Characterisation of \( \mu, \kappa \) and \( \delta \) opioid receptor selective antagonists by \textit{in vitro} radioligand binding assays

Ph.D. Thesis
by
Árpád Márki

Szeged
2002
## CONTENTS

1. INTRODUCTION .................................................................................. 2
   1.1. OPIOID RECEPTORS ....................................................................... 2
       1.1.1. Mu receptors ........................................................................ 2
       1.1.2. Kappa receptors .................................................................. 4
       1.1.3. Delta receptors ................................................................... 5
   1.2. MOLECULAR CLONING OF OPIOID RECEPTORS ..................... 5
   1.3. OPIOID LIGANDS .......................................................................... 6
       1.3.1. Norbinaltorphimine ............................................................. 6
       1.3.2. Cyprodime .......................................................................... 6
       1.3.3. β-Funaltrexamine ............................................................... 6
       1.3.4. Nepenthone and thevinone derivatives .............................. 6

2. AIMS .................................................................................................. 6

3. METHODS .......................................................................................... 6
   3.1. CHEMICALS .................................................................................. 6
   3.2. ANIMALS ....................................................................................... 6
   3.3. MEMBRANE PREPARATION ....................................................... 6
   3.4. RADIOLIGAND BINDING ASSAYS ............................................. 6
       3.4.1. Association and dissociation studies ................................. 6
       3.4.2. Saturation binding experiments ....................................... 6
       3.4.3. Competition experiments ............................................... 6
   3.5. [(35)S]GTPyS BINDING ............................................................... 6
   3.6. DATA ANALYSIS ......................................................................... 6

4. RESULTS ............................................................................................. 6
   4.1. NORBINALTORPHIMINE ............................................................ 6
   4.2. CYPRODIME ................................................................................ 6
       4.2.1. Unlabelled cyprodime ....................................................... 6
       4.2.2. [3H]cyprodime .................................................................. 6
       4.2.3. [(35)S]GTPyS binding ...................................................... 6
   4.3. β-FUNALTREXAMINE ANALOGUES ........................................ 6
   4.4. NEPENTHONE AND THEVINONE DERIVATIVES ..................... 6

5. DISCUSSION ....................................................................................... 6
   5.1. NORBINALTORPHIMINE ........................................................... 6
   5.2. CYPRODIME ................................................................................ 6
   5.3. β-FUNALTREXAMINE ANALOGUES ........................................ 6
   5.4. NEPENTHONE AND THEVINONE DERIVATIVES ..................... 6

6. CONCLUSIONS .................................................................................... 6

7. ACKNOWLEDGEMENTS ...................................................................... 6

8. LIST OF ABBREVIATIONS .................................................................. 6

9. REFERENCES ....................................................................................... 6

10. LIST OF PUBLICATIONS ................................................................. 6
    10.2. ABSTRACTS IN REFERRED JOURNALS ................................ 6

APPENDIX .............................................................................................. 6
1. INTRODUCTION

1.1. Opioid receptors

There is convincing evidence for three major classes of opioid receptors in the central nervous system (Martin et al., 1976, Gilbert et al., 1976), designated μ, κ and δ, as well as indications of subtypes within each class. Most endogenous opioids and synthetic ligands do not possess absolute specificity for a given receptor type, but interact with more than one opioid receptor. The situation is further complicated by the fact that multiple receptor types may coexist within a single tissue, or even a cell (Borsodi, 1991). Receptor binding studies reveal distinct selectivity profiles for each class, whereas functional studies have established their unique pharmacological profiles (Pasternak, 1993) (Table I).

1.1.1. Mu receptors

Mu receptors initially were defined by their affinity for morphine (Martin et al., 1976). Several groups have identified endogenous morphine in the brain, raising the possibility that it may be the natural ligand for this site (for review see Benyhe, 1994 and Stefano et al., 2000). Endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂, EM-1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH₂, EM-2) are peptides recently isolated from mammalian brain that show the highest affinity and selectivity for the μ (morphine) opiate receptor of all the known endogenous opioids (Zadina et al., 1997). The endomorphins have potent analgesic and gastrointestinal effects. They activate G-proteins and inhibit adenylyl cyclase in both cellular and animal models. Both tetrapeptides activate G-proteins and inhibit adenylyl cyclase activity in membrane preparations from cells stably expressing the μ opioid receptor, an effect reversed by the μ receptor antagonist CTAP, but they have no influence on cells stably expressing the δ opioid receptor (Monory et al., 2000). Support for their role as endogenous ligands for the μ-opioid receptor includes their localisation by radioimmunoassay and immunocytochemistry in central nervous system regions of high μ receptor density. Intense EM-2 immunoreactivity is present in the terminal regions of primary afferent neurones in the dorsal horn of the spinal cord and in the medulla near high densities of μ receptors. Chemical (capsaicin) and surgical (rhizotomy) disruption of nociceptive primary afferent depletes the immunoreactivity, implicating the primary afferents as the source of EM-2. Thus, EM-2 is well-positioned to serve as an endogenous modulator of pain in its earliest stages of perception.
Table I. Classification of opioid receptor subtypes and actions from animal models. Table was taken from Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th edition, Edited by J.G. Hardman and L.E. Limbird

In contrast to EM-2, which is more prevalent in the spinal cord and lower brainstem, EM-1 is more widely and densely distributed throughout the brain. The distribution is consistent with a role for the peptides in the modulation of diverse functions, including autonomic, neuroendocrine, and reward functions as well as modulation of responses to pain and stress (for review see Zadina et al., 1999). β-Endorphin has also high affinity for μ receptors, which also have high affinity for the enkephalin. Dynorphin A also binds to μ receptors potently, but not as potently as to κ₁ receptors. Although highly selective agonists have been developed for μ receptors (DAMGO, sufentanil, methadone), antagonists (CTOP, CTAP, cyprodime, naloxonazine) have been most useful in defining the pharmacological actions of μ receptors. β-Funaltrexamine (β-FNA) irreversibly blocks μ receptors (Portoghese et al., 1980, Recht and
Pasternak, 1987), while naloxonazine selectively antagonises \( \mu \) receptors (Pasternak et al., 1980, Hahn et al., 1982, Ling et al., 1986, Pick et al., 1991). Using these antagonists, investigators have established in animal models that morphine can elicit analgesia either spinally (\( \mu_2 \)) or supraspinally (\( \mu_4 \)).

The \( \mu \) receptors are thought to mediate the opiate phenomena classically associated with morphine, including analgesia, opiate dependence, cardiovascular and respiratory functions, and several neuroendocrine effects (Table I.).

### 1.1.2. Kappa receptors

Several \( \kappa \) receptor subtypes have been proposed from results of binding assays and pharmacological studies. \( \kappa_1 \) Receptors are characterised by selective labelling with the agonist U50,488 (Kosterlicz et al., 1981) and antagonised by norbinaltorphimine (norBNI) (Takemori et al., 1988). Administration of U50,488 elicits analgesia in animal model which can not be antagonised by antagonists selective for \( \mu \) or \( \delta \) receptors. The U50,488H-insensitive \( \kappa_2 \) receptor was proposed from binding studies (Zukin et al., 1988, Benyhe et al., 1990), but their pharmacological properties remain unknown. The other U50,488H-insensitive binding site - the so called \( \kappa_3 \) receptor - also were first identified in binding studies using the novel opiate derivative, NalBzoH (Clark et al., 1989, Price et al., 1989, Gistrak et al., 1989, Paul et al., 1990), but its pharmacological properties are moderately well established. Unlike \( \kappa_1 \) receptors, which produce analgesia spinally, \( \kappa_3 \) receptors relieve pain through supraspinal mechanism. Although effects mediated by \( \kappa_3 \) receptors are readily reversed by a number of opioid antagonists, no \( \kappa_3 \)-selective antagonists have been identified. Evidence now indicates that nalorphine elicits its analgesic responses through \( \kappa_3 \) receptors (Paul et al., 1991) thus \( \kappa_3 \) receptors correspond to Martin's nalorphine (N) receptors (Paul et al., 1991).

The \( \kappa \) receptors mediate a spectrum of unique and distinctive functions, including the modulation of drinking, water balance, food intake, gut motility, temperature control, and various endocrine functions (Table I.). The binding profile of the \( \kappa \) receptor is relatively unique among the opioid receptors, while those of the \( \mu \) and \( \delta \) receptors are more similar to each other.
1.1.3. Delta receptors

Enkephalins are the endogenous ligands for δ receptors. Our understanding of δ receptor pharmacology has relied heavily on the development of highly selective agonists e.g. deltorphin II (Erspamer et al., 1989), DPDPE (Mosberg et al., 1983, Delay-Gojet et al., 1985), DSLET (David et al., 1982) and antagonists, such as naltrindole (Portoghese et al., 1988). Using these drugs, investigators have established δ analgesia both spinally and supraspinally, although the spinal system appears to be more robust. Two subclasses, δ₁- and δ₂-opioid receptors, have been proposed based on their differential sensitivity to blockade by several novel antagonists (Sofuoglu et al., 1991 and 1993). The agonist [D-Pro²,Glu⁴]deltorphin and DSLET preferentially bind to δ₂ receptors, whereas DPDPE has higher affinity for δ₁ receptors (Mattia et al., 1991 and 1992, Jiang et al., 1991).

The δ receptors are thought to mediate analgesia, gastrointestinal motility, as well as a number of hormonal functions (Table I.).

1.2. Molecular cloning of opioid receptors

Members of each class of opioid receptor have been cloned from animal and human (Knapp et al., 1994, Manson et al., 1994, Wang et al., 1994) cDNA and their predicted amino acid sequence obtained. The first opioid receptor to be cloned was the δ-opioid receptor (Kieffer et al., 1992, Evans et al., 1992), which was identified from cDNA libraries derived from NG-108-15 neuroblastoma glyome hybrid cells by expression cloning. The μ-opioid receptor was cloned using probes against putative conserved transmembrane regions of the δ-opioid receptor to screen a rat brain cDNA library (Chen et al., 1993). Yasuda and colleagues cloned the κ- as well as the δ-opioid receptors by screening a mouse brain cDNA library with probes selective for the cloned somatostatin receptors (Yasuda et al., 1993). All the opioid receptors have the putative structure of seven transmembrane domains, extracellular N terminus with multiple glycosylation sites, third intracellular loop with multiple amphiphatic α-helices, and fourth intracellular loop formed by putative palmitoylation sites at the carboxyl tails (Fig. 1.). The amino acid sequences of opioid receptors are approximately 65% identical to each other. The regions of highest similarity in sequence are the sequences predicted to lie in the seven transmembrane-spanning regions (73-76%) and the intracellular loops (86-100%). Regions of amino acid sequence divergence are the amino (9-10%) and carboxy (14-20%) termini and the second and third extracellular loops (14-72%). The extracellular regions
that differ in amino acid sequence may contain the unique ligand binding domains of each receptor, whereas the different intracellular domains may be involved in their differential regulation and may contribute to variations in their coupling to effector systems. The number of multiple receptor subtypes as defined by pharmacological or biochemical binding studies appears to far exceed the number of cloned receptors and their genes. The existence of \( \mu_1 \), \( \mu_2 \), \( \mu_3 \), \( \delta_1 \), \( \delta_2 \), \( \kappa_1 \), \( \kappa_2 \) and \( \kappa_3 \)-opioid receptors has long been postulated. The presence of introns within the receptor genes allows for the generation of splice variants, and probable subtypes of the receptors (Law et al., 2000).

**Fig. 1.** Serpentine model of the \( \kappa \)-opioid receptor. The black lines represent the boundaries of the membrane. Filled circles indicate the residues that are conserved among \( \delta \), \( \kappa \) and \( \mu \) types. The highly conserved cysteine residues of extracellular loop 1 and extracellular loop 2 are shown linked by a disulphide bond. The putative disulphide bridge within the amino terminal extension is indicated between non conserved cysteine residues. Each transmembrane region is indicated with Roman numeral. The Arabic numbers in the membrane denote the position of each residue from the N-terminal end of each region using the generic GPCR numbering scheme. Glycosylation sites on the N-terminus and palmitoylation site on the C-terminus are indicated. IL = intracellular loop, EL = extracellular loop. Figure was taken from CORD Web Site (http://www.opioid.umn.edu/).

Though a splice variant of the \( \mu \)-opioid receptor cDNA was isolated (Zimprich et al., 1995), the pharmacology of this splice variant resembled that of the wild-type MOR. With extensive low-stringency hybridisation procedures, no opioid receptor type other than the cloned \( \mu \)-, \( \delta \)- and \( \kappa \)-opioid receptors could be isolated (Law et al., 2000).
The functional significance of the cloned receptors has been established with antisense approaches in rodent model systems. Using short oligodeoxynucleotides complementary to the mRNA of the various subtypes, it is possible to attenuate selectively the expression of individual subtypes of receptors in vivo as a means to reveal their functional significance (Pasternak, 1996, Lai and Porreca, 1996).

Using the homologous recombination method to disrupt receptor transcription, several groups have successfully generated strains of mice in which the μ- (Matthes et al., 1996, Sora et al., 1997) κ- (Simonin et al., 1998) or δ-opioid (Zhu et al., 1999) receptor was knocked out. Either by radioactive ligand binding studies, immunofluorescence studies, or quantitative autoradiographic studies (Kitchen et al., 1997), these receptor knockout animals exhibited the specific reduction in the receptor protein levels without the alteration of other opioid receptor types. The overall behaviour of the receptor knockout animals remains similar to that of wild-type, with some minor changes (Matthes et al., 1996, Simonin et al., 1998).

The μ, δ and κ receptors in endogenous neuronal settings are coupled to pertussis toxin-sensitive GTP-binding protein (G/Gp) (Fig. 2.) and can regulate the same spectrum of effectors, which include adenylyl cyclase, inwardly rectifying K+ channels (Henry et al., 1995), the N-type (Tallent et al., 1994) and L-type (Piros et al., 1995 and 1996) Ca2+ channels, phospholipase C (Johnson et al., 1994, Spencer et al., 1997) and mitogen-activated protein kinases ERK1 and ERK2 (Li et al., 1996, Fukuda et al., 1996).

The cloned receptors also couple to these same effector systems when expressed in heterologous cells (Kieffer et al., 1992, Fukuda et al., 1993, Meng et al., 1993). The hyperpolarization of the membrane potential by K+ current activation and the limiting of Ca2+ entry by suppression of Ca2+ currents are both tenable mechanisms for explaining opioid blockade of neurotransmitter release and pain transmission in various neuronal pathways.

1.3. Opioid ligands

The most frequently used ligands (naloxone, naltrexone) are potent and universal antagonists; they are capable of antagonising the agonist effects mediated by multiple opioid receptor types. In order to determine the functional correlates of receptor activation, to study interaction of endogenous opioid peptides with opioid receptor types and subtypes and to determine the receptor selectivity of new opioid agonists, selective antagonists must be used. In the last few years highly selective antagonists for delta opioid receptors (e.g. naltirindole...
(NTI), (Portoghese et al., 1988); naltriben, (Portoghese et al., 1991); naltrindole 5'-isothiocyanate, (Portoghese et al., 1990); TIPP (Tyr-Tic-Phe-Phe-OH), (Schiller et al., 1992); TIPP[Ψ] (Tyr-Tic[Ψ]-Phe-Phe-OH), (Schiller et al., 1993); mu (cyprodime, Schmidhammer et al., 1989; CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2), Pelton et al., 1986) and kappa (norbinaltorphimine, Portoghese et al., 1987) opioid receptor have been developed. Some of these compounds became available in radiolabeled form as well NTI (Yamamura et al., 1992, Borsodi et al., 1993), TIPP[Ψ] (Nevin et al., 1994), cyprodime (Ötvös et al., 1992) and norBNI (Márki et al., 2000).

![G protein activation/inactivation cycle](http://www.awlonline.com)

Fig. 2. The G protein activation/inactivation cycle. G protein-linked receptors contain seven transmembrane segments that form a ligand-binding site on the outside of the cell and a G protein-binding site on the inside. (1) When the ligand binds, (2) the receptor activates a G protein by causing the $G_\alpha$ subunit to release GDP and acquire GTP. (3) The $G_\alpha$ and $G_{\beta\gamma}$ subunits then separate and (4) initiate signal transduction events. (5) The GTP-$G_\alpha$ subunit eventually hydrolyses its bound GTP, converting the subunit back to its inactive GDP-$G_\alpha$ form. (6) The inactive GDP-$G_\alpha$ subunit then recombines with $G_{\beta\gamma}$ to form the inactive G heterotrimer. The figure was taken from http://www.awlonline.com

### 1.3.1. Norbinaltorphimine

The first selective κ-opioid receptor antagonist was the β-naltrexamine derivative bivalent ligand, TENA. The term "bivalent ligand" has been given to molecules that contain two recognition units linked through a spacer. It was demonstrated that the potency and selectivity of κ-selective antagonist bivalent ligands is a function of the length and flexibility.
of the spacer (Erez et al., 1982, Portoghese et al., 1986). This was based on that idea that immobilisation of the antagonist’s pharmacophores in an orientation that permits binding to a unique site, that is proximal to the opioid receptor, would confer high selectivity by increasing the affinity of the ligand to a specific opioid receptor type. Norbinaltorphimine (norBNI) (Fig. 3.) is the most potent and selective κ-opioid receptor bivalent ligand - developed by Portoghese and associates (Portoghese et al., 1987) – possesses very high κ-opioid receptor antagonist potency and selectivity both in analgesic assays in mice (Portoghese et al., 1987) and receptor binding studies in guinea pig brain membrane (Takemori et al., 1988).

![Chemical structure of norBNI](image)

NorBNI has been found to selectively block κ mediated antinociception (Czlonkowski et al., 1987) and diuresis (Takemori et al., 1988a). It was found that only one pharmacophor is required for the κ-opioid antagonist selectivity of norBNI. In smooth muscle preparations, the meso isomer of norBNI was more potent than norBNI and about half as selective κ antagonist. Therefore the κ selectivity of norBNI and its meso isomer is derived from the portions of the second halves of these molecules in that they mimic key address components of dynorphin at κ-opioid receptors. This address element conferring selectivity in norBNI has been suggested to be a basic function (N17’) that mimics the guanidinium moiety of Arg⁷ and possibly Arg⁶ in dynorphin (Lin et al., 1993).

NorBNI was radiolabelled with tritium using 1,1’-dibromo-norBNI as leading compound resulting in high specific radioactivity (47.24 Ci/mmol) (Márki et al., 2000).
1.3.2. Cyprodime

Cyprodime (Fig. 4.) has been synthesised and pharmacologically investigated by Schmidhammer and his collaborators (Schmidhammer et al., 1989). The pure and selective \(\mu\)-opioid antagonism of cyprodime was verified \textit{in vitro} in opioid receptor binding assays, in bioassays (GPI, MVD and RVD) and \textit{in vivo} (e.g. acetic acid writhing test in mice, opioid-type withdrawal jumping precipitation test in morphine-dependent mice).

![Fig. 4. Chemical structure of N-cyclopropylmethyl-4,14\(\beta\)-dimethoxy-morphinan-6-one (cyprodime).](image)

Cyprodime showed an antagonist potency of about one-tenth of that of naloxone \textit{in vivo}. In receptor binding, cyprodime exhibited about one half the affinity of naloxone, and in MVD, about one-fortieth of the \(\mu\) potency of naloxone, but in contrast to naloxone, it showed good \(\kappa/\mu\) and especially good \(\delta/\mu\) selectivity ratios. Cyprodime was able to antagonise sufentanil-induced respiratory depression in the dog. It was found that the onset of cyprodime does not exhibit sympathicotonic stimulation as does naloxone. The cyprodime has been radiolabelled in isotope laboratory of Biological Research Centre, Szeged (Ötvős et al., 1992) resulting [1-\(^3\)H]cyprodime with 31.6 Ci/mmol specific activity.

1.3.3. \(\beta\)-Funaltrexamine

\(\beta\)-Funaltrexamine (Fig. 5.), a fumaramate methyl ester of naltrexamine, was synthesised by Portoghese and his co-workers (Portoghese et al., 1980). It was found to have reversible \(\kappa\) agonist and irreversible \(\mu\) antagonist activities \textit{in vivo} and \textit{in vitro} (Takemori et al., 1985).

Binding of \(\beta\)-FNA to opioid receptors in tissue membrane preparations \textit{in vitro} has also been characterised. \(\beta\)-FNA binds to brain \(\mu\), \(\kappa\) and \(\delta\) receptors with IC\(_{50}\) values of 2.2, 14 and 78 nM, respectively (Tam et al., 1986). There is a general agreement in the literature that \(\beta\)-FNA binds reversibly, and not irreversibly, to \(\kappa\)-opioid receptors.
It binds irreversibly to μ-opioid receptors (Recht and Pasternak, 1987) and at low concentration (1-10 nM) [3H]β-FNA covalently labels only μ-opioid binding sites with high specificity (Liu-Chen et al., 1987, 1990, 1991). The antinociceptive actions in the mouse writhing and tail flick tests were of short duration and appeared to be κ receptor mediated (Ward et al., 1982, 1982a).

Fig 6. Structure of 6β-chloroacetamido-4,5α-epoxy-7,8-didehydro-morphinan (13, 14, 16, 18) and 6β-monomethylfumaramido-4,5α-epoxy-7,8-didehydro-morphinan (15) analogues.
Fourteen β-FNA analogues (for structures and analytical data see Simon et al., 1997) were synthesised by Csaba Simon at the ICN Alkaloida Company and five compounds (Fig. 6.) out of them were selected for characterisation in radioligand binding assays.

1.3.4. Nepenthone and thevinone derivatives

In the search for clinically useful narcotic agonists and antagonists a large number of compounds were synthesised in both oripavine and thebain series (Lewis et al., 1971, Michne et al., 1977, Cowan et al., 1977, Richards and Sadee, 1986). The compounds are structurally similar to morphine and dihydromorphine but contain a C_6-C_4 etheno bridge and C_7 substituents. Among other reasons, the series became a focus of attention because some of the compounds had unexpectedly high agonist and antagonist potencies, and these potencies differ between diastereoisomers at C_{19}. Also, some of the relative agonist/antagonist potencies of N-substituted compounds were dramatically sensitive to substitution at C_7 and some of the compounds showed unique pharmacological profiles (Lewis et al., 1973). The nepenthone, thevinone and their derivatives (Fig. 7.) are oripavine and thebaine analogues as diprenorphine and buprenorphine are, and they were planed to be δ-selective opioid ligands.
Fig. 7. Chemical structures of nepenthone, thevinone and their analogues. It is continued on the next page.
2. AIMS

The development of pharmacological agonists and antagonists that can distinguish the opioid receptors has facilitated investigations of their distinct functional roles. Such compounds are necessary, because most tissues express more than one opioid receptor type, which has made it difficult to study each receptor individually to reveal any unique biological actions. This problem has now partly been overcome by the cloning of the three opioid receptor classes, since each receptor can now be expressed in a separate cell line and their pharmacological, functional and biochemical characteristics can be studied independent of the other receptors. In the present work, focused on the opioid receptor antagonists, we demonstrate the characterisation of two newly radiolabelled opioid antagonist, namely norbinaltorphimine (norBNI), a κ opioid receptor selective antagonist and cyprodime, a μ opioid receptor selective antagonist on rat, guinea pig and frog brain membrane preparations. We also present the characterisation of five β-FNA and ten nepenthone/thevinone analogues on rat brain membrane preparation. The following specific aims were set:

1. Characterisation of tritiated norbinaltorphimine.
   a. Determination of optimal receptor binding parameters (time, temperature, buffer, concentration of radioligand) of $[^{3}H]$norBNI on rat, guinea pig and frog brain membrane preparation.
   b. Kinetic investigation of $[^{3}H]$norBNI; determination of equilibrium dissociation constant ($K_d$) based on association rate constant ($k_{+1}$) and dissociation rate constant ($k_{-1}$) on guinea pig brain membrane preparation.
   c. Determination of equilibrium dissociation constant ($K_d$) and maximal number of binding sites ($B_{max}$) of $[^{3}H]$norBNI on rat and guinea pig membrane preparation by saturation binding experiments.
   d. Determination of type and/or subtype selectivity of $[^{3}H]$norBNI on three different tissues using opioid receptor selective ligands.

2. Characterisation of tritiated cyprodime.
   a. Determination of agonist/antagonist character and opioid receptor selectivity of
cyprodime by radioligand binding assay on rat brain membrane.

b. Determination of agonist/antagonist character of cyprodime by $[^{35}\text{S}]{\text{GTP}}\gamma\text{S}$ functional assay

c. Determination of optimal receptor binding parameters of $[^{3}\text{H}]$cyprodime (time, temperature, buffer, concentration of radioligand).

d. Determination of equilibrium dissociation constant ($K_d$) and maximal number of binding sites ($B_{\text{max}}$) of $[^{3}\text{H}]$cyprodime on rat membrane by saturation binding experiments.

e. Investigation of selectivity profile of $[^{3}\text{H}]$cyprodime using $\mu$, $\delta$ and $\kappa$ opioid receptor selective ligands.

3. Determination of agonist/antagonist properties of $\beta$-FNA analogues using $[^{3}\text{H}]$naloxone in presence and absence of 100 mM Na$^+$-ions and investigate their selectivity profile with highly selective tritiated ligands.

4. Characterisation of nepenthone and thevinone derivatives in competition experiments using highly selective tritiated opioid ligands to affirm or reject their $\delta$-selectivity.
3. METHODS

3.1. Chemicals

Unlabelled cyprodime (Schmidhammer, 1989) as synthesised and kindly provided by Prof. Schmidhammer (Innsbruck University). (±)Ethylketocyclazocine methanesulphonate (EKC) was supplied by Sterling Winthrop Research Institute (Rensselaer, NY). Naltrindole (NTI) and norbinaltorphimine were provided by Prof. Portoghese (Portoghese, 1990), [Ile<sup>5,6</sup>]deltorphin II (Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH<sub>2</sub>) (Nevin et al, 1994), TIPP (Tyr-Tic-Phe-Phe-OH) (Nevin et al, 1993) TIPP[Ψ] (Tyr-Tic[Ψ]-Phe-Phe-OH) (Nevin et al, 1994), dihydromorphine (Tóth et al, 1982) and MERF (Tyr-Gly-Gly-Phe-Met-Arg-Phe) (Wolleman et al, 1994) were synthesised in the Isotope Laboratory of Biological Research Center. U69,593 (5α,7α,8β-(−)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-benzeneacetamide) and US0,488 (trans-3,4-dichloro-N-methyl-1-N-1-pyrrolidinyl-(cyclohexyl)-benzeneacetamide) was from the Upjohn Co. Kalamazoo (MI). CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>), DSLET ([D-Ser<sup>2</sup>, Leu<sup>5</sup>, Thr<sup>6</sup>]enkephalin and DAMGO ([D-Ala<sup>2</sup>- (Me)Phe<sup>4</sup>-Gly<sup>1</sup>-ol]enkephalin) were generous gifts of National Institute of Drug Abuse Drug Supply System Rockville, MD. Bacitracin, benzamidine, bestatin, ethylenediaminetetraacetic acid (EDTA), ethylenebis-(oxyethylenenitrilo)-tetraacetic acid (EGTA), phenylmethyl-sulfonyl fluoride (PMSF), soybean trypsin inhibitor, GTP<sub>γ</sub>S, GDP, TRIS and thiorphan were purchased from Sigma Chemicals. Gordox® (aprotinin) and captopril were from Gedeon Richter Ltd (Hungary, Budapest).

<sup>[3]H</sup>Cyprodime (31.6 Ci/mmol) (Ötvös et al., 1992), <sup>[3]H</sup>naloxone (72 Ci/mmol) (Tóth et al., 1982), <sup>[3]H</sup>deltorphin II (20 Ci/mmol) and <sup>[3]H</sup>Ille<sup>5,6</sup>deltorphin II (49.5 Ci/mmol) were synthesised in the Isotope Laboratory of the Biological Research Center. <sup>[3]H</sup>EKC (15 Ci/mmol), <sup>[3]H</sup>U69,593 (43 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA, USA) and <sup>[3]H</sup>DAMGO (59 Ci/mmol) was from Amersham. <sup>[35]S</sup>GTP<sub>γ</sub>S was purchased from Isotope Institute Ltd, Budapest.

3.2. Animals

Frogs (*Rana esculenta*) weighing about 50 - 150 g were captured by Tisza Fishing Co. (Szeged, Hungary) and sacrificed immediately. Rats (PVG/C and Wistar strain, 200-250 g, male) were from our Animal House. Guinea pigs (350-400 g, male) were from the Semmelweis Medical University, Budapest. Rats and guinea pigs were housed in groups of
six under controlled illumination (lights on from 07-19 h), temperature (22-24 °C) and had free access to food and water.

3.3. Membrane preparation

The frog, guinea pig whole brain and rat brain (without cerebellum) membrane fractions were prepared according to Benyhe et al (1992). The brains were removed and then homogenised in twenty volumes (wt/vol) of ice-cold buffer (Tris-HCl 50 mM, pH 7.4) with Braun teflon-glass homogeniser, the suspension was filtered on four layers of gauze and centrifuged with Sorvall RC5C centrifuge with 40,000 x g at 4 °C for 20 minutes. The pellet was resuspended in Tris-HCl buffer and incubated at 37 °C for 30 minutes. The centrifugation step was repeated. The pellet was resuspended in 5x volumes of buffer (50 mM Tris-HCl, 0.32M sucrose, pH 7.4) and stored at -70 °C. Before using, membranes were diluted and centrifuged (40,000 x g, 4 °C, 20 min) and then the pellet was resuspended in 50 ml buffer (Tris-HCl 50 mM, pH 7.4) giving approximately 0.3 mg/ml protein concentration determined by the method of Bradford (19..).

3.4. Radioligand binding assays

The frozen membranes were thawed at room temperature and centrifuged with 40,000 x g, for 20 minutes at 4 °C in Tris-HCl buffer. Ligand binding experiments were carried out in buffer D (1000 ml 50 mM Tris-HCl contained 300 mg ethylenediaminetetraacetic acid (EDTA), 380 mg ethylenesbis-(oxyethylenenitrilo)-tetraacetic acid (EGTA), 20 mg bacitracin, 9 mg bestatin, 2 mg thiorphan, 156 mg benzamidin, 4 mg soybean trypsin inhibitor, 2 mg leupeptin, 2.2 mg captopril, 10⁻⁶ M phenylmethyl-sulfonyl fluoride (PMSF) and 40 KIU/ml aprotinin [Gordox®]; pH 7.4) at the final volume of 1 ml containing 100 mM Na⁺-ions and 0.3 - 0.5 mg protein. The concentration of 0.1 nM tritiated norBNI was used for competition and kinetics assays and 0.01-0.7 nM for saturation experiments. Incubations were started by the addition of membrane suspension and continued in a shaking water bath until steady-state was achieved (25 °C, 60 min). The GF/C filters were pre-treated with 50 mM Tris-HCl buffer containing 10 µM norBNI one hour prior to the assay to decrease binding the radioligand to the filter. In the case of cyprodime, ligand binding experiments were carried out in 50 mM Tris-HCl buffer (pH 7.4) with or without 100 mM NaCl at 25 °C for 40 min. The membranes were incubated with 2 nM (competition experiments) and 0.1 - 20 nM (saturation
experiments) of \[^3H\]cyprodime. The radioligand binding properties such as time and temperature of incubation, type of the glass fibre and the specific activity of the other tritiated ligands are summarised in Table II. When the radioligand was a peptide (\[^3H\]DAMGO, \[^3H\]deltorphin II, \[^3H\]Ile\(^5\)-\[^3H\]deltorphin II, \[^3H\]DPDPE, \[^3H\]DSLET) the buffer D was used during the radioligand binding assays.

<table>
<thead>
<tr>
<th>tritiated ligand</th>
<th>time, [min]</th>
<th>temperature, [°C]</th>
<th>separation filter type</th>
<th>specific activity, [Ci/mmol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>naloxone</td>
<td>60</td>
<td>0</td>
<td>B</td>
<td>35.0</td>
</tr>
<tr>
<td>DAMGO</td>
<td>45</td>
<td>35</td>
<td>C</td>
<td>47.4</td>
</tr>
<tr>
<td>cyprodime</td>
<td>40</td>
<td>25</td>
<td>B</td>
<td>31.6</td>
</tr>
<tr>
<td>deltorphin II</td>
<td>45</td>
<td>35</td>
<td>C</td>
<td>20.0</td>
</tr>
<tr>
<td>Ile(^5)-deltorphin II</td>
<td>45</td>
<td>35</td>
<td>C</td>
<td>49.5</td>
</tr>
<tr>
<td>DPDPE</td>
<td>150</td>
<td>25</td>
<td>B</td>
<td>21.6</td>
</tr>
<tr>
<td>DSLET</td>
<td>45</td>
<td>25</td>
<td>C</td>
<td>42.0</td>
</tr>
<tr>
<td>norBNI</td>
<td>25</td>
<td>60</td>
<td>C</td>
<td>47.2</td>
</tr>
<tr>
<td>EKC</td>
<td>40</td>
<td>24</td>
<td>B</td>
<td>20.8</td>
</tr>
<tr>
<td>U69,593</td>
<td>30</td>
<td>30</td>
<td>B</td>
<td>47.0</td>
</tr>
</tbody>
</table>

Table II. The radioligand binding properties of the tritiated ligands used in radioligand binding assays on guinea pig, rat and frog brain membranes.

The reaction was terminated by rapid filtration on Brandel M24R cell harvester through Whatmann GF/C filters and washed with 3x5 ml of ice-cold Tris-HCl (pH 7.4) buffer. The filters were dried at 37 °C, and the bound radioactivity was determined in a toluene based scintillation cocktail in Wallac 1409 liquid scintillation counter. The total binding was defined as that measured in the absence of a competing agent. Non-specific binding was determined in the presence of 10 µM unlabelled naloxone and was about 40 percent of total binding. The relative affinity (RA) constant values were determined with the following equation:

\[
RA = \frac{(IC_{50,\text{rel}})^{-1}}{(IC_{50,\text{rel}})^{-1} + (IC_{50,\text{rel}})^{-1} + (IC_{50,\text{rel}})^{-1}}
\]
3.4.1. Association and dissociation studies

The association and dissociation kinetics parameters of $[^3H]$norBNI were determined in guinea whole brain membrane preparation. The membrane was incubated with 0.1 nM $[^3H]$norBNI for different time interval (1-110 min). $10^{-5}$ M naloxone was added to the incubation mixture at 60 min inducing dissociation of $[^3H]$norBNI from kappa opioid receptors. The reaction was terminated by rapid filtration on Brandel M24R cell harvester through Whatmann GF/C filters and washed with 3x5 ml of ice-cold Tris-HCl (pH 7.4) buffer. The filters were dried at 37 °C, and the bound radioactivity was determined in a toluene based scintillation cocktail in Wallac 1409 liquid scintillation counter.

The association and dissociation rate constant values were calculated using the following equations:

$$\ln(B_{eq}/(B_{eq} - B_0)) = k_{ob} t$$  \hspace{1cm} (2)

$$k_{ob} = k' + k_1$$  \hspace{1cm} (3)

$$k' = k_1[L]$$  \hspace{1cm} (4)

where $B_{eq}$ is the concentration of bound ligand at equilibrium, $B_0$ is the concentration of bound ligand at any time t, $k_{ob}$ is the observed rate constant, $k_1$ is the association rate constant and the $k_1$ is the dissociation rate constant. The equation (3) gives a straight line when the term $\ln(B_{eq}/(B_{eq} - B_0))$ is plotted against time, and the slope of the line is the experimentally observed apparent rate constant, $k_{ob}$. The $k_1$ value should be determined from a dissociation kinetics study, where (5) gives a straight line when the term $\ln(B/B_0)$ is plotted against time.

$$\ln(B/B_0) = k_1 t$$  \hspace{1cm} (5)

3.4.2. Saturation binding experiments

The saturation binding experiment is an appropriate assay to determine the equilibrium dissociation constant, $K_d$, and the maximal number of binding sites, $B_{max}$, of a radioligand. It was performed by incubating cell membranes with a range of concentrations of tritiated norBNI (0.01 - 0.7 nM) and $[^3H]$cyprodime (0.1 - 20 nM) at 25 °C for 60 and 45 minutes, respectively. The observed total binding consists of specific binding to the receptor itself, plus non-specific binding to non-receptor sites. The non-specific binding was measured with 10 $\mu$M unlabelled naloxon. The specific binding was calculated as a difference between total and non-specific binding, and was plotted in function of the free radioligand concentration. The $K_d$ and $B_{max}$ values were calculated with GraphPad Prism software.
3.4.3. Competition experiments

A radioligand binding assay in which a single concentration of radioligand is used for every assay point is a suitable method to determine the $K_i$ values of the unlabelled molecules. In this experiments the concentration of the $[^3H]norBNI$ was 0.1 nM and tritiaded cyprodime concentration was 2 nM while the concentration of the unlabelled ligands were varied between $10^{-2}$ M and $10^{-12}$ M. The radioligand binding properties of the other tritiated ligands are shown in Table II.

3.5. $[^35S]GTP\gamma S$ binding

For $[^35S]GTP\gamma S$ binding the same membrane preparation was used as in radioligand binding assays. Tubes contained 10 µg of protein, 30 µM GDP, $10^{-10}$-$10^{-5}$ M opioid ligands, and 0.05 nM $[^35S]GTP\gamma S$, all in 50 mM Tris-HCl buffer containing 1 mM EGTA and 3 mM MgCl$_2$ in a final volume of 1 ml. Tubes were incubated for 1 h at 30 °C. Total activity was measured in the absence of tested compounds, non-specific binding was measured in the presence of 100 µM non-labelled GTP\gamma S. The incubation was terminated by filtration the samples through Whatman GF/B glass fibre filters. Filters were washed three times with ice-cold buffer in a Millipore filtration instrument, then dried. Radioactivity was measured in a Wallac 1409 scintillation counter using a toluene based scintillation cocktail. Stimulation is given as percentage of basal specific binding. Data were calculated from three or four separate experiments done in triplicate.

3.6. Data analysis

All assays were carried out at least three times in duplicate, and values are given as means ± SEM. The binding capacity ($B_{max}$) and the equilibrium dissociation constants ($K_i$) of $[^3H]norBNI$ and $[^3H]cyprodime$ were calculated according to Rosenthal (1967). Data of the radioligand and GTP\gamma S binding experiments were analysed using the program LIGAND (Munson and Rodbard, 1980) and GraphPad Prism 2.0 software, utilising a non-linear regression analysis.
4. RESULTS

4.1. Norbinaltorphimine

The kinetic studies were carried on guinea pig whole brain membrane preparation which carries high concentration of \( \kappa \)-opioid receptors. The association \( (k_+\text{)} \) and dissociation \( (k_-\text{)} \) rate constants of \([^3H]\)norBNI were determined at 25°C. The binding reached steady state in 60 min (Fig. 8). The value of 3.28±0.43 sec\(^{-1}\) for \( k_-\) and 1.09±0.30\( \cdot 10^{10} \) sec\(^{-1}\)M\(^{-1}\) for \( k_+\) was calculated. The \( K_d \) was found to be 0.30 nM in this assay. The association binding studies were also carried out on rat and frog brain membranes and steady state was reached in 60 min in both preparations.

![Association and dissociation binding kinetics study of [\(^3H\)]norBNI on guinea pig brain membranes. The membrane was incubated with 0.1 nM [\(^3H\)]norBNI for different time interval (1-110 min). 10\(^{-5}\) M naloxone was added to the incubation mixture at 60 min inducing dissociation of [\(^3H\)]norBNI from kappa opioid receptors (A). The \( k_{ob} \) value was determined from the transformed association binding kinetics curve (B).](image-url)
The results of the saturation experiments on guinea pig and rat brain membrane preparations are shown on Fig. 9. The concentration of radioligand varied between 0.01 and 0.7 nM. The binding was saturable and the Scatchard plot of data suggested single binding site in both tissues.

Fig. 9. Representative saturation binding isotherm and Scatchard plot of [3H]norBNI bound to rat (A) and guinea pig (B) brain membranes, respectively. Specific binding was measured at 11 concentrations (0.01-0.7 nM) using 10 μM naloxone to define non-specific binding.
[\(^{3}\text{H}\)]norBNI labels kappa receptors with high potency in guinea pig (K\(_d\) 0.10±0.015 nM) as well as in rat (K\(_d\) 0.10±0.013 nM) brain membranes. This value is comparable to K\(_d\) value obtained from binding kinetic studies. The maximal number of binding sites are 115.7±6.66 fmol/mg protein in guinea pig and 36.3±0.81 fmol/mg protein in rat brain membranes.

In competition experiments the selectivity of tritiated norBNI was examined with mu, delta and kappa selective unlabelled ligands in frog, guinea pig and rat brain membrane preparations (Table III.).

<table>
<thead>
<tr>
<th>Unlabelled ligands</th>
<th>Rat (K_i) [nM]</th>
<th>Guinea pig (K_i) [nM]</th>
<th>Frog (K_i) [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>norBNI</td>
<td>0.19 ± 0.12</td>
<td>0.22 ± 0.09</td>
<td>13.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>17.1 ± 2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EKC</td>
<td>2.4 ± 2.3</td>
<td>2.9 ± 2.6</td>
<td>41.6 ± 29.7</td>
</tr>
<tr>
<td>dynorphin (1-13)</td>
<td>50.1 ± 30.5</td>
<td>9.4 ± 5.2</td>
<td>11.0 ± 2.5</td>
</tr>
<tr>
<td>U50,488</td>
<td>4.7 ± 2.3</td>
<td>5.4 ± 0.4</td>
<td>246 ± 114</td>
</tr>
<tr>
<td></td>
<td>476 ± 9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MERF</td>
<td>282 ± 77</td>
<td>742 ± 369</td>
<td>499 ± 186</td>
</tr>
<tr>
<td>cyprodime</td>
<td>307 ± 207</td>
<td>4107 ± 6</td>
<td>248 ± 18</td>
</tr>
<tr>
<td>CTAP</td>
<td>1781 ± 992</td>
<td>(\approx) 10000</td>
<td>1521 ± 614</td>
</tr>
<tr>
<td>DAMGO</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
<td>167 ± 40</td>
</tr>
<tr>
<td>naltrindole</td>
<td>61.1 ± 1.7</td>
<td>257 ± 32</td>
<td>13.9 ± 1.2</td>
</tr>
<tr>
<td>Ile(^{A\delta})-deltorphin II</td>
<td>&gt;10000</td>
<td>(\approx) 10000</td>
<td>386 ± 53</td>
</tr>
<tr>
<td>TIPP[(\Psi)]</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
</tbody>
</table>

Table III. [\(^{3}\text{H}\)]norBNI type specificity in rat, guinea pig and frog brain membrane preparations. The brain membranes were incubated with 0.1 nM of [\(^{3}\text{H}\)]norBNI and eleven concentration \((10^{-5}-10^{-12})\) of competing ligands. All assays were carried out at least three times in duplicate, the given values are means ± S.E.M.

All tested kappa ligands (EKC, dynorphin (1-13), U50,488) proved to be good competitors \((K_i: 3-10 \text{nM})\) of [\(^{3}\text{H}\)]norBNI in guinea pig brain membrane except MERF, which inhibited labelled norBNI binding at high nanomolar concentration \((K_i 742 \text{nM})\) while mu and delta specific ligands possessed very low affinities \((K_i \geq 10,000 \text{nM})\). The norBNI and U50,488 recognised two receptor types with high \((K_i 0.31 \text{ and } 4.7 \text{nM})\) and low \((K_i 17.1\text{ and } 476 \text{nM})\) in rat brain. The K\(_i\) value of dynorphin (1-13) were higher in rat than in guinea pig brain, while MERF, cyprodime, CTAP and naltrindole were better competitors of tritiated
norBNI in rat brain compared to guinea pig brain. The kappa ligands, except MERF, displayed lower affinity to norBNI-sensitive kappa receptors in frog brain than in guinea pig brain. The \( K_i \) value of norBNI from homologous displacement studies was found to be lower by two order of magnitude in frog brain compared to rat and guinea pig brain. Most of the delta and mu ligands proved to be better competitors of labelled norBNI in frog than in guinea pig or in rat brain.

4.2. Cyprodime

4.2.1. Unlabelled cyprodime

The selectivity of unlabelled cyprodime was tested in rat brain membranes using highly selective radioligands for each receptor (\( \mu \), \( \delta \) and \( \kappa \)) (Table IV.). The \( \mu \)-opioid receptor selective peptide, \([^3H]DAMGO\), was readily displaced by cyprodime (\( K_i \) value 5.4 nM). Cyprodime showed much less affinity for \( \delta \) binding sites, which were labelled with \([^3H]DPDPE\). More than 40-fold difference was observed in the \( K_i \) values (244.6 for DPDPE vs. 5.4 nM for DAMGO competition) when compared in competing \([^3H]DAMGO\) sites. A similar low affinity (\( K_i \) 213.7 nM) was found when cyprodime competed for the \( \kappa \) binding sites labelled with \([^3H]U69,593\).

Further characteristics of cyprodime in rat brain were investigated, when \([^3H]naloxone\), a general opioid receptor antagonist, was displaced in the absence and presence of 100 mM NaCl. Without the salt, a \( K_i \) value of 14.95±1.01 nM was estimated, which did not change upon addition of NaCl (\( K_i \) value 14.70±1.92 nM). This result is a biochemical proof for the antagonistic property of cyprodime.

<table>
<thead>
<tr>
<th>Tritiated ligands</th>
<th>( K_i ) [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>([^3H]DAMGO) (( \mu ))</td>
<td>5.4 ± 2.4</td>
</tr>
<tr>
<td>([^3H]DPDPE) (( \delta ))</td>
<td>244.6 ± 23.1</td>
</tr>
<tr>
<td>([^3H]U69,593) (( \kappa ))</td>
<td>213.7 ± 42.3</td>
</tr>
</tbody>
</table>

Table IV. Affinity of cyprodime for opioid receptors labelled with different tritiated ligands. Rat and guinea pig brain membranes were incubated with \([^3H]DAMGO\) for 45 min at 35 °C, \([^3H]DPDPE\) for 150 min at 25 °C and \([^3H]U69,593\) for 30 min at 30 °C with eleven concentrations of cyprodime (10\(^{-5}\)-10\(^{-13}\)). Values represent means ± SEM from 3 separate experiments.
4.2.2. $[^3\text{H}]$cyprodime

Binding of $[^3\text{H}]$cyprodime to rat brain membranes reached equilibrium at 25 °C in 40 min and was stable for at least 90 min. In the saturation experiments, a single class of binding site was detected with a $K_d$ value 3.83±0.18 nM. The binding capacity was found to be 87.1±4.83 fmol/mg protein in Wistar rats (Fig. 10.). The same affinity (3.84±0.12 nM) was observed when another strain (PVG/C) was used. However, it is interesting to note that the $B_{\text{max}}$ value was significantly (p<0.01) higher (124±13 fmol/mg protein) in these animals.

![Specific binding](image)

**Fig. 10.** Representative saturation binding isotherm and Scatchard plot (insert) of $[^3\text{H}]$cyprodime bound to rat brain membranes. Specific binding was measured at 12 concentrations (0.1-20 nM) using 10 µM naloxone to define non-specific binding.

Specifically bound $[^3\text{H}]$cyprodime could be displaced from rat brain membranes by unlabelled cyprodime ($K_i$ 8.1 nM) and by the µ-opioid receptor agonists dihydromorphine ($K_i$ 0.4 nM) and DAMGO ($K_i$ 1.1 nM) (Table V.). The µ-opioid receptor selective somatostatin analogue, CTAP, showed less affinity ($K_i$ 43.8 nM). On the other hand, the mixed opioid receptor antagonist, naloxone, exhibited high affinity ($K_i$ 0.9 nM). Only weak interaction with of δ- and κ-opioid receptors was confirmed by the low affinities of the δ- (deltorphin II and Ile$^{5,6}$deltorphin II) and κ- (norbinaltorphimine and U50,466) opioid receptor selective ligands. The highly δ-opioid receptor-specific agonists, deltorphin II and Ile$^{5,6}$deltorphin II exhibited very low affinity ($K_i$ values 1186 nM and 1900 nM, respectively). The δ-opioid receptor specific TIPP was even less potent ($K_i$ 2827 nM). DSLET, the fairly δ-opioid receptor
selective ligand, which shows cross reactivity with µ-opioid receptor (Gacel et al., 1988) competes with relatively high affinity ($K_i$ 9.8 nM) for $[^3]$Hcyprodime. The affinity of the highly selective κ-opioid receptor agonist U50,488 is much lower ($K_i$ 288.1 nM) and the κ-opioid receptor selective antagonist norbinaltorphimine showed a comparable low affinity ($K_i$ 171.6 nM).

<table>
<thead>
<tr>
<th>Competing ligands</th>
<th>$K_i$ [nM] rat brain (Wistar)</th>
<th>$K_i$ [nM] guinea pig brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>dihydromorphine</td>
<td>0.4 ± 0.1</td>
<td>6.0 ± 2.3</td>
</tr>
<tr>
<td>naloxone</td>
<td>0.9 ± 0.03</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>DAMGO</td>
<td>1.1 ± 2.3</td>
<td>2.6 ± 1.9</td>
</tr>
<tr>
<td>cyprodime</td>
<td>8.1 ± 1.8</td>
<td>26.6 ± 3.9</td>
</tr>
<tr>
<td>CTAP</td>
<td>43.8 ± 33.0</td>
<td>48.1 ± 26.9</td>
</tr>
<tr>
<td>DSLET</td>
<td>9.8 ± 2.4</td>
<td>6.3 ± 3.7</td>
</tr>
<tr>
<td>deltorphin II</td>
<td>1186 ± 104</td>
<td>2878 ± 1242</td>
</tr>
<tr>
<td>Ile$^5$6-deltorphin II</td>
<td>1900 ± 98</td>
<td>n.t.</td>
</tr>
<tr>
<td>TIPP</td>
<td>2827 ± 1243</td>
<td>7060 ± 850</td>
</tr>
<tr>
<td>norBNI</td>
<td>171.6 ± 57.0</td>
<td>769.8 ± 321.8</td>
</tr>
<tr>
<td>U50488</td>
<td>288.1 ± 5.5</td>
<td>303.7 ± 112.8</td>
</tr>
<tr>
<td>U69,593</td>
<td>1070 ± 159</td>
<td>52.1 ± 33.2</td>
</tr>
</tbody>
</table>

Table V. Displacement of $[^3]$Hcyprodime by opioid ligands in membranes of rat and guinea-pig brain. n.t. means not tested. Membranes were incubated with 2 nM $[^3]$Hcyprodime for 40 min at 25 °C with eleven concentrations ($10^{-5}$-$10^{-12}$) of each competing ligand. Values represent mean ± SEM from three observations.

In guinea pig brain, all ligands except DSLET, showed higher $K_i$ values than in rat brain. Cyprodime itself had about three times less affinity ($K_i$ 26.6 nM), and DAMGO displayed about two times less affinity ($K_i$ 2.6 nM) than in rat brain. Naloxone still exhibited high affinity ($K_i$ 1.4 nM) whereas dihydromorphine showed somewhat decreased affinity ($K_i$ 6.0 nM). DSLET exhibited about the same affinity in guinea pig ($K_i$ 6.3 nM) as in rat brain ($K_i$ 9.8 nM). The δ-opioid receptor selective agonist deltorphin II showed a $K_i$ value in the micromolar range ($K_i$ 2878 nM) while the δ-opioid receptor antagonist peptide TIPP was even less potent ($K_i$ 7060 nM). The κ-opioid receptor specific ligands displayed higher affinities.
than the δ-opioid receptor ligands, where $K_i$ values for U50,488 and norbinaltorphimine were found to be 303.7 nM and 769.8 nM, respectively.

4.2.3. $[^{35}S]GTP\gamma S$ binding

We first examined the effects of increasing concentrations of cyprodime on $[^{35}S]GTP\gamma S$ binding. Morphine, a potent μ-opioid receptor agonist, was used as a reference compound. The responses in this assay were detected in the concentration range from $10^{-9}$ to $10^{-4}$ M (Fig. 11.). Cyprodime caused a slight, but not significant increase in the incorporation of $[^{35}S]GTP\gamma S$ in rat brain membranes. Thus, the maximal stimulation induced by cyprodime was about 10 % above the basal line, while morphine reached a plateau at 155 %.

![Fig. 11. Effect of different concentrations of morphine (△) and cyprodime (■) on $[^{35}S]GTP\gamma S$ binding to G proteins in crude rat brain membrane preparations. Total binding [without any stimulating agent] is 100%. Data points achieved by the addition of cyprodime do not differ significantly from the basal line. Assay tubes contained 10 µg of protein, 50 pmol $[^{35}S]GTP\gamma S$, 30 µmol GDP, 1 mM EGTA, 3 mM MgCl$_2$ in Tris-HCl buffer, pH 7.4. Incubation was carried out for 60 min at 30 °C. Experiments were done three times in triplicates. Data are mean ± S.E.M.

In further experiments, the effects of two different concentrations of cyprodime were studied (Fig. 12.). Morphine was incubated either with buffer (negative control), 1 µM cyprodime, 10 µM cyprodime or 1 µM naloxone (positive control). When morphine was incubated with buffer alone, an EC$_{50}$ of 244 nM was detected with 155 % maximal stimulation, in agreement with the results of the previous experiment. 1 µM naloxone abolished the stimulating effects of morphine almost completely (EC$_{50}$ ≈ 100 µM). 1 µM
cyprodime had no significant effect on the morphine dose-response curve when incubated at lower concentration (1 μM). However, a higher dose of cyprodime (10 μM) dramatically reduced the stimulatory responses of morphine on [35S]GTPγS binding. At this concentration of cyprodime, the dose-response curve of morphine shifted to the right, revealing a 500-fold increase of the EC$_{50}$.

Fig. 12. Stimulation of [35S]GTPγS binding in crude rat brain membranes by various concentrations of morphine in the presence of 0 [△], 1 [■], 10 [□] μM cyprodime or 1 μM naloxone [★]. Stimulation of [35S]GTPγS (50 pmol) binding to crude rat brain membranes (10 μg/tube). Assays were performed in the presence of 30 μM GDP for 60 min at 30°C. Points represents mean ± S.E.M. from three separate experiments done in triplicates.

4.3. β-Funaltrexamine analogues

A number of β-funaltrexamine analogues (6β-chloroacetamido- and 6β-monomethylfumaramido-4,5α-epoxy-7,8-didehydro-morphinans) were synthesised by Csaba Simon, in the ICN Alkaloida Company Ltd. (Fig. 6.). Five novel compounds (13-16 and 18) were characterised in radioligand binding assays using neuronal membrane fractions prepared from rat brain. Firstly, opioid binding affinities of the compounds were determined by heterologous competition experiments using the general opioid antagonist, [3H]naloxone. These studies also included the estimation of the so-called 'sodium index'. In the presence of 100 mM NaCl the affinity of agonists, but not antagonists, is significantly decreased (Pert et al., 1974). Data obtained in [3H]naloxone displacement studies are shown in Table VI. The compounds have high affinities in competing tritiated naloxone binding, although the IC$_{50}$
values are about one order of magnitude higher than those measured for naltrexone and levorphanol. N17-methyl derivatives (13 and 14) are agonists on the basis of their sodium index, whereas the N17-allyl (16) and N17-propyl (14 and 15) compounds show antagonist properties.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC\textsubscript{50} values [nM]</th>
<th>Na\textsuperscript{+}-index</th>
<th>Suggested agonist/antagonist character</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-\text{Na}^+)</td>
<td>+\text{Na}^+</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>2.1(\pm)0.5</td>
<td>54.5(\pm)10</td>
<td>26</td>
</tr>
<tr>
<td>14</td>
<td>2.1(\pm)0.4</td>
<td>2.0(\pm)0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>15</td>
<td>5.4(\pm)1.1</td>
<td>4.7(\pm)0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>16</td>
<td>1.4(\pm)0.5</td>
<td>2.7(\pm)0.4</td>
<td>1.9</td>
</tr>
<tr>
<td>18</td>
<td>2.8(\pm)0.1</td>
<td>71.2(\pm)14</td>
<td>25</td>
</tr>
<tr>
<td>naltrexone</td>
<td>0.2(\pm)0.05</td>
<td>0.1(\pm)0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>levorphanol</td>
<td>0.5(\pm)0.05</td>
<td>25.3(\pm)8</td>
<td>50</td>
</tr>
</tbody>
</table>

Table VI. Inhibition of \([\textsuperscript{3}H]\)naloxone binding on rat brain membranes by eleven concentration (10\(^{-5}\) to 10\(^{-12}\) M) of unlabelled \(\beta\)-FNA analogues, naltrexone and levorphanol (reference compounds). The sodium index were calculated by the following expression: \(\text{IC}_{50}^{+\text{Na}^+}/\text{IC}_{50}^{-\text{Na}^+}\).

Data from heterologous competition experiments using \(\mu\)-, \(\kappa\)- and \(\delta\)-selective tritiated ligands (\([\textsuperscript{3}H]\)DAMGO for \(\mu\), \([\textsuperscript{3}H]\)DSLET for \(\delta\) and \([\textsuperscript{3}H]\)U69,593 for \(\kappa\) opioid receptors) are listed in Table VII. Relative affinity values, introduced by Kosterlitz and co-workers (Kosterlitz et al., 1980), as a measure of receptor-type selectivity (calculated using Eq. 1.) are also presented. The highest affinities were obtained in \([\textsuperscript{3}H]\)DAMGO binding assays indicating that all ligands bind preferably to \(\mu\)-opioid receptors. At the \(\delta\)-receptor, labelled with \([\textsuperscript{3}H]\)DSLET, significantly lower affinities were found. The good \(\mu/\delta\) selectivity of the compounds is demonstrated by their relative affinity values (Table VII.). Most of the ligands showed only moderate IC\textsubscript{50} values in \([\textsuperscript{3}H]\)U69,593 binding studies, although compound 15 and 16 exhibited quite good affinity, so substantial interaction with the \(\kappa\)-opioid receptors occurred. This is consistent with the data reported by Ward and co-workers (Ward et al., 1982), who described considerable effects mediated by \(\kappa\)-receptors in the case of \(\beta\)-FNA.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>[3H]DAMGO [nM]</th>
<th>[3H]DSLET [nM]</th>
<th>[3H]U69,593 [nM]</th>
<th>μ</th>
<th>δ</th>
<th>κ</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>0.77±0.1</td>
<td>56±11</td>
<td>49±8</td>
<td>97</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>0.99±0.3</td>
<td>53±17</td>
<td>20±5</td>
<td>94</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td>1.60±0.3</td>
<td>96±24</td>
<td>7.9±3</td>
<td>81</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>16</td>
<td>0.89±0.3</td>
<td>35±12</td>
<td>3.3±1</td>
<td>76</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>18</td>
<td>1.48±0.1</td>
<td>170±48</td>
<td>101±25</td>
<td>98</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>β-FNA</td>
<td>2.2a</td>
<td>78a</td>
<td>14.0a</td>
<td>84</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>morphine</td>
<td>2.8±0.5</td>
<td>48±16</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSLET</td>
<td>33±8</td>
<td>5.0±1.9</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U69,593</td>
<td>n.d.</td>
<td>n.d.</td>
<td>2.7±0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table VII. Inhibition of the binding of receptor-type selective radioligands to rat brain membranes by unlabelled β-FNA analogues, morphine, DSLET and U69,593 (reference compounds). Rat brain membranes were incubated with [3H]DAMGO for 45 min at 35 °C, [3H]DSLET for 45 min at 25 °C and [3H]U69,593 for 30 min at 30 °C with eleven concentrations of unlabelled ligands (10⁻¹⁻10⁻¹²). Values represent means ± SEM from 3 separate experiments. aIC₅₀ values were taken from publication of Tarn et al., 1986.

4.4. Nepenthone and thevinone derivatives

First, the delta receptor affinity of the compounds were examined using δ opioid receptor selective peptide radioligand, the tritiated deltorphin II because these compounds were expected to bind preferably to the δ opioid receptors. If the inhibition constant value of a molecule was higher than 100 nM determined in [3H]deltorphin II competition assays, further investigation was not carried out. Only four compounds (6h, 6b, 5o and 5p) showed Kᵢ values greater than 100 nM. In that case, when Kᵢ values were less than 100 nM further displacement studies were performed with [3H]DAMGO and [3H]EKC to specify the selectivity profile of the remaining molecules. The calculated inhibition constant values and the relative affinities (calculated by Eq. 2.) of the tested ligands are listed in Table VIII. The remaining six compounds possess high affinity (Kᵢ 5-60 nM) toward the delta, mu and kappa opioid receptors as well. These ligands did not show any opioid receptor type selectivity except 6c (δ/μ 15.0, κ/μ 5.0) and 11d (δ/μ 17.7, κ/μ 17.0), which are somewhat mu selective ligands.
<table>
<thead>
<tr>
<th>Unlabelled compounds</th>
<th>Kᵢ, [nM]</th>
<th>Relative Affinity, [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[³H]DT II</td>
<td>[³H]DAMGO</td>
</tr>
<tr>
<td>5m</td>
<td>32.5</td>
<td>27.5</td>
</tr>
<tr>
<td>5o</td>
<td>280.5</td>
<td>-</td>
</tr>
<tr>
<td>5p</td>
<td>194.5</td>
<td>-</td>
</tr>
<tr>
<td>6h</td>
<td>262.8</td>
<td>-</td>
</tr>
<tr>
<td>6b</td>
<td>106.4</td>
<td>-</td>
</tr>
<tr>
<td>6c</td>
<td>61.4</td>
<td>4.1</td>
</tr>
<tr>
<td>6i</td>
<td>38.9</td>
<td>7.2</td>
</tr>
<tr>
<td>6v</td>
<td>14.8</td>
<td>1.0</td>
</tr>
<tr>
<td>11d</td>
<td>5.3</td>
<td>0.3</td>
</tr>
<tr>
<td>11c</td>
<td>7.2</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table VIII. Inhibition of the binding of receptor-type selective radioligands to rat brain membranes by nepenthone and thevinone analogues. Rat brain membranes were incubated with [³H]DAMGO for 45 min at 35 °C, [³H]deltorphin II for 45 min at 35 °C and [³H]EKC for 40 min at 24 °C with eleven concentrations of unlabelled ligands (10⁻⁵-10⁻¹²). Values represent means ± SEM from 3 separate experiments.
5. DISCUSSION

5.1. Norbinaltorphimine

Takemori et al. (1988) have previously reported that unlabelled norBNI proved to be highly selective (mu/kappa: 169, delta/kappa: 153) antagonist and it possessed very good affinity (K_i 0.47 nM) to κ-opioid receptors in guinea pig brain membrane preparation. In this study we have examined the binding properties of tritiated norBNI in three different tissues. We found that the labelled norbinaltorphimine is even more selective in our conditions (mu/kappa: >18600, delta/kappa: >1170) than in that reported by Takemori et al. (1988). The lower the density of kappa, receptor (guinea pig > rat > frog) (Wollemann et al., 1993) the lower the selectivity and affinity of the labelled norBNI. The K_i values and Hill coefficients of kappa ligands indicated that the tritiated norBNI labelled only one receptor type (kappa,;) in guinea pig brain (nH 0.98 - 1.03) and (kappa,;) in frog brain (nH 0.80 - 1.11). In contrast two sites were recognised in rat brain (nH 0.50 - 0.57). In each case there is about two orders of magnitude difference for the favour of kappa, site. The calculated B_max values with tritiated norBNI are very similar to those obtained by others for the kappa, receptor subtype in rat (κ, 16 fmol/mg, κ: 111 fmol/mg) and guinea pig brain (κ: 80 fmol/mg) measured with [³H]EKC (Zukin et al., 1988), while the maximal number of binding sites of [³H](d)-N-allylnormetazocine and [³H]bremazocine (non-selective kappa ligands) were much higher: 260 fmol/mg (Itzhak et al., 1985) and 378 fmol/mg (Rothman et al., 1985) in rat brain, respectively. On the basis of these observation we suggest that [³H]norBNI has a preference for the κ, site.

It appears from Table III. that non-peptide mu and delta selective ligands are better competitors of [³H]norBNI than the peptides. The reason for this is likely due to the 100 mM Na^+ content of buffer D, which decreases the affinity of opioid agonist ligands. However, this is inconsistent with the K_i values of CTAP which has a weaker affinity to norBNI sensitive binding sites than cyprodime. The real explanation is probably that the κ-opioid receptors have different binding domains for peptide and non-peptide ligands (Xue et al., 1994). It is conspicuous that naltrindole is the best non-kappa competitor of [³H]norBNI (Table III.) to such a degree that the K_i value of NTI is equal to the same value of norBNI in frog brain. These two ligands have a very common ‘message’ part because both of them are naltrexone derivatives, but their ‘address’ portions are different; the 14'-N is missing in NTI which is a responsible part for kappa selectivity (Lin et al., 1993). Several studies have highlighted the
importance of Glu297 in enabling the κ selectivity of norBNI or 5'-guanidinonaltrindole (GNTI, Jones et al., 2000) (Hjort et al., 1995, Larson et al., 2000). It was also demonstrated that GNTI as well as norBNI displayed κ-like affinity for the μ receptor when a glutamate residue was incorporated into the top of transmembrane region VI at position VI:26 of the μ receptor (Jones et al., 1998). When a glutamate was introduced at the same position of the δ receptor, affinities for both norBNI and GNTI are enhanced relative to that for the δ wild-type. However, the magnitude of the enhancement for both compounds is significantly less than that observed for the corresponding mutant μ receptor (Metzger et al., 2001).

5.2. Cyprodime

The present findings with [3H]cyprodime strongly support previous bioassay data which indicated cyprodime to be a highly selective μ-opioid receptor antagonist (Schmidhammer et al., 1989). The μ/κ selectivity ratio in the guinea pig ileum was found to be 37, while in the isolated mouse vas deferens preparation it was 28, which were 2 to 3 times greater values than with naloxxone. In the mouse vas deferens preparation the μ/δ selectivity ratio was 15 times greater than with naloxxone (Schmidhammer et al., 1989). In the present radioligand binding assays highly selective compounds [3H]DAMGO, [3H]DPDPE and [3H]U69,593 were used in rat brain membranes for labelling μ-, δ- and κ-opioid receptors, respectively. Unlabelled cyprodime displaced [3H]DAMGO with high affinity (Kd 5.4 nM). The affinity of cyprodime for δ and κ sites was more than 40 times less (Table IV.). It is interesting to note that the same rank order of affinity was seen in guinea pig and frog brain (data not shown) as in rat. The antagonist property of cyprodime was shown in binding assays where [3H]naloxxone was displaced by unlabelled cyprodime.

Cyprodime was labelled with tritium resulting in a specific radioactivity of 31.6 Ci/mmol (Ötvös et al., 1992). The detailed binding properties of this ligand were investigated. The binding was saturable and a single binding site was detected with high affinity: Kd value of 3.8 nM in two different strains of rats (PVG/C and Wistar). The maximal number of binding sites were 87 ± 4.8 and 124 ± 13 fmol/mg protein in Wistar and PVG/C strains, respectively. Although cyprodime labels a single population of receptors in rat brain, the density is less than that measured by [3H]DAMGO (Bmax 222±5 fmol/mg) and may indicate that cyprodime is only labelling a subset of μ-opioid receptor, possibly supporting the concept of μ-opioid receptor heterogeneity (Varga et al., 1987).
The selectivity of tritiated cyprodime was tested in rat and guinea pig brain. The unlabelled cyprodime, the mu agonist dihydromorphine and DAMGO, as well as naloxone showed affinities in the low nanomolar range ($K_i$: 0.4 - 8.1 nM, Table V.). Somewhat lower potency was detected with CTAP ($K_i$: 43.8 nM), which is a peptide derivative analogue of somatostatin. In guinea pig brain all of the above unlabelled ligands showed affinities in the nanomolar range with somewhat higher $K_i$ values (2.6 - 26.6 nM, Table V.). These differences might be related to the different ratio of mu and kappa receptors in the two species (Benyhe et al., 1992). A number of delta selective ligands (including DSLET, TIPP, deltorphin II, Ile$^{5,6}$deltorphin II) were applied to compete for tritiated cyprodime. Low affinities were observed ($K_i$ values in the micromolar range) when using these compounds in rat as well as in guinea pig brain (Table V.). These findings confirm the high selectivity of tritiated cyprodime. Low affinities were also measured using the $\kappa$-opioid receptor selective agonist U50,488 ($K_i$ 288 and 303 nM) and the $\kappa$-opioid receptor selective antagonist norbinaltorphimine ($K_i$ 171 and 769 nM) representing $\kappa$-opioid receptor specific compounds (Table V.).

The most currently used non-peptide antagonists, naloxone and naltrexone, do not exhibit high selectivity for any of the opioid receptors. $\beta$-funaltrexamine ($\beta$-FNA) (Takemori et al., 1986) and CTAP (Pelton et al., 1986) were found to be $\mu$-opioid receptor selective antagonists. However, $\beta$-funaltrexamine is a non-competitive ligand for $\mu$-opioid receptor and CTAP, which does not cross the blood-brain barrier, also has high affinity for somatostatin receptors. Such inconveniences limit the application of these compounds. The basic pharmacological properties of cyprodime have been previously described (Schmidhammer et al., 1989). The selective antagonistic properties of this ligand were shown in the guinea pig ileal longitudinal muscle, mouse vasa deferentia and rat vasa deferentia preparations. High selectivity ratios for cyprodime were shown in these assays ($\mu/\delta$: 74 in rat vasa deferentia and 100 in mouse vasa deferentia; $\mu/\kappa$: 28 in mouse vasa deferentia and 37 in guinea pig ileal longitudinal muscle preparation).

When used as a tritiated ligand, cyprodime labels a population of receptors in rat brain confirmed to be $\mu$-receptors by competition assays. The high selectivity for $\mu$-opioid receptor found with $[^3H]$cyprodime in the binding studies is supported by the high selectivity of cold cyprodime also reported in rat brain membranes. These results extend the previous findings.
Schmidhammer et al., 1989) suggesting that cyprodime can be a useful pharmacological tool to characterise the μ-opioid receptor.

Besides in vitro binding experiments, functional biochemical assays were also performed. The effects of cyprodime on agonist stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding in crude rat brain membrane preparations were studied. These functional experiments confirm the finding that cyprodime is an antagonist at μ opioid binding sites, since the compound reversed the stimulatory effect of morphine in the \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding assay at a concentration of 10 μM. Inhibition could not be detected at lower concentrations showing a weaker antagonist property in comparison with naloxone, which is in agreement with the previous findings in in vivo pharmacological assays. Indeed, cyprodime exhibited about one-tenth the potency of naloxone to antagonise morphine-induced antinociception in the acetic acid writhing test in mice, and a similar one-tenth ratio was obtained to modify respiratory activity parameters in rabbits and to precipitate withdrawal syndrome in morphine dependent mice (Schmidhammer et al., 1989). Cyprodime itself produced a negligible and non-significant stimulatory response on \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding, which may reflect a very small degree of inherent agonist property of the compound. In binding assays, cyprodime exhibited a lower potency than naloxone or naltrexone to displace \[^{3}\text{H}]\text{naloxone in the presence of NaCl, but the binding properties of cyprodime were strongly impaired in the absence of NaCl which suggests a pronounced antagonistic activity in this test (Schmidhammer et al., 1989).}

5.3. β-Funaltrexamine analogues

Three of five tested β-FNA analogues (14, 15, 16) proved to be opioid antagonist (Na-index: 0.9, 0.9 and 1.9, respectively) and the remaining two (13, 18) were characterised as agonist (Na-index: 26 and 25, respectively) in \[^{3}\text{H}]\text{naloxone competition experiments (Table VI.). These findings are in agreement with the previous literature data on several N17-substituted epoxymorphinans (Gero, 1985). In that study, ligands with small substituents on N17 atom, such as H- or methyl-, were found to be agonists, however, compounds having bulky substituents (e.g. allyl- or cyclopropylmethyl-) behaved as pure antagonists. The IC\textsubscript{50} value of compound 15 is significantly higher than that of the other β-FNA analogues (p < 0.05: 18, p < 0.01: 13, 14, 16) and the reference compounds naltrexone and levorphanol (p < 0.001). This is based on the different structure of compounds, because 13, 14, 16, 18 possess CH\textsubscript{2}Cl substituent on C19 while 15 has fumarate group on the same carbon atom (Fig 6.).
Using $\mu$, $\delta$ and $\kappa$ opioid receptor selective radioligands in displacement studies we found that the newly synthesised morphinan analogues possess quite a good affinities toward the $\mu$ binding sites. The calculated relative affinity values prove that these ligands are $\mu$-selective compounds, two of them (15, 16) also act on $\kappa$ opioid receptors and the interactions of all these ligands with the $\delta$-binding sites are less pronounced (Table VII.). It is interesting to note, that the two agonist compounds have the highest selectivity profile, because of the increased IC$_{50}$ values toward to $\kappa$ opioid receptors. The presence of 14-OH group significantly decrease the affinity of compound 18 to $\delta$ and $\kappa$ opioid receptors while the $\mu$ affinity is not changed significantly, yielding the most selective $\beta$-FNA analogue in this series.

5.4. Nepenthone and thevinone derivatives

The tested ten compounds possess different binding affinity and selectivity toward the opioid receptors. There are four molecules (6b, 6h, 6c, 6i) which are $\Delta^{18,19}$-derivatives of the parent compounds. Two of them (6b, 6h) possess a phenyl function on 17N atom resulting a very low $\delta$ opioid receptor affinity while the 17N-n-propyl analogues (6c, 6i) possess moderate $\delta$- and good $\mu$-affinity (Table VIII.). Two of the three 3,6-dimethoxy compounds (5o, 5p) have inhibition constants higher than 100 nM on $\delta$ opioid receptors while the remaining molecule (5m) binds to the three different opioid receptor types only with moderate affinity. The 6v, which is the 18,19-dihydro-derivative of 6i, binds to the $\mu$ and $\kappa$ opioid receptors with high affinity. The 11c and 11d compounds, which are 17N-methyl derivatives, produce the best $\delta$-affinity but they are $\mu$ opioid receptor selective ligands. We characterised two pairs of diastereoisomer molecules, which have different configurations at the C20 atom (R: 6c, 11c; S: 6i, 11d). The 20S conformers possess better affinity values than the 20R conformers indicating that the structure of these molecules are closer to the $\delta$-selective NTI or especially 7-(spiroindano)oxymorphone (SIOM) structure. The ‘address’ part of these molecules also contains a phenyl ring but it is fixed in a rigid structure guaranteeing the $\delta$-selectivity. The important role of the ‘address’ moiety were proved making mutations in all three types of opioid receptors with the focus on two positions at the extracellular end of transmembrane regions VI and VII (Metzger et al., 2001). When the amino acids with bulky aromatic group (Trp318 in $\mu$ and Tyr312 in $\kappa$ receptors) were changed to alanine the affinity of NTI and SIOM were increased toward mutated $\mu$ and $\kappa$ receptors compared to the wild types. These results confirm the hypothesis of Metzger group, that the presence of the large
aromatic side chain at this position inhibits the docking of the 'address' portion of these ligands (Metzger et al., 1996). The decreased δ-selectivity of our tested ligands could based on the flexible 'address' part, in which the phenyl ring is capable of interacting with the phenyl ring of Trp318 in μ and Tyr312 in κ opioid receptors.
6. CONCLUSIONS

In this work we characterised two tritiated and twelve unlabelled non-peptide opioid ligands. On the basis of our results and the data found in scientific publications we establish the following conclusions:

1. \(^{3}\text{H}\)norBNI binds to the opioid receptor and its binding is saturable. The highest specific binding can be achieved with incubation at 25 °C for 60 min in the presence of 100 mM Na\(^+\)-ions using 0.1 nM of the tritiated norBNI. The non-specific binding was 35-40 % in all tissues (rat, guinea pig and frog brain membranes) compared to the total binding.

   According to the kinetic experiments we concluded that \(^{3}\text{H}\)norBNI labels a homogenous receptor population with high affinity. The formation of the receptor-ligand complex is fast, the steady-state develops during 40 min and the equilibrium reached after 90 min.

   In the saturation assays we verified that \(^{3}\text{H}\)norBNI recognises a homogenous receptor population with high affinity in guinea pig and rat brain membrane preparations. The \(K_d\) value is in agreement with to the value calculated in kinetic studies. The density of the tritiated norBNI binding sites indicates that the tritiated ligand binds selectively to the \(\kappa_1\) opioid receptors, which is supported by the other results found in scientific publications.

   Using opioid receptor type or subtype selective peptide and non-peptide ligands in competition experiments we proved that \(^{3}\text{H}\)norBNI binds selectively to the \(\kappa_1\) opioid receptors in rat, guinea pig and frog brain membrane preparations.

2. We verified the pure antagonist feature of cyprodime using \(^{3}\text{H}\)naloxone in competition experiments in the presence or absence Na\(^+\)-ions.

   We demonstrated that cyprodime is an antagonist at \(\mu\) opioid binding sites, since it reversed the stimulatory effect of morphine in the \(^{35}\text{S}\)GTP\(\gamma\)S binding assay at a concentration of 10 \(\mu\)M but it possesses a weaker antagonist property in comparison with naloxone. Cyprodime itself produced a negligible and non-significant stimulatory response on \(^{35}\text{S}\)GTP\(\gamma\)S binding, which may reflect a very small degree of intrinsic agonist activity of the compound.
The $[^3]H$cypromide binds to the opioid receptor and its binding is saturable. The highest specific binding can be achieved with incubation at 25 °C for 40 minutes using 2.0 nM of the tritiated cypromide. The non-specific binding compared to the total binding was 15-25 % in all tissues investigated.

In equilibrium saturation experiments we demonstrated that $[^3]H$cypromide labels a homogenous receptor population with high affinity in rat brain. The density of the binding sites was different in PVG/C and Wistar strain of rats and significantly lower than that was detected using the $\mu$-selective opioid peptide $[^3]HDAMGO$.

The tritiated cypromide labels a population of opioid receptors in rat brain that confirmed to be $\mu$-receptors by competition assays. The high selectivity of $[^3]H$cypromide for $\mu$-opioid receptor found in the binding studies is further supported by the high selectivity of unlabelled cypromide also reported in rat brain membranes.

3. In the competition assays performed with $[^3]H$naloxone in the presence or absence of 100 mM Na$^+$-ions we found that the $\beta$-FNA derivatives containing methyl function on N17 behaved as agonists and ligands with n-propyl or allyl function on the same nitrogen atom proved to be antagonists. Using $\mu$, $\delta$ and $\kappa$ opioid receptor selective radioligands in displacement studies we found that the newly synthesised morphinan analogues possess quite good affinities toward the $\mu$ binding sites. The calculated relative affinity values verify that these ligands are $\mu$-selective compounds.

4. The tested ten nepenthone and thevinone derivatives show different binding affinity and selectivity toward the opioid receptors. The competition experiments with highly selective tritiated opioid ligands did not affirm the rationale that these compounds could behave as $\delta$-selective opioid ligands. Among the tested molecules are two compounds, which have 5-15 fold higher affinity toward the $\mu$ than for $\delta$ or $\kappa$ opioid receptors.
7. ACKNOWLEDGEMENTS

I would like to express my warmest thanks to my supervisor, Prof. Anna Borsodi, for offering me the possibility to work in her group and for many years providing the professional and financial background for my studies. I am also thankful to Prof. Mária Wolleman and Dr. Sándor Benyhe for their valuable help and support.

I am grateful to Prof. George Falkay, head of the Institute of Pharmacodynamics and Biopharmacy, for his support and encouragement. He provided all facilities for writing my Ph.D. thesis.

I also wish to thank my co-authors and colleagues for the pleasant co-operation, inspiring discussions and stimulating me to complete this thesis.

I am likewise grateful to Zsuzsanna Canjavec for her technical assistance in the experiments.
8. LIST OF ABBREVIATIONS

β-FNA  β-Funaltrexamine
B_{max}  maximal number of binding sites
CTAP  D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂

cyprodime  N-cyclopropylmethyl-4,14β-dimethoxy-morphinan-6-one

DAMGO  [D-Ala²-(Me)Phe⁴-Gly⁵-ol]enkephalin
DPDPE  [D-Pen³,D-Pen⁵]enkephalin
DSLET  D-Ser², Leu³, Thr⁴enkephalin
dynorphin (1-13)  Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys
EDTA  ethylenediaminetetraacetic acid
EGTA  ethylenbis-(oxyethylenenitrolo)-tetraacetic acid

EKC  (±)Ethylketocyclazocine methanesulfonate
G-protein  heterotrimeric guanine nucleotide binding regulatory protein
GDP  guanosine-5'-diphosphate

GNTI  5'-guanidinonaltrindole
GTP  guanosine-5'-triphosphate
GTPyS  guanosine-5'-O-(γ-thio)triphosphate
IC_{50}  the concentration of the inhibitor that reduces the concentration of the

receptor bounded radioligand with 50 %

[Ile⁵⁶]delorphin II  Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH₂
k_{i1}  association rate constant
k_{i}  dissociation rate constant
K_{i}  equilibrium dissociation constant
K_{i}  inhibition constant
MERF  Tyr-Gly-Gly-Phe-Met-Arg-Phe

norbinaltorphimine  17, 17'bis(cyclopropylmethyl)-6,6',7,7'-tetradehydo-4,4',5,5'-
diepoxy-6,6'-(imino)(7,7'-bimorphinan)-3,3'14,14'-tetrol

(norBNI)  17-(cyclopropylmethyl)-4,5α-epoxy-1'Η-indolo[2',3':6,7]
morphinan-3,14-diol

naltrindole, NTI  17-(cyclopropylmethyl)-4,5α-epoxy-1'Η-indolo[2',3':6,7]
morphinan-3,14-diol

PMSF  phenylmethylsulfonyl fluoride
SIOM  7-(Spiroindano)oxymorphone

Tic  trans-3,4-dichloro-N-methyl-1-N-1-pyrrolidinyl-(cyclohexyl)-
benzeneacetamide

TIPP  Tyr-Tic-Phe-Phe-OH

TIPP[ψ]  Tyr-Tic[Ψ]-Phe-Phe-OH
Tris-HCl  tris(hydroxymethyl)aminomethane

U50,488  trans-3,4-dichloro-N-methyl-1-N-1-pyrrolidinyl-(cyclohexyl)-

benzeneacetamide

U69,593  5α,7α,8β(-)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-
yl]-benzeneacetamide
9. REFERENCES


Number of cited references: 125
10. LIST OF PUBLICATIONS

   Mixed azines of naloxone with dihydromorphinone derivatives.
   If: 2.463

   Structurally novel group of ligands selective for kappa opioid receptors.
   If: 2.173

3. Marton, J., Simon, Cs., Hosztafi, S., Szabó, Z., Márki Á., Borsodi, A., Makleit, S.
   New nepenthone and thevinone derivatives
   If: 1.500

4. Simon, Cs., Hosztafi, S., Makleit, S., Márki, Á., Benyhe, S., Borsodi, A.
   Synthesis and opioid binding properties of new β-funaltrexamine (β-FNA) analogues
   If: 0.896

5. Márki, Á., Monory, K., Ötvös, F., Tóth, G., Schmidhammer, H., Traynor, J.R., Maldonado, R., Roques, B.P. and Borsodi, A.
   Mu opioid receptor specific antagonist cyprodime: Characterisation by in vitro
   radioligand and[^35]S[GTPyS] binding assays
   If: 1.992

   Tritiated kappa receptor antagonist norbinaltorphimine: Synthesis and in vitro binding in
   three different tissues
   If: 1.937

10.2. Abstracts in referred journals

1. Borsodi, A., Márki, A., Tóth, G. and Hosztafi, S.
   Characterization of mu and kappa opioid receptors with new antagonist radioligands
   If: 4.234

   Characterization of kappa opioid receptors with tritiated norBNI
   *Analgesia* 1(4-6):557-560, 1995
   If: -