHAPTER 1 INTRODUCTION

1.1 Opioid receptors and their ligands

Morphine (Figure 1.1) has been used for many centuries to relieve pain. However, very little progress was made to answer the question of how morphine was able to elicit pain relief, or its myriad of other pharmacological effects, until the early 1970's.

![Figure 1.1 Morphine](image)

In 1973 several groups, working independently, published results demonstrating that opiates bind to stereospecific receptors in central nervous system tissues [Pert & Snyder, 1973; Simon et al., 1973; Wong & Hong, 1973]. These findings initiated the search for endogenous opioid ligands. Two years later Hughes et al. [1975] isolated and characterised two pentapeptides, later named [Met\(^5\)]-and [Leu\(^5\)]-enkephalin (Figure 1.2), from porcine brain. These peptides mimicked the action of morphine in \textit{in vitro} pharmacological assays, in particular using electrically stimulated smooth muscle preparations. Subsequently the larger peptide \(\beta\)-endorphin (Figure 1.2) containing the [Met\(^5\)]-enkephalin sequence at its N-terminus [Bradbury et al., 1976]
and the dynorphins (Figure 1.2) containing [Leu\(^5\)]-enkephalin at their N-terminus [Goldstein et al., 1975] were discovered and extensively investigated.

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

(c) Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Glu-Thr-Pro-Leu-Val-
    Thr-Leu-Phe-Lys-Asn-Ala-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln

(d) Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln

Figure 1.2 (a) [Met\(^5\)]-enkephalin, (b) [Leu\(^5\)]-enkephalin, (c) \(\beta\)-endorphin and
(d) dynorphin 17

It is now generally accepted that there are three types of opioid receptor through which the physiological functions of opioids are mediated. The concept of multiple opioid receptors was first postulated by Martin et al. [1976] following behavioural and neurophysiological experiments in the chronic spinal dog. These authors suggested that there were at least three types of opioid receptor which they termed \(\mu\), \(\kappa\) and \(\sigma\). Morphine was classified as a typical \(\mu\)-agonist and ketocyclazocine was proposed as a \(\kappa\)-agonist, while N-allylnormetazocine (SKF10,047) was purported to act as an agonist at \(\sigma\)-receptors. Many of the effects of \(\sigma\)-agonists, however, are not reversed by the opioid antagonist naloxone, and it is now commonly accepted that \(\sigma\)-sites are not opioid receptors. Experiments using isolated tissue preparations and receptor ligand binding studies supported the concept of opioid receptor heterogeneity. The observation that the rank order of potency of morphine and the enkephalins was not the same in the guinea-pig ileum and the mouse vas deferens preparations, and the
drugs were differentially sensitive to the opioid antagonist naloxone in the two preparations, led to the discovery of the δ receptor. Thus, three types of opioid receptor μ, δ and κ are now recognised [Lord et al., 1977]. There is also some evidence that subtypes of opioid receptors may exist, although this is still controversial [Traynor, 1994].

A number of studies have examined the opioid receptor selectivity of endogenous opioid peptides. The endogenous ligands with some selectivity for δ-receptors are [Leu^5]- and [Met^5]-enkephalin, although these compounds also act at μ-receptors [Lord et al., 1977]. β-Endorphin acts at both μ- and δ receptors with equal affinity [Lord et al., 1977]. The dynorphins act preferentially at κ-receptors [Goldstein et al., 1981].

Synthetic compounds acting selectively at the three types of opioid receptor with a higher degree of stability to metabolism than the endogenous ligands have been developed. Widely used μ-agonists include morphine itself and the synthetic peptide DAMGO ([D-Ala^2, MePhe^4, Gly-o1^5]enkephalin) [Handa et al., 1981], the latter being the most selective μ-agonist currently available with very low affinity for δ-and κ-receptors [Kosterlitz & Paterson, 1981]. Commonly used δ agonists include DADLE ([D-Ala^2, D-Leu^5]enkephalin) [Magnan et al., 1982], DSLET([D-Ser^2, Leu^5, Thr^6]enkephalin) [Gracel et al., 1980] and DPDPE ([D-Pen^2, D-Pen^5]enkephalin) [Mosberg et al., 1983]. The latter is highly selective and therefore usually used to label the δ-receptor binding site and examine δ-receptor mediated functions. Selective κ-agonists include U69,593 [Lahti et al., 1985] and CI977 [Hunter et al., 1990] (Figure 1.3).

Agents acting as antagonists have also been synthesised. Most studies have employed the opioid antagonists naloxone and naltrexone, which unfortunately do not clearly
discriminate between different types of opioid receptor. More recently, the selective μ-antagonist cyprodime [Schmidhammer et al., 1989], κ-antagonist norbinaltorphimine (nor-BNI) [Portoghese et al., 1987] and δ-antagonist naltrindole (NTI) [Portoghese et al., 1988] have been synthesised and these provide useful tools for further characterising receptors and screening new opioid ligands (Figure 1.4).

Figure 1.3 (a) U69,593 and (b) CI977
Figure 1.4 (a) naloxone, (b) naltrindole and (c) norbinaltorphimine
1.2 Molecular biology of the opioid receptors

μ-, δ- and κ- Opioid receptors have been extensively studied in various tissues and cell lines. However, the lack of highly selective ligands, in particular labelled antagonists for each receptor type, combined with the lack of animal tissues which express a single type of opioid receptor, has hindered further characterisation. Recently, several groups have reported the molecular cloning of the δ, μ and κ receptors [Kieffer et al. 1992; Evans et al.,1992; Chen et al. 1993; Yasuda et al. 1993]. The availability of the cloned individual receptor types should allow for the identification of the structural features of ligand-receptor interactions.

The mouse δ receptor contains 372 amino acids and was cloned using a strategy which involved extraction of cDNA from neuroblastoma x glioma NG108-15 hybrid cells, which contain a high density of δ receptors [Chang & Cuatrecasas, 1979]. The pharmacological profile of the cloned receptor in subsequently transfected cells confirmed that it was a δ receptor. In addition, the cloned receptor showed the ability to mediate agonist inhibition of cyclic AMP formation, which indicated that the receptors were functionally coupled to adenylyl cyclase. Subsequently, molecular cloning and functional expression of a μ-opioid receptor from rat brain and a κ-opioid receptor from mouse brain was achieved. The μ- and κ-opioid receptors consist of 398 and 380 amino acids respectively. More recently the amino acid sequences of the human μ and δ opioid receptors have been published [Wang et al.,1994; Knapp et al., 1994]. The amino acid sequences of the three opioid receptor types are given in Figure 1.5.

All three types of opioid receptor belong to the superfamily of seven transmembrane domain G-protein coupled receptors. Thus their general structure is of an extracellular
amino terminus, 7 transmembrane regions, 3 extracellular loops, 3 intracellular loops and an intracellular carboxyl terminus, as outlined in the diagram (Figure 1.6).

Consideration of the amino acid sequences of the opioid receptors shows that 57% of the amino acid sequences of the three types of opioid receptors are identical. Much homology is seen in transmembrane domains 2(84%) > 3(82%) > 5(71%) > 6(50%) > 1(45%) > 4(30%). The sequences of the intracellular loops are also highly conserved; loop I shows 90%, loop III shows 91% and loop V shows 78% sequence homology respectively, whereas the extracellular loops are generally much more divergent; loop II shows 67% loop IV shows 24% and loop VI shows 7% sequence homology. There is very little sequence or size homology at either the amino or carboxyl termini. The divergent extracellular regions of the receptors may prove to be vital for ligand binding and therefore explain ligand selectivity profiles.

Knowledge of the amino acid sequence and therefore the structure of the opioid receptors should lead to a better understanding of their function. Several groups are currently investigating chimeric receptors [Meng et al., 1994; Xue et al., 1994], in which, for example, a sequence from the \( \mu \) receptor replaces a specific sequence in the \( \kappa \) receptor. The binding and function of this chimeric receptor is then compared to the original receptor to elucidate which regions of the receptor may be responsible for determining ligand specificity. Alternatively the effects of mutagenesis or deletion of certain amino acids can be tested [Hong et al., 1994]. In the long-term these kinds of studies should provide information useful for the rational design of opioids.
Figure 1.5: Sequence alignment of the mouse δ [Evans et al., 1992], mouse κ [Yasuda et al., 1993] and rat μ [Chen et al., 1993] opioid receptors. Underlined regions indicate conserved amino acids between the receptors. (Adapted from Reisine and Bell, 1993).

<table>
<thead>
<tr>
<th>Transmembrane 1</th>
<th>Transmembrane 2</th>
</tr>
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<tbody>
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<td>SAS-IALALIAITALYSAVCAYGLCNVLVMFGTIVRYLT KLKTATNITYFNALADALATS</td>
<td>100 δ</td>
</tr>
<tr>
<td>SAS-IALPALV liTVS VVFVGLVGS LVMFVT IVTRY TKMTATNITYFNALADALVTT</td>
<td>110 κ</td>
</tr>
<tr>
<td>TGFSMVT IALIYIANTY CVVGLFONFLVMYIVLYVTRKMTKMTATNITYFNALADALATS</td>
<td>119 μ</td>
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<tr>
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</tr>
<tr>
<td>TLPPOSAYLMNSHPGDVLKCLIVISIDYNFTSTPTTLTMSSVDRYI AVCHPVKALDFR</td>
</tr>
<tr>
<td>TLPPOSVNYLMGTHPGETLCKIVISIDYNFTSTPTTLCMSSVDRYI AVCHPVKALDFR</td>
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</tr>
<tr>
<td>TPKKLINICINVLASGSVAIVLGGTGKTVREDVVDVECSLOPPDIDEYSW-WDLFMKIC</td>
</tr>
<tr>
<td>TParkinCVCVNCSATGLPEVMFMATKYROGS--IDCTLTFSHP-- TKYWENLKLIC</td>
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<th>Transmembrane 6</th>
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<td></td>
</tr>
<tr>
<td>VFEAPV VIP LITTVY CYTMLRRSLRVSLSGSKREKRLRRTITKLVLYVVAVFCWFCPT</td>
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</tr>
<tr>
<td>THYVITY VeALGASTHSTA-ALSSYFCIALGYTNSSSLNPVLYAFLDENFRCFROQLCRTP</td>
</tr>
<tr>
<td>THYVITYKALITI-PETTFQTVSVHFCIALGYNSSCLNPVLYAFLDENFRCFROQLCRTP</td>
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</table>

| CGBQEPGSLRRPRQATRERTVTACTPSD-- GPPGGAAAAA |
| KMMRQSTNRVRN-TVQDPAS-- MRDVGGMNKEV |
| SSTIEQQNSREVFRONT-REPHESTANTVDRTNHQLENLEAETAELP |

41 δ 59 μ 100 δ 110 κ 119 μ 160 δ 170 μ 179 μ 216 δ 229 κ 235 μ 276 δ 289 κ 295 μ 336 δ 348 κ 354 μ 372 δ 380 κ 398 μ
Figure 1.6  β receptor diagram
1.3 Receptor-Effector Coupling

1.3.1 G proteins

The interaction of agonist with specific receptors at the cell surface represents only the first step in a cascade of molecular events that underlies transmembrane signalling. Stimulation of these receptors results in activation of effector proteins such as enzymes or ion channels, which synthesise or mobilise chemical 'second messengers' that initiate characteristic actions within the cell. In many cases, a group of proteins, known as G proteins, play an essential transducing role in linking cell-surface receptors to effector proteins at the plasma membrane.

G proteins are heterotrimers, composed of three distinct subunits: α, β and γ. The βγ-subunit exists as a tightly associated complex that functions as a unit. The α-subunit has a single, high affinity binding site for guanine nucleotides. The α-subunits clearly differ between members of the G protein family while the βγ units appear to be shared among some α-subunits to form specific oligomers [Gilman, 1986]. At present, G proteins are classified on the basis of their amino acid sequences into major families, namely Gs, Gi, Go, Gq and G12 [Hepler & Gilman, 1992]. The G proteins differ in their effects on regulation of intracellular effector systems.

G proteins act as switches turning the transmembrane signal on and off. They are able to achieve this function by a cycle of events known as the G protein turnover cycle (Figure 1.7). In the resting state, a receptor is unoccupied by ligand and likely to be unassociated with other components of the pathway. The G protein exists as an unattached αβγ trimer, with GDP (guanine diphosphate) occupying the nucleotide site on the α-subunit. When a receptor is occupied by an agonist molecule, a conformational change occurs, presumably involving the cytoplasmic domain of the
receptor, causing it to interact with αβγ. Association of αβγ with the receptor causes the bound GDP to dissociate and to be replaced with GTP (guanine triphosphate), which in turn causes dissociation of α-GTP from the βγ subunits. Both the GTP bound α subunit and βγ can associate with various enzymes and ion channels, causing activation or inactivation of downstream events. The process is terminated when the hydrolysis of GTP to GDP occurs through the intrinsic GTPase activity of the α-subunit. The resulting α-GDP dissociates from the effector and reassociates with βγ, completing the cycle. This mechanisms results in amplification of the signal because a single agonist-receptor complex can activate several G-protein molecules, and each of these in turn can associate with an effector enzyme for long enough to produce many molecules of second messenger.

The α subunits of the various G proteins differ in structure. In some cases, α subunits possess specific residues that can be covalently modified by bacterial toxins. Cholera toxin catalyses the transfer of the ADP-ribose moiety of NAD (nicotinamide adenine dinucleotide) to a specific Arg residue in the Gα protein α subunit. Similarly, pertussis toxin ADP-ribosylates Gα subunits that possess a specific Cys residues near the carboxyl terminus. Therefore modification of α-subunits by cholera toxin causes constitutive activation inhibiting GTPase activity and lengthening the life time of active α-GTP, whereas modification by pertussis toxin prevents receptor-mediated activation of G proteins [Hepler & Gilman, 1992]. Not all G protein are toxin-sensitive, for example, G proteins insensitive to either of these toxins are thought to regulate receptor-mediated activation of phospholipase C (either Gq and /or Gi2).

1.3.2 Effector systems

G protein coupled effector systems include many membrane enzymes, such as adenylyl cyclase, guanylyl cyclase, phospholipase C and phospholipase A2, as well as a variety
of ion channels [Strader et al., 1994]. Opioid receptors have been shown to couple to adenylyl cyclase, phospholipase C, and K⁺ and Ca²⁺ ion channels.

**Adenylyl cyclase** Cyclic-AMP (cAMP) is a nucleotide synthesised from ATP by the action of the enzyme adenylyl cyclase and hydrolysed to 5'-AMP by the action of phosphodiesterase enzymes. Many different drugs, hormones and neurotransmitters produce their effects through G proteins by increasing or decreasing the catalytic activity of adenylyl cyclase and thus changing the intracellular concentration of cAMP. cAMP has a regulatory action on many intracellular functions, including, for example, energy metabolism, cell division and cell differentiation in addition to ion channel function and ion transport, leading to changes in neuronal excitability and in contractile proteins in smooth muscle. These varied effects are caused through the common mechanism of activation of various protein kinases by cAMP. These enzymes catalyse protein phosphorylation and thereby regulate the function of target proteins.

Opioid receptors have been shown to be negatively coupled to adenylyl cyclase via Gi and G½ proteins [North et al., 1987]. A direct influence of opioids on intracellular levels of cAMP was first shown in the δ receptor containing NG108-15 cells, in which the cAMP forming enzyme, adenylyl cyclase, was inhibited by the binding of opioids to cell surface receptors [Klee & Hvrenaberg, 1976]. Furthermore, activation of μ and κ opioid receptors results in inhibition of cAMP formation as demonstrated for example, in SH-SY5Y cells and guinea-pig brain [Sadee, et al. 1988; Lambert et al. 1993;]. The ability of opioids to inhibit adenylyl cyclase has been widely used as a measure of opioid agonist activity.

**Phospholipase C** Many hormones and neurotransmitters can activate phosphoinositide-specific phospholipase C, an effector enzyme that catalyses hydrolysis of the minor lipid phosphatidylinositol 4,5- biphosphate to form two second messengers, inositol 1,4,5-triphosphate (IP₃) and diacylglycerol. This receptor-
mediated activation of phospholipase C involves functional G proteins [Heper & Gilman, 1992]. IP₃ acts very effectively to release calcium from intracellular stores, by binding to a receptor on the membrane of the endoplasmic reticulum. The action of calcium depends on its ability to regulate the function of various enzymes, contractile proteins and ion channels. Diacylglycerol also acts as a second messenger since it directly changes the activity of membrane-bound protein kinase C, and thus controls protein phosphorylation.

Evidence for the coupling of opioid receptors to phospholipase C and the generation of IP₃ is controversial [Yu & Sadee, 1986; Barg et al., 1992], but growing [Okajima et al., 1993; Chen & Huang 1991J. Recently, μ-Opioid receptor mediated activation of phospholipase C via a pertussis toxin-sensitive G protein has been successfully demonstrated in human neuroblastoma SH-SY5Y cells [Smart et al., 1994].

Regulation of ion channels  G protein coupled receptors can interact with Ca²⁺ and K⁺ ion channels. There is good evidence in support of G protein involvement [North et al., 1987]. The μ selective ligand DAMGO and δ selective ligand DPDPE are capable of increasing the conductance of the inwardly rectifying potassium channels and thus lead to membrane hyperpolarization. Furthermore, κ and μ receptors are linked to voltage dependent Ca²⁺ channels and the activation of κ and μ receptors leads to a decrease of Ca²⁺ entry. Thus, it has been suggested that opioid receptor activation results in the regulation of cation conductance via G proteins, which in turn induces membrane hyperpolarization and this leads to a reduction in neurotransmitter release.
Figure 1.7 Cartoon depicting G protein-mediated transmembrane signalling. A detailed explanation is given in the text (Adapted from Rang and Dale, 1991).
1.4 Theoretical consideration in receptor-ligand interactions

The agonist activity of an opiate or opioid depends upon the principles of affinity and efficacy.

Affinity is the ability of a drug to bind to its receptor, which is defined as the reciprocal of the equilibrium dissociation constant of the drug for the receptor.

Efficacy is a parameter that varies between different drugs and expresses the ability of the drug-receptor complex to elicit a physiological response. Full agonists which can produce maximal effects, have high efficacy; for a pure antagonist efficacy is zero; agonists which can produce only submaximal effects have intermediate efficacy, and are termed partial agonists. The concept of efficacy was originally defined by Stephenson [1956].

In the 'occupation' theory of drug-receptor interaction, it is assumed that the magnitude of the biological response is linearly proportional to fractional receptor occupancy such that

\[
\frac{ED}{EM} = \frac{[RD]}{[Rt]}
\]  

(1)

where \(ED\) the observed response, \(EM\) is the maximal response possible, \([RD]\) is the concentration of drug-receptor complex, and \([Rt]\) is the total concentration of receptors. Assuming a simple bimolecular interaction, the binding of the drug to its receptor may be described by the law of Mass Action, such that

\[
\frac{[RD]}{[Rt]} = \frac{[D]}{[D] + KD}
\]  

(2)

where \([D]\) is the concentration of drug.
Later modifications of receptor theory recognized that a nonlinear relationship exists between receptor occupation and tissue response [Stephenson, 1956]. Stephenson suggested that the measured response is some function of the stimulus (S) which is generated by interaction of ligand and receptor. Thus

\[
\frac{ED}{EM} = f(S) = f \left( \frac{e[R]}{[R_t]} \right) = f \left( \frac{e[D]}{[D] + KD} \right) \tag{3}
\]

where \( e \) is efficacy, the parameter that relates stimulus to occupancy, and \( f \) represents the transducer function which describes the characteristics of the responding system. The concept of receptor reserve (or spare receptors) is implicit in this model, since an agonist with a high efficacy value needs to occupy only a fraction of the total receptor population to elicit a maximal response. Thus, the concentration which produces a 50% maximal response is less than the concentration which is necessary to occupy 50% of total receptor pool.

Furchgott [1966] has argued that efficacy (e), as defined in equation (3) is a drug and tissue-dependent term, which reflects both the ability of the agonist to induce an active receptor-effector complex and the total number of receptors in the system, i.e.

\[
e = E[R_t] \tag{4}
\]

where \( E \) is 'intrinsic efficacy', a strictly drug-related property, and \([R_t]\) is the total concentration of receptors. Intrinsic efficacy (E) should be constant for a given drug-receptor pair across species and tissues [Kenakin, 1983], whereas efficacy (e) varies with receptor density. Therefore, agonist activity is dependent upon factors which are specific to the drug (\( K_D \), drug dissociation constant, and \( E \) intrinsic efficacy) as well as upon factors which are specific to the target tissue (\( f \), the function relating stimulus to response, and \([R_t]\) total receptor concentration). When receptor density is high, the value \( e \) will be high, and the agonist may produce a maximal response by occupying a small proportion of the total receptor population (i.e., \( ED_{50} < K_D \)). Where receptor
density is low, the value of e will be low, and the same agonist may occupy the total receptor population without producing a maximal response i.e., behave as a partial agonist.

Another widely used concept is ‘intrinsic activity’, which describes the ability of the drug-receptor complex to elicit the observed pharmacological response. This was defined by Ariens [1954] as:

$$E = \alpha [DR]$$ (5)

where E is observed response, \(\alpha\) is intrinsic activity, and [DR] is the concentration of drug-receptor complex.

This definition is strictly based on the assumption of occupation theory, i.e. the linear relationship between receptor occupation and tissue response. So the magnitude of intrinsic activity is equal to the magnitude of the maximal response. Thus a partial agonist producing a maximal response that was 40% of the tissue maximal response has \(\alpha = 0.4\). The measurement of intrinsic activity is a widely used method for quantifying the ability of a drug to produce a response. However, caution should be used since that this parameter is also drug and tissue dependent and cannot be used to classify drugs without awareness of the tissue factors. In reality the observed pharmacological response is not a linear function of receptor occupancy, therefore, quantification of agonism by intrinsic activity depends on the recognition of the efficiency of stimulus-response coupling.
1.5 The oripavines: a family of alkaloid derivatives containing highly potent analgesics

Chemical modification of opioid analgesics have been carried out for many years to design compounds that have greater pain-killing capacity and less side-effects than morphine. Much of this work has been based on the naturally occurring alkaloids. Thebaine is one of the components of the opium poppy and serves as the starting point in the synthesis of the thevinols and orvinols series of agonists and antagonists (Figure 1.9).

Bentley and coworkers prepared many derivatives of thebaine using the Diels-Alder reaction. The idea for synthesising this series of compounds stemmed from the hypothesis that if morphine and related compounds bind to a receptor in order to initiate their response, molecular flexibility would permit a molecule to fit a number of different receptor types. This would lead not only to the required analgesic effects, but also a number of undesirable side-effects. Therefore it was thought that greater molecular rigidity would restrict receptor fit and afford a greater degree of selectivity towards the opioid receptor. Although such a view is probably an oversimplification, and devised before our knowledge of opioid receptor types, useful compounds resulted from this approach. In particular etorphine (Figure 1.8) was found, in animal studies, to be 2000-10,000 times more potent than morphine, depending on the test. It has been used in veterinary practice and in the immobilisation and capture of wild animals. Antagonists were also discovered in this series of compounds, such as diprenorphine (Figure 1.8) which is about 100 times more potent than nalorphine as a morphine antagonist in the mouse writing test [Blane, 1967]. Subsequently, the mixed agonist and antagonist buprenorphine (Figure 1.8) was synthesised, which is 75 times more potent than morphine as an agonist and 4 times more potent than nalorphine as an antagonist in rodents [Lewis, 1985].
Many chemical modifications have been made in the exploration of the structure-activity relationships of the thebaine Diels-Alder products (Figure 1.9) [Lewis et al., 1973; Casy & Parfitt 1986]. Some of these are summarized below:

**A ring modifications** These have not been extensively studied, but in general additional aromatic substituents tend to reduce potency below that of the parent orvinols (which contain 3-OH) or thevinols (which contain 3-OMe). Compounds lacking an oxygen function at C-3 lie between orvinols and thevinols in potency.

**D ring modifications** Variation of the N-substituent are most often entailed. In this series the pattern of activity is much the same as that in other rigid opioids. Groups such as n-propyl, allyl, dimethylallyl, and cyclopropylmethyl afford antagonists in the thevinol series. However, with orvinols, the type of pharmacological response elicited depends heavily upon the nature of C-ring substitution in addition to the N-substituent.

**C ring variations** These have the most significant influence on pharmacological responses in this series. Originally the high potency of these compounds was thought to be due to the C-19 OH, which could undergo hydrogen-bonding interaction with the receptor protein. However, it was discovered that extremely high potency analgesics could exist in the oripavine series without a C-19 OH. Lipophilic substituents at C-19, preferably with a C-19 OH of appropriate geometry, gives some orvinols and thevinols a high level of opioid agonist activity, with potency greatly influenced by the size of the C-7 substituent [Casy and Parfitt, 1986].

Isomers with the R configuration at C-19 are more potent than the corresponding S isomers. For example, etorphine (19R) has 1000 times the agonist potency of morphine, whereas its diastereoisomer, although still with antinociceptive activity, is only 20 times potent than morphine. This was suggested to result from intramolecular
hydrogen bonding between the C-19 OH and the C-6 methoxy oxygen atom in the R isomer, which would direct the C-19 lipophilic group towards its receptor site [Lewis et al., 1971; Loew & Berkowitz, 1979]. However, with the 6-deoxy analogue a similar difference in analgesic activity between the diastereoisomers, has been found [Hutchins, 1981]. This indicates that intramolecular hydrogen bonding does not play a significant role in establishing the conformational preference for any R diastereoisomer-lipophilic site interaction. However, Hutchins and Rapoport [1984] examined 19-deoxy- and 6,20-epoxy orvinols and thevinols in which the high level of activity of some R isomers could also be explained by intermolecular H-bonding.

To account for these various observations, Hutchins and Rapoport [1984] proposed two binding sites for the C-ring, one for the C-7 lipophilic side chain located below C-8 and approaching the 6,14-etheno bridge, and another, a hydrophilic receptor site to receive the C-19 hydroxy above the C-ring. Some compounds in the series could interact with both sites, and others would only present a lipophilic interaction. Thus, the sites proposed would engage in synergistic and competitive binding of ligands, the response elicited being commensurate with a fit to one, or both, of the sites (Figure 1.10).
Figure 1.8 (a) etorphine (b) diprenorphine and (c) buprenorphine

(where CPM = cyclopropylmethyl)
Figure 1.9 Structures of the thevinol and orvinol series of compounds (showing the position of C atoms and A, B, C, ring system)

Figure 1.10 Proposed orvinol conformer with greatest agonist activity. The pendant alcohol is oriented for maximum interaction with the hydrophilic (H) and lipophilic (L) subsites (adapted from Hutchins & Rapoport, 1984).
1.6 Pharmacological effects of buprenorphine

The oripavine derivative buprenorphine has been widely used in the clinic as an analgesic for many years, particularly in the treatment of postoperative and cancer pain [Jasinski, 1979]. Because of its lower physical dependence liability, it is being evaluated for use in morphine dependent subjects in the treatment of addiction [Cowan & Lewis, 1995].

*In vivo*, buprenorphine (Figure 1.8) produces similar effects to morphine with a lower ceiling due to its partial agonist activity at μ receptors [Cowan et al., 1977]. However, in *in vitro* binding assays buprenorphine has high affinity at μ, δ and κ opioid binding sites [Villiger, 1984].

The main features of the pharmacology of buprenorphine are its potent analgesic activity with long duration of action and low physical dependence liability [Jasinski, 1979]. *In vitro* studies [Rance & Dickens, 1978; Manara, et al., 1978] have demonstrated that buprenorphine has extremely slow kinetics, which could explain its slow onset and long duration of the action. The rate at which a drug leaves its receptor sites on challenge with an antagonist or on abrupt withdrawal of the opioid seems to be of importance in determining the severity of the abstinence syndrome produced. Buprenorphine dissociates extremely slowly from the receptor and this could balance the disturbance of homeostasis which results from abrupt withdrawal and therefore produce only a mild abstinence syndrome [Sharma, et al., 1975].

In animal tests, a bell-shaped dose-response curve for buprenorphine has been observed [Cowan et al., 1977]. Thus as the concentration of buprenorphine is increased the observed agonist action is reversed. This could be explained by postulating an action of buprenorphine at two different opioid receptor systems with
different characteristics, i.e. buprenorphine interacts with one type of receptor to produce an agonist activity, whereas it interacts with a second type of receptor to produce an antagonist effect. This is referred to as non-competitive auto-inhibition [Ariens et al., 1964]. However, this idea has not been properly examined at the biochemical level.
1.7 Aims of the project

As discussed earlier, the stereochemistry at C-19 in buprenorphine and related oripavines, which controls the position of the t-Butyl group and the tertiary OH, is important in determining the pharmacological profile of the compounds. In order to investigate the role of the tertiary alcohol function at C-19, a series of buprenorphine analogues in which the position of the alcohol function is fixed by incorporation into a ring structure have been synthesised by J.W.Lewis and colleagues at the University of Bristol.

The aims of this study were to examine the affinity and efficacy of such conformationally constrained analogues in several biological test systems. This should allow determination of the importance of the position of the tertiary alcohol function in this series of compounds, and thus help to point out important features, both in the molecule and in the binding pocket of opioid receptors, which control affinity and efficacy.

Compounds examined are given in the Figure 1.11. These include the isomeric pairs BU46 & BU47 and BU95 & BU96 in which the OH is held in a 5-membered ring structure. In addition BU95 and BU96 contain methyl substituents, similar to buprenorphine. Two further compounds BU48 in which the OH is fixed in a 6-membered ring and BU61 with same stereochemistry as BU47 but with introduction of a methyl group were also examined. For comparison a compound BU4 with the N-Me group was evaluated.
Figure 1.11 Structures of buprenorphine analogues
CHAPTER 2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Radiochemicals

\( ^{[3]} \text{H} \)-DAMGO ([(D-Ala\textsubscript{2}, MePhe\textsubscript{4}, Gly(ol)\textsubscript{5}] enkephalin) (2.22TBq/mmol; 60Ci/mmol), was from Amersham International plc., Aylesbury.

\( ^{[3]} \text{H} \)-DPDPE ([D-Pen\textsubscript{2},D-Pen\textsubscript{5}] enkephalin)(1.5TBq/mmol; 40.7Ci/mmol) was from Dupont NEN Research Products, Stevenage.

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

\( ^{[3]} \text{H} \)-Diprenorphine (1.1TBq/mmol; 30Ci/mmol) was bought from Amersham International plc., Aylesbury.

\( ^{[3]} \text{H} \)-cAMP (3',5'- cyclic adenosine monophosphate) (TBq/mmol; Ci/mmol) was from Dupont, NEN Research Products, Stevenage.

\( ^{[35]} \text{S} \)-GTP\textsubscript{γS} (Guanosine5'-[γ-thio] triphosphate) (46.1TBq/mmol; 1245Ci/mmol) was purchased from Dupont, NEN Research Products, Stevenage.

Structures of the radiochemicals are given in Figure 2.1
Figure 2.1 (a) $[^3\text{H}]$DAMGO, (b) $[^3\text{H}]$DPDPE, (c) $[^3\text{H}]$Cl977, (d) $[^3\text{H}]$diprenorphine, (e) $[^3\text{H}]$cAMP and (f) $[^35\text{S}]$GTP$\gamma$S.
2.1.2 Chemicals

HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), Trizma base (Tris-[hydroxy-methyl]aminomethane), bovine serum albumin, Folin's reagent, adenosine triphosphate (ATP), adenosine diphosphate (ADP), guanosine 5'-[γ-thio]triphosphate (GTPγS) and norit activated charcoal were purchased from Sigma, Poole.

CaCl₂·2H₂O, ethylenediaminetetraacetic acid (EDTA), Glucose, KCl, KH₂PO₄, MgSO₄·7H₂O, NaCl, NaHCO₃, NaOH, NaCO₃, Na₂HPO₄, CuSO₄, HCl and KNaC₄H₄O₆·4H₂O were purchased from Fisons, Loughborough.

Ecoscint scintillation fluid was from National Diagnostics, Georgia, USA.

2.1.3 Drugs

Fentanyl citrate was gift from Janssen, Belgium. Naloxone hydrochloride was purchased from Sigma, Poole. Naltrindole and norbinaltorphine were purchased from Semat. Morphine hydrochloride, codeine phosphate, methadone hydrochloride, and heroin hydrochloride were gifts from McFarlane Smith Edinburgh.

Buprenorphine and its analogues were synthesised by Dr. J. W. Lewis, Bristol University, UK.

2.1.4 Peptides and cAMP-binding protein

DAMGO [D-Ala², MePhe⁴, Gly(ol)⁵]enkephalin and DPDPE [D-Pen², D-Pen⁵]enkephalin were purchased from Sigma, Poole.
The cAMP-binding protein prepared from bovine adrenal glands was a gift from Dr. D. G. Lambert, Department of Anaesthesia, Leicester University.

2.1.5 Cell culture media

Minimum Essential Medium (with Eagle’s salts), Dulbecco’s Modified Eagle’s Medium, L-Glutamine, penicillin/streptomycin, fungizone, HAT supplement (hypoxanthine, aminopterin and thymidine), trypsin/EDTA, foetal calf serum and newborn calf serum were purchased from Gibco Laboratories, Paisley.

2.1.6 Buffers

The composition of Krebs solution used in the isolated tissue studies was as follows (mM):

\[
\text{NaCl (118), NaHCO}_3 (29), \text{KCl (4.7), CaCl}_2 \cdot 2\text{H}_2\text{O (2.5), MgSO}_4 \cdot 7\text{H}_2\text{O (4.0), KH}_2\text{PO}_4 (1.2) and Glucose (11.1), pH 7.4. MgSO}_4 \cdot 7\text{H}_2\text{O was omitted for the mouse vasa deferentia.}
\]

The Krebs/HEPES buffer used in cAMP assays and whole cell ligand binding assays contained (mM):

\[
\text{NaCl (118), NaHCO}_3 (25), \text{KCl (4.7), CaCl}_2 \cdot 2\text{H}_2\text{O (2.5), MgSO}_4 \cdot 7\text{H}_2\text{O (1.2), KH}_2\text{PO}_4 (1.2) and Glucose (11.7) and HEPES (10), pH 7.4.}
\]

The buffer used for membrane binding assays was Tris-HCl (50mM), pH 7.4.

The buffer used in \[^{35}\text{S}-\text{GTP} \gamma \text{S}\] binding assays contained (mM):

\[
\text{NaCl (100), MgCl}_2 \cdot 6\text{H}_2\text{O (10) and HEPES (20), pH 7.4.}
\]

Adsorption buffer used in cyclic AMP assays contained (mM):

\[
\text{EDTA (4), BSA (4mg/ml), and Tris (50) adjusted to pH 7.4 using concentrated hydrochloric acid.}
\]
2.1.7 Animals

Male Dunkin-Hartley guinea-pigs (250-500g), were bought from David Hall, Newchurch, Burton-on-Tent.

Male CSI mice (25-30g), were purchased from Nottingham University Medical School.

Animals were fed on a standard laboratory diet and kept on 12h light/dark cycle at a temperature of 20°C.

2.1.8 Equipment

Isolated tissue assays:

Grass 88 Stimulator, Grass Medical Instruments, Quincy, Mass., U.S.A.

Ligand binding and intracellular assays:

Brandel cell harvester M-48R, Gaithersburg, U.S.A. Liquid scintillation counter, either MINAXI TRI-CARB 4000 series, United Technologies Packard, Pangbourne or 1215 Rackbeta, LKB Wallac, Milton Keynes.
Polytron PT 10-35 Homogeniser, Kinematica GmbH, Littau, Switzerland.
Tissue tearor, Biospec products, Bartlesville, OK, U.S.A.
Ultracentrifuge, Optima TL100, Beckman, High Wycombe.
Centrifuge, Universal 1200 Httich, Tuttlingen, Germany.
Micro-centrifuge, Model 320, Quickfit Instrumentation, Stone.
Whatman GF/B filter strips, Whatman, Maidstone.
Cell culture:

Centrifuge, FP-510, Labsystems Oy, Finland.

Class II Microbiological Safety Cabinet, Walker Safety Cabinets Ltd, Glossop, Derbyshire.

Incubator, GC4, Grant Instruments, Cambridge.

Powerpette, Jencons Scientific Ltd, Leighton Buzzard.

Tissue culture plastics, Gibco Laboratories, Paisley.
2.2 Methods

2.2.1 Isolated tissue studies

(a) Mouse vas deferentia

Male CSI albino mice (25-30g) were killed by cervical dislocation. The vasa deferentia were removed and mounted in 3ml organ baths, containing Krebs solution (minus MgSO₄.7H₂O). An initial resting tension was set up at 0.5g. Tissues were bathed constantly in buffer solution at 37°C, and aerated with 5% CO₂ in 95% O₂.

After a recovery period of 90 minutes, field stimulation between platinum ring electrodes at upper and lower ends of the bath was initiated, consisting of three pulses at supramaximal voltage of 1s duration at intervals of 250 ms. The contractions were recorded isotonically.

(b) Guinea-pig myenteric plexus-longitudinal muscle

Male Dunkin-Hartley guinea-pigs (300-400g) were killed by cervical dislocation. The ileum was immediately removed and placed in Krebs solution. Strips of myenteric plexus-longitudinal muscle were prepared and mounted (approximately length of 5 cm) in 3ml organ bath. The resting tension was set at 1.0g. Tissues were bathed in Krebs solution at a constant 37°C and aerated with 5% CO₂ in 95% O₂.

After a recovery period of 90 minutes, tissues were stimulated using square wave 0.4ms pulses of supramaximal voltage, and a frequency of 0.16 Hz. The contractions were recorded isotonically.
(c) Determination of agonist potencies

Tissues were stimulated as described above until even contractions were maintained. Agonists were then added cumulatively, until the inhibition of contraction was maximal; then the stimulation was stopped and the tissue was washed with an overflow of Krebs solution. The tissue was stimulated at regular intervals, during which continuous washing was undertaken, until maximal contraction was restored. The tissue was allowed to rest for approximately 15 min prior to the next application of agonist.

The concentration of agonist required to reduce the twitch height to half the maximal value (IC$_{50}$) was calculated from dose-response curves, to give agonist potencies.

(d) Measurement of antagonist affinities

The tissue was incubated with the antagonist for the following lengths of time prior to the addition of the agonist:

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naloxone</td>
<td>15 min (mouse vas deferens)</td>
</tr>
<tr>
<td>Naltrindole</td>
<td>15 min (mouse vas deferens)</td>
</tr>
<tr>
<td>nor-BNI</td>
<td>30 min (guinea-pig ileum)</td>
</tr>
</tbody>
</table>

Dose-response curves of the agonists were obtained before the addition of the antagonists and then repeated in the presence of antagonist. Antagonist affinities were determined as equilibrium dissociation constants (Ke values). Ke values were determined with a single dose of antagonist using the equation:

$$Ke = \frac{[\text{antagonist}]}{DR - 1}$$
where $\text{DR} = \text{IC}_{50}$ in the presence of antagonist / $\text{IC}_{50}$ in the absence of antagonist [Kenakin, 1983]

2.2.2 Ligand-binding studies

(a) Preparation of homogenates

Mouse brain

Mice were killed by cervical dislocation and the brains removed, weighed and homogenised in Tris buffer for 10 seconds using a polytron homogeniser set at unit 7.0. After centrifugation (25000g, 15 min) at 4°C, the pellet was resuspended in 10×volume Tris buffer and incubated at 37°C for 30 minutes to remove endogenous ligands. The homogenates were recentrifuged as above and then the pellet resuspended in 1/60 weight/volume in Tris buffer. The final protein concentration of brain tissue was determined by the Lowry method and adjusted to approximately concentration of 0.5mg/ml [Lowry et al., 1951].

SH-SY5Y human neuroblastoma cells

The SH-SY5Y cell line was kindly donated by Dr. D Lambert, Department of Anaesthesia, Leicester University. Cells were grown in Minimum Essential Medium (MEM) supplemented with 2% foetal calf serum, 10% new born calf serum, 2.5μg/ml amphotericin B (fungizone), 50U/ml penicillin, 50μl streptomycin and L-glutamine (254μg/ml) at 37°C in a humidified 5% CO$_2$ atmosphere. Cells were passaged when confluent (using 500μg/ml trypsin and 200μg/ml EDTA in a physiological solution) and fed three times weekly.
NG108-15 neuroblastoma x glioma hybrid cells

NG108-15 cells were kindly provided by Dr. M. Keen, Dept. of Pharmacology, University of Birmingham. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with HAT [hypoxanthine (13.6μg/ml), aminopterin (0.17μg/ml) and thymidine (3.88μg/ml)] at 37°C in a 5% CO₂ atmosphere. Cells were passaged when confluent (by gentle mechanical agitation) and fed every day, with the exception of Day 1 after sub-culture.

Cell membrane preparations

Confluent monolayers of SH-SY5Y cells were harvested in HEPES (20mM) buffered saline containing 0.02% (w/v) EDTA. NG108-15 cells were harvested in Dulbecco's Modified Eagle Medium. Harvested cells were centrifuged at 250g for 2 min at room temperature and the pellet of cells was resuspended in the appropriate buffer (Tris buffer or Tris buffer plus Na⁺ and GTPγS or [³²S]-GTPγS assay buffer). Membranes were prepared by treatment with a tissue tearor (2×5s, 30,000rpm), collected by centrifugation (50,000g, 15min, 4°C). The pellet was resuspended in the appropriate buffer using the tissue tearor (2×1s, 5000rpm), and then recentrifuged as before. The resulting pellet of membranes was resuspended in the appropriate assay buffer (approximately one flask to 35mls), as required, at a protein concentration of 0.25 mg/ml [Lowry et al., 1951].

(b) Competitive binding assays

To 960μl of the final appropriate homogenate prepared as above, 20μl aliquots of unlabelled competing ligand in increasing concentrations, and 20μl of a labelled ligand were added to a final volume of 1ml. Total binding was determined by adding 20μl of
the labelled ligand to the homogenate in the absence of any competing ligand, and non-
specific binding was determined in the presence of 10μM naloxone. The
concentrations of labelled ligand used in the assays were approximately as follows:
[^3H]DAMGO: 1nM,[^3H]DPDPE: 2nM,[^3H]Cl977: 0.5nM,[^3H]diprenorphine: 0.5nM.

The assay solutions were incubated for 40 min at 25°C, then filtered rapidly through
Whatman GF/B glass filters under vacuum (the filter paper was pre-soaked in Tris-HCl
buffer for at least 15 min) and washed twice with 4mIs of ice-cold Tris-HCl buffer.
The filter papers were placed in scintillation vials to which Ecoscnt was added and the
amount of bound tritiated ligand was determined by using a liquid scintillation counter.
The IC\textsubscript{50} value for test compounds were determined from the displacement curves
which were drawn using " Cricket " graph. The equilibrium inhibition constants (Ki
values) were calculated according to the equation of Cheng and Prusoff [1973].

\[ Ki = \frac{IC50 \times KD}{KD + [L^*]} \]

where \( K_D \) is the equilibrium dissociation constant of the labelled ligand and \([L^*]\) is
the concentration of labelled ligand used in the assay.

(c) Saturation binding assays

SH-SY5Y or NG 108-15 cell membrane homogenates were incubated at 25°C for 40
min with varying concentration of tritiated ligand (0.005-20nM) in the absence or
presence of 10μM naloxone to define non-specific binding. The remaining procedures
were the same as described for the displacement binding assay.
The binding capacities ($B_{\text{max}}$) and equilibrium dissociation constant ($K_D$ values) were calculated from the computer programme LIGAND [Munson and Rodbard, 1980], and represented graphically as Scatchard plot, thus:

$$\frac{B}{F} = \frac{B_{\text{max}}}{K_D} - \frac{B}{K_D}$$

where $B$ is radioligand bound to receptor, $F$ is free radioligand, $K_D$ is equilibrium dissociation constant of radioligand and $B_{\text{max}}$ is the maximal number of binding sites. A plot of $B/F$ versus $B$ gives a straight line with a slope of $1/K_D$ and an intercept with the abscissa of $B_{\text{max}}$.

2.2.3 $[^{35}\text{S}]$-GTPγS binding assays

SH-SY5Y cell membrane preparations were prepared as described in section 2.2. Membrane homogenates (100-200μg) were incubated in “GTPγS binding assay buffer” containing 100pM $[^{35}\text{S}]$-GTPγS, 3μM GDP and varying concentration of opioid ligand, for 1 hour at 30°C. The solution was then rapidly vacuum-filtered and washed twice with 4 mls of ice-cold buffer. The filter papers were placed in Ecoscint scintillation fluid, and the amount of bound radioactivity was determined by counting in the C-14 channel of a liquid scintillation counter.

The amount of stimulated $[^{35}\text{S}]$-GTPγS binding is given as a percentage of the stimulation evoked by 3μM fentanyl. The $EC_{50}$ (concentration affording a 50% response) value was calculated from the concentration-response curve (drawn by “Cricket” graph) and the basal $[^{35}\text{S}]$-GTPγS binding was expressed as fmol $[^{35}\text{S}]$-GTPγS bound/mg protein.
2.2.4 Cyclic AMP assays

SH-SY5Y cells were harvested by a brief exposure to HEPES (10mM) buffered saline containing EDTA (0.5mM), and centrifuged at 500g for 2min. The pellets were resuspended in Krebs/HEPES buffer at a protein concentration of approximate 2mg/ml, then the whole cell suspension (approximately 300μg) were incubated in the total volume 300μl containing isobutymethyxanthine (1mM), forskolin (10μM) and various concentrations of opioid agonist. The incubation was performed at 37°C for 15 min. The reaction was terminated by addition of 20μl HCl (10M), 20μl NaOH (10M) and 180 μl Tris (1M) pH 7.4. After centrifugation (1500g, 2min), cAMP in the supernatant was determined using a protein binding method [Brown et al.1971].

The protein binding method relies on the competition between [3H]-cAMP and unlabeled cAMP in the sample for a crude cAMP-binding protein prepared from bovine adrenal glands. Typically, 50μl of reaction mixture or standard cAMP solution was incubated with 2 pmol [3H]-cAMP and binding protein at 4°C in the dark overnight. Non-specific binding of [3H]-cAMP was determined using a 125-fold excess of unlabelled cAMP. Free [3H]-cAMP was adsorbed by activated charcoal (10mg/ml) suspended in adsorption buffer and removed by centrifugation (700g, 2min, room temperature). The amount of bound [3H]-cAMP in the supernatant was measured by counting an aliquot of the supernatant in the tritium channel of a liquid scintillation counter.

cAMP content of the unknown samples was determined by interpolation from a standard curve and the measure of inhibition of cAMP formation was given as a percentage of the basal cAMP level. The IC_{50} was calculated manually from the concentration-effect curves.
2.2.5 Molecular modelling:

SYBYL (version 6.1, Tripos Associates, St. Louis, USA) was used to model the structures of buprenorphine and analogues using an Evans and Sutherland workstation with a unix computer. This allowed distances and angles in the structures to be accurately measured.
CHAPTER 3 RESULTS

3.1 Isolated tissue studies

3.1.1 Standard compounds

The mouse vas deferens, containing \( \mu \), \( \delta \) and \( \kappa \) opioid receptors and guinea-pig ileum containing functional \( \mu \) and \( \kappa \) opioid receptors, were used as in vitro models for the analysis of opioid receptor interactions [Kosterlitz and Waterfield, 1975]. The affinity and selectivity of several synthetic compounds were assayed in these test preparations.

Standard selective agonists (DAMGO, DPDPE and U69593); and selective, non-selective antagonists (naloxone, naltrindole and nor-BNI ) were tested in isolated tissue systems in order to obtain reference data and also to show the sensitivity of the tissue for use in the determination of agonist efficacy and selectivity.

In the mouse vas deferens the compounds DAMGO, DPDPE, and U69593 all acted as full agonists, and afforded \( IC_{50} \) values of 17.6±2.0 nM, 0.93±0.04 nM and 28.4±3.6 nM respectively. The affinity (as equilibrium dissociation constant values, \( K_e \) values) of naloxone, naltrindole and nor-BNI against these agonists were determined using single-dose method [Kosterlitz & Watt, 1968]. The data are summarised in Table 3.1.

In the guinea-pig ileum, DAMGO and U69593 afforded \( IC_{50} \) values of 5.6±1.8 nM and 2.4±0.9 nM respectively. The affinity (\( K_e \) values) of naloxone and nor-BNI against both compounds were obtained. The data are summarised in Table 3. 2
Table 3.1. The IC₅₀ values of DAMGO, DPDPE and U69593 & Ke values of naloxone, naltrindole and nor-BNI in the mouse vas deferens.

<table>
<thead>
<tr>
<th></th>
<th>IC₅₀(nM)</th>
<th>Ke(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>naloxone</td>
</tr>
<tr>
<td>DAMGO</td>
<td>17.6±2.0</td>
<td>2.8±0.36</td>
</tr>
<tr>
<td>DPDPE</td>
<td>0.93±0.04</td>
<td>54.4±2.1</td>
</tr>
<tr>
<td>U69593</td>
<td>28.4±3.6</td>
<td>19.6±2.3</td>
</tr>
</tbody>
</table>

Values represent means ± s.e. mean where n≥3. *data are taken from previous work in our laboratory [Bell, 1994].

Table 3.2. The IC₅₀ values of DAMGO and U69593; and Ke values of naloxone and nor-BNI in the guinea-pig myenteric plexus-longitudinal muscle.

<table>
<thead>
<tr>
<th></th>
<th>IC₅₀(nM)</th>
<th>Ke(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Naloxone</td>
</tr>
<tr>
<td>DAMGO</td>
<td>5.6±1.8</td>
<td>2.09±0.54*</td>
</tr>
<tr>
<td>U69593</td>
<td>2.4±0.9</td>
<td>31.4±9.69*</td>
</tr>
</tbody>
</table>

Values represent means ± s.e. mean where n≥3. *data are taken from previous work in our laboratory [Bell, 1994].
Figure 3.1 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by DAMGO (□), DPDPE (■) and U69593 (●).

Figure 3.2 Dose-response curve for the inhibition of electrically induced contractions of guinea-pig ileum by DAMGO (□) and U69593 (■).
3.1.2 Buprenorphine analogues

A series of buprenorphine analogues were examined for their ability to inhibit the electrically-induced contractions in the mouse vas deferens and the guinea-pig ileum using the methods described in section 2.2.1.

All compounds showed potent agonist activity in the mouse vas deferens (Figures 3.3-3.14). There was little difference in potency between isomeric pair BU46 and BU47; and BU95 and BU96 (Table 3.3). In order to determine the receptor through which the compounds were acting, the non-selective antagonist naloxone was used. The dose-response curves for the agonists and the shifts induced in the agonist dose-response curves are shown in Figures 3.3-3.8. The naloxone affinity values obtained (Ke values) listed in Table 3.3 were in the range 14.8nM to 50.8nM, which suggested that these compounds acted via δ or κ receptors. For further confirmation, the δ selective antagonist naltrindole was employed in this assay. The results shown in Table 3.3 (Figures 3.9-3.14), are consistent with δ-mediated responses, since the naltrindole Ke values were in the range 0.06 nM to 0.17 nM [Rogers et al., 1990]
Table 3.3 Agonist properties of buprenorphine analogues in the mouse vas deferens and their antagonism by naloxone and naltrindole.

<table>
<thead>
<tr>
<th>BU compound</th>
<th>IC50 (nM)</th>
<th>Ke (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Naltrindole</td>
<td>Naloxone</td>
</tr>
<tr>
<td>46</td>
<td>1.3±0.3</td>
<td>0.17±0.07</td>
</tr>
<tr>
<td>47</td>
<td>3.6±0.3</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td>48</td>
<td>0.2±0.1</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>95</td>
<td>4.1±1.6</td>
<td>0.03±0.02</td>
</tr>
<tr>
<td>96</td>
<td>7.2±0.2</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>61</td>
<td>2.5±0.8</td>
<td>0.06±0.02</td>
</tr>
</tbody>
</table>

Values represent means ± s.e.mean where n≥3.

In the guinea-pig ileum assay, the compounds were also potent with IC50 values ranging from 0.30nM to 3.70nM (Figures 3.15-3.20) as given in Table 3.4. There was a marked difference (10 fold) between the isomers BU46 and BU47. In order to determine the agonist site through which the compounds were acting, the selective κ antagonist nor-BNI was used. The Ke values for nor-BNI determined against the various buprenorphine analogues are listed in Table 3.4 and are in line with κ mediated response [Frankin & Traynor, 1991], demonstrating that all of the buprenorphine analogues tested were acting via κ receptors in this tissue.
Table 3.4 Agonist properties of buprenorphine analogues in the guinea-pig myenteric plexus-longitudinal muscle and their antagonism by nor-BNI.

<table>
<thead>
<tr>
<th>BU compound</th>
<th>$IC_{50}$ (nM)</th>
<th>nor-BNI $K_e$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td>0.30±0.23</td>
<td>0.03±0.03</td>
</tr>
<tr>
<td>47</td>
<td>3.64±1.3</td>
<td>0.04±0.01</td>
</tr>
<tr>
<td>48</td>
<td>1.35±0.23</td>
<td>0.10±0.05</td>
</tr>
<tr>
<td>95</td>
<td>2.02±0.2*</td>
<td>0.15±0.07*</td>
</tr>
<tr>
<td>96</td>
<td>3.70±1.2*</td>
<td>0.09±0.01*</td>
</tr>
<tr>
<td>61</td>
<td>1.70±0.5*</td>
<td>0.12±0.02*</td>
</tr>
</tbody>
</table>

Values represent means ± s.e.mean where $n \geq 3$, or *where $n = 2$ ± range.
Figure 3.3 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU46 in the absence (□) and presence (■) of 100 nM naloxone.

Figure 3.4 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU47 in the absence (□) and presence (■) of 100 nM naloxone.
Figure 3.5 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU48 in the absence (□) and presence (■) of 100 nM naloxone.

Figure 3.6 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU95 in the absence (□) and presence (■) of 100 nM naloxone.
Figure 3.7 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU96 in the absence (□) and presence (■) of 100 nM naloxone.

Figure 3.8 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU61 in the absence (□) and presence (■) of 100 nM naloxone.
Figure 3.9 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU46 in the absence (□) and presence (●) of 1nM naltrindole.

Figure 3.10 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU47 in the absence (□) and presence (●) of 1nM naltrindole.
Figure 3.11 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU48 in the absence (□) and presence (■) of 1 nM naltrindole.

Figure 3.12 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU95 in the absence (□) and presence (■) of 1 nM naltrindole.
Figure 3.13 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU96 in the absence (□) and presence (■) of 1nM naltrindole.

Figure 3.14 Dose-response curve for the inhibition of electrically induced contractions of mouse vas deferens by BU61 in the absence (□) and presence (■) of 1nM naltrindole.
Figure 3.15 Dose-response curve for the inhibition of electrically induced contractions of guinea-pig ileum by BU46 in the absence (□) and presence (■) of 1nM n-BNI.

Figure 3.16 Dose-response curve for the inhibition of electrically induced contractions of guinea-pig ileum by BU47 in the absence (□) and presence (■) of 1nM n-BNI.
Figure 3.17 Dose-response curve for the inhibition of electrically induced contractions of guinea-pig ileum by BU48 in the absence (□) and presence (■) of 1nM n-BNI.

Figure 3.18 Dose-response curve for the inhibition of electrically induced contractions of guinea-pig ileum by BU95 in the absence (□) and presence (■) of 1nM n-BNI.
Figure 3.19 Dose-response curve for the inhibition of electrically induced contractions of guinea-pig ileum by BU96 in the absence (♦) and presence (■) of 1nM n-BNI.

Figure 3.20 Dose-response curve for the inhibition of electrically induced contractions of guinea-pig ileum by BU61 in the absence (♦) and presence (■) of 1nM n-BNL.
3.2 Ligand-binding assays.

3.2.1 Determination of affinity of buprenorphine and analogues at opioid receptors in brain tissue and in cultured cells.

3.2.1 (a) Displacement of the μ, δ and κ selective ligands: 


The competitive displacement of[^3]H]-DAMGO,[^3]H]-DPDPE and[^3]H]-CI977 by buprenorphine and analogues were conducted in mouse brain (or guinea-pig brain in the case of buprenorphine) homogenates according to the method described in section 2.2. The graphs derived from these experiments are given in Figures 3.21-3.38. Ki values were calculated from IC50 values and are listed in Table 3.5. The results indicated that the compounds all showed high affinity for μ, δ and κ receptors in brain tissue.
Table 3.5 Binding affinity of buprenorphine and analogues at μ, δ and κ opioid receptors in mouse brain homogenates

<table>
<thead>
<tr>
<th>Compound</th>
<th>μ</th>
<th>δ</th>
<th>κ</th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td>0.60±0.05</td>
<td>0.86±0.08</td>
<td>1.02±0.1</td>
</tr>
<tr>
<td>47</td>
<td>0.88±0.20</td>
<td>1.45±0.19</td>
<td>2.75±0.1</td>
</tr>
<tr>
<td>48</td>
<td>0.36±0.05</td>
<td>1.41±0.30</td>
<td>1.56±0.1</td>
</tr>
<tr>
<td>95</td>
<td>0.48±0.19</td>
<td>1.41±0.09</td>
<td>3.20±1.0</td>
</tr>
<tr>
<td>96</td>
<td>1.50±0.15</td>
<td>1.31±0.39</td>
<td>2.99±0.5</td>
</tr>
<tr>
<td>61</td>
<td>0.55±0.06</td>
<td>1.78±0.11</td>
<td>1.79±0.1</td>
</tr>
<tr>
<td>buprenorphine</td>
<td>0.57±0.05*</td>
<td>1.30±0.03*</td>
<td>2.00±0.3*</td>
</tr>
</tbody>
</table>

Values represent means ± s.e. mean n≥3.

*Experiments were conducted in guinea-pig brain in our laboratory by J. Elliot.

3.2.1 (b) Displacement of [³H]-DAMGO by buprenorphine and analogues in SH-SY5Y cell membranes

The competitive displacement of [³H]-DAMGO by buprenorphine and analogues was conducted in SH-SY5Y cell membranes to examine the affinity of the compounds at the human μ receptor. The displacement curves are given in Figures 3.39-3.43. The Ki values obtained from these data listed in Table 3.6 were consistent with the data obtained from mouse brain tissue and confirmed all compounds to have high affinity at μ receptors.
Table 3.6 Binding affinity of buprenorphine and analogues at \( \mu \) opioid receptors in SH-SY5Y cell membranes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ki (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU 46</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td>47</td>
<td>0.37±0.07</td>
</tr>
<tr>
<td>95</td>
<td>0.69±0.03</td>
</tr>
<tr>
<td>96</td>
<td>0.75±0.02</td>
</tr>
<tr>
<td>buprenorphine</td>
<td>0.18±0.01</td>
</tr>
</tbody>
</table>

Values represent means ± s.e. mean where \( n \geq 3 \).

3.2.1 (c) Displacement of \(^{3}H\)-diprenorphine by buprenorphine in NG108-15 cell membranes

The competitive displacement of the opioid antagonist \(^{3}H\)-diprenorphine by buprenorphine was also studied at \( \delta \) receptors in NG108-15 cell membranes in Tris buffer or Tris buffer containing 100 mM NaCl and 10 \( \mu \)M GTP\( \gamma \)S. The results are shown in Figure 3.44. The displacement of \(^{3}H\)-diprenorphine (0.5 nM) by buprenorphine in Tris buffer alone afforded a Ki of 0.21±0.06 nM. When 100mM NaCl plus 10\( \mu \)M GTP\( \gamma \)S was included in the incubation buffer, the Ki value was only slightly increased to 0.37±0.08 nM. The results demonstrated that binding affinity of buprenorphine at \( \delta \) receptors does not alter in buffers which contain physiological concentrations of Na\(^{+}\) ions.
3.2.2 Displacement of $[^3H]$-diprenorphine by morphine, heroin, methadone and codeine in SH-SY5Y cell membranes

The displacement of $[^3H]$-diprenorphine by morphine, heroin, methadone and codeine was conducted in a binding assay buffer containing 100 mM NaCl, 10 mM MgCl$_2$ in order to compare the binding affinity (Ki values) with the EC$_{50}$ values obtained in 'functional' assays. The obtained Ki values for morphine, heroin, methadone and codeine were 624±34 nM, 1335±316 nM, 133±21 nM and 28145±1634 nM, respectively (Figure 3.45).

3.2.3 Saturation binding assays in SH-SY5Y cell membranes

There are many stocks of human neuroblastoma SH-SY5Y cells throughout the world and the receptor population of these may differ. The SH-SY5Y cell line is known to express both μ- and δ-opioid receptors, with μ-opioid receptors predominating (Yu & Sadee, 1988). However, earlier passage number (17-20) SH-SY5Y cells are reported to show different properties from later passage number SH-SY5Y cells (70-90) in electrophysiology experiments (G. Henderson, unpublished data). In order to use the cells for future assays of μ-receptor affinity and efficacy, experiments were designed to determine the opioid receptor population and characteristics on earlier passage number cells compared with the later passage number cells. The study employed $[^3H]$-DAMGO and $[^3H]$-DPDPE as radioligands. The results summarised in Table 7 showed that the earlier passage number SH-SY5Y cells possessed a larger population of δ receptors than that of μ receptors, which is opposite to the situation in the later passage SH-SY5Y cells. Later passage cells containing higher population of μ receptors were used in future experiments described in this thesis.
Table 3.7 Radioligand saturation binding in SH-SY5Y cell membranes

<table>
<thead>
<tr>
<th>Cell number</th>
<th>[^{3}H]DAMGO</th>
<th>[^{3}H]DPDPE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bmax (fmol/mg protein)</td>
<td>Bmax (fmol/mg protein)</td>
</tr>
<tr>
<td>earlier</td>
<td>34±8.6</td>
<td>162±16</td>
</tr>
<tr>
<td>later</td>
<td>140±11*</td>
<td>56±11*</td>
</tr>
</tbody>
</table>

\(B_{\text{max}}\) and \(K_{D}\) values are expressed as means ± s.e. mean \(n\geq3\). Assays were conducted at 25°C for 40 min, Tris-HCl buffer pH 7.4. *Experiments performed in our laboratory by J. Elliot.
Figure 3.21 Competitive displacement of specifically bound [*H]DAMGO (InM) by BU46 in mouse brain homogenates.

Figure 3.22 Competitive displacement of specifically bound [*H]DAMGO (InM) by BU47 in mouse brain homogenates.
Figure 3.23 Competitive displacement of specifically bound \[^3\text{H}\]DAMGO (1nM) by BU48 in mouse brain homogenates.

Figure 3.24 Competitive displacement of specifically bound \[^3\text{H}\]DAMGO (1nM) by BU95 in mouse brain homogenates.
Figure 3.25 Competitive displacement of specifically bound $[^3H]DAMGO$ (1nM) by BU96 in mouse brain homogenates.

Figure 3.26 Competitive displacement of specifically bound $[^3H]DAMGO$ (1nM) by BU61 in mouse brain homogenates.
Figure 3.27 Competitive displacement of specifically bound [3H] DPDPE (2nM) by BU46 in mouse brain homogenates.

Figure 3.28 Competitive displacement of specifically bound [3H] DPDPE (2nM) by BU47 in mouse brain homogenates.
Figure 3.29 Competitive displacement of specifically bound \[^3H\) DPDPE (2nM) by BU48 in mouse brain homogenates.

Figure 3.30 Competitive displacement of specifically bound \[^3H\) DPDPE (2nM) by BU95 in mouse brain homogenates.
Figure 3.31 Competitive displacement of specifically bound \[^{[H]}\]DPDPE (2nM) by BU96 in mouse brain homogenates.

Figure 3.32 Competitive displacement of specifically bound \[^{[H]}\]DPDPE (2nM) by BU61 in mouse brain homogenates.
Figure 3.33 Competitive displacement of specifically bound [\(^3\)H] CI977 (0.5nM) by BU46 in mouse brain homogenates.

Figure 3.34 Competitive displacement of specifically bound [\(^3\)H] CI977 (0.5nM) by BU47 in mouse brain homogenates.
Figure 3.35 Competitive displacement of specific bound \(^{\text{3}}\text{H}\) CI977 (0.5nM) by BU48 in mouse brain homogenates.

Figure 3.36 Competitive displacement of specifically bound \(^{\text{3}}\text{H}\) CI977 (0.5nM) by BU95 in mouse brain homogenates.
Figure 3.37 Competitive displacement of specifically bound \[^3^H\] CI977 (0.5nM) by BU96 in mouse brain homogenates.

Figure 3.38 Competitive displacement of specifically bound \[^3^H\] CI977 (0.5nM) by BU61 in mouse brain homogenates.
Figure 3.39 Competitive displacement of specifically bound[^H]DAMGO (1nM) by buprenorphine in the SH-SY5Y cell membranes.

Figure 3.40 Competitive displacement of specifically bound[^H]DAMGO (1nM) by BU46 in the SH-SY5Y cell membranes.
Figure 3.41 Competitive displacement of specifically bound \[^{3}H\]DAMGO (1nM) by BU47 in the SH-SY5Y cell membranes.

Figure 3.42 Competitive displacement of specifically bound \[^{3}H\]DAMGO (1nM) by BU95 in the SH-SY5Y cell membranes.
Figure 3.43 Competitive displacement of specifically bound $[^3H]$DAMGO (1nM) by BU96 in the SH-SY5Y cell membranes.
Figure 3.44 Competitive displacement of specifically bound $[^3H]$diprenorphine (0.5nM) from membranes of NG108-15 cell by buprenorphine in Tris buffer (■) or Tris buffer containing 100mM NaCl and 10μM GTPγS (□).

Figure 3.45 Competitive displacement of specifically bound $[^3H]$diprenorphine (0.5nM) from membranes of SH-SY5Y cells by methadone (■), morphine (●), heroin (▲) and codeine (■) in $[^35S]$GTPγS binding assay buffer.
Figure 3.46 A representative graph showing saturation $[^{3}H]$ DAMGO binding to earlier passage number SH-SY5Y cell membranes. Inset is the corresponding Scatchard plot.
Figure 3.47 A representative graph showing saturation \([^3]H\) DPDPE binding to earlier passage number SH-SY5Y cell membranes. Inset is the corresponding Scatchard plot.
3.3 Intracellular assays using SH-SY5Y cells

3.3.1 cyclic AMP assays

3.3.1(a) Use of DAMGO as a standard compound to determine the sensitivity of cAMP assays

In SH-SY5Y cells, inhibition of cAMP formation by a range of μ opioids has illustrated that cAMP assays offer a good system in which the functional response of μ opioid agonists can be measured [Lambert et al., 1993].

In this experiment, the μ agonist DAMGO has been used as a standard compound to show the sensitivity of the assay. Assays were conducted using the method described in section 2.3. The maximal inhibition of forskolin stimulated cAMP formation by DAMGO was about 50% of the basal level, and the IC₅₀ value was 90 nM [Figure 3.48], which is in agreement with the results obtained by Lambert et al. [1993].

3.3.1(b) Buprenorphine analogues evaluated using cAMP assay

Two isomer pairs BU46 and BU47; BU95 and BU96 were tested in the cAMP assay. All compounds inhibited forskolin stimulated cAMP levels (Figures 3.49-3.52). Both the maximal inhibition and the IC₅₀ values obtained are listed in Table 3.8 and compared with DAMGO. The data showed that the efficacy of all compounds tested was lower than that of DAMGO in this cell system. From the IC₅₀ values, it can be seen that all the buprenorphine analogues tested are potent agonists at μ opioid receptors.
Table. 3.8  IC$_{50}$ values and maximal inhibition of cAMP formation in SH-SY5Y cells by DAMGO and buprenorphine analogues

<table>
<thead>
<tr>
<th>compound</th>
<th>IC$_{50}$ (nM)</th>
<th>maximal effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMGO</td>
<td>96±4.6</td>
<td>1</td>
</tr>
<tr>
<td>BU46</td>
<td>15±4.5</td>
<td>0.6</td>
</tr>
<tr>
<td>BU47</td>
<td>17±3.5</td>
<td>0.6</td>
</tr>
<tr>
<td>BU95</td>
<td>20±4.2</td>
<td>0.5</td>
</tr>
<tr>
<td>BU96</td>
<td>28±3.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Values represent mean ± range n=2 with exception of DAMGO where n=3.
Figure 3.48 Inhibition of forskolin-stimulated adenylyl cyclase activity by DAMGO in SH-SY5Y whole cells. Forskolin stimulated adenylyl cyclase activity was 63±4.3 fmol/mg protein/min.

Figure 3.49 Inhibition of forskolin-stimulated adenylyl cyclase activity by BU46 in SH-SY5Y whole cells. Forskolin stimulated adenylyl cyclase activity was 67.8±5.6 fmol/mg protein/min.
Figure 3.50 Inhibition of forskolin-stimulated adenylyl cyclase activity by BU47 in SH-SY5Y whole cells. Forskolin stimulated adenylyl cyclase activity was 67.8±5.6 fmol / mg protein / min.

Figure 3.51 Inhibition of forskolin-stimulated adenylyl cyclase activity by BU95 in SH-SY5Y whole cells. Forskolin stimulated adenylyl cyclase activity was 70.2±4.3 fmol / mg protein / min.
Figure 3.52 Inhibition of forskolin-stimulated adenylyl cyclase activity by BU96 in SH-SY5Y whole cells. Forskolin stimulated adenylyl cyclase activity was 70.2±4.3 fmol/mg protein/min.
3.3.2 $[^{35}S]$-GTP$\gamma$S binding assay

3.3.2 (a) $[^{35}S]$-GTP$\gamma$S binding assay as a sensitive method for the determination of opioid efficacy

μ Opioid agonists have been shown to modulate $[^{35}S]$-GTP$\gamma$S binding in SH-SY5Y cell membranes in a naloxone-reversible manner [Traynor & Nahorski, 1995]. Therefore, using this assay the efficacy and potency of μ opioids can be quickly determined in SH-SY5Y cells and the assay can potentially be used to screen large numbers of opiates.

Initial experiment were conducted to demonstrate that similar results could be achieved to those originally reported by Traynor & Nahorski [1995]. The μ opioid agonist DAMGO afforded an EC$_{50}$ value of 8.3±0.8 nM (Figure 3.53).

In the same manner, concentration-effect curves for the μ opioid agonists morphine, methadone, heroin and codeine were conducted (Figure 3.54). The maximal stimulation of $[^{35}S]$-GTP$\gamma$S binding and the potency of the compounds obtained are listed in Table 3.9.

3.3.2 (b) Buprenorphine and analogues examined using the $[^{35}S]$-GTP$\gamma$S binding assay

The maximal effects (compare to fentanyl) and EC$_{50}$ values obtained for buprenorphine and analogues are listed in Table 3.10. From the data, it can be seen that buprenorphine and analogues are all potent partial agonists at μ opioid receptors. However, the efficacy of buprenorphine in this system is slightly higher then that of its analogues [Figures 3.55-3.62]. The agonist activity of BU47 was readily blocked by the antagonist naloxone affording a Ke value of 3.3±0.7 nM, indicating the effect is mediated via μ receptors.
For comparison a further derivative BU4 (Figure 1.11), which contains an N-Me instead of N-CPM was examined. This compound was a potent full agonist.

Table 3.9 EC$_{50}$ values and maximal effects of stimulation of $[^{35}\text{S}]$-GTP$_{Y}$S binding to membranes from SH-SY5Y cells by DAMGO, morphine, methadone, heroin and codeine

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$(nM)</th>
<th>Maximal effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMGO</td>
<td>8.4±1.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Morphine</td>
<td>31.8±3.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Methadone</td>
<td>19.9±2.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Heroin</td>
<td>316±28</td>
<td>1.1</td>
</tr>
<tr>
<td>Codeine</td>
<td>5309±430</td>
<td>1.1</td>
</tr>
<tr>
<td>Fentanyl*</td>
<td>15.2±3.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Values represent mean ± s.e. n≥3. *Values are normalized to the stimulation of $[^{35}\text{S}]$-GTP$_{Y}$S binding caused by 3 μM fentanyl (maximal effect =1.0). *Fentanyl data are taken from Traynor & Nahorski [1995].
Table 3.10 EC$_{50}$ values and maximal effects of stimulation of [$^{35}$S]-GTPyS binding to membranes from SH-SY5Y cells by buprenorphine and analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$</th>
<th>Maximal effect</th>
<th>Naloxone Ke</th>
</tr>
</thead>
<tbody>
<tr>
<td>buprenorphine</td>
<td>0.18±0.10</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>BU46</td>
<td>0.21±0.04</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>BU47</td>
<td>0.40±0.07</td>
<td>0.44</td>
<td>3.3±0.7</td>
</tr>
<tr>
<td>BU48</td>
<td>0.23±0.04</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>BU95</td>
<td>0.33±0.06</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>BU96</td>
<td>0.56±0.08</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>BU61</td>
<td>0.26±0.05</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>BU4</td>
<td>0.18±0.03</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.mean  n≥3. Values are normalized to the stimulation of [$^{35}$S]-GTPyS binding caused by 3μM fentanyl (maximal effect = 1.0)
Figure 3.53 Stimulation of $[^{35}S]$GTPyS binding to membranes of SH-SY5Y cells by DAMGO. Control binding of $[^{35}S]$GTPyS was 40±3.6 fmol/mg protein in the absence of fentanyl and 76±5.7 fmol/mg protein in the presence of fentanyl (3μM).

Figure 3.54 Stimulation of $[^{35}S]$GTPyS binding to membranes of SH-SY5Y cells by methadone (■), morphine (□), heroin (▲) and codeine (▲). Control binding of $[^{35}S]$GTPyS was 40±3.6 fmol/mg protein in the absence of fentanyl and 76±5.7 fmol/mg protein in the presence of fentanyl (3μM).
Figure 3.55 Stimulation of $[^{35}S]$GTPyS binding to membranes of SH-SY5Y cells by buprenorphine. Control binding of $[^{35}S]$GTPyS was 39±3.2 fmol/mg protein in the absence of fentanyl and 63±3.6 fmol/mg protein in the presence of fentanyl (3µM).

Figure 3.56 Stimulation of $[^{35}S]$GTPyS binding to membranes of SH-SY5Y cells by BU4. Control binding of $[^{35}S]$GTPyS was 40±3.6 fmol/mg protein in the absence of fentanyl and 76±5.7 fmol/mg protein in the presence of fentanyl (3µM).
Figure 3.57 Stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding to membranes of SH-SY5Y cells by BU46. Control binding of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ was 44±4.7 fmol/mg protein in the absence of fentanyl and 78±6.3 fmol/mg protein in the presence of fentanyl (3μM).

Figure 3.58 Stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding to membranes of SH-SY5Y cells by BU95. Control binding of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ was 42±2.2 fmol/mg protein in the absence of fentanyl and 78±4.2 fmol/mg protein in the presence of fentanyl (3μM).
Figure 3.59 Stimulation of $[^{35}S]GTP\gamma S$ binding to membranes of SH-SY5Y cells by BU47 in the absence (○) and presence (■) of 10 nM naloxone. Control binding of $[^{35}S]GTP\gamma S$ was 44±4.7 fmol/mg protein in the absence of fentanyl and 78±6.3 fmol/mg protein in the presence of fentanyl (3μM).

Figure 3.60 Stimulation of $[^{35}S]GTP\gamma S$ binding to membranes of SH-SY5Y cells by BU48. Control binding of $[^{35}S]GTP\gamma S$ was 49±5.2 fmol/mg protein in the absence of fentanyl and 83±7.3 fmol/mg protein in the presence of fentanyl (3μM).
Figure 3.61 Stimulation of $[^{35}S]$GTP$\gamma$S binding to membranes of SH-SY5Y cells by BU96. Control binding of $[^{35}S]$GTP$\gamma$S was 42±2.2 fmol/mg protein in the absence of fentanyl and 78±4.2 fmol/mg protein in the presence of fentanyl (3μM).

Figure 3.62 Stimulation of $[^{35}S]$GTP$\gamma$S binding to membranes of SH-SY5Y cells by BU61. Control binding of $[^{35}S]$GTP$\gamma$S was 40±3.3 fmol/mg protein in the absence of fentanyl and 79±4.8 fmol/mg protein in the presence of fentanyl (3μM).
Chapter 4.0 Discussion and Conclusions

The presence of two lipophilic agonist binding sites for the N-cyclopropymethyl orvinols; one above the ring system associated with \( \mu \) agonism and one below \( C_8 \) associated with \( \kappa \) agonism, has been proposed [Coop, 1995]. Buprenorphine is an N-cyclopropylmethyl orvinol which is a potent analgesic agent. *In vivo*, this compound is a partial agonist at \( \mu \) opioid receptors and a potent antagonist at \( \kappa \) receptors [Rance, 1979]. To investigate factors determining this unique \( \mu \) partial agonism / \( \kappa \) antagonism profile, analogues of buprenorphine where the conformation about C(7)-C(19) is constrained into rings, have been synthesised. This should allow examination of the influence of the spatial position of the tertiary alcohol function. In the present studies seven conformationally restrained analogues have been tested for their affinity and efficacy in *in vitro* experiments. In the constrained molecules the OH and additional alkyl ring are fixed below the buprenorphine ring system and hence the ring methylenes are available to interact with the putative \( \kappa \)-agonist site, but not the proposed \( \mu \)-agonist site (Diagram 4.1).

![Diagram 4.1 Partial structure of BU47 showing how the additional alkyl ring might interact with a proposed \( \kappa \)-lipophilic binding site.](image_url)
(1) Binding assays

(a) buprenorphine

The *in vivo* κ-antagonist activity of buprenorphine occurs over the same dose range that buprenorphine exhibits partial μ agonist activity in rats [Leander, 1987]. In several *in vivo* assays buprenorphine exhibits a bell-shaped dose-response curve with the agonist response being reversed at higher concentration [Cowan et al., 1977]. This observed pharmacological phenomenon is unlikely to be explained by differential affinity for opioid receptor types, since *in vitro* buprenorphine has a similar high affinity for μ, δ and κ receptors, all being in the low nanomolar range, namely 0.57±0.05 nM at μ receptors, 1.30±0.03 nM at δ receptors and 2.00±0.30 nM at κ receptors respectively [Elliot, 1991]. However these values were obtained in Tris buffer which favours high agonist affinity states of the receptor and does not necessarily indicate affinity under more physiological condition [Carroll et al., 1988].

The affinity of buprenorphine at both μ and κ receptors shifts just 2-fold in the presence of 25 mM Na⁺ suggesting buprenorphine may have same affinity at μ and κ receptors under physiological conditions [Elliot, 1991].

The activity of buprenorphine at δ receptors, particularly in buffers containing Na⁺ has not been much studied to date, hence an attempt was made to explain the bell-shaped dose-response curves seen *in vivo* by an action of buprenorphine at δ receptors. The hypothesis was proposed that at the lower concentrations, buprenorphine acts via μ receptors to elicit agonist activity whereas in the higher concentrations, buprenorphine could act via an agonist action at δ receptors to elicit the reversal effect by a mechanism involving physiological antagonism. This would require buprenorphine to have a lower affinity at δ receptors and thus require higher concentrations to show an effect. The assays were performed for the following two reasons. Firstly, the true
differences in affinity of buprenorphine at opioid receptor types may not be revealed in the normal binding assay conditions of low ionic strength Tris buffer [Carroll et al., 1988]; secondly, the agonist and antagonist properties of opioids may be revealed by the effects of guanyl nucleotides and sodium on the affinity of the compound for their receptors. Data from binding assays confirmed that at δ receptors buprenorphine had a high affinity, similar to its affinity at μ receptors, in Tris buffer. The binding affinity of buprenorphine at δ receptors under more physiological conditions, i.e. in a buffer containing the guanosine-5′-triphosphate stable analogue GTPγS and a physiological concentration of Na⁺ was therefore tested.

The results showed that the Ki value of buprenorphine at δ receptors in Tris buffer alone was 0.21±0.06 nM, but in the buffer containing GTPγS and Na⁺ was 0.37±0.08 nM; that is no significant difference was found. This result demonstrated that the binding affinity of buprenorphine at δ receptors did not alter in the physiological conditions similar to the observations at μ and κ receptors. This is in line with the results of similar experiments using mouse brain carried out by Villiger et al. [1984] which yielded the conclusion that binding affinity of buprenorphine is either increased or not affected by guanosine-5′-triphosphate and physiological concentrations of Na⁺ at μ, δ or κ receptors. Thus the in vivo pharmacological effects of buprenorphine are unlikely to be readily explained by simple analysis of ligand-binding assays, even in physiological buffers.

(b) Buprenorphine analogues

The binding affinity of the ring constrained buprenorphine analogues was examined in homogenates of mouse brains and SH-SY5Y human neuroblastoma cells in Tris buffer. All the buprenorphine analogues tested showed similar binding profiles. The compounds possessed high affinity at the three opioid receptors, with Ki values in the
low nM range between 0.36±0.05 nM for compound BU48 at μ receptors to 3.20±1.0 nM for compound BU95 at κ receptors. All six compounds were slightly μ selective, and affinity was in the order μ>δ>κ. This was consistent with the parent compound buprenorphine [Elliot, 1991]. It can be concluded that the position of tertiary alcohol function does not play an important role in controlling receptor-binding affinity.

(2) Isolated tissue assays

(a) Assays in the mouse vas deferens preparation

The compounds all showed potent agonist activity with IC₅₀ values in the range 0.2 nM for BU48 to 7.2 nM for BU96. The compounds were all full agonists in this preparation, since the maximum inhibition of the electrically-induced contractions of the mouse vas deferens was always greater than 90% of the control twitch height. Since the mouse vas deferens preparation contains μ, δ and κ receptors with δ receptor predominating [Leslie, 1987], compounds with agonist activity at different receptors are likely to show responses via δ receptors preferentially.

The non-selective antagonist naloxone was used initially to determine the selectivity of the compounds. Antagonism by naloxone afforded Ke values in the range 14.8±0.3 nM for compound BU96 to 50.8±23 nM for compound BU47, which suggested that the action of these compounds is associated with δ or κ receptors, but certainly not μ receptors where the expected Ke value is in the range 1-3 nM [Leslie, 1987]. The δ selective antagonist naltrindole was then chosen to discriminate δ actions from κ mediated effects. The naltrindole Ke values were in the range 0.03 nM for compound BU96 to 0.17 nM for compound BU46, which were consistent with antagonism of δ-mediated responses [Rogers et al., 1990]. The conclusion can be drawn that the
buprenorphine analogues tested were all potent δ full agonists in the mouse vas deferens preparation. However, there was no obvious difference in efficacy and potency between the isomeric pairs, although the least strained ring derivative compound BU48, where a 6-membered ring is included rather than a 5-membered ring, was the most potent with its IC₅₀ value (0.2±0.1 nM) being much lower than its Ki value (1.41±0.3 nM) at δ receptors in the binding assay. This is in contrast with the other compounds in which the Ki and IC₅₀ values were similar (Table 4.1). Hence the position of the tertiary OH may play a role in the determination of efficacy and potency at δ receptors, although the higher potency and efficacy of BU48 may related to the 6-membered ring itself in this compound.

Table 4.1 IC₅₀ / Ki of buprenorphine analogues at δ and κ receptors

<table>
<thead>
<tr>
<th>BU compound</th>
<th>IC₅₀ / Ki (δ)</th>
<th>IC₅₀ / Ki (κ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU46</td>
<td>1.51</td>
<td>0.29</td>
</tr>
<tr>
<td>BU47</td>
<td>2.50</td>
<td>1.32</td>
</tr>
<tr>
<td>BU48</td>
<td>0.14</td>
<td>0.87</td>
</tr>
<tr>
<td>BU95</td>
<td>2.90</td>
<td>0.63</td>
</tr>
<tr>
<td>BU96</td>
<td>5.49</td>
<td>1.25</td>
</tr>
<tr>
<td>BU61</td>
<td>1.40</td>
<td>0.95</td>
</tr>
</tbody>
</table>
(b) Assays in the guinea-pig ileum preparation

The parent compound buprenorphine shows an agonist profile in the guinea-pig preparations which is relatively resistant to naloxone [Schulz & Herz, 1976]. However, much in vivo evidence suggests a μ agonism and κ antagonism profile for buprenorphine.

In the guinea-pig ileum preparation all ring constrained buprenorphine analogues tested acted as a potent full agonists with the IC₅₀ values in the range 0.30±0.23 nM for compound BU46 to 3.70±0.20 nM for compound BU96. Since the guinea-pig ileum preparation contains μ and κ receptors, the κ selective antagonist nor-BNI was used to show the receptor through which the compounds were acting. The nor-BNI Ke values were in the range 0.03±0.03 nM to 0.15±0.07 nM, which is in line with κ-mediated responses [Franklin & Traynor, 1991].

The isomers BU46 and BU47 showed a 10-fold difference in potency with the IC₅₀ values being 0.30±0.23 nM for BU46, and 3.64±1.30 nM for BU47. In contrast, only a 2-fold difference was seen in binding affinity with Ki values being 1.02±0.10 nM for BU46, and 2.75±0.10 nM for BU47. This suggests that the position of tertiary OH plays a role in the determination of efficacy and potency at κ sites. However, this is not the case for the isomers BU95 and BU96 that had similar potency which was in line with the weaker BU47. Thus the introduction of methyl groups in compounds BU95 and BU96 appears to mask differences in potency and efficacy.

Since the guinea-pig ileum preparation possesses a higher μ receptor density than κ receptor density [Leslie, 1987], the fact that the κ activity of the buprenorphine analogues was seen in this tissue suggests that the compounds have no, or only weak
activity, at μ receptors. In order to examine whether the compounds possess any intrinsic activity at μ receptors, intracellular assays were performed as a measure of function in a system not possessing κ receptors, namely human neuroblastoma, SH-SY5Y cells.

(3) Intracellular assays

The most commonly used intracellular assay for opioid agonism is the inhibition of stimulated cyclic AMP accumulation. Although the assay indicates the efficacy and potency of the opioid compounds in several cell lines and brain tissues, the sensitivity of the assay is not always very good. Moreover, this assay does not lend itself to large scale drug screening due to the small magnitude of the response [Costa et al., 1991].

In SH-SY5Y cells, which possess μ opioid receptors predominantly, the signal to noise ratio for cyclic AMP assay was small, even for a full μ-agonist such as DAMGO. The maximum inhibition caused by DAMGO was only about 50% of the basal levels, which is in agreement with previous studies [Lambert et al., 1993], hence compounds with weak partial agonist activity cannot be distinguished readily in this assay. As an alternative, the [35S]-GTPγS binding assay has been investigated at opioid receptors recently [Traynor & Nahorski, 1995]. In this assay, the ability of μ opioid agonists to activate G proteins, has been measured as the binding of [35S]-GTPγS to membranes from human neuroblastoma SH-SY5Y cells. In these cells the maximum stimulation of [35S]-GTPγS binding caused by μ full agonist fentanyl (3μM) is about 70-100% increase over the basal levels, and it has been shown that DAMGO and fentanyl are full agonists and pentazocine is a partial agonist at μ receptors in this system [Traynor & Nahorski, 1995].

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The standard compounds, morphine, heroin, codeine and methadone were examined using the \[^{35}\text{S}\]-GTP\gamma S binding assay in SH-SY5Y cells. The results showed that morphine, heroin, codeine and methadone were all full agonists at \(\mu\) receptors, since the maximal stimulation of the \[^{35}\text{S}\]-GTP\gamma S binding caused by these compounds was similar to that of the full agonist fentanyl. However, codeine was the least potent and \(EC_{50}\) values of 19.9±2.9 nM, 31.8±3.2 nM, 316±28 nM and 5309±430 nM for methadone, morphine, heroin and codeine respectively were obtained. In ligand-binding assays \(K_i\) values for methadone, morphine, heroin and codeine under the conditions of the GTP\gamma S assay were 133±21 nM, 624±34 nM, 1335±316 nM, and 28145±1634 nM. The different potency and the different \(K_i\) values in binding of morphine, heroin and codeine indicated that heroin and codeine are not metabolised to morphine to elicit their pharmacological effects under these assay conditions. Indeed, no metabolism of these compounds was observed in SH-SY5Y cell homogenates [Naweed, 1995]. Thus the replacement of the 3-OH in morphine to 3-OMe in codeine reduces the potency but a maximal effect is still obtained. This is in contrast to some N-allyl series of thebaine derivatives in which 3-OMe compounds are agonists but their counterpart 3-OH oripavines are antagonists [Lewis, 1978].

Both the cyclic AMP and the \[^{35}\text{S}\]-GTP\gamma S intracellular assays were used for testing several buprenorphine analogues including the two isomeric pairs: BU46 and BU47; and BU95 and BU96. All compounds showed partial agonist activity with similar efficacy in both assays. However, the potency of the compounds was different in the two assays being 10-fold lower in the cyclic AMP assay. This may be attributed to the different assay conditions. The cyclic AMP assays were performed in Krebs solution using whole cell preparations, but the \[^{35}\text{S}\]-GTP\gamma S binding assays were conducted in cell membranes and in a non-physiological buffer containing HEPES, Mg\(^{2+}\) and Na\(^+\). Indeed, lower \(IC_{50}\) values have been obtained for cAMP assays conducted in whole cells than in cell membranes. For example DAMGO has an \(IC_{50}\) 20nM in cell membranes and an \(IC_{50}\) 90 nM in whole cell preparations [Sadee et al., 1991; Lambert
et al., 1993]. These data perhaps suggest a more efficient coupling in the ‘artificial’ membrane system. In addition, in binding assays, the occupancy curve for the opioid agonist DAMGO in Krebs solution is on the right side (approximately 2 log units) of that in the [³⁵S]-GTPγS binding assay buffer, indicating that μ opioid receptors are in a low agonist affinity state in Krebs solution, but in a higher agonist affinity state and therefore more tightly coupled to effectors in the [³⁵S]-GTPγS binding assay buffer [Traynor & Nahorski, 1995].

The low efficacy profile of the buprenorphine analogues at μ opioid receptors, as indicated by the lack of μ agonism in the guinea-pig ileum, was confirmed by the [³⁵S]-GTPγS binding and cAMP assays in SH-SY5Y cells. Although this cell line possesses a large population (approximately 200 fmol/mg protein) of μ opioid receptors [Traynor & Nahorski, 1995], all compounds showed rather low efficacy, affording values between 31.6% - 53% of the maximum response seen with fentanyl, even at concentration of 10 μM, thus indicating that all compounds were partial agonists at μ receptors in this system. Indeed, all of the analogues had slightly lower efficacy than the parent compound buprenorphine which itself gave 70% of the response seen with the full agonist fentanyl. This explains why the ring constrained buprenorphine analogues show agonism via κ-receptors rather than μ-receptors in the guinea-pig ileum preparation.

Replacement of N-CPM by N-Me (BU4) did give increased agonist activity with maximal response increasing from 40% to 100% of the fentanyl response. This is in agreement with the theory that a cyclopropyl group on the nitrogen atom of opiates confers antagonist activity [Casy & Parfit, 1986].

In spite of the observed low efficacy compared to fentanyl, all of the compounds were potent with EC₅₀ values of less than 1nM in the [³⁵S]GTPγS binding assay. No
significant differences were seen between the ring constrained compounds in efficacy and potency suggesting the position of the tertiary OH is not crucial for μ activity, though the compounds were less active than buprenorphine where free rotation about the C7-C19 bond is allowed.

The results described above are consistent with a pattern of low efficacy at μ receptors with high efficacy κ-agonism and δ-agonism. All the compounds were similar in potency and efficacy except for the isomers BU46 and BU47 which showed a 10-fold difference in potency at κ receptors in the guinea-pig ileum, and compound BU48 which was the most potent δ-agonist examined. This information needs to be considered in relationship to the proposals for the interaction of the oripavines with opioid receptors.

κ and μ receptors

The proposed model for receptor binding (Diagram 4.1) suggests the need for a μ-agonist binding site above the plane of the oripavine ring and for a κ-agonist binding site below the ring. The results which show that all compounds are κ agonists and possess low efficacy at μ receptors, are in line with the structural features required of this model i.e. the additional alkyl ring in the buprenorphine analogues is fixed below the plane of the ring of the oripavine.
Diagram 4.2 The structure of BU47 showing the distances from the C_{19}-OH to the tertiary N atom, Q-OH and C_6-OMe; and angles between C_{19}-OH to C_{19}-C_7 bond (alpha) and C_{19}-C_{20} bond (beta).

Although in purely distance terms the 19-OH group is not in a dissimilar position in relation to other functional groups in the different molecules (Diagram 4.2) (Table 4.2), the actual location in space is very different as shown by the torsion angles (Diagram 4.3). Thus for example the tertiary OH group in BU46 could bind to a suitably located hydrogen bond donor or acceptor which would not be available for the same OH in BU47 (Diagram 4.4). This is obviously important for potency presumably by interaction with a vital hydrogen binding site and resultant contribution to conformational changes in the κ receptor as a first step in the transduction of an agonist response. However, it makes little difference to the affinity, probably due to contributions to the binding affinity from many other groups in the molecule or there may be a suitably placed hydrogen bonding site for BU47 in the receptor, but which cannot lead to the transduction of a response.

These ideas would directly support the binding model of Hutchins and Rapoport [1984] with the presence of a hydrophilic site (i.e., the hydrogen bonding site) ‘above’ the molecule and a lipophilic site ‘below’ the molecule.
Diagram 4.3 Partial structure of BU47 (looking down the C$_{20}$-C$_{19}$ axis) showing torsion angles between C$_{19}$-OH and C$_{20}$ substituents.

Diagram 4.4 Partial structure of BU46 (1) and BU47 (2) showing the H bond could form between the OH in BU46 and the kappa hydrophilic binding site but not the OH in BU47. The 19-OH in BU47 could form a H-bond with another site on the receptor which is not important for the transduction of an agonist response. Dotted lines indicate potential hydrogen bond.
The same argument should apply to the isomeric pair BU95 and BU96 which contain two methyl substituents at C₂₀ and therefore are highly substituted as in buprenorphine. However, this is not the case. Indeed the potency of BU95, where the OH is in the same position as the more active isomer BU46, was found to be equivalent to the potency of BU96 and both were similar to the potency of the weaker BU47. The changes are not due to differences induced in the molecule by the methyl group, since the position of the 19-OH group relevant to other atoms in BU46 and BU95; and BU47 and BU96 is very similar (Table 4.2). This suggests that the methyl groups in BU95 are interfering, either directly or indirectly by altering receptor fit, with the ability of the 19-OH in BU95 to bind to the receptor by H-bonding (Diagram 4.5). Indeed the latter explanation would seem to be the case, since the methyl substituted derivative BU96 has somewhat reduced affinity than the less substituted BU47. In both compounds the 19-OH is not in a position to interact with the hydrogen bonding site which is important for the κ agonist potency of BU46.

Diagram 4.5 Partial structure of BU95 showing possible interference of C₂₀-methyls with receptor fit.
Since all the buprenorphine analogues tested showed δ agonist activity with similar binding affinity, it seems that the δ agonist binding site is not differentiated by the compounds and may also, like the κ site have a lipophilic region located below C₈. There was no difference in potency and efficacy between the isomeric pairs examined. However, the dimethyl substituent derivatives BU95 and BU96 (IC₅₀ 4.1±1.6 nM and 7.2±0.2 nM respectively) possess slightly lower potency than the corresponding unsubstituted BU46 and BU47 (IC₅₀ 1.3±0.3 nM and 3.6±0.3 nM respectively). It is therefore likely that the extra methyl groups again do not fit the δ agonist binding site better than the H atoms.

In contrast, BU48 has higher potency and efficacy at δ receptors. This must be due to the 6-membered ring in this compound (Diagram 4.6), in contrast with the other compounds which have a 5-membered ring. This could relate to improved binding due to the extra C atom leading to a shift in the position of the 19-OH group in BU48 which may be more favourable for combination via H-bonding to the δ receptor (Diagram 4.6). i.e. the exact position of the OH in BU48 may be extremely critical. Alternatively it may be that the 6-membered ring itself i.e. its extra C atom, interacts in providing additional hydrophobic interaction which contribute to the observed effects.

In contrast to the functional responses, binding assays for all compounds at δ receptors are similar, including compound BU48. This suggests the position of the OH, even in the highly active BU48 is not important for affinity, unlike its apparent role in efficacy.
Diagram 4.6 Partial structure of BU48 showing H-bonding may increase delta intrinsic activity or the six membered ring itself contributes to agonist response. Dotted line indicates potential hydrogen bond.
Table 4.2. The relative position of C₁₉-OH function to other groups in the different buprenorphine analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Distance (Å) between OH and 3-OH</th>
<th>Distance (Å) between OH and 6-OMe</th>
<th>Angle (°) between OH bond and C₇-C₉ bond</th>
<th>Angle (°) between OH bond and C₁₉-C₂₀ bond</th>
<th>Torsion angle (°) between OH bond and C₂₀-OH (Me) bond</th>
<th>Torsion angle (°) between OH bond and C₂₀ OH (Me) bond</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU46</td>
<td>6.743</td>
<td>8.475</td>
<td>3.204</td>
<td>111.48</td>
<td>112.53</td>
<td>-46.5</td>
</tr>
<tr>
<td>BU47</td>
<td>6.106</td>
<td>7.417</td>
<td>4.169</td>
<td>110.64</td>
<td>110.51</td>
<td>-151.3</td>
</tr>
<tr>
<td>BU48</td>
<td>6.275</td>
<td>7.992</td>
<td>3.151</td>
<td>114.34</td>
<td>108.68</td>
<td>-179.6</td>
</tr>
<tr>
<td>BU95</td>
<td>6.734</td>
<td>8.458</td>
<td>3.179</td>
<td>110.65</td>
<td>113.75</td>
<td>-42.8</td>
</tr>
<tr>
<td>BU96</td>
<td>6.216</td>
<td>7.545</td>
<td>2.858</td>
<td>109.66</td>
<td>112.32</td>
<td>-162.8</td>
</tr>
<tr>
<td>BU61</td>
<td>6.089</td>
<td>7.752</td>
<td>2.980</td>
<td>110.00</td>
<td>109.18</td>
<td>-170.7</td>
</tr>
</tbody>
</table>
Overview

Buprenorphine has a distinct pharmacological profile. The aims of the present investigation were to determine the importance of the C7 substituent in the activity of buprenorphine (Figure 1.8) with regard to affinity for opioid receptors and the ability to cause an agonist response.

Obviously in buprenorphine the C7-C19 bond can rotate freely and only limited information can be gained of the recognition site for the C7 substituents from studying this compound. However, the use of constrained analogues in which the spatial arrangement of atoms around C19 are fixed as described in this thesis can enable us to learn about the topography of the ligand recognition site and features which are important for both ligand binding and signal transduction.

The results obtained suggest that the 19-OH function in buprenorphine and analogues does not play an important role in differentiating ligand affinity for μ-, δ-, and κ-opioid receptors. However this OH function may be more important in events related to agonist activation of the receptor, as determined by the ability to activate G-proteins in the cell assay or muscle contraction in the bioassay.

The findings substantiate the view that μ and κ opioid binding sites differ in their interaction with the oripavine group of molecules, and show that δ-receptor interactions are more allied to κ-receptor interactions. Indeed, the compounds all appear to be more efficacious at δ and κ receptors and only partial agonists at μ receptors.

The binding site for the rigid alkaloids is believed to be in the transmembrane domains; indeed a conserved aspartic acid residue in the third transmembrane domain is believed to be important for the ionic interaction with the tertiary nitrogen function of these molecules [Uhl, et al., 1994]. See figure(4.7) below and receptor diagram (4.8).
Potential lipophilic sites to accommodate the alkyl substituents on buprenorphine and its analogues is presumably made up of amino-acids such as Phe, Leu, Val which are abundant in the hydrophobic α-helical regions of the receptor [Uhl et al., 1994]. Potential hydrogen bonding sites are also numerous. However, within the transmembranes domains likely amino-acid substituents with hydrogen bonding potential do vary between receptor types. Examples of potential H bonding substituents include serine, cysteine, aspartic acid, lysine. These occur in transmembrane domains but are generally conserved across the three receptor types and thus are unlikely to contribute to differences in the effects of the oripavines. Those residues that differ between receptors and could contribute to different H-bonding are highlighted in the receptor diagram (4.8).

The approaches used in this thesis, together with modelling of the opioid receptor by groups such as Hruby, Mosberg, Akil and Portoghese allied to site-directed mutagenesis studies should lead to a much more informed approach to drug design.
Diagram 4.8 Sequence alignment of opioid receptors. ↑ conserved aspartic acid residue in transmembrane III. ° Potential H bonding sites found in κ and δ but not μ receptors. × Potential H bonding sites found in κ receptor. □ Potential H bonding sites found in δ receptor.
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