Biochemical and pharmacological studies of morphine-6-glucuronide and related compounds

This item was submitted to Loughborough University's Institutional Repository by the/an author.

Additional Information:

- A Doctoral Thesis. Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University.

Metadata Record: https://dspace.lboro.ac.uk/2134/13901

Publisher: © Jason Lewis Martin

Please cite the published version.
This item was submitted to Loughborough University as a PhD thesis by the author and is made available in the Institutional Repository (https://dspace.lboro.ac.uk/) under the following Creative Commons Licence conditions.

For the full text of this licence, please go to:
http://creativecommons.org/licenses/by-nc-nd/2.5/
BIOCHEMICAL AND PHARMACOLOGICAL STUDIES OF MORPHINE-6-GLUCURONIDE AND RELATED COMPOUNDS

by

Jason Lewis Martin

A Doctoral Thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of the Loughborough University of Technology.

June 1994

© by Jason Lewis Martin (1994)
ACKNOWLEDGEMENTS

Primarily I would like to thank my supervisor, Dr. John Traynor for his help, guidance and encouragement throughout this project.

I would also like to thank British Technology Group, especially Dr. Terry Smith, for funding the project.

I am also indebted to Mr. John Brennan, Miss Jill Thorley, Mrs. Helen Tidmas and Mrs. Wendy Jones for their technical assistance.
DEDICATION

To my mum and dad

I would like to thank my mother and father for their continual support and understanding while I have been studying, and I therefore dedicate this thesis to them.
Daring ideas are like chessman moved forward; they may be beaten, but they may start a winning game

JOHANN WOLFGANG VON GOETHE
ABSTRACT

Biochemical and pharmacological studies of morphine-6-glucuronide and related compounds

Jason L. Martin

Keywords: morphine, morphine-6-glucuronide, morphine-3-glucuronide, synthetic opiates, brain, spinal cord, ligand-binding assays, isolated tissue bioassays, analgesia, metabolism and distribution.

Morphine-6-glucuronide is a minor metabolite, representing 5% of an administered dose of morphine. The metabolite has analgesic activity exceeding that of morphine and may contribute to analgesia following morphine administration.

The aims of the study were to attempt to identify the reasons behind the improved activity of morphine-6-glucuronide over the parent compound and to examine a series of 6-substituted compounds, based on 6-substituted benzoate esters, as potential mimics of morphine-6-glucuronide.

Morphine-6-glucuronide was seen to have similar affinity to morphine at µ-opioid receptors as assessed by ligand-binding assays in mouse brain homogenates. However a three-fold improved affinity at δ-opioid receptor binding sites was observed and a ten-fold reduction in affinity at κ-opioid sites. Using in vitro bioassay systems, the glucuronide showed a two-fold improved potency over morphine, in both the guinea-pig ileum and the mouse vas deferens preparations. Following in vivo (s.c.) administration in the mouse the glucuronide was seen to be equipotent with morphine in the tail-flick test, but was of much longer duration, lasting up to 9 hours. Ex-vivo binding assays confirmed that morphine-like material was still present in the central nervous system six hours after administration of the glucuronide, but was not observed at a similar time after morphine administration. Activity was retained if the hydroxyl groups of the sugar moiety of the glucuronide were protected as esters. In contrast the more prevalent morphine metabolite, morphine-3-glucuronide, was inactive in all in vitro and in vivo tests used, and did not antagonise morphine in vitro or in vivo. A group of 3-substituted derivatives containing saturated and unsaturated substituents, did show affinity for opioid receptors, but no agonist activity of the compounds could be demonstrated in vitro.
A series of synthetic 6-substituted compounds showed a variety of affinities for, and agonist potencies at, opioid receptors, though low affinity at κ-opioid receptors was a general finding. For example, morphine-6-nitrobenzoate was μ-opioid receptor preferring, while morphine-6-phthaleate had improved δ-opioid receptor affinity and acted via δ-opioid receptors in the mouse vas deferens and in vivo. However the compounds were weaker than morphine and the duration of action in vivo was shorter than morphine-6-glucuronide.

The conclusions from these studies are that morphine-6-glucuronide and morphine have similar in vitro affinities at the μ-receptor, although morphine-6-glucuronide has somewhat improved binding affinity for δ receptor sites, it has less affinity for κ receptor sites. Pharmacokinetic reasons are probably responsible for the improved activity and duration of action of morphine-6-glucuronide over morphine. None of the synthetic compounds examined are potentially useful as direct mimics of the glucuronide because morphine-6-glucuronide is more potent and has a longer duration of action than the synthetic derivatives, though alteration at the 6-position of the morphine nucleus can lead to dramatic changes in selectivity and potency of ligands for the differing opioid receptors.
**Abbreviations used in this thesis**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxy methyl cellulose</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CPM</td>
<td>Cyclopropylmethyl</td>
</tr>
<tr>
<td>DADL</td>
<td>[D-Ala&lt;sub&gt;2&lt;/sub&gt;,D-Leu&lt;sup&gt;5&lt;/sup&gt;] enkephalin</td>
</tr>
<tr>
<td>DALCE</td>
<td>[D-Ala&lt;sub&gt;2&lt;/sub&gt;, Lys&lt;sup&gt;4&lt;/sup&gt;,Cys&lt;sup&gt;5&lt;/sup&gt;] enkephalin</td>
</tr>
<tr>
<td>DAMGO</td>
<td>[D-Ala&lt;sub&gt;2&lt;/sub&gt;,MePhe&lt;sup&gt;4&lt;/sup&gt;,Gly-ol&lt;sup&gt;5&lt;/sup&gt;] enkephalin</td>
</tr>
<tr>
<td>DPDPE</td>
<td>[D-Pen&lt;sup&gt;2&lt;/sup&gt;,D-Pen&lt;sup&gt;5&lt;/sup&gt;] enkephalin</td>
</tr>
<tr>
<td>DSLET</td>
<td>[D-Scr&lt;sup&gt;2&lt;/sup&gt;, Leu&lt;sup&gt;5&lt;/sup&gt;] enkephalin-Thr&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-FNA</td>
<td>β-Funaltrexamine</td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>5′ guanylimidodiphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GPI</td>
<td>Guinea-pig ileum</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N-ethanesulphonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>i.t.</td>
<td>Intrathecal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>M6G</td>
<td>Morphine-6-glucuronide</td>
</tr>
<tr>
<td>M3G</td>
<td>Morphine-3-glucuronide</td>
</tr>
<tr>
<td>MPLM</td>
<td>Myenteric plexus longitudinal muscle</td>
</tr>
<tr>
<td>MVD</td>
<td>Mouse vas deferens</td>
</tr>
<tr>
<td>NEM</td>
<td>N-Ethylmaleimide</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal grey</td>
</tr>
<tr>
<td>PAPS</td>
<td>3′-phosphoadenosine-5′-phosphosulphate</td>
</tr>
<tr>
<td>p.o.</td>
<td>Oral administration</td>
</tr>
<tr>
<td>RVD</td>
<td>Rat vas deferens</td>
</tr>
<tr>
<td>sem</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction

1.1 Historical 1

1.2 Opioid receptors and endogenous peptides 3
  1.2.1 Endogenous opioids 6
  1.2.2 Distribution of opiate receptor types in the CNS 8
  1.2.4 Intracellular mechanism of opioid action 10

1.3 Morphine analogues and related drugs 13

1.4 Pharmacokinetics and metabolism of morphine 16
  1.4.1 Species differences in morphine metabolism 19

1.5 Pharmacological properties of morphine metabolites 21
  1.5.1 M6G 21

1.6 Aims of the project 25

CHAPTER 2

Materials and Methods

2.1 Materials 29

2.2 Methods 29
  2.2.1 Ligand binding assays 29
    i) Brain homogenate 29
    ii) Guinea-pig myenteric-plexus homogenate 30
  2.2.2 Alkylation by β-funaltrexamine 30

2.3 Isolated tissue studies 31
2.3.1 Tissue preparation
   a) Guinea-pig myenteric plexus-longitudinal muscle (MPLM) bioassay
   b) Mouse, rat and hamster vas deferens preparation

2.3.2 Experimental
   a) Agonist potencies
   b) Antagonist affinities
   c) Alkylation by β-FNA in the MPLM

2.4 Metabolism studies
   2.4.1 Preparation of liver microsomes
   2.4.2 Morphine metabolism

2.5 Separation of morphine and its metabolites
   2.5.1 Methods available for studying morphine metabolites
   2.5.2 Separation analysis using HPLC

2.6 Lipophilicities of morphine and related compounds

2.7 Stability of morphine and related compounds

2.8 In vivo experiments
   2.8.1 Antinociceptive properties of morphine and related compounds
   2.8.2 Distribution of morphine and M6G into the CNS
CHAPTER 3

Analysis of binding and isolated tissue data

3.1 Radioreceptor assays
3.1.1 Determination of inhibitor binding constants

3.2 In vitro bioassays
3.2.1 Antagonist potencies
3.2.1.1 Using the Schild plot
3.2.1.2 Antagonist potencies where partial agonism is shown in the rat vas deferens
3.2.2 Determination of agonist potencies using Furchgott's analysis
3.2.2.1 Efficacy and spare receptors
3.2.2.2 Furchgott's method

CHAPTER 4

In vivo antinociceptive activity of morphine and its glucuronide
4.1 Introduction

4.2 Results
4.2.1 Antinociceptive properties as measured by the tail-flick test

4.3 Distribution of morphine-like activity in the brain and spinal cord after s.c. administration of morphine and M6G

4.4 Metabolism of morphine
4.4.1 Metabolism in liver homogenates

4.5 Discussion
CHAPTER 5

Binding and isolated studies of morphine and its glucuronide

5.1 Characterisation of the binding of µ, δ, and κ opioid ligands to homogenates of mouse brain 75

5.2 Receptor binding profile of M6G and morphine 77
5.2.1 Binding of [3H]DAMGO at low concentrations of µ ligands 79
5.2.2 Binding to opioid κ receptors in guinea-pig cerebellum 80
5.2.3 Binding studies in mouse spinal cord 81
5.2.4 Binding in the myenteric plexus longitudinal muscle (MPLM) 82
5.2.5 Binding at µ sites in brain under different buffer conditions 82
5.2.6 Competition studies using [3H] CTOP in mouse brain 91

5.3 Isolated tissue studies 93
5.3.1 Myenteric plexus longitudinal muscle 93
5.3.2 The rat vas deferens preparation 95
5.3.3 Furchgott's analysis in the MPLM 97
5.3.4 The mouse vas deferens preparation 101
5.3.5 The hamster vas deferens preparation 104
5.3.6 Alkylation of µ receptors 105

5.4 Discussion 107
5.4.1 Binding 107
5.4.2 Isolated tissue bioassays 114
5.4.3 Overview 117
CHAPTER 6

Synthetic morphine-6-substituted compounds as analogues of morphine-6-glucuronide

6.1 Introduction

6.2 Compounds under study

6.3 Ligand binding assays

6.4 Isolated tissue studies

6.5 Stability studies

6.6 Lipophilicities of morphine and related compounds

6.6.1 Rates of onset and offset of morphine and derivatives as measured in the MPLM

6.6.2 Lipophilicities as determined using an HPLC system

6.7 Antinociceptive activity of morphine-6-substituted compounds in vivo

6.8 Discussion

6.9 Investigation of 3-position substituents

6.10 Overview

CHAPTER 7

References
LIST OF FIGURES
(N.B. First number indicates chapter in which the figure may be found).

1.1 Morphine 2
1.2 Codeine 2
1.3 Thebaine 2
1.4 Narcotine 2
1.5 Naloxone 3
1.6 Ketocyclazocine 4
1.7 N-allylnormetazocine 4
1.8 The enkephalins 6
1.9 G-protein regulatory cycle 11
1.10 Heroin 12
1.11 Etorphine 13
1.12 Pethidine 13
1.13 Methadone 13
1.14 Nalorphine 14
1.15 Pentazocine 14
1.16 Buprenorphine 14
1.17 M6G 15
1.18 M36G 16
1.19 Normorphine 16
1.20 N6G 16
1.21 M3ESO4 16
1.22 M3G 17
1.23 UDP-GA 17
1.24 Formation of morphine glucuronide 18
1.25 Morphine metabolism in the guinea-pig 21

2.1 [3H]DAMGO 28
2.2 [3H]DPDPE 28
2.3 [3H]U69593 28
2.4 [3H]CI977 28
2.5 Effect of acetonitrile concentration on retention time for normorphine, M3G, M6G & morphine 35
2.6 HPLC gradient system used 36
2.7 Calibration curve for morphine 37
2.8 Calibration curve for M6G 37
2.9 Calibration curve for M3G 38
2.10 Calibration curve for normorphine 38
3.1 Scatchard analysis
3.2 Hill plot
3.3 Determination of antagonist activity ($K_e$)
4.1 Time course for morphine (5mg/kg s.c.) using the mouse tail-flick test
4.2 Dose-response curve for morphine after 60 mins in the mouse tail-flick test
4.3 Time course for M6G (5mg/kg s.c.) using the mouse tail-flick test
4.4 Dose-response curve for M6G after 60 mins in the mouse tail-flick test
4.5 Potency of morphine and M6G in the mouse tail-flick test
4.6 M6G (protected)
4.7 Time course for morphine (5mg/kg s.c.) using the mouse tail flick test
4.8 Dose-response curve for M6G(p) after 60 mins in the mouse tail-flick test
4.9 Time course for M3G (10, 30 & 100mg/kg s.c.) using the mouse tail-flick test
4.10 Effect of M3G (30mg/kg) on morphine induced analgesia
4.11 Time course for codeine (30mg/kg s.c.) and C6G (30mg/kg s.c.) using the mouse tail-flick test
4.12 Dose-response curve for codeine and codeine-6-glucuronide after 60 mins in the mouse tail-flick test
4.13 Calibration curve for morphine versus [$^3$H]DAMGO in mouse brain
4.14 Calibration curve for morphine versus [$^3$H]DAMGO in mouse spinal cord
4.15 Time course for morphine metabolism in mouse, rat, guinea-pig and rabbit liver homogenates
4.16 M6G in a. folded conformation
b. extended conformation
5.1 Displacement of tritiated DAMGO, DPDPE in mouse & U69593 in guinea-pig brain by M6G
5.2 Displacement of tritiated DAMGO, DPDPE in mouse & U69593 in guinea-pig in guinea-pig brain by morphine
5.3 Displacement of tritiated DAMGO by subnanomolar concentrations of $\mu$-ligands
5.4 Displacement of $[^3\text{H}]$naloxone by M6G in rat brain in the presence and absence of Gpp(NH)p(50μM) and NaCl (100mM) 82

5.5 Displacement of $[^3\text{H}]$naloxone by morphine in rat brain in the presence and absence of Gpp(NH)p(50μM) and NaCl (100mM) 83

5.6 Displacement of $[^3\text{H}]$naloxone by DAMGO in rat brain in the presence and absence of Gpp(NH)p(50μM) and NaCl (100mM) 83

5.7 CTOP 85

5.8 Time course for $[^3\text{H}]$CTOP binding in mouse brain 86

5.9 Time course for $[^3\text{H}]$CTOP binding in the presence of Gpp(NH)p(50μM) and NaCl (100mM) in mouse brain 86

5.10 Specific binding of $[^3\text{H}]$CTOP to homogenates of brain from mouse and rat 87

5.11 Scatchard analysis for $[^3\text{H}]$CTOP in mouse and rat brain 88

5.12 Specific binding of $[^3\text{H}]$DAMGO to rat brain homogenate 89

5.13 Scatchard analysis for $[^3\text{H}]$DAMGO in rat brain 89

5.14 Sodium shift for DAMGO in mouse brain homogenate using $[^3\text{H}]$CTOP, in the presence and absence of Gpp(NH)p(50μM) and NaCl (100mM) 91

5.15 Inhibition of electrically induced contractions in the guinea-pig MPLM by M6G in the absence and presence of varying concentrations of naloxone 93

5.16 Schild plot for naloxone versus M6G 93

5.17 Inhibition of electrically induced contractions in the guinea-pig MPLM by morphine in the absence and presence of varying concentrations of naloxone 94

5.18 Schild plot for naloxone versus morphine 94

5.19 Inhibitory potency of $\mu$-ligands in the RVD 95

5.20 Effect of calcium concentration and half calcium in the RVD for DAMGO 96

5.21 Effect of $\beta$-FNA pre-treatment on the potency of M6G in the MPLM 98

5.22 Furchgott's analysis for M6G 98
5.23 Effect of β-FNA pre-treatment on the potency of morphine in the MPLM
5.24 Furchgott's analysis for morphine
5.25 Effect of β-FNA pre-treatment on the potency of DAMGO in the MPLM
5.26 Furchgott's analysis for DAMGO
5.27 Inhibition of electrically induced contractions in the MVD by M6G in the absence and presence of varying concentrations of naloxone
5.28 Schild plot for Naloxone versus M6G
5.29 Inhibition of electrically induced contractions in the MVD by morphine in the absence and presence of varying concentrations of naloxone
5.30 Schild plot for naloxone versus morphine
5.31 Inhibition of contraction of the field stimulated MPLM and MVD by morphine-6-O-protected glucuronide
5.32 Potencies of DPDPE, morphine and M6G in the hamster vas deferens preparation
5.33 Degree of alkylation of µ receptors by M3G, M6G and β-FNA
6.1 Desomorphine
6.2 Levorphanol
6.3 Morphine-6-sulphate
6.4 Scheme showing the reactivity of morphine-6-conjugates with lithium chloride or piperidine in boiling acetone or benzene
6.5 Morphine-6-nicotinate
6.6 Morphine-6-hemisuccinate
6.7 Acetyltiomorphine
6.8 6-Azidomorphine
6.9 Morphine-6-phthalate
6.10 Morphine-6-benzoate
6.11 Morphine-6-succinate
6.12 3-TIPS-morphine (12)
6.13 M6G (protected) (17)
6.14 Inhibition of contraction of the field stimulated MPLM and MVD by morphine-6-nitrobenzoate (5)
6.15 Inhibition of contraction of the field stimulated MPLM and MVD by morphine-6-phthalate (18)
6.16 Inhibition of contraction of the field stimulated MPLM and MVD by morphine-6-succinate (20)
6.17 Inhibition of contraction of the field stimulated MPLM and MVD by morphine-6-hydroxybenzoate (16) 133

6.18 Inhibition of contraction of the field stimulated MPLM and MVD by morphine-6-benzoate (19) 133

6.19 Inhibition of contraction of the field stimulated MPLM and MVD by morphine-6-fluorobenzoate (13) 134

6.20 Inhibition of contraction of the field stimulated MPLM and MVD by morphine-6-chlorobenzoate (14) 135

6.21 Inhibition of contraction of the field stimulated MPLM and MVD by morphine-6-bromobenzoate (15) 135

6.22 Calibration curve of log P against log k' for standard compounds morphine, heroin and codeine 140

6.23 Time course for the antinociceptive action of morphine-6-nitrobenzoate(30mg/kg) and saline controls in 0.25% CMC, assessed by the tail-flick test 144

6.24 Dose-response curve for morphine-6-nitrobenzoate (10mg/kg) and saline controls for 60 mins assessed by the tail-flick test 144

6.25 Time course for the antinociceptive action of morphine-6-phthalate (10mg/kg) and saline controls in 0.25% CMC assessed by the tail-flick test 145

6.26 Dose-response curve for morphine-6-phthalate (10mg/kg s.c) and saline controls for 60 mins assessed by the tail-flick test 145

6.27 Effect of naltrindole (1mg/kg s.c) on morphine-6-phthalate (10mg/kg s.c.) and morphine (5mg/kg s.c.) analgesia as determined by the mouse tail-flick test 146

6.28 BW373U86 150

6.29 Potency of BU 19, 20, 21, 23 & 25 in the MPLM 157
LIST OF TABLES

(N.B. First number indicates chapter in which the table may be found)

1.1 Pharmacological actions of morphine 3
1.2 Possible major pharmacological effects mediated by opioid receptor types 8
1.3 Pharmacokinetic parameters of morphine, M3G & M6G after i.v. and oral (p.o.) injection 23
1.4 Distribution of morphine and M6G in the body of rats 23
4.1 Antinociceptive activity of morphine, M6G and M6G(p) in the mouse tail-flick test 59
4.2 Antinociceptive activity of codeine and C6G in the mouse tail-flick test 61
4.3 Comparison of the penetration of morphine and M6G into the CNS 64
4.4 Retention times for morphine, M6G, M3G and normorphine 65
4.5 Glucuronidation of morphine in phenobarbital induced mouse livers 67
4.6 Potencies of morphine and M6G in the mouse tail-flick test 69
5.1 Binding characteristics for $[^3H]$DAMGO,$[^3H]$DPDPE and $[^3H]$U69593 in mouse brain 75
5.2 Effective inhibition constants (K_i/nM) for morphine, its metabolites, and some control opioids 76
5.3 Selectivities of M6G, morphine and DAMGO for μ, δ, or κ-sites 77
5.4 Slopes for displacement of $[^3H]$DAMGO,$[^3H]$DPDPE and $[^3H]$U69593 by morphine, M6G, M6G (p), DAMG0, DPDPE & U69593 77
5.5 Comparison of κ binding in guinea-pig brain and cerebellum for morphine and M6G 80
5.6 Effective dissociation constants for morphine & M6G in mouse spinal cord for the μ and δ receptor 80
5.7 Effect of tissue concentration on % specific binding using $[^3H]$DAMGO at 25°C for 40 mins 81
5.8 Affinities (Ki/nM) of M6G, morphine and DAMGO for μ receptors, using $[^3H]$naloxone, in the presence or absence of Gpp(NH)p (50μM ) and NaCl(100mM) in rat brain homogenates 82
5.9 Affinities of M6G, morphine and DAMGO for μ receptors, using \([^3H]\text{naloxone}\) in the presence and absence of Gpp(NH)p and NaCl for
a) μ component 84
b) non-μ component 84

5.10 Effect of BSA and PMSF on specific binding of \([^3H]\text{CTOP}\) 85

5.11 Characteristics of binding of \([^3H]\text{CTOP}\) in mouse and rat brain homogenates 87

5.12 Comparison of B\text{max} values (pmoles/g) for DAMGO and CTOP in mouse and rat brain 90

5.13 Affinities (K\text{A}nM) of M6G, morphine, naloxone, fentanyl and DAMGO for μ receptors, using \([^3H]\text{CTOP}\), in the presence or absence of Gpp(NH)p (50μM) and NaCl(100mM) 90

5.14 Potency (IC\text{50}) and naloxone affinity constants (K\text{e}) in MPLM for a series of opiates and related compounds 92

5.15 IC\text{50}'s(nM) obtained for μ-ligands in the rat vas deferens 95

5.16 Dissociation constants [K\text{e}(nM)] for M6G and morphine in the rat vas deferens versus DAMGO 97

5.17 Furchgott's analysis for μ-ligands in MPLM using β-FNA as an irreversible μ-antagonist 97

5.18 Comparison of affinities obtained using either NEM or β-FNA 101

5.19 Potencies [IC\text{50}(nM)] of various compounds and naloxone K\text{e}'s in the mouse vas deferens preparation 101

5.20 Potencies of morphine and M6G in the hamster vas deferens preparation 105

5.21 Affinities of M6G and Morphine measured in the rat vas deferens against the agonist DAMGO, and in the MPLM, following a reduction of μ receptors by β-FNA and in Tris, Tris-Na and HEPES/Krebs and in the plexus 117

6.1 Affinities(K\text{A}nM) of 6-substituted morphine derivatives at μ, δ and κ opioid binding sites in mouse brain homogenates 128

6.2 Selectivities of 6-substituted compounds for μ, δ, or κ sites 129

6.3 Potency of 6-substituted morphine derivatives on the guinea-pig ileum (MPLM) and mouse vas deferens (MVD) preparations 130

6.4 Antagonist equilibrium affinity constants (K\text{e}, nM) for naloxone and cyprodime against 6-substituted morphine derivatives 136
6.5 *Antagonist equilibrium affinity constants (K_e, nM) of morphine-6-substituted compounds in the MVD and MPLM*

6.6 *TLC analysis of morphine and related synthetic compounds and stability following treatment for 30 mins at 37°C in Tris-HCl buffer or mouse brain homogenate*

6.7 *Rates of onset and offset of action of morphine and M6G in MPLM of the guinea-pig ileum*

6.8 *Liphophilicities (log P_oct) of morphine and related compounds*

6.9 *Liphophilicities (log P) of synthesised morphine-6-substituted compounds derived from log k' values*

6.10 *Rates of onset and offset of action of morphine and morphine-6-nitrobenzoate (5) in MPLM of the guinea-pig ileum*

6.11 *Comparative antinociceptive properties of morphine, M6G and M6G (protected) and phthalate and nitrobenzoate*

6.12 *Affinities K_i (nM) of 3-substituted BU compounds at μ, δ and κ opioid binding sites*

6.13 *Selectivities of BU compounds for μ, δ, or κ sites*
CHAPTER 1

INTRODUCTION
1.1 Historical

Pain is a subjective experience encountered by every individual during some stage of their life, though some people have higher thresholds to pain than others. Most people will experience acute pain, some will be unlucky enough to experience chronic pain. The cause of the pain cannot always be removed, so relief is the next best option and thus drugs accomplishing this task are extremely useful and constantly sought.

Pain is usually a reflex and conscious reaction, which suggests an injurious (nociceptive) stimulus is present. Pain saves many lives because it compels those who can to avoid harm and to seek treatment when harm has been done. Some individuals however are congenitally insensitive to pain, and often sustain severe injury, sometimes leading to death. The relief of pain is always desirable, but the use of drugs for this purpose is dangerous if it makes diagnosis more difficult, or if it is allowed to take the place of more fundamental treatment.

Opium (from the Greek word for juice) is the dried exudate from unripe seed capsules of the oriental opium poppy, *Papaver Somniferum*. It relieves pain and induces ‘euphoria’. Opium and its derivatives have been the established treatment of pain for many thousands of years. Opium’s psychological effects were known to the ancient Babylonians (4000 BC), and it was used to pacify children in ancient Egypt before 2000 BC. Arabian traders introduced the drug to the Orient mainly for the treatment of diarrhoea. Paracelsus, in the sixteenth century gave the name ‘laudanum’ (Latin, laudare = to praise) to preparations containing opium. Thomas Sydenham in 1680 introduced opium into Britain.

In the eighteenth century opium smoking became popular in the Orient following the prohibition of tobacco smoking. The invention of the hypodermic syringe increased the problem of dependence because opiates administered intravenously give rise to more severe dependence. The recognition of this serious dependence liability stimulated a search for potent analgesics, which whilst retaining analgesic activity did not induce dependence.
In 1803, a German pharmacist, Serturner, isolated and described an opium alkaloid that he named morphine after the Greek god of dreams. The discovery of other alkaloids in opium quickly followed morphine. Noscapine, by Robiguet, in 1817, codeine in 1832, thebaine by Pelletier in 1835 and papaverine by Merck in 1848. By the middle of the nineteenth century pure alkaloids were taking the place of preparations of opium in medicinal use.

Opium contains about twenty-five different alkaloids, the most prevalent of which are morphine (Figure 1.1), codeine (Figure 1.2), thebaine (Figure 1.3) and narcotine (Figure 1.4).

Figure 1.1 Morphine

Figure 1.2 Codeine

Figure 1.3 Thebaine

Figure 1.4 Narcotine

Morphine has become the most exploited analgesic known to man. In addition to analgesia morphine possesses a range of pharmacological actions (Bowman and Rand, 1980) both centrally and peripherally mediated. These are summarised in Table 1.1.
Table 1.1 Pharmacological actions of morphine

1. Analgesia  
2. Euphoria or dysphoria  
3. Sedation  
4. Tolerance and dependence  
5. Depression of coughing centre  
6. Depression of the vasomotor centre with hypotension  
7. Vomiting  
8. Miosis  
9. Release of antidiuretic hormone  
10. Increase in tone and reduction in motility of the gastrointestinal tract  
11. Constriction of the bronchi  
12. Dilation of blood vessels  
13. Itching  
14. Muscle rigidity  
15. Respiratory depression

1.2 Opioid Receptors and endogenous opioid peptides

'Opioid' is a general term for agents with morphine-like properties. Analgesia associated with opioids results from interaction with specific opioid receptors distributed heterogeneously within the CNS. Thus the definition of an opioid effect, caused via action at an opioid receptor is that it must be antagonised by an opioid antagonist eg. naloxone (Figure 1.5).

![Image of Naloxone](image)

Figure 1.5 Naloxone

Martin (1976) suggested the existence of three different opioid receptors, namely, the mu (µ) receptor, kappa (κ) receptor and the sigma (σ) receptor based on observations using the chronic spinal dog preparation. Prototype agonists for these receptors are morphine (µ), ketocyclazocine (κ) (Figure 1.6) and N-allylnormetazocine (SKF 10047)(σ)(Figure 1.7) respectively.
Further *in vitro* work using isolated tissue preparations, e.g. the guinea pig ileum, confirmed the presence of the \( \mu \) receptor site characterised by morphine and the \( \kappa \) site characterised by ketocyclazocine. The \( \sigma \) receptor, however is no longer considered of the opioid type because it is not blocked by the opioid antagonist naloxone. Hallucinogenic drugs e.g. phencyclidine (angel dust) act at the \( \sigma \) receptor.

Following the discovery of the endogenous opioid peptides, the enkephalins, a third receptor was confirmed to account for the actions of these peptides. This was named the \( \delta \) receptor and was first identified by electrically stimulating the mouse vas deferens (MVD) preparation (Lord *et al.*, 1977). The presence of these three receptors has recently been confirmed by cloning methods. The \( \mu \) receptor (Chen *et al*, 1993) was cloned from rat brain and the \( \delta \) (Yasuda *et al*, 1993) and \( \kappa \) (Yasuda *et al*, 1993) from mouse brain, making use of complementary DNA techniques.

1.2.1 Endogenous opioids

Kosterlitz and Hughes (Kosterlitz & Hughes, 1975) isolated substances possessing opioid-like activity from porcine brain, termed 'enkephalins'. The enkephalins are two pentapeptides, namely methionine enkephalin ([Met\(^5\) enkephalin) and [Leu\(^5\] enkephalin (Figure 1.8) present in the ratio 3:1 and differing only in the carboxy-terminal amino acid residue. Extended compounds were soon discovered. \( \beta \)-endorphin was one of the earliest extended [Met\(^5\] enkephalin to be isolated, and found to be 30 times more potent than [Met\(^5\] enkephalin (Holiday & Loh, 1981). Goldstein *et al* (1981) later discovered another group of endogenous opioid peptides named
the dynorphins, which were extended [Leu$^5$] enkephalins; showing high potency in isolated tissue preparations. The opioid peptides are derived from three large precursor molecules. Thus pre-proenkephalin, pre-proopiomelanocotin and pre-prodynorphin respectively are cleaved to yield the pro-peptides initially and then the opioid peptides (Akil et al, 1984).

A problem with peptides like the enkephalins and other small opioid peptides is that they are readily degraded and therefore care has to be taken when studying these molecules. Two possible solutions are available to overcome this problem, namely, substitution of stable amino acids or the use of specific enzyme inhibitors to reduce proteolysis (Corbett et al, 1982). In the enkephalin series substitution of glycine residue in position 2 by D-alanine and leucine or methionine at position 5 by the corresponding D-isomer produces an increase in potency, whilst not changing the receptor selectivity (Pert, 1976; Kosterlitz et al, 1980). Unfortunately this method cannot be employed for dynorphins because the glycine residue at position 2 is essential for receptor selectivity. As an alternative enzyme inhibitors are used to increase dynorphin stability (Corbett et al, 1982).
Figure 1.8 The enkephalins
1.2.2 Distribution of opioid receptor types in the CNS

Using \textit{in vitro} binding and competition experiments (Simon \textit{et al}, 1973), the distribution of \(\mu\), \(\delta\), and \(\kappa\) - opiate binding sites has been studied in several species.

Opioid receptors are widely, but heterogeneously, distributed throughout the CNS. The regions of the brain rich in opioid receptors are areas closely associated with analgesia (eg. the periaqueductal grey).

In rat brain a population of virtually pure \(\mu\)-sites is found in the thalamus, whereas \(\delta\)-sites are found in the frontal cortex. In cow brain \(\mu\) sites are found predominately in the substantia nigra and the thalamus. The highest proportion of \(\delta\) sites exists in the frontal cortex and the hippocampus. In human brain distribution is similar to that in rat and cow. Most regions of the human brain have a high proportion of \(\kappa\) sites. The highest level is found in the hypothalamus. In guinea-pigs the highest density of \(\kappa\)-sites is found to occur in layers V and VI of the cerebral cortex and in the pyriform cortex (Mansour \textit{et al}, 1987; Shariff & Hughes, 1989).

Receptors concerned with the processing of sensory information are found on neurones in the cortex and in the dorsal horn of the spinal cord, which pass messages to the brain stem, thalamus and cortical centres. \(\mu\) Receptors are especially found in areas receiving visual, olfactory and nociceptive information. The presence of \(\mu\) receptors in the thalamus, brain stem and colliculi may correlate with a role for \(\mu\)-agonists in sedation and respiratory depression in addition to analgesia, whereas the sedative actions of \(\kappa\)-agonists may relate to the presence of \(\kappa\) receptors in deep layers of the cortex. In the striatum and substantia nigra \(\mu\) receptors show a diffuse distribution, which may be related to modulating dopamine transmission in nigrostriatal pathways, with large doses of opiates inducing muscle rigidity and cataleptic behaviour. Dense distribution of opioid receptors in the limbic system may relate to the behavioural effects of \(\delta\)-opioids eg. convulsions. Hypothalamic nuclei and the posterior pituitary gland are rich in \(\kappa\)-sites and \(\kappa\)-opioids have a role in neuroendocrine regulation eg. \(\kappa\)-agonists cause diuresis caused by an inhibition of vasopressin release (Mansour \textit{et al}, 1987; Shariff & Hughes, 1989). The possible major pharmacological effects mediated by opioid action at \(\mu\), \(\delta\) and \(\kappa\) receptors are shown in Table 1.2.
Table 1.2 Possible major pharmacological effects mediated by opioid receptor types

<table>
<thead>
<tr>
<th>Opioid receptor</th>
<th>Typical ligand</th>
<th>Possible effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>μ</td>
<td>morphine</td>
<td>Analgesia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Respiratory depression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Addictive liability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Miosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Muscle rigidity</td>
</tr>
<tr>
<td>δ</td>
<td>[Leu]enkephalin</td>
<td>Analgesia</td>
</tr>
<tr>
<td></td>
<td>[Met] enkephalin</td>
<td>Behavioural effects</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>κ</td>
<td>ethylketocyclazone</td>
<td>Analgesia</td>
</tr>
<tr>
<td></td>
<td>dynorphin</td>
<td>Sedation/dysphoria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Miosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diuresis</td>
</tr>
</tbody>
</table>

The different receptor subtypes are located at different levels of the neuraxis. Intrathecal (i.t.) administration of μ, δ, and κ-receptor selective opioid compounds produces strong analgesia, whereas with intracerebroventricular (i.c.v.) administration, μ selective drugs are the most active (Porreca et al., 1984). μ-Receptors therefore are important in both spinal and supraspinal analgesia (Pasternak, 1983).

All clinically used opiates are μ-agonists. μ-Receptors have been divided into 2 distinct subtypes: μ₁-receptors which bind both opiates and most enkephalins with similar high affinities and μ₂-receptors which bind morphine (Goodman & Pasternak, 1985). Experiments performed using selective and long-lasting μ₁-receptor antagonists, such as naloxazine and naloxonazine (Pasternak & Hahn, 1980), have implicated μ₁-receptors in a number of opiate actions, including supraspinal analgesia, but not others, such as respiratory depression and gastrointestinal transit (Paul & Pasternak, 1988). Respiratory depression and decreased gastrointestinal
transit are considered to be \( \mu_2 \) actions. For example Bodnar et al (1988) showed that \( \mu_1 \)-receptors mediate analgesia produced by microinjection of receptor-selective opiate peptides into the periaqueductal grey (PAG) of rat brain. A role for \( \mu_1 \)-receptors was inferred since the analgesic effect of the \( \mu \)-agonist, morphine was attenuated by naloxonazine pre-treatment. The peptide derivative \([D-\text{Scr}^2, \text{Leu}^5] \text{enkephalin-Thr}^6 \) (DSLET) binds to \( \mu_1 \) and \( \delta \) receptors and produces naloxonazine reversible analgesia, further supporting a role for \( \mu_1 \) receptors.

Also Heyman et al (1988) demonstrated that naloxonazine attenuated the analgesic effect of i.c.v. administered \( \mu \)-receptor agonists morphine and DAMGO, but did not affect the analgesia produced by the \( \delta \)-selective agonist, DPDPE. When the opiates were given i.t. naloxonazine did not affect analgesia suggesting \( \mu_1 \) receptors are more important for supraspinal, than for spinal analgesia.

1.2.3 Intracellular mechanism of opioid action

Opioid receptors belong to a superfamily of receptors, characterised by seven particularly hydrophobic stretches, consensus sites for N-linked glycosylation near the amino terminus, three short extracellular loops, two short, cationic cytoplasmic loops, and a longer cytoplasmic loop that connects spans five and six. They are also all of similar size (40-50kD plus carbohydrate) (Ross, 1989). These receptors are linked to second messenger systems via G-proteins. Adenylate cyclase (AC), the enzyme that converts ATP to cyclic adenosine monophosphate (cAMP), constitutes part of a regulatory system that controls the actions of hormones and neurotransmitters at their sites of action. A family of GTP-binding regulatory (G) proteins exist that conveys information between the ligand activated receptors and AC. They include \( G_i \), \( G_o \) and \( G_s \), which lead to inhibition \( (G_i \) or \( G_o \) ) or stimulation \( (G_s \) ), respectively of AC. It is now known that \( G_o \) protein is very concentrated in neural growth cones and is involved in changing the electrical activity of neurones (Hepler & Gilman, 1992). Many different \( \alpha, \beta \) and \( \gamma \) sub-units exist, making it possible for over 1,000 theoretical G-proteins to exist and this number is increasing.

Gilman(1984) showed that \( G_s, G_i \) and \( G_o \) proteins were all \( \alpha\beta\gamma \) heterotrimers. The \( \alpha \) subunit contains a single GTP binding site, GTPase activity and sites for modification by either cholera toxin for a subunit of \( G_s \) (\( \alpha_s \)) or pertussis
toxin for $\alpha_1$ and $\alpha_3$. The $\alpha$ subunits differ among the various G-proteins, as do $\beta$(35 or 36kd) and $\gamma$ (6-10kd) subunits. In contrast to receptors, G-proteins do not seem to include strongly hydrophobic regions that would explain how the proteins attach to the plasma membrane, but it has been reported that a lipophilic portion is present. One end of the gamma protein is bound to an isoprenoid. This lipid probably anchors the G-protein to the cell membrane or it helps the G-protein to attach to other proteins in the membrane. The $\alpha$-subunits of some G-proteins are also bound to the membrane with the assistance from a second lipid, myristic acid (Linder & Gilman, 1992).

The G-proteins interact with the receptor-ligand complex, bind GTP, and become activated. They then interact with AC to alter the rate of cAMP synthesis along with other second messengers and finally, they terminate their effect on AC by hydrolysing GTP to GDP. The mechanism of action of G-proteins was proposed by Cassel and Selinger (1977) in the form of a G-protein regulatory cycle (Figure 1.9). Ross & Gilman (1980) modified this slightly. The hormone-receptor complex dissociates GDP and promotes GTP binding and activation of all G-proteins ($G_s$, $G_i$ and $G_o$). Activated $\alpha$-GTP dissociates from $\beta\gamma$, and both $\alpha$-GTP and $\beta\gamma$ can then activate downstream processes, including AC. Hydrolysis of GTP to GDP by $\alpha$, and reassociation of $\alpha$ with $\beta\gamma$, returns systems to resting state. Hydrolysis resistant analogues of GTP; namely, guanylimidodiphoshate (Gpp(NH)p) and guanosine-5'-O-(3-thiodiphosphate) (GTPyS) are often used for experimental purposes as stable GTP analogues to convert the receptor to a low affinity state.

Opioids decrease cAMP levels, as shown in mouse neuroblastoma x rat glioma cells (NG108-15) by inhibiting the activity of AC in these cells (Klee & Nirenberg, 1974). Other mechanisms for intracellular activity as well as AC do exist and are via ions, namely $Ca^{2+}$ and $K^+$. $\mu$ and $\delta$ Agonists increase $K^+$ conductance and decrease $Ca^{2+}$ levels, whereas $\kappa$-agonists close the $Ca^{2+}$ channel and thus lower $Ca^{2+}$ levels (Dickenson, 1991).

Carter and Medzihradsky (1993) reported that the $\mu$-opioid receptor couples to AC via $G_o$ protein in SH-SY5Y human neuroblastoma cells, whereas Laugwitz et al (1993) suggested an action via the $G_i$ subtype $G_i3$ protein for the $\mu$ receptor, using photoaffinity labelling experiments with the GTP analogue [$\alpha^{32}P$] GTP azidoanilide. McKenzie and Milligan (1990) reported
that the δ-opioid receptor mediated inhibition of AC is transduced by G_12 protein in mouse neuroblastoma x rat glioma hybrid cells (NG108-15). In SHSY-5Y cells the δ response is via G_11 protein, according to Laugwitz et al (1993), using photoaffinity experiments.

Figure 1.9 G protein regulatory cycle

The presence of GTP is essential for the coupling of opioid receptors to effectors (Schwartz, 1979). In turn GTP can control opioid binding as can ions. Pert and Synder (1974) showed that the presence of sodium ions reduced the affinity of opiate agonists, whilst having no effect on the binding of opiate antagonists. Ions and nucleotides are thought to induce a conformational change in the μ receptor (Simon et al, 1975) converting between a 'high agonist affinity' conformation in the absence of sodium and GTP to a 'low agonist affinity' conformation in the presence of sodium and GTP.

The majority of early data (Creese & Synder, 1975) based on comparisons with estimates of pharmacological potency, implicated that the 'low agonist affinity' conformation, as defined by binding in the presence of sodium ions, as the physiologically relevant conformation. Carroll and Goodfriend (1984) compared data from binding in the presence and absence of sodium
ions and Gpp(NH)p with data from isolated tissue preparations. Affinities for a range of μ-agonists were calculated from binding experiments run in Krebs/HEPES buffer and correlated extremely well with antagonist equilibrium constants ($K_e$) determined by antagonism of [D-Ala$^2$,MePhe$^4$Gly-ol$^5$]enkephalin (DAMGO) in the rat vas deferens preparation. These results suggest that it is the 'low agonist affinity' conformation that represents the relevant state of the μ-receptor.

The purpose of the high agonist affinity conformation is unclear. It is likely to represent a short-lived coupled conformation of the receptor, but it may represent an uncoupled form of the receptor present in non-physiological conditions (Kent et al, 1979).

1.3 Morphine analogues and related drugs

The pharmacological properties of morphine have already been discussed. Morphine is the recommended choice of treatment for moderate to severe pain by the World Health Organisation. However many attempts to improve on morphine have been carried out. Heroin (Figure 1.10) (diacetyl morphine) has been used for pain relief. It is however a more highly addictive drug and is often abused.

![Heroin](image)

**Figure 1.10 Heroin**

Oripavines eg. etorphine (Figure 1.11) are very potent analgesics, but the World Health Organisation (W.H.O.) condemned Etorphine in 1966 because of the risk of dependence and the ease of illicit trafficking. Synthetic morphine-like analgesics, include pethidine (Figure 1.12) and methadone (Figure 1.13). Pethidine is used in labour and post-operative pain. Methadone is used to treat addicts because of its longer duration of action, which means the withdrawal syndrome is relatively slow in onset, longer in duration, and much less intense.
Substitution of an allyl group on the nitrogen atom in many cases produces compounds which are antagonists eg. naloxone which antagonise the analgesic, euphoric and respiratory depressant effects of opiates and may precipitate withdrawal symptoms in addicts.

Nalorphine (Figure 1.14) and pentazocine (Figure 1.15) are narcotic analgesic-antagonists. Whilst maintaining analgesic activity, such compounds cause much less tolerance, respiratory depression, constipation or vomiting than morphine. Thus the potential for abuse by addicts is less. They do however have psychotomimetic effects.
A synthetic programme of the N-substituted analgesic-antagonist group of compounds led to the introduction of buprenorphine (Figure 1.16) into clinical use. Buprenorphine is a partial agonist, being potent and long lasting with low physical dependence and low abuse potential. It is non-psychotomimetic, but does cause vomiting.

1.4 Pharmacokinetics and metabolism of morphine

Morphine is readily absorbed from all routes of administration, except transdermal. Peak plasma levels are achieved within 15-20 mins of intramuscular and subcutaneous administration, and within 30-90 mins after oral administration. Peak levels after administration are much lower than after parenteral routes, since oral morphine undergoes extensive
first-pass metabolism in the liver (Glare & Walsh, 1991). With repeated administration, the oral-parenteral relative potency ratio is 1:3. After absorption, morphine is rapidly and widely distributed and crosses the blood -brain barrier. With therapeutic doses, plasma protein binding is only 20-35%, and the volume of distribution is 1.6 l. The primary site of morphine metabolism is the liver. Morphine can also be administered spinally. Epidural morphine enters the subarachnoid space, but is also absorbed into the systemic circulation. Only 5% of a dose crosses the dura. When morphine is administered in the lumbar region it is quickly redistributed in the cerebrospinal fluid in a rostral direction, explaining the high incidence of systemic side effects following spinal administration.

Drug metabolism can be divided into two parts, namely, Phase I reactions, eg. oxidation, reduction and hydrolysis, and Phase II reactions, which increase the water solubility of the compounds, and allow for easier excretion eg. acetylation, sulphation, methylation, amino acid conjugation and glucuronidation. Drugs can be eliminated from the body by a variety of routes, urinary, biliary and in the form of faeces. Elimination of drugs is increased by four factors, namely by metabolites which have reduced lipid solubility, low volume of distribution, do not bind to plasma proteins and are not actively transported.

Morphine is metabolised in man by the cytochrome P-450 system in liver to give a variety of metabolites, including morphine-6-glucuronide (M6G) (Figure 1.17), morphine-3-glucuronide (M3G), morphine-3,6-glucuronide (M3,6,G) (Figure 1.18), normorphine (Figure 1.19), normorphine-6-glucuronide (Figure 1.20), and morphine-3-ethereal sulphate (M-3-ES) (Figure 1.21).

![Figure 1.17 M6G](image-url)
Glucuronidation is the major metabolic process present and M3G (Figure 1.22) is the major metabolite accounting for over 50% of administered morphine. Woods (1954) isolated and identified M3G from canine urine, and later M3G was isolated from human addicts urine by Fujimoto and Way (1957, 1958). M6G is a minor metabolite, accounting for about 5% of an administered dose of morphine (Peterson et al, 1990; Sawe et al, 1983 ii) UDP-glucuronyl transferase, the enzyme responsible for performing glucuronidation, exists in multiple forms A to D. Morphine is glucuronidated by the B form of glucuronyl transferase enzyme (Burchell, 1981), mainly in the liver, to a limited extent in the intestine and kidney, and to a lesser extent in the placenta (Miettinen, 1963). Dutton and Storey (1954) demonstrated the presence of UDP-glucuronic acid (Figure 1.23) and glucuronyltransferase in liver tissue. Strominger (1957) characterised UDP-glucuronic acid dehydrogenase and the reduced
diphosphopyridine nucleotide system as essential contributors to glucuronide synthesis. The formation of morphine glucuronides is shown in Figure 1.24.

Figure 1.22 Morphine-3-glucuronide

Figure 1.23 UDP-GA
Morphine glucuronide is excreted into the bile and hence the intestine. However, the intestine contains significant amounts of β-glucuronidase, resulting in the formation of free drug, which is then reabsorbed, transported to the liver and then undergoes re-conjugation and re-excretion. This enterohepatic recirculation may make a significant contribution, prolonging the half-life in the body, resulting in the potentiation of the pharmacological action of morphine. In addition, Fishman and Green (1957) reported in vitro the transferase activity of β-glucuronidase. Thus a second possible pathway of M6G formation may occur through β-glucuronidase catalysis of morphine.

Sulphation to form 3-ethyl sulphate of morphine requires an active donor, 3’-phosphoadenosine-5’-phosphosulphate (PAPS), produced from ATP and sulphate. Many drugs that can be glucuronidated can also be sulphated, thus leading to the possibility of competition for substrate between 2 pathways. Generally, sulphate conjugation predominates at low substrate concentration and glucuronide conjugation at high concentration, due to the kinetics of the reactions, and the limited supply of PAPS in the cell.
compared with UDP-glucuronic acid.

Morphine and its metabolites are excreted by the kidney. Urinary free morphine accounts for <10% of an administered dose. The elimination half-life of morphine is approximately 2 h and is independent of route of administration. Total body clearance is 21 ml/min/kg, the same as hepatic blood flow (Table 1.3). The relationship between plasma morphine concentrations and pharmacological effects is not clearly defined currently, but is influenced by pharmacodynamic factors, such as development of tolerance and individual pain thresholds (Glare & Walsh, 1991).

Table 1.3 Pharmacokinetic parameters of morphine, M3G & M6G after i.v. and oral (p.o) injection

<table>
<thead>
<tr>
<th></th>
<th>MORPHINE</th>
<th>M3G</th>
<th>M6G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>iv</td>
<td>po</td>
<td>iv</td>
</tr>
<tr>
<td>(t_{0.5}^{\text{elim}}) (h)</td>
<td>1.7-3.5</td>
<td>1.3-3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>(t_{\text{max}}) (h)</td>
<td>-</td>
<td>0.5-1.5</td>
<td>0.25</td>
</tr>
<tr>
<td>(V_d) (l)</td>
<td>3.2-5.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(C) (ml/min/kg)</td>
<td>11-33</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(t_{0.5}^{\text{elim}}\) - elimination half life  
\(t_{\text{max}}\) - time to reach maximum concentration  
\(V_d\) - volume of distribution  
\(C\) - clearance  
(Taken from Glare & Walsh, 1991)

1.4.1 Species differences in morphine metabolism

Substantial interspecies differences in drug metabolism frequently occur and this can be a good indication of the nature and duration of pharmacological and toxicological activities.

Yeh isolated and detected morphine metabolites from the urine of several mammalian species (Yeh et al, 1979). M3G and M6G are found in humans, guinea-pigs, rats, rabbits (Kuo et al, 1991), monkeys (Rane et al, 1984), dogs (Misra, 1970). In cats, M3G is a minor metabolite and M6G is not observed (Yeh et al, 1979). Morphine ethereal sulphate was observed in humans and
cats. Oguri et al. (1970) also showed that in rabbits and guinea-pigs morphine was excreted as conjugates, whereas in rats morphine was excreted as the free base. Dihydromorphinone is observed in guinea-pigs, rats, rabbits and monkeys. α- and β- dihydromorphines were observed in guinea-pigs. Hydroxylated morphine is observed in guinea-pigs, rats, rabbits, and cats. More recent work performed in the guinea-pig (Kumagai et al., 1990) shows the presence of other metabolites namely, morphinone, a morphinone-glutathione adduct, morphine-glutathione adduct and morphinone-cysteine adduct as shown in Figure 1.25.

Dechelotte et al. (1993) investigated the glucuronidation of morphine at the 3- and the 6-position in cells from the stomach, intestine, colon and liver of the guinea-pig. Morphine was taken up by all cell types in a time-dependent manner. There was evidence for a carrier-mediated accumulation in liver cells, but not in other cell types. The epithelium of the small and large intestine along with the liver contribute to the formation of the 'active' M6G, whereas gastric and intestinal epithelia are involved in the inactivation of morphine to M3G. The M3G/M6G ratio averaged 3.5, 4.7 and 5.4 for colonic, intestinal and liver cells respectively.

Kuo et al. (1991) also detected M6G and M3G in the urine of guinea-pigs and rabbits in the metabolism of codeine using HPLC. Yue et al. (1990) showed that glucuronidation of morphine and codeine proceeded similarly in both human liver and kidney microsomes.

Excretion ratios of the M3G to M6G are approximately 4:1 and 50:1 in guinea-pigs and rabbits respectively. The urinary excretion of M6G in mice and rats was too small to be determined (Kuo et al., 1991). The ratios of UDPGT activities toward 3- and 6- hydroxyl groups of morphine in liver microsomes of mice, rats, guinea-pigs, rabbits and rhesus monkeys were approximately 300:1, 90:1, 4:1, 40:1 and 50:1 respectively (Rane et al., 1984). In rats natural (-) morphine is only glucuronidated at position 3, forming (-) M3G, whereas the unnatural (+) morphine is glucuronidated preferentially at position 6, forming (+) M6G (Rane et al., 1985).

Morphine glucuronidation is deficient in premature human neotates and M3G is detected, but M6G is not (Hartley et al., 1993).
Figure 1.25 Morphine metabolism in the guinea-pig

1.5 Pharmacological properties of morphine metabolites

1.5.1 M6G

The metabolite morphine-6-glucuronide (M6G) is reputed to possess a more potent analgesic activity than morphine (Paul et al., 1989 i). It is possible that M6G accounts for a major component of the analgesia when morphine is administered. M6G avoids first-pass metabolism, and as a result of this gives more predictable kinetics, whilst retaining its analgesic activity.
Morphine appears to produce an exaggerated clinical response in patients with renal disease (Chan & Matzke, 1987). The presence of renal impairment significantly reduces the patients' dosage requirements of morphine, possibly because of the accumulation of M6G, rather than morphine. Osborne et al (1986) described that patients with renal failure experienced respiratory depression, following morphine treatment. Svensson et al (1982) showed by HPLC that M6G and M3G, but not morphine were the only compounds detectable. Hence the classic signs of morphine intoxication were attributed to accumulation of M6G, which is usually renally excreted. Previous workers have linked the respiratory depression observed with morphine administration to M6G (Christensen & Jorgensen, 1987), supported by M6G causing a dose-dependant ventilatory depression in conscious dogs (Ronald, 1989). Gong et al (1991) observed respiratory depression in rats and postulated that the potent ventilatory depression induced by M6G is related to its antinociceptive effect. Hasselstrom et al (1989) reported on a 7 year old girl with renal failure who experienced long lasting respiratory depression after morphine was administered intravenously. The plasma concentrations of the active metabolite M6G were more than 10 times those normally seen.

Hanks (1991) investigated the oral to parenteral relative potency ratios for morphine. He reports that a ratio of 1:6 applies to single oral administration of morphine, and a ratio of 1:3 to repeated administration. The difference may be attributed to the presence of the active metabolite M6G and the enterohepatic circulation of morphine. In contrast Osborne reports after oral administration, the mean M6G/morphine area under the curve of a graph plotted of concentration versus time (AUC) ratio in normal patients was 9.7:1, greater than 3:1 previously reported by McQuay et al (1987), whereas the mean M3G/morphine AUC ratio was 56:1 (Osborne et al, 1990), greater than 20:1 reported by Sawe et al (1983 i).

Hanna et al (1990) reported that in patients with chronic cancer, intrathecal M6G has analgesic activity exceeding that of intrathecal morphine and of longer duration. The mean potency ratio for M6G : morphine calculated from cumulative pethidine requirements was 2 : 1.

Osborne et al (1992) suggests that M6G given intravenously offers significant advantages over morphine because M6G does not induce nausea and vomiting like morphine does. Another advantage of M6G over morphine is that the safety margin of M6G was about 3 times that of
morphine using the $ED_{50}/LD_{50}$ ratio (Shimomura et al., 1971).

Hand et al. (1987) measured CSF levels of morphine, M6G and M3G after i.m. and oral routes and suggested M6G to account for 85% of the analgesic effect of morphine. Peterson et al. (1990) reported a mean M6G/morphine plasma concentration ratio of 7:1 in cancer patients receiving chronic therapy with oral morphine. It was established that accumulation of M6G with chronic morphine therapy is related to the renal function of the patient. M6G contributes to the clinical response to morphine treatment because of extensive first-pass glucuronidation of morphine and therefore, higher ratios of M6G/morphine in plasma.

The activity observed with M6G is surprising because of the presence of the polar hydrophilic sugar group would be expected to reduce the lipopholicity of the drug. This would suggest that M6G does not penetrate the blood-brain barrier and predicts an increased elimination from the body via biliary and urinary routes. This is obviously not the case.

Yoshimura et al. (1973) investigated the penetration of M6G and morphine into the brain of rats using $[^{14}C]$-M6G and $[^{14}C]$-morphine. Results from these experiments indicated that even though M6G was a very polar compound, it did penetrate the blood-brain barrier and interact with the 'analgesic receptor' without hydrolysis of the glucuronide. This was shown by the presence of only M6G in the brain confirmed by TLC. Highest levels achieved were 1/5 of morphine. Levels of $[^{14}C]$-M6G and $[^{14}C]$-morphine were also found in the liver, kidneys and blood (Table 1.4).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Brain (%)</th>
<th>Liver</th>
<th>Kidney (%)</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>0.028</td>
<td>0.44</td>
<td>2.36</td>
<td>0.028</td>
</tr>
<tr>
<td>M6G</td>
<td>0.0057</td>
<td>0.68</td>
<td>0.91</td>
<td>0.043</td>
</tr>
</tbody>
</table>

23
M3G was shown to penetrate the blood-brain barrier (BBB) as well as M6G because significant amounts of M3G were found in the brain and plasma of rats at 45 mins after i.p. injection of $[^{14}C]$morphine. The radioactivity determined to be M3G accounted for 10% and 30% of the total radioactivity in the brain and plasma respectively. M3G did not show any analgesic effect after s.c. or i.c.v. injection, suggesting M3G reaches but does not interact with the 'analgesic receptor' in the brain (Shimomura et al, 1971).

The lack of analgesic activity of M3G has been confirmed (Yoshimura et al 1973; Pasternak et al 1987). Pasternak et al (1987) also showed that M3G had no affinity for $\mu$, $\delta$ or $\kappa$ opioid receptors. However Smith et al (1990) showed that M3G is a potent antagonist of morphine and M6G induced analgesia when administered i.c.v. Gong et al (1992) also showed M3G to antagonize M6G's actions, namely its analgesic and ventilatory depression effects, when administered i.c.v or i.t. It was also found that M3G stimulated ventilation implying M3G is an antagonist of morphine and M6G (Smith et al, 1990). In fact M3G produces hyperalgesia in rats when administered by the intrathecal and i.c.v. routes (Yaksh & Harty, 1988).

The question still remains as to how M3G and M6G enter the CNS. Partridge (1988) quotes four ways in which drugs could be transported into the brain. These are :-

1. Carrier-mediated transport eg. glucose carrier
2. Receptor-mediated transport eg. receptors for peptides
3. Plasma protein-mediated transport eg. steroids are transported through the BBB by binding to circulating plasma proteins, such as albumin or specific globulins.
4. Drug delivery eg. invasive, pharmacologic (lipid soluble prodrugs), or physiologic (chimeric peptides).
1.6 Aims of the project

The aim of the project was firstly to attempt to explain the reasons behind the increased analgesic activity of M6G and its longer duration of action over morphine. M6G avoids first pass metabolism, thereby having more predictable kinetics and this is obviously a major advantage over morphine in terms of drug administration. Unfortunately M6G may retain the unwanted side effects of morphine, such as dependance and respiratory depression.

Secondly the project was to evaluate synthetic analogues of M6G designed in the hope that they would give a more predictable level of pain relief, similar to M6G, and would retain potency over a long duration. It would be hoped that these compounds would also show a reduced incidence of side-effects.

The approach was to determine the affinity of M6G and 6-derivatives for opiate receptors and the efficacy of M6G and related compounds in various isolated tissue models. Also to study the in vivo activity of compounds in antinociceptive tests and correlate this with distribution studies within the CNS.
CHAPTER 2
MATERIALS AND METHODS
2.1 Materials

Equipment

Brandell cell harvester M-48R (Brandell, U.S.A.);
Minaxi Tricarb 4000 Series Liquid Scintillation Counter (United Technologies, Packard USA);
LKB Bromma 2330 Beckman ultraspin 55 centrifuge, USA (Scientific and Research Instruments (SRI);
Square wave stimulators; Grass S88; Square Wave Stimulator (Grass medical instruments, Quincy, Mass., U.S.A.);
Washington 400 MD2R chart recorder; Isotonic transducers (Harvard bioscience);
Kinematica Polytron PTA 10TS homogeniser (Kinematica of Switzerland);
Gilson HPLC system with Gilson Holochrome UV variable wavelength detector, Altex ODS C₈ reverse phase column and C₁₈ reverse phase column (Anachem, Herts).

Animals

Male mice (CSI strain) (30-50g), male Wistar rats (200-250g) and male syrian hamsters (140-200g) were from Nottingham University Medical School. Male Dunkin-Hartley guinea-pigs (400-500g) were from Arnold Hall, Burton-on-Trent.
All animals were fed on a standard laboratory diet and kept on a 12 hr light/dark cycle at a temperature of 20°C.

Chemicals

Peptides

Tyr-[D-Ala²GlyNMePhe⁴Gly-oISjenkephalin (DAMGO) and

Tyr-[D-Pen-Gly-Phe-D-Pen]enkephalin (DPDPE) (Sigma).

Drugs

Morphine hydrochloride (a gift from Macfarlan Smith); morphine-6-glucuronide (Ultrasine chemicals); morphine-3-glucuronide, naloxone (Sigma chemical company); normorphine (a gift from Prof. H.W. Kosterlitz); β-funaltrexamine (a gift from Glaxo Pharmaceuticals); (-)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzene acetamide
Novel opiate compounds were synthesised at Loughborough University (Anna DiPretoro), or at Bristol University (John Lewis).

All drugs and peptides were made up as 10mM stock solutions in water, except for the compounds synthesised at Loughborough University, which were made up in dimethyl sulphoxide (DMSO) and diluted with water or buffer as appropriate, and stored at -20°C. For in vivo studies drugs were made up in sterile saline or if less soluble, in saline containing 0.25% carboxymethyl cellulose.

Buffers and Biochemicals
Tris[hydroxymethyl]aminomethane hydrochloride (Tris), N-ethylmaleimide (NEM); N-2-hydroxyethylpiperazine-N'ethanesulphonic acid (HEPES); 5'-guanylimidodiphosphate (Gpp(NH)p); Isocitrate (trisodium salt); isocitrate dehydrogenase; β-nicotinamide adenine dinucleotide (β-NADP); uridine 5'-diphosphoglucuronic acid; nicotinamide; magnesium chloride. All chemicals were of analytical grade and obtained from Sigma chemical company.

Other chemicals
Ecoscint scintillation fluid (National Diagnostics); acetonitrile and trichloroacetic acid (both HPLC grade) (Fisons plc.).

Radiochemicals
[^3]H]DAMGO (60Ci/mmole) (Figure 2.1),[^3]H]DPDPE (40Ci/mmole) (Figure 2.2),[^3]H]U69593(60Ci/mmole)(Figure 2.3), and[^3]H]Naloxone (52Ci/mmole) were from Amersham International plc.,[^3]H]CTOP (64.4Ci/mmole)(Figure 2.4) (New England Nuclear) and[^3]H]CI977 (21.1Ci/mmole) (Figure 2.5) (Amersham International plc).
Tritiated ligands used:

\[ ^3H \]3,5 Tyr-D-AlaGlyNMePheGly-ol

Figure 2.1 \([^3H]DAMGO\)

\[ ^3H \]3,5 Tyr-D-Pen-Gly-Phe-D-Pen

Figure 2.2 \([^3H]DPDPE\)

\[ ^3H \]D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2

Figure 2.4 \([^3H]CTOP\)

\[ ^3H \]D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2

Figure 2.3 \([^3H]U69593\)

\[ ^3H \]CI977

Figure 2.5 \([^3H]CI977\)
Solutions

Tris buffer was prepared as 50mM in distilled water and the pH adjusted to 7.4 with HCl(4N). Tris-NaCl buffer contained 100mM NaCl.

Krebs buffer solution for the myenteric plexus longitudinal muscle (MPLM) and the rat vas deferens (RVD) preparations comprised the following:
- NaCl (6.92g/l), KCl (0.35g/l), KH₂PO₄ (0.16g/l), CaCl₂.2H₂O (0.375g/l for MPLM and 0.188g/l for RVD), NaHCO₃ (2.1g/l), MgSO₄.7H₂O (0.29g/l) and glucose (2g/l). The buffer was gassed with 95% O₂, 5% CO₂.

Krebs solution for the mouse vas deferens (MVD) preparation was as above, but with the omission of MgSO₄.7H₂O (Ward et al. 1986). Krebs/HEPES buffer was made up as Krebs buffer (above) with HEPES at a concentration of 25mM adjusted to pH 7.4 with 0.88M ammonia solution.

2.2 Methods

2.2.1 Ligand binding assays

(i) Brain homogenates

Brains were removed from male CSI mice (discarding the cerebellum) and homogenised in Tris buffer (50mM, pH 7.4) at 10% tissue w/v. The homogenate was centrifuged at 25,500g for 20 mins. The supernatant was discarded and the pellet resuspended in buffer. The suspension was incubated at 37°C for 30 mins, then recentrifuged. The pellet obtained was resuspended in buffer to obtain a 1:60 tissue w/v ratio. This dilution corresponds to a protein concentration of approximately 1mg/ml as determined by Lowry's method (Lowry et al. 1951).

For saturation binding assays, tubes were set up containing 20µl of tritiated ligand and 960µl of brain homogenate in a total volume of 1ml. 20µl of naloxone (10µM) was added to each tube to determine the non-specific binding. For competition assays, tubes contained labelled ligand (usually 1.0nM final concentration) plus increasing concentrations of competing cold ligand, or 20µl of water or 20µl of naloxone (10µM), which represented the total bound ligand and non-specifically bound ligand values respectively. Assay tubes were incubated at 25°C for 40 mins, unless stated...
otherwise. At the end of the incubation period the tube contents were filtered through glass filter papers (Whatman GFB) which were presoaked in either Tris buffer pH 7.4 or Tris-buffer pH 7.4 containing polyethyleneimine (0.1%) to reduce non-specific binding to filters. The tubes were washed three times with 3ml of ice cold Tris buffer and the washings were also filtered. The filters were placed in scintillation vials, ecoscint scintillant fluid added, and the filters soaked for 8 h. The radioactivity remaining on the filters was counted in a Minaxi Tricarb 4000 Series Liquid Scintillation Counter at an efficiency of 58%.

Binding parameters $K_D$ and $B_{max}$ were obtained using the LIGAND programme, following Scatchard analysis using the EBDA programme (McPherson, 1985). IC$_{50}$ values for competing ligands were determined using a logistic curve fitting programme developed by Barlow (1991).

(ii) Guinea-pig myenteric plexus longitudinal muscle homogenate

Guinea-pigs were killed by cervical dislocation and the ileum rapidly removed and placed in ice-cold Krebs solution. The myenteric plexus-longitudinal muscle was carefully detached from the ileum, washed, chopped and homogenised in ice-cold Tris buffer (50mM, pH 7.4). The homogenate was prepared as above but the final resuspension corresponded to approximately 2mg/ml of protein. Binding assays were performed as above.

2.2.2 Alkylation by β-funaltrexamine (β-FNA)

Homogenates of mouse brain were prepared in Tris buffer and Tris/NaCl buffer as described in 2.2.1(i) and incubated for 60 mins at 37°C with β-FNA (100nM). Ligand binding assays were performed after four washes in Tris buffer, including an incubation at 37°C for 60 mins to remove non-irreversibly bound β-FNA.
2.3 Isolated tissue studies

2.3.1 Tissue preparation

a) Guinea-pig myenteric plexus-longitudinal muscle (MPLM) bioassay

Male Dunkin-Hartley guinea-pigs (400-500g) were killed by cervical dislocation. The ileum was removed and immediately placed in aerated Krebs solution at room temperature (Ward et al. 1986). After flushing out the contents, strips of myenteric plexus longitudinal muscle (MPLM) were removed and mounted, under a tension of 1g, in 3ml organ baths previously coated with silicon to reduce adsorption of peptides onto the glass surface. Tissues were bathed in Krebs solution at 37°C, aerated with 5% CO₂ in 95% O₂. After allowing a recovery period of 1 h, each tissue was stimulated through platinum ring electrodes using square wave pulses at supramaximal voltage at a frequency of 0.16Hz and a pulse width of 400μs.

b) Mouse, hamster and rat vas deferens preparation

Male C57 mice 930-50g), male Syrian hamsters (140-200g) or male Wistar rats (200-250g) were killed by cervical dislocation. The vasa deferentia were removed immediately, and mounted under a tension of 0.5g in 1.8ml organ baths, previously coated with silicon to reduce adsorption losses of peptides. Tissues were bathed in Krebs with MgSO₄ (rat) or without MgSO₄ (mouse and hamster) at 37°C, aerated with 5% CO₂ in 95% O₂. After allowing a recovery period of 1 h, each vas deferens was stimulated through platinum ring electrodes using square wave pulses at supramaximal voltage at a frequency of 0.1Hz and a pulse width of 250μs (rat) or using a train of 3 square wave pulses of 1ms duration and 250ms delay at supramaximal voltage at a frequency of 0.1Hz (mouse and hamster).

2.3.2 Experimental

For all in vitro preparations the same procedure was performed as follows:-

a) Agonist potencies

Agonists were added to the organ baths in a cumulative way such that when the response to any one dose reached a maximum the next dose was administered, until approximately 80% inhibition of twitch height was
attained after about four cumulative doses. The tissues were washed by overflow with Krebs solution until the original twitch height was restored. The potency of agonists was assessed by measurement of IC$_{50}$'s, the concentration of agonist causing 50% inhibition of the electrically evoked twitch.

b) **Antagonist affinities**

Antagonists were preincubated with the appropriate tissue for 15 mins, prior to the addition of an agonist. Dose-response curves for agonists were obtained before the addition of an antagonist and then repeated in the presence of varying concentrations of the antagonist (normally 10, 30, 100nM). Dose-ratios were calculated at 50% inhibition and Schild plots constructed. Antagonists were removed from the tissue by continuous washing until the response to the added agonist was fully recovered. In some experiments antagonist K$_e$ values were calculated using a single-dose method. The antagonist equilibrium dissociation constant (K$_e$) is a measure of affinity and was determined for a partial agonist in the rat vas deferens by pre-incubating the test compound for 15 mins and observing the effect on the dose-response curve for the full μ agonist DAMGO. Antagonist equilibrium dissociation constants (K$_e$) were obtained by analysing the results according to Kosterlitz and Watt (1968) (see Chapter 3).

c) **Alkylation by β-FNA and NEM in the MPLM**

The agonist potency of ligands were first determined, then tissues washed and β-FNA(100nM) added for a contact time of 60 mins. The β-FNA was then removed by continuous washing for about 60 mins. The agonist potency of ligands was then remeasured. The effect of β-FNA on agonist potency was expressed as a dose-ratio shift. This was also used to determine the affinity of compounds by the method of Furchgott (1966) (see Chapter 3). In some experiments NEM was used as an alkylating agent. Tissues were treated with 10μM NEM as for β-FNA.
2.4 Metabolism studies

2.4.1 Preparation of liver microsomes

Livers taken from various male animals (CSI mice, (30-50g), Wistar rats (200-250g), Dunkin-Hartley guinea-pigs (400-500g) and New Zealand white rabbits (1500-1750g) were washed in 0.25M sucrose, and blotted dry on filter paper. The livers were chopped using scissors, diluted four-fold with 0.25M sucrose, homogenised with a glass pestle and centrifuged at 12,500g for 10 mins. The supernatant was taken and recentrifuged for 60mins at 100,000g to afford a microsomal fraction. The pellet was resuspended in Tris (2.5X w/v) (50mM, pH7.4). The microsomes were either used immediately or stored in liquid nitrogen. For some experiments mice were treated with phenobarbital (80mg/kg i.p.) for 4 days, before the livers were removed (Puig & Tephly, 1974).

2.4.2 Morphine metabolism

The method of Sanchez and Tephly (1974) was used with some modifications: -

The assay conditions were UDPGA (5.0mM), MgCl₂ (5.0mM), Tris HCl (50mM), pH 7.4 at 37°C, and microsomal protein (4.0mg), morphine (1.5mM) in a total volume of 1.0ml. In some experiments Triton X-100 (2.5%) was also used. Control reactions had no UDPGA or contained boiled protein. Reactions took place on a shaking water bath (37°C) and were stopped after 30 mins by the addition of 100µl of 10% trichloroacetic acid to a 50µl aliquot of the assay mixture, and centrifuged briefly on an ultracentrifuge to precipitate protein. Supernatants (10µl) used for HPLC were injected onto the HPLC column, after addition of a spike (10µg of morphine), to enable the assay to be quantitative.

2.5 Separation of morphine and its metabolites

2.5.1 Methods available for studying morphine metabolites

Several sensitive methods exist for the determination of low levels of morphine and its metabolites, in biological fluids. Radioimmunoassays can detect levels down to picogram amounts, but until relatively recently lacked specificity and could not differentiate between morphine and structurally related morphine metabolites (Berkowitz et al, 1974). Thus the
need for specific antisera. However Hand et al (1987) reported the analysis of morphine, M3G and M6G by differential radioimmunoassay, using iodinated labels and three different antisera in man. Lee et al (1991) described a sensitive and specific RIA for morphine. The limit of detection was 0.3ng/ml in plasma for morphine, but with cross-reactivity occurring at 0.7% with hydromorphone-3-glucuronide.

Spectrofluorometric and radiochemical morphine assays also have low specificity unless morphine is purified before analysis (Garrett & Gurkan, 1978), although Colbert et al (1988) described a simple polarisation fluoroimmunoassay to detect the opiate group of drugs in urine. Gas chromatography (GC) with electron-capture detection (ECD) required extraction and the formation of a volatile morphine derivative before measurements can be taken (Wallace et al, 1980). Tasker & Nakatsu (1986) described a GC-MS method, but the equipment was expensive. Wallace (1980) also reported the use of HPLC with electrochemical detection with ng/ml sensitivity, greater than GC-ECD. Todd et al (1982) used HPLC-ECD to detect down to 20pg of morphine. Svensson et al (1982) reported the use of ion-pair HPLC to determine morphine, M6G, and normorphine. This procedure was sensitive to interference and only allowed the tentative identification of M6G. More recently Svensson (1986) detected morphine, M6G, and normorphine, but the quantitative detection of these compounds using ECD was not possible due to the differing redox potentials of the compounds. Venn et al (1989) reported on HPLC with native fluorescence detection. The limit of detection quoted was 0.5ng. It was a successful method of separating morphine, M3G, M6G and codeine, being very fast and specific. A variation of this method, with UV detection was used:

5.5.2 Separation analysed using HPLC

Metabolites were actually separated using an Altex Ultrasphere RP C8 column, with a precolumn of the same material, using a flow rate of 1ml/min. HPLC was used with UV detection. The E\text{max} for morphine and normorphine is reported to be 285nm in aqueous acid (Clarke, 1986), but 225nm was found to be the most sensitive wavelength. The solvent system consisted of a gradient containing different proportions of CH\textsubscript{3}CN (0.1%TFA) and H\textsubscript{2}O (0.1%TFA), over a time period of 30 mins.

The initial objective was to find a suitable method for separating morphine and its metabolites. For quantitative purposes a spike was used in samples
before injection (morphine, 10μg), after the use of phenytoin and phenobarbital as an internal standard (Lear et al, 1991) proved to be inappropriate due to extremely long retention times.

Figure 2.5 Effect of acetonitrile concentration on retention time for normorphine (open circles), M3G (closed circles), M6G (closed circles) & morphine (open squares) under isocratic elution

From Figure 2.5 it can be seen that 2% CH₃CN gave the greatest peak separation of morphine and its metabolites, but the retention times were long. At concentrations above this the retention times were shorter, but inadequate separation occurred. A gradient system was thus devised (Figure 2.6) to give maximum separation of the compounds, and varied from 2 to 10% and back to 2% acetonitrile over 25 mins. Retention times obtained using this gradient system were 8.2±0.2, 9.8±0.1, 12.0±0.2 and 14.0±0.2 mins for M3G, normorphine, M6G and morphine respectively.
Calibration curves were obtained for morphine (Figure 2.7), M6G (Figure 2.8), M3G (Figure 2.9) and normorphine (Figure 2.10), and these four compounds were successfully separated using this gradient system (Figure 2.6). A less sensitive setting was used for morphine because of the quantities of morphine to be detected.
Figure 2.7 Calibration curve for morphine

\[ y = -2.6521 + 1.4707x \quad R^2 = 0.997 \]

Figure 2.8 Calibration curve for M6G

\[ y = 1.0192 + 1017.9x \quad R^2 = 1.000 \]
Figure 2.9 Calibration curve for M3G

Figure 2.10 Calibration curve for normorphine
2.6 Lipophilicities of morphine and related compounds

Drugs were made up in phosphate buffer pH 7.4 (1mg/ml) and 1ml of octanol was added, the flask stoppered, and shaken for 24 h at room temperature, centrifuged and the UV absorbance of the resulting two layers was read at 285nM. \( \log P \) is a measure of a compounds lipophilicity and was calculated as the logarithm of the ratio of the absorbance in octanol to that of phosphate buffer.

In order to determine lipophilicities of a variety of compounds the retention times of test compounds were determined using a Gilson HPLC system with a C18 RP column at 280nm. An isocratic solvent system was used of 16% CH3CN in H2O at a flow rate of 1ml/min. A graph was constructed of \( \log P \) against \( \log k' \) (\( \log k' \) is the capacity factor for the column and is defined as the difference between the retention time for test and reference compounds, divided by the retention time for the reference compound) for morphine, codeine, heroin and methadone and from this \( \log P \) values were interpolated from \( \log k' \) values for the test compounds. The shortest retention time is used as the reference compound, and in this case is morphine-6-fluorobenzoate.

2.7 Stability of morphine and related compounds

The stability of several synthetic compounds was qualitatively determined by TLC using silica gel 60 F254 as the stationary phase and a mobile phase of 75% CHCl3/25% MeOH/3 drops NH3. Compounds to be examined from aqueous solution were extracted by the method of Shimomura et al (1971) as follows: - 100\( \mu l \) of 10mM stock solution of compound was mixed with 900\( \mu l \) of homogenate (1:60 w/v tissue concentration) or Tris buffer pH 7.4 in a glass stoppered test tube. After 30 mins at 25°C, 3 drops of ammonia were added, then 1ml chloroform:isopropanol (3:1) and the tube vortexed. The organic (bottom) layer was taken and the extraction step was repeated on the aqueous layer. The organic extracts were combined and dried over magnesium sulphate. An aliquot (500\( \mu l \)) was taken, the solvent was blown off with nitrogen and the residue resuspended in 100\( \mu l \) of methanol and spotted on a TLC plate.
2.8 *In vivo* experiments

2.8.1 Antinociceptive properties of morphine and related compounds

Morphine sulphate and related compounds were administered s.c. to C57Bl/6 mice. Six control mice (injected with vehicle) and 6 test mice were used for each study. Analgesic activity was assessed using the tail-flick test with tail immersion in water at 50°C. The cut off time was 10s. The time to reach maximal analgesia was deduced and ED50 values were obtained for each drug as a measure of potency. The saline points were subtracted from the test points and the dose required to give 50% of the maximal tail-flick latency (ED50) was deduced.

2.8.2 Distribution of morphine and M6G into the CNS

The distribution of morphine and morphine-6-glucuronide into the brain and spinal cord was determined using an *ex-vivo* binding assay as follows: Mice were killed by cervical dislocation 60 mins after s.c. injection of morphine (5mg/kg) or M6G (5mg/kg). The brains and spinal cords were rapidly removed and placed in liquid nitrogen. Control brains from saline injected animals were pooled, homogenised and used to construct a morphine calibration curve. This calibration curve was used to determine the amount of morphine present in the test brain, by interpolating the specific binding value obtained from the test brain to give a morphine concentration. Control cords were treated in a similar manner, but a full dose-response curve was not obtained because the mouse spinal cords did not weigh very much. Analgesia was assessed using the tail flick test at 50°C as above. Tail flick tests were performed before dosing and before the mice were killed. Six mice were used for controls (saline) and six for treatments. The penetration of compounds into the CNS was assessed in terms of 'morphine equivalents' present in nM, which was interpolated from a calibration curve of % specific [3H]DAMGO bound versus morphine concentration performed in mouse brain or spinal cord homogenate.
CHAPTER 3

ANALYSIS OF BINDING AND ISOLATED TISSUE DATA
3.1 Radioreceptor assays

Binding assays are based on the principle of the Law of Mass Action where a simple bimolecular interaction between a drug and its receptor are considered. Assuming the binding of the ligand (L) to its receptor (R) follows this law then at equilibrium for a reversible ligand-receptor interaction, where [LR] = concentration of ligand-receptor complex and

\[ k_{+1} \]

\[ a[L] + b[R] \xrightarrow{k_{-1}} c[LR] \]  \hspace{1cm} [1]

\[ a, b, \text{and} c \text{ represent the stoichiometry of the reaction. At steady state or equilibrium, the rate of the forward reaction equals the rate of the reverse reaction} \]

\[ k_{+1}[L]a[R]b = k_{-1}[LR]c \]  \hspace{1cm} [2]

Therefore the association binding constant, \( K_A \), and the dissociation binding constant, \( K_D \), are as below

\[ K_A = k_{+1} = \frac{[LR]c}{[L]a[R]b} \quad \quad K_D = k_{-1} = \frac{[L]a[R]b}{[LR]c} \]  \hspace{1cm} [3] \& [4]

so \( K_A \) and \( K_D \) can also be determined under steady-state conditions. \( K_D \) is the equilibrium dissociation constant and is an indication of the affinity of the radioligand for its binding site.

There is a maximum number of specific receptor sites per unit of tissue, \( B_{\text{max}} \), such that :-

\[ [LR] + [R] = B_{\text{max}} \]  \hspace{1cm} [5]
\[ [LR] = \frac{B_{\text{max}}[L]}{[L] + K_D} \]  

Now if \([RL]\) is bound ligand = B and, \([L]\) is free ligand = F

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

we get the Scatchard plot (Figure 3.1)

The assumption is made that :-

i) \([L]\) = mass / volume,

ii) one molecule of L combines with one molecule of R,

iii) the response is proportional to \([LR]\), and,

iv) both L and R sites are homogeneous species.

There are 2 stages in the determination of the equilibrium constant \((K_D)\) and apparent maximum number of binding sites \((B_{\text{max}})\) for a given tissue receptor and radioligand:
i) incubate the radioligand at various concentrations with a fixed concentration of tissue, 

ii) determine the amount of bound and free ligand by employing a suitable separation technique - usually filtration as used in this study or centrifugation.

Unfortunately radiolabelled ligands often bind non-specifically to both biological and non-biological matter, e.g. non-receptor tissue, filters and test tubes. A measure of the specific binding is given by the difference between total binding and the binding that occurs in the presence of a large excess of unlabelled ligand. The unlabelled competitive ligand is better if it is chemically very different to the radioligand so that it does not interact at the same non-specific receptor site. In current studies all non-specific binding is defined using the opioid antagonist naloxone at a concentration of 10μM.

As mentioned previously $K_D$ is a ratio of the binding reaction's reverse and forward rate constants ($K_D = k_{-1} / k_{+1}$). It is possible to determine these rate constants and thus $K_D$ can be verified.

3.1.1 Determination of inhibitor binding constants ($K_i$)

Dissociation constants determined as inhibition constants ($K_i$) are obtained from competition curves which are generated by incubating a fixed concentration of radioligand with increasing concentrations of unlabelled ligand and finding the concentration of unlabelled ligand required to displace 50% of specific radioligand binding, the IC$_{50}$:

$$K_i = \frac{IC_{50}}{1 + [L]/K_D}$$

[8]

if labelled and unlabelled ligand interact competitively at the receptor site (where $K_i$ is the binding affinity of the unlabelled ligand).

The IC$_{50}$ can be obtained from a Hill plot of the data (Figure 3.2) or by curve-fitting (Barlow, 1991).
Figure 3.2 Hill plot

where Bo = no unlabelled competing ligand, and
B = with competing ligand

The concentration of free competing ligand is difficult to determine and is approximated to the value of the total concentration of competing ligand. This can be done if the amount of bound competing ligand is low compared to $K_i$ (Bowman & Rand, 1980 ii).

3.2 In vitro bioassays

3.2.1 Antagonist potencies

3.2.1.1 Using the Schild Plot

The Schild Plot

In order to define a receptor at which an agonist acts it is necessary to employ an antagonist, and the ability of the antagonist to compete with an agonist at that receptor is determined as the value described either as a $K_e$ or as a $pA_2$. These values are a measure of the affinity of the antagonist for the receptor. The $K_e$ value can act as an indicator of the receptor selectivity for the agonist.
A competitive antagonist can be regarded as a drug that interacts reversibly with a set of receptors to form a complex, but unlike an agonist-receptor complex, it fails to elicit a response (i.e., it exhibits no intrinsic activity). The antagonist-receptor complex can therefore be characterised by a dissociation constant, and expressed as:

\[
\begin{array}{c}
\text{Antagonist + Receptor} \\
\text{[A] [R]} \\
\hline
\text{Antagonist-Receptor Complex} \\
\text{[AR]} \\
\end{array}
\]

\[
K_1 \frac{[A][R]}{[AR]} = K_2
\]

where \( K_1 \) and \( K_2 \) are the association and dissociation rates for the complex.

According to the law of mass action, the rates of the forward and the reverse reactions are the same once equilibrium is attained therefore:

\[
K_1 [A][R] = K_2 [AR]
\]

and:

\[
K_1 = K_c = \frac{[A][R]}{[AR]}
\]

where \( K_c \) is the antagonist dissociation constant.

When both an antagonist \([A]\) and an agonist \([L]\) are present, the various interactions with receptors can be expressed:

\[
[L] + [A] + [R] \rightleftharpoons [AR] + [LR]
\]

where \([L]\) is the concentration of agonist and \([LR]\) is the concentration of the agonist-receptor complex.

If the total number of receptors is \([R_t]\), then the number of free receptors \([R]\) can be expressed as:
\[ [R] = [R_t] - [AR] - [LR] \]

Dividing through by \([LR]\)

\[ \frac{[R]}{[LR]} = \frac{[R_t]}{[LR]} - \frac{[AR]}{[LR]} - 1 \]

\[ \Rightarrow \frac{[R]}{[LR]} = \frac{[R_t]}{[LR]} - \frac{AR}{[LR]} - I \]

\[ [LR] = [LR] \]

The principles applied to the antagonist are valid for an agonist therefore:

\[ K_D = \frac{[L][R]}{[LR]} \quad \text{ie} \quad [R] = K_D \]

\[ \frac{[L]}{[LR]} \quad \frac{[R]}{[LR]} \quad \frac{[L]}{[LR]} \]

\[ [11] \]

where \(K_D\) is the dissociation constant for the agonist.

Substituting equations \([11]\) and then \([9]\) into \([10]\):

\[ K_D = \frac{[R_t]}{[L]} - \frac{[AR]}{[LR]} - 1 \]

\[ \frac{[L]}{[LR]} \quad \frac{[R]}{[LR]} \quad \frac{[L]}{[LR]} \]

Since \[ [AR] = [A][R] \quad \text{(from [9])} \]

\[ K_D = \frac{[R_t]}{[L]} - \frac{[A]K_D}{[LR]} - 1 \]

\[ \frac{[L]}{[LR]} \quad \frac{[R]}{[LR]} \quad K_D[K_c] \]

which reduces and rearranges to:


\[ \frac{[R_t]}{[LR]} + \frac{K_D[A]}{K_c[L]} \]

\[ [12] \]

The reciprocal of this equation gives the fraction of receptors occupied by an agonist in terms of concentrations and dissociation constants of agonist and antagonist. Assuming that \([LR]/[R_t].\text{ie} \) the proportion of receptors occupied by the agonist is equal to \(E/E_{\text{max}}\) (the ratio of effect produced by a
given dose of agonist to the maximal possible effect) then the reciprocal of the equation expresses any given response to an agonist as a fraction of the maximum possible response. When the concentration of the antagonist is zero then equation [12] simplifies to:

\[
\frac{[L_R]}{[R_i]} = \frac{[L]}{[L] + K_D}
\]  

Equation [12] also predicts that the linear portion of the agonist concentration response curves carried out in the presence and absence of a competitive antagonist will be parallel, but displaced to the right in the presence of antagonist. The important feature of competitive antagonism is that it may be overcome by increasing concentrations of agonist, i.e. the maximum response is not affected by a competitive antagonist. The degree of shift to the right of the agonist logarithmic concentration response curve is proportional to the antagonist concentration and to the affinity of the antagonist for the receptor.

The affinity of the antagonist for any given receptor type is inversely proportional to the antagonist-receptor dissociation constant $K_e$. The value of the $K_e$ can be determined from the concentration of agonist producing equal responses in the absence $[L_0]$, and the presence $[L_A]$ of antagonist. Since the response to the agonist is equal, the proportion of receptors occupied is assumed to be the same.

Therefore from equations [12] and [13]:

\[
\frac{[L_0]}{[L_0] + K_D} = \frac{K_e [L_A]}{K_D K_e + K_D [A] + K_e [L_A]}
\]

which can be rearranged and reduced to:

\[
\frac{[L_A]^{-1}}{[L_0]} = \frac{[A]}{K_e}
\]
The value of the $K_e$ is independent of the agonist used provided the agonist competes for the receptor. When the concentration of antagonist ($[A_2]$) is such that:

$$[L_A] = 2[L_0]$$

then

$$[A_2] = K_e$$

ie the value of the dissociation constant for the antagonist is the concentration of antagonist with which the ratio of concentrations of agonist producing equal responses in its presence $[L_A]$ and absence $[L_0]$ equals 2.

The negative logarithm of the molar concentration of antagonist with which the ratio of equi-effective concentrations of agonist in the presence and absence of antagonist is 2, has been designated by Schild as the $pA_2$ value, thus:

$$pA_2 = -\log[A_2] = \log[1/[A_2]] \quad [14]$$

Equation [14] can be converted to a form containing $pA_x$ where $pA_x$ is the negative logarithm of the molar concentration of antagonist in the presence of which the potency of the agonist is decreased $x$ times.

$$x - 1 = \frac{[A_x]}{K_e}$$

taking logs

$$\log[x - 1] = \log[A_x] - \log K_e \quad [15]$$

Equation [15] predicts that the plot of $\log[x - 1]$ versus $\log[A_x]$ is a straight line, the intercept on the $\log[A]$ axis giving the value of $\log K_e$ or $-pA_2$. The slope of the line is 1 provided the agonist is acting at a single receptor class. Deviation from the unit slope is consistent with a system of more than one receptor class. It should be noted that the slope of the Schild plot ought to be unity if a selective agonist has been displaced from a single receptor (Bowman & Rand, 1980 ii).
Departures from linearity for the double log relationship can be due to:
(a) Tissue uptake of agonist,
(b) The use of insufficient incubation times for the antagonist resulting in a non-equilibrium situation,
(c) The use of antagonists that are toxic.
The first two problems result in slopes that are less than unity, whereas problem (c) gives a slope greater than unity.

3.2.1.2 Antagonist potencies where partial agonism is shown in the rat vas deferens (Figure 3.3)

Kosterlitz & Watt (1968) showed that all narcotic analgesic drugs have dual agonist and antagonist actions, whether they are used clinically as 'agonists' or 'antagonists'.

The equilibrium constant ($K_e$) as an indication of the drugs antagonist activity is defined as:

$$K_e = \frac{a(1-y)}{y} \quad [16]$$

where $a$ is the molar concentration of the antagonist and $y$ is the fraction of receptors occupied which is determined from the dose-ratio (DR), that is, the ratio of the concentration of the agonist, morphine, required to depress the twitch in the presence or absence of a given concentration of an antagonist. Since:

$$y = \frac{DR-1}{DR} \quad K_e = a \quad [17] & [18]$$

The dose-ratio is determined as shown in Figure 3.3.
Figure 3.3 Measurement of the antagonist affinity of a partial agonist by the determination of its equilibrium constant, $K_e$. At the arrow marked antagonist, the partial agonist is added and produces a depression of the twitch equal to a depression caused by a full agonist (e.g. DAMGO) in concentration $M_1$. The full agonist was added 20 mins later to give a concentration $M_3$, the total depression was equal to a reduction in the height of the twitch caused by the full agonist in a concentration $M_2$ in the absence of the antagonist.

\[
DR = \frac{M_3}{M_2 - M_1} \quad [19]
\]
3.2.2 Determination of agonist potency using Furchgott's analysis

### 3.2.2.1 Efficacy and spare receptors

Assuming the Law of Mass Action is obeyed

\[
[LR] = \frac{[L]}{R_1} \quad [20]
\]

where \([L]\) is the concentration of agonist

\(K_e\) is the equilibrium dissociation constant of drug for its receptor

However only fractional occupancy of the total receptor population may be required to elicit a maximal response. Stephenson (1956) suggested the biological response was a function of stimulus \((S)\) generated by interaction of ligand and receptor. The relationship between stimulus and response is 50% of the maximal response achievable, thus :-

\[
E_a = \frac{f(s)}{E_m} = \frac{fe[LR]}{fe[L]} \quad [21]
\]

where \(f(s)\) is a function of the stimulus and \(e\) is the efficacy, relating stimulus to occupancy. Efficacy may range from 0 to 1. If \(e=0\) then \(f(s) = 0\) and the drug will bind but cause no biological response i.e. act as an antagonist.

Experimental evidence has provided support for the spare receptor concept. Exposure of isolated tissues to irreversible receptor antagonists e.g. \(\beta\)-funaltrexamine (\(\beta\)-FNA), and \(\beta\)-chloralnaltrexamine (\(\beta\)-CNA) causes parallel shifts in opioid potency with no change in maximal response until high dosage (Williams & North, 1984; Lajtha, 1989).

Furchgott (1966) argued that efficacy, as defined in equation [21], is drug and tissue dependent, and reflects the ability of the agonist to induce an active receptor-effector complex. Thus:

\[
e = \frac{E[R_1]}{E} \quad [22]
\]
Intrinsic efficacy, E, should be constant for a given drug-receptor pair across species and tissues, whereas e will vary with receptor density.

The various tissue and drug-related factors associated with agonist response may be summarised as follows

\[
\frac{E_a}{E_m} = \frac{f E[R] [L]}{[L] + K_c} \tag{23}
\]

Agonist activity is apparently dependent upon the following factors:

1. Agonist dissociation constant \(K_c\).
2. Intrinsic activity, E, of the agonist.
3. The function \(f\) relating stimulus (s) to response.
4. The total receptor concentration \([R]\).

3.2.2.2 Furchgott’s method

Considering equation (23) it is clear that IC\(_{50}\) values are not a reliable indication of agonist affinity. The Furchgott method (Chapter 2, page 32) involves partial inactivation of receptor numbers, using an irreversible antagonist, to measure \(K_c\). The basis of this approach is that equiactive concentrations of agonist before ([A]) and after([A']) inactivation produce an equivalent biological stimulus (Furchgott, 1966).

Thus

\[
\frac{E_a}{E_m} = \frac{E_a'}{E_m'} \tag{24}
\]

then

\[
f(s) = f(s') \tag{25}
\]

Assuming only receptor number is changed by the irreversible ligand then
\[
e [A] = qe [A'] \quad [26]
\]

where \( q \) is the fraction of free receptors (ie. those not blocked). This simplifies to

\[
\frac{1}{[A]} = \frac{1}{q [A']} + \frac{1 - q}{K_e} \quad [27]
\]

A plot of \( 1/[A] \) versus \( 1/[A'] \) will yield a straight line with a slope of \( 1/q \) and an intercept of \( 1 - q/K_e \).

Thus:

\[
K_e = \frac{\text{slope} - 1}{\text{intercept}} \quad [28]
\]
CHAPTER 4

IN VIVO ANTINOCICEPTIVE ACTIVITY OF MORPHINE AND ITS GLUCURONIDE
4.1 Introduction

M6G is reputed to be much more potent than morphine when administered centrally, either i.c.v. or i.t., (Paul et al, 1989 i; Frances et al, 1992) whereas when given peripherally they are reported as being equipotent in the mouse tail-flick test (Paul et al, 1989 i). The time course of M6G analgesia is also much longer. The potencies were compared when administered s.c. as was the ability of the compounds to penetrate the CNS. A preliminary investigation of the ability of tissues from various species, including the mouse, to metabolise morphine to M6G was also investigated.

4.2 Results

4.2.1 Antinociceptive properties as measured by the tail-flick test

Morphine and morphine-6-glucuronide (M6G)

Morphine sulphate (5mg/kg) administered s.c. to mice reached the cut off maximum (10 s cut off) analgesia in 60 mins and this level of analgesia was maintained for 60 mins. This can be seen from Figure 4.1. Using a time of 60 mins a dose-response curve to various concentrations of administered (s.c.) morphine was constructed (Figure 4.2). The dose required to afford the maximally allowed analgesia was 5 mg/kg, with a half-maximum (ED$_{50}$) of 2.2mg/kg. ED$_{50}$ values were calculated by subtracting the saline controls from the test curves and the curve re-drawn and is the concentration of drug required to produce a half-maximum tail-flick latency.

In contrast a similar dose of M6G reached maximum in 30 mins, but significant analgesia was still observed after 9 h (Figure 4.3), and maximum allowed analgesia was maintained for 5.5 h. However the potency of M6G was similar to that of morphine (Figure 4.4), affording maximal effect at 5mg/kg and a half-maximum at 1.9mg/kg. The two compounds are compared in Figure 4.5.
Figure 4.1 Time course for morphine (closed circles, 5mg/kg s.c.) and saline controls (open circles) assessed using the mouse tail-flick test. Six mice were used for each point. * Represents $p < 0.05$ indicating that the morphine points are significantly different from the saline points, as determined using the Wilcoxon signed rank test.

Figure 4.2 Dose-response curve for morphine (closed circles) and saline controls (open circles) assessed using the mouse tail-flick test 60 mins after s.c. injection. Six mice were used for each point. * Represents $p < 0.05$ indicating that the morphine treatment is significantly different from the saline treatment, as determined using the Wilcoxon signed rank test.
Figure 4.3 Time course of M6G (closed circles, 5mg/kg s.c.) and saline controls (open circles) assessed using the mouse tail-flick test. Six mice were used for each point. * Represents $p < 0.05$ indicating that the M6G points are significantly different from the saline points, as determined using the Wilcoxon signed rank test.

Figure 4.4 Dose-response curve for M6G (closed circles) induced antinociception and saline controls (open circles) for 60 mins assessed using the mouse tail-flick test. Six mice were used for each point. * Represents $p < 0.05$ indicating that the M6G treatment are significantly different from the saline treatment, as determined using the Wilcoxon signed rank test.
Figure 4.5 Potency of morphine (closed circles), M6G (open circles) compared in the tail flick test.

M6G in which the glucuronide hydroxyls and acid functions were protected as esters, i.e. morphine-6-protected glucuronide (17) (Figure 4.6) was also examined (Figure 4.7).

In vivo analysis of this compound gave very similar results to that of M6G. The time courses were the same (Figure 4.7), as were the ED50's for the two compounds and shape of the dose-response curves were the same (Figure 4.8, Table 4.1).
Figure 4.7 Time course for M6G (protected sugar) (closed circles, 5mg/kg) induced antinociception and saline (0.25% CMC) controls (open circles) assessed using the mouse tail-flick test. Six mice were used for each point. * Represents p < 0.05 indicating that the M6G (protected sugar) treatment is significantly different from the saline (0.25% CMC) treatment, as determined using the Wilcoxon signed rank test.

Figure 4.8 Dose-response curve for M6G (protected sugar) (closed circles, 5mg/kg) and saline (0.25% CMC) controls (open circles) for 60 mins assessed using the mouse tail-flick test. Six mice were used for each point. * Represents p < 0.05 indicating that the M6G (protected sugar) points are significantly different from the saline (0.25% CMC) points, as determined using the Wilcoxon signed rank test.
Table 4.1 Antinociceptive activity comparison of morphine, M6G and M6G (protected). Onset and duration are taken for peak analgesia

<table>
<thead>
<tr>
<th></th>
<th>Onset (mins)</th>
<th>Offset (mins)</th>
<th>Total Duration (mins)</th>
<th>Active Duration (h)</th>
<th>ED50 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>30</td>
<td>270</td>
<td>90</td>
<td>4</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>M6G</td>
<td>30</td>
<td>1050</td>
<td>330</td>
<td>11</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>M6G (protected)</td>
<td>60</td>
<td>1050</td>
<td>300</td>
<td>11</td>
<td>1.5±0.4</td>
</tr>
</tbody>
</table>

*No significant difference was determined by the t-test (p>0.05), † represented the duration of maximal (cut-off) antinociception, and # the length of time latency was significantly above the saline controls. n=6. The onset is the time taken to reach maximal tail-flick latency, whereas the offset is the time taken to go from maximal latency to the saline baseline.

**Morphine-3-glucuronide (M3G)**

The lack of an effect after M3G administration on the tail-flick response over 4 h is shown in Figure 4.9. This confirmed M3G administered on its own did not appear to act as an antinociceptive agent, its profile being not significantly different from that of saline. The effect of M3G co-administration with 3mg/kg morphine s.c is shown in Figure 4.10. No antagonism of morphine analgesia was seen under the conditions employed.

![Figure 4.9 Time course for M3G 10 (open circles), 30 (closed squares) & 100mg/kg (open squares) administered s.c. in the tail-flick test p>0.05 for all concentrations of M3G versus saline (closed circles) using the t-test](image-url)
Figure 4.10 Effect of M3G (30mg/kg) on morphine (closed circles, 3mg/kg) induced analgesia. Saline+morphine (open circles, 3mg/kg) and saline (open squares)

**Codeine and codeine-6-glucuronide (C6G)**

Codeine is a frequently used analgesic and also often present in antitussive preparations. Because M6G had an increased potency over morphine, codeine-6-glucuronide (C6G) may also have an increased potency over codeine. Codeine and C6G were tested in the mouse tail flick test and were seen to have similar duration of action (Figure 4.11, Table 4.2) and potencies (Figure 4.12, Table 4.2), with peak analgesia maintained for 2 h, and an onset of action of 1 h. Codeine retained its analgesia for longer than C6G, but the compounds were equipotent, affording ED50's of 7.8 and 7.2mg/kg respectively (Table 4.2).
Table 4.2 Comparison of the antinociceptive properties of codeine and C6G in the mouse tail-flick test, following s.c. administration

<table>
<thead>
<tr>
<th></th>
<th>ED$_{50}$ (mg/kg)</th>
<th>active (h)</th>
<th>duration (h)</th>
<th>onset (h)</th>
<th>offset (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeine</td>
<td>7.8±0.6</td>
<td>3-4</td>
<td>2.0±0.2</td>
<td>1.0</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>C6G</td>
<td>7.2±0.4</td>
<td>2</td>
<td>2.0±0.2</td>
<td>1.0</td>
<td>1.3±0.2</td>
</tr>
</tbody>
</table>

The active period was the total time that the drug was antinociceptive, whereas the duration of action was the time that the drug gave its maximal permitted analgesia.

Figure 4.11 Time course for codeine (open squares, 30mg/kg), C6G (closed circles, 30mg/kg) and saline controls (open circles) assessed using the mouse tail-flick test. Six mice were used for each point. * Represents p < 0.05 indicating that the codeine and C6G points are significantly different from the saline points, as determined using the Wilcoxon signed rank test.
Figure 4.12 Dose-response curve for codeine (open squares), C6G (closed circles) and saline controls (open circles) assessed 60 mins after s.c administration, using the mouse tail-flick test. Six mice were used for each point. * Represents p < 0.05 indicating that the codeine and C6G treatment are significantly different from the saline treatment, as determined using the Wilcoxon signed rank test.

4.3 Distribution of morphine-like activity in the brain and spinal cord after s.c. administration of morphine and M6G

A direct measure of CNS penetration of a drug was obtained by using ex-vivo binding techniques to assess the brain levels achieved following systemic administration. Investigation of morphine distribution in the brain and spinal cord was determined using an ex-vivo binding assay.

Calibration curves were constructed in mouse brain and spinal cord for a range of morphine concentrations and are shown in Figures 4.13 & 4.14. Slopes for the two displacement curves in mouse brain and spinal cord homogenate are the same. The displacement curve in mouse spinal cord is incomplete because mouse cords did not weigh as much as mouse brains. Thus the points for the cord calibration curve were chosen in the region that was thought to be most relevant.
Figure 4.13 Calibration curve for displacement by morphine of $[^3H]$DAMGO (1.0nM) in mouse brain homogenate for 40 mins at 25°C.

Figure 4.14 Calibration curve for displacement by morphine of $[^3H]$DAMGO (1.0nM) in mouse spinal cord homogenate for 40 mins at 25°C.
From the control curves for mouse brain and spinal cord quantities of morphine in test tissues were determined by interpolating off the curve. A concentration of $6.3\pm1.8\text{nM}$ morphine was found in the brain (this corresponded to a total brain level of $216\text{pmoles/whole brain}$) and $2.7\pm0.8\text{nM}$ morphine was present in the spinal cord 1 h after morphine (5mg/kg) was administered s.c.

Following M6G (5mg/kg s.c.) administration for 1 h $1.8\pm0.2\text{nM}$ and $2.0\pm0.2\text{nM}$ morphine equivalents were found in the brain and spinal cord respectively. Thus about three times as much morphine penetrated into the brain compared to M6G, whereas equivalent amounts of morphine and M6G were found in the spinal cord. The relative CNS penetration, determined by dividing the brain level (in ng/mg protein) by the dose administered, for morphine and M6G, and gave ratios of 7.2 and 2.5 in brain, and 3.1 and 2.8 in spinal cord (Table 4.3). Thus morphine penetrated the brain to a greater extent than M6G, as expected, but this was not found to be the case in the spinal cord.

Table 4.3 Comparison of morphine and M6G in their ability to penetrate into the CNS

<table>
<thead>
<tr>
<th>CNS</th>
<th>Dose (mg/kg)</th>
<th>Level (nM)</th>
<th>Level (ng/mg protein)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Brain</td>
<td>5</td>
<td>$6.3\pm1.8$</td>
<td>36.0</td>
</tr>
<tr>
<td>M6G</td>
<td>Brain</td>
<td>5</td>
<td>$1.8\pm0.2$</td>
<td>12.5</td>
</tr>
<tr>
<td>M</td>
<td>Cord</td>
<td>5</td>
<td>2.7</td>
<td>15.4</td>
</tr>
<tr>
<td>M6G</td>
<td>Cord</td>
<td>5</td>
<td>2.0</td>
<td>13.8</td>
</tr>
</tbody>
</table>

Six mice are used for each experiment, except for the cord where n=1, pooled from 6 mice.

From the time course experiments it was apparent that M6G was much more longer acting than morphine, whilst retaining similar potency, after peripheral administration. M6G was still fully active after 6 h, whereas morphine started to lose activity after 2 h. To determine if M6G's longer duration of action was due to the continued presence of M6G or an alternative mechanism, *ex-vivo* binding assays were also performed 6 h after administration of morphine (5mg/kg), M6G (5mg/kg) or saline. After 6 h $2.5\pm0.4\text{nM}$ morphine equivalents were present in brains of M6G-
treated animals, whereas <0.1nM morphine equivalents were present in brains from the morphine treated animals. Thus morphine-like material was still present 6 h after M6G administration to produce its effects, but not with morphine.

The experiment was repeated following a high dose of morphine sulphate (50mg/kg s.c.) to mice. The animals were killed after 30 mins, at the time of maximal analgesia. A latency of >10s (n=6) was observed for morphine, with a saline control of 3.2±0.2s (n=6). In treated mice 72.9±7.7% (n=6) of [³H]DAMGO was specifically displaced from mouse brain, which corresponded to a morphine concentration of 52.2±6.3nM (n=6). In the spinal cord 70.3±1.1% of [³H]DAMGO was specifically displaced, which corresponded to a morphine concentration of 58.6±2.1nM. Thus when morphine is administered at this higher dose of 50mg/kg to mice by the subcutaneous route, equivalent amounts of morphine penetrated into both the brain and the spinal cord. The difference observed between the two dose of morphine used (5 or 50mg/kg) may be due to the different times of determination used and different kinetics of distribution.

4.4 Metabolism of morphine
Morphine metabolites were separated by HPLC, using the gradient system developed, as described in the methods (Chapter 2). The retention times for M3G, normorphine, M6G and morphine are given in Table 4.4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time/mins (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3G</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>normorphine</td>
<td>9.8 ± 0.1</td>
</tr>
<tr>
<td>M6G</td>
<td>12.0 ± 0.2</td>
</tr>
<tr>
<td>morphine</td>
<td>14.0 ± 0.2</td>
</tr>
</tbody>
</table>

4.4.1 Metabolism in liver homogenates
Glucuronidation of morphine was investigated in livers from several species, namely mouse, guinea-pig, rat and rabbit. Under the conditions used no significant glucuronidation was observed in any of these liver homogenates (Figure 4.15). Glucuronidation was also investigated in other tissue homogenates, namely brain, kidneys and intestines, but no conversion of morphine was observed here either.
Figure 4.15 Lack of morphine metabolism in mouse (black), rat (grey), guinea-pig (black hashed lines) and rabbit (white hashed lines) liver homogenates. Boiled enzyme controls after 6 h afforded values of 98±3, 96±3, 98±2 and 94±6% of the % control morphine values in mouse, rat, guinea-pig and rabbit liver respectively. Values were not significantly different from 100%, as assessed in the student t-test.

Following this livers from phenobarbital pre-treated mice were used (Sanchez & Tephly, 1974; Puig & Tephly, 1974). Livers were removed from mice pretreated with phenobarbital (80mg/kg i.p. for 4 days). The results of a 6 h time course of glucuronide formation in homogenates of these livers are summarised in Table 4.5. Results showed a 20% conversion to M3G, but the 6-glucuronide was detected in only very small quantities (approximately 1.0%).
Table 4.5 Glucuronidation of morphine in homogenates of livers from phenobarbital treated mice

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% recovery of morphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>92.3±2.1</td>
</tr>
<tr>
<td>0.5</td>
<td>92.3±2.1</td>
</tr>
<tr>
<td>1</td>
<td>90.5±3.8</td>
</tr>
<tr>
<td>3</td>
<td>82.9±7.3</td>
</tr>
<tr>
<td>6</td>
<td>72.0±9.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% recovery of M3G</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.2±2.7</td>
</tr>
<tr>
<td>0.5</td>
<td>9.2±0.6</td>
</tr>
<tr>
<td>1</td>
<td>16.4±7.2</td>
</tr>
<tr>
<td>3</td>
<td>25.2±8.8</td>
</tr>
<tr>
<td>6</td>
<td>32.2±8.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% recovery of M6G</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>1</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td>3</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td>6</td>
<td>0.8±0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% recovery of Morphine (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>n.t.</td>
</tr>
<tr>
<td>0.5</td>
<td>101.3±8.8</td>
</tr>
<tr>
<td>1</td>
<td>100.7±0.7</td>
</tr>
<tr>
<td>3</td>
<td>97.8±4.1</td>
</tr>
</tbody>
</table>

n.t. denotes not tested
4.5 Discussion

No glucuronidation of morphine was detected in studies performed here in mouse, guinea-pig, rat or rabbit liver or in mouse brain, kidney or ileum. Possibly 4mg of protein quoted by Sanchez and Tephly (1974) to give them optimal glucuronidation was not adequate in experiments performed here. Lawrence et al (1992) reports that morphine metabolism within the guinea-pig liver produces both M3G and M6G in a ratio 1:4, although the use of the detergent Brij 58 and magnesium ions (15mM) is necessary for optimal production. The findings in mouse kidney are in contrast to Yue et al (1988), that the human kidney does glucuronidate morphine to M3G and M6G, whilst Wahlstroem et al (1988) reports that brain microsomes glucuronidate morphine. Glucuronic acid, a co-substrate for glucuronidation, has been identified by Shashoua et al (1986) in rat brain. The possibility of glucuronidation of morphine within the CNS is an interesting area, and thus needs to be re-examined.

Use of livers from phenobarbital-treated mice did produce glucuronidation. Other authors have shown the need for phenobarbital treatment, either p.o. in the animals drinking water (Hanna et al, 1993) or i.p. (Sanchez & Tephly, 1974; Puig & Tephly, 1974; Rane et al, 1985) or a combination of both (Bock et al, 1980). Phenobarbital increases glucuronyl transferase-type 2(GT2) enzyme glucuronidation by 3-10 fold depending on the method used. Using phenobarbital (80mg/kg i.p. for 4 days) morphine metabolism was observed in vitro, in mouse liver, affording the 3-glucuronide (up to 25% of recovered metabolites after 6 h), but only approximately 1% of the 6-glucuronide. Thus there is no evidence that M6G is important in the mouse. In confirmation of this Yeh et al (1979) reports the amount of M6G to be too small to be determined quantitatively. However in vivo data does show M6G in the urine of humans, rats, monkey, dog, guinea-pigs and rabbits (Yeh et al, 1979; Kuo et al, 1991; Rane et al, 1984; Misra et al, 1970).

Morphine and M6G had similar potencies in the mouse tail flick test, when injected s.c. (ED50's of 2.2 and 1.9mg/kg respectively), but M6G did have a much longer duration of action than morphine, 9 h compared to 2 h. M6G(protected) had a similar potency to M6G (1.5mg/kg) and a similar in vivo profile. It is likely that the protected sugar is rapidly metabolized to M6G in vitro and in vivo and this could be followed up in future. The protected glucuronide could thus be used as a useful replacement to M6G, acting as a prodrug. The synthesis is much easier and more economical (Di
The results for M6G and morphine in the mouse tail-flick test agreed with those of Shimomura et al. (1971) who reported that M6G was 4 times as potent in mice in the hot plate test, with twice the duration (Shimomura et al., 1971), compared with morphine when injected s.c. The duration of the analgesic effect may be a consequence of a lower rate of metabolism of M6G than morphine (Frances et al., 1990). The potencies of M6G and morphine are shown in Table 4.6 for different routes of administration. Morphine and M6G have similar potency when administered s.c. or p.o. However when injected i.c.v. M6G is reported to be 330 times as potent as morphine. In agreement, Paul quotes M6G to be 90 times as potent i.c.v. (20 times in rats) and 650 times as potent as morphine i.t. (Paul et al., 1989), as does Gong et al. (1991), who find M6G to be 200 times more potent when administered centrally and assayed in rats using the tail-flick and hot plate tests, but only 9 times in the writhing test. When given i.c.v. in rats, M6G is 60 times more potent than morphine when examined in the formalin test (Abbott & Palmour, 1988; Abbott & Franklin, 1991). In electrophysiological experiments M6G is 13-fold more potent than morphine when given i.t., in inhibiting C-fibre-evoked activity in rats (Sullivan et al., 1989). In summary M6G is more more potent when given centrally (i.t. > i.c.v.) than morphine, whereas both compounds exhibit similar potencies when administered systemically. As can be seen wide variations are evident in the assessment of the analgesic potencies of M6G and morphine, from the different authors. Possible reasons for these differences are due to species and strain differences as well as different analgesic tests used.

Table 4.6 Potencies (ED_{50}’s nmoles/mouse) of morphine and M6G in the mouse tail flick test

<table>
<thead>
<tr>
<th></th>
<th>s.c.</th>
<th>i.c.v.</th>
<th>p.o.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>79.8</td>
<td>1.65</td>
<td>918</td>
</tr>
<tr>
<td>M6G</td>
<td>49.3</td>
<td>0.005</td>
<td>504</td>
</tr>
<tr>
<td>M/M6G</td>
<td>1.62</td>
<td>330</td>
<td>1.82</td>
</tr>
</tbody>
</table>

In vivo binding studies show that M6G exerts its antinociceptive effect at low (<1%) fractional occupancy of [^3H]diprenorphine-specific binding.
sites, by co-injecting M6G with \[ ^{3} \text{H} \] diprenorphine. In contrast morphine needs to occupy 9.5 (writhing) to 47 (tail-flick) times more opioid binding sites to produce the same antinociceptive activity (Frances et al., 1992).

Frances et al. (1992) suggests that the higher pharmacological potency of M6G (i.c.v) compared to morphine is either due to its enhanced stimulation of 'opiod receptor-transducer-effect or complexes' ie. second messenger systems or to elevated local concentrations in brain regions involved in antinociception. The same group has since reported that the analgesic potency of M6G is related to a LiCl-sensitive pathway and not to adenylate cyclase inhibition, thus indicating that a phosphoinositide pathway could be preferentially involved (Frances et al., 1993). Lambert & Hirst (1993) investigate binding and inhibition of cyclic AMP formation in intact SH-SY5Y neuroblastoma cells for morphine, M6G and M3G. M6G is about 4 times weaker than morphine in binding, but is equipotent in inhibiting cyclic AMP formation. M3G is ineffective in both assays. Christensen et al. (1991) show that morphine and M6G inhibit dopamine-stimulated cyclic AMP formation in rat striatal membranes, affording values of 1 and 5\(\mu\)M respectively. These findings do not however explain M6G's higher antinociceptive action.

It is suggested by Frances that thermal analgesia at the level of the spinal cord, is mediated mainly through \(\delta\) receptors, since DPDPE is more potent when given i.t., whereas DAMGO is more effective when given i.c.v., and morphine is equipotent by both routes (Frances et al., 1992). The stimulation of \(\delta\) spinal sites by i.t. M6G could account for the high degree of antinociception elicited in thermal nociceptive tests, due to its higher affinity for \(\delta\) receptors as discussed in Chapter 5.

The difference in activity between 6-OH and 6-glucuronide was not observed with the codeine equivalents. Codeine and C6G had an almost identical \textit{in vivo} profile, their potencies were the same, as were their onsets and offsets of action. The only difference was that codeine was slightly longer acting than C6G.

Following s.c. administration, three times as much morphine penetrated the brain as did M6G, whereas both compounds penetrated the cord to an equivalent extent, 1 h after systemic administration. M6G thus seemed to act much more strongly after penetration into the brain and to be removed
more slowly from the brain as compared with morphine, because M6G had a longer duration of action than morphine. Indeed 6 h after systemic administration, <0.1 nM morphine equivalents were present after morphine, but 2.5 nM morphine equivalents were present after M6G. These findings are in agreement with Frances et al (1992), who shows M6G to be 10-fold less effective at penetrating the brain than morphine. These differences might be explained by a more hydrophilic nature of M6G, due to the sugar moiety.

The present results show that after s.c. administration of morphine, twice as much gets into the brain as the cord, whereas with M6G equivalent amounts get into both the brain and the cord. This contradicts findings after i.v. injection of morphine. Bhargava et al (1992) quotes the level of morphine to be highest in the spinal cord. The highest concentration in the brain was found in the hypothalamus and the lowest in the amygdala. However with larger dosing equivalent amounts were found.

Retention times obtained using HPLC indicated that morphine and M6G had similar lipophilicities. Carrupt et al (1991) also suggest that the lipophilicities of M6G and morphine are not that dissimilar. This is explained by the fact that M6G may exist in a predicted folded conformation (Figure 4.16a,) as opposed to the extended conformation (Figure 4.16b, both figures produced using the Evans and Sutherland workstation), thereby masking the polar hydrophilic groups, so the groups are thus unexposed and the compound appears more lipophilic to biological membranes. M3G can also exist in folded and extended conformations, explaining its ability to penetrate the blood-brain barrier, but to a lesser degree than M6G.

Morphine is still a relatively hydrophilic compound, hence both M6G and morphine have relatively long durations of action. The drugs are disposed of more slowly than a more lipophilic compound such as methadone, which penetrates the blood-brain barrier rapidly and its duration is determined by its slow release from receptor sites. Because M6G is more hydrophilic than morphine, the onset and offset would be predicted to be longer in vivo. Antinociception is seen at a similar time after administration, suggesting they have the same onset rate. M6G (protected) in which the hydrophilic groups are protected as esters would be predicted to have faster kinetics than M6G, because it is more lipophilic. In fact M6G (protected) takes longer to show analgesia, but this may be because of the use of saline + 0.25% carboxy methyl cellulose as the vehicle, as opposed
Figure 4.16 M6G in (a) the folded conformation using the Evans & Sutherland ($\Delta H_f = 32.61\text{kcal/mole}$)
(b) extended conformation ($\Delta H_f = 28.54$ kcal/mole)
to saline for M6G, and the drug takes longer to diffuse out of the polymer. However it could be the need for removal of the protecting groups. It thus appears likely that the morphine protected sugar was metabolised to M6G, and the results obtained here are due to formed M6G. However, this suggestion was not confirmed by stability studies performed in mouse brain homogenate, which suggests that the protected sugar was stable (see page 138). This could mean that the carboxy and hydroxy groups on the sugar in M6G are not essential for potency or duration of action. Before any definite conclusions can be deduced from these results though, M6G must be injected in 0.25% CMC to directly compare the results obtained from the M6G (protected sugar) in 0.25% CMC.

In contrast to M6G, M3G was shown to be totally inactive up to doses of 100mg/kg s.c. in the mouse tail-flick test in agreement with Shimomura et al (1971). In addition M3G was shown not to antagonise antinociception induced by morphine. These results differed from those of Smith et al (1990), who showed M3G to be hyperanalgesic, and Gong et al (1991) also reported that M3G stimulated ventilation. There are contradicting views as to whether M3G is an antagonist to morphine and/or M6G analgesia. Smith et al (1990) reports that this is the case when M3G is administered i.c.v. or i.p. to rats. Interestingly M3G causes allodynia when administered i.t. or i.c.v., as does morphine itself, a condition in which an ordinarily innocuous stimulus is perceived as being painful. M3G is about thirty times more potent than morphine in causing this effect when administered i.t. The response causes aggressive behaviour, indicative of a pain state. This response is however a non-opioid effect (Yaksh & Harty, 1988).
CHAPTER 5

BINDING AND ISOLATED TISSUE STUDIES OF MORPHINE AND ITS GLUCURONIDE
In vivo studies confirmed the antinociceptive activity of M6G and in particular its longer duration of action compared to morphine. To determine if this was due to differences in receptor activity of the two compounds, studies were performed in vitro.

5.1 Characterisation of the binding of μ, δ, and κ opioid ligands to homogenates of mouse brain

The saturation binding of [³H]DAMGO (μ), [³H]DPDPE (δ) and [³H]U69593 (κ) to mouse brain homogenate was determined at 25°C for 40 mins (Table 5.1).

Table 5.1 Binding characteristics for [³H]DAMGO, [³H]DPDPE, and [³H]U69593 in mouse brain

<table>
<thead>
<tr>
<th></th>
<th>DAMGO</th>
<th>DPDPE</th>
<th>U69593</th>
</tr>
</thead>
<tbody>
<tr>
<td>KD (nM)</td>
<td>1.37±0.21</td>
<td>5.75±0.62</td>
<td>1.67±0.32</td>
</tr>
<tr>
<td>Bmax (pmoles/g)</td>
<td>8.49±0.93</td>
<td>11.36±1.3</td>
<td>9.30±1.30</td>
</tr>
</tbody>
</table>

Values are means ± sem of ≥3 experiments

The high affinity of each ligand for respective receptors confirms the presence of μ, δ, and κ sites in mouse brain. From Table 5.1 the % of sites was determined as μ (29.1), δ (39.0) and κ (31.9), thus there are similar levels of μ and κ receptors, but slightly more δ receptors. Analysis of [³H]U69593 binding in guinea-pig brain afforded similar affinity to that obtained in mouse brain, namely a KD of 0.95±0.24nM, but a reduced Bmax of 3.31±0.25pmoles/g.

The ability of morphine, M6G, M3G and some selective opioid ligands to displace selective tritiated ligands for μ (DAMGO), δ (DPDPE) and κ (U69593) opioid binding sites in mouse or guinea-pig brain homogenates was determined. Results are shown in Figures 5.1 and 5.2 and Table 5.2. As expected morphine was selective for μ receptor binding sites (Kᵢ 6.2nM), but also had greater affinity for κ than δ binding sites. M6G on the other hand was less selective for μ over δ binding sites, but had much lower affinity for κ-opioid binding sites (ten-fold). The selectivities of M6G, morphine and DAMGO are shown in Table 5.3, the lower the number, the more selective the drug was for the μ site. Results from binding assays
showed morphine to be more μ/δ selective, whereas M6G was more μ/κ selective.

The O-protected glucuronide analogue of M6G showed a very similar receptor profile to that of M6G. In contrast M3G had no affinity for μ, δ, or κ sites.

Displacement of [3H]DPDPE by both morphine (Figure 5.2) and M6G (Figure 5.1) was biphasic with the first component representing approximately 20% displacement of specifically bound [3H]DPDPE. This was particularly clear with M6G (refer to Figure 5.2). Thus taken over the whole range of concentrations the slope of the displacement was 0.7. The slopes of the other displacement curves were all approximately unity, consistent with activity at single sites (Table 5.4).

5.2 Receptor binding profile of M6G and morphine

Table 5.2 Effective inhibition constants (K_i/nM) for morphine, some metabolites and control opioids in brain homogenates

<table>
<thead>
<tr>
<th></th>
<th>μ (mouse)</th>
<th>δ (mouse)</th>
<th>κ (mouse)</th>
<th>guinea-pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>6.22±0.86</td>
<td>218±41.2</td>
<td>84.7±4.01</td>
<td>40.83±3.23</td>
</tr>
<tr>
<td>M-6-G</td>
<td>7.90±1.48</td>
<td>*75.1±15.6</td>
<td>*850.15±118</td>
<td>*766±27.3</td>
</tr>
<tr>
<td>M6G (p)</td>
<td>11.2±2.6</td>
<td>33.0±3.24</td>
<td>820±26.3</td>
<td></td>
</tr>
<tr>
<td>M-3-G</td>
<td>&gt;10,000*</td>
<td>&gt;10,000†</td>
<td>10,000*</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>DAMGO</td>
<td>2.45±0.40</td>
<td>42.5±7.88</td>
<td>2218±518</td>
<td></td>
</tr>
<tr>
<td>DPDPE</td>
<td>&gt;10,000</td>
<td>4.76±1.44</td>
<td>600±124</td>
<td></td>
</tr>
<tr>
<td>U69593</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>2.51±1.22</td>
<td>1.80±0.81</td>
</tr>
</tbody>
</table>

Values are means ± sem of ≥3 experiments. * Significantly different from results for morphine p< 0.005 (student's t-test). 10,000nM M3G displaced 27.9% of [3H]DAMGO (1.16±0.11nM, •); 30.3% of [3H]DPDPE (0.74±0.02nM, †) and 25.8% of [3H]U69593 (1.07±0.13nM, #). M6G (p) is the O-protected glucuronide of M6G (compound 17).
Table 5.3 Selectivities of M6G, morphine and DAMGO for μ, δ or κ-sites, taken from Table 5.2

<table>
<thead>
<tr>
<th></th>
<th>$K_i/μ/K_δ$</th>
<th>$K_i/μ/K_κ$</th>
<th>$K_iδ/K_κ$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6G</td>
<td>0.105</td>
<td>0.010</td>
<td>0.088</td>
</tr>
<tr>
<td>M6G(p)</td>
<td>0.330</td>
<td>0.010</td>
<td>0.040</td>
</tr>
<tr>
<td>Morphine</td>
<td>0.029</td>
<td>0.152</td>
<td>2.580</td>
</tr>
<tr>
<td>DAMGO</td>
<td>0.006</td>
<td>0.006</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Table 5.4 Slopes for displacement of $[^3H]DAMGO$, $[^3H]DPDPE$ and $[^3H]U69593$ by morphine, M6G, M6G (p), DAMGO, DPDPE & U69593

|        |  |  |  |
|--------|  |  |  |
| Morphine | 1.03±0.03 | 0.97±0.04 | 0.95±0.08 |
| M6G    | 0.93±0.07 | 1.05±0.08# | 1.05±0.05 |
| M6G (p) | 0.95±0.08 | 1.10±0.09# | 1.02±0.06 |
| DAMGO  | 0.98±0.08 | 0.94±0.00 | 0.89±0.04 |
| DPDPE  | 0.89±0.04 |  | 0.90±0.06 |
| U69593 |  |  |  |

# represents the linear portion of the displacement curve, from 15 to 85% displacement. IC$_{50}$ values were re-calculated, assuming that 85% is now 100% control, and a new curve plotted.
Figure 5.1 Displacement of tritiated DAMGO (closed circles, 1.12±0.09nM), tritiated DPDPE (open circles, 0.74±0.02nM) and tritiated U69593 (closed squares, 0.50±0.01nM) in mouse brain by M6G.

Figure 5.2 Displacement of tritiated DAMGO (closed circles, 1.19±0.20nM), DPDPE (open circles, 0.74±0.02nM) and U69593 (closed squares, 1.07±0.26nm) in mouse brain homogenate by morphine.
5.2.1 Binding of $[^3\text{H}]$DAMGO at low concentrations of $\mu$ ligands

Abbott & Pailmour (1988) reported effects of M6G on $\mu$-binding at low (subnanomolar) concentrations. Competition studies against $[^3\text{H}]$DAMGO as low as $10^{-11}$M competing ligands were carried out. As can be seen in Figure 5.3 the binding of $[^3\text{H}]$DAMGO was unaffected at these lower concentrations of the $\mu$ ligands morphine, M3G or M6G.

![Figure 5.3 Displacement of $[^3\text{H}]$DAMGO (0.99±0.06nM) by subnanomolar concentrations of morphine (closed circles), M3G (open circles) and M6G (open circles).](image)

5.2.2 Binding to opioid $\kappa$ receptors in guinea-pig cerebellum

Since the $\kappa$-receptor binding of morphine and M6G in mouse brain were so different further binding studies were performed in guinea-pig cerebellum, a tissue containing predominantly $\kappa$-receptors, for comparative purposes. Morphine again showed a higher affinity for $\kappa$ receptor binding sites than M6G, though the absolute $K_i$ values depended upon the tritiated ligand used. When using the antagonist $[^3\text{H}]$diprenorphine or the agonist $[^3\text{H}]$U69593 to label $\kappa$ sites, differences
were obtained of approximately 11 and 27-fold respectively. However morphine had 4 and M6G had approximately 10-fold higher affinity for \(\kappa\) receptors in brain compared to cerebellum, suggesting tissue differences.

### Table 5.5 Comparison of \(\kappa\) binding in guinea-pig brain and cerebellum for morphine and M6G

<table>
<thead>
<tr>
<th></th>
<th>(\kappa) (nM)</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cerebellum</td>
<td>cerebellum</td>
</tr>
<tr>
<td>[(3^H)]diprenorphine</td>
<td>[(3^H)]U69593</td>
<td>[(3^H)]U69593</td>
</tr>
<tr>
<td>M6G</td>
<td>6170±622.6</td>
<td>850.15±117.8</td>
</tr>
<tr>
<td>Morphine</td>
<td>546.8±59.89</td>
<td>84.67±4.01</td>
</tr>
</tbody>
</table>

* 10,000nM corresponded to 71.4±2.3% control [\(3^H\)]U69593 binding.

5.2.3 Binding studies in mouse spinal cord

In mouse spinal cord homogenate morphine showed a slightly higher affinity for \(\mu\) receptor sites than M6G with \(K_i\) values of 5.4 and 14.8nM respectively (Table 5.6), but values were similar to brain. Again as in brain tissue, M6G had a 6-fold higher affinity for the \(\delta\) receptor than morphine (68.7 compared to 405.2nM). These values were of the same order as the \(K_i\) values determined in brain.

### Table 5.6 Effective dissociation constants for morphine & M6G at the \(\mu\) and \(\delta\) receptors in mouse spinal cord.

<table>
<thead>
<tr>
<th></th>
<th>(K_i) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu)</td>
</tr>
<tr>
<td>Morphine</td>
<td>5.4±0.60</td>
</tr>
<tr>
<td>M6G</td>
<td>14.8±0.42</td>
</tr>
</tbody>
</table>

Values were means ± sem of ≥ 3 experiments. Labelling of \(\mu\) and \(\delta\) receptor sites was done with [\(3^H\)]DAMGO or [\(3^H\)]DPDPE. * \(p<0.005\) in the students t-test (significantly different from M6G). \(\cdot \) \(K_i\) for M6G was significantly different in the brain (Table 5.2) compared to the cord \((p<0.001)\).
5.2.4 Binding in the myenteric plexus longitudinal muscle (MPLM)

The binding properties of morphine and M6G were also studied in the MPLM (Table 5.7). To obtain an acceptable degree of specific binding initial studies varying tissue concentration were performed.

Table 5.7 Effect of tissue concentration on % specific binding using \[^{3}\text{H}]\text{DAMGO}\ \text{(2.67±0.42nM)}\ \text{at 25°C for 40 mins}

<table>
<thead>
<tr>
<th>Dilution (w/v)</th>
<th>% Specific binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>14.9±4.3</td>
</tr>
<tr>
<td>30</td>
<td>41.5±3.8</td>
</tr>
<tr>
<td>60</td>
<td>62.8±5.2</td>
</tr>
</tbody>
</table>

A dilution of 60 times w/v, approximating to 1mg/ml of protein, was chosen for these studies. Affinities of Morphine and M6G in MPLM measured by the displacement of \[^{3}\text{H}]\text{DAMGO}\ were assessed, and afforded similar \(K_i\) values of 3.1±0.6 and 2.4±0.8nM respectively.

5.2.5 Binding at \(\mu\) sites in brain under different buffer conditions

Affinities of M6G, morphine and DAMGO were determined against the antagonist \[^{3}\text{H}]\text{naloxone}\ using Tris buffer pH 7.4 in the presence and absence of the stable GTP analogue, guanylimidodiphosphate (Gpp(NH)p, 50\mu M) and NaCl (100mM).

Results showed that M6G and morphine had similar affinity for \(\mu\) receptors under these conditions (Table 5.8). Displacement curves are shown in Figures 5.4-5.6. DAMGO had a significantly higher affinity (189nM) than the alkaloids.

Affinities determined in HEPES/Krebs for M6G, morphine and DAMGO against \[^{3}\text{H}]\text{naloxone}\ gave values of 101.2±20.1, 121.3±33.8 and 118.4±16.2nM respectively.

81
Table 5.8 Affinities (K_i/nM) of M6G, morphine and DAMGO for \( \mu \) receptors, using \( [3H]naltrexone (0.44\pm0.04nM) \), in the presence or absence of Gpp(NH)p (50\( \mu \)M) and NaCl(100mM) in rat brain homogenates.

<table>
<thead>
<tr>
<th></th>
<th>Na-Gpp(NH)p</th>
<th>Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6G</td>
<td>10.39( \pm )4.33</td>
<td>*423.97( \pm )50.82</td>
</tr>
<tr>
<td>morphine</td>
<td>2.83( \pm )0.29</td>
<td>340.20( \pm )51.48</td>
</tr>
<tr>
<td>DAMGO</td>
<td>5.97( \pm )2.19</td>
<td>189.32( \pm )76.02</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) sem of \( \geq 3 \) experiments. *p<0.05 in the students t-test) compared with DAMGO. The KD for naltrexone was the same in the presence or absence of Gpp(NH)p and NaCl and was 2.00nM.

Figure 5.4 Displacement of \( [3H]naltrexone (0.44\pm0.04nM) \) by M6G in rat brain in the presence (open circles) and absence (closed circles) of Gpp(NH)p (50\( \mu \)M) and NaCl (100mM)
Figure 5.5 Displacement of $[^3H]$naloxone (0.44±0.04nM) by morphine in rat brain in the presence (open circles) and absence (closed circles) of Gpp(NH)p (50μM) and NaCl (100mM).

Figure 5.6 Displacement of $[^3H]$naloxone (0.44±0.04nM) by DAMGO in rat brain in the presence (open circles) and absence (closed circles) of Gpp(NH)p (50μM) and NaCl (100mM).

The displacement curves did have slopes of 1.0, probably due to the ability of $[^3H]$naloxone to label other sites, i.e. δ and κ, in addition to μ. However the
affinity of morphine and M6G in the presence of sodium and Gpp(NH)p were similar, and much lower than in Tris-buffer.

Computer analysis of the lines allowed for comparison only of the μ-component, using logistic curve fitting (Barlow, 1991). The results are shown in Table 5.9, with the two components (μ and non-μ) of the displacement curve shown. As can be seen DAMGO, morphine and M6G had similar affinities for the high affinity component, though the affinity for the non-μ component varied from 600 nM for DAMGO to 5199 nM for M6G, in the presence of NaCl and Gpp(NH)p. The relative proportion of μ to non-μ sites was 78.4 : 21.6 %. The non-μ component being made up of 14.3 and 7.3 % for δ and κ sites respectively. Thus it is unlikely that the non-μ component is a μ subtype.

Table 5.9 Affinities of M6G, morphine and DAMGO for μ receptors, using [3H]naloxone in the presence and absence of Gpp(NH)p and NaCl

a) Higher affinity (μ) component

<table>
<thead>
<tr>
<th></th>
<th>Ki (nM)</th>
<th>Tris</th>
<th>Tris-Na-Gpp(NH)p</th>
<th>shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6G</td>
<td>2.52±0.14</td>
<td>63.5±3.40</td>
<td>25.2</td>
<td></td>
</tr>
<tr>
<td>morphine</td>
<td>1.76±0.31</td>
<td>89.1±4.56</td>
<td>50.6</td>
<td></td>
</tr>
<tr>
<td>DAMGO</td>
<td>0.87±0.20</td>
<td>51.4±4.65</td>
<td>59.1</td>
<td></td>
</tr>
</tbody>
</table>

b) Lower affinity (non-μ) component

<table>
<thead>
<tr>
<th></th>
<th>Ki (nM)</th>
<th>Tris</th>
<th>Tris-Na-Gpp(NH)p</th>
<th>shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6G</td>
<td>358±52.6</td>
<td>5199±301</td>
<td>14.5</td>
<td></td>
</tr>
<tr>
<td>morphine</td>
<td>89.1±4.5</td>
<td>1001±54.3</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>DAMGO</td>
<td>198±30.1</td>
<td>600±32.6</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

Because [3H]naloxone binds to all three receptors it was decided to use a more selective ligand for the μ receptor, namely the cyclic somatostatin analogue, [3H]CTOP(Figure 5.7), which is reported to be a highly selective μ antagonist (Yamamura et al, 1989). Prior to displacement experiments, the binding of this ligand was studied.
Experiments were performed to investigate the effect of protein concentration on the specific binding of \(^{3}\text{H}\)CTOP. Values ranged from 49 to 69%. A 1/100 dilution (9.6mg tissue weight) of brain was chosen to perform future experiments, as this gave the maximum specific binding.

Yamamura et al (1989) reports the use of other compounds to facilitate \(^{3}\text{H}\)CTOP binding, namely bovine serum albumin (BSA) and phenyl methyl sulphonyl fluoride (PMSF). From the results shown in Table 5.10. It can be observed that the use of BSA and/or PMSF did not produce any improvement to the % specific binding. Consequently \(^{3}\text{H}\)CTOP binding was performed under conditions used for other opioid ligands, ie in Tris buffer pH 7.4, without any additions.

Table 5.10 Effect of BSA and PMSF on specific binding of \(^{3}\text{H}\)CTOP

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% Specific binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>52.7±5.4</td>
</tr>
<tr>
<td>BSA</td>
<td>58.8±4.2</td>
</tr>
<tr>
<td>PMSF</td>
<td>50.8±3.7</td>
</tr>
<tr>
<td>BSA+PMSF</td>
<td>54.7±4.1</td>
</tr>
</tbody>
</table>

The time taken for \(^{3}\text{H}\)CTOP binding to reach equilibrium was determined at both 25°C and 37°C in mouse brain homogenates. Equilibrium binding was reached at 120 and 60 mins respectively (Figure 5.8). It was noticeable that binding at 25°C was greater than that at 37°C. In the presence of Na/Gpp(NH)p, equilibration was reached after 60 mins (Figure 5.9), but the binding was much reduced (8-fold).
Figure 5.8 Time course for specific \( [^3\text{H}]\text{CTOP}(0.35\pm0.05\text{nM}) \) binding in mouse brain homogenates at 25°C (closed circles) and 37°C (open circles). \( n=4 \)

Figure 5.9 Time course for \( [^3\text{H}]\text{CTOP} \) (0.16±0.01nM) specific binding in the presence of \( \text{NaCl} \) (100mM) and Gpp(NH)p (50μM) at 25°C
Characteristics of the binding of \(^{3}\text{H}\)CTOP in mouse and rat brain in the presence and absence of \(\text{Na}^+\) are reported in Table 5.11. As can be seen the \(K_D\) for \(^{3}\text{H}\)CTOP shifts in \(\text{NaCl}\) and Gpp(NH)p to lower affinity by greater than 10-fold. Saturation binding results for \(^{3}\text{H}\)CTOP were different in mouse and rat brain homogenates (Table 5.11, Figure 5.10). Scatchard analysis of the data in mouse and rat brain is shown in Figure 5.11.

**Table 5.11 Characteristics of binding of \(^{3}\text{H}\)CTOP in mouse and rat brain homogenates**

<table>
<thead>
<tr>
<th></th>
<th>(K_D/\text{nM})</th>
<th>(B_{\text{max}}/\text{fmoles/mg})</th>
<th>(K_D/\text{nM})</th>
<th>(B_{\text{max}}/\text{fmoles/mg})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>0.30±0.03</td>
<td>97.8±10.9</td>
<td>7.62±0.83</td>
<td>77.5±6.32</td>
</tr>
<tr>
<td>Rat</td>
<td>0.65±0.07</td>
<td>59.7±7.3</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

Values are means ± sem of ≥3 experiments. Binding was performed at 25°C for 40 mins. n.t. denotes not tested.

**Figure 5.10** Specific binding of \(^{3}\text{H}\)CTOP to homogenates of brain (9.6mg tissue weight) from mouse (closed circles) and rat (closed triangles) at 25°C and for 40 mins (representative experiment)
At higher concentrations of $[^3\text{H}]$CTOP the specific binding seemed to increase for rat homogenate and resulted in a complex Scatchard Plot. Due to this the values for the rat are approximate, but fitted as a single site by LIGAND. In mouse homogenate a plateau was maintained.

![Scatchard analysis for $[^3\text{H}]$CTOP](image)

**Figure 5.11** Scatchard analysis for $[^3\text{H}]$CTOP in mouse (closed circles) and rat brain (9.6mg tissue weight) (closed triangles) at 25°C and for 40 mins

For comparative purposes the binding of $[^3\text{H}]$DAMGO binding was studied in rat brain homogenate (Figures 5.12 and 5.13), and afforded a $K_D$ of $0.80\pm0.12\text{nM}$ and a $B_{\text{max}}$ of $67.9\pm5.6\text{fmol/mg}$. 

88
Figure 5.12 Specific binding of $[^3\text{H}]\text{DAMGO}$ to rat brain homogenate (9.6mg) at 25°C for 40 mins

Figure 5.13 Scatchard analysis of $[^3\text{H}]\text{DAMGO}$ binding in rat brain homogenate (9.6mg tissue weight) at 25°C for 40 mins

As can be seen from the results in Table 5.12 the maximal binding capacity ($B_{\max}$) obtained for $[^3\text{H}]\text{DAMGO}$ and $[^3\text{H}]\text{CTOP}$ was comparable in both mouse and rat brain homogenate.
Table 5.12 Comparison of $B_{\text{max}}$ values (fmol/mg protein) for [$^3$H]DAMGO and [$^3$H]CTOP in mouse and rat brain homogenate

<table>
<thead>
<tr>
<th></th>
<th>DAMGO</th>
<th>CTOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>82.6</td>
<td>97.8</td>
</tr>
<tr>
<td>Rat</td>
<td>67.9</td>
<td>59.7</td>
</tr>
</tbody>
</table>

5.2.6 Competition studies using [$^3$H] CTOP in mouse brain

The binding of [$^3$H]CTOP was studied in the presence and absence of NaCl (100mM) and Gpp(NH)p (50μM). Competition studies with opioids were performed to compare displacement curves under high (Tris) and low (NaCl, Gpp(NH)p) agonist affinity states of the $\mu$-receptor. The results are summarised in Table 5.13. The difference between displacements (sodium shift) for DAMGO in mouse brain against [$^3$H]CTOP is shown in Figure 5.14, as a representative graph of these results. The results were somewhat confusing because no significant shifts was observed for the $\mu$ agonist ligands (Table 5.13). Affinities obtained in Tris with [$^3$H]CTOP for $\mu$-ligands were much lower than those obtained with [$^3$H]naloxone (Table 5.8) and as a result of this the shifts are much less with [$^3$H]CTOP. Also the fact that [$^3$H]CTOP itself shifts 10 times, probably mask any agonist shifts. In addition shallow slopes were obtained for the displacements, as shown for DAMGO in Figure 5.14.

Table 5.13 Affinities (K$_i$/nM) of M6G, morphine, naloxone, fentanyl and DAMGO for $\mu$ receptors using [$^3$H]CTOP (0.38±0.02nM) in the presence or absence of Gpp(NH)p (50μM) and NaCl(100mM)

<table>
<thead>
<tr>
<th></th>
<th>Tris</th>
<th>Tris-Na</th>
<th>Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>17.1±5.21</td>
<td>18.0±3.21</td>
<td>1.1</td>
</tr>
<tr>
<td>M6G</td>
<td>19.7±4.12</td>
<td>37.3±6.47</td>
<td>1.9</td>
</tr>
<tr>
<td>Naloxone</td>
<td>3.12±0.45</td>
<td>1.72±0.62</td>
<td>0</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>13.1±3.82</td>
<td>12.9±2.91</td>
<td>0</td>
</tr>
<tr>
<td>DAMGO</td>
<td>24.7±5.32</td>
<td>81.8±6.82</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 5.14 Sodium shift for DAMGO in mouse brain using $[^3H]CTOP$ (0.38±0.02nM) in the presence (open circles) and absence (closed circles) of Gpp(NH)p (50μM) and NaCl (100mM)
5.3 Isolated tissue studies

5.3.1 Myenteric plexus longitudinal muscle (MPLM)

The potency of various agonists including morphine and M6G was assessed in the MPLM (Table 5.14). Graphs are shown in Figures 5.15-5.18.

Table 5.14 Potency (IC$_{50}$'s) and naloxone equilibrium constants (K$_e$) in MPLM for a series of opiates and related compounds

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$/nM</th>
<th>K$_e$(Nx)/nM</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6G</td>
<td>*58.0±4.30</td>
<td>3.00±0.80</td>
<td>0.94±0.01</td>
</tr>
<tr>
<td>M6G(p)</td>
<td>40.6±4.32</td>
<td>3.05±0.92</td>
<td>0.89±0.05</td>
</tr>
<tr>
<td>Morphine</td>
<td>130±7.20</td>
<td>3.45±0.59</td>
<td>1.02±0.03</td>
</tr>
<tr>
<td>U69593</td>
<td>2.20±0.41</td>
<td>9.00±2.11</td>
<td>0.97±0.06</td>
</tr>
<tr>
<td>DAMGO</td>
<td>11.8±1.20</td>
<td>1.60±0.06</td>
<td>0.98±0.04</td>
</tr>
<tr>
<td>Codeine</td>
<td>1600±150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M3G</td>
<td>&gt;10,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± sem of ≥3 experiments. * p<0.001 (Students t-test) shows IC$_{50}$'s for M6G and morphine are significantly different. Schild plots were employed to calculate K$_e$ values.

M6G (IC$_{50}$ 58nM) showed a 2-fold increase in potency over morphine (IC$_{50}$ 130nM). Naloxone K$_e$'s derived from Schild plots of antagonist data (Figures 5.17 & 5.19) gave values against both agonists of approximately 3nM, i.e. in the expected range for $\mu$ receptors. Slopes of 1.0 were obtained confirming an action solely via a single receptor population. M6G (protected) (IC$_{50}$ 41nM) showed similar potency to that of M6G (IC$_{50}$ 58nM). DAMGO and U69593 are selective $\mu$ and $\kappa$ agonists respectively and both were very potent in this preparation. M3G had no effect in the MPLM up to 10,000nM. Similarly codeine had a much reduced potency compared to morphine, but was considerably more potent than M3G.
Figure 5.15 Inhibition of electrically induced contractions of the guinea-pig MPLM by M6G in the absence (open circles) and presence of varying concentrations, 10 (closed circles), 30 (closed squares) and 100nM (closed triangles) of naloxone.

Figure 5.16 Representative Schild plot for naloxone antagonism of the response to M6G.
Figure 5.17 Inhibition of electrically induced contractions of guinea-pig MPLM by morphine in the absence (open circles) and presence of varying concentrations, 10 (closed circles), 30 (closed squares) and 100nM (closed triangles) of naloxone

Figure 5.18 Representative Schild plot for naloxone antagonism of the response to morphine
5.3.2 The rat vas deferens preparation

The rat vas deferens contains a reduced μ receptor population compared to the guinea-pig MPLM or the mouse vas deferens (Smith & Rance, 1983) and therefore partial agonists act as antagonists and only highly efficacious μ compounds produce a response. Consequently such a preparation can be used as an indication of efficacy in a series of compounds. For example, morphine acts as a partial agonist (Smith & Rance, 1983). This also allowed for the determination of affinity constants, by determining shifts observed against more efficacious compounds, which acted as full agonists in this preparation (eg. DAMGO).

![Graph showing inhibitory potency of μ-agonists DAMGO (closed circles), M6G (open circles) and morphine (closed squares) in the rat vas deferens preparation in Krebs with 0.188g/l Ca^{2+}](image)

Table 5.15 IC\textsubscript{50} 's(nM) obtained for μ-ligands in the rat vas deferens

<table>
<thead>
<tr>
<th></th>
<th>IC\textsubscript{50} (nM)</th>
<th>Max % inhibition observed</th>
<th>IC\textsubscript{20} (nM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMGO</td>
<td>306.8±45.8</td>
<td>91±4</td>
<td>110±9.3</td>
<td>6</td>
</tr>
<tr>
<td>M6G</td>
<td>&gt;10,000</td>
<td>40±2</td>
<td>850±45</td>
<td>4</td>
</tr>
<tr>
<td>M3G</td>
<td>&gt;3000</td>
<td>0</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Morphine</td>
<td>&gt;10,000</td>
<td>32±3</td>
<td>2000±203</td>
<td>3</td>
</tr>
<tr>
<td>Codeine</td>
<td>&gt;100,000</td>
<td>0</td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

Values are means ± sem of ≥ 3 experiments
Figure 5.19 shows the relative potency of μ ligands. M6G and morphine do not reach 50% inhibition, whereas DAMGO reached >90% inhibition, but a large reduction in potency for DAMGO is observed compared with the MPLM (IC50 of 12 nM in the MPLM to 307 nM in the RVD). The calcium chloride concentration (0.188 g/l) used in the RVD was half that used in the MPLM. M6G and morphine thus had a much reduced maximum effect observed compared with values in the MPLM, and were also shifted dramatically (58 nM and 120 nM in the MPLM to >10,000 nM in the RVD) (Table 5.15, n values are given in Table). Codeine showed no agonist effect at all. The relative efficacies of morphine and M6G were 18.0±8.0 and 7.8±3.0 times less potent than DAMGO, using a modification of the Furchgott analysis (Chapter 3, page 52).

In this preparation therefore M6G and morphine can be used as antagonists and thus affinity values were obtained for the compounds at μ receptors. Assays were done using 1000 nM morphine and M6G against DAMGO as an agonist and results were analysed using the technique developed by Kosterlitz & Watt (1968). To ensure that DAMGO acted as a full agonist, the tissues were bathed in Krebs solution with half the normal Ca^{2+} present (Sheehan et al, 1988) (With a quarter Ca^{2+} no response was observed) (Figure 5.20). The shifts in DAMGO dose-response curves are given in Table 5.16 together with the apparent Ke values determined.

Figure 5.20 Effect of calcium concentration, 0.375 g/l (closed circles) and 0.188 g/l (open circles) in the RVD for DAMGO
Table 5.16 Dissociation constants \( [K_e (\text{nM})] \) for M6G and morphine in the rat vas deferens versus DAMGO

<table>
<thead>
<tr>
<th>Dose-ratio</th>
<th>( K_e / \text{nM} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6G</td>
<td>9.7±2.6</td>
</tr>
<tr>
<td>Morphine</td>
<td>2.2±0.4</td>
</tr>
</tbody>
</table>

Values are means ± sem of ≥3 experiments

5.3.3 Furchgott analysis of \( \mu \)-agonists in the myenteric plexus longitudinal muscle

In order to obtain a measure of affinity for M6G and morphine in the MPLM, the method of Furchgott was used (refer to methods pages 52-3). \( \beta \)-FNA at dose of 100nM for 1 h was employed as an alkylating agent to reduce \( \mu \) receptor number (Figures 5.21-5.26). Furchgott analysis were constructed by interpolating from the linear portions of the dose-response curves values for \( A \) and \( A' \). As can be seen the effect of \( \beta \)-FNA was least pronounced for the DAMGO dose-response curve, whereas it caused the M6G curve to tip over markedly. From these curves the affinity for \( \mu \) receptors (\( K_e \)) and the percentage of \( \mu \) receptors remaining (\( q \)) were determined and are shown in Table 5.17.

Table 5.17 Furchgott analysis for \( \mu \)-ligands in myenteric plexus longitudinal muscle using \( \beta \)-FNA as an irreversible \( \mu \)-antagonist

<table>
<thead>
<tr>
<th>100nM</th>
<th>( K_e / \mu \text{M} )</th>
<th>( q/% )</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6G</td>
<td>0.90±0.10</td>
<td>5.33±0.95</td>
</tr>
<tr>
<td>Morphine</td>
<td>1.90±0.41</td>
<td>5.57±0.43</td>
</tr>
<tr>
<td>DAMGO</td>
<td>0.83±0.1</td>
<td>6.18±0.87</td>
</tr>
</tbody>
</table>

Values are means ± sem of ≥3 experiment

From this M6G had twice the affinity of morphine for the \( \mu \) receptor; affinities of 0.9\( \mu \text{M} \) and 1.9\( \mu \text{M} \) respectively were determined (Table 5.17).
Figure 5.21 Effect of β-FNA (100 nM, open circles) pre-treatment on the potency of M6G (closed circles) in the MPLM.

Figure 5.22 Furchgott analysis for M6G. A is the concentration of M6G before inactivation, whereas A' is the concentration of M6G after inactivation with β-FNA.
Figure 5.23 Effect of β-FNA (100nM, open circles) pre-treatment on the potency of morphine (closed circles) in the MPLM.

Figure 5.24 Furchgott analysis for morphine. A is the concentration of morphine before inactivation, whereas A' is the concentration of morphine after inactivation with β-FNA.
Figure 5.25 Effect of β-FNA (100nM, open circles) pre-treatment on the potency of DAMGO (open circles) in the MPLM.

Figure 5.26 Furchgott analysis for DAMGO. A is the concentration of DAMGO before inactivation, whereas A' is the concentration of DAMGO after inactivation with β-FNA.
Affinity constants were similarly obtained using N-ethyl malemide (NEM) as an alternative alkylating agent to β-FNA and the results compared. It can be seen that affinity constants obtained following 10μM NEM treatment were of a similar order to those obtained following β-FNA pretreatment (Table 5.18).

Table 5.18 Comparison of affinities obtained using either NEM or β-FNA

<table>
<thead>
<tr>
<th></th>
<th>NEM</th>
<th>β-FNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_a (μM)</td>
<td>q(%)</td>
</tr>
<tr>
<td>DAMGO</td>
<td>0.3±0.1</td>
<td>6.2±0.9</td>
</tr>
<tr>
<td>Morphine</td>
<td>1.6±0.3</td>
<td>8.2±1.1</td>
</tr>
<tr>
<td>M6G</td>
<td>0.8±0.2</td>
<td>2.1±2.4</td>
</tr>
<tr>
<td>U69593</td>
<td>0.02±0.004</td>
<td>25.0±2.3</td>
</tr>
</tbody>
</table>

5.3.4 Mouse vas deferens preparation

M6G and morphine were also studied using the MVD preparation (Table 5.19, Figures 5.27-5.30). This preparation contains mainly δ opioid receptors, but also has populations of μ and κ receptors.

Table 5.19 Potencies (IC_{50} (nM)) of various compounds and naloxone K_e values, in the mouse vas deferens preparation

<table>
<thead>
<tr>
<th></th>
<th>IC_{50}/nM</th>
<th>K_e/nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMGO</td>
<td>24.7±3.0</td>
<td>1.9±0.6</td>
</tr>
<tr>
<td>M6G</td>
<td>104±17.1</td>
<td>2.6±0.9</td>
</tr>
<tr>
<td>M6G (p)</td>
<td>140±11.3</td>
<td>2.9±0.6</td>
</tr>
<tr>
<td>Morphine</td>
<td>173±62.8</td>
<td>3.6±0.8</td>
</tr>
<tr>
<td>DPDPE</td>
<td>2.8±0.6</td>
<td>20.4±2.6</td>
</tr>
</tbody>
</table>

Values are means ± sem of ≥3 experiments.

M6G and morphine showed a similar potency in the MVD, affording IC_{50}'s of 104 and 173nM respectively. Both actions were antagonised by naloxone affording K_e's in the range expected for μ receptors. Morphine-6-0-protected glucuronide M6G(protected) exhibited a similar potency to that of
M6G. This was inagreement with the similar receptor profile to M6G and similar IC\textsubscript{50} values obtained in the MPLM and MVD (Figure 5.31).

![Figure 5.27](image)

**Figure 5.27** Inhibition of electrically induced contractions of the mouse vas deferens by M6G in the absence (open circles) and presence of varying concentrations, 10 (closed circles), 30 (closed squares) and 100nM (closed triangles) of naloxone

![Figure 5.28](image)

**Figure 5.28** Representative Schild plot for naloxone antagonism of the response to M6G

102
Figure 5.29 Inhibition of electrically induced contractions of the mouse vas deferens by morphine in the absence (open circles) and presence of varying concentrations, 10 (closed circles), 30 (closed squares) and 100nM (closed triangles) of naloxone.

Figure 5.30 Representative Schild plot for naloxone antagonism of the response to morphine.
5.3.5 The hamster vas deferens preparation

The hamster vas deferens contains solely opioid receptors of the δ-type. Since M6G had a 3 times higher affinity for the δ receptor than morphine in binding the potencies of the two compounds were compared in this isolated tissue preparation. DPDPE was shown to be a partial agonist, reaching a maximum inhibition of 81% (Figure 5.32). Morphine and M6G also only acted as partial agonists, the maximum inhibition reached was 40±4 and 38±3% for M6G and morphine respectively when extrapolated from the dose-response curves up to 3,000nM (Barlow, 1991) (Figure 5.32). M6G was seen to be slightly more potent than morphine, though both were only weak partial agonists (Table 5.20).
Table 5.20 Potencies of morphine and M6G in the hamster vas deferens preparation

<table>
<thead>
<tr>
<th>Agonist</th>
<th>IC50 (nM) ± SD</th>
<th>IC25 (nM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPDPE</td>
<td>52.8±4.30</td>
<td>7.52±1.25</td>
</tr>
<tr>
<td>M6G</td>
<td>171±12.6</td>
<td>286±15.3</td>
</tr>
<tr>
<td>Morphine</td>
<td>283±14.3</td>
<td>715±36.4</td>
</tr>
</tbody>
</table>

* expressed as concentration required to give 50% of the maximum effect of each agonist, * represents 25% of the maximal effect of DPDPE.

Figure 5.32 Potencies of DPDPE (closed circles) morphine (closed squares) and M6G (open circles) in the hamster vas deferens preparation

5.3.6 Alkylation of μ receptors

A possible explanation for the longer duration of action of M6G over morphine is that it binds irreversibly or at least very tightly to μ-receptors (Yoshimura et al., 1976). If this is the case it should be possible to demonstrate this in similar experiments to those using β-FNA. From Figure 5.33 it can be seen that in mouse brain homogenate, β-FNA (100nM, 60 mins) alkylated 50% of μ receptors, whereas under similar conditions M3G(1μM) and M6G(1μM) did not affect binding following washout, not
deviating significantly from 100%.

Also in the presence of NaCl (100mM) and Gpp(NH)p (50μM), when the receptor is likely to be in a more physiological state no alkylation with M6G was evident. Thus M6G and M3G did not appear to act as irreversible μ antagonists in conditions promoting high or low affinity states of the receptor.

Figure 5.33 Degree of alkylation of μ receptors by M3G, M6G and β-FNA, H2O represents vehicle controls.
5.4 Discussion

5.4.1 Binding

Characterisation of opioid binding sites, in the brain of CSI mouse, showed similar proportions of μ, δ and κ receptors, (ie 29, 39, and 32% respectively). These percentages corresponded to \( B_{\text{max}} \) values of 8.49, 11.36 and 9.30 pmoles/g respectively. Robson et al (1985) investigated the relative proportions of μ:δ:κ receptors in two other strains of mice, namely DBA/2 mice and C57BL/10 mice, and finds them to be 51 : 29 : 20 and 44 : 35 : 21 % respectively. These two strains of mice having a higher proportion of μ receptors than in the CSI mice, suggesting strain differences. In guinea-pig brain the proportions of receptors is reported by Kosterlitz to be 20-24 (μ) : 32-40 (δ) : 40-44 (κ) % (Kosterlitz et al, 1981), whereas in the rat brain it is 46 : 42 : 12 % (Gillan & Kosterlitz, 1982) and in rabbit brain 43 : 19 : 38 %. Thus both species differences and strain differences exist (Robson et al, 1985), and so the majority of the investigations and evaluation of M6G and related compounds in biochemical experiments were carried out using CSI mouse brain homogenates to complement in vivo experiments in the same strain of mice.

Yoshimura (1977) suggests that some morphine-6-substituted compounds act as irreversible alkylating agents in rat CNS. A possible explanation of M6G's increased analgesic activity and longer duration of action over morphine, could be that it irreversibly alkylates μ-receptors as does β-FNA. It was shown that in mouse brain homogenate β-FNA alkylates 50% of μ receptors, which agrees with many authors including Franklin and Traynor (1992), who find this level of alkylation in guinea-pig brain homogenates. However no alkylation was observed with M3G or M6G, either in the presence or absence of NaCl (100mM) and Gpp(NH)p (50μM). Thus M6G and M3G did not act as irreversible μ antagonists at high or low affinity states of the μ receptor. A slight increase in \(^{3}H\)DAMGO binding was observed in the presence of M6G in the high affinity receptor state, but this was not significantly different from 100%, and therefore could not be interpreted as any alteration of receptors. It may be that nucleophilic reactions carried out by Yoshimura (1977) cannot be directly comparable to the method employed here and indeed to the in vivo situation.
Binding studies of M6G and morphine have previously been performed in various species, though not using the same species to study all three opioid receptor types. In the present studies in mouse brain homogenate M6G had the same affinity (Kᵢ = 7.90nM) for the μ receptor as morphine (Kᵢ = 6.22nM). In mouse spinal cord, the determined affinity of M6G for the μ receptor was half that for morphine, with Kᵢ's of 14.8 and 5.4nM respectively. This was not statistically different from affinities obtained in mouse brain. These values are similar to those obtained by Frances et al (1992), using the rabbit cerebellum for μ, where a Kᵢ for M6G of 20nM is obtained. Previous workers (Paul et al, 1989 (i); Pasternak et al, 1987; Frances et al, 1992; Pasternak & Wood, 1986) show morphine has 4-fold higher affinity for μ receptors than M6G, affording Kᵢ's of 2.5-5.6 and 10.6-20.3nM respectively, while Christensen & Jorgensen (1987) report that M6G has a three times lower affinity for the opioid receptor than morphine as measured by the displacement of [³H]naloxone in bovine brain (IC₅₀'s of 90 and 30nM respectively).

No effects were obtained on [³H]DAMGO binding at subnanomolar concentrations of M6G. This disagrees with Abbott and Palmour's (1988) findings, that at low concentrations M6G enhances the binding of [³H]etorphine, [³H]dihydromorphine, and [³H]naloxone to rat brain membrane receptors by 30%, whilst at higher concentrations M6G displaced the radiolabelled compounds, giving Kᵢ's of about 30nM, compared with 3nM for morphine. This implies that the effect occurs at concentrations that would be expected in extracellular space (15-20% brain volume) after i.c.v. injection of a 0.01mg dose. Abbott's findings suggest that either linked agonist binding sites share a common antagonist component, or receptor subtypes are arranged in complexes such that binding at one site, alters the affinity of binding at the second site, and receptor subtypes interconvert as the physiological environment alters. However the present findings lend no support for this.

Although morphine showed a similar affinity for μ receptors as M6G, this relative affinity in binding studies contrasts with the reputedly far greater potency of M6G in vivo following central administration. This was possibly because binding studies can never truly reflect physiological conditions, such as the intra- and extracellular orientation of the membranes to different concentrations of ions, nucleotides and other factors which have
not yet been determined, which may have greatly influenced receptor function *in vivo*. Importantly, binding studies only indicated the affinity of a compound for the receptor under specified conditions. These do not give an indication of the efficacy of the compound in producing a physiological response. However even in the isolated tissue preparations the potencies of the two compounds were of a similar order.

No evidence for the existence of multiple μ subtypes was evident from binding studies, but has been suggested by many workers (Pasternak and Wood, 1986; Franklin and Traynor, 1992; Rothman *et al.*, 1989). Hucks *et al.* (1992) finds M6G (82nM) to have a 4-fold lower affinity for the μ2 receptor than morphine (17nM). The μ2 receptor is postulated to be responsible for mediating the respiratory depression and gastrointestinal effects after morphine administration (Pasternak & Wood, 1986). It is expected therefore from these findings, that after M6G administration in man, reduced respiratory depression and vomiting would be seen. Unfortunately though M6G has been linked as a possible component to the respiratory depression caused by morphine administration (Osborne *et al.*, 1986; Christensen & Jorgensen, 1987; Ronald, 1989). The predicted reduced incidence of nausea and vomiting is confirmed by Osborne *et al.* (1992) who reports that after i.v. administration no nausea or vomiting is observed.

In contrast the affinity of morphine and M6G for δ receptors, labelled with tritiated DPDPE, differed by 3-fold, M6G having the higher affinity. These results are comparable to those obtained by Frances *et al.* (1992) who used rat brain to study the δ receptor, thus indicating that species variation may not affect opiate binding of M6G, and indicates the label is binding similar sites, even though Frances used tritiated DTLET to label δ sites. Similarly, Oguri *et al.* (1970) shows that M6G has more cross-reactivity with the δ receptor than morphine, though using the non-selective (μ/δ) ligand [3H] [Leu5] enkephalin. Therefore by glucuronidating morphine at the 6-position (M6G), the affinity for the μ receptor is somewhat reduced, but the affinity for the δ receptor is enhanced. From this data the enhanced *in vivo* potency of M6G may be related to an enhanced interaction with the δ receptor, though isolated tissue studies (discussed later, 5.4.2). do not show great differences.

M6G displayed a biphasic dose-displacement curve against [3H]DPDPE. The reasons for this were unknown, but may indicate the ability of M6G to
differentiate two subtypes of δ receptor (Cotton et al., 1985; Traynor & Elliott, 1993). In rat spinal cord Traynor et al. (1990) suggest the possibility of a common high affinity site for δ- and μ-opioid receptor agonists, for the displacement of [3H]DPDPE by DAMGO and morphine. This could be extended to imply μ-δ interactions existing. In support of such a concept, Qi et al. (1990) found that at sub-antinociceptive doses δ-agonists can modulate antinociceptive responses to μ-opioid receptor agonists in the mouse tail-flick test and Russell et al. (1987) reports that after i.t. administration of μ and δ agonists, cross-tolerance exists between both agonists. From binding studies, Rothman et al. (1988) divides δ-receptors into two subtypes, those that are associated with μ-receptors (called δ_complexed or δcx) and those not associated (called δ_noncomplexed or δncx). Based on studies with different agonists and antagonists, another subdivision of δ receptors has been proposed not related to δcx or δncx, namely δ1 and δ2 receptors. δ1 receptors have been nominated as those activated by DPDPE and blocked by [D-Ala2, Lys4,Cys5] enkephalin (DALCE), and δ2 receptors as those activated by [D-Ala2] deltorphin II and [D-Ser2, Leu5] enkephalin-Thr6 (DSLET) and blocked by the noncompetitive derivative of naltrindole, naltrindole 5'-isothiocyanate (Jiang et al., 1991).

The ability of M6G to act at one particular δ-opioid receptor may be responsible for its enhanced potency. This was also shown in mouse spinal cord, where M6G showed a 6-fold higher affinity for the spinal δ receptor than morphine, with Ki's of 68.7±4.2 and 402.2±42.6nM respectively. This observation could partly contribute towards the explanation for Paul and co-workers (1989 i) findings, that M6G showed a 650 times increased potency over morphine, when both compounds were injected intrathecally.

It has been suggested above that M6G's effectiveness after i.t. administration may be due to an enhanced δ-mediated effect. However Paul et al. (1989 ii) suggests that the effect is due to a mechanism involving μ2 receptors. This is on the basis that the μ1-selective antagonist naloxonazine blocks the analgesic effect of systemic and i.c.v. M6G and morphine, but not after i.t. administration, suggesting a μ2 mechanism within the spinal cord. However the results do indicate that M6G elicits its analgesic actions through the same receptor mechanisms as morphine.

Morphine and M6G differentiated κ receptors to varying extents, as
demonstrated by the data obtained in mouse brain homogenates. Morphine had a 10-fold higher affinity than M6G. These results compared to those obtained by Frances et al (1992), who uses the guinea-pig cerebellum to study the κ receptor and reports that M6G has 19 times less affinity for κ sites than morphine, whereas M6G is 11 times more μ/κ selective than morphine, being comparable to DAMGO (Table 5.3) (Martin et al, 1993). Further binding studies in the guinea-pig cerebellum, which contains a largely (84%) κ-population (Robson et al, 1984), confirmed this finding, though the actual magnitude of the difference depended on the tritiated ligand used. Values of 11 and 27-fold differences were obtained when using [³H]diprenorphine and [³H]U69593 respectively. Both M6G and morphine had higher affinity for κ receptors in brain compared to cerebellum, whereas there was no significant difference in affinity between the two compounds in guinea-pig cerebellum, using [³H]diprenorphine or [³H]U69593. Therefore the difference in affinities of morphine and M6G in brain and cerebellum may indicate tissue differences, possibly differences in receptor characteristics. The existence of κ-subtypes has been suggested by several authors. Such subtypes have been classified as κ₁, those receptor subtypes that preferentially bind arylacetamide κ opioids such as U69593, or κ₂, those that bind benzomorphan κ opioids such as bremazocine and EKC (Horan et al, 1993; Wollemann et al, 1992). Although this classification is by no means final, it does offer a possible explanation to some of the anomalous findings. However more extensive studies need to be performed to establish if real differences do exist between affinities measured in brain or cerebellum.

Opioid receptors are allosterically modulated by ions, in particular sodium, and linked to G-proteins. Thus in the presence of Na⁺ (100mM) and Gpp(NH)p (50μM) the receptor population is largely converted to a low agonist affinity state, which may represent the uncoupled form of the receptor. Richardson et al (1992) report that both receptor-coupled and uncoupled G-proteins exist. Naloxone as an antagonist with no efficacy should recognise all states of the receptor, but under conditions promoting low agonist affinity, the ability of agonists to compete with [³H]naloxone is greatly decreased (Carroll et al, 1988). In the present studies the affinity of morphine, M6G and DAMGO for μ sites, were decreased and afforded similar low affinities, approximately 400nM, as measured against [³H]naloxone in rat brain homogenate in the presence of sodium ions and
guanylimidodiphosphate. Some workers suggest that this low affinity state is the physiological relevant receptor state (Carroll et al., 1988). If so, then both compounds have similar affinities for the μ receptor under physiological conditions. However present results may indicate that the low affinity state is not the physiological relevant receptor state, as differences were observed between affinities obtained in the presence of sodium ions and guanylimidodiphosphate and affinities obtained in isolated tissue preparations. The affinity of M6G in Tris-sodium and guanylimidodiphosphate (400nM) correlated reasonably with that obtained in the guinea-pig MPLM (900nM) and the RVD (200nM), but the affinity of morphine in Tris-sodium and guanylimidodiphosphate (300nM) did not correlate well with that obtained in the guinea-pig MPLM (1900nM) and the RVD (1000nM). Carroll et al. (1988) showed a good correlation between affinities obtained in the guinea-pig ileum and binding assays performed in Krebs/HEPES for a range of μ-agonists. Morphine though gave similar values to those obtained here of 1788 and 416nM in the guinea-pig ileum and binding assays respectively, thus not correlating particularly well. So it seems the correlation does not fit for every μ-ligand, but for some μ-ligands the low affinity state is the physiological relevant receptor state.

The affinities of morphine and M6G were also investigated by binding assays in Tris buffer pH 7.4, in a peripheral tissue, namely the MPLM, and afforded Kᵢ values of 3.1 and 2.4nM respectively. Corbett et al. (1985) report a μ : δ : κ binding ratio of 25 : 26 : 49 % respectively and an affinity for [³H]DAMGO at the μ receptor of 2.27nM, comparing well with the values obtained here. In the present studies M6G afforded similar affinities for μ receptors in both brain, cord and plexus (7.9, 14.8 & 2.4nM) as did morphine (6.2, 5.4 and 3.1nM).

The displacement profiles in the assays performed, under conditions promoting low affinity receptor states, did not have slopes of 1.0 against naloxone, probably due to the ability of naloxone to label other sites, δ and κ, in addition to μ receptors. The M6G displacement curve was shallower than morphine, and indicated a greater selectivity and thus differentiation between receptor types, especially over κ.

In order to avoid the problem caused by the lack of selectivity of [³H]naloxone, studies using the more μ-selective ligand, [³H]CTOP were performed. [³H]CTOP, like naloxone is an antagonist and would be expected
to label both high and low affinity states with similar affinities.

\[ ^3\text{H} \]CTOP binding was investigated in mouse brain and an equilibration time of 120 mins at 25°C and 60 mins at 37°C were obtained. The kinetics for \[ ^3\text{H} \]CTOP were slightly at variance with Yamamura et al (1989) who reports a quicker equilibration time of 90 mins at 25°C and 25 mins at 37°C. It was interesting that higher binding was observed at 25°C than 37°C, which is again in agreement with Yamamura et al (1989).

The maximal \( \mu \) binding capacity \( B_{\text{max}} \) as measured using DAMGO in rat and mouse brain was 67.9 and 82.6 fmoles/mg respectively. \( B_{\text{max}} \)'s determined using \[ ^3\text{H} \]CTOP in rat and mouse brain were 59.7 and 97.8 fmoles/mg respectively. Thus the \( \mu \) antagonist CTOP and the \( \mu \) agonist DAMGO appeared to label a similar population of sites. The affinity (\( K_D \)) of \[ ^3\text{H} \]CTOP for these \( \mu \)-sites in mouse brain was 0.30nM, and in rat brain 0.65nM. Yamamura et al (1989) quotes a \( B_{\text{max}} \) of 94 fmoles/mg for \[ ^3\text{H} \]CTOP in rat brain and a \( K_D \) of 0.16nM and Kosterlitz and Paterson (1990) report \[ ^3\text{H} \]CTOP to label a single class of binding site, with a \( K_D \) of 0.12±0.01nM.

Saturation analysis for \[ ^3\text{H} \]CTOP in Tris buffer for the rat gave a low correlation coefficient for one site, but a high coefficient for one site in mouse brain homogenate. However displacement studies in the mouse did not afford monophasic curves. From the nature of the dose-response curves for the \( \mu \)-ligands, it was possible that two separate sites were involved. As \[ ^3\text{H} \]CTOP is a highly selective \( \mu \)-antagonist (Yamamura et al, 1989), this may indicate the ability of \[ ^3\text{H} \]CTOP to bind to high and low affinity \( \mu \)-opioid receptor states in mouse brain. However using the logistic curve-fitting programme of Barlow (1991) it was not possible to differentiate more than 1-site. Shifts obtained in NaCl and guanylimidodiphophate using \[ ^3\text{H} \]CTOP were inconclusive because of the shallow slopes obtained and the lack of shifts produced. Affinities obtained in Tris with \[ ^3\text{H} \]CTOP for \( \mu \)-ligands were much lower than those obtained with \[ ^3\text{H} \]naloxone (Table 5.8) and as a result of this the shifts are much less with \[ ^3\text{H} \]CTOP and this could explain why no shifts were observed. Also though \[ ^3\text{H} \]CTOP itself was seen to be sensitive to NaCl and Gpp(NH)p, thus
making it difficult to determine any shift with the competing agonists. Thus in the presence of NaCl and guanylimidodiphosphate, a decrease in affinity of $[3^H]$CTOP was observed to 7.62nM, and a reduction in $\mu$ receptor density to 78 fmoles/mg. This confirms the findings of Yamamura et al (1989), that NaCl reduces the number of binding sites by 31% and the affinity by 20-fold, whereas Gpp(NH)p (30μM) reduced the affinity three-fold, but not the number of binding sites.

As expected by virtue of the 3-substituent M3G had no measurable affinity for opioid receptors, $\mu$, $\delta$ or $\kappa$, agreeing with Paul et al (1989 i) who find it to have very low affinities ( $>500$ at $\mu$, $>1000$ at $\delta$ in cow brain and $>250$ at $\kappa$ in guinea-pig brain) and Christensen et al (1991), who reports an affinity of $>10,000$ nM for $\mu$ receptors in cow brain. All these findings disagree with Chen et al (1991), who quotes an affinity of 37.1nM for M3G at the $\mu$ receptor in rat brain homogenate. This discrepancy is difficult to explain, though minor methodological differences are present.

### 5.4.2 Isolated tissue bioassays

*In vitro* work was performed in the MPLM and MVD. There is much evidence to support that receptors in the these peripheral tissues are very similar to the brain receptors that mediate the analgesic action of the narcotic analgesic (Kosterlitz et al, 1975). In support of this similar binding affinities were obtained for agonists in mouse brain and guinea-pig MPLM.

In the mouse vas deferens preparation an IC$_{50}$ value was obtained for morphine of 174.4nM, which is lower than the 794.3nM quoted by Schulz (1979) and 492nM by Kosterlitz & Watt (1968), while Smith (1984) reports that morphine acts as a partial agonist in the MVD preparation. The differences are probably due to different strains of mice used. M6G shows a somewhat higher potency (IC$_{50}$ 104nM) than morphine. Similar affinities for naloxone were obtained, giving $K_e$ values of 2.6nM and 3.6nM against M6G and morphine respectively, confirming the compounds are acting *via* $\mu$-opioid receptors in this tissue.

In the guinea-pig MPLM, potency was determined for morphine and M6G. It was shown that M6G had twice the potency of morphine affording IC$_{50}$'s of 58 and 130nM respectively. Naloxone $K_e$ values for both compounds
were in the range corresponding to an interaction with \( \mu \) receptors (3.00 and 3.45nM). This agrees with Kosterlitz & Watt who quoted an IC\(_{50}\) of 68.2nM for morphine and a naloxone \( K_e \) of 1.22nM (Kosterlitz & Watt, 1968), and Schulz (1979) who obtained an IC\(_{50}\) for morphine of 125.9nM. During the writing of this thesis Schmidt et al (1994) report that M6G and morphine are equipotent in the guinea-pig ileum, acting via \( \mu \) receptors, thus supporting findings here.

M3G was inactive as an agonist in the guinea-pig MPLM up to 10,000nM. Previous workers (Gong et al, 1991; Smith et al, 1990) report that M3G is an antagonist at \( \mu \) receptors. The potential antagonist nature of M3G was investigated in vitro, in the MPLM. No functional antagonism was seen against either DAMGO or morphine at doses of M3G up to 10,000nM. This is consistent with results obtained in binding assays obtained here and by other workers (Paul et al, 1989 i), which show M3G to have no affinity for opioid receptors whether \( \mu \), \( \delta \) or \( \kappa \) probably because it lacks the 3-phenolic hydroxy group essential for binding to opiate receptors (Casy & Parfitt, 1986). Studies were also made of similar weaker compounds e.g. codeine in the MPLM. Codeine was relatively inactive in this tissue compared with morphine. The reason why codeine possesses some potency, whilst M3G does not, is probably due to steric effects i.e. the glucuronide moiety is much larger than the methoxy group. The low potency of codeine in the guinea-pig MPLM as compared with its antinociceptive effect in the whole animal, may be due to the metabolic formation of morphine in the whole animal (Casy & Parfitt, 1986).

The higher affinity of M6G for the \( \delta \) receptor in binding assays was confirmed in the hamster vas deferens preparation, where M6G shows a slightly higher potency than morphine (IC\(_{50}\)'s of 286 & 715nM respectively). IC\(_{50}\) values were predicted by interpolation using Barlow's programme (1991), as 50% inhibition was not reached. However both compounds only showed partial agonism, and reached a maximum inhibition of 40 and 44% for morphine and M6G respectively. Miller and Shaw (1985) report that morphine shows no agonist properties in this preparation and Sheehan et al (1986) reports that normorphine and fentanyl were both inactive as agonists at doses up to 10,000nM in the same strain of hamster.

An IC\(_{50}\) is a measure of potency, so in order to study the affinity of a compound in isolated tissue preparations it is necessary to use a
preparation in which the compounds can be shown to be antagonists. Such a preparation is the rat vas deferens (RVD). The RVD contains a μ receptor population which possesses a high intrinsic activity requirement for activation, i.e., it has a low receptor reserve such that partial agonists (including morphine) demonstrate antagonist properties, for example Schulz (1979) quotes an IC50 of >100,000nM for morphine.

In the RVD full agonist potency could not be obtained with either M6G or morphine; both compounds reaching a maximum of 30% inhibition. However M6G had twice the potency of morphine at the IC20 level. The relative efficacies of morphine and M6G were found to be 18.0 and 7.8 times less than DAMGO.

Since morphine and M6G acted as weak partial agonists the Ke values could be determined against DAMGO as an agonist. M6G (194nM) had an affinity five times that of morphine (1007nM). Carroll et al (1988) quote a similar Ke for morphine of 1500±230nM and Smith and Ranee (1983) obtain a Ke value of 1226nM.

The affinity of morphine was also determined in the MPLM, following reduction in the number of μ receptors using β-FNA. Results in the MPLM (after 100nM β-FNA treatment) gave affinity values of 900nM, 1900nM and 830nM for M6G, morphine and DAMGO respectively i.e. M6G had twice the affinity of morphine. Using N-ethylmaleimide (NEM) as an alkylating agent M6G again afforded twice the affinity for the μ receptor compared to morphine (800 compared to 1600nM), in agreement with results obtained using β-FNA. Although the results agree with others (Carroll et al, 1988; Smith & Rance, 1983), they do not however agree with those obtained by Kosterlitz. He reports an affinity of morphine (Ke) of 87.5±18.1nM in the MPLM. The reason for this discrepancy may relate to experimental differences. Kosterlitz did not use Furchgott analysis, but used a method developed by himself and other co-workers (See Chapter 3, page 49, Kosterlitz & Watt, 1968).

After β-FNA pre-treatment, a much greater flattening of the dose-response curve for M6G than morphine was seen. This may be that as few μ receptors were present, morphine exerts part of its action via κ receptors (Franklin & Traynor, 1992), whereas the M6G response was solely due to the remaining μ receptors, as it lacks affinity for the κ receptor.
5.4.3 Overview

The results showed that morphine and M6G had comparable affinities by Furchgott analysis, in the MPLM. Binding assays in Tris-Na and HEPES/Krebs gave similar results for morphine and M6G. However results in the rat vas deferens preparation may indicate a slightly higher \( \mu \)-affinity for M6G (Table 5.21). The fact that morphine and M6G had similar affinities for the \( \mu \) receptor in both high and low affinity states, indicated that the glucuronic acid part of the molecule does not interfere with binding and consequently there is probably an area within the receptor, into which the sugar moiety can be accommodated. This means that the 6-position of morphine is relatively free for chemical manipulation as demonstrated by the existence of many 6-substituted compounds eg. \( \beta \)-FNA, some of which have been mentioned earlier (Vizi et al., 1974; Gebhart & Spratt, 1976; Lidner & Raab, 1981; Takayaruji et al., 1988). It is interesting that the sugar group increased the \( \delta \) affinity, but reduced the \( \kappa \) affinity. This may suggest that the sugar can be accommodated in the \( \delta \)-site, but not the \( \kappa \)-site, however lipophilic differences between \( \delta \) and \( \kappa \)-sites, may explain the findings, though again the HPLC studies did not show any marked overall differences in the overall lipophilicity of the compounds.

From these studies it appeared that neither affinity or efficacy were responsible for M6G’s increased activity over morphine. The especially high potency of M6G administered directly into the CNS, along with its ability to elicit analgesia when given peripherally are not explained by receptor differences. Thus pharmacokinetic studies eg. distribution of M6G may be responsible.

Table 5.21 Affinities of M6G and morphine measured in the rat vas deferens against the agonist DAMGO, and in the MPLM, following a reduction of \( \mu \) receptors by \( \beta \)-FNA and in Tris, Tris-Na and HEPES/Krebs and in the plexus.

<table>
<thead>
<tr>
<th>Affinities (( \mu M ))</th>
<th>BRAIN</th>
<th>MPLM</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_D ) (MPLM)</td>
<td>( K_c ) (RVD)</td>
<td>( K_i ) Tris</td>
</tr>
<tr>
<td>M6G</td>
<td>0.9±0.10</td>
<td>0.2±0.01</td>
</tr>
<tr>
<td>Morphine</td>
<td>1.9±0.41</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>DAMGO</td>
<td>0.83±0.10</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± sem of data from \( \geq 3 \) experiments.
CHAPTER 6

SYNTHETIC MORPHINE-6-SUBSTITUTED COMPOUNDS AS ANALOGUES OF MORPHINE-6-GLUCURONIDE

- INCLUDING A STUDY OF SOME 3-MORPHINE ETHERS
Many extensive reviews have covered structure activity relationships of the morphine skeleton (for example see Casy & Parfitt, 1986). Several points are relevant to the 6-position. Substitution at C-6 of the morphine skeleton is not essential for analgesic activity. For example desomorphine (Figure 6.1) is 10 times more potent than morphine as measured in the mouse, using the hot plate test, and is much more rapid in onset (Eddy et al., 1957).

![Desomorphine](image)

Figure 6.1 Desomorphine

Similarly (-)-levorphanol (Figure 6.2), which also lacks the 4,5-ether bridge, is 2 to 3 times more potent than morphine. Unlike morphine it is almost as effective by p.o. as by s.c. or i.m. administration (Bowman & Rand, 1980 i).

![Levorphanol](image)

Figure 6.2 Levorphanol

However whilst 6-substitution is not a requirement, analgesic activity can be manipulated by 6-substitutions as indicated by the analgesic activity of M6G, discussed in Chapter 5. Also there are other morphine-6-substituted compounds in addition to M6G. Yoshimura et al (1973) reported that morphine-6-sulphate (M6S) (Figure 6.3), a metabolite of morphine in mice, had analgesic activity in mice of the same order of magnitude as M6G, being much more potent than morphine and of longer duration. Morphine-3-sulphate was shown to be inactive (Yoshimura et al, 1973).
Analgesia observed after M6S administration is not attributable to free morphine, which may have been liberated in vivo from M6S, because the potencies of morphine and M6S are so dissimilar in the two tests (Yoshimura et al., 1976), as is the case with M6G (Shimomura et al., 1971).

The activities of some 6-substituted compounds have also been examined in vitro. Yoshimura (1977) studied the inhibitory effect of morphine, morphine-3-X, and morphine-6-X, X being glucuronides, ethereal sulphates and phosphates, on narcotine-induced contraction of the isolated guinea-pig ileum. The potency of the compounds decreased in the order:- Morphine-6-sulphate > M6G > morphine = morphine-6-phosphate > M3G = morphine-3-phosphate.

Treatment of morphine-6-conjugates with lithium chloride or piperidine readily forms 6-chloro-6-deoxy-morphine (2) and 8-piperidino-6-deoxy-6-morphine (1), respectively, as shown in Figure 6.4 (Yoshimura, 1977). It is interesting to note that the reaction rates of the conjugates are roughly parallel with analgesic activity decreasing in the order :- sulphate > glucuronide = acetate > phosphate > morphine. Thus this nucleophilic substitution reaction gives an indication of the compounds analgesic potency.

Some of the reported 6-substituted derivatives are of particular interest in relation to studies of M6G in that they contain a functional group linked by an oxygen to the alkaloid D-ring. Lidner and Raab (1981) studied the pharmacokinetics of morphine-6-nicotinate (Figure 6.5) following i.v. administration in rats. The strong analgesic activity of this compound corresponds with a high morphine-6-nicotinate level in brain. Indeed the transport of free morphine across the blood brain barrier decreases in the presence of morphine-6-nicotinate in the blood.
Figure 6.4 Scheme showing the reactivity of morphine-6-conjugates with lithium chloride or piperidine in boiling acetone or benzene.

Figure 6.5 Morphine-6-nicotinate

Morphine-6-hemisuccinate (Figure 6.6) is reportedly similar in potency to morphine and is 6 times less potent than morphine-6-sulphate in the hot plate test in mice when administered i.p. The peak analgesia and duration
of action being the same for morphine-6-hemisuccinate and morphine-6-
sulphate (Gebhart & Spratt, 1976). Codeine-6-succinate is also reported to
retain its potency both \textit{in vitro} (MPLM) and \textit{in vivo} (mice), while having
less antitussive potency in cats (Harris, 1987).

\begin{center}
\includegraphics[width=0.3\textwidth]{morphine-6-hemisuccinate.png}
\end{center}

\textbf{Figure 6.6} Morphine-6-hemisuccinate

In 1988 Takayaaruji \textit{et al} found \(-\)-6\(\beta\) acetyltiomorphine (Figure 6.7) to
be \(\mu\)-selective, with the same affinity as morphine for the \(\mu\)-opioid binding
site, but twice the potency in the guinea-pig MPLM preparation and 5 times
the potency \textit{in vivo} in the paw pressure test in rats.

\begin{center}
\includegraphics[width=0.3\textwidth]{acetyltiomorphine.png}
\end{center}

\textbf{Figure 6.7} Acetyltiomorphine

Compounds may also contain nitrogen in place of oxygen in the 6-position.
6-Azidomorphine (Figure 6.8) is more potent than morphine, affording an
IC\(_{50}\) in the MPLM of 2.0nM, compared to a value for morphine of 58.0nM.
This improved activity can also be seen \textit{in vivo} (Vizi \textit{et al}, 1974).
Azidomorphine and morphine afforded ED\(_{50}\) values in the rat as measured
in the hot plate test of 0.036 and 4.0mg/kg respectively (Vizi \textit{et al}, 1974).
Many other 6-substituted derivatives exist. β-funaltrexamine (β-FNA) (Portoghese et al., 1980; Ward et al., 1982) and β-chlornaltrexamine (β-CNA) (Portoghese et al., 1987) are two such compounds containing large functional groups that act as irreversible alkylating agents. The size of these substituents and others, in particular the glucuronides, suggests considerable space exists in the receptor for exploitation by synthetic derivatives.

The aim of the experiments described in this chapter was to evaluate a series of synthesised compounds designed as mimics of M6G. Such compounds would hopefully have greater potency and give more predictable kinetics over morphine and should be long lasting and preferably orally active for medical usage. The compounds were synthesised by Anna Di Pretoria at Loughborough University in a collaborative project. Previous studies show some 3-substituted ether compounds to possess antinociceptive potency (Mohacsi et al., 1982), so an investigation of the 3-position was carried out using a set of compounds supplied by Dr. Lewis (Bristol University), coded as BU18-BU25 inclusively.

### 6.2 6-substituted compounds under study

The derivatives (Table 6.2) were studied by ligand-binding assay and isolated tissue bioassay to investigate their opioid receptor affinities and potencies respectively.
<table>
<thead>
<tr>
<th>No.</th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>2</td>
<td>Me</td>
<td>H</td>
</tr>
<tr>
<td>3</td>
<td>Me</td>
<td>phthalate</td>
</tr>
<tr>
<td>4</td>
<td>Me</td>
<td>succinate</td>
</tr>
<tr>
<td>5</td>
<td>H</td>
<td>p-nitrobenzoate</td>
</tr>
<tr>
<td>6</td>
<td>H</td>
<td>p-fluorobenzoate</td>
</tr>
<tr>
<td>7</td>
<td>H</td>
<td>p-bromobenzoate</td>
</tr>
<tr>
<td>8</td>
<td>H</td>
<td>p-chlorobenzoate</td>
</tr>
<tr>
<td>9</td>
<td>H</td>
<td>p-bromobenzoate</td>
</tr>
<tr>
<td>10</td>
<td>H</td>
<td>p-hydroxybenzoate</td>
</tr>
<tr>
<td>11</td>
<td>H</td>
<td>O-protected glucuronide*</td>
</tr>
<tr>
<td>12</td>
<td>H</td>
<td>phthalate (Figure 6.9)</td>
</tr>
<tr>
<td>13</td>
<td>H</td>
<td>benzoate (Figure 6.10)</td>
</tr>
<tr>
<td>14</td>
<td>H</td>
<td>succinate (Figure 6.11)</td>
</tr>
<tr>
<td>15</td>
<td>H</td>
<td>O-protected glucuronide* (Figure 6.13)</td>
</tr>
<tr>
<td>16</td>
<td>Me</td>
<td>O-glucuronide</td>
</tr>
<tr>
<td>17</td>
<td>Me</td>
<td>O-glucuronide</td>
</tr>
<tr>
<td>18</td>
<td>H</td>
<td>3-TIPS</td>
</tr>
</tbody>
</table>

* sugar hydroxyl groups protected as acetates and the acidic function as a methyl ester (Figure 6.13). 3-TIPS is triisopropylsilyl.

Figure 6.9 Morphine-6-phthalate
Figure 6.10  Morphine-6-benzoate

Figure 6.11  Morphine-6-succinate

Figure 6.12  3-TIPS-morphine (12)
6.3 Ligand binding assays

The binding of the various 6-substituted compounds was studied at pH 7.4 in Tris buffer (Table 6.1). M6G ($K_i$ 8nM) showed a similar high affinity binding to the $\mu$ receptor as morphine ($K_i$ 6nM). The 6-substituted morphines, the nitrobenzoate (5) and halogenated, hydroxybenzoate, protected glucuronide, phthalate, benzoate and succinate (13)-(20) also showed high affinities for the $\mu$ receptor, having $K_i$'s in the range 2nM for the 6-hydroxybenzoate derivative (16) to 30nM for the 6-nitrobenzoate derivative (5).

As shown in chapter 5, M6G had a 3 times higher affinity for the $\delta$ receptor than morphine. At the $\delta$-site other 6-substituted morphine derivatives, namely the hydroxybenzoate, protected glucuronide, phthalate, benzoate and succinate (16)-(20) had higher affinity than morphine with $K_i$'s in the range 14nM for morphine-6-phthalate to 33nM for the M6G-protected sugar (17) (chapter 5). Indeed morphine-6-phthalate (18) had a higher $\delta$ than $\mu$-affinity. Other 6-substituted compounds (5), (18) and halogenated compounds (13)-(15) afforded affinities in the range similar to morphine and M6G ie. 69-205nM.

The 6-substituted morphine derivatives had lower affinities for the $\kappa$-receptor than the $\mu$-receptor. This is similar to M6G which showed a 20-fold decrease in affinity over morphine. Indeed all of the morphine compounds (6) and (11)-(20) had $\kappa$-affinities less than morphine, ranging from 135-2774nM. The nitrobenzoate (5) had a very low affinity of $>10,000nM$. 

Figure 6.13 M6G (protected) (17)
Codeine (2) had weak affinity at both μ and δ sites. Codeine phthalate (3) and codeine succinate (4) had similarly weak affinities for μ, but affinity for δ was higher than codeine, especially for the codeine phthalate (3), which had a 30-fold higher affinity for the δ receptor, having an affinity in between morphine and M6G.

The immediate synthetic precursors to all of the 6-substituted morphine compounds were their 3-silyl ethers. Several of these were evaluated in the binding assays. Surprisingly in view of the site of substitution all of the compounds had some degree of affinity for the μ receptor. Indeed 3-O-silyl morphine, with a Kᵢ of 2.5nM, had higher affinity even than morphine. The phthalate (7) also had high affinity for δ receptors with a Kᵢ of 4.10nM, whilst 3-O-silyl morphine (6), (175nM) and benzoate (8), (485nM) also bound reasonably well at the δ-site. 3-O-silyl morphine (6), (135nM) was the only silyl compound with affinity for the κ receptor (Table 6.1). Even 3-TIPS morphine (12), with a very large 3-substituent, showed affinity for opioid receptors, having highest affinity for the μ (107nM), κ (356nM) then δ (3048nM) receptor.

The selectivities of the various 6-substituted compounds for the opioid receptor types, as derived from binding experiments, are shown in Table 6.2. The most highly μ/δ selective compounds were 3-O-silyl morphine (6), 3-O-TIPS morphine (12), morphine-6-hydroxybenzoate (16), the protected codeine glucuronide (21) and the codeine glucuronide (22). All being comparable with morphine, having a preference for μ over δ of >10-fold.

Compounds selective for μ over κ include the morphine-6-substituted compounds p-nitrobenzoate (5) and bromobenzoate, hydroxybenzoate, protected glucuronide, phthalate, benzoate and succinate, protected codeine glucuronide and codeine glucuronide (15)-(22) and 3-silylmorphine (6), 3-silylmorphine-6-phthalate (7) and 3-silylmorphine-6-benzoate (8). All having a preferred affinity of μ over κ of at least 17 times, being comparable to M6G. Compounds with a similar ratio to that of morphine include 3-TIPS morphine (12) and morphine-6-chlorobenzoate (14).
Compounds selective for $\delta$ over $\kappa$ include morphine-6-nitrobenzoate (5), 3-silylmorphine-6-phthalate (7), 3-silylmorphine-6-benzoate (8), and morphine-6-protected glucuronide, phthalate, benzoate and succinate (17)-(20), having $\kappa/\delta$ ratios of $<0.05$, being comparable to M6G. In contrast the only compounds which have a higher affinity for the $\kappa$ receptor than the $\delta$ receptor, were 3-silylmorphine-6-nitrobenzoate (11), and 3-TIPS morphine (12).

Compounds 3-silylmorphine-6-fluorobenzoate (9), 3-silylmorphine-6-bromobenzoate (10) and codeine glucuronide (22) were largely inactive and thus non-selective between $\delta$ and $\kappa$ receptors. Indeed some compounds showed no affinity for $\kappa$ receptor binding sites up to 10,000nM.
### Table 6.1 Affinities ($K_i$ in nM) of 6-substituted morphine derivatives at $\mu$, $\delta$ and $\kappa$ opioid binding sites in mouse brain homogenates

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (nM)</th>
<th>$\mu$</th>
<th>$\delta$</th>
<th>$\kappa$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-6-G</td>
<td>7.90±1.48</td>
<td>75.1±15.6</td>
<td>*850±118</td>
<td></td>
</tr>
<tr>
<td>(1) morphine</td>
<td>6.22±0.86</td>
<td>218±41.2</td>
<td>*84.7±4.01</td>
<td></td>
</tr>
<tr>
<td>(2) codeine</td>
<td>2700±302</td>
<td>&gt;10,000</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>(3) codeine derivative</td>
<td>4500±513</td>
<td>182.0±21.3</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>(4) codeine derivative</td>
<td>2010±298</td>
<td>3,001±231</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>30.1±1.40</td>
<td>68.5±4.61</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>(6) silyl derivative $^x$</td>
<td>2.50±0.41</td>
<td>175±5.60</td>
<td>135±14.1</td>
<td></td>
</tr>
<tr>
<td>(7) silyl derivative $^x$</td>
<td>17.5±2.80</td>
<td>4.10±0.30</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>(8) silyl derivative $^x$</td>
<td>310±22.1</td>
<td>485±24.5</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>(9) silyl derivative $^x$</td>
<td>1,477±251</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>(10) silyl derivative $^x$</td>
<td>5,081±428</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>(11) silyl derivative $^x$</td>
<td>1282±87.1</td>
<td>&gt;10,000</td>
<td>737±52.6</td>
<td></td>
</tr>
<tr>
<td>(12)</td>
<td>107±15.8</td>
<td>3,048±247</td>
<td>356±20.2</td>
<td></td>
</tr>
<tr>
<td>(13)</td>
<td>16.6±3.2</td>
<td>83.3±6.8</td>
<td>193.2±14.1</td>
<td></td>
</tr>
<tr>
<td>(14)</td>
<td>17.7±2.8</td>
<td>84.9±8.4</td>
<td>149.9±12.2</td>
<td></td>
</tr>
<tr>
<td>(15)</td>
<td>28.4±5.2</td>
<td>205.0±10.2</td>
<td>832.9±24.1</td>
<td></td>
</tr>
<tr>
<td>(16)</td>
<td>1.73±0.26</td>
<td>22.2±3.41</td>
<td>157±12.6</td>
<td></td>
</tr>
<tr>
<td>(17)</td>
<td>11.2±2.6</td>
<td>33.0±3.24</td>
<td>820±26.3</td>
<td></td>
</tr>
<tr>
<td>(18)</td>
<td>27.8±3.8</td>
<td>14.2±2.9</td>
<td>2,774±205</td>
<td></td>
</tr>
<tr>
<td>(19)</td>
<td>9.25±3.65</td>
<td>20.7±2.65</td>
<td>161.1±12.6</td>
<td></td>
</tr>
<tr>
<td>(20)</td>
<td>9.83±2.68</td>
<td>32.2±3.56</td>
<td>1187±156</td>
<td></td>
</tr>
<tr>
<td>(21)</td>
<td>125.9±10.3</td>
<td>4,100±36.4</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>(22)</td>
<td>430.1±23.6</td>
<td>7,964±126</td>
<td>8,392±206</td>
<td></td>
</tr>
</tbody>
</table>

$^x$ Sites were labelled with [3H]DAMGO (1.02±0.19nM), $\delta$ sites by [3H]DPDPE (1.60±0.29nM) and $\kappa$ sites by [3H]CI977 (0.15±0.03nM, except * where tritiated U6953 (1.07±0.13nM) was used. $K_i$ values were determined from $K_D$ values of the ligands at $\mu$ (DAMGO 1.37nM), $\delta$ (DPDPE 5.75nM) and $\kappa$ (CI977 0.25nM) (Chapter 5). Experiments were performed at 25°C for 40 mins in Tris-HCl buffer pH 7.4. In all cases Hill Coefficients were not significantly different from unity. Values are means ± sem of > 3 experiments unless stated, nt denotes not tested and $^x$ 3-O-silyl substituted.
Table 6.2 Selectivities of 6-substituted compounds for μ, δ, or κ sites, taken from Table 6.1

<table>
<thead>
<tr>
<th></th>
<th>Kᵢ μ/Kᵢδ</th>
<th>Kᵢ μ/Kᵢκ</th>
<th>Kᵢ δ/Kᵢκ</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6G</td>
<td>0.11</td>
<td>0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>(1) morphine</td>
<td>0.03</td>
<td>0.15</td>
<td>5.34</td>
</tr>
<tr>
<td>(2)</td>
<td>&lt;0.27</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(3)</td>
<td>24.73</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(4)</td>
<td>0.67</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(5)</td>
<td>0.44</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(6)</td>
<td>0.01</td>
<td>0.02</td>
<td>1.30</td>
</tr>
<tr>
<td>(7)</td>
<td>4.27</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(8)</td>
<td>0.64</td>
<td>&lt;0.03</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(9)</td>
<td>0.15</td>
<td>&lt;0.15</td>
<td>-</td>
</tr>
<tr>
<td>(10)</td>
<td>&lt;0.51</td>
<td>&lt;0.51</td>
<td>-</td>
</tr>
<tr>
<td>(11)</td>
<td>0.13</td>
<td>1.74</td>
<td>13.57</td>
</tr>
<tr>
<td>(12)</td>
<td>0.04</td>
<td>0.30</td>
<td>8.56</td>
</tr>
<tr>
<td>(13)</td>
<td>0.20</td>
<td>0.09</td>
<td>0.43</td>
</tr>
<tr>
<td>(14)</td>
<td>0.21</td>
<td>0.12</td>
<td>0.57</td>
</tr>
<tr>
<td>(15)</td>
<td>0.14</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>(16)</td>
<td>0.08</td>
<td>0.01</td>
<td>0.14</td>
</tr>
<tr>
<td>(17)</td>
<td>0.33</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>(18)</td>
<td>1.96</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(19)</td>
<td>0.45</td>
<td>0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>(20)</td>
<td>0.31</td>
<td>&lt;0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>(21)</td>
<td>0.03</td>
<td>&lt;0.01</td>
<td>&lt;0.41</td>
</tr>
<tr>
<td>(22)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.95</td>
</tr>
</tbody>
</table>

6.4 Isolated tissue studies

Binding assays only give a measure of the affinity of a drug for a receptor. They give no indication of possible agonist or antagonist nature, i.e., efficacy at various receptor sites. In order to study this, compounds were evaluated in two *in vitro* isolated tissue preparations, namely the guinea-pig myenteric plexus longitudinal muscle (MPLM) and the mouse vas deferens (MVD). The results are shown in Table 6.3.
Table 6.3 Potency of 6-substituted morphine derivatives (unless indicated) on the guinea-pig ileum (MPLM) and mouse vas deferens (MVD) preparations

<table>
<thead>
<tr>
<th>Compound</th>
<th>MPLM (nM)</th>
<th>MVD (nM)</th>
<th>MPLM/MVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-6-G</td>
<td>58.0±4.30</td>
<td>104±17.1</td>
<td>0.56</td>
</tr>
<tr>
<td>(1) morphine</td>
<td>130±7.8</td>
<td>173±62.8</td>
<td>0.75</td>
</tr>
<tr>
<td>(2) codeine</td>
<td>1600±403</td>
<td>&gt;1000</td>
<td>&gt;1.60</td>
</tr>
<tr>
<td>(3) codeine derivative</td>
<td>3200±123</td>
<td>nt</td>
<td>-</td>
</tr>
<tr>
<td>(4) codeine derivative</td>
<td>1000±205</td>
<td>nt</td>
<td>-</td>
</tr>
<tr>
<td>(5)</td>
<td>132.4±11.85</td>
<td>1166±467</td>
<td>0.11</td>
</tr>
<tr>
<td>(6) silyl derivative x</td>
<td>302.0±21.2</td>
<td>279±45.0</td>
<td>1.08</td>
</tr>
<tr>
<td>(7) silyl derivative x</td>
<td>429±4.31</td>
<td>41.0±8.60</td>
<td>10.46</td>
</tr>
<tr>
<td>(8) silyl derivative x</td>
<td>&gt;10,000</td>
<td>2.633±502</td>
<td>&gt;3.80</td>
</tr>
<tr>
<td>(9) silyl derivative x</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>-</td>
</tr>
<tr>
<td>(10) silyl derivative x</td>
<td>&gt;10,000</td>
<td>5.625±618</td>
<td>&gt;1.78</td>
</tr>
<tr>
<td>(13)</td>
<td>1010±102</td>
<td>1325±262</td>
<td>0.76</td>
</tr>
<tr>
<td>(14)</td>
<td>1506±161</td>
<td>1162±151</td>
<td>1.30</td>
</tr>
<tr>
<td>(15)</td>
<td>1800±183</td>
<td>1281±186</td>
<td>1.41</td>
</tr>
<tr>
<td>(16)</td>
<td>9004±106</td>
<td>230±18.6</td>
<td>39.1</td>
</tr>
<tr>
<td>(17)</td>
<td>40.6±4.32</td>
<td>140±11.3</td>
<td>0.29</td>
</tr>
<tr>
<td>(18)</td>
<td>350±25.3</td>
<td>24.3±3.54</td>
<td>14.40</td>
</tr>
<tr>
<td>(19)</td>
<td>10,000±265</td>
<td>280±21.6</td>
<td>35.7</td>
</tr>
<tr>
<td>(20)</td>
<td>158.5±2.65</td>
<td>171±12.8</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Values are means ± sem of ≥3 experiments, unless stated otherwise, nt denotes not tested. x 3-O-silyl substituted.

In the MPLM, morphine-6-nitrobenzoate (5), M6G (protected) (17), morphine-6-phthalate (18) and morphine-6-succinate (20) had IC50 values in the range 132-350nM, similar to morphine, while the morphine-6-halogenated compounds (13)-(15) and hydroxybenzoate (16) and morphine-6-benzoate (19) had IC50 values in the range 1010-10,000nM. All these compounds acted as agonists. In the MVD the morphine-6-substituted
hydroxybenzoate, protected sugar, phthalate, benzoate and succinate (16)-(20) had IC50 values in the range 24-280nM, potencies similar to morphine. The morphine-6-halogenated compounds (13)-(15) had IC50 values in the range 1162-1325nM. Compounds of particular interest with good potency include morphine-6-nitrobenzoate (5) (Figure 6.14), which showed a higher potency in the MPLM (132nM) as opposed to the MVD (1166nM), where (5) was a partial agonist. In contrast morphine-6-phthalate (18) was much more active in the MVD (24nM) than in the MPLM (350nM) (Figure 6.15), while morphine-6-succinate (20) was non-selective between the MPLM (159nM) and the MVD (171nM) (Figure 6.16). Other compounds which had higher potency in the MVD as opposed to the MPLM included morphine-6-hydroxybenzoate (16) (Figure 6.17) and morphine-6-benzoate (19) (Figure 6.18), and acted as full agonists in the guinea-pig MPLM. The compounds appeared to be only partial agonists in the MPLM at the levels used, and was due to the shallow slopes obtained.

Figure 6.14 Inhibition of contractions of the field stimulated MPLM (closed circles) and MVD (open circles) by morphine-6-nitrobenzoate (5)
Figure 6.15 Inhibition of contractions of the field stimulated MPLM (closed circles) and MVD (open circles) by morphine-6-phthalate (18).

Figure 6.16 Inhibition of contractions of the field stimulated MPLM (closed circles) and MVD (open circles) by morphine-6-succinate (20), showing a lack of selectivity between the 2 preparations.
Figure 6.17 Inhibition of contractions of the field stimulated MPLM (closed circles) and MVD (open circles) by morphine-6-hydroxybenzoate (16).

Figure 6.18 Inhibition of contractions of the field stimulated MPLM (closed circles) and MVD (open circles) by morphine-6-benzoate (19), showing weaker activity in the MPLM.
The 6-substituted derivatives in which the phenyl ring was substituted with a p-halogen [morphine-6-fluoro (13) (Figure 6.19), chloro (14) (Figure 6.20) and bromo benzoate (15) (Figure 6.21)] showed weak agonist action in both the MPLM and the MVD, with IC$_{50}$ values of $>1000\text{nM}$, in spite of showing similar binding profiles to M6G. This implied that the compounds had high affinity, but low efficacy.

![Figure 6.19](image_url)

**Figure 6.19** Inhibition of contractions of the field stimulated MPLM (closed circles) and MVD (open circles) by morphine-6-fluorobenzoate (13), showing a lack of selectivity between the two preparations.
Figure 6.20 Inhibition of contractions of the field stimulated MPLM (closed circles) and MVD (open circles) by morphine-6-chlorobenzoate (14)

Figure 6.21 Inhibition of contractions of the field stimulated MPLM (closed circles) and MVD (open circles) by morphine-6-bromobenzoate (15)
Of the 3-O-silyl compounds, only silyl morphine (6) and silyl phthalate (7) afforded a reasonable potency. Silyl morphine (6) afforded IC$_{50}$'s of 302 and 279nM in the MPLM and MVD respectively. In contrast 3-silyl phthalate (6) had a much greater effect in the MVD than the MPLM. (IC$_{50}$ values of 41 and 429nM respectively). The 3-silyl-6-benzoate (8), 6-fluorobenzoate (9) and 6-bromobenzoate (10) had no effect in the MPLM and the 6-benzoate (8) and 6-bromobenzoate (10) only had a partial agonist effect in the MVD.

In order to determine with which receptors the various 6-substituted morphines were interacting, in the two tissues, the antagonist affinity constants ($K_e$) for two antagonists, the relatively non-selective naloxone and the $\mu$-selective cyprodime were determined (Table 6.4).

**Table 6.4 Antagonist equilibrium affinity constants ($K_e$, nM) for naloxone and cyprodime against 6-substituted morphine derivatives**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_e$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naloxone</td>
</tr>
<tr>
<td></td>
<td>MPLM</td>
</tr>
<tr>
<td>M-6-G</td>
<td>3.00±0.80</td>
</tr>
<tr>
<td>(1) morphine</td>
<td>3.45±0.59</td>
</tr>
<tr>
<td>(5)</td>
<td>3.50±0.31$^\dagger$</td>
</tr>
<tr>
<td>(6)</td>
<td>5.91±0.52$^\dagger$</td>
</tr>
<tr>
<td>(7)</td>
<td>4.12±1.40</td>
</tr>
<tr>
<td>(16)</td>
<td>3.21±0.35</td>
</tr>
<tr>
<td>(19)</td>
<td>3.10±0.62</td>
</tr>
</tbody>
</table>

Values are means ± sem of ≥ 3 experiments, unless stated otherwise. Naloxone $K_e$'s were determined using Schild plots, unless marked by a $^\dagger$, which represent a single concentration (30nM) of naloxone. Cyprodime $K_e$'s were obtained using a single concentration (300nM) of cyprodime. nt denotes not tested.

The results suggested that M6G, morphine (1) and 3-O-silylmorphine (6) were acting via $\mu$ receptors in both the MPLM and MVD, all affording naloxone $K_e$ values of approximately 3nM, whereas morphine-6
nitrobenzoate (5) and 3-O-silyl-morphine-6-phthalate (7) acted via $\mu$ receptors in MPLM and $\delta$ receptors in the MVD. The values for 3-O-silylmorphine (6) suggested the compounds acted mainly by $\mu$ receptors, but the $K_e$ values indicated a mixed receptor population. However cyprodime $K_e$ values confirmed $\mu$ receptor involvement.

Some of the morphine-6-substituted compounds showed affinity for opioid receptors in binding assays, but possessed only weak efficacy in isolated tissue preparations. These compounds may act as antagonists in some situations. Morphine-6-halogenated and hydroxybenzoate compounds (13), (14), (15) & (16) were tested for their antagonist nature at concentrations varying between 30-1000nM against DAMGO, DPDPE and U69593 as agonists for $\mu$, $\delta$, and $\kappa$ receptors respectively in the MVD and MPLM. The affinity values ($K_e$) obtained are given in Table 6.5. It should be noted that these compounds did exhibit weak agonist properties and so antagonist affinity constants were determined using the method devised by Kosterlitz & Watt (1968) for partial agonists, as described in Chapter 3.

Table 6.5 Antagonist equilibrium affinity constants ($K_e$ nM) of morphine-6-substituted compounds in the MVD and MPLM

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$K_e$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MVD</td>
</tr>
<tr>
<td></td>
<td>$\mu$</td>
</tr>
<tr>
<td>F Benzoate (13)</td>
<td>21.5±3.0</td>
</tr>
<tr>
<td>Cl Benzoate (14)</td>
<td>288±25.3</td>
</tr>
<tr>
<td>Br Benzoate (15)</td>
<td>193±23.3</td>
</tr>
<tr>
<td>OH Benzoate (16)</td>
<td>86.7±6.5</td>
</tr>
<tr>
<td>NO$_2$ Benzoate (5)</td>
<td>nt</td>
</tr>
</tbody>
</table>

Compounds tested at 30nM(#), 300nM(¶), or 1,000nM(•).
- indicates no antagonist action at this receptor type, ie no shift in the dose-response curve observed at a concentration of 1,000nM. nt denotes not tested.

All of the compounds tested acted as antagonists, but to varying degrees. Compound (5) (6-p-nitrobenzoate) and (16) (6-p-hydroxybenzoate) showed a high degree of $\mu$ antagonism in the MPLM. It is interesting to note that (5) afforded a $K_e$ of 0.7nM in the MPLM, but a $K_i$ of 30nM in mouse brain.
homogenate (Table 6.1). The reason for this is at present unexplained. In the MVD however compounds (13)-(16) acted as both \( \mu \) and \( \delta \) antagonists, morphine-6-chlorobenzoate (14) had the highest affinity for the \( \delta \) receptor. The hydroxybenzoate analogue (16) was the only compound tested to demonstrate antagonism at the \( \kappa \)-receptor site.

6.5 Stability studies
The observed activity of some of the 3-O-silyl compounds (6 and 7) was unexpected, as a 3-OH group is believed important for activity (Chen et al., 1991). Studies were performed in mouse brain homogenate to determine whether activity was due to loss of the 3-O-silyl group. Compounds were evaluated by TLC before and after incubation with homogenate for 30 mins at 37°C. \( R_F \) values of the compounds are shown in Table 6.6.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R_F ) value</td>
<td>Buffer alone (( R_F ) in brackets)</td>
</tr>
<tr>
<td>Morphine (1)</td>
<td>0.30</td>
<td>Stable (0.30)</td>
</tr>
<tr>
<td>3OSi morphine (6)</td>
<td>0.70</td>
<td>Unstable (0.30)</td>
</tr>
<tr>
<td>M6G(prot.sug) (17)</td>
<td>0.70</td>
<td>Stable (0.70)</td>
</tr>
<tr>
<td>M-6-NO(_2)benzoate (5)</td>
<td>0.63</td>
<td>Stable (0.63)</td>
</tr>
<tr>
<td>3OSiM-6-NO(_2) benzoate (11)</td>
<td>0.86</td>
<td>Stable (0.86)</td>
</tr>
<tr>
<td>M-6-Fbenzoate (13)</td>
<td>0.50</td>
<td>Stable (0.50)</td>
</tr>
<tr>
<td>3OSiM-6Fbenzoate (9)</td>
<td>0.80</td>
<td>Stable (0.80)</td>
</tr>
<tr>
<td>M-6-Brbenzoate (15)</td>
<td>0.53</td>
<td>Stable (0.53)</td>
</tr>
<tr>
<td>3OSiM-6Brbenzoate (10)</td>
<td>0.81</td>
<td>Stable (0.81)</td>
</tr>
</tbody>
</table>

A solvent system of composition 80:20 % dichloromethane:water with silica as the stationary phase was used.

All of the compounds tested were stable to the buffer, the extraction process and mouse brain homogenate, except for 3-O-silyl morphine, which was broken down to the parent compound. This occurred even in the absence of homogenate. It would thus appear that the observed activity of (6) was due to the loss of the 3-O-silyl group to afford morphine. However this did not seem to be the case for other 3-O-silyl compounds.
6.6 Lipophilicities of morphine and related compounds

6.6.1 Rates of onset and offset of morphine and derivatives as measured in the MPLM

To determine whether the change in lipophilicity afforded by glucuronidation is reflected in kinetics, the onset and offset times of drugs in reducing the electrically induced twitch were assessed. Half-times of onset and offset of action (t_{0.5}) were measured in the MPLM for 3 different doses of the drug. The drug doses were chosen to give between 30 and 70% inhibition of the twitch. Results for morphine and M6G are given in Table 6.7 and show M6G to have the same onset and only slightly shorter offset of action compared with morphine.

Table 6.7 Rates of onset and offset of action of morphine and M6G in MPLM of the guinea-pig ileum

<table>
<thead>
<tr>
<th></th>
<th>Onset t_{0.5} (s)</th>
<th>Offset t_{0.5} (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6G</td>
<td>25.7±4.4</td>
<td>67.1±10.2</td>
</tr>
<tr>
<td>Morphine</td>
<td>26.8±3.1</td>
<td>90.0±12.4</td>
</tr>
</tbody>
</table>

6.6.2 Lipophilicities as determined using an HPLC system

The lipophilicities of morphine and three other selected, related compounds were analysed using an octanol:water solvent system (see Chapter 2, page 39). The lipophilicities were compared as log P values. Log P is defined as the logarithm of the ratio of the absorbance in octanol to the absorbance in phosphate buffer pH 7.4 (Leo et al., 1971). the higher the number the more lipophilic the drug. The determined lipophilicities of the compounds are shown in Table 6.8. The order of lipophilicity is thus methadone > heroin > codeine > morphine. The retention times of these compounds were determined by HPLC (Table 6.9) and a calibration curve constructed of log P against log k' where k' is the capacity factor and is defined as the difference in retention times between the test compound and morphine-6-fluorobenzoate, divided by the retention time of morphine-6-fluorobenzoate (13) (Figure 6.22). This was used as it had the shortest retention time.
Table 6.8 Lipophilicities (log P\textsubscript{oct}) of morphine and related compounds

<table>
<thead>
<tr>
<th></th>
<th>log P</th>
<th>Lit. values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>0.70±0.04</td>
<td>0.76 (Hansch &amp; Anderson, 1967)</td>
</tr>
<tr>
<td>Methadone</td>
<td>1.85±0.06</td>
<td>1.65^† (Kutter et al, 1970)</td>
</tr>
<tr>
<td>Codeine</td>
<td>0.92±0.04</td>
<td>1.01 (Bashilova &amp; Figuroski, 1959)</td>
</tr>
<tr>
<td>Heroin</td>
<td>0.95±0.02</td>
<td>1.07^* (Valette &amp; Eichererry, 1958)</td>
</tr>
</tbody>
</table>

- Solvent used is ethylloleate
- Solvent used is heptane

To determine the lipophilicities of other synthetic compounds without direct measurement, the retention times of the test compounds were determined using HPLC, and the log P value interpolated from the calibration curve of log P against log k' (Terada, 1986) (Table 6.9).

Figure 6.22 Calibration curve of log P against log k' for standard compounds morphine (M), heroin (H) and codeine (C). (Methadone was not added due to its very long retention time).
Table 6.9 Lipophilicities (log P) of synthesised morphine-6-substituted compounds derived from log k' values

<table>
<thead>
<tr>
<th>Retention time (mins)</th>
<th>log k'</th>
<th>log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6succ(20)</td>
<td>3.6±0.1</td>
<td>-1.23</td>
</tr>
<tr>
<td>M6CIB(14)</td>
<td>3.6±0.1</td>
<td>-1.23</td>
</tr>
<tr>
<td>M6BrB(15)</td>
<td>3.8±0.1</td>
<td>-0.93</td>
</tr>
<tr>
<td>M6ph(18)</td>
<td>3.8±0.1</td>
<td>-0.93</td>
</tr>
<tr>
<td>M3G</td>
<td>4.0±0.2</td>
<td>-0.75</td>
</tr>
<tr>
<td>M6G (p)(17)</td>
<td>4.0±0.2</td>
<td>-0.75</td>
</tr>
<tr>
<td>C6G (p)(21)</td>
<td>4.2±0.1</td>
<td>-0.63</td>
</tr>
<tr>
<td>M6G</td>
<td>5.1±0.1</td>
<td>-0.30</td>
</tr>
<tr>
<td>M6FB(13)</td>
<td>3.4±0.1</td>
<td>0</td>
</tr>
<tr>
<td>Morphine(1)</td>
<td>7.0±0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>M6B(19)</td>
<td>7.4±0.1</td>
<td>0.07</td>
</tr>
<tr>
<td>M6OHB(16)</td>
<td>8.4±0.2</td>
<td>0.17</td>
</tr>
<tr>
<td>C6G(22)</td>
<td>8.8±0.1</td>
<td>0.20</td>
</tr>
<tr>
<td>CPE(3)</td>
<td>9.0±0.2</td>
<td>0.22</td>
</tr>
<tr>
<td>Codeine(2)</td>
<td>16.8±0.2</td>
<td>0.60</td>
</tr>
<tr>
<td>M6NB(5)</td>
<td>16.6±0.2</td>
<td>0.59</td>
</tr>
<tr>
<td>Heroin(H)</td>
<td>22.0±0.3</td>
<td>0.74</td>
</tr>
<tr>
<td>CSE(4)</td>
<td>22.6±0.2</td>
<td>0.75</td>
</tr>
<tr>
<td>Methadone *2703</td>
<td>2.90</td>
<td>1.85</td>
</tr>
</tbody>
</table>

*The retention time of methadone was determined from the log k' value, which was interpolated off the calibration curve from the log P value. Morphine-6-fluorobenzoate was used as the reference compound, as it had the shortest retention time.

The order of lipophilicities of morphine-6-substituted compounds in increasing order is as follows:

Morphine-6-chlorobenzoate (14) = morphine-6-succinate (20), morphine-6-phthalate (18) = morphine-6-bromobenzoate (15), M3G = M6G(protected) (17), C6G(protected)(21), M6G, morphine-6-fluorobenzoate (13), morphine (1), morphine-6-benzoate (19), morphine-6-hydroxybenzoate (16), C6G(22), codeine phthalic ester(3), morphine-6-nitrobenzoate (5) = codeine (2), heroin, codeine succinic ester (4), methadone.

141
Methadone was the most lipophilic compound tested here. The only morphine-6-substituted compound tested which was more lipophilic than morphine was morphine-6-nitrobenzoate. Morphine-6-benzoate (19), morphine-6-hydroxybenzoate (16) and morphine-6-fluorobenzoate (13) had similar lipophilicities, whilst all of the others were less lipophilic than morphine. Compounds with closest lipophilicities to M6G (0.57) were morphine-6-benzoate (0.72) and morphine-6-hydroxybenzoate (0.75). Drugs less lipophilic than M6G included morphine-6-chlorobenzoate (0.22), morphine-6-succinate (0.22), morphine-6-bromobenzoate (0.33), and morphine-6-phthalate (0.33).

This altered lipophilic nature can be seen in isolated tissue preparations. It was noticeable that the onset and offset of action of morphine-6 nitrobenzoate was much longer than that of morphine in isolated tissue preparations (Table 6.10).

Table 6.10 Rates of onset and offset of action of morphine and morphine-6-nitrobenzoate (5) in MPLM of the guinea-pig ileum

<table>
<thead>
<tr>
<th></th>
<th>Onset t₀.₅ (s)</th>
<th>Offset t₀.₅ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5)</td>
<td>270±18.2</td>
<td>&gt;1200</td>
</tr>
<tr>
<td>morphine</td>
<td>26.8±3.1</td>
<td>90.0±12.4</td>
</tr>
</tbody>
</table>

6.7 Antinociceptive activity of morphine-6-substituted compounds in vivo

From the in vitro studies compounds were chosen for further investigation. The criteria for selecting compounds was that the compounds should possess affinity in binding assays and potency in isolated tissue preparations. Thus the compounds chosen were morphine-6-glucuronide 'protected' (17), morphine-6-p-nitrobenzoate (5), morphine-6-phthalate (18) and morphine-6-succinate (20). The in vivo potency and duration of action of these was evaluated. The compounds were all free base and insoluble in aqueous solvents, and so for injection a suspension was made up in saline containing 0.25% carboxy methyl cellulose.

The model used was the mouse tail-flick test, following s.c. administration of the drug. Time course and dose-response curves are shown for morphine-6-nitrobenzoate and morphine-6-phthalate in Figures 6.23-6.26.
The nitrobenzoate (5) (Figure 6.23) showed a different time course to morphine, reaching peak analgesia slower, i.e., after 90 mins compared to 30 mins for morphine. As soon as the peak analgesia was reached, the effect decreased gradually with time, returning to control values after 6 h. This compared with morphine, which maintained maximal allowed analgesia (i.e., 10s cut off) for 2 h and suggested a lesser activity for the nitrobenzoate. The reduced activity of morphine-6-nitrobenzoate (5) was confirmed by constructing a dose-response curve at 60 mins (Figure 6.24).

The phthalate also showed a slower time course than that of morphine and reached peak analgesia after 120 mins compared to 30 mins for morphine (Figure 6.25). After 120 mins the antinociceptive effect decreased gradually with time, and showed no antinociception after 6 h. The dose-effect curve showed the phthalate to be weaker than morphine, and did not attain maximal allowable time flick latency, even at 30mg/kg (Figure 6.26), although maximal tail flick latency was achieved in Figure 6.27.

Since morphine-6-phthalate acted via δ receptors \textit{in vitro} (MVD/MPLM ratio of 14.4, Table 6.3), it was tested \textit{in vivo} in animals pretreated with naltrindole (1mg/kg), a selective δ antagonist. The \textit{in vivo} analgesia induced by morphine-6-phthalate was blocked by naltrindole, whereas morphine induced analgesia was unchanged (Figure 6.27).
Figure 6.23 Time course for the antinociceptive action of morphine-6-nitrobenzoate (closed circles) (30mg/kg) and saline controls in 0.25% carboxy methyl cellulose (CMC) (open circles) assessed by the tail-flick test. * p < 0.05 [Wilcoxon signed rank test] indicating that the response in the morphine-6-nitrobenzoate treated animals is significantly different from the saline in 0.25% carboxy methyl cellulose (CMC) treatment.

Figure 6.24 Dose-response curve for morphine-6-nitrobenzoate (closed circles) and saline in 0.25% carboxy methyl cellulose (CMC) controls (open circles) at 60 mins assessed by the tail-flick test. * p < 0.05 [Wilcoxon signed rank test] indicating that the response in the morphine-6-nitrobenzoate treated animals is significantly different from the saline in 0.25% carboxy methyl cellulose (CMC) treatment.
Figure 6.25 Time course for the antinociceptive action of morphine-6-phthalate (closed circles) (10mg/kg) and saline controls in 0.25% CMC (open circles) assessed by the tail-flick test. * p < 0.05 [Wilcoxon signed rank test] indicating that the response in the morphine-6-phthalate treated animals is significantly different from the saline in 0.25% CMC treatment.

Figure 6.26 Dose-response curve for morphine-6-phthalate (closed circles) and saline controls for 60 mins assessed by the tail-flick test. * p < 0.05 [Wilcoxon signed rank test] indicating that the response in the morphine-6-phthalate treated animals is significantly different from the saline in 0.25% CMC treatment.
Figure 6.27 Effect of naltrindole (N) (1mg/kg s.c) on morphine-6-phthalate (phthalate) (10mg/kg s.c.) and morphine (5mg/kg s.c.) analgesia as determined by the mouse tail-flick test. Naltrindole was injected 15 mins prior to the agonist and the responses were tested after 90 mins for morphine and 120 mins for the phthalate. * p > 0.05 [Wilcoxon signed rank test] indicating that the treatment is not significantly different from the pretest saline treatment and # p > 0.05 [Wilcoxon signed rank test] indicating not significantly different from the morphine treatment. n=6 mice in each category.
6.8 Discussion

Initially 6-substituted codeine derivatives were investigated as target compounds due to their easier synthesis. However such derivatives (2-4) had low affinity for opioid receptors, and were comparatively weak agonists in the MPLM. This agrees with previous studies that codeine compounds are inactive due to the lack of a 3-phenolic hydroxy group, which is essential for binding to the opioid receptor (Chen et al., 1991). However two codeine-6-substituted compounds, namely the phthalate (3) and the succinate (4) showed a similar affinity for the μ receptor as codeine (2), indeed codeine phthalate (3) showed improved δ receptor affinity and efficacy. Consequently studies concentrated on 6-substituted morphines.

The synthesised compounds were essentially 6-substituted aromatic esters of morphine designed as potential mimics of M6G. The parent compound morphine-6-benzoate (19) had a similar affinity to morphine and M6G for the μ receptor (9nM as compared to 6nM and 8nM respectively). However (19) had a 3-fold higher affinity over M6G for the δ receptor (21nM as compared to 75nM), and a 5-fold higher affinity for the κ receptor than M6G (162nM as compared to 850nM). Isolated tissue studies performed on (19) showed it to have approximately half the potency to that of M6G in the MVD (IC50 of 280nM), but to be essentially inactive in the MPLM (IC50 of 10,000nM), where M6G was a potent agonist (IC50 58nM). This would suggest that compound (19) was acting as a δ agonist in the MVD, confirmed by a naxoxone K_e of 18.4nM, but that it may also act as a μ antagonist since it showed affinity for the μ receptor, with extremely low potency in the MPLM.

Introduction of a para-OH group onto the benzene ring gave a compound, morphine-6-hydroxy benzoate (16), which showed a very similar receptor binding profile to the parent benzoate, at δ and κ sites, but with improved μ affinity (K_i 2nM). In isolated tissue preparations, it afforded similar potency and appeared like the parent morphine-6-benzoate, to be acting via δ receptors in the MVD, but was essentially inactive in the MPLM.

Morphine-6-hydroxy benzoate (16) evaluated in vivo did show antinociceptive activity as measured in the mouse tail flick test, giving a tail flick latency of 7.2±1.3s at a dose of 10mg/kg, compared with the saline control of 3.0s. A similar dose of M6G would afford long lasting ‘cut-off’ analgesia. The in vitro data would appear to suggest that this analgesic
activity may be via an action on δ receptors in this test.

Introduction of a para-nitro group afforded morphine-6-p-nitrobenzoate (5). This showed somewhat reduced μ-affinity compared to M6G, resulting in a compound which was relatively nonselective between μ or δ receptors. There was no measurable affinity for the κ-opioid receptor. The 6-p-nitrobenzoate derivative (5) had agonist activity in the MPLM comparable to M6G, though potency in the MVD was much reduced. The equilibrium dissociation constants (K_e) for naloxone and cyprodime confirmed that morphine-6-p-nitrobenzoate acted via μ-receptors in the MPLM and via δ-receptors in the MVD. Morphine-6-nitrobenzoate (5) was a highly lipophilic drug compared to morphine, and thus showed slow kinetics in the MPLM. This was reflected in the onset and offset times for the drug, which were much longer than that of morphine, whereas M6G had the same onset and only slightly shorter offset of action compared with morphine. M6G and morphine were not that dissimilar in terms of their lipophilicities, confirmed by similar retention times determined by HPLC, giving values of 12 and 14 mins respectively (see Chapter 4). These findings are consistent with the findings of Kosterlitz et al (1975) that more hydrophilic compounds have faster kinetics. In vivo experiments however show the opposite. Herz & Teschemacher (1971) found that in vivo the rate of onset and offset is directly proportional to a drugs lipid solubility. The values obtained here for the onset and offset of morphine and M6G are similar. Morphine-6-nitrobenzoate (5) showed in vivo analgesic activity in the mouse tail flick test, but reached a maximal tail-flick latency of only 5.3s and peak analgesia was reached after 90 mins (Table 6.11). The onset t_{0.5} was 40 mins in vivo, longer than M6G (30 mins). The offset t_{0.5} (measured as the time taken for analgesia to be no longer evident divided by two) for (5) was 90 mins in vivo, whereas for M6G it was 1050 mins.
Table 6.11 Comparative antinociceptive properties of morphine, M6G, M6G (protected) and the 6-phthalate (18) and 6-nitrobenzoate (5). Onset and duration are taken for peak analgesia

<table>
<thead>
<tr>
<th></th>
<th>max analgesia (10s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>ED50 (mg/kg)</th>
<th>onset (mins)</th>
<th>offset (mins)</th>
<th>duration (mins)</th>
<th>active (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>2.2±0.3</td>
<td>30</td>
<td>270</td>
<td>90</td>
<td>4</td>
</tr>
<tr>
<td>M6G</td>
<td>1.9±0.3</td>
<td>30</td>
<td>1050</td>
<td>330</td>
<td>11</td>
</tr>
<tr>
<td>M6G (protected)</td>
<td>1.5±0.4</td>
<td>60</td>
<td>1050</td>
<td>300</td>
<td>11</td>
</tr>
<tr>
<td>M-6-nitrobenzoate</td>
<td>16.2±2.6</td>
<td>90</td>
<td>150</td>
<td>*</td>
<td>3</td>
</tr>
<tr>
<td>M-6-phthalate</td>
<td>2.6±0.4</td>
<td>120</td>
<td>120</td>
<td>X</td>
<td>3</td>
</tr>
</tbody>
</table>

* maintains >6s tail-flick latency for 2.5 h
X maintains >6s tail-flick latency for 1.5 h

Introduction of an ortho-carboxyl group as in the phthalate afforded a closer chemical mimic of M6G and also a closer pharmacological mimic. Thus morphine-6-phthalate (18) had a good affinity for μ and δ receptors in ligand binding assays, with a 5-fold higher affinity for δ receptors than M6G and a 3-times lower affinity for μ receptors. Its affinity for κ receptors (2774nM) was 3 times lower than M6G. In vitro bioassay experiments, showed (18) was more potent than the parent benzoate and had an improved activity at the δ receptor. Also it was 14-fold more potent in the MVD than the MPLM (Table 6.2, Figure 6.15) and 4-fold more potent than M6G in the MVD. It was the most potent compound tested in the MVD and together with (16) and (19) had greater activity in the MVD compared to the MPLM. However unlike (16) and (19), (18) was a full agonist in the MPLM.

Analysis of morphine-6-phthalate (18) in vivo, afforded a a lower ceiling than morphine, with a maximum tail flick latency of 8.2s at 10mg/kg. The time to reach peak analgesia was 120 mins. The effect of naltrindole (1mg/kg), the δ selective antagonist (Rogers et al, 1990) on morphine-6-phthalate (10mg/kg) and morphine (5mg/kg) analgesia was investigated. In this experiment peak analgesia was reached and naltrindole antagonised morphine-6-phthalate (18), but not morphine, demonstrating that analgesia produced by morphine-6-phthalate was δ-mediated (Figure 6.27).
This confirmed *in vitro* findings and implies that morphine-6-phthalate produces its analgesia *via* δ receptors, whereas morphine, as is well known, exerts its actions not *via* δ, but by μ receptors (Paul *et al*, 1989; Frances *et al*, 1992).

The *in vivo* δ-activity of the morphine-6-phthalate could be an important finding since the majority of δ-selective ligands reported to date are peptides (Corbett *et al*, 1984), for example the δ-agonist DPDPE and the δ-antagonist N,N-diallyl-Tyr-Alb-Aib-Phe-Leu-OH (ICI 174864). Portoghese *et al* (1990) reports on non-peptide naltrindole analogues with δ agonist and antagonist properties, but the only non-peptidic δ-opioid agonist reported to date is BW373U86 (Figure 6.28) (Childers *et al*, 1993). It shows a high degree of selectivity for the δ receptor in both *in vitro* and *ex vivo* binding assays, affording Kᵢ's of 85, 1.8 and 34nM for μ, δ, and κ receptors respectively and an IC₅₀ of 0.2nM in the mouse vas deferens (Chen *et al*, 1993). BW373U86 was reported not to be antinociceptive in the mouse tail-flick test (Comer *et al*, 1993) and not to induce any physical dependance (Lee *et al*, 1993), but it has been shown to produce brief, non-lethal convulsions in mice when administered systemically (Comer *et al*, 1993).

![Figure 6.28 BW373U86](image)

The activity of the morphine-6-substituted benzoates indicated that the benzene ring can be accommodated in opioid μ and δ receptors. Of the compounds tested, all had electron-withdrawing groups at the para position (and the phthalate having electron withdrawing groups ortho). The hydroxy and nitro groups possessing lone pairs of electrons, increasing the negative charge delocalisation. It is surprising though that the halogenated benzoates (13)-(15) were inactive as they also had electron withdrawing groups. It is possible that the halogenated compounds may act
as antagonists, because they possess affinity, but not efficacy.

The only non-aromatic ester in the current study was the 6-succinate. This can take up a similar shape to that of the phthalate. Morphine-6-succinate (20) had a similar affinity to morphine and M6G for the μ receptor (10nM as compared to 6nM and 8nM respectively) and a 2-fold higher affinity over M6G for the δ receptor (32nM as compared to 75nM), but no affinity for the κ receptor (>10,000nM). Isolated tissue work performed on (20) showed it to have a very similar potency to morphine and M6G (IC50 values of 159nM and 171nM in the MPLM and MVD respectively). Unfortunately the synthesis of the 6-maleic derivative was unsuccessful and so could not be tested. The maleic derivative is important because it has a double bond, which means the position of the carboxyl group is more fixed and this could provide invaluable information about stereochemistry. This provides scope for additional work in the future.

It is very notable that all of the 6-substituted compounds tested had a low affinity for the κ-receptor (ranging from 150nM to >10,000nM). This is similar to M6G, which also has a very low affinity for the κ receptor. This does suggest that constraints in the κ-receptor for the 6-position are greater at the κ-site. In support of this is the fact that β-FNA is an irreversible μ (and perhaps δ) antagonist, but a reversible agonist at the κ receptor site which does suggest differences at this position. However this low κ affinity of the 6-substituted compounds could be a therapeutic advantage in novel drug design because of the reduction in κ mediate adverse effects eg. sedation and psychotomimetic effects (Wollemann et al, 1992).

Although the codeine derivatives and M3G are inactive, 3-silyl derivatives were synthesised as intermediates (protecting groups) in the synthesis of the 6-substituted analogues. It was decided to examine these for their ability to interact with opioid receptors.

It was found that 3-silylmorphine (6) had a similar profile in binding assays to the parent compound morphine (Table 6.1). This was unexpected in view of the supposed necessity of a 3-phenolic hydroxy group for binding to opiate receptors. 3-Silyl morphine (6) did however have reduced potency in the bioassays (MPLM and MVD), which may indicate a lower efficacy. In addition this potentially suggests two properties of the opioid receptor : (i) that it must be able to accomodate the bulky silyl
group; and (ii) that hydrogen bonding due to the OH is not necessary for binding affinity. The much larger 3-substituted compound, 3-
triisopropylsilyl morphine (12) also had affinity for opioid receptors being most active for the μ receptor ($K_i = 107 \text{nM}$). However the affinity was reduced (by 10 times compared with morphine) at each receptor type (Table 6.2), suggesting steric constraints do exist at this position. This agreed with much previous work which demonstrates that bulky groups are not tolerated and dramatically reduce affinity. Mohacsi et al (1982) reports that 3-tBu morphine has a much reduced affinity (1000 times less) and potency (50 times less as measured in the writhing test) than morphine. However studies showed that 3-silyl morphine was metabolised to morphine, and this was responsible for its effects.

Nevertheless the 3-silyl 6-substituted compounds did have some opioid properties and were not metabolised, especially the phthalate (7). Presumably the binding of 6-phthalate enhances the binding of 3-silyl-6-phthalate derivative, so overcoming the absence of a 3-OH.

Introduction of phthalic acid into the 6-position of 3-silyl morphine gave improved δ affinity compared with M6G and morphine. Since the affinity of 3-silyl morphine-6-phthalate (7) for the μ-receptor was less than M6G and it had no affinity for the κ-receptor the selectivity of this compound was different from M6G with a preference, albeit small, for the δ-receptor. The improved affinity for the δ receptor was confirmed in isolated tissues, since it was 10-fold more potent in the MVD compared to the MPLM. Naloxone and cyprodime $K_e$ values confirmed that 3-silylmorphine-6-phthalate (7) acted via μ-receptors in the MPLM and δ-receptors in the MVD. Thus it acted like its parent compound, morphine-6-phthalate (18).

Alteration of the ester grouping in the 6-position had a marked effect on the activity of the 3-silylmorphines (Tables 6.1 and 6.2). Thus 3-silylmorphine-6-benzoate (8) had a much reduced affinity for μ- and δ-receptors, and was inactive at the κ-receptor. It showed no agonist activity in the MPLM and was only weakly active in the MVD. 3-Silylmorphine-6-p-fluorobenzoate (9), 6-p-bromobenzoate (10) and 6-p-nitrobenzoate (11) also had a much reduced affinity for the μ-receptor and essentially no measurable affinity for δ- and κ-receptors. The 3-silyl 6-p-fluorobenzoate (9) was inactive as an agonist in both the MPLM and MVD, whereas the 3-silylmorphine 6-p-bromobenzoate (10) did have a very slight effect in the MVD.
6.9 Investigation of 3-position substituents

It is reported that morphine-3-substituted compounds like codeine and M3G are devoid of analgesic activity because of the absence of the 3-OH group which is reputed to be essential for binding to opioid receptors (Casy & Parfitt, 1986). The 3-substituted OH-protected morphine analogue 3-tBu morphine had a much reduced affinity (1000 times less) and potency (50 times less as measured in the writhing test) than morphine (Mohacsi et al, 1982), as did 3-TIPS morphine in this study.

Interestingly as reported above, 3-silyl morphine showed a similar receptor profile to that of morphine, but was less potent, though this appears to be due to metabolism to free morphine. 3-Tri isopropylsilyl morphine also had affinity for opioid receptors, showing the highest affinity for the \( \mu \) receptor \( (K_i=107\text{nM}) \). However other compounds remain active even if apparently not metabolised. Similarly a lack of any substituent at the 3-position (eg. 3-deoxymorphine) results in a reduced affinity by 13 times and 8-fold reduced \textit{in vivo} potency as measured in the hot plate test, compared with morphine, showing that the 3-OH is not absolutely essential for analgesic activity (Reden et al, 1979). These observations prompted an investigation of the 3-position more fully. A group of compounds supplied by Dr. Lewis (Bristol University), BU18-BU25 inclusively, were used for these studies and were of the general structure shown below, \( R_3 \) is the variable moiety.
CPM denotes cyclopropylmethyl. Binding of these compounds to opioid receptors was evaluated in rat brain homogenates. Dissociation constants ($K_i$) are shown in Table 6.12, and the selectivities for $\mu$, $\delta$ and $\kappa$ receptors in Table 6.13.
Table 6.12 Affinities $K_i$ (nM) of 3-substituted BU compounds for $\mu$, $\delta$ and $\kappa$ opioid binding sites

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (nM)</th>
<th>$\mu$</th>
<th>$\delta$</th>
<th>$\kappa$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU18</td>
<td>9.0±2.1</td>
<td>12.5±2.2</td>
<td>5.4±1.1</td>
<td></td>
</tr>
<tr>
<td>BU19</td>
<td>8.9±1.6</td>
<td>65.4±3.1</td>
<td>17.9±2.2</td>
<td></td>
</tr>
<tr>
<td>BU20</td>
<td>12.4±1.9</td>
<td>89.5±2.6</td>
<td>18.0±2.1</td>
<td></td>
</tr>
<tr>
<td>BU21</td>
<td>7.6±1.2</td>
<td>56.5±2.1</td>
<td>131.2±4.2</td>
<td></td>
</tr>
<tr>
<td>BU22</td>
<td>24.6±2.4</td>
<td>82.6±2.5</td>
<td>9.5±1.0</td>
<td></td>
</tr>
<tr>
<td>BU23</td>
<td>1.6±0.3</td>
<td>21.3±1.0</td>
<td>19.1±0.9</td>
<td></td>
</tr>
<tr>
<td>BU24</td>
<td>7.6±1.3</td>
<td>24.8±1.3</td>
<td>95.4±4.6</td>
<td></td>
</tr>
<tr>
<td>BU25</td>
<td>39.8±4.2</td>
<td>96.4±2.2</td>
<td>14.9±1.8</td>
<td></td>
</tr>
</tbody>
</table>

$\mu$ sites were labelled with $[^3\text{H}]$DAMGO (0.97±0.11nM), $\delta$ sites by $[^3\text{H}]$DPDPE (1.83±0.23nM) and $\kappa$ sites by $[^3\text{H}]$U69593 (1.13±0.22nM). In all cases Hill coefficients were not significantly different from unity. Values are means ± sem of $>3$ experiments.

<table>
<thead>
<tr>
<th></th>
<th>$K_i \mu/K_i \delta$</th>
<th>$K_i \mu/K_i \kappa$</th>
<th>$K_i \delta/K_i \kappa$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU18</td>
<td>0.72</td>
<td>1.67</td>
<td>2.31</td>
</tr>
<tr>
<td>BU19</td>
<td>0.14</td>
<td>0.50</td>
<td>3.65</td>
</tr>
<tr>
<td>BU20</td>
<td>0.14</td>
<td>0.69</td>
<td>4.97</td>
</tr>
<tr>
<td>BU21</td>
<td>0.13</td>
<td>0.06</td>
<td>0.43</td>
</tr>
<tr>
<td>BU22</td>
<td>0.30</td>
<td>2.59</td>
<td>8.69</td>
</tr>
<tr>
<td>BU23</td>
<td>0.08</td>
<td>0.08</td>
<td>1.12</td>
</tr>
<tr>
<td>BU24</td>
<td>0.31</td>
<td>0.08</td>
<td>0.26</td>
</tr>
<tr>
<td>BU25</td>
<td>0.41</td>
<td>2.67</td>
<td>6.47</td>
</tr>
</tbody>
</table>
All of the compounds showed high affinity for opioid receptors, despite being 3-substituted. None of the compounds tested above were exclusively selective for a particular opioid receptor site, though most showed a slightly higher affinity for one receptor type. Thus BU19 (R3=propene), BU20 (R3=cyanoethane), BU22 (R3=propane) and BU25 (R3=isopropyl) showed a relatively lower affinity for the δ receptor as compared with the μ or κ receptors. BU21 (R3=CPM) showed a much lower affinity at κ receptors than μ or δ receptors. BU21 and BU23 (R3=propyne) had some preference for the μ receptor.

As can be seen from the binding data, the introduction of an alkyl group substituent at R3, replacing OH in BU18 caused a loss in affinity for the δ receptor and no significant change in affinity for the κ receptor [except for BU21 (R3=CPM) and BU24 (R3=ester)]. Some changes were seen at the μ receptor. Thus BU23 (R3=propyne) had a higher affinity than BU18 (R3=H), which was equivalent with BU19 (R3=allyl), BU20 (R3=cyano), BU21 (R3=CPM) and the BU24 (R3=ester), which has higher affinity than BU22 (R3=propyl), which in turn had a higher affinity for BU25 (R3=isopropyl). In order of decreasing affinities for the μ receptor: 23>18=19=20=21=24>22>25. Thus alkyl substituted compounds are less active than their parent compound and introduction of multiple bonds or heteroatoms causes no change or improved μ affinity. Overall this set of compounds were either branched or unsaturated. The substituent at the 3-position could be interacting in some way with the receptor to overcome loss of the 3-OH.

The agonist potencies of these compounds were assessed in the guinea-pig MPLM (Figure 6.29). None of the compounds reached 50% inhibition even at 1000nM and BU18, BU22 and BU24 showed no agonism at all. The compounds did not washout after continual washing for 4 h, suggesting they bind very tightly to opioid receptors. The remaining compounds (BU19,20,21,23 and 25) acted as agonists at lower doses, but at higher doses as antagonists, showing bell-shaped dose-response curves. This is similar to the situation with buprenorphine, which is a partial agonist, acting at low doses as an agonist at one site, and at higher doses, interacting with the second site of lower affinity, counteracting the effects of the high-affinity site. This is known as non-competitive autoinhibition (Rance et al, 1980). It is not clear though whether this action is due to non-specific, possibly cholinergic effects or to a more intricate opioid mechanism, such as allosteric interactions among opioid receptor subtypes (Rothman &
Another hypothesis assumes the existence of only one type of receptor site and bell-shaped dose-response curves may be generated either by multiple subsites of drug attachment within the receptor (De Leon et al., 1979) or by cooperative binding interactions (Dum & Herz, 1981).

These BU compounds are not the only 3-ether series to possess opioid activity, since 14-p-nitrocinnamoyl derivatives also have activity. A series of 3-substituted 14-p-nitrocinnamoyl amino morphinones (R$_2$=C$_6$H$_4$NO$_2$ in BU type structure on page 154) showed affinities for $\mu$, $\delta$ and $\kappa$ opioid receptors and some 14-p-nitrocinnamoyl amino 7,8-dihydromorphinones acted as $\mu$ antagonists after i.c.v. administration (Sebastian et al., 1993). This contradicts findings that the 3-O-cyclopropylmethyl ethers of buprenorphine, naltrexone and diprenorphine all have low affinities, compared to the 3-OH parents (Lewis, 1994). It could be that the BU compounds are metabolised to 3-OH, but this is unlikely as one might expect the 3-O-cyclopropylmethyl ethers of buprenorphine, naltrexone and diprenorphine to also show activity if this were the case.

![Figure 6.29 Potency of BU compounds 19 (closed circles), 20 (open circles), 21 (open triangles), 23 (+) & 25 (x) in the MPLM](image-url)
M6G has a much longer duration of action than morphine. This, allied to a lack of first-pass metabolism potentially means a better control of analgesia. An interesting finding was the very low affinity for κ compared to μ and δ receptors. This was seen in both ligand binding and bioassay preparations. However the measured properties were mediated via μ receptors, suggesting a similar pharmacological profile to that of morphine, possibly with reduced potential for side-effects mediated via κ receptor activation. Thus, in order to take advantage of these properties the emphasis must be towards morphine-6-substituted compounds in the hope of finding compounds with similar analgesic potency and duration to M6G, but with simpler synthesis and increased stability to air and light, and therefore more cost effective production.

The newly synthesised compounds morphine-6-phthalate (18) and morphine-6-nitrobenzoate (5) studied in this thesis did exhibit good analgesic activity, but had a lower antinociceptive ceiling than morphine or M6G, and a shorter length of action. Their stability to metabolism has not been studied. Also identified in this work was morphine-6-hydroxybenzoate (16). Morphine-6-hydroxybenzoate (16) had a similar binding profile to M6G, but a much reduced efficacy, in vitro as measured in isolated tissue preparations, and in vivo. Morphine-6-nitrobenzoate (5) was relatively non-selective for μ or δ receptors. It was active in isolated tissue preparations, being more potent in the MPLM, but acted as a partial agonist in vivo. Morphine-6-phthalate (18) showed a higher affinity for δ >> μ >> κ receptors, although δ/μ selectivity was not great. This was confirmed in the in vitro isolated tissue preparation the MVD, where an IC₅₀ of 24nM was obtained and was antagonised by naltrindole, a selective δ antagonist. Morphine-6-phthalate (18) acted as a good agonist in vivo, where its activity was seen to occur via δ receptor activation. This may prove to be a suitable lead compound in the development of a non-peptide δ active agonist exhibiting analgesic activity, and hopefully with an improved side-effect profile over μ-agonists.

Differentiation between μ and κ receptors and δ and κ receptors is an important consideration. Morphine-6-phthalate and morphine-6-nitrobenzoate had μ/δ and μ/κ ratios of <0.01. This selectivity for μ/δ and μ/κ was greater than that of M6G, and may allow the development of compounds without the unwanted κ side effects, such as sedation and
psychotomimetic effects.

This project has thus enabled a better understanding of antinociception induced by M6G and related synthetic derivatives. The reported improved \textit{in vivo} activity of M6G over morphine is not due to improved receptor affinity or efficacy of the compound, at least as examined in the systems used here, in ligand binding and isolated tissue preparations. It is likely that pharmacokinetic differences between M6G and morphine will reveal why M6G is much more potent and long lasting. The next step in a continuation of this study would therefore be to investigate pharmacokinetic parameters, for example, the assays of CNS levels reported in this thesis only concern 'morphine-like' material. It is important to identify this. Indeed when administered centrally M6G is very potent. A possible explanation would be that extremely high local concentrations of M6G are in the CNS, which diffuse away very slowly.

The involvement of second messenger systems could also play a major role in the effectiveness of M6G and this could also be investigated, by studying efficacy in CNS preparations, for example by measuring changes in cyclic AMP levels in brain slices, to probe possible differences between the effectiveness of M6G at opioid systems in the periphery compared to centrally exist.

3-Substituted opioids, eg. codeine are generally weakly active agonists and confirmed to have low affinity for \(\mu\), \(\delta\) and \(\kappa\) receptors. However a series of 3-substituted derivatives investigated, containing saturated and unsaturated substituents, did show affinity for opioid \(\mu\), \(\delta\) and \(\kappa\) receptors. Unfortunately though only very weak agonist activity and bell-shaped dose-response curves were shown \textit{in vitro}. Nevertheless the results demonstrate that the 3-phenolic hydroxyl group is not absolutely essential for high affinity receptor binding and opens up possibilities for beneficial structure activity relationships of opioid receptors within this group.

Synthetic derivatives at positions 6 and also at 3 do appear to be able to control both affinity selectivity and efficacy. There is still scope for a tremendous number of of compounds to be evaluated and studied. In particular, it would be interesting to extend the structure-activity relationship study to try to develop the \(\delta\)-activity of morphine-6-phthalate, since this group of compounds are relatively new, and the pharmacology of non-peptide \(\delta\)-compounds is only in its infancy.
What is now proved
was once only imagined

WILLIAM BLAKE
REFERENCES


DI PRETORIA, A. Personal communication, 1994.


LEWIS, J. Personal communication, 1994.


PASTERNAK, G.W. Brain Res. 71, 152-6, 1983.


YOSHIMURA, H. I. Yakugaku Zasshi. 95(5), 564-569, 1975.


