In vitro and in vivo characterisation of buprenorphine and other long-lasting opioids

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IN VITRO AND IN VIVO CHARACTERISATION OF BUPRENORPHINE AND OTHER LONG-LASTING OPIOIDS

by

Claire L. Neilan

A Doctoral thesis submitted in partial fulfillment of the requirements for the award of

Doctor of Philosophy of Loughborough University

March 1999

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Abstract

Buprenorphine is a promising medication for the treatment of opiate abuse. The pharmacology of buprenorphine has been studied in vitro using radioligand binding and [35S]GTPγS assays, and in vivo using assays of antinociception in rodents. A number of compounds with potential similar pharmacology have also been characterised. These are an iso-morphinan pyrrolidine derivative, and long-lasting 14-aminomorphinones and codeinones, in particular clocinnamox (C-CAM), a pure μ-antagonist and methoclocinnamox (MC-CAM), which has some agonist properties.

Buprenorphine, a partial μ-agonist and κ-antagonist, displays a unique bell-shaped dose-response curve in assays of antinociception in rodents. Pre-treatment of rats with the antagonist naltrexone shifted the ascending, but not the descending, phase of the dose-response curve. Investigation of the time-course of various doses of buprenorphine revealed that higher doses produced a rapid on- and offset of analgesia compared to lower doses, and this may explain the bell-shaped dose-response curve.

The morphinan-pyrrolidine derivative, BU72, is shown to be a highly efficacious μ-opioid agonist in C6 glioma cells transfected with the cloned μ-opioid receptor. In the mouse warm water tail withdrawal assay BU72 was 400 times more potent than morphine, and considerably longer-lasting. BU72 exhibited delayed μ-antagonist action in both the warm water tail withdrawal and writhing assays, blocking the effects of a maximal dose of morphine for up to 2 weeks in the writhing assay. This compound may represent an improvement over buprenorphine for the treatment of opiate abuse.

MC-CAM and a series of 3-substituted alkyl ether derivatives showed varying efficacy in SH-SY5Y human neuroblastoma cells. All displayed high affinity for the μ-opioid receptor and with the exception of the iso-propyl ether, BU25, all the analogues displayed biphasic binding profiles. BU25 was shown to be fully reversible in wash resistance assays. The results support 'pseudoirreversible' binding of these compounds to the μ-opioid receptor.

Further in vitro studies of C-CAM confirmed μ-antagonist action but have also shown it to be an inverse agonist at the δ-opioid receptor. Also identified as inverse agonists were 7-benzylidenenaltrexone (BNTX), and the peptide ICI 174,864. Treatment of C6 glioma cells with pertussis toxin confirmed the presence of
constitutive activity at the δ- but not the μ-receptor. C-CAM did not inhibit basal
\[^{35}\text{S}]\text{GTPγS}\) binding in \text{C6µ}\) or \text{C6}\) wild-type cells.

Keywords: Opioid receptors, μ-opioid receptor, buprenorphine, antinociception, irreversible antagonist, clocinnamox (C-CAM), multiple binding sites, affinity, efficacy, \text{SH-SY5Y}\) cells, \text{C6}\) glioma cells, inverse agonist, constitutive activity.
Acknowledgements

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<tr>
<td>AIDS</td>
<td>acquired immuno-deficiency syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β-FNA</td>
<td>β-funaltrexamine</td>
</tr>
<tr>
<td>BNTX</td>
<td>benzylidenenaltrexone</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>C-CAM</td>
<td>clocinnamox</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DADLE</td>
<td>[D-Ala², Leu⁵]enkephalin</td>
</tr>
<tr>
<td>DAMGO</td>
<td>[D-Ala², MePhe⁴Gly(ol)⁵]enkephalin</td>
</tr>
<tr>
<td>DPDPE</td>
<td>[D-Pen², D-Pen⁵]enkephalin</td>
</tr>
<tr>
<td>DSLET</td>
<td>[D-Ser², Leu⁵, Thr⁶]enkephalin</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration (USA)</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTP₇S</td>
<td>guanosine 5'-[γ-thio]triphosphate</td>
</tr>
<tr>
<td>G protein</td>
<td>guanosine triphosphate binding protein</td>
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<tr>
<td>GppNHp</td>
<td>guanylylimidophosphate</td>
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<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HIV</td>
<td>human immuno-deficiency virus</td>
</tr>
<tr>
<td>ICI 174,864</td>
<td>N, N-diallyl-Tyr-Aib-Aib-Phe-Leu</td>
</tr>
<tr>
<td></td>
<td>(Aib = α-aminoisobutyric acid)</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.t.</td>
<td>intrathecal</td>
</tr>
<tr>
<td>LAAM</td>
<td>levo-alpha-acetylmethadol</td>
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<tr>
<td>[Leu⁵]enkephalin</td>
<td>leucine enkephalin (Tyr-Gly-Gly-Phe-Met)</td>
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<tr>
<td>M-CAM</td>
<td>methocinnamox</td>
</tr>
<tr>
<td>MC-CAM</td>
<td>methoclocinnamox</td>
</tr>
<tr>
<td>[Met⁴]enkephalin</td>
<td>methionine enkephalin (Tyr-Gly-Gly-Phe-Met)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Nor-BNI</td>
<td>nor-binaltorphimine</td>
</tr>
<tr>
<td>NTB</td>
<td>naltriben</td>
</tr>
<tr>
<td>NTI</td>
<td>naltrindole</td>
</tr>
<tr>
<td>NTX</td>
<td>naltrexone</td>
</tr>
<tr>
<td>NX</td>
<td>naloxone</td>
</tr>
<tr>
<td>ORL1</td>
<td>opioid-receptor-like 1</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-[hydroxymethyl]-aminomethane</td>
</tr>
<tr>
<td>7TM-GPCR</td>
<td>7-transmembrane-G-protein coupled receptor</td>
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## Amino acid structures

Structure and coding of amino acids of the general structure: \( \text{H}_2\text{N-CH(R)-CO}_2\text{H} \)

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

* Proline is an imino acid of the structure:
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CHAPTER 1
GENERAL INTRODUCTION
1.1 Historical Perspective

The control of pain continues to be one of the most important and challenging aspects of medical care. The perception of pain may be categorized into two distinct subjective measures – a sensory component and an emotional component. Analgesic drugs may act by altering either or both of these elements. Although non-opioid analgesics can afford relief from mild to moderate pain, the opioid analgesics have the added advantage of reducing the emotional component, namely anxiety, tension, and fear. Therefore opioid analgesics can enable the patient to cope with relatively severe pain. However, neuropathic pain i.e. pain caused by damage to neuronal structures, often resulting in supersensitivity, does not respond well to the opioid analgesics.

Opium, the latex obtained from the poppy {Papaver somniferum}, is the source of many pharmacologically important alkaloids. Morphine, named after Morpheus, the Greek God of dreams, was first isolated in 1803 by the German pharmacist Serturner. Well reputed for its pain relieving properties, morphine rapidly established itself in medicine as an analgesic.

![Figure 1.1 The structure of morphine.](image)

Little was known about the mechanisms underlying the extensive pharmacological actions of morphine or other opiates until the 1970's. In 1971 Goldstein proposed that radiolabeled compounds might be used to demonstrate the existence of opioid receptors [Goldstein et al., 1971]. Direct evidence that opiates are recognized by specific receptors came from binding studies in neuronal membranes [Pert & Snyder, 1973; Wong & Hong, 1973, Simon et al., 1973]. These receptors were henceforth named opiate receptors. It soon became clear from experimental work that peptides
may be endogenous ligands for the opioid receptor, and in 1975 Kosterlitz and co-workers isolated two pentapeptides with opiate-like activity from porcine brain [Hughes et al., 1975]. These peptides were named methionine enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu), and differed only in the C-terminus amino acid. Both peptides mimicked the action of morphine in in vitro bioassays by inhibition of electrically stimulated contractions of smooth muscle. Two other families of opioid peptides are the endorphins [Bradbury et al., 1976], and the dynorphins [Goldstein et al., 1979]. Both of these classes of peptides contain the Tyr-Gly-Gly-Phe-X sequence, where X = Leu or Met.

In vivo studies of the wide range of actions of different opiates implied that multiple receptor types might be involved. Indeed in 1976 multiple receptor types were established [Martin et al., 1976]. The proposed receptor types were μ, at which morphine is the prototype agonist; κ, at which ketocyclazocine is the prototype agonist, and σ, at which N-allylnormetazocine is the prototype agonist. Evidence for a fourth type of receptor came from Kosterlitz and his colleagues [Lord et al., 1977]. They discovered that in mouse vas deferens and guinea-pig ileum morphine was not equipotent, nor were the enkephalins. Furthermore naloxone, an important opioid antagonist, was 10 times more potent in blocking opioid responses in the guinea pig ileum than in the mouse vas deferens. It was concluded that another type of receptor, christened δ, predominated in the mouse vas deferens. σ-Receptors are insensitive to antagonism by naloxone and so are no longer classed as opioid receptors. Of the endogenous ligands, the enkephalins and endorphins bind to both the μ- and δ-receptors, whereas the dynorphins bind more selectively to the κ-receptor. Other classes of peptides more recently discovered are the dermorphins and deltorphins, which were isolated from South American frog skin [Montecucchi et al., 1981, Kreil et al., 1989, Erspamer et al., 1989]. The dermorphins are highly selective for the μ-receptor, and, as their name suggests, the deltorphins are highly selective for the δ-receptor. Also recently discovered are the endomorphins 1 and 2 [Zadina et al., 1997] which are selective for the μ-receptor. The structures of the above named opioid peptides are shown in Fig. 1.2.

The structure of the opioid receptors remained largely unknown until 1992, when two groups published reports of the expression cloning of cDNA encoding the δ-receptor from the neuroblastoma x glioma (NG108-15) cell line [Evans et al., 1992;
Kieffer et al., 1992). A rapid succession of molecular cloning reports were to follow: cloning of the rat μ-opioid receptor [Chen et al., 1993; Fukuda et al., 1993; Wang et al., 1993], the rat κ-opioid receptor [Chen et al., 1993b; Minami et al., 1993; Li et al., 1993; Meng et al., 1993], the mouse κ-opioid receptor [Yasuda et al., 1993], and the rat δ-opioid receptor [Fukuda et al., 1993]. The amino acid sequences of all three human opioid receptors have now been published, μ- [Wang et al., 1994], δ- [Knapp et al., 1994; Simonin et al., 1994], and κ- [Simonin et al., 1995].

Several research groups also reported the discovery of another, ORL1 (opioid-receptor-like) opioid receptor [Mollereau et al., 1994, Bunzow et al., 1994, Fukuda et al., 1994, Chen et al., 1994, Wang et al., 1994]. This receptor was found to be similar in sequence to the other opioid receptors, but shares greatest homology with the κ-receptor. The pharmacological effects mediated by the ORL1 receptor are not yet fully understood. It mediates the inhibition of adenylyl cyclase as do μ, δ, and κ-receptors, however it appears to also mediate anti-opioid actions, such as the inhibition of opioid induced analgesia. The endogenous ligand for the orphanin receptor is orphanin FQ, also known as nociceptin [Meunier et al., 1995, Reinscheid et al., 1995]. The opioid peptides and opiate alkaloids have low affinity for this receptor.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met-enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Met</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Leu</td>
</tr>
<tr>
<td>Dynorphin 1-17</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn</td>
</tr>
<tr>
<td>Dermorphin</td>
<td>Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH₂</td>
</tr>
<tr>
<td>Deltorphin 1</td>
<td>Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂</td>
</tr>
<tr>
<td>Endomorphin 1</td>
<td>Tyr-Pro-Trp-Phe-NH₂</td>
</tr>
</tbody>
</table>

Figure 1.2 The structures of various endogenous peptides for the opioid receptors.
1.2 Molecular Biology of the Opioid Receptor

The opioid receptors belong to the superfamily of 7-transmembrane domain-spanning G-protein coupled receptors (7TM-GPCR's). Similar to the other members of this receptor class, the opioid receptors consist of a single polypeptide chain of approximately 400 amino acids in length, and possess 7 transmembrane \( \alpha \)-helices (see Figure 1.3). The majority of 7TM-GPCR's share a degree of sequence homology with rhodopsin, the light-sensing molecule that binds the chromophore retinal. Since rhodopsin was the first G-protein coupled receptor to be cloned, a great deal of our understanding of the structure and function of this family of receptors is due to the detailed investigation of this molecule. It is believed that the ligand binding domain of the 7TM-GPCR's lies deep within one or more of the transmembrane segments, and site-directed mutagenesis has revealed that the third cytoplasmic loop is responsible for the binding of the G-protein [Bluml et al., 1994]. The totally conserved arginine residue of the third intracellular loop has been implicated in the binding of the G-protein upon activation of the receptor [Schwartz, 1996].

There is a large degree of sequence homology between the three opioid receptor types, \( \mu \), \( \delta \), and \( \kappa \), with 57 % of the amino acid sequence conserved. The greatest sequence homology is seen in the first and second intracellular loops (90 %). The third intracellular loop shares approximately 78 % homology. Of the transmembrane domains, greatest sequence homology is seen in transmembrane domains II and III (84 % and 82 %) Transmembrane domains V and VII are also highly conserved (71 %), however domains I, IV and VI show less homology. The amino and carboxy termini, and the extracellular loops, are not well conserved. The ORL1 receptor shares the greatest sequence homology with the \( \kappa \)-receptor due to its acidic second extracellular loop.

The opioid receptors, like other 7TMD GPCR's, contain potential sites for phosphorylation by protein kinase enzymes. For example in the \( \mu \)-opioid receptor phosphorylation of residues such as serine-263 or -365 or threonine-281 may be important in receptor desensitization leading to a reduced agonist response upon receptor occupation [Arden et al., 1995]. The \( \mu \)-opioid receptor also contains cysteine residues in positions 142 and 219 of the first and second intracellular loops which may be able to form a disulfide bridge. It is possible that this disulfide bridge controls receptor conformation changes and contributes to the overall structural stability of the
receptor [Simon & Groth, 1975, Smith & Simon, 1980, Ofri & Simon, 1992]. These cysteine residues are also present in δ and κ receptors.

Point mutations of the receptor can also allow us to deduce which amino-acids in particular are responsible for the binding of ligands to, and activation of, the receptor. For example replacing an aspartate residue at position 116 of transmembrane domain II with an asparagine residue (and thus removing a carboxylate function) reduces the binding affinity of agonists, but not antagonists, for the receptor [Surratt et al., 1996]. Replacement of a serine residue at position 198 in transmembrane domain IV with a leucine residue converts agonist into antagonists as measured by coupling to adenylyl cyclase and K⁺ channels [Claude et al., 1996].

The construction of chimeric receptors offers another approach to the investigation of the role of specific regions of the opioid receptor. A chimeric receptor is one in which certain sequences of amino-acids are replaced by the sequence from another receptor subtype. The function of the chimeric receptor is then compared to the original receptor in order to elucidate the regions of the receptor involved in ligand specificity and G-protein coupling. For example, by this method it has been shown that a region of the third extracellular loop and transmembrane domain VI of the δ-receptor is important for the binding of δ-selective ligands [Meng et al., 1996].

It has been proposed that subtypes of the μ-receptor exist [Pasternak & Wood, 1986]. The μ₁ receptor binds both the opiates and enkephalins with high affinity and the μ₂ receptor, a lower affinity site, is responsible for the binding of the opiates only. Bare et al. [1994], reported the existence of a splice variant of the human μ-receptor, and Holtt and his colleagues [Zimprich et al., 1995] reported the cloning and expression of an isoform of the rat μ-receptor. Splice variants therefore could represent μ-receptor subtypes. For further discussion of μ-receptor subtypes see Chapter 5, section 5.3.

The existence of δ-opioid receptor subtypes was first suggested by the groups of Portoghese and Porreca [Sofuoglu et al., 1991, Jiang et al., 1991] based on in vivo antagonism studies using naltrindole and its analogues. This led to the proposal of δ₁ and δ₂ subtypes. The peptides [D-Pen²,D-Pen⁵]enkephalin (DPDPE) and [D-Ala²,D-Leu⁵]enkephalin (DADLE) are agonists at the δ₁ subtype and the peptide [D-Ser²,Leu⁵,Thr⁶]enkephalin (DSLET) is an agonist at the δ₂ subtype. Similarly selective antagonists exist for both subtypes, 7-benzylidenenaltrexone (BNTX) is an
antagonist at δ₁ and naltriben (NTB) is an antagonist at δ₂. The question of κ-receptor subtypes has also been raised [Dhawan et al., 1996]. Competition binding assays using brain membranes revealed biphasic inhibition curves suggesting that the arylacetamide agonists CI977, U50,488H and U69,593 interact with κ₁ receptors whereas the benzomorphan ligands also interact with κ₂ and κ₃ subtypes.

As yet however no cloning data is available to support the existence of any of the opioid receptor subtypes.
Figure 1.3 Cartoon showing the 7-transmembrane spanning domains of the rat \( \mu \)-opioid receptor. The amino-acid sequence data was obtained from the Center for Opioid Research and Design, Department of Medicinal Chemistry, University of Minnesota.
1.3 The G-Protein

Attached to the inner surface of the cell membrane, G-proteins act on membrane bound intermediaries called effectors, either enzymes or ion channels. So called because of their interaction with the guanine nucleotides GDP and GTP, the G-proteins represent the ‘middle management’ by relaying messages from the receptor to the effector. There are three main G-protein coupled effector systems:

a) The adenylyl cyclase/cyclic AMP system
b) The phospholipase C/inositol phosphate system
c) The regulation of ion channels

Each G-protein consists of three subunits: α, β, and γ. Guanine nucleotides bind to the α-subunit. The β- and γ-subunits exist as a tightly associated complex that functions as a single unit, and serves to anchor the G-protein to the membrane. In the resting state, the αβγ trimer is bound by GDP and is uncoupled from the receptor (Fig. 1.4). When an agonist binds to the receptor a conformational change in the receptor causes it to actively associate with the receptor-G-protein complex. This association causes an exchange of GDP for GTP, which in turn causes dissociation of the α-GTP complex from the βγ subunits. This α-GTP complex is the activated form of the G-protein that diffuses into the membrane, thus relaying its message by activating or inactivating enzymes or ion channels. An intrinsic GTPase property of the α-subunit catalyzes the hydrolysis of the terminal phosphate of bound GTP to form GDP, thereby deactivating the α-subunit. The Ga-GDP complex then re-associates with the βγ complex, which is then available for activation following agonist occupation of the receptor.

Different types of G-protein exist which interact with different receptors and control different effectors. The four major families are Gs, Gq, Gi/Go and G12, of which there are many subsets. The G-protein families are grouped together according to the degree of amino acid conservation of the α-subunit, and to a certain extent these families also have functional correlates in terms of receptor and effector interaction. The Gs and Gq families stimulate adenylyl cyclase whereas the Gi/Go family inhibits this enzyme. Similarly the voltage-gated Ca²⁺ channel is positively modulated by Gs and negatively modulated by Go. Opioid receptors couple to the Gi/Go family. Most of the α-subunits of opioid receptor-coupled G-proteins are
substrates of one or both of two bacterial toxins, the toxin of *Vibrio cholera* (cholera toxin) or *Bordatella pertussis* (pertussis toxin). In the presence of GTP, cholera toxin catalyses the ADP-ribosylation of a specific arginine residue of the Gs protein α-subunit. This causes persistent activation of the G-protein and consequently uncontrolled activation of adenylyl cyclase. In the presence of ATP pertussis toxin ADP-ribosylates a specific cysteine residue of the Gi or Gq α-subunit. The Gq family of G-proteins (which is not bacterial toxin sensitive) is known to stimulate phospholipase C, which catalyses the formation of the second messengers IP3 (inositol 1,4,5-triphosphate) and diacylglycerol.
Effectors

Agonist occupied receptor

~

GTP or GDP

GTP's

Effector proteins are activated.

Figure 1.4 Schematic diagram representing G-protein-mediated transmembrane signalling. Adapted from Rang & Dale, 1991.
Structural features
Although opioid ligands vary in their size and molecular skeleton, certain pharmacophoric groups are essential for binding to the opioid receptors, namely an aromatic feature and a basic centre permitting protonation at physiological pH. The clinically useful opioids can be subdivided into groups according to their structure: polycyclic molecules of the morphine type, the benzomorphans e.g. pentazocine, the phenyl piperidines e.g. fentanyl and meperidine, and the acyclic diarylamines e.g. methadone. The structures of some of the opioids are shown in Fig. 1.5.

Pharmacological actions – Effects on the Central Nervous System
Analgesia - Morphine mediates analgesia via its actions at spinal and supraspinal sites in the central nervous system (see Fig. 1.6). The opioid analgesics selectively inhibit various nociceptive reflexes and cause profound analgesia when injected intrathecally, by inhibiting transmission of nociceptive impulses through the dorsal horn of the spinal cord. Injection of morphine directly into the periaqueductal gray and nucleus raphe regions also produces analgesia by enhancing the activity of the descending aminergic pathways that inhibit the processing of nociceptive information in the spinal cord. δ- and κ-opioid receptors and their agonists also contribute to analgesia. δ-Agonists mediate antinociception by directly inhibiting the actions of selective agonists on the release of substance P from nociceptive afferent neurons. In addition there is evidence that supraspinal δ-sites mediate antinociception [Mathiasen & Vaught, 1987, Heyman et al., 1988]. Antinociceptive effects of κ-agonists have been shown in rhesus monkeys and rodents [Porreca et al., 1987, France et al., 1994], and appear to be mediated via spinal receptor sites. There is a degree of controversy surrounding the existence of supraspinal κ-sites which mediate antinociceptive effects [Porreca et al., 1987, Millan et al., 1989].

Euphoria - Euphoria is an important component of the analgesic effect of morphine, since the patient is relieved of the distress associated with the sensation of pain. The activation of dopaminergic neurons that project to the nucleus accumbens is postulated to be a critical element in the reinforcing effects of opioids, and
therefore, the euphoria induced by these compounds. However, \( \kappa \)-agonists inhibit the firing of dopaminergic neurons and thus produce dysphoria.

\textbf{Respiratory depression} - The primary mechanism behind respiratory depression involves a reduction in responsiveness of the respiratory centres in the brainstem to increased arterial \( PCO_2 \). All phases of respiratory activity, including tidal exchange, minute volume, and rate, are depressed. There is some evidence to suggest that respiratory depression is mediated through the \( \mu_2 \) sub-population of receptors, [Ling \textit{et al.}, 1983, 1985] due to the ability of the \( \mu_1 \) selective antagonist naloxonazine to antagonize the antinociceptive, but not respiratory depressive, effects of morphine in rats. However, Gatch \textit{et al.}, [1996], have shown that in rhesus monkeys naloxonazine antagonized both the respiratory depressive and antinociceptive effects of the \( \mu \)-agonist levorphanol to a similar degree, suggesting that the antinociceptive and ventilatory effects of levorphanol are mediated \textit{via} a similar population of receptors. Again there is controversy surrounding the ability of \( \delta \)- and \( \kappa \)-opioid receptor agonists to cause respiratory depression [Wang \textit{et al.}, 1993, Hepburn \textit{et al.}, 1997, Shook \textit{et al.}, 1990].

\textbf{Cough reflex} - Morphine acts as an antitussive agent by blocking the cough reflex centre in the medulla. There is no relationship between respiratory depressive effects of morphine and the suppression of coughing. Codeine is one of the few opioid analgesics used as an antitussive agent. Effective doses are generally lower than those required to produce analgesia.

\textbf{Pupillary constriction} - This is a centrally-mediated effect caused by activation of the nucleus of the oculomotor nerve by \( \mu \)- and \( \kappa \)-receptors.

\textbf{Nausea and vomiting} - Nausea and vomiting can occur in up to 40 \% of patients receiving morphine. This is caused by a direct action on the chemoreceptor trigger zone (CTZ) of the medulla, where chemical stimuli of many kinds may initiate vomiting. There is also an increase in vestibular sensitivity, which contributes to the nausea. These effects of morphine usually disappear with repeated administration.

\textbf{Endocrinological effects} – Morphine acts on the hypothalamus to cause release of antidiuretic hormone (ADH), which leads to a decrease in urinary output. Conversely, \( \kappa \)-agonists inhibit the release of ADH thus causing diuresis.
Effects on the gastrointestinal system
Morphine causes an increase in the resting tone of the stomach, small intestine and large intestine. In the stomach there is decreased motility and a delay in emptying of gastric contents to the small intestine. There is a delay in digestion of food in the small intestine, and in the large intestine, propulsive peristaltic contractions are slower or even abolished after morphine administration. As a result of these actions, constipation occurs, which may be severe. Morphine also causes a decrease in biliary secretions and an increase in biliary tract pressure.

Effects on the cardiovascular system
At therapeutic doses morphine does not have a marked effect on the cardiovascular system. At higher doses morphine can cause bradycardia and hypotension via a direct action on the medulla. Hypotension is also a result of peripheral vasodilatation and reduced peripheral resistance. Morphine induces histamine release, which may contribute to hypotension, but it also decreases the reflex vasoconstriction caused by increased PCO₂.

Metabolism
Morphine undergoes considerable first pass metabolism and so is not as effective orally compared to intravenous or intramuscular injection. The majority of metabolic transformation occurs in the liver. Morphine is conjugated at the 3-OH and/or the 6-OH position with glucuronide to give either the monoglucuronide or the diglucuronide. The conjugates are excreted in the urine and the bile.

Tolerance and dependence
Tolerance can be defined as an increase in the dose of drug needed to produce a given pharmacological effect. Tolerance occurs to most of the pharmacological effects of morphine, including analgesia, euphoria, and respiratory depression. Little tolerance develops to the gastrointestinal effects and pupillary constriction. The exact mechanisms behind the development of tolerance still remain elusive, although it is believed that the chronic effects of opioids in vitro involve the following changes at the cellular level:
a) desensitisation – the receptor becomes uncoupled from the effector such as adenylyl cyclase hence giving a reduction in agonist affinity.
b) down-regulation - receptors are removed from the cell surface (internalised), passing into the cell where they are either degraded or later recycled to the cell surface. This results in a loss of receptor number or binding sites, and although this has been demonstrated for opioids acting on various cell lines, attempts to show this in brain homogenates from tolerant animals have failed.

c) Adaptive changes in effector systems - it has been suggested that tolerance might be associated with an increase in activity of adenylyl cyclase within the cell. Chronic opioid treatment results in a reduction of opioid inhibition of adenylyl cyclase, which gradually returns to normal. Removal of opioid agonist then results in an increase of cAMP production, known as a 'cAMP overshoot'.

Continued use frequently results in physical and psychological dependence; physical dependence manifested by a characteristic abstinence syndrome induced on abrupt withdrawal of the opioid. Diarrhoea, fever, chills, vomiting, abdominal cramps and lacrimation are common signs of withdrawal. Peak withdrawal symptoms occur around 36 – 72 hr, and usually disappear after 8 – 10 days. Some symptoms may persist for weeks. Re-administration of morphine (or other opioid agonist) will abolish the abstinence syndrome.

Psychological dependence is more complex, but plays an important part in compulsive drug-taking behaviour. Behavioural models have been developed to study this phenomenon, in particular self-administration assays using rhesus monkeys or rats whereby the reinforcing effects of the drugs can be studied.

For a detailed review of the pharmacology of morphine and other opioids see Martin, 1984.
Morphine: $R = OH$, $R_1 = OH$
Codeine: $R = OH$, $R_1 = CH_3O$
Heroin: $R = OOCH_3$, $R_1 = OOCH_3$

Naloxone

Buprenorphine

Etorphine

Fentanyl

Methadone

Pentazocine

Figure 1.5 The structures of various opioid ligands.
Figure 1.6 Diagram representing the descending control pathway, showing postulated sites of action of opioids on pain transmission. Opioids excite neurons in the periaqueductal grey (PAG) and in the nucleus reticularis paragigantocellularis (NRPG), which in turn project to the nucleus raphe magnus (NRM). From the NRM, enkephalin-containing neurons exert an inhibitory effect on transmission in the substantia gelatinosa of the dorsal horn. Opioids can also have a direct action on the dorsal horn. Adapted from Rang & Dale, 1995.
1.5 Opioid Addiction: The Drug Abuse Problem

Opioid abuse is one of the most significant public health problems facing our society today. Heroin, an acetylation product of morphine that causes intense craving and physical dependence, is the most commonly abused opioid. The National Household Survey on Drug Abuse in the U.S.A reported that in 1996 an estimated 2.4 million people use heroin at some stage in their lives. According to the 1997 bulletin of drug misuse statistics for Scotland, 66% of new attenders at drug services in the U.K. for the period of 6 months up to March 1997 were using heroin. In Scotland alone the percentage of people using heroin as their main drug of abuse has increased from 39% in 1992/1993 to 49% in 1996/1997. In addition reports of HIV infection amongst injecting drug users has risen from 744 in 1987 to 1176 in 1997. More than one third of the AIDS cases registered in 1997 were due to parental drug abuse.

Street heroin is most commonly found as a brownish powder or as 'black tar heroin', which, as its name suggests, is of a black sticky consistency. It can be injected, sniffed or snorted, and smoked. Street heroin is cut with a variety of different substances, brown sugar, talc, flour, cocoa, quinine, or procaine (a local anaesthetic). The average street purity of heroin is now approximately 5%. Many addicts do not know the strength of their obtained heroin and so are at risk from overdose, which unfortunately is a frequent occurrence in the drug scene. Death occurs as a result of respiratory depression. Long-term effects of heroin use include collapsed veins, bacterial infections, abscesses, and arthritis due to immune reactions to batch contaminants. Sharing of injection equipment also leads to the transmission of AIDS.

Prior to the development of substitution medication for the treatment of opioid abuse, addicts were treated by abrupt withdrawal or gradual discontinuation of their drug. Although addicts were institutionalized for prolonged periods of time, and the programs were successful during the residential phase, frequent relapse upon discharge occurred. It became clear that a new type of approach to opioid abuse treatment was required.

The ideal drug abuse treatment would be to alleviate the craving, prevent withdrawal symptoms, and avoid relapse. At present there are three substances approved by the Food and Drug Administration (FDA) of the U.S which are used to treat heroin abuse - methadone, LAAM (levo-alpha-acetylmethadol) and naltrexone.
Clonidine, which is used to treat hypertension, is also sometimes used in the initial stages of detoxification. Both methadone and LAAM are μ-opioid receptor agonists, whereas naltrexone is an μ-antagonist and clonidine is an α-adrenergic agonist.

Methadone, a synthetic opioid, was first reported as a successful substitution therapy for opioid dependence in 1965 by Dole & Nyswander. It is currently the most widely used treatment and has been shown to decrease illicit drug use and criminal activity as well as preventing further spread of HIV amongst intravenous drug users [Gerstein & Harwood, 1990]. Unfortunately methadone is not without disadvantages. Daily visits to the clinic for supervision of methadone ingestion are inconvenient for the patient and also interfere with the ability of the person to become truly integrated with a typical drug-free lifestyle. The introduction of take-home methadone resulted in the sale and illicit use of this treatment drug. It too causes physical dependence and has abuse potential comparable to that of heroin. For this reason methadone treatment programs are strictly controlled.

LAAM, (levo-alpha-acetylmethadol), an analogue of methadone, was formally approved as a pharmacotherapy in the U.S in 1993. The effects of LAAM have been described as being more 'subtle' than those of methadone. LAAM has a slower onset of action, and a longer-lasting effect, therefore clinic visits can be reduced to three or four times weekly. The lack of take-home doses of LAAM may aid in preventing creation of a street market for the drug.

The opioid antagonist naltrexone is also used to treat opioid dependence, although this type of compound can precipitate severe withdrawal symptoms and so is normally restricted to either treating opioid overdose, or is used as a continuing treatment drug after the patient has been drug-free for one to two weeks.

One of the most promising alternative medications currently being considered for clinical use is the partial μ-opioid receptor agonist buprenorphine, which has pharmacological properties that appear to be ideal for the treatment of heroin abuse.
1.6 Buprenorphine: A Novel Treatment for Opioid Abuse?

Buprenorphine is currently marketed as Temgesic in the U.K (Buprenex in the U.S.A), and is used as an analgesic for the treatment of moderate to severe pain. At present buprenorphine is being developed as a daily-dosing pharmacotherapy for the treatment of opioid abuse. It is anticipated that the Food and Drug Administration of the U.S.A will grant approval for this indication in the near future. Indeed buprenorphine is already used in France for the treatment of opioid dependency [Henrion, 1997].

Buprenorphine, a derivative of the morphine alkaloid thebaine, was first synthesized and tested by Bentley & Hardy [1967]. Despite the need for further characterization of this drug, there are a number of unique pharmacological features of buprenorphine that suggest it to be a viable treatment for heroin or other opioid dependencies. It has a high affinity for the \( \mu \)-receptor with a low intrinsic activity; therefore it is mixed partial \( \mu \)-agonist/antagonist. The partial agonist nature of buprenorphine confers a ceiling of effect thus limiting the possibility of overdose, and increasing the margin of safety. Although buprenorphine has a high degree of lipophilicity it has a long duration of action, due to extremely slow receptor dissociation kinetics [Hambrook & Rance, 1976]. Hence buprenorphine also has the ability to block any subsequently self-administered opioid.

Another unique feature of buprenorphine that has perplexed researchers for several years is its characteristic biphasic dose-response curve that is observed in a variety of in vivo assays, particularly those of antinociception. This will be discussed in more detail in Chapter 3.

1.6.1 In vitro studies

In vitro data has shown that buprenorphine has high affinity in the nM range for the \( \mu \), \( \delta \) and \( \kappa \) binding sites, although with somewhat reduced affinity for the \( \delta \)-binding site [Grayson et al., 1991]. The order of potency of buprenorphine for the binding sites is \( \kappa_1=\mu>\delta>\kappa_2 \). These affinity results correspond with the data obtained in vivo by Richards & Sadee [1985a]. Interestingly, a comparison of the affinities of buprenorphine for the recently available cloned opioid receptors reveals that it has highest affinity for the \( \delta \)-receptor [Reisine & Bell, 1993].
Pharmacokinetic analysis of the binding of $[^3\text{H}]$buprenorphine to opioid receptors has shown that it has a slow receptor association and dissociation as compared to other opiate ligands [Boas & Villiger, 1985, Hambrook & Rance, 1976]. Indeed the dissociation of buprenorphine is biphasic with an initial rapid phase followed by a very slow phase where approximately 50% of the tritiated ligand still remains bound. This persistent binding probably accounts for the long duration of action in vivo (in excess of 6 hr) that does not parallel its plasma half-life time of approximately 3 – 5 hr. Villiger & Taylor [1981] also reported that large concentrations of naloxone were required to displace $[^3\text{H}]$buprenorphine, and in studies using rhesus monkeys it has been shown that the discriminative effects of buprenorphine are prevented, but not reversed, by naltrexone [France et al., 1984].

More recently techniques have been developed which enable the measurement of agonist efficacy in vitro. Two such techniques are the use of stimulation of $[^3\text{S}]\text{GTP}\gamma\text{S}$ binding [Traynor & Nahorski, 1995], and the inhibition of forskolin-stimulated cyclic AMP [Law et al., 1983]. It has now been shown using the $[^3\text{S}]\text{GTP}\gamma\text{S}$ assay that buprenorphine exhibits partial agonist activity in membranes prepared from rat C6 glioma cells expressing the cloned rat $\mu$-opioid receptor (see Section 4.2, Chapter 4) and in membranes prepared from SH-SY5Y cells [Traynor & Nahorski, 1995]. There is limited information regarding the interaction of buprenorphine with the $\delta$-receptor. Recently it has been shown using the $[^3\text{S}]\text{GTP}\gamma\text{S}$ assay that buprenorphine has no agonist activity at the cloned rat $\delta$-receptor expressed in C6 glioma cells [Lee et al., submitted for publication] nor at the $\delta$-receptor in NG108-15 cells [our laboratory, unpublished observations]. However, buprenorphine has been shown to inhibit the accumulation of cAMP in human embryonic kidney (HEK) 293 cells expressing the cloned mouse $\delta$-receptor [Blake et al., 1997]. Buprenorphine shows very low efficacy at the cloned $\kappa$-receptor expressed in Chinese hamster ovary (CHO) cells [Zhu et al., 1997].

1.6.2 In vivo studies

The first experiments using buprenorphine were carried out by Martin et al. [1976] using the chronic spinal dog preparation. This was closely followed by the work of
Cowan et al. [1977a,b] who reported a thorough pharmacological characterization of the compound. From these studies came several valuable findings:

a) In the mouse writhing assay and the rat tail pressure test buprenorphine was 25 – 40 times more potent than morphine.

b) The dose-response curve for buprenorphine was biphasic in rodent tail withdrawal assays; test of catalepsy, and gastrointestinal transit assays.

c) Buprenorphine induced locomotor activity in mice, showed an antitussive action in guinea-pigs, reduced heart rate but had no significant effect on arterial blood pressure in conscious rats and dogs, and increased arterial $P_{CO_2}$ and $P_{O_2}$ values in conscious rats.

d) It suppressed urine output in water loaded rats.

e) Buprenorphine antagonized the antinociceptive effects of morphine in the rodent tail withdrawal assay, but not the rat tail pressure test.

f) It precipitated signs of abstinence in morphine-dependent mice and monkeys but not in rats.

g) Withdrawal symptoms were relatively low in monkeys chronically treated with buprenorphine.

From this early work it was clear that buprenorphine was showing partial agonist actions at the $\mu$-opioid receptor. This was also supported by the work of Hayes et al. [1986], who showed that pre-treatment of rats with the irreversible $\mu$-antagonist $\beta$-funaltrexamine, ($\beta$-FNA), resulted in the dose-response curve for buprenorphine having a decreased slope and maximal antinociceptive effect in the paw pressure test.

There is now substantial evidence that buprenorphine has antagonist properties at the $\kappa$-receptor. For instance the dose-response curve for bremazocine-induced diuresis in rats is displaced to the right following administration of buprenorphine [Richards & Sadee, 1985b]. There are very few reports concerning the effects of buprenorphine at the $\delta$-receptor, in part due to the lack of appropriate pharmacological tools. With the advent of naltrindole, the $\delta$-selective antagonist [Portoghese et al., 1988], studies of the interaction of buprenorphine with the $\delta$-receptor should be easier.

The most interesting feature of the animal pharmacology of buprenorphine must surely be the characteristic bell-shaped dose-response curve, which was first observed in rat tail withdrawal assay [Cowan et al., 1977a]. Having perplexed researchers for
many years, the cause of this unique effect still remains to be determined. To date it has been explained in terms of a two-receptor model (non-competitive autoinhibition) [Sadee et al., 1982], a two-point attachment to a single receptor [De Lean et al., 1979], and cooperative receptor interactions [Dum & Herz, 1981]. Perhaps the most widely accepted model is that of non-competitive autoinhibition whereby agonist effects are mediated by one receptor, at the lower dose range, and a second inhibitory receptor, at the higher dose range, counteracts the effects of the first. Sadee et al. [1983] proposed that the second inhibitory site may be δ-mediated, but as yet no evidence has been found to support this. Speculation has also arisen as to the second phase of the curve being κ-mediated [Rothman et al., 1995], however the possibility of the κ\textsubscript{1} receptor being responsible has been eliminated [Leander, 1983, Rothman et al., 1995] although the κ\textsubscript{2} receptor still remains to be investigated. Studies have shown that the non-selective antagonists naloxone and naltrexone can shift the dose-response curve to the right symmetrically, therefore both antagonists must have similar affinity for both sites and are able to interact with these sites in a competitive manner.

Another interesting aspect of the pharmacological actions of buprenorphine is that its antinociceptive action can easily be prevented, but not reversed, by opioid antagonists [France et al., 1984]. This is indicative of an irreversible component of receptor binding. This is in accordance with in vitro studies where it was found that [\textsuperscript{3}H]buprenorphine dissociation from the receptor was extremely slow and a portion of the tritiated ligand remained persistently bound.

Opioid agonists typically produce physical dependence upon chronic administration. It was therefore surprising to discover that buprenorphine failed to produce the manifestations of physical dependence when administered to animals for prolonged periods of time [Cowan et al., 1997a]. It is generally believed that the tight interaction of buprenorphine with the opioid receptor maintaining a long duration of action helps to counteract the sudden biochemical imbalance on sudden withdrawal of the drug.
An important feature of buprenorphine as a pharmacotherapy for opioid abuse is its ability to block the physiological effects of subsequently administered opioid agonists such as heroin. Early studies with opioid addicts indicated that buprenorphine blocked the effects of subsequently administered doses of morphine [Jasinski et al., 1978], and hydromorphone [Bickel et al., 1988a], presumably due to its partial agonist nature and long-lasting receptor interaction.

As regards to its clinical efficacy and promotion of treatment compliance, it has been reported that a comparison of fixed doses of buprenorphine and methadone showed that buprenorphine was as effective as methadone for the treatment of opioid dependence [Johnsone et al., 1992, Bickel et al., 1988b]. Another study comparing the efficacy of methadone and buprenorphine in a flexible dosing procedure also recommended that buprenorphine was as efficacious as methadone [Strain et al., 1994]. However a study by Kosten et al. [1993] reported that methadone may be more effective. As a substitution therapy it may be that buprenorphine is ideal for those addicts who have a less chronic form of opioid addiction, whereas those more resistant to dependence treatment may be better served by methadone.

Reports concerning physical dependence caused by chronic treatment with buprenorphine indicate that compared to methadone, buprenorphine does not induce physical dependence to the same degree [Jasinski et al., 1978, Fudala et al., 1990]. Heroin and methadone-dependent subjects given buprenorphine as a substitution therapy did not experience a typical methadone abstinence syndrome, but noted mild withdrawal symptoms during the transition period. Subsequent termination of buprenorphine precipitated a mild abstinence syndrome that peaked after 3 days [Jasinski et al., 1983, 1984]. Challenge with naloxone was found to be not nearly as effective in precipitating abstinence in subjects treated chronically with buprenorphine as compared to morphine dependent subjects [Jasinski & Preston, 1995].

Unfortunately, the fact that buprenorphine does have significant reinforcing effect and appears to maintain treatment compliance gives the compound potential for abuse. In several countries, where the use of buprenorphine is less restricted, there have emerged reports of the abuse of buprenorphine. For example in Scotland [Gray et al., 1988, Robertson & Bucknell, 1986], in England [Strang, 1985], and in Australia
and New Zealand [Quigley et al., 1984, Harper, 1983]. This led to the development of a buprenorphine/naloxone combination product marketed in New Zealand. The route of administration is an important consideration where abuse liability is concerned, as it was reported that sublingual preparations were being injected intravenously [Sakol et al., 1989]. It is believed that the non-injectable sublingual preparation is the better drug-delivery system [Jasinski et al., 1989].

From the evidence thus far it would seem that buprenorphine would be an effective medication for the treatment of opiate dependence. Given its morphine-like subjective effects, combined with the low level of physical dependence and mild withdrawal syndrome, buprenorphine appears to be acceptable to opiate-dependent subjects. Its unusual, yet promising, pharmacological profile has also now provided a basis from which opioid researchers can continue to develop novel treatments for drug abuse.
1.7 General Aims

The preceding discussion suggests that buprenorphine may be useful as an alternative pharmacotherapy for the treatment of opioid abuse. Buprenorphine has unique pharmacological characteristics which include mixed partial \( \mu \)-agonist/\( \kappa \)-antagonist, long duration of action and the ability to block subsequently self-administered opioids. Despite its promising profile, buprenorphine still may not be ideal, in particular the degree of reinforcing effect may not be sufficient to maintain certain addicts in treatment programs. The aim of this work is to study buprenorphine and a number of its analogues in order to ascertain the mechanisms behind their unique pharmacology and to determine if the existing characteristics of buprenorphine can be further improved. To achieve this a combination of \textit{in vitro} and \textit{in vivo} techniques will be used as follows:

a) study the antinociceptive profile of buprenorphine in the rat warm water tail withdrawal assay,

b) examine the pharmacology of a novel morphinan pyrrolidine derivative at both the cellular level and \textit{in vivo} using mice, and assess its potential as a treatment drug,

c) examine the binding profile and efficacy at the \( \mu \)-opioid receptor of analogues of clocinnamox, a long-lasting 3-substituted cinnamoylamino compound, using homogenates prepared from SH-SY5Y cells and C6 glioma cells transfected with the cloned rat \( \mu \)-opioid receptor,

d) evaluate constitutive activity at the \( \mu \)- and \( \delta \)-opioid receptors in C6 glioma cells and investigate the inverse agonist effects of clocinnamox at these receptors.
CHAPTER 2
MATERIALS AND METHODS
2.1 Materials

2.1.1 Radiochemicals

\([^3]H\)DAMGO (\([D-Ala^2, MePhe^4, Gly(ol)^5\]enkephalin\) (54 Ci mmol\(^{-1}\)), \([^3]H\)diprenorphine (45 Ci mmol\(^{-1}\) and 58 Ci mmol\(^{-1}\)), \([^3]H\)DPDPE (\([D-Pen^2, D-Pen^5\]enkephalin\) (39 Ci mmol\(^{-1}\)), \([^3]H\)naltrindole (33 Ci mmol\(^{-1}\)), \([^3]H\)U69,593 (54 Ci mmol\(^{-1}\) ) and \([^{35}S]GTP\gammaS\) (1250 Ci mmol\(^{-1}\)) were all purchased from DuPont NEN Boston, MA, USA.

2.1.2 Chemicals

Acetic acid, EDTA (ethylenediaminetetraacetic acid), Folin & Ciocalteu’s phenol reagent, GDP (guanosine diphosphate), GTP\(\gammaS\) (guanosine triphosphate), GppNHp (guanylylimidophosphate), HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], MgCl\(_2\cdot6\)H\(_2\)O, MgSO\(_4\cdot7\)H\(_2\)O, pertussis toxin, Trizma base (tris[hydroxymethyl]aminomethane) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

CaCl\(_2\), KCl, K\(_2\)HPO\(_4\)·3H\(_2\)O and NaCl were purchased from Mallinckrodt, St. Louis, MO, USA.

DMSO (dimethylsulfoxide) was from Fisher Scientific, Pittsburgh, PA, USA.

NaHCO\(_3\) was from Columbus Chemical Industries, Inc., Columbus, WI, USA.

UltimaGold liquid scintillation fluid was from Packard Bioscience, Groningen, Holland.

2.1.3 Drugs

The following drugs were generous gifts from the National Institute on Drug Abuse, Rockville, MD, USA: buprenorphine HCl, ciocinnamox (C-CAM), fentanyl HCl, methadone HCl, methocinnamox (M-CAM), methoclocinnamox (MC-CAM), naloxone HCl, naltrexone HCl, and SNC80.
7-Benzyldenedenaltrexone (BNTX), D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP), naltrindole (NTI) and nor-binaltorphimine (nor-BNI) were a kind gift from NIH, Bethesda, MD, USA.

Bremazocine was from Sandoz, Basel, Switzerland.

BW373U86 was from Burroughs Wellcome, Research Triangle Park, NC, USA.

β-Funaltrexamine was purchased from Research Biochemicals Inc., SEMAT, St. Albans, UK.

ICI 174,864 was purchased from Tocris Cookson, Ballwin, MO, USA.

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

All of the BU (Bristol University) compounds were synthesized by John Lewis and co-workers at the Department of Chemistry, University of Bristol, UK.

2.1.4 Cell culture media

Dulbecco's Modified Eagle Medium, Minimum Essential Medium, foetal calf serum, trypsin, EDTA, and geneticin were all from Gibco Life Sciences, Grand Island, NY, USA.

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

2.1.5 Buffers

The composition of the Krebs/HEPES buffer used to maintain whole cells during wash resistant binding studies was (mM): NaCl (118), NaHCO₃ (25), KCl (4.7), CaCl₂ (2.5), MgSO₄·7H₂O (1.2), KH₂PO₄ (1.2), Glucose (11.7) and HEPES (10), pH 7.4.

The buffer used in [³⁵S]GTPγS assays was (mM): HEPES (20) MgCl₂·6H₂O (10) and either NaCl (100) for standard assays or KCl (100) for assays using inverse agonists. The pH was adjusted to 7.4 with NaOH.

The buffer used in membrane binding studies was Trizma base (50 mM) acidified with HCl to pH 7.4.
Male Dunkin-Hartley guinea pigs (250 - 500g) were bought from David Hall, Newchurch, Burton-on-Trent, UK. Male NIH Swiss mice (25 - 30 g) and male Wistar rats (250 - 300 g) were purchased from Harlan Sprague Dawley Inc., Indianapolis, IN, USA. Mice were housed in groups of 10 - 12 and rats were housed in groups of 3 in a temperature-controlled room maintained on a 12 hr light-dark cycle. Food and water were available ad libitum until the time of the experiment. For in vivo experiments each subject was tested only once and studies were carried out in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The experimental protocols were approved by the University of Michigan's University Committee on the Use and Care of Animals.
2.2 Methods

2.2.1 Cell culture and membrane preparation

The SH-SY5Y human neuroblastoma cell line was kindly donated by Dr. D. Lambert, Department of Anaesthesia, Leicester University, UK. The cells were grown in Minimum Essential Medium (MEM) supplemented with 10 % foetal calf serum, 2.5 μg ml⁻¹ amphotericin B (fungizone), 50 μg ml⁻¹ penicillin/streptomycin, and 250 μg ml⁻¹ L-glutamine at 37°C in a humidified 5 % CO₂ atmosphere.

C6 glioma cells transfected with either the cloned rat μ- or δ-receptor were a kind donation from Dr. Huda Akil, Mental Health Research Institute, University of Michigan, MI, USA. Cells were cultured under a 5 % CO₂ atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10 % fetal calf serum. For subculture one flask from each passage was grown in the presence of 1 mg ml⁻¹ Geneticin. Cells used for experiments were grown in the absence of Geneticin with no significant reduction in receptor number.

Once cells had reached confluency they were harvested in HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], 20 mM pH 7.4)-buffered saline containing 1 mM EDTA, dispersed by agitation and collected by centrifugation at 1600 rpm (Centra CL2 centrifuge, International Equipment Co.). The cell pellet was suspended in 50 mM Tris-HCl buffer pH 7.4, and homogenized with a tissue tearor (Biospec Products). The resultant homogenate was centrifuged for 15 min at 18000 rpm at 4°C (Beckman centrifuge model J2-21) and the pellet collected, resuspended and recentrifuged. The final pellet was resuspended in 50 mM Tris-HCl buffer pH 7.4; separated into 0.5 ml aliquots (0.75 - 1.0 mg protein) and frozen at -80°C.

For wash resistance studies whole cell suspensions were incubated in Krebs/HEPES buffer (pH 7.4) in the presence of drug for 1 hr at 37°C. Both treated and control cells were washed four times to remove any unbound drug. Cell membranes were then prepared as described above.

For pertussis toxin (PTX) treatment cells were incubated with 100 ng ml⁻¹ PTX for 24 hr prior to harvesting.
2.2.2 Preparation of brain homogenates

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

Protein concentration for both cell membrane and mouse brain membrane preparations was determined by the method of Lowry et al. [1951], using a bovine serum albumin standard.

2.2.3 Ligand binding assays

An important method for the study of receptors and their ligands is the radioligand binding assay. A great advantage of this method is that it does not require the receptor of interest to be purified; relatively crude tissue preparation will suffice. However there also exists a second binding component, namely non-specific binding, where the radioligand binds to non-receptor tissue, the test tubes, or filters. The total specific binding is therefore determined by the difference between the binding in the absence of, and the binding in the presence of, a large excess of an unlabelled, competitive ligand. In most instances this ligand will be naloxone, which has high affinity for the opioid receptors.

The specificity of opioid receptor binding is demonstrated by the findings that compounds which show opiate-like activity generally have a much higher affinity for opioid binding sites than non-opioids. Pharmacological observations have established that stereospecificity plays a major role in opioid receptor binding; for instance the (-) isomer of some alkaloids can compete for the binding site 1000 times more effectively than the (+) isomer.

The specific binding of an opioid is characterized by two parameters:
a) the affinity constant of the ligand (or its reciprocal, the dissociation constant).
b) the number of binding sites (Bmax).
When examining a radioligand, saturation analysis enables us to obtain both parameters. When examining a non-radiolabeled ligand its affinity constant is obtained through competition studies. This is done by measuring the ability of the ligand to displace a radiolabeled opioid with a known affinity constant. The affinity of the unlabelled ligand can be estimated either as an IC$_{50}$ or as a $K_i$. These two values are related by the Cheng and Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + ([L] / K_d)}$$

where $[L]$ = concentration of radioligand, IC$_{50}$ = concentration of unlabelled ligand at which 50 % inhibition of binding of radioligand is achieved, $K_d$ = dissociation constant of radiolabeled ligand.

Although $K_i$ values provide a more accurate assessment some assumptions have to be made - one assumes that the radioligand is labeling a single site and that all the assumptions regarding mass law hold. No assumptions need to be made regarding IC$_{50}$ values; unlike the $K_i$ values these are dependent on the radioligand used and its concentration.

Hill plots can be used to assess binding site heterogeneity in competition studies. Hill coefficient values determine if there is a possibility that the radioligand is labeling more than one site. A value of unity suggests one site, however a value of less than 1 is indicative of receptor heterogeneity.

**Competition binding assays** - C6 glioma cell membranes (30 - 60 µg protein), SH-SY5Y cell membranes (100 - 150 µg protein), or mouse/guinea-pig brain membranes (1 mg ml$^{-1}$ protein) were incubated at 25°C in 50 mM Tris-HCl buffer, pH 7.4 for 1 hr with radiolabelled ligand and varying concentrations of unlabeled ligand to give a final volume of 1 ml. Non-specific binding was defined with 10 µM naloxone. The reaction was terminated by filtering the samples through glass fiber filters (Whatman GF/C, Schleicher and Schuell #32) mounted in a Brandel 24 well harvester. The filters were subsequently washed 3 times with ice-cold Tris-HCl, pH 7.4 and radioactivity determined by scintillation counting (Beckman LS6800 scintillation counter) after addition of 3 ml of liquid scintillation fluid. IC$_{50}$ and $K_i$ values were determined using GraphPad Prism, version 2.01 (GraphPad, San Diego, CA), using
$K_D$ values of 0.2 nM for $[^3H]$diprenorphine and 1.0 nM for $[^3H]$DAMGO, $[^3H]$DPDPE, and $[^3H]$U69,593 as determined by saturation assay.

**Saturation binding assays** – Membrane homogenates (1 mg ml$^{-1}$) were incubated at 25°C for 1 hr with varying concentrations of tritiated ligand (10 – 0.005 nM) in the presence of either water (control) or 10 μM naloxone to determine total specific binding. The reaction was terminated by rapid filtration and filters subjected to liquid scintillation counting as above. Binding capacities (Bmax) and equilibrium dissociation constants ($K_D$) were calculated from non-linear regression using GraphPad Prism, San Diego, CA, USA.

$[^35S]$GTP$\gamma$S binding – Membrane homogenates were incubated for 1 hr at 30°C in the presence of $[^35S]$GTP$\gamma$S (100 pM), GDP (10 μM for SH-SY5Y cell homogenates, 30 μM for C6 glioma cell homogenates) and various concentrations of unlabeled ligand. Maximal stimulation was determined using fentanyl (10 μM) for μ-receptor studies, and SNC80 ((+)-4-[(αR)-α-(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide, 10 μM for δ-receptor studies). The tissue was again rapidly filtered and filters subjected to liquid scintillation counting as before. The amount of stimulated $[^35S]$GTP$\gamma$S binding is given as a percentage of the maximal stimulation evoked by either fentanyl or SNC80. The EC$_{50}$ values (effective concentration producing a 50 % maximal response) were calculated using GraphPad Prism.

2.2.4 **In vivo studies – Warm water tail withdrawal assay**

A modification of the warm water tail-withdrawal assay procedure of Janssen et al. [1963] was employed. Each mouse was placed in a cylindrical restraint with the tail fully exposed. Approximately one-third of the distal portion of the tail was immersed in water at 50°C, and latency to complete tail-withdrawal was measured. Baseline latencies were typically 2 - 4 sec. Only mice meeting this criterion were used. Agonists and vehicles were administered intraperitoneally (i.p.) (26 gauge needle, Becton Dickinson and Co.) and except for time course studies, latencies were measured 25 min later. Antagonists were administered by the intraperitoneal route at
the following times prior to agonist: M-CAM, 1 hr; naltrindole, 15 min; nor-BNI, 24 hr. A cut-off latency of 20 sec was used to prevent injury to the tail. Mice that did not respond within this time were removed and assigned a score of 20 sec.

Experiments using rats were essentially the same, except that rats were wrapped in a surgical towel instead of being placed in a restraint. Baseline latencies were typically 4 – 6 sec. All drugs were administered subcutaneously (s.c.).

The percent maximum possible effect (% MPE) at each point was calculated according to the following formula:

\[
\% \text{ MPE} = \frac{(\text{test latency} - \text{baseline latency})}{(20 - \text{baseline latency})}
\]

ED50 and 95 % confidence limits were determined using linear regression according to the method of Tallarida and Murray (1981, procedure 8). Dose-effect curves were considered to be significantly different when the range of values contained within the 95 % confidence limits did not overlap.

**Acetic Acid Induced Writhing Assay** - The acetic acid induced writhing assay [Koster et al., 1959] was used as another method for determining antinociception. Mice received agonist or vehicle subcutaneously, and except for the time course, this was followed 20 min later by 0.6 % acetic acid (0.4 ml per animal) given i.p. Mice were placed in individual Plexiglass boxes (18 x 28 x 13 cm) for observation. Five minutes after injection of acid an observation period began where the number of writhes during a 5 min period were counted. A writhe is typically a wave of contraction of the abdominal muscles followed by an extension of the hind legs. Control values were determined for each new batch of mice. Data were calculated as mean number of writhes ± S.E.M. for each treatment group. Student's t-test was employed to determine significant differences between treatment groups.

**Measurement of Locomotor Activity** - Experiments were performed with a Digiscan Micro System, Omnitech Electronics, Columbus, OH, USA, with four transparent Plexiglas activity monitor cages (46 x 24 x 18.5 cm) each placed in a soundproof chamber. A Plexiglass plate drilled with 8 to 12 airholes covered each cage. Locomotor activity in the horizontal plane was measured with a set of 16 photocells projecting horizontal infrared beams 2.5 cm apart and 2.4 cm above the cage floor. One unit or count of activity registered each time a light beam was broken. Data were
automatically recorded and processed by the Digiscan Micro Analyzer. Individual points were then compared for significant differences using Student’s t-test, GraphPad Prism.
CHAPTER 3
AN INVESTIGATION OF THE BELL-SHAPED DOSE-RESPONSE CURVE OF BUPRENORPHINE
3.1 Introduction

Buprenorphine is a promising novel pharmacotherapy for the treatment of opiate abuse [Bickel & Amass, 1995]. It has a unique pharmacological profile, which may offer superiority over currently existing treatment drugs such as methadone or LAAM. As discussed in section 1.6, buprenorphine produces morphine-like subjective effects, although because it is a μ-partial agonist it can block the euphoria caused by higher efficacy opiates [Jasinski et al., 1978]. In addition the unpleasant and often dangerous side effects associated with traditional full μ-opioid agonists, such as respiratory depression, are limited with the use of buprenorphine [Jasinski et al., 1978], and only a mild withdrawal syndrome is observed on cessation of its use [Fudala et al., 1990]. Therefore the use of buprenorphine in treatment programs can provide the addict with a relatively comfortable and rapid detoxification [Jasinski et al., 1978; Mello et al., 1982]. Additionally buprenorphine has a long duration of action due to extremely slow dissociation kinetics, which provides for the attenuation of the effects of any subsequently self-administered heroin or other opioid.

In many in vivo assays using either rodents or primates, buprenorphine exhibits a unique biphasic dose-response relationship, for example in the mouse hot plate assay [Tyers, 1980], the rat charcoal meal [Cowan et al., 1977b], and the monkey tail dip assay [Woods et al., 1992]. In assays of low noxious stimulus such as the late phase flinching of the formalin paw test buprenorphine is fully efficacious and exhibits a normal sigmoidal dose-response relationship.

Current literature attempts to explain the unusual biphasic relationship in terms of non-competitive autoinhibition and the existence of two functionally related opioid receptor subtypes [Sadee et al., 1982, Rance et al., 1980, Cowan, 1992]. As yet, however, no definitive explanation for this mechanism has been reported. In order to elucidate the mechanism behind the unusual phenomenon, a study of the antinociceptive effect of buprenorphine in rats using the warm water tail withdrawal assay at 50°C was carried out. In particular, time-courses of antinociceptive effects of various doses of buprenorphine were determined. This set of experiments test the hypothesis that the biphasic dose-response is dependent upon the time-course of the drug. The ability of the antagonist naltrexone to inhibit the dose-response curve was also investigated.
3.2 Results

In the rat warm water tail withdrawal assay at 50°C buprenorphine acted as a partial agonist (Fig. 3.1), and compared to morphine, EC$_{50}$ 4.2 mg kg$^{-1}$ (95% CI = 3.1 - 5.7 mg kg$^{-1}$) it was 140 times more potent, EC$_{50}$ 0.03 mg kg$^{-1}$ (95% CI = 0.01 - 0.08 mg kg$^{-1}$). Buprenorphine showed a bell-shaped dose-response curve with maximal effect (68.8 ± 9.3 % MPE) reached at a dose of 0.32 mg kg$^{-1}$. At higher doses a plateau effect is reached at approximately 35 % MPE.

![Cumulative dose-response curves of morphine and buprenorphine in the rat warm water tail withdrawal assay (50°C). Each point represents mean ± S.E.M for at least 6 rats.](image)

Figure 3.1 Cumulative dose-response curves of morphine and buprenorphine in the rat warm water tail withdrawal assay (50°C). Each point represents mean ± S.E.M for at least 6 rats.

Time-courses of the antinociceptive effects of various doses of buprenorphine are shown in Figs. 3.2 and 3.3. Lower doses appear to have antinociceptive action for up to 8 - 12 hr (Fig. 3.2), whereas the higher doses retain some antinociceptive activity 12 hr after administration (Fig. 3.3.).
Figure 3.2 Time course of antinociceptive effect of buprenorphine, 0.032 mg kg$^{-1}$ and 0.1 mg kg$^{-1}$. Values represent mean ± S.E.M for 6 rats.

Figure 3.3 Time course of antinociceptive effect of buprenorphine, 0.32 mg kg$^{-1}$ and 1.0 mg kg$^{-1}$. Values represent mean ± S.E.M for six rats.
A closer evaluation of the initial time-course of effect revealed that at a higher dose (1.0 mg kg\(^{-1}\)) there was a rapid onset of antinociception, reaching a near maximal effect within 15 min of administration (Fig. 3.4). This was followed by a rapid offset of antinociception, and after 1.5 hr the antinociceptive effect had decreased to 25.5 % MPE. Conversely at lower doses (0.1 - 0.32 mg kg\(^{-1}\)) the onset of antinociception was much slower, reaching peak effect at 1 hr after administration of 0.1 mg kg\(^{-1}\) and 30 min after administration of 0.32 mg kg\(^{-1}\). The offset of antinociception was also considerably slower with the lower doses of buprenorphine.

![Graph showing the time-course of antinociceptive effect of various doses of buprenorphine. Values represent mean ± S.E.M for 6 rats.](image)

Figure 3.4 Initial time-course of antinociceptive effect of various doses of buprenorphine. Values represent mean ± S.E.M for 6 rats.

A plot of % MPE against dose of buprenorphine for the different testing time intervals (i.e. the time elapsing between administration of drug and test of antinociception), revealed that the bell-shape of the dose-response curve was dependent upon the testing time interval (Fig. 3.5). Measurement of antinociception 15 min after administration does not show a biphasic curve over the dose range tested, whereas antinociceptive measurements taken 30 min or 1 hr after administration show a bell-shape dose-response. The peak effect also occurs at different doses depending on the testing time interval. Employing a testing time interval of 1 or 1.5 hr results in
the peak effect occurring at 0.1 mg kg\textsuperscript{-1}, whereas the peak effect occurs at 0.32 mg kg\textsuperscript{-1} if a testing time interval of 30 min is used.

![Graph showing dose-effect curve](image)

Figure 3.5 Plot of dose-effect curve for buprenorphine using different testing time intervals. Testing time interval is defined as the time elapsing between administration of drug and test of antinociception. Values represent mean ± S.E.M for 6 rats.

Due to the ability of the larger dose of buprenorphine (1.0 mg kg\textsuperscript{-1}) to produce a rapid on-set, followed by a rapid off-set, of antinociception, a possible hypothesis to explain this was that the subsequent rapid decline in antinociceptive effect was a result of receptor desensitization. Rats were pre-treated with 1.0 mg kg\textsuperscript{-1} buprenorphine and after 1.5 hr, at which time the antinociceptive effect had decreased to a plateau level of approximately 35 % MPE, rats were given a second dose of 1.0 mg kg\textsuperscript{-1} buprenorphine (Fig. 3.6). Fifteen min after the second dose there was only a slight increase in antinociception to 51 %, which was maintained for at least 60 min. A dose-response to fentanyl, a high efficacy μ-agonist, was also determined after pre-treatment with 1.0 mg kg\textsuperscript{-1} buprenorphine for 1.5 hr (Figs. 3.7 and 3.8). Even after normalization of values to account for the antinociception caused by buprenorphine it can be seen that there was not a significant shift in the dose-response to fentanyl (Fig. 3.8).
Figure 3.6 Antinociceptive effect of a 1.0 mg kg\(^{-1}\) dose of buprenorphine administered after pre-treatment of rats with 1.0 mg kg\(^{-1}\) buprenorphine for 1.5 hr. Each point represents mean ± S.E.M for 6 rats.

Figure 3.7 Dose-effect curve of fentanyl after pre-treatment of rats for 1.5 hr with 1.0 mg kg\(^{-1}\) of buprenorphine. Each point represents mean ± S.E.M for 6 rats.
Figure 3.8 Dose-effect curve of fentanyl following pre-treatment with 1.0 mg kg\(^{-1}\) buprenorphine for 1.5 hr, as in Fig. 3.7. In this graph the baseline level of antinociception caused by the buprenorphine pre-treatment has been subtracted from the fentanyl curve. Each point represents the mean ± S.E.M for 6 rats.

In order to examine the effect of the antagonist naltrexone on the bell-shape dose-response curve of buprenorphine, rats were pre-treated with various doses of naltrexone for 15 min prior to administration of buprenorphine (Fig. 3.9). It can be seen that naltrexone is able to shift the ascending, but not the descending, phase of the dose-response curve. The magnitude of the shift increased with increasing dose of naltrexone. The EC\(_{50}\) for buprenorphine in the presence of 0.1 mg kg\(^{-1}\) naltrexone was 0.14 mg kg\(^{-1}\) (95% CI = 0.12 - 0.16 mg kg\(^{-1}\)) and in the presence of 1.0 mg kg\(^{-1}\) was 0.81 mg kg\(^{-1}\) (95% CI = 0.49 - 1.35 mg kg\(^{-1}\)). Pre-treatment with 10 mg kg\(^{-1}\) naltrexone almost completely flattened the dose-response curve, hence the EC\(_{50}\) could not be calculated.
Figure 3.9  Dose-response curve of buprenorphine following pre-treatment with various doses of naltrexone for 30 min. A cumulative dosing procedure was employed. Values again represent mean ± S.E.M for at least 6 rats.
3.3 Discussion

The bell-shaped dose-response curve of buprenorphine has puzzled researchers for many years. The above study was carried out in an attempt to shed further light on the mechanism behind this unusual effect. Initial experiments using a cumulative dosing paradigm in the rat warm water tail withdrawal assay at 50°C confirmed that buprenorphine was a partial agonist, and indeed showed a biphasic dose-response relationship with maximal effect occurring at 0.32 mg kg\(^{-1}\). This is consistent with previous findings in rats using the radiant heat tail-flick assay [Rance et al., 1980], the tail withdrawal assay at 55°C [Cowan et al., 1977a], and the electrically induced vocalization test [Dum & Herz, 1981].

A time-course of various doses of buprenorphine revealed that the highest dose tested, 1 mg kg\(^{-1}\), a dose that forms part of the descending phase of the dose-response curve, produced a rapid, near maximal antinociceptive effect which appeared to occur within 15 min of administration. This was followed by a rapid offset of antinociceptive effect. The opposite was observed with lower doses of buprenorphine, i.e. a slow onset of antinociception followed by an equally slow offset. Dum & Herz [1981] reported similar results in the rat electrically induced vocalization test, i.e. larger doses of buprenorphine produced a rapid peak of antinociception whilst the lower doses showed a slower onset of antinociceptive activity. Interestingly, in the present study, following administration of a larger dose of buprenorphine the rapid decline in antinociceptive activity did not reach zero % MPE, but rather reached a plateau around 25 % which was maintained for several hours. This also correlates with the data of Dum & Herz. A subsequent plot of buprenorphine dose vs % MPE for each different testing interval (the time elapsing between administration and test for antinociception) showed that the biphasic curve was dependent upon this time interval. Testing for antinociception 15 min after administration did not reveal a bell-shaped dose-response curve due to the fact that at higher doses of buprenorphine an initial, rapid, peak of antinociception occurred. The dose of buprenorphine producing peak antinociception also changed depending upon testing time interval. For example, if the test for antinociception was to be employed 30 min after buprenorphine administration the peak effect would be seen at 0.32 mg kg\(^{-1}\). However, if the test of antinociception were to be employed 1.5 hr after
administration, the peak effects would be seen at 0.1 mg kg\(^{-1}\). A likely explanation for the rapid decline in antinociceptive effect seen at high doses of buprenorphine is receptor desensitization. Alternatively, buprenorphine is interacting with a second site on the receptor that modulates the agonist actions of the first site, and this second interaction is more rapid with a high dose of buprenorphine. Both of these explanations will be discussed in greater detail below.

Blake *et al.* [1997], have shown that acute buprenorphine treatment of HEK 293 cells expressing the cloned mouse \(\mu\)-opioid receptor resulted in a desensitization reaction characterized by a reduced ability of morphine and buprenorphine to inhibit forskolin-stimulated cyclic AMP accumulation. Buprenorphine is known to have very slow kinetics of dissociation resulting in a long-lasting interaction with the receptor [Boas & Villiger, 1985, Hambrook & Rance, 1976]. Additionally, it is difficult to reverse the effects of buprenorphine once established [Cowan *et al.*, 1977a, France *et al.*, 1984]. Therefore, despite the fact that buprenorphine is only a partial agonist, the 'pseudoirreversible' binding may be responsible for a rapid desensitization of the \(\mu\)-opioid receptor, as this persistent binding could cause functional desensitization. If this were so, then it should be testable by administration of a further dose of \(\mu\)-agonist when the agonist effects of buprenorphine had declined. Administration of a second 1.0 mg kg\(^{-1}\) dose of buprenorphine following pre-treatment for 1.5 hr with an initial 1.0 mg kg\(^{-1}\) dose did not result in any significant increase in antinociceptive effect over and above that which remained. The ability of a high efficacy \(\mu\)-opioid agonist, fentanyl, to evoke an antinociceptive response was unchanged. This would argue against desensitization.

It is possible that the decreased ability of buprenorphine and morphine to inhibit cAMP accumulation following pre-treatment of cells with buprenorphine in the study by Blake *et al.* [1997] is simply a result of being unable to wash buprenorphine from the receptors. Buprenorphine is a highly lipophilic molecule and for this reason is known to persistently bind to the \(\mu\)-receptor [Hambrook & Rance, 1976]. The inability of a second dose of buprenorphine to evoke a significant antinociceptive response may be because the initially administered buprenorphine has formed a tight interaction with the receptor and is therefore simply antagonizing itself. However, if this were the case then one would expect the antinociceptive effect of fentanyl to be blocked also. The fact that fentanyl is a high efficacy \(\mu\)-opioid agonist, thus requiring
less receptor occupancy to evoke a full response, could account for its ability produce an antinociceptive effect. The receptors may not be fully saturated after treatment with 1.0 mg kg⁻¹ buprenorphine as there has been conflicting evidence as to what dose of causes receptor saturation [Dum & Herz, 1981, Rance et al., 1980]. But again we would still expect to see a shift in the dose-response curve to fentanyl following buprenorphine pre-treatment. A possible explanation is that fentanyl is binding to a different receptor site. A recent study by Heerding et al. [1994] has suggested that buprenorphine has different μ-receptor binding requirements than other μ-agonists. Site directed mutagenesis experiments revealed that mutation of key amino-acids required for morphine, DAMGO and levorphanol binding did not affect the binding of buprenorphine. Additionally Bot et al. [1988] found that mutation of a histidine residue in transmembrane VI of the μ-receptor greatly reduced the binding affinity of diprenorphine and buprenorphine, but only a small shift in the affinity of naloxone and morphine for the receptor was observed. Recent results in our lab have also suggested that the oripavines may have different binding modes which are not altered by Na⁺ ions, and are different from morphine and fentanyl [Lee et al., submitted for publication]. This may explain why the production of antinociception by fentanyl was unaffected by buprenorphine pre-treatment as the two compounds may have different receptor interactions. It may be useful to examine the effects of another μ-agonist with structural similarity, such as etorphine, following buprenorphine pre-treatment.

An alternative explanation for the bell-shaped dose-response curve is that buprenorphine exerts its agonist and antagonist effects at different receptors, and that it accesses the inhibitory receptors at higher doses. Evidence exists that over the agonist dose-range buprenorphine produces its effects via the μ-receptor [Martin et al., 1976, Cowan et al., 1977a,b, Ward & Takemori, 1983], although more recently it has been shown that buprenorphine inhibits cyclic AMP accumulation in COS cells and HEK 293 cells transfected with the cloned mouse δ-opioid receptor [Kong et al., 1993, Bot et al., 1998]. The findings that buprenorphine has agonist action at the δ-receptor, however, are not consistent, for example in our laboratory it was found that buprenorphine did not stimulate the binding of [³⁵S]GTP⁺S in C6 glioma cells transfected with the cloned rat δ-receptor [Lee et al., submitted for publication], nor in membranes prepared from NG108-15 cells (unpublished observations). Indeed it has
been reported that pre-treatment of mice with the δ-selective antagonist naltrindole or the κ-selective antagonist nor-binaltorphimine (nor-BNI) had no effect on the antinociception produced by buprenorphine in the radiant heat tail flick assay [Kamei et al., 1997], and there is evidence that buprenorphine acts as a κ-antagonist in vivo [Richards & Sadee, 1985b, Negus & Dykstra, 1988]. As yet the identity of the receptor whereby buprenorphine might exert its antagonist actions is largely unknown. There are a number of explanations involving different receptors that could account for the unusual antagonist actions of higher doses of this drug. Firstly, as proposed by Cowan et al. [1977], Rance et al. [1980], and Sadee et al. [1982], non-competitive autoinhibition may be occurring. Buprenorphine exerts its agonist actions at one receptor site, and then at higher doses buprenorphine binds to the second, lower affinity site that counteracts the effects of the first, higher affinity site. It has been suggested that this second site may be a δ-opioid receptor site [Sadee et al., 1983] although this has never been confirmed. Another suggestion comes from DeLean et al. [1979], who proposed that buprenorphine forms a two-point attachment to a single receptor. Dum & Herz [1981] have also postulated that the biphasic curve may be a result of cooperative receptor interaction at higher occupancy, of a homogenous population of receptors.

In our experiments, at doses of both 0.1 and 1.0 mg kg⁻¹, naltrexone shifted the ascending, but not the descending, phase of the dose-response curve. This is inconsistent with the previous findings and suggests differences between single dosing and cumulative dosing paradigms. In the studies by Dum & Herz [1981], Cowan [1992], and Rance et al. [1980], it was found that either naloxone or naltrexone shifted the both phases in a parallel fashion, implying that naltrexone and naloxone are competitive antagonists at both sites. However, both Dum, Herz, and Cowan employed a single dosing procedure as opposed to a cumulative dosing procedure used in our study and the study of Rance et al. Furthermore, it is interesting to note that statistical analysis of the parallel shift of the dose-response curve generated by Rance et al. showed only one point to be significantly different. The inability of naltrexone to shift the descending arm of the curve suggests that naltrexone is not a competitive antagonist at this site. This implies that the second site of buprenorphine action is one that is inaccessible to naltrexone. Aside from its agonist action, the only major difference between the binding of naltrexone and
buprenorphine to the opioid receptors is that buprenorphine can bind to the \( \mu \)-opioid receptor in a 'pseudoirreversible' manner \cite{Lewis, 1985}. It is therefore possible that the second phase of the dose-response curve represents this long-lasting interaction of buprenorphine with the \( \mu \)-receptor. It may be that there is not a distinct second site involved, but rather a subsequently induced receptor conformational change. This may relate to the irreversible nature of buprenorphine such that naloxone cannot reverse the effects of buprenorphine once established.

Further evidence that the second phase of buprenorphine dose-response is connected to 'pseudoirreversible' \( \mu \)-binding comes from a paper published by Husbands \textit{et al.} \cite{1988}, who report that the 14-cinnamoylamino codeinone methoclocinnamox (MC-CAM), a partial \( \mu \)-agonist \cite{Woods \textit{et al.}, 1995} and other alkyl ether derivatives with \( \mu \)-agonist activity exhibit biphasic dose-response curves similar to buprenorphine. It has been shown that MC-CAM is a long-lasting 'pseudoirreversible' \( \mu \)-antagonist \cite{Woods \textit{et al.}, 1995, Butelman \textit{et al.}, 1996}.

We propose that the descending, antagonist phase of the bell-shaped dose-response curve of buprenorphine is due to the onset of 'pseudoirreversible' binding and a subsequent induction of an antagonist conformation of the \( \mu \)-receptor (Fig. 3.10). It is possible that the development of this tight interaction is slower than the initial binding interaction that enables agonist activation. This accounts for the fact that at higher doses a peak of antinociception is observed but is followed by a rapid decrease as higher receptor occupancy enables the change of more receptors into the antagonist conformation. This second conformation of the receptor would, however, appear to allow some agonist action resulting in the persistent 25 – 35% antinociception seen in our experiments. Indeed it may also be that there is a percentage of receptors which are not susceptible to the buprenorphine-induced receptor conformation change. At lower doses, the inhibition of agonist activity induced by the change in receptor conformation has a much slower time course due to overall less receptor occupancy. Once a certain level of receptor occupation is reached and the secondary, long-lasting inhibitory interaction of buprenorphine occurs, the inhibitory interactions of naltrexone are simply overcome. This model may also address the issue of differences seen between single and cumulative dosing procedures. Under a cumulative dosing paradigm buprenorphine is given more time to form its secondary interaction with the receptor, and so ultimately more of the receptors undergo the
conformation change within the time limits of the experiment. However, a single dosing procedure does not allow this, hence there are never enough receptors occupied tightly enough by buprenorphine to block the competitive actions of naltrexone.

From this and previous studies we can see that the biphasic nature of the buprenorphine response is one of great complexity that clearly warrants further study. It may be of great importance to determine the exact mechanism by which buprenorphine exerts its unique effects as ultimately this may be the key to the development of novel pharmacotherapies for the treatment of opioid abuse.
Figure 3.10 Schematic representation of buprenorphine binding to the μ-opioid receptor. Phase A shows the receptor in its normal resting state. Phase B shows buprenorphine binding to the receptor and causing an agonist response. The binding of buprenorphine at this first stage is reversible. Phase C depicts the receptor adapting an antagonist conformation as the binding of buprenorphine becomes 'irreversible' with time. Fentanyl interacts with a different site on the receptor.
CHAPTER 4
BU72: A NOVEL, HIGHLY EFFICACIOUS, OPIOID AGONIST WITH LONG-LASTING ANTAGONIST PROPERTIES
4.1 Introduction

A mentioned previously in sections 1.6 and 3.1, buprenorphine is a promising alternative to methadone and LAAM for the treatment of opioid abuse. Much research has focused around the mixed agonist/antagonist profile of buprenorphine [Cowan et al., 1977a, 1977b; Bickel & Amass, 1995], with the belief that a combination of these characteristics produces an efficacious compound for patient compliance, together with the ability to attenuate any subsequently self-administered heroin or other opioid.

Despite its desirable pharmacological characteristics, buprenorphine is not ideal as a treatment drug. In particular the degree of reinforcing effect afforded by buprenorphine is less than methadone, and may not be sufficient to maintain certain opiate addicts in treatment [Fudala et al., 1995]. With this in mind we have sought to characterize and develop more efficacious and longer lasting analogues of buprenorphine. A promising candidate is the novel morphinan pyrrolidine derivative, BU72 (Fig. 4.1) [Husbands & Lewis, 1995]. BU72 is an iso-morphinan derivative, bridged 6,14- with an etheno group and 5,7- with an aminobenzyl group which forms a substituted pyrrolidine ring. When compared structurally to buprenorphine the phenyl ring corresponds to the t-butyl group but is attached β- to C7 whereas the t-butyl group in buprenorphine is attached α- to C7. BU72 has a piperidine N-methyl group in contrast to the N-cyclopropylmethyl group of buprenorphine which is more usually associated with opioid antagonism [Lewis, 1995].

In the following study the pharmacology of BU72 is characterized, both in vitro and in vivo using mice. The binding profile of BU72 has been studied in mouse brain and µ-efficacy has been determined in a rat glioma cell line stably expressing the cloned µ-opioid receptor [Thompson et al., 1993]. The antinociceptive actions of BU72 were studied in the mouse warm water tail-withdrawal assay at 50°C, and the acetic acid-induced writhing assay. The results show that BU72 is a highly efficacious µ-agonist with delayed µ-receptor antagonist action, and may, therefore, exhibit a better pharmacological profile for a treatment drug than either methadone or buprenorphine.
Figure 4.1. The structure of BU72.
4.2 Results

4.2.1 *In vitro* studies

*Competition binding assays*

As can be seen in Table 4.1 the morphinan-pyrrolidine BU72 displayed high affinity in the sub-nanomolar range for μ-, δ-, and κ-opioid binding sites in mouse brain membranes as measured by the inhibition of \(^{3}\text{H}\)DAMGO, \(^{3}\text{H}\)DPDPE, and \(^{3}\text{H}\)U69,593 binding respectively. BU72 also showed high affinity for the μ-opioid binding site in C6 glioma cell membranes as measured by the Ki derived from the inhibition of \(^{3}\text{H}\)diprenorphine binding (Table 4.2 and Fig. 4.2). This affinity was similar to buprenorphine but 100-fold higher than morphine or methadone. Hill coefficients for the binding of BU72, buprenorphine, methadone, and morphine to C6 glioma cell membranes and mouse brain membranes were all close to unity.

<table>
<thead>
<tr>
<th>Ki (nM)</th>
<th>μ ((^{3}\text{H})DAMGO)</th>
<th>δ ((^{3}\text{H})DPDPE)</th>
<th>κ ((^{3}\text{H})U69,593)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU72</td>
<td>0.36 ± 0.07</td>
<td>0.46 ± 0.09</td>
<td>0.15 ± 0.06</td>
</tr>
</tbody>
</table>

Table 4.1 Ki values for the inhibition of \(^{3}\text{H}\)DAMGO, \(^{3}\text{H}\)DPDPE, and \(^{3}\text{H}\)U69,593 binding in mouse brain membranes. Data represent mean Ki ± S.E.M. for three experiments performed in duplicate.
Figure 4.2 Displacement of $[^3H]$diprenorphine (0.2 nM) specific binding by BU72, buprenorphine, morphine and methadone in membranes prepared from C6 glioma cells. Each data point represents mean ± S.E.M for at least three experiments performed in duplicate. SB = specific binding.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU72</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Morphine</td>
<td>6.33 ± 2.55</td>
</tr>
<tr>
<td>Methadone</td>
<td>5.85 ± 2.39</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>0.12 ± 0.03</td>
</tr>
</tbody>
</table>

Table 4.2. Binding potencies of BU72, morphine, methadone, and buprenorphine at the $\mu$-opioid receptor in C6 glioma cell membranes. Data are expressed as the mean $K_i$ ± S.E.M. for three determinations performed in duplicate.
BU72 acted as a potent full agonist in the \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) assay as compared to the \(\mu\)-opioid receptor agonist fentanyl, affording an \(EC_{50}\) value of \(0.05 \pm 0.01\) nM and a maximal stimulation of \(70.4 \pm 23.6\) fmol \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) bound mg\(^{-1}\) protein (Fig. 4.3). This represented 116\% of the fentanyl response. Morphine and methadone afforded \(EC_{50}\) values of \(30.10 \pm 4.20\) nM and \(77.60 \pm 18.90\) nM, and maximal stimulations of 96\% and 105\% of the fentanyl response respectively. Buprenorphine was a partial agonist representing 33\% of the fentanyl response with an \(EC_{50}\) value of \(0.21 \pm 0.19\) nM.

![Figure 4.3 Stimulation of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding by BU72, buprenorphine, morphine and methadone in membranes of C6 glioma cells. Values shown are mean ± S.E.M for at least three experiments performed in duplicate.](image)
4.2.2 In vivo studies

1. Agonist Actions – Antinociceptive effect

BU72 administered i.p. produced dose-dependent antinociception in the mouse warm water tail-withdrawal assay at 50°C (Fig. 4.4). BU72 was approximately 400 times more potent than morphine in this assay, affording an ED$_{50}$ value of 0.07 mg kg$^{-1}$ (95% CI = 0.05 - 0.09 mg kg$^{-1}$) as compared to 27.14 mg kg$^{-1}$ (95% CI = 40.92 - 17.99 mg kg$^{-1}$) for morphine. At single doses administered s.c. BU72 also produced potent, dose-dependent, antinociception in the mouse acetic acid-induced writhing assay (Fig. 4.5).

![Graph showing antinociceptive effects of morphine and BU72 in the mouse warm water tail withdrawal assay at 50°C. Values represent the mean ± S.E.M. of all mice tested (n=5) at each particular dose of drug.](image)

Figure 4.4 Antinociceptive effects of cumulative doses of morphine and BU72 in the mouse warm water tail withdrawal assay at 50°C. Values represent the mean ± S.E.M. of all mice tested (n=5) at each particular dose of drug.
Figure 4.5 Antinociceptive effect of morphine and BU72 in the mouse acetic acid-induced writhing assay. Values represent mean ± S.E.M for 6 mice
Control writhing = 11.4 ± 1.9 writhes.

To determine the receptor through which BU72 was exerting its antinociceptive action in the tail withdrawal assay, selective antagonists were used (Fig. 4.6). Pre-treatment of mice i.p. with the δ-antagonist naltrindole (10 mg kg⁻¹, 15 min) or the κ-antagonist nor-BNI (32 mg kg⁻¹, 24 hr) had no effect on the antinociceptive effect of BU72. In contrast, pre-treatment i.p. with the long-lasting μ-antagonist M-CAM (1.8 mg kg⁻¹, 1 hr) [Broadbear et al., submitted for publication] fully inhibited the antinociception produced by BU72. The doses of antagonists used have been shown in our laboratory to be selective for the respective opioid receptor subtype (J. Broadbear, unpublished observations, see Table 4.3). In addition, administration of BU72 induced behavioural phenomena typically associated with μ-opioid agonists, inducing a Straub tail and an increase in locomotor activity [Aceto et al., 1969; Rethy et al., 1971]. Diuresis and sedation, behavioral effects associated with κ-agonist action [Leander et al., 1987], were observed after administration of a high dose (10 mg kg⁻¹) of BU72. These κ-agonist effects were blocked by pre-treatment with nor-BNI (32 mg kg⁻¹).
Table 4.3  Potencies of morphine (μ), bremazocine (κ) and BW373U86 (δ) in the mouse acetic acid-induced writhing assay and their antagonism by M-CAM. Values in parentheses represent the 95% confidence limits. Data from Broadbear et al., submitted for publication.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Morphine $ED_{50}$ (mg kg$^{-1}$)</th>
<th>Bremazocine $ED_{50}$ (mg kg$^{-1}$)</th>
<th>BW373U86 $ED_{50}$ (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.55 (0.40-0.77)</td>
<td>0.012 (0.01-0.015)</td>
<td>2.81 (1.60-4.93)</td>
</tr>
<tr>
<td>M-CAM</td>
<td>40.8 (19.5-85.8)</td>
<td>0.009 (0.006-0.014)</td>
<td>5.58 (4.18-8.18)</td>
</tr>
<tr>
<td>1.8 mg kg$^{-1}$, 1 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.6  Antinociceptive effects of cumulative doses of BU72 before and after pretreatment with the selective opioid antagonists M-CAM, 1.8 mg kg$^{-1}$, 1 hr, naltrindole, 10 mg kg$^{-1}$, 15 min; and nor-BNI, 32 mg kg$^{-1}$, 24 hr in the warm water tail withdrawal assay. Values represent the mean ± S.E.M for 5 mice.
2. Agonist actions – Time course of antinociception

The antinociceptive time course of BU72 relative to morphine and buprenorphine was determined in the tail withdrawal assay administered i.p. and writhing assay administered s.c. (Figs. 4.7 and 4.8). The doses of drug chosen were those which produced maximum antinociceptive effect in each assay system. In the tail withdrawal assay BU72 (0.32 mg kg\(^{-1}\)) still retained some antinociceptive activity at 12 hr as compared to morphine (100 mg kg\(^{-1}\)) which had a duration of action of less than 5 hr. The antinociceptive effect of buprenorphine (3.2 mg kg\(^{-1}\)) lasted approximately 8 hr. A higher dose of BU72 (10 mg kg\(^{-1}\)) maintained antinociception for up to 24 hr, and in the writhing assay lasted for up to 48 hr.

![Graph of time-course of antinociceptive effects](image)

**Figure 4.7** Time-course of the antinociceptive effects of bolus doses of morphine, buprenorphine, and BU72 in the warm water tail withdrawal assay. Values represent mean ± S.E.M for 5 mice.
3. Agonist actions – Reversibility

Due to the inability of naloxone to reverse the effects of buprenorphine once established [e.g. France et al., 1994], it was thought that perhaps the long-lasting antinociception produced by BU72 may be a result of a tight interaction with the receptor, similar to buprenorphine. The ability of naloxone and naltrexone to reverse the antinociceptive effect of BU72 was investigated in the tail withdrawal assay (Fig. 4.9). Although naloxone and naltrexone (10 mg kg\(^{-1}\)) were unable to reverse the antinociceptive effect of a high dose of BU72 (10 mg kg\(^{-1}\)) administered 1 hr previously, both antagonists were able to fully reverse the antinociceptive effect of BU72 at a dose of 0.32 mg kg\(^{-1}\). However BU72 attenuated antinociception once the naloxone was no longer effective. This was approximately 2 - 3 hr after
administration of the antagonist. Naltrexone was longer lasting than naloxone and blocked the antinociceptive effect of BU72 for at least 7 hr.

![Graph showing % MPE over time after BU72 injection](image)

**Figure 4.9** Reversal of the antinociceptive effect produced by BU72 (0.32 mg kg\(^{-1}\) or 10 mg kg\(^{-1}\)) using either naloxone or naltrexone, both 10 mg kg\(^{-1}\) i.p., in the tail withdrawal assay. Values represent mean ± S.E.M for 5 mice. NX = naloxone, NTX = naltrexone.

Naloxone and naltrexone were unable to reverse the observed analgesia produced by a large dose (10 mg kg\(^{-1}\)) of BU72 (Fig. 4.9). It was noted however that both antagonists appeared to block the sedation produced by this dose of BU72. To quantify this effect the locomotor activity of the mice was examined by counting the number of times the mouse crossed a light beam either in the horizontal or vertical plane (Fig. 4.10). After a habituation period during which time a sterile water injection was administered, mice were injected i.p. with BU72. A complete decrease in locomotor activity counts was observed as the mice became sedated. The mean total locomotor count 20 min after a sterile water injection was 78, this decreased to 13 counts after administration of BU72. One hr later, naloxone (10 mg kg\(^{-1}\), i.p.) was given. Approximately 15 min after the naloxone injection a normal level of
locomotor activity was observed with the mean count returning to 93. Sedation was seen to occur at 2 hr when naloxone was no longer effective.

Figure 4.10 Measurement of total locomotor activity counts before and after administration of BU72, 10 mg kg$^{-1}$ and reversal by naloxone, 10 mg kg$^{-1}$. Each point represents the mean count of 8 mice.

4. Antagonist actions

Kamei et al., [1996] have shown that the highly efficacious opioid, dihydroetorphine (DHE), is able to block the effects of subsequently administered morphine in mice. Since BU72 is also highly efficacious and longer lasting than DHE, it was of interest to determine if the antinociceptive effect of morphine was blocked following BU72 administration, particularly since the aim of this study was to evaluate the potential of BU72 as a treatment drug. The dose-response curve for morphine in the tail withdrawal assay was determined before and after pre-treatment with BU72 (0.32 or 10 mg kg$^{-1}$). When the antinociceptive effect of a 0.32 mg kg$^{-1}$ i.p. dose of BU72 had fully diminished (18 hr) a 3-fold shift in the dose-response curve to morphine was
observed from a control ED$_{50}$ of 27.1 mg kg$^{-1}$ (95 % CI = 18.0 - 40.9 mg kg$^{-1}$), to an ED$_{50}$ at 18 hr of 72.4 mg kg$^{-1}$ (95 % CI = 41.8 - 125.4 mg kg$^{-1}$) (Fig. 4.11). A similar shift in the morphine dose-response curve was observed 24 hr after pre-treatment but due to a larger 95 % confidence interval the ED$_{50}$ was found to be not statistically significantly different from the ED$_{50}$ for morphine control. No significant shift was observed after pre-treatment for 48 or 72 hr. A higher dose of BU72 (10 mg kg$^{-1}$, i.p.) produced antinociception for 28 hr, after which time the dose-response curve of morphine was observed to give an ED$_{50}$ of 58.8 mg kg$^{-1}$ (95 % CI = 39.5 - 87.5 mg kg$^{-1}$) (Fig. 4.12). After a pre-treatment time of 72 hr this shift increased to 7-fold greater than control, giving an ED$_{50}$ of 188 mg kg$^{-1}$. Due to fatalities at higher doses of morphine the upper limit of the 95 % CI could not be calculated. A week after single dose pre-treatment the ED$_{50}$ for morphine (24.5 mg kg$^{-1}$ (95 % CI = 10.92 - 54.98 mg kg$^{-1}$)) had returned to control value. Administration of a single s.c. dose of BU72 (10 mg kg$^{-1}$) produced a larger shift (ED$_{50}$ = 468 mg kg$^{-1}$ after 72 hr) than the equivalent i.p. dose.

An acute dose of BU72 (0.32 mg kg$^{-1}$) did not cause a shift in the dose-response curve of BU72. The ED$_{50}$ for BU72 in naïve animals was 0.07 mg kg$^{-1}$, (95 % CI = 0.05 - 0.09 mg kg$^{-1}$) and 18 hr after BU72 administration the ED$_{50}$ remained the same (0.07 mg kg$^{-1}$, 95 % CI = 0.04 - 0.12 mg kg$^{-1}$). In contrast to BU72, 5 hr after administration of a maximal dose of methadone (32 mg kg$^{-1}$), at which time methadone was no longer effective, the degree of antinociception produced by morphine was unchanged from control mice, ED$_{50}$ = 24.0 mg kg$^{-1}$ (95 % CI = 11.3 - 51.0 mg kg$^{-1}$).
Figure 4.11 Antinociceptive effect of morphine before and after pre-treatment with BU72 (0.32 mg kg\(^{-1}\)) for 18 hr, 24 hr, 48 hr, and 72 hr in the tail withdrawal assay. Values represent mean ± S.E.M for 5 mice.

Figure 4.12 Antinociceptive effect of morphine before and after pre-treatment with BU72 (10 mg kg\(^{-1}\)) for 28 hr, 48 hr, 72 hr and 1 week in the tail withdrawal assay. Values represent mean ± S.E.M for 5 mice.
The antagonist effect of BU72 was also determined in the writhing assay. In naïve animals morphine (3.2 mg kg\(^{-1}\)) completely blocked writhing but 24 hr after a pre-treatment with a low dose of BU72 (0.32 mg kg\(^{-1}\)) the antinociceptive effect of morphine was partially blocked; returning to 25 % of control, and 86 % of control after 72 hr. The antinociceptive effect of morphine returned to normal after 1 week (2 % of control) (Fig. 4.13). After a 72 hr pre-treatment with a high dose of BU72 (10 mg kg\(^{-1}\)) the antinociceptive effect of morphine was antagonized to 73 % of control. This value was not significantly different from control. Morphine was still ineffective after 1 week (86 % of control, not significantly different from control) and even after 14 days the antinociception produced by morphine was still partially blocked (24 % of control) (Fig. 4.14).

![Graph showing the antinociceptive effect of morphine in the writhing assay](image)

**Figure 4.13** Antinociceptive effect of morphine (3.2 mg kg\(^{-1}\)) in the writhing assay before and after pre-treatment with BU72 (0.32 mg kg\(^{-1}\)) for 24 hr, 48 hr, 72 hr, and 1 week. Control writhing = 14.0 ± 1.9 writhes. Values represent mean ± S.E.M for 6 mice. **P< 0.01, *P< 0.05 vs morphine alone, Students' unpaired t-test.**
Figure 4.14  Antinociceptive effect of morphine (3.2 mg kg\(^{-1}\)) in the writhing assay before and after pre-treatment with BU72 (10 mg kg\(^{-1}\)) for 24 hr, 48 hr, 72 hr, 1 week and 2 weeks. Control writhing = 14.8 ± 1.9 writhes. Values represent mean ± S.E.M for 6 mice. *** P< 0.005, ** P< 0.01, vs morphine alone. Students' unpaired t-test.

The effect of BU72 pre-treatment on the antinociception produced by the \(\delta\)-agonist BW373U86 and the \(\kappa\)-agonist bremazocine was also examined (Fig. 4.15). Seventy-two hr after pre-treatment with a single 10 mg kg\(^{-1}\) s.c. dose of BU72, when morphine action is maximally blocked, the antinociceptive effect of a maximally effective dose of BW373U86 (10 mg kg\(^{-1}\)) was reduced giving 39 % of control writhing. The antinociception produced by a maximally effective dose of bremazocine (0.1 mg kg\(^{-1}\)) was unaffected.
Figure 4.15 Antinociceptive effect of morphine (0.32 mg kg\(^{-1}\)), BW373U86 (10 mg kg\(^{-1}\)), and bremazocine (0.1 mg kg\(^{-1}\)), before and after pre-treatment with BU72 (10 mg kg\(^{-1}\)). Control writhing = 10.7 ± 2.4 writhes. Values represent mean ± S.E.M for 6 mice. ** P< 0.01 vs agonist alone, Students’ unpaired t-test.
4.3 Discussion

The present study indicates that the morphinan-pyrrolidine BU72 is a highly efficacious μ-opioid receptor agonist with subsequent μ-antagonist properties, which are particularly striking in the writhing assay. Similar to buprenorphine, BU72 binds with high affinity to the μ-opioid receptor, but is relatively non-selective in agreement with previous data obtained in guinea-pig brain membranes [Husbands & Lewis, 1995]. It is interesting to note that the $K_i$ value for the binding of BU72 to membranes from C6 cells expressing the cloned μ-receptor was lower than the $K_i$ for the binding of BU72 to the μ-receptor in mouse brain membranes. This may reflect differences in the competing tritiated ligand, as $[^3H]$DAMGO was used in competition assays using mouse brain membranes and $[^3H]$diprenorphine was used for competition binding using C6μ cells. Results from our laboratory suggest that these two ligands have different binding requirements [Lee et al., submitted for publication]. In C6μ cells BU72 was a more efficacious agonist than fentanyl, 600 times more potent than morphine and approximately 40 times more potent than buprenorphine which, as expected, was only a partial agonist.

In accordance with its high in vitro μ-efficacy BU72 produced dose-dependent antinociception in both the tail withdrawal and writhing assays. On administration of 0.1 mg kg$^{-1}$ and above behavioral effects typically associated with μ-opioid receptor ligands were observed [Aceto et al., 1979; Rethy et al., 1971], such as an increase in locomotor activity and Straub tail. Confirmation that the antinociception was mediated through the μ-opioid receptor was obtained by inhibition of the antinociceptive response by pre-treatment with the irreversible μ-selective antagonist M-CAM. Neither the δ-selective antagonist naltrindole, nor the κ-selective antagonist nor-BNI had any effect on the antinociception produced by BU72. Diuresis and sedation; behavioral effects associated with κ-agonist action [Leander et al., 1987], were observed after administration of a high dose (10 mg kg$^{-1}$) of BU72 and blocked by pretreatment with nor-BNI (32 mg kg$^{-1}$). This is consistent with the high affinity of the compound for the κ-receptor, but the higher doses required to observe this effect agree with the preferential activity of the compound at μ-, rather than κ-receptors, in the tail withdrawal assay.
It is interesting to note that in one experiment a dose of 32 mg kg\(^{-1}\), that is 100 times the dose needed to produce antinociception in the tail withdrawal assay, was administered safely. This is in contrast to our findings with morphine and methadone, where a dose 10 times that needed to produce maximum antinociception is lethal in the NIH strain of mice used in this study.

An important feature of a treatment drug is a long duration of action, this being one of the characteristics of buprenorphine that makes it particularly attractive alternative to methadone. BU72 has a duration of action longer than both morphine and buprenorphine. It is thought that buprenorphine is long-lasting because the C\(_7\) t-butyl group forms a tight lipophilic interaction with the receptor, thus slowing down its dissociation kinetics [Hambrook and Rance, 1976]. It is difficult to reverse the agonist action of buprenorphine once it is established [Cowan et al., 1977a, France et al., 1984], so it was of interest to determine if the reason for the long duration of action of BU72 was due to irreversible or pseudo-irreversible binding to the \(\mu\)-opioid receptor. However, the antinociceptive effect of BU72 (0.32 mg kg\(^{-1}\)) was completely reversed by both naloxone and naltrexone. Furthermore BU72 still had access to \(\mu\)-receptors several hr after administration as antinociception was once more observed when the effect of naloxone had dissipated. Although the antinociception produced by a high dose of BU72 (10 mg kg\(^{-1}\)) could only be partially reversed by naloxone, the level of sedation, as measured by locomotor activity, was fully reversible. These data would suggest that the mechanism behind the long duration of action is different from that of buprenorphine, and probably involves the continued presence of BU72 in the central tissue rather than a persistent binding to the \(\mu\)-opioid receptor.

The ability to block the effects of subsequently administered opioids is also an important pharmacological characteristic of a treatment drug. The antagonist action of BU72 was therefore studied in both the tail withdrawal and writhing assays. Once an antinociceptive effect of BU72 was no longer apparent a shift in the dose-response curve of subsequently administered morphine in the tail withdrawal assay was observed. This antagonism appeared to develop over time, and was more pronounced in the writhing assay. In the tail withdrawal assay, the greatest shift was seen at 18 - 24 hr in the case of 0.32 mg kg\(^{-1}\) BU72 and at 48 - 72 hr in the case of 10 mg kg\(^{-1}\) BU72. In the writhing assay a maximal dose of morphine was partially suppressed for up to 3 days after pre-treatment with a single dose of 0.32 mg kg\(^{-1}\) BU72. Using a
higher dose of BU72 (10 mg kg\(^{-1}\)) the antinociceptive action of morphine was completely suppressed 3 days after a single dose and still not fully effective after two weeks. At this dose of BU72 an antagonist effect against the \(\delta\)-agonist BW373U86 was observed but the antinociceptive effect of the \(\kappa\)-agonist bremazocine was unaffected. However it is known that BW373U86 does have a \(\mu\)-agonist component [A. Alt, our laboratory, personal communication], which would be susceptible to antagonism by BU72. The ability of BU72 to produce full antagonism of morphine for several days is comparable to the effects seen with irreversible \(\mu\)-antagonists such as clocinnamox (C-CAM) [Zernig \textit{et al.}, 1995].

Initially it was thought that BU72 was binding to the \(\mu\)-receptor in an irreversible manner similar to buprenorphine, hence the long-duration of action. However, the data has shown that BU72 is reversible by antagonist administration in both antinociception and locomotor assays. It is possible that BU72 is causing down-regulation of the \(\mu\)-receptor, leading to reduced effectiveness of subsequently administered \(\mu\)-agonists. \textit{In vivo} regulation of \(\mu\)-opioid receptors by agonists has yielded variable results, some reporting up-regulation and some reporting down-regulation [Tempel \textit{et al.}, 1988; Brady \textit{et al.}, 1989, Yoburn \textit{et al.}, 1993], but it has been shown that chronic treatment for longer periods of time with high (usually toxic) doses of high efficacy agonists such as etorphine and fentanyl can produce down-regulation of opioid receptors [Tao \textit{et al.}, 1987, Yoburn \textit{et al.}, 1993]. We would perhaps, therefore, not expect a single dose of BU72 to produce down-regulation of the \(\mu\)-opioid receptor. Another possibility is desensitization without down-regulation, although it has also been shown that acute treatment with etorphine does not affect either receptor density or affinity of the agonist for the receptor [Yoburn \textit{et al.}, 1993; Tao \textit{et al.}, 1987]. In spite of this the most likely explanation for delayed antagonist action of BU72 is the production of tolerance to morphine. This also allows an explanation as to why pretreatment with BU72 does not prevent the subsequent agonist action of BU72, i.e. tolerance to BU72 is not evident as the highly efficacious nature of the compound means only a few receptors need to be activated to evoke a full antinociceptive response.

These results are similar to data obtained with dihydroetorphine (DHE), an oripavine-thebaine derivative 12000 times more potent than morphine [Bentley & Hardy, 1967]. It has been reported that DHE possesses antagonist properties at the \(\mu\)-
opioid receptor [Kamei et al., 1996]. DHE, despite its high efficacy, apparently has low physical dependence capacity and has been successfully used to detoxify more than 3000 opiate addicts in China [Wang et al., 1992]. Using the radiant heat tail-flick assay in mice, Kamei et al. showed that an i.p. injection of 10 μg kg⁻¹ DHE produced an antinociceptive effect for up to 90 min after administration. After this time the antinociceptive effect of subsequently administered morphine was significantly reduced for up to 6 hr. A similar antagonism of morphine was seen using buprenorphine instead of DHE. The authors concluded that either acute tolerance to μ-opioid receptor agonist-induced antinociception was occurring, or that DHE and buprenorphine were interacting with a separate ‘antagonist’ recognition site which is allosterically coupled to the μ-receptor ‘agonist’ binding site [Portoghese & Takemori, 1983]. The results obtained with DHE and buprenorphine are similar to the present finding obtained with BU72, suggesting that a common mechanism of action may be responsible for the favourable profile of these compounds as potential treatment drugs.

If tolerance development at the μ-opioid receptor by BU72 is the reason for loss of efficacy of morphine, then the question arises as to why BU72 is more effective at antagonizing morphine in the writhing than the tail-withdrawal assay; when based on receptor theory [Kenakin, 1997] we would expect to see greater antagonism at the system requiring higher efficacy i.e. the tail withdrawal assay. One possibility is that different μ-receptor populations are involved. This would explain why BU72 has similar potency in the tail-withdrawal and writhing assays when morphine differentiates the two assays, and is 50-times more potent in the latter assay. However, a simpler explanation may relate to the pharmacokinetics of BU72, following the different injection routes employed in the tail-withdrawal assay (i.p.) and the writhing assay (s.c.) leading to a greater tolerance following s.c. administration. In support of this, the antagonist action of BU72 was greater following s.c. than i.p. administration in the tail withdrawal assay.

In conclusion BU72 is a highly efficacious μ-opioid receptor agonist with subsequent properties that inhibit the antinociceptive action of morphine for up to one week in mice. If this transfers to man this would provide efficacy for patient compliance, followed by blockade of the reinforcing effects of any subsequently

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administered opiate. Experiments in non-human primates are in progress to answer this question.
CHAPTER 5
CHARACTERISATION OF 3-ALKYL ETHER DERIVATIVES OF METHOCLOCINNAMOX
5.1 Introduction

The 14-cinnamoylamino codeinone, methoclocinnamox, (MC-CAM; BU28, Fig.5.1), has aroused interest in recent years due to its potential as a therapeutic agent for the treatment of opioid abuse [Woods et al., 1995]. MC-CAM is a member of an unusual series of compounds comprising the long-lasting 'pseudoirreversible' antagonist clocinnamox (C-CAM; BU18, Fig. 5.1) [Aceto et al., 1989, Comer et al., 1992] and other similarly 14-substituted cinnamoylamino morphinones and codeinones [e.g. Jiang et al., 1993]. These compounds are relatively selective for the μ-receptor but display varying degrees of efficacy [Lewis et al., 1988]. MC-CAM itself exhibits initial partial agonist activity followed by long-lasting antagonism at the μ-receptor in a number of in vivo assays including the warm water tail withdrawal assay in rhesus monkeys [Butelman et al., 1996], and the mouse writhing assay and tail withdrawal assay [Aceto et al., 1989]. This may be due to metabolism of MC-CAM to its corresponding morphinone, C-CAM [Woods et al., 1995]. Indeed in our hands MC-CAM also exhibits a bell-shaped dose-response curve similar to that observed with buprenorphine in the rat tail withdrawal assay [our laboratory, unpublished observations, see also Husbands et al., 1998]. Taken together these features suggest a common mechanism of action which may be important as a potential treatment drug.

More recently this series of compounds has been extended to include ethers of C-CAM, other than the methyl ether MC-CAM, in which the 3-OH is replaced with larger alkyl groups. These compounds have been investigated for their efficacy and long-lasting antagonism in vitro and in vivo [Husbands et al., 1998]. In rat brain homogenates all compounds bound with high affinity to the μ-opioid receptor, despite the presence of the 3-alkyl substituents. In the mouse warm water tail withdrawal assay several of the compounds displayed a higher degree of efficacy than MC-CAM, with the exception of the cyclopropylmethyl ether which showed no agonist activity, but a profile similar to that of C-CAM. As observed with MC-CAM, those ethers showing agonist activity also displayed delayed, long-lasting, μ-antagonist activity.

In order to further characterize these compounds and to understand this unique mechanism of action similar to what has been previously observed with BU72 in Chapter 4, the binding profile of the ethers and the in vitro efficacy using the
[\textsuperscript{35}S]GTP\gamma S assay has been examined using membranes prepared from SH-SY5Y human neuroblastoma cells.
Figure 5.1 The structures of the 3-substituted 14-cinnamoylamino compounds.

BU18 (C-CAM)  \( R = H \)
BU19  \( R = \text{CH}_2\text{-CH}=\text{CH}_2 \)
BU20  \( R = \text{CH}_2\text{-C≡N} \)
BU21  \( R = \text{CPM} \)
BU22  \( R = \text{CH}_2\text{CH}_2\text{CH}_3 \)
BU23  \( R = \text{CH}_2\text{C≡CH} \)
BU24  \( R = \text{CH}_2\text{CO}_2\text{CH}_3 \)
BU25  \( R = \text{CH}(\text{CH}_3)_2 \)
BU28 (MC-CAM)  \( R = \text{CH}_3 \)
5.2 Results

5.2.1 Radioligand Binding Assays

C-CAM (BU18) and its 3-alkyl ethers (BU19 – 25, 28) (Fig. 5.1) showed high affinity for the \( \mu \)-receptor in membranes prepared from SH-SY5Y human neuroblastoma cells (Figs. 5.2 – 5.6). All of the ethers, with the exception of the isopropyl ether BU25, displayed a biphasic displacement curve for the inhibition of \([^3H]DAMGO\) binding to SH-SY5Y cell membranes giving Hill coefficients (Table 5.1) of less than unity except for that obtained for BU25, which was closest to unity at 0.77. Displacement curves were subsequently analyzed using GraphPad Prism and a best fit to a two-site binding model was confirmed (all \( P \) values < 0.0005) for all the compounds with the exception of BU25. The fraction of binding to the high affinity site varied from 32 % for the cyclopropylmethyl ether BU21 to 69 % for the propargyl ether BU23. The irreversible \( \mu \)-ligand \( \beta \)-funaltrexamine (\( \beta \)-FNA) showed high affinity binding that was monophasic.

The rank order of affinity of the 3-alkyl ethers as determined by radioligand binding is as follows: For site 1 (high affinity site): C-CAM > propargyl (BU23) > nitrile (BU20) > allyl (BU19) > methyl (MC-CAM) > cyclopropylmethyl (CPM) (BU21) > \( n \) propyl (BU22) > ester (BU24). For site 2 (low affinity site): C-CAM > methyl > isopropyl > allyl > nitrile > ester > CPM > propargyl > \( n \) propyl.
Figure 5.2 The displacement of $[^{3}H]$DAMGO by C-CAM (BU18) and MC-CAM (BU28) in SH-SY5Y cell membranes. Values for this and each subsequent graph represent means ± S.E.M for at least three experiments performed in duplicate. SB = specific binding.

Figure 5.3 The displacement of $[^{3}H]$DAMGO by BU19 and BU20 in SH-SY5Y cell membranes.
Figure 5.4 The displacement of $[^3]$H DAMGO by BU21 and BU22 in SH-SY5Y cell membranes.

Figure 5.5 The displacement of $[^3]$H DAMGO by BU23 and BU24 in SH-SY5Y cell membranes.
Figure 5.6 The displacement of [³H]DAMGO by BU25 in SH-SY5Y cell membranes.

Table 5.1 Ki values and Hill coefficients for the displacement of [³H]DAMGO by BU compounds in SH-SY5Y cell membranes. Values represent means ± S.E.M for at least three experiments performed in duplicate. N/A = not applicable, single site competition.
In order to determine if the biphasic nature of the displacement curves obtained was particular to \( \mu \)-receptors in the SH-SY5Y cell line, the displacement of the selective \( \mu \)-ligand \([^3H]DAMGO\) by C-CAM, the cyclopropylmethyl ether, BU21, and the isopropyl ether, BU25, was investigated in guinea-pig brain membranes (Figs. 5.7 and 5.8). Again a biphasic displacement curve for C-CAM was found, whereas the curves for BU21 and BU25 were best fit to a one-site model. The affinities of these ligands for the \( \mu \)-receptor sites in guinea-pig brain homogenate were similar to the affinities for the low affinity site obtained in SH-SY5Y cell membranes. \( K_i \) values for the displacement of \([^3H]DAMGO\) in guinea-pig brain membranes by C-CAM, BU21, and BU25 are given in Table 5.2. However, the fraction of C-CAM binding to the receptor population in the higher affinity site is considerably lower in guinea-pig membranes (16 \%) as compared to SH-SY5Y cell membranes (47 \%) (Table 5.2).

In membranes prepared from C6 glioma cells expressing only the \( \mu \)-opioid receptor, C-CAM also showed biphasic displacement with approximately 46 \% of the binding to the higher affinity site (Fig. 5.9). Since guinea-pig brain homogenates contain \( \mu \)-, \( \delta \)-, and \( \kappa \)-receptor subtypes, and the SH-SY5Y cell line contains \( \mu \)- and \( \delta \)-receptors, the use of the C6 cell line expressing only the \( \mu \)-receptor eliminated the possibility of an interaction with another opioid receptor type.

![Figure 5.7](Image)

Figure 5.7 The displacement of \([^3H]DAMGO\) by C-CAM and BU25 in guinea-pig brain membranes.
Figure 5.8 The displacement of $[^3]H$DAMGO by BU21 in guinea-pig brain membranes.

<table>
<thead>
<tr>
<th></th>
<th>$K_i$ 1 (nM)</th>
<th>$K_i$ 2 (nM)</th>
<th>Fraction site 1</th>
<th>Fraction site 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU18</td>
<td>0.0125 ± 0.009</td>
<td>2.6 ± 0.4</td>
<td>0.16</td>
<td>0.84</td>
</tr>
<tr>
<td>BU21</td>
<td>N/A</td>
<td>15.0 ± 1.3</td>
<td>N/A</td>
<td>1.0</td>
</tr>
<tr>
<td>BU25</td>
<td>N/A</td>
<td>9.3 ± 1.9</td>
<td>N/A</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 5.2 $K_i$ values for the displacement of $[^3]H$DAMGO by C-CAM (BU18), BU21 and BU25 in guinea-pig brain membranes.

N/A = not applicable, single site competition.

In order to determine if the biphasic curves were a result of a single binding site existing in multiple affinity states, for example, coupled and uncoupled forms of the receptor, the binding was determined in the presence of NaCl and the GTP analogue GppNHp. This serves to uncouple the G-protein resulting in only a low affinity state of the receptor. To test this the displacement of $[^3]H$diprenorphine by C-CAM was
investigated in membranes prepared from C6 glioma cells (Fig. 5.9). Diprenorphine is an antagonist at the μ-receptor and possesses equal affinity for both high and low affinity sites. Under both conditions C-CAM still recognized two components of binding, and there was no difference in $K_i$ values in the presence or absence of Na$^+$ ions confirming the antagonist action of C-CAM.

![Graph showing the displacement of $[^3]$H]diprenorphine by C-CAM in membranes prepared from C6 cells expressing the cloned μ-opioid receptor, with and without the presence of NaCl and the GTP analogue, GppNHp present in the binding buffer.](image)

Figure 5.9 The displacement of $[^3]$H]diprenorphine by C-CAM in membranes prepared from C6 cells expressing the cloned μ-opioid receptor, with and without the presence of NaCl and the GTP analogue, GppNHp present in the binding buffer.

A time-course of the displacement of $[^3]$H]DAMGO in SH-SY5Y cell membranes was undertaken to determine if the biphasic nature of the curves varied with incubation time. For this study C-CAM was used and compared to BU25 (Fig. 5.10). Membranes were incubated with drug and radioligand for 1, 2, and 3 hr. Variation of incubation time did not affect the displacement curve of BU25, however a flattening of the displacement curve was observed with time for C-CAM. After a 3 hr incubation period C-CAM at a concentration of 3 pM was able to displace 61 % of specific $[^3]$H]DAMGO binding, compared with 14 % after 1 hr. The $K_i$ values for the displacement of $[^3]$H]DAMGO by C-CAM from the higher affinity site increased with time, changing from 4.1 ± 1.0 pM after a 1 hr incubation to 0.52 ± 0.09 pM after a 3
hr incubation. The fraction of binding to the higher affinity site also increased with time, changing from 47 % to 74 % and 78 % for 2 hr and 3 hr incubation times respectively.

![Graph showing displacement of [3H]DAMGO by C-CAM and BU25 in SH-SY5Y cell membranes after different periods of incubation.]

Figure 5.10 The displacement of [3H]DAMGO by C-CAM and BU25 in SH-SY5Y cell membranes after different periods of incubation.

A number of studies have shown that changes in membrane microviscosity result in profound effects on opioid receptor binding [Creese et al., 1975, Remmers & Medzihradsky, 1991, Zhang & Yang, 1989]. The displacement of [3H]diprenorphine by C-CAM and BU25 in C6μ cell membranes was carried out at 37°C for an incubation time of 1 hr, and at 4°C for an incubation time of 4 hr (Fig. 5.11), in order to promote or hinder access to receptor binding sites by increasing or decreasing membrane fluidity. It should also be noted that an increase or decrease in incubation temperature can simply speed up, or slow down, the attainment of equilibrium in binding. Neither temperature change affected the nature of the displacement curve for C-CAM, although incubation at 37°C increased the fraction of the binding to the higher affinity site to 87 %, from 46 % at 25°C for 1 hr, and also significantly
increased the affinity for this site ten-fold (Kᵢ = 0.4 ± 0.1 pM, P = 0.02, Students’ unpaired t-test). There was no change in fraction of binding to the higher affinity site after incubation at 4°C (45 %), nor was there a significant change in affinity (Kᵢ = 4.8 ± 4.4 pM). The binding of the isopropyl ether BU25 was unaffected by changes in incubation temperature (data not shown).

Figure 5.11 The displacement of [³H]diprenorphine by C-CAM in C6µ cell membranes. Incubation temperatures of 4°C for 4 hr and 25°C or 37°C for 1 hr were used.

It has been shown previously that a µ-opioid receptor population is resistant to alkylation by the irreversible ligand β-funaltrexamine (β-FNA) (Elliott et al., 1994). Fig. 5.12 shows the results from a wash-resistance study, where pre-treatment of C6µ cells with 100 nM β-FNA resulted in a partial knock-out of receptor number. The percentage of receptors still able to bind [³H]DAMGO following β-FNA treatment was 42.6 ± 8.0 %, confirming the results of Elliott et al. To examine the possibility that the β-FNA-resistant population was related to one of the sites recognized by C-CAM and its 3-alkyl ethers, binding studies using C-CAM were carried out in C6µ cells which had been pre-treated with this maximal dose (100 nM) of β-FNA (Fig.
5.13. C-CAM once again exhibited a biphasic displacement curve indicating that the β-FNA-resistant population is not identical to one of the sites recognized by C-CAM.

Figure 5.12 Wash-resistant binding of β-FNA to the µ-receptor in C6 glioma cells, as determined by a reduction in specific binding of [3H]DAMGO. Cells were incubated for 1 hr at 37°C with vehicle control or drug and then washed 5 times prior to membrane preparation. Values represent mean ± S.E.M for four experiments carried out in duplicate.

Figure 5.13 The displacement of [3H]DAMGO by C-CAM in C6µ cell membranes before and after treatment of the cells with 100 nM β-FNA.
C-CAM is a long-lasting 'pseudoirreversible' antagonist for the µ-opioid receptor, as is MC-CAM. The unusual binding profile of these compounds may therefore relate to their ability to form tight hydrophobic interactions with the µ-receptor over time. If this is indeed the case, then one might expect BU25 to be a fully reversible since it does not show the same binding pattern as the other compounds in this series. The ability of BU25 to bind in a wash-resistant manner to the µ-opioid receptor expressed in C6 glioma cells was examined and compared to C-CAM and MC-CAM (Fig. 5.14). Cells were incubated for 1 hr with drug and subsequently washed 5 times to remove any unbound drug prior to membrane preparation. As can be seen pre-treatment of cells with 100 nM C-CAM and MC-CAM resulted in a complete block of [³H]DAMGO binding. In contrast, BU25 pre-treatment, up to a concentration of 100 nM, did not affect the binding of [³H]DAMGO to the µ-receptor.

![Graph showing wash resistant binding of C-CAM, MC-CAM and BU25 to C6µ cells](image)

**Figure 5.14** Wash resistant binding of C-CAM, MC-CAM and BU25 to C6µ cells, as determined by reduction in specific [³H]DAMGO binding to cell membranes. Cells were incubated for 1 hr at 37°C with either vehicle control or drug and then were washed 5 times before membrane preparation.
5.2.2 \([^{35}S]GTP\gamma S\) binding assays

The 3-alkyl ethers were tested up to a concentration of 1 \(\mu M\) in the \([^{35}S]GTP\gamma S\) assay. The compounds varied in efficacy ranging from antagonist, (C-CAM and the isopropyl ether BU25), to partial agonist, giving up to 50% of the fentanyl response (Figs. 5.15 – 5.19). \(EC_{50}\)'s and maximal stimulation relative to that produced by 10 \(\mu M\) fentanyl are given in Table 5.3. The rank order of potency of the ethers as obtained from the \([^{35}S]GTP\gamma S\) assay was as follows: methyl (BU28, MC-CAM) > allyl (BU19) > propargyl (BU23) > nitrile (BU20) >> ester (BU24) > \(n\) propyl (BU22) > CPM (BU21).

<table>
<thead>
<tr>
<th></th>
<th>(EC_{50}) (nM)</th>
<th>% Maximal Stimulation (relative to fentanyl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU18 (C-CAM)</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>BU19</td>
<td>0.10 ± 0.03 nM</td>
<td>50.6 ± 4.2</td>
</tr>
<tr>
<td>BU20</td>
<td>5.93 ± 1.98</td>
<td>15.7 ± 6.3</td>
</tr>
<tr>
<td>BU21</td>
<td>158.0 ± 58.1</td>
<td>32.8 ± 1.2</td>
</tr>
<tr>
<td>BU22</td>
<td>111.4 ± 80.7</td>
<td>41.0 ± 7.0</td>
</tr>
<tr>
<td>BU23</td>
<td>1.18 ± 0.87</td>
<td>32.2 ± 4.4</td>
</tr>
<tr>
<td>BU24</td>
<td>72.7 ± 68.8</td>
<td>13.8 ± 2.8</td>
</tr>
<tr>
<td>BU25</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>BU28 (MC-CAM)</td>
<td>0.03 ± 0.02</td>
<td>21.9 ± 3.4</td>
</tr>
<tr>
<td>DAMGO</td>
<td>10.2*</td>
<td>97.0*</td>
</tr>
</tbody>
</table>

Table 5.3 \(EC_{50}\) and \% maximum stimulation values for the stimulation of \([^{35}S]GTP\gamma S\) binding by the BU compounds. Values represent mean ± S.E.M. of at least three experiments performed in duplicate. * = mean of one experiment performed in duplicate. N/A = not applicable, zero stimulation measured.
Figure 5.15 Stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding by C-CAM in SH-SY5Y cell membranes relative to the full $\mu$-agonist fentanyl. Values for this and subsequent figures represent the mean $\pm$ S.E.M for at least three experiments performed in duplicate.

Figure 5.16 Stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding by BU19 and BU20 in SH-SY5Y cell membranes.
Figure 5.17 Stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding by BU21 and BU22 in SH-SY5Y cell membranes.

Figure 5.18 Stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding by BU23 and BU24 in membranes prepared from SH-SY5Y cells.
Figure 5.19 Stimulation of [35S]GTPγS binding by BU25 and BU28 (MC-CAM) in SH-SY5Y cell membranes.
5.3 Discussion

The results clearly demonstrate that all of the tested 3-alkyl ether 14-cinnamoylamino compounds bind to the \( \mu \)-opioid receptor in cultured cells and in guinea-pig brain with high affinity. This correlates well with the data of Husbands et al., 1998, who reported on the binding affinities of the 3-alkyl ethers to rat brain membranes. However, with the exception of the iso-propyl ether, BU25, all of the compounds in this series showed biphasic displacement of \( ^{3}\text{H}] \text{DAMGO} \) from membranes prepared from SH-SY5Y human neuroblastoma cells. C-CAM was also confirmed to show biphasic displacement in C6\( \mu \) cell membranes. Husbands et al. did not report biphasic curves. Interestingly, in guinea-pig brain membranes the fraction of binding to the high affinity site was much lower than in SH-SY5Y and C6 glioma cell membranes, suggesting that the degree of biphasic binding observed is dependent on the system used. It is possible that in brain homogenate the time of access to the receptor site is longer. Additionally, the displacement curve for BU21 was monophasic in guinea-pig brain homogenates. This may explain why the previous studies of Husbands et al. did not uncover this biphasic binding in rat brain membranes. There are at least three possible explanations for the biphasic binding curves seen for these ligands: a) the compounds recognize a single site on the receptor existing in both high and low affinity states, b) the compounds are binding to two distinct sites of the \( \mu \)-opioid receptor or differentially to the same receptor site, or c) the compounds recognize different opioid receptors. Experiments in C6 glioma cells expressing only the \( \mu \)-opioid receptor enable us to eliminate the possibility of one of the sites being an interaction with another (\( \delta \)- or \( \kappa \)-) opioid receptor.

To test the hypothesis that the biphasic binding was a result of the ability of the drugs to distinguish between high and low affinity states of the receptor, competition binding assays were carried out using a C6 glioma cell line transfected with the cloned \( \mu \)-opioid receptor, in the presence of NaCl and the GTP analogue, GppNHp. Addition of NaCl and guanine nucleotides serves to uncouple the receptor from G-protein hence promoting an inactive, low affinity state of the receptor and abolishing high affinity binding. Clearly from the results obtained the higher affinity binding component has been unaffected. Moreover, \( ^{3}\text{H}] \text{diprenorphine} \) and C-CAM, the
ligands used in this assay, are both antagonists at the \( \mu \)-receptor and so should recognize coupled and uncoupled states of the receptor with equal affinity.

One feasible explanation is the presence of subtypes of the \( \mu \)-receptor. This concept of multiple subtypes has been of interest to many researchers for several years. Evidence from binding [Wolozin & Pasternak, 1981, Nishimura et al., 1984] and autoradiographic studies [Goodman & Pasternak, 1986] suggest that opiates and enkephalins bind to a common high affinity \( '\mu_1' \) site, and that the opiates (but not the enkephalins) also bind to a lower affinity \( '\mu_2' \) site. \textit{In vivo} studies also support the presence of multiple subtypes of the \( \mu \)-receptor. Naloxonazine and naloxazone, which irreversibly bind to the high affinity \( \mu_1 \) site [Pasternak \textit{et al.}, 1980], have proved useful tools not only in binding experiments but in determining the specific pharmacological actions mediated by \( \mu_1 \) and \( \mu_2 \) subtypes \textit{in vivo} [Ling & Pasternak, 1983, Heyman \textit{et al.}, 1988]. It has been shown that naloxonazine selectively antagonizes supraspinal morphine analgesia without affecting spinal morphine analgesia [Ling & Pasternak, 1983]. Additionally, naloxonazine does not antagonize the respiratory depressant actions of morphine [Ling \textit{et al.}, 1985]. The authors ruled out the involvement of \( \delta \)-mechanism and concluded that respiratory depression was mediated \textit{via} the \( \mu_2 \) sub-population of receptors. Bare \textit{et al.}, 1994, reported that a splice variant of the human \( \mu \)-receptor exists. This form of the receptor differs at the C-terminus and is eight amino-acids shorter than the originally established structure of the \( \mu \)-receptor. Hollt and his colleagues also reported the on the cloning and expression of an isoform of the rat \( \mu \)-receptor [Zimprich \textit{et al.}, 1995], and have shown more recently that the distribution of these isoforms of the \( \mu \)-receptor is very different, suggesting cell-specific receptor expression [Schulz \textit{et al.}, 1998]. Splice variants therefore could represent \( \mu \)-receptor subtypes. However it is unlikely that subtypes exist in the C6 glioma cell line transfected with a single cDNA. Therefore it would also seem unlikely that the 3-alkyl ethers are binding to two distinct structurally different subtypes of the \( \mu \)-receptor if biphasic curves are observed in membranes prepared from the C6\( \mu \) cell line. Caveat that \( \mu \)-receptor subtypes could exist due to post-translational processing or coupling to different effector systems.

If the biphasic binding curves do not arise as a result of a single binding site existing in multiple affinity states, and are probably not due to the existence of
multiple μ-receptor subtypes, this leaves us with the possibility that the alkyl ethers have different μ-receptor binding modes. If this is the case, an important question is why the iso-propyl ether BU25 does not exhibit biphasic binding. This could be due to the fact that BU25 is a pure antagonist. However, this would also hold true for C-CAM, yet C-CAM does exhibit biphasic binding. One possible explanation may be that one of the sites recognized by the other compounds in this series is a second site on the same receptor protein, probably that which is responsible for 'pseudoirreversible' binding. Indeed wash-resistance studies confirm that BU25 does not ‘pseudoirreversibly’ bind to the receptor in C6 cells, while C-CAM and MC-CAM do bind in a wash-resistant manner.

Preliminary molecular modeling studies have shown that all of the 3-alkyl substituents have similar molecular volumes. However, the steric hindrance caused by a branched chain substituent, such as the isopropyl group of BU25, may preclude access to the relevant site on the receptor. Displacement assays were carried out at different temperatures in order to increase or decrease membrane fluidity [Remmers & Medzihradsky, 1991, Creese et al., 1975, Zhang & Yang, 1989], in order to examine if this had any effect on access of either C-CAM or BU25 to the receptor sites. BU25 was unaffected by temperature changes, however the fraction of binding to, and the affinity of, C-CAM for the higher affinity binding site increased when experiments were carried out at 37°C. This would suggest a greater ease of access of C-CAM to the higher affinity site with increased membrane fluidity. It may also be that the binding to the higher affinity site takes a longer time to reach equilibrium, in which case an increase in temperature results in quicker attainment of that equilibrium. This may also be an important point when considering why there is less binding to the higher affinity site in guinea-pig brain membranes, as access to the higher affinity site may be more hindered in this preparation.

Further evidence for irreversible interactions of C-CAM (and presumably the other 3-alkyl ethers of C-CAM) comes from a time course study of the displacement of [3H]DAMGO from the μ-receptor in SH-SY5Y cell membranes by C-CAM and BU25. Whilst the increase in incubation time has no effect on the displacement of [3H]DAMGO by BU25, C-CAM becomes increasingly more effective at displacing [3H]DAMGO from the receptor with time, presumably as a tighter interaction with the μ-opioid receptor develops (Fig. 5.20).
As can also be seen from the results of the wash resistance study, the alkylating agent β-FNA does not appear to irreversibly bind to all of the receptors. This confirms the findings of Elliott et al., 1994, who proposed that there is a population of the μ-receptor resistant to alkylation by this ligand. In order to examine if the site available to β-FNA alkylation was the same site of the receptor for 'pseudoirreversible' binding of the ethers, the displacement of [3H]DAMGO by C-CAM was carried out after cells had been exposed to 100 nM β-FNA for 1 hr. However the biphasic displacement was still not resolved, indicating that the β-FNA-resistant population still contained both sites available to C-CAM binding.

A study by Klein & Nelson, 1992, has shown that a series of 3-O-methacrylate ethers derived from a number of opioids (naltrindole, naltrexone, oxymorphone, etorphine, diprenorphine, and nor-binaltorphimine) undergo substantial O-dealkylation to give the parent phenol, accounting for the activity of the ethers observed in radioligand binding assays. The authors also tested a saturated ether under the same conditions, and showed that it did not possess high affinity for any of the opioid receptors, presumably due to the inability of the ether to undergo dealkylation. It is highly unlikely however, that the 3-alkyl ethers of this series could undergo nucleophilic attack in the same manner as the 3-O-methacrylate ethers described by Klein & Nelson. It would also be unlikely that O-dealkylation can explain the observation of two-site binding as C-CAM itself, which contains the 3-OH group, exhibits biphasic binding.

Results from [35S]GTPγS assays show that the ethers have limited efficacy. The rank order of potency as determined by this assay is slightly different from the order obtained from radioligand binding studies, although the ester, CPM, and n propyl ethers share the lowest potency in the [35S]GTPγS assay and for both binding sites in the radioligand binding studies. The propargyl ether BU23 appears to have reduced potency for the second, lower affinity binding site yet has high potency for the first binding site and exhibits high potency in the [35S]GTPγS assay. C-CAM and BU25 do not stimulate the binding of [35S]GTPγS above basal levels, and BU20 and BU24 are of very weak agonist character. BU19 is the most efficacious of the series. The overall [35S]GTPγS data obtained correlates with the data of Husbands et al., 1998, who found that BU19, BU20, and BU23 were the most potent in the mouse warm water tail withdrawal assay. The data of Husbands et al. also shows that BU19 and
BU23 are the most efficacious. MC-CAM is a weak partial agonist in the [35S]GTPγS assay, affording only 22% of the maximal stimulation produced by 10 μM fentanyl. This is consistent with previous findings in vivo, where it has been shown that MC-CAM is of weak partial agonist nature [Woods et al., 1995, Aceto et al., 1989, Butelman et al., 1996]. MC-CAM is fully effective in the mouse phenylquinone writhing assay but inactive in the mouse tail flick and hot plate tests [Aceto et al., 1989], and it produces analgesia in the tail withdrawal assay at 50°C, but not 55°C, in rhesus monkeys [Woods et al., 1995]. We can therefore conclude that modification of the substituent in the 3-position can have a definite effect on the efficacy of the compound, but does not interfere with binding.

From the above experiments it is clear that the 3-alkyl ethers of C-CAM display intriguing binding characteristics, which may provide us with useful information to ascertain the mechanisms behind the long-lasting μ-receptor antagonism produced by these ligands. Modification of the 3-substituent appears not only to change the efficacy of the compound, but, as is the case with BU25, changing the substituent has also had a profound effect on its binding profile relative to the other 3-alkyl ethers of C-CAM in this series. Compounds like this with somewhat higher efficacy may provide us with useful treatment drugs based upon our hypothesis that a sufficient degree of μ-efficacy followed by a long-lasting μ-antagonism, probably attributed to an irreversible binding interaction such as with buprenorphine, confer upon the compound suitable characteristics for use as a pharmacotherapy for the treatment of opioid abuse.
Figure 5.20  Schematic representation of C-CAM binding to the mu receptor. DAMGO binds to sites A and B with equal affinity. Sites A and B could represent two points of attachment for the drug molecule in the same binding pocket of the receptor. C-CAM binds to both sites A and B, but has a preference for site A, and exerts a non-competitive interaction with this site. C-CAM exerts a competitive interaction with site B, resulting in two possible outcomes: 1) DAMGO may remain bound, or 2) C-CAM displaces DAMGO and if able to access site A it again undergoes a non-competitive interaction. Hence with time C-CAM irreversibly binds to more receptors thus reducing the receptor number available for DAMGO binding.
CHAPTER 6
CONSTITUTIVE ACTIVITY AT $\mu$- AND $\delta$-OPIOID RECEPTORS AND THE IDENTIFICATION OF NOVEL INVERSE AGONISTS
6.1 Introduction

Opioid receptors belong to the superfamily of seven transmembrane domain, G-protein coupled receptors. In this family of receptors signal transduction is achieved by agonist occupation of receptors activating one or more G-proteins (See Chapter 1, section 1.3). It is now widely accepted that G-protein coupled receptors can exist in equilibrium between an active and an inactive state of the receptor [Leff, 1995]. Even in the absence of agonist, these receptors can maintain an active conformation and display constitutive activity by interacting with the G-protein [Costa & Herz, 1989, Lefkowitz et al., 1993] (Fig. 6.1). Ligands that preferentially stabilize the inactive form of the receptor abolish this spontaneous, agonist-independent activity and are termed inverse agonists. Neutral antagonists bind to the receptor but do not alter the equilibrium between active and inactive states of the receptor. The only known opioid receptor inverse agonists are the δ-selective peptides ICI 174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH) [Costa & Herz, 1989] and the structurally related ICI 154,129 (N, N-Bisallyl-Tyr-Gly-Gly-ψ-(CH₂S)-Phe-Leu-OH) [Shaw et al., 1982] and diallyl-G (N, N-Diallyl-Tyr-D-Leu-Gly-Tyr-Leu-OH) [Georgoussi & Zoidrou, 1993]. No non-peptide δ- inverse agonists have been identified to date. Moreover, no ligand has been discovered to have inverse agonist activity at the μ- or κ-opioid receptor, although Wang et al. [1994], have proposed that naloxone exhibits negative intrinsic activity in SH-SY5Y cells under conditions of narcotic tolerance. The observation that clocinnamox (C-CAM, see Chapter 5) gave a dose-dependent reduction in [³⁵S]GTPγS binding in rat C6 glioma cells transfected with the cloned rat δ-receptor led us to investigate constitutive activity and inverse agonism in this cell line and also in C6 glioma cells transfected with rat cloned µ-opioid receptor. To study constitutive activity we have used the agonist-independent stimulation of [³⁵S]GTPγS binding in the absence of sodium ions to provide a higher level of spontaneous activity [Costa & Herz, 1989, Szekeres & Traynor, 1997]. The findings confirm constitutive activity associated with the cloned δ-receptor and identify C-CAM and 7-benzylidenenaltrexone (BNTX), a δ₁-receptor antagonist [Portoghese et al., 1992], as two novel non-peptide inverse agonists. Under similar conditions the µ-receptor exhibits limited constitutive activity.
Figure 6.1 The two-state model according to Leff [1995]. The receptor exists in equilibrium between inactive (R) and active (R*) states. The equilibrium constant, L, determines the distribution of receptors between the two states in the absence of agonist. Interaction of an agonist, A, will shift the equilibrium in favour of one of the two states. If it has higher affinity for R*, the ligand will be an agonist, but if it has higher affinity for R, the resting state, it will be an inverse agonist.
6.2 Results

6.2.1 Saturation binding of $[^3H]$diprenorphine to C6 glioma cell membranes

Saturation binding experiments were carried out using $[^3H]$diprenorphine in order to determine the receptor number in C6δ and C6μ cells. Bmax values and dissociation constants are given in Table 6.1 and representative saturation binding plots are shown in Figs. 6.2 and 6.3.

<table>
<thead>
<tr>
<th></th>
<th>Bmax (fmols mg$^{-1}$ protein)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6δ</td>
<td>728 ± 18</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>C6μ LOW</td>
<td>420 ± 94</td>
<td>0.24 ± 0.11</td>
</tr>
<tr>
<td>C6μ HIGH</td>
<td>1733 ± 83</td>
<td>0.18 ± 0.10</td>
</tr>
</tbody>
</table>

Table 6.1 Bmax and $K_D$ values obtained from the saturation binding of $[^3H]$diprenorphine to membranes prepared from C6δ cells and C6μ cells expressing both high and low receptor number. Values represent mean ± S.E.M for three experiments carried out in duplicate.

Figure 6.2 Representative graph showing saturation binding of $[^3H]$diprenorphine to C6δ cell membranes. Bmax = 732 fmols mg$^{-1}$ protein and $K_D$ for diprenorphine = 0.39 nM.
Figure 6.3 Representative graphs showing saturation binding of $[^3]$H]diprenorphine to C6µ cell membranes (A) = with lower receptor number, (B) = with higher receptor number. $B_{\text{max}} = 306$ and $1650$ fmols mg$^{-1}$ protein respectively, and $K_D$ values for diprenorphine = 0.13 and 0.28 nM respectively.
6.2.2 Constitutive activity of cloned opioid receptors expressed in C6 glioma cells.

In the presence of Na⁺ ions the basal binding of [³⁵S]GTPγS to membranes from C6δ cells was 41.3 ± 1.8 fmols mg⁻¹ protein (Fig. 6.4). This increased to 72.3 ± 9.5 fmols mg⁻¹ protein when Na⁺ ions were replaced with K⁺ ions. Basal binding of [³⁵S]GTPγS to C6μ cell membranes was also increased from 22.4 ± 0.1 to 37.1 ± 1.8 fmols mg⁻¹ protein.

Treatment of C6δ cells with pertussis toxin (PTX) reduced basal levels of [³⁵S]GTPγS binding by 51.9 ± 6.1 fmols mg⁻¹ protein to 20.4 ± 3.9 fmols mg⁻¹ protein confirming the presence of constitutively active receptors activating G₁/G₀ proteins (Fig. 6.5). In contrast PTX treatment reduced basal [³⁵S]GTPγS binding in C6 wild-type cells by only 8.6 ± 3.1 fmols mg⁻¹ protein, to 17.30 ± 1.70 fmols mg⁻¹ protein. A similar low reduction was observed in C6μ cells, regardless of receptor expression level. In the clone expressing a receptor number of 427 fmols mg⁻¹ protein, PTX treatment reduced the basal [³⁵S]GTPγS binding by 10.0 ± 3.5 fmols mg⁻¹ protein, and
in the clone expressing the receptor number of 1703 fmols mg\(^{-1}\) protein the reduction in basal binding was 14.6 ± 3.3 fmols mg\(^{-1}\) protein (Fig. 6.5). The reduction in basal binding in both C6 clones was not statistically significantly different from the reduction in wild-type C6 cells (Students’ unpaired t-test). One-way ANOVA to compare basal [\(^{35}\)S]GTP\(_{\gamma}\)S binding in C6\(\mu\) cells of low and high receptor expression number following PTX treatment also showed no statistically significant differences between the means of each clone.

Figure 6.5 Basal [\(^{35}\)S]GTP\(_{\gamma}\)S binding in membranes prepared from C6 glioma cells pre-treated with pertussis toxin (100 ng ml\(^{-1}\), 24 hr). Values represent the mean ± S.E.M. of at least three experiments performed in duplicate. ** \(P < 0.01\), significantly different reduction of basal binding compared to that seen in C6 wild-type cells, Students’ unpaired t-test.
6.2.3 Identification of non-peptide inverse agonists at the δ-receptor

The previously reported peptide inverse agonist ICI 174,864 inhibited basal \([^{35}S]GTP\gamma S\) binding by \(11.3 \pm 0.3 \text{ fmols mg}^{-1} \text{ protein}\) at a concentration of \(1 \mu M\) (Fig. 6.6).

![Graph](image)

Figure 6.6 Inhibition of \([^{35}S]GTP\gamma S\) binding by various antagonists at the cloned δ-opioid receptor in membranes prepared from C6δ cells. Values represent mean ± S.E.M. for at least three experiments performed in duplicate.

Investigation of the antagonist nature of various compounds revealed that 7-benzylidenanltrexone, (BNTX), a δ₁ receptor antagonist, and the 14-cinnamoylamino morphinone clocinnamox, (C-CAM), were able to inhibit the basal binding of \([^{35}S]GTP\gamma S\) in C6δ cell membranes by \(10.0 \pm 3.1\) and \(20.3 \pm 3.4 \text{ fmols mg}^{-1} \text{ protein}\) respectively. The partial µ-agonist buprenorphine, the δ-selective antagonist naltrindole, and the non-selective antagonist naloxone all acted as neutral antagonists in this system (Fig. 6.6).
Figure 6.7 Dose-dependent inhibition of $[^{35}S]$GTPγS binding by the peptide ICI 174,864 in membranes prepared from C6δ cells. All values represent mean ± S.E.M. for at least three experiments performed in duplicate.

Figure 6.8 Dose-dependent inhibition of $[^{35}S]$GTPγS binding by C-CAM in membranes prepared from C6δ cells. Values represent mean ± S.E.M from at least three experiments carried out in duplicate.
Figure 6.9 Dose-dependent inhibition of $[^{35}\text{S}]$GTPγS binding by BNTX in membranes prepared from C6δ cells. Values represent mean ± S.E.M. for at least three experiments carried out in duplicate.

Figure 6.10 Inhibition of $[^{35}\text{S}]$GTPγS binding by BNTX, C-CAM and ICI 174,864 (all 1 μM) in C6δ, C6μ and C6 wild-type membranes. Values represent mean ± S.E.M. for at least three experiments performed in duplicate. **P < 0.01, *P < 0.05, comparing inverse agonists in C6δ to C6WT and C6μ, one way analysis of variance.
Figure 6.11 Reversal of inverse agonist activity by the \( \delta \)-neutral antagonist naltrindole (10 \( \mu \)M) in C6\( \delta \) cell membranes. Values represent mean ± S.E.M for at three experiments performed in duplicate. * \( P < 0.05 \), Students’ unpaired t-test.

No inhibition of basal \( ^{35}S \)GTP\( \gamma \)S binding was observed in membranes prepared from wild-type C6 cells or C6\( \mu \) cells (Fig. 6.10). Concurrent addition of the \( \delta \)-opioid neutral antagonist naltrindole blocked the inverse agonist response of BNTX, C-CAM, and ICI 174,864 (all 1 \( \mu \)M) in C6\( \delta \) cell membranes (Fig. 6.11). Basal \( ^{35}S \)GTP\( \gamma \)S binding values in the presence of naltrindole were 2.7 ± 2.4, -2.0 ± 4.0, and 2.0 ± 2.1 fmols mg\(^{-1} \) protein for BNTX, C-CAM, and ICI 174,864 respectively.

It is well documented that the addition of NaCl and guanine nucleotides to binding assay buffer serves to uncouple the G-protein from the receptor resulting in a low affinity form of the receptor [Pasternak et al., 1975, Childers & Snyder, 1980]. In this study the \( K_i \) values for the inhibition of \( ^{3}H \)naltrindole binding to C6\( \delta \) cell membranes were obtained for ICI 174, 864, C-CAM, and BNTX with and without NaCl and GppNHp, a GTP analogue, present in the binding buffer (Table 6.1). To calculate the affinities a \( K_D \) of 0.12 nM for \( ^{3}H \)naltrindole was used (A. Alt, our laboratory, personal communication). Addition of NaCl and GppNHp afforded a 7-
fold leftward shift in the competition curve was observed for ICI 174,864 (Fig. 6.12). As seen in Chapter 5 with studies at the µ-receptor, C-CAM also displayed a biphasic binding profile for the displacement of [³H]naltrindole from C6δ cell membranes. Addition of NaCl and GppNHp afforded a 5-fold leftward shift of the lower affinity site of the competition curve (Fig. 6.13), but the leftward shift of the higher affinity site was much less pronounced (1.4 fold), and was not significant. The $K_i$ value for the inhibition of [³H]naltrindole binding by BNTX was unchanged (Fig. 6.14).

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_i$ (nM) in Tris Buffer</th>
<th>$K_i$ (nM) in Tris Buffer + NaCl + GppNHp</th>
<th>$K_{i_L} / K_{i_H}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICI 174,864</td>
<td>266 ± 9.0</td>
<td>37.0 ± 9.9</td>
<td>7.2</td>
</tr>
<tr>
<td>C-CAM</td>
<td>0.37 ± 0.28 pM</td>
<td>0.26 ± 0.13 pM</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>1.3 ± 0.05</td>
<td>0.25 ± 0.10</td>
<td>5.2</td>
</tr>
<tr>
<td>BNTX</td>
<td>1.6 ± 0.3</td>
<td>2.1 ± 1.0</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Table 6.2 $K_i$ values for the inhibition of [³H]naltrindole binding (0.2 nM) to C6δ cell membranes, with and without the addition of NaCl and the non-hydrolyzable GTP analogue, GppNHp to the assay buffer.
Figure 6.12 The displacement of \(^{3}\text{H}n\text{altrindole} (0.2 \text{ nM})\) by ICI 174, 864 in C6\(\delta\) cell membranes, with and without NaCl and GppNHp in binding buffer. Values represent mean ± S.E.M of three experiments performed in duplicate.

Figure 6.13 The displacement of \(^{3}\text{H}n\text{altrindole} by C-CAM in C6\(\delta\) cell membranes, with or without NaCl and GppNHp to the assay buffer. Values represent mean ± S.E.M for three experiments performed in duplicate.
Figure 6.14 The displacement of $[^3]$Hnaltrindole by BNTX in C6δ cell membranes, with and without NaCl and GppNHp in the assay buffer. Values represent mean ± S.E.M for at least three experiments performed in duplicate.
6.3 Discussion

Previous literature suggests that there is a degree of constitutive activity associated with the δ-opioid receptor, due to the ability of the peptide ICI 174,864 to inhibit basal GTPase activity [Costa & Herz, 1989, Mullaney et al., 1996] and enhance forskolin-stimulated cAMP accumulation [Chiu et al., 1996]. However, tools to investigate constitutive activity at δ-opioid receptors are scarce and as yet there have been no reports of similar findings with the μ-opioid receptor. An initial observation in our laboratory that clocinnamox (C-CAM) (See Chapter 5) appeared to exhibit negative intrinsic activity at the cloned δ-receptor, but not at the cloned μ-receptor, in rat C6 glioma cells led us to evaluate the level of constitutive activity associated with these receptors and compare it to the level of constitutive activity in wild-type rat C6 glioma cells. In order to improve chances of observing constitutive activity experiments were carried out in GTPγS binding buffer containing K+ ions instead of Na+ ions. It has been shown previously [Costa & Herz, 1989] that the inverse agonist effect of ICI 174,864 on GTPase activity is much more pronounced in buffer in which the Na+ ions had been replaced by K+. Constitutive activity occurs when receptors exist in the active (R*) state, and therefore can couple to G-proteins in the absence of agonist. Thus, because Na+ reduces basal [35S]GTPγS binding by lowering the affinity of 'empty' opioid receptors for G-protein, it serves to reduce the constitutive activity of the receptor and therefore masks any observable inverse agonist activity. Substitution of Na+ ions with K+ ions thus provides a higher level of spontaneous G-protein coupling [Costa & Herz, 1989, Szekeres & Traynor, 1997], hence enabling the observation of any reduction in agonist-independent receptor activation.

Treatment of C6 wild-type cells with pertussis toxin (PTX) alone reduced basal binding of [35S]GTPγS suggesting a tonic level of G-protein activation. However in C6δ cells the reduction in basal levels by PTX was much more pronounced confirming an increased level of constitutive activity associated with the δ-receptor. In contrast C6 cells expressing the μ-opioid receptor showed a similar small reduction of basal [35S]GTPγS binding by PTX as seen with wild-type cells, suggesting a lack of constitutive activity associated with the μ-opioid receptor. A high receptor expression level is thought to lead to constitutive activity [Lefkowitz et al., 1993, Samama et al., 1993] due to enhanced collision probability of R* with G [Stickle & Barber, 1992].
However, the lack of constitutive activity observed at the μ-receptor does not appear to be due to receptor expression level, as similar levels of basal \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding were seen with C6μ cells expressing a higher receptor number. The PTX-induced reduction in basal binding in wild-type and C6μ cells must be due to the presence of other non-opioid \(G_\alpha\) coupled receptors.

In addition to ICI 174,864, the irreversible μ-antagonist clocinnamox (C-CAM) and the δ-selective antagonist 7-benzylidenenaltrexone (BNTX) also acted as inverse agonists in C6δ cell membranes affording dose-dependent reduction in \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding. The dose-response curve for C-CAM was shallow which may reflect its binding to a higher affinity site of the receptor as observed in competition binding assays at the δ-receptor expressed in C6 glioma cells, and the μ-opioid receptor in SH-SY5Y cell membranes, C6μ cell membranes, and guinea-pig brain membranes (see discussion in chapter 5). The inverse agonist activity of these compounds was not observed in wild-type or C6μ cell membranes, suggesting that the response was mediated via the δ-opioid receptor. This was supported by the reversal of inverse agonist activity by the δ-antagonist naltrindole.

None of the inverse agonists were able to inhibit basal \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding to the same extent as pre-treatment of cells with pertussis toxin. This is due to the presence of non-opioid constitutively active receptors, as seen by PTX-reduction of activity in the wild-type cells, but also suggests that the present compounds can only partially reverse constitutive activity at the δ-receptor, i.e. they are partial inverse agonists. Szekeres & Traynor [1997] reported similar findings using ICI 174,864 on membranes prepared from NG108-15 neuroblastoma x glioma hybrid cells. However, Mullaney et al. [1996] reported that in rat 1 fibroblasts expressing the cloned mouse δ receptor, ICI 174,864 did inhibit basal binding of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) to the same extent as pre-treatment of cells with pertussis toxin. The authors concluded that ICI 174,864 was an inverse agonist of high negative intrinsic efficacy.

According to the two state model of receptor activation, [Leff, 1995, Fig. 6.1], inverse agonists preferentially stabilise the inactive conformation of the receptor. Therefore, one would predict that these compounds would have a higher affinity for the uncoupled state of the receptor. It was hypothesised that BNTX, C-CAM, and ICI 174,864 would have higher affinity for the δ-receptor when uncoupled from G-
protein. Competition binding assays were performed with the addition of NaCl and the non-hydrolyzable GTP analogue, GppNHp, to the buffer. The addition of Na\(^+\) ions and guanine nucleotides alters the equilibrium between R and R\(^*\) towards the inactive form of the receptor (R). Thus the affinity of an agonist for the receptor is decreased whereas the affinity of an antagonist is unchanged, and if our hypothesis is correct, the affinity of an inverse agonist would be increased. This held true for both C-CAM and ICI 174,864. Indeed Appelmans et al. [1986], have shown that the affinity of ICI 174,864 for the δ-opioid binding site is increased 8 – 16 fold upon addition of 25 mM NaCl to the reaction buffer. However, the affinity of BNTX for the receptor did not change. This suggests that the ability of BNTX to inhibit \(^{35}\)S\text{GTPyS}\ binding does not relate to its affinity for the δ-receptor under conditions promoting coupled (active) and uncoupled (inactive) forms of the receptor. Additional evidence for this comes from other studies using dopaminergic systems, where it was found that several inverse agonists at the D\(_3\) receptor, including haloperidol, bound with the same affinity to untreated and PTX treated D\(_3\) receptors [Malmberg et al., 1998]. Together these findings question the hypothesis that inverse agonism arises due to greater affinity of a ligand for uncoupled (R), as opposed to coupled (R\(^*\)), forms of the receptor.

Studies of inverse agonism at the δ-receptor have also been extended to include whole cell preparations. Merkouris et al. [1997], presented some interesting data concerning the effects of ICI 174,864 on cAMP accumulation in intact rat-1 fibroblasts. Prior treatment of cells with PTX inhibited, but did not mimic, the effects of ICI 174,864 on the enhancement of forskolin stimulated cAMP. Similar results were reported by Chiu et al. [1996] using human embryonic kidney cells expressing the cloned δ-receptor. If the receptor exists in a constitutively active, coupled state, then one would expect that forskolin is having to work against a tonic inhibition of cAMP accumulation. Therefore PTX treatment should abolish this tonic inhibition, thus allowing an apparent greater stimulation of cAMP by forskolin. However this was not seen. This again questions the hypothesis that constitutive activity is dependent upon an active (coupled) form of the receptor, and, together with the binding data for BNTX and the study using haloperidol, this suggests that perhaps other mechanisms are in place, for example tonic activation arising from the G-protein itself.
It would be of considerable interest to examine more closely the possible physiological consequences of administration of these opioid inverse agonists *in vivo*. C-CAM has been well characterised as a long-lasting 'pseudoirreversible' opioid antagonist devoid of agonist properties *in vivo* [Comer *et al.*, 1992, Zernig *et al.*, 1994, 1996]. However Zernig *et al.* [1995], reported that C-CAM caused hyperalgesia in mice in the acetic acid-induced writhing assay. If C-CAM were able to produce an inverse agonist effect *in vivo*, one might expect hyperalgesia to occur. Administration of C-CAM would be expected to shift the equilibrium of the δ-opioid receptors to an inactive state hence reducing the tonic level of G-protein activation, thereby reducing the baseline level of δ-receptor mediated analgesia.

The therapeutic benefit of inverse agonists is largely unknown. Speculation arises as to whether inverse agonists may be of use in the treatment of certain diseased states occurring from constitutively active receptors as a result of point mutations in the genome [Milligan *et al.*, 1995, Spiegel *et al.*, 1993]. For example precocious puberty has been shown to occur as a result of constitutive activation of adenylate cyclase by the luteinizing hormone receptor, and mutations of the thyrotropin receptor are thought to be responsible for hyperfunctioning thyroid adenomas. To date there have been no reports of disease states arising from mutations of opioid receptors, therefore it would seem that inverse agonists at opioid receptors would have no clinical utility. However, the presence, or lack of, constitutive activity at opioid receptors and the characterisation of opioid inverse agonists warrants further investigation in order to further understand the physiological relevance of tonic opioid receptor/G-protein interactions.

The results shown indicate that constitutive activity within the opioid receptor family may be dependent upon receptor subtype. The lack of constitutive activity at the μ-opioid receptor, even under optimum conditions of replacing Na⁺ ions with K⁺ ions, is intriguing but caution must be exercised as experiments have only been carried out on the δ-receptor expressed in C6 glioma cells, albeit at two different expression levels. However, it may explain why, when so many μ-ligands are known of other chemical types, including the competitive antagonists such as naltrexone, naloxone, cyprodime, CTOP, CTAP etc, that no μ-inverse agonists have been discovered to date.
CHAPTER 7
OVERVIEW
OVERVIEW

The advent of buprenorphine has provided researchers with a useful lead to develop novel pharmacotherapies for the treatment of opioid abuse. The mixed agonist/antagonist actions of buprenorphine, together with its ability to attenuate the effects of any subsequently self-administered opioid, appear to offer significant advantages over currently existing therapies such as the full $\mu$-agonists methadone and LAAM. More recently, there have been preliminary reports that the long-lasting 14-cinnamoylamino codeinone, methoclocinnamox (MC-CAM), may be useful as a treatment drug. In a manner similar to buprenorphine, MC-CAM exhibits initial agonist activity followed by a long-lasting antagonism of morphine.

The characteristic bell-shaped dose-response curve produced by buprenorphine *in vivo* is probably an important contributing factor to the overall safety profile of the drug. An investigation into the antinociceptive actions of buprenorphine using the rat warm water tail withdrawal assay at 50°C revealed that the unique bell-shaped dose-response curve of buprenorphine is time-dependent. Higher doses of buprenorphine show a rapid peak of near-maximal antinociception within 15 min of administration. This is followed by an equally rapid decline in antinociceptive effect. In contrast lower doses show a slow onset and offset of antinociceptive action. Therefore if the antinociceptive measure is recorded 15 min after administration of buprenorphine, a bell-shaped dose-response curve is not observed. The possibility of receptor desensitization following administration of higher doses of buprenorphine was examined, but the results obtained suggest this not to be the case. Although the subsequent actions of buprenorphine were blocked following pre-treatment for 1.5 hr with a high (1.0 mg kg$^{-1}$) dose of buprenorphine, the antinociceptive effect of the full $\mu$-agonist fentanyl was unchanged under similar conditions. Since buprenorphine binds very tightly to the $\mu$-opioid receptor, this may reflect different binding requirements of fentanyl and buprenorphine for the $\mu$-receptor, and indeed there is evidence to support this [Heerding *et al*., 1994]. It is clear from the data obtained that the mechanism behind the bell-shaped dose-response is of great complexity. The inability of naltrexone to shift both the ascending and descending phases of the curve contradicts previous literature reports, however this may be explained in terms of cumulative dosing paradigms in the current investigation. In order to attempt to
explain the mechanism behind the unique biphasic dose-response curve, a model has been proposed whereby buprenorphine initially binds to the μ-opioid receptor producing agonist actions, and then over time is able to form a tight lipophilic interaction with the receptor, thus inducing an antagonist conformation and inhibiting further agonist activation. Indeed this would agree with evidence that approximately 50% of buprenorphine still remains bound to the receptor [Boas & Villiger, 1985].

Although buprenorphine is the most promising alternative to methadone or LAAM for the treatment of heroin abuse, the degree of reinforcing effect may not be sufficient enough to maintain the most experienced of addicts in a treatment program. The morphinan-pyrrolidine derivative, BU72, appears to offer that quality. In radioligand binding assays using C6 glioma cells transfected with the μ-opioid receptor, BU72 displayed high affinity for the μ-receptor and was a full agonist in the \[^{35}S\]GTP\gammaS assay affording 116% of the fentanyl response. In vivo BU72 produced dose-dependent antinociception in the mouse warm water tail-withdrawal assay and was approximately 400 times more potent than morphine. Similar results were obtained in the acetic-acid induced writhing assay. Unlike buprenorphine however, the agonist effects of BU72 were readily reversed by the non-selective antagonists naloxone and naltrexone. Administration of a high dose of BU72 afforded long-lasting antinociception after which time antagonism of morphine was observed in both tail withdrawal and writhing assays. In the writhing assay a maximal dose of morphine was still fully antagonized after 6 days. Thus, BU72 is a highly efficacious μ-opioid agonist with subsequent antagonist properties and as such represents an important lead for the development of therapies for the treatment of opiate abuse.

The reports that MC-CAM may also be useful as a treatment drug has led to the development of 3-alkyl ethers of MC-CAM. It has been shown that variation of the substituent of the 3-position offers a wider range of efficacies without altering the long-lasting antagonist properties of this series of compounds. This was confirmed in \[^{35}S\]GTP\gammaS assays using membranes prepared from SH-SY5Y human neuroblastoma cells. An investigation into the binding profile of the 3-alkyl ethers using SH-SY5Y and C6μ cells revealed that with the exception of the iso-propyl ether BU25, all of these drugs bound to the μ-opioid receptor in a biphasic manner. Experiments carried out to determine the mechanism behind this unusual phenomenon showed that the biphasic binding was unaffected by the addition of NaCl to the assay buffer, hence the
two components of binding did not represent a single receptor site existing in high and low affinity states. Pre-treatment of cells with the alkylating agent β-FNA also did not change the binding profile, suggesting that the population of cells previously shown to be resistant to β-FNA alkylation still contained both sites available to C-CAM binding. The degree of biphasic binding appeared to be system dependent, and incubation for longer periods of time or at an elevated temperature increased the fraction of binding to the higher affinity site. It is suggested that the biphasic binding exhibited by the series of 3-alkyl ethers represents two components of binding to the μ-opioid receptor; a reversible, lower affinity component and an irreversible, higher affinity component. The steric hindrance caused by the bulky iso-propyl substituent of BU25 may prevent it accessing the higher affinity site and thus this compound shows monophasic binding. Clearly modification of the 3-substituent has a profound effect on the overall pharmacological profile of these compounds.

The morphinone derivative of MC-CAM, clocinnamox (C-CAM), is an antagonist devoid of agonist activity. It too displays biphasic binding not only at the μ-receptor, but also at the δ-opioid receptor. Surprisingly, studies of [35S]GTPγS binding in C6μ and C6δ cells revealed that C-CAM acted as an inverse agonist at the δ-, but not the μ-receptor. This prompted an investigation into constitutive activity at these opioid receptors, where it was discovered that constitutive activity was present at the δ-receptor, but not at the μ-receptor. This may explain why, to date, no inverse agonists have been shown for the μ-receptor. Moreover it was discovered that the δ1 antagonist 7-benzylidenenaltrexone (BNTX) also acted as an inverse agonist at the δ-receptor in C6 glioma cells. Together with C-CAM these are the first non-peptide δ-receptor inverse agonists to be found.

In conclusion, the characterization of buprenorphine and other long-lasting opiates has provided a useful lead to the development of alternative treatment drugs. The morphinan pyrrolidine derivative BU72 appears to be a useful candidate for further evaluation. Studies of MC-CAM and its derivatives have revealed a unique binding profile related to their ability to form long-lasting interactions with the μ-receptor. Although the exact mechanisms behind the ‘pseudoirreversible’ interactions of buprenorphine and the 14-cinnamoylamino compounds have not been fully elucidated, it is clear that this aspect of their pharmacology is of great importance in their profile as a potential treatment drug.


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