Simultaneous targeting of multiple opioid receptors: a strategy to improve side-effect profile

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Opioid receptors are currently classified as μ (mu: μOP), δ (delta: dOP), κ (kappa: kOP) with a fourth related non-classical opioid receptor for nociceptin/orphain FQ, NOP. Morphine is the current gold standard analgesic acting at MOP receptors but produces a range of variably troublesome side-effects, in particular tolerance. There is now good laboratory evidence to suggest that blocking DOP while activating MOP produces analgesia (or antinociception) without the development of tolerance. Simultaneous targeting of MOP and DOP can be accomplished by: (i) co-administering two selective drugs, (ii) administering one non-selective drug, or (iii) designing a single drug that specifically targets both receptors; a bivalent ligand. Bivalent ligands generally contain two active centres or pharmacophores that are variably separated by a chemical spacer and there are several interesting examples in the literature. For example linking the MOP agonist oxymorphone to the DOP antagonist naltrindole produces a MOP/DOP bivalent ligand that should produce analgesia with reduced tolerance. The type of response/selectivity produced depends on the pharmacophore combination (e.g. oxymorphone and naltrindole as above) and the space between them. Production and evaluation of bivalent ligands is an emerging field in drug design and for anaesthesia, analgesics that are designed not to be highly selective morphine-like (MOP) ligands represents a new avenue for the production of useful drugs for chronic (and in particular cancer) pain.

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Acute pain is a very common presenting complaint in primary and secondary care. Chronic pain, on the other hand, is a rather complex symptom associated with a range of diseases and is particularly difficult to treat effectively. Chronic pain is one of the most common symptoms associated with cancer, with a prevalence of approximately 30–50% among patients who are undergoing active treatment for a solid tumour and 70–90% among those with advanced disease.2 19 Records show that 88% of cancer patients in the last year of their life are in pain and 47% of those treated for pain by their general practitioner said their treatment only partially controlled their pain.2 19 From this description of chronic cancer pain it is fair to state that, despite a wide range of potential therapeutic targets, the control of cancer pain is poor.

Current classification of opioids and their receptors (MOP; μ, DOP; δ, and KOP; κ) is summarized in Table 1, where it is worthy of note that the main target for analgesia is the MOP receptor. Indeed, in an elegant study by Kieffer and colleagues, mice in which the MOP gene was deleted did not display morphine-induced analgesia.36

A fourth receptor was identified by cloning,16 56 which was abbreviated as NOP, while the identification of its endogenous peptide followed shortly, named nociceptin/orphanin FQ or N/OFQ. The structure of the NOP receptor and the transduction pathways after its activation show

Declaration of interest. R.G., S.S. and G.C. are founders of a university spin-out company, University of Ferrara Peptides (UFP), that is involved in the development of opioid ligands. N.D., D.J.R., and D.G.L. are collaborators with UFP.
that it has been evolved from the opioid receptor family. Selective ligands have been synthesized for the NOP receptor, that is J-113397 and UFP-101. Pharmacologically this is not a classical opioid receptor, as non-selective opioid antagonists (i.e. naloxone) display negligible affinity. The International Union of (basic and clinical) Pharmacology (IUPHAR) database of receptors proposes that the NOP receptor is considered as a non-opioid branch of the opioid receptor family.

Opioids are the gold standard for acute pain and are often used in chronic pain. However, there is a trade-off between good analgesia and poor side-effect profile. Indeed, tolerance to opioids (morphine) develops such that dose escalation is required which increases the prevalence and severity of side-effects. There is a need for morphine-like molecules with reduced side-effects such as (i) lower liability, (ii) depressed respiration, and (iii) reduced gastrointestinal motility.

In 1994 in an editorial in this journal we stated that 'As far as clinical anaesthetists are concerned, the “holy grail” of profound analgesia without significant side-effects has yet to be realized.' While there have been significant advances in this time, the “holy grail” remains unrealized. In that editorial we also suggested that highly selective MOP receptor agonists were the future for modern analgesics. Using the vast amount of post-cloning pharmacology data now available we question the wisdom of developing more morphine-like analogues and explore the properties of low-selectivity mixed opioid molecules (multi-targeting) in the hope that these may produce new drug prototypes to address clinical need in chronic pain.

**Why is multi-targeting important?**

As noted above, long-term clinical use of opioids (mainly MOP receptor agonists) can cause a wide range of side-effects such as respiratory depression, constipation, tolerance, and possibly dependence. Tolerance leads to dose escalation with the potential to produce increased side-effects and a vicious cycle develops with a sick patient at the centre. Selective KOP or DOP receptor agonists do not possess some morphine-like side-effects such as constipation, respiratory depression, and addiction, but have a side-effect profile of their own, that is diuresis, sedation, and dysphoria for KOP receptor ligands.

In animal studies over the last decade, it has been shown that when a MOP receptor agonist (i.e. morphine) is co-administered with a DOP receptor antagonist (i.e. naltrindole), then increased analgesia results with an improved side-effect profile (tolerance and dependence). Moreover, in DOP receptor knock-out, antisense oligodeoxynucleotide knock-down and preproenkephalin knock-out mice, reduced morphine tolerance was reported. In addition, highly selective KOP receptor agonists produce more side-effects than some of the newer mixed KOP/MOP receptor agonists. One of the most potent MOP receptor agonists (MDAN-21) is not a selective MOP receptor agonist but rather a newly designed mixed MOP-agonist/DOP-antagonist, being 50 times more potent than morphine. Collectively, these data indicate a far more complex pharmacology for opioid receptors than it has previously been suggested. The challenge ahead is to develop this multi-target strategy to produce new analgesics that retain a favourable side-effect profile in man.

From a practical perspective, multiple targeting can take several forms and these are illustrated in Figure 1. The simplest mechanism is to administer two drugs separately. This can be accomplished with the existing battery of ligands, for example oxymorphone as a MOP agonist and naltrindole as a DOP antagonist. An alternative is to use a non-selective single ligand that will interact with both targets, for example UFP-505 (Table 2, compound 4) that interacts both with MOP receptor as an agonist and DOP receptor as an antagonist. Finally, and representing more of a medicinal chemistry challenge, is to design a single bivalent ligand that interacts with both targets, for example an

### Table 1: Classification and basic characteristics of opioid receptors, including endogenous and selective exogenous opioid ligands

<table>
<thead>
<tr>
<th>Receptor nomenclature*</th>
<th>Most common roles and functions</th>
<th>Most common location in the CNS†</th>
<th>Endogenous agonists§</th>
<th>Selective ligands§</th>
</tr>
</thead>
<tbody>
<tr>
<td>µ, μ, MOP, OP3</td>
<td>Analgesia, intestinal transit, feeding, mood, hormone secretion, thermoregulation, cardiovascular function</td>
<td>Thalamus, neocortex, amygdala, dorsal horn, inferior and superior colliculi</td>
<td>β-Endorphin, enkephalins, endomorphin (no precursor identified)</td>
<td>Morphine (A), DAMGO (A), β-FNA (N), DALDA (N)</td>
</tr>
<tr>
<td>δ, δ, DOP, OP1</td>
<td>Analgesia, mood, gastrointestinal motility, behaviour, cardiovascular regulation</td>
<td>Offactory bulb, thalamus, neocortex, caudate putamen, nucleus accumbens, amygdala, dorsal horn</td>
<td>Dynorphin A (1–13), enkephalins, β-endorphin</td>
<td>Naltrindole (N), DPDPE (A), TIPP (N)</td>
</tr>
<tr>
<td>κ, κ, KOP, OP2</td>
<td>Analgesia in inflammation, diuresis, feeding, neuroprotection, neuroendocrine functions</td>
<td>Cerebral cortex, nucleus accumbens, claustrum, hypothalamus</td>
<td>Enkephalins, neoeendorphin, dynorphin A (1–13)</td>
<td>KCN (A), bremaazine (A), norBNI (N)</td>
</tr>
<tr>
<td>ORL1/LC132, NOP, OP4</td>
<td>Spinal analgesia, anxiety, mood, memory, feeding, locomotor activity</td>
<td>Hippocampus, hypothalamus, amygdala, substantia nigra, dorsal horn, lateral septum</td>
<td>N/OFQ</td>
<td>UFP-101 (N), UFP-102 (A), Ro64-6198 (A)</td>
</tr>
</tbody>
</table>

*Recommended and alternative nomenclature for opioid receptors as defined by NC-IUPHAR. †For full information on receptor location in the CNS, see the NC-IUPHAR database on www.iuphar-db.org. ‡As published in IUPHAR receptor database. § Denoted as (A) for agonist and (N) for antagonist.
oxymorphone/naltrindole ligand (see also Fig. 2). It is true to say that the whole animal endpoint would, in general, be the same and using the examples above all combinations have the potential to produce tolerance-free analgesia. One important point to be considered in the design and use of bivalent ligands is that their potency (agonist, antagonist, or both) for the two targets should be roughly similar.

**Multi-targeting ligands and their pharmacophores**

*Bivalent, bifunctional, or simply non-selective?*

A pharmacophore is defined by the International Union of Pure and Applied Chemistry (IUPAC) as ‘an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response’ (IUPAC Recommendations 1998). Ligands that have two distinct binding properties can be found in the literature under two different names; bivalent and bifunctional. A bivalent ligand is a compound that possesses two distinct pharmacophores in its structure (Fig. 2). On the other hand, a bifunctional ligand is a non-selective compound that acts at two different targets and thus presents two distinct binding properties. These terms are often used interchangeably and in this review we will use the terms bivalent and non-selective to describe the properties of several interesting ligands. Two sub-categories of bivalent ligands also exist: homobivalent ligands, where both pharmacophores are of the same structure and heterobivalent ligands, where the two pharmacophores are different. It is possible that a bivalent ligand may have only one function if both of its pharmacophores are selective for the same receptor. In contrast, it may also be possible that a bifunctional ligand can have only one pharmacophore which is non-selective and thus acts on two different targets. For example, the non-selective opioid receptor agonist butorphan acts on KOP and MOP receptors and as it has only one pharmacophore, it is regarded as a bifunctional (non-selective) ligand. Similarly, the monovalent opioid-receptor ligands cyclorphan and pentazocine act as agonists at KOP receptors and antagonists at the MOP receptors are also bifunctional (non-selective) ligands. Morphy and Rankovic have proposed a further chemical sub-classification depending on whether the pharmacophores are merged, conjugated, or overlapping.

**Opioid bivalent ligands and pharmacophores**

There is a wide range of bivalent ligands studied with a wide range of properties. A detailed review of the chemistry of these molecules has been recently published and is beyond the scope of this article. Bivalent ligands with a variety of different opioid pharmacophores have been developed, including ligands for mixed MOP/NOP receptor binding. Some of the main groups are covered below and a schematic oxymorphone/naltrindole bivalent ligand is illustrated in Figure 2.

**Dmt-Tic pharmacophores**

Dmt-Tic (Dmt: 2',6'-dimethyl-l-tyrosine; Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) is a pharmacophore that facilitates ligand recognition of both MOP and DOP

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Table 2 Receptor binding (Ki) and general pharmacological profile of various bifunctional (non-selective) ligands at the opioid receptors. Table shows data for the (µ (MOP) and δ (DOP) receptors. Key: 2',6'-dimethyl-l-tyrosine (Dmt), tetrahydroisoquinoline-3-carboxylic acid (Tic), benzimidazole (Bid), phenyl (Ph), phenylalanine (Phe), glycine (Gly), tyrosine (Tyr), glycine (Gly), alanine (Ala), tyrosine (Tyr), tryptophan (Trp), prolín (Pro), 3-[1-naphthyl-D-Ala] (d-1-Nal).

<table>
<thead>
<tr>
<th>Non-selective ligands (bifunctional)</th>
<th>Ki (µ) (nM)</th>
<th>Ki (δ) (nM)</th>
<th>Pharmacological profile*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Butorphan</td>
<td>0.23</td>
<td>5.90</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 Naltrexone</td>
<td>0.23</td>
<td>38.0</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 Cyclophoran</td>
<td>0.06</td>
<td>1.90</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 H-Dmt-Tic-Gly-NH-CH&lt;sub&gt;2&lt;/sub&gt;-Ph</td>
<td>0.16, pEC&lt;sub&gt;50&lt;/sub&gt; 7.70**</td>
<td>0.03, pA&lt;sub&gt;2&lt;/sub&gt; 9.25**</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 H-Dmt-Tic-Gly-NH-Ph</td>
<td>0.16, pEC&lt;sub&gt;50&lt;/sub&gt; 8.59**</td>
<td>0.04, pEC&lt;sub&gt;50&lt;/sub&gt; 8.52**</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>6 H-Dmt-Tic-NH-CH&lt;sub&gt;2&lt;/sub&gt;-Bid</td>
<td>0.50, pEC&lt;sub&gt;50&lt;/sub&gt; 7.57**</td>
<td>0.03, pEC&lt;sub&gt;50&lt;/sub&gt; 9.90**</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 H-Dmt-Tic-Gly-NH-CH&lt;sub&gt;2&lt;/sub&gt;-Bid</td>
<td>20.5, pEC&lt;sub&gt;50&lt;/sub&gt; 6.45**</td>
<td>0.06, pA&lt;sub&gt;2&lt;/sub&gt; 9.00**</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>8 H-Dmt-Tic-Phe-Phe-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.19</td>
<td>0.12</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>9 H-Tmt-Tic-Phe-Phe-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>4.08</td>
<td>0.39</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 H-Dmt-Tic-Ψ(CH&lt;sub&gt;3&lt;/sub&gt;NH)-Phe-Phe-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.94</td>
<td>0.48</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>11 H-Tyr-Tic-Phe-Phe-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>78.8</td>
<td>3.00</td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
<tr>
<td>12 H- Dmt-Pro-Trp-D-1-Nal-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>pA&lt;sub&gt;2&lt;/sub&gt; 8.59**</td>
<td></td>
<td>MOP&lt;sup&gt;δ&lt;/sup&gt; – DOP&lt;sup&gt;δ&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
receptors and can be embedded in structures of various ligands. Affinity and bioactivity of simple DOP and MOP receptor ligands is enhanced by the addition of the Dmt structure: (i) [Dmt]deltorphin-B shows a higher agonist affinity at DOP and MOP receptors compared with the simple selective DOP receptor agonist deltorphin-B, (ii) [Dmt]DALDA shows a higher agonist affinity for the DOP and MOP receptors compared with the selective MOP receptor agonist DALDA, and (iii) [Dmt] endomorphin-1 and -2 shows a higher agonist affinity at the MOP receptor compared with the selective MOP receptor agonists endomorphin-1 and -2. Studies show that the Dmt moiety facilitates ligand recognition for MOP and DOP receptors and therefore is believed to play the ‘address’ role in the ‘message-address’ concept for ligand structure. The ‘message-address’ concept is often regarded as the extension of the ‘key-lock’ theory of receptor-ligand binding. Dmt promotes DOP/MOP receptor agonist activity, while Tic is responsible for the promotion of a DOP-antagonist activity when incorporated alone into position 2 of enkephalin (a DOP receptor agonist), dermorphin (a MOP receptor agonist), and dynorphin-A (1–11) NH2 (a KOP receptor agonist). Using the Dmt-Tic pseudodipeptide as a chemical template, several interesting series of ligands have been generated. UFP-505 (Table 2, compound 4) is a simple non-selective ligand with a Dmt-Tic pharmacophore, linked to a phenyl group (Ph) through the Gly-NH-CH2 linker. This ligand is a MOP receptor agonist and DOP receptor antagonist. A similar Dmt-Tic-containing molecule (Table 2, compound 6) has been shown to be a highly potent DOP receptor agonist.

Butorphan and the MCL series of ligands

Butorphan (3-hydroxy-N-cyclobutylmethyl morphinan or MCL-101) is a non-selective (bifunctional) monovalent opioid agonist, acting at KOP and MOP receptors (Table 2, compound 1). Its promising pharmacological profile led to
the synthesis of a series of bivalent ligands with two butorphan pharmacophores and varying linker lengths (MCL-139, MCL-144, MCL-145). These bivalent compounds have receptor affinities that are dependent on the type or length of the spacer between the pharmacophores. MCL-139 and MCL-144 have 4- and 10-carbon atom linkers, respectively, and both were full KOP receptor agonists and partial agonists at the MOP receptor. The long-linker bivalent ligand MCL-144 has the highest affinity for KOP receptors (Ki=0.049 nM; Table 3, compound 4) and its diastereoisomer MCL-193 (with the one pharmacophore being enantiomerically inactive) has reduced KOP receptor affinity (Ki=1.2 nM; Table 3, compound 5). MCL-450 is a bivalent ligand with Dmt-Tic and butorphan pharmacophores (Table 3, compound 6). Although its profile shows affinity for all opioid receptors, there are distinguishable differences in affinity when compared with its monomeric pharmacophores, Dmt-Tic and butorphan. MCL-450 has lower affinity for MOP receptors than butorphan, lower affinity for DOP receptors than Dmt-Tic (Table 3, compound 18) but higher affinity for DOP receptors than butorphan. However, its affinity for the KOP receptor is vastly increased compared with that of Dmt-Tic, although lower than that of butorphan (data not shown).

**Biphalin and related pharmacophores**

Biphalin (Table 3, compound 2) is a bivalent ligand with two enkephalin analogues (H-Tyr-d-Ala-Gly-Phe-) positioned at mirror-image positions on either side of an amino-linker (-NH-NH-). It is known that endogenous opioid peptides have a common tetrapeptide sequence (Tyr-Gly-Gly-Phe), one amino acid different from the pharmacophores in biphalin, and is a target for enzymatic hydrolysis by aminopeptidases and enkephalinas. Replacement of Gly in position 2 of the opioid tetrapeptide sequence, with a D-Ala, protects biphalin from enzymatic breakdown. Biphalin has been shown to possess higher in vitro affinity than met-enkephalin and produce higher in vivo analgesia than morphine. Its actions are attributed to activation of both MOP and DOP receptors, while it shows no affinity for the KOP receptors. Alterations of biphalin’s pharmacophores to exclude the D-Ala and Gly amino acids causes a vast decrease in affinity for all receptors (Table 3, compound 1). Alteration of the structure of biphalin to include a CH2-CH2-CH2 chain between the -NH-NH- linker, produces a compound that has much lower biological activity indicating that the spacer plays a vital role in activity. Elongation of the linker causes further loss of MOP receptor affinity, while DOP receptor affinity remains unaffected. Indeed, in vivo testing of biphalin analogues with the linker formula -(NH-(CH2)6-NH- (with n=2–12), has shown inadequate antinociception. This observation confirms the key role of MOP receptor affinity in analgesia, as all biphalin analogues with longer linkers than biphalin showed a reduced MOP/DOP receptor activity ratio. Further in vivo tests have shown that biphalin diminishes physical dependence after chronic i.p. morphine, a result that is attributed to DOP receptor activation. Biphalin has increased metabolic stability [T1/2 is 87 min (serum) and 112 min (brain)] and

| Table 3 Receptor binding (Ki) and general pharmacological profile of various bivalent ligands, monovalent ligands, and endogenous peptides at the |  |
|---|---|---|---|---|---|
| Pharmacophore 1 | Linker | Pharmacophore 2 | Ki (μ) (nM) | Ki (m) (nM) | Ki (α) (nM) | Pharmacological profile** |
| Bivalent ligands | | | | | | |
| 1 H-Tyr-d-Phe | NH-NH | d-Phe-Tyr-H | 31.0 | 187 | 360 | MOPDOP 47 |
| 2 H-Tyr-d-Ala-Gly-Phe | NH-NH | Phe-Gly-d-Ala-Tyr-H | 1.40 | 2.60 | | MOPDOP 49 |
| 3 Butorphan | fumaryl ester | Butorphan | 0.20 | 9.40 | 0.08 | MOPDOP 61 |
| 4 Butorphan | 10-C linker | Butorphan | 0.09 | 4.20 | 0.05 | MOPDOP 66 |
| 5 (–)Butorphan | 10-C linker | (–)Butorphan | 2.20 | 23.0 | 1.20 | |
| 6 Dmt-Tic-OH | β-Ala | Butorphan | 0.69 | 1.50 | 0.28 | mixed-N 60 |
| 7 N,N-dimethyl-Dmt-Tic | diaminoalkyl-2(1H)-pyrazinon | N,N-dimethyl-Dmt-Tic | 1.68, pA2 7.7* | 0.29, pA2 10.4* | | MOPDOP 43 |
| 8 N,N-dimethyl-Dmt-Tic | NH-(CH2)3 NH- | N,N-dimethyl-Dmt-Tic | 2.21, pA2 8.3* | 0.06, pA2 11.3* | | MOPDOP 43 |
| 9 Dmt | Aminobuccin | Dmt | 0.04 | 14.8 | | |
| 10 Endomorphin-2 | ethylendiamine | Dmt-Tic (no linker) | 1.03 | 1.45 | | |
| 11 JD | Ttc | | 3.73 | 301 | 0.32 | |
| Reference compounds | | | | | | |
| 12 Dermorphin | – | – | 0.28 | 82.5 | 1.10 | MOP 8 |
| 13 Endomorphin-2 | – | – | 0.69 | 9233 | 5240 | MOP 29, 29,88 |
| 14 Morphine | – | – | 0.88 | 140 | 24 | MOP 65 |
| 15 Deltaorphin C | – | – | 387 | 0.21 | 0.08 | DOP 8 |
| 16 Dynorphin A (1–13) | – | – | 0.50 | 4.40 | 0.11 | DOP 51 |
| 17 Naloxone | – | – | 0.79 | 76.0 | 0.25 | mixed-N 66 |
| 18 Dmt-Tic-OH | – | – | 50 | 0.29, pA2 7.95* | 34.1 | DOPN 43 |
| 19 NTI | – | – | | | | |

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increased resistance to enzymatic degradation when compared with Met- enkephalin, two characteristics that relate to biphain’s long analgesic activity.\textsuperscript{12} Mouse-tail flick tests have shown that when biphain is administered s.c. or i.v., it produces inferior analgesia to morphine. The opposite is true for the intrathecal route, where biphain has increased potency when compared with morphine,\textsuperscript{57, 82} suggesting that s.c. or i.v. biphain undergoes some enzymatic breakdown in the periphery before entering the central nervous system (CNS) and therefore shows decreased activity, compared with intrathecal administration.\textsuperscript{82} Drugs such as biphain, with limited CNS activity through the s.c. route, may be useful as local analgesics where central effects are undesirable.\textsuperscript{58, 82}

\textbf{Morphine-related analogue pharmacophores}

Oxymorphone (ED\textsubscript{50} 0.043 nmol)\textsuperscript{22} pharmacophores have been used to synthesize bivalent opioid ligands with various linker lengths. Using the same linker length, a similar bivalent ligand has been synthesized, which links a non-active (a ‘+’ enantiomer) with an active oxymorphone pharmacophore (a ‘−’ enantiomer).\textsuperscript{71} The ligand incorporating the (+)enantiomer pharmacophore showed 20\% of the activity of that containing both active pharmacophores, a similar observation to butorphan bivalent ligands (Table 3, compounds 4 and 5). The reduction in bioactivity shows that the bivalent molecule activates two different receptors and therefore the ligand bridges two receptor sites through binding. It is unclear whether these two bridged receptor sites are part of a receptor dimer complex (dimerization is discussed in a later section) or simply neighbouring receptors.\textsuperscript{12} Other morphine-related pharmacophores have also been used in the synthesis of bivalent compounds (Table 3, compound 10) in order to increase the activity of an additional target (i.e. DOP or KOP receptor). Similarly, some bifunctional compounds that are structurally-related to morphine have also shown an interesting activity profile when combined with morphine. For example, nalbuphine, a MOP antagonist and a KOP agonist, is almost as potent an analgesic as morphine.\textsuperscript{50} This bifunctional compound has shown analgesic synergy and reduced pruritus in patients, when combined with morphine at a 1:1 ratio.\textsuperscript{87} The mechanism behind the cross-linking of the activity of MOP antagonists with MOP agonists after co-administration needs further investigation.

\textbf{Naltrexone-related pharmacophores}

Naltrexone and naloxone (Table 3, compound 17) are two structurally related non-selective opioid antagonists. When two naltrexone pharmacophores are linked by a glycy1-rich spacer, a remarkably long-lasting antagonism of the antinociceptive activity of morphine results.\textsuperscript{83} Norbinaltorphimine (norBNI) is a rigid opioid bivalent ligand with two naltrexone pharmacophores linked by a pyrrole ring.\textsuperscript{72} NorBNI is a potent selective KOP receptor antagonist, structurally similar to binaltorphimine (BNI) which has a methyl group linked in the pyrrole ring, rather than hydrogen compared with norBNI.\textsuperscript{70} Although naltrexone is a non-selective opioid antagonist, norBNI and BNI are more KOP receptor selective, probably because of the presence of the short non-flexible pyrrole ring as a spacer. A bivalent ligand that includes one inactive (+)-naltrexone enantiomer has shown 1/30th of the potency shown for the (−)/(−)-naltrexone bivalent ligand.\textsuperscript{67} This increase in affinity may suggest a significant role for the pharmacophoric spatial orientation in bivalent ligands.

\textbf{MDAN series of ligands}

Naltrindole (NTI) (Table 3, compound 19) is a potent DOP receptor antagonist, structurally related to naltrexone. Co-administration of morphine and NTI has been shown to suppress opioid-related physical dependence and tolerance in mice, along with increasing analgesia.\textsuperscript{3} Independent studies using DOP receptor knock-out mice have shown reduced opioid tolerance without an effect on analgesia.\textsuperscript{62} These data led to the synthesis of a series of bivalent ligands, that bear one oxymorphone pharmacophore (a MOP receptor agonist) and one NTI pharmacophore, denoted as the MDAN series of bivalent ligands (M for MOP receptor, D for DOP receptor, A for agonist, N for antagonist).

MDAN-21, a compound with the specified pharmacophores and a 21-atom linker between them, has shown that it reduces tolerance and physical dependence of morphine in vivo, while showing a 50-fold increase in potency at MOP receptors, compared with that of morphine (MDAN-21 i.c.v. ED\textsubscript{50} 0.08 nM, morphine i.c.v. ED\textsubscript{50} 4.1 nM).\textsuperscript{22}

Chronic administration of MDAN-21 abolishes conditioned place preference (CPP) used to predict drug addiction and abuse.\textsuperscript{41} Each one of the pharmacophores used in the MDAN-21 ligand, when attached individually to a linker (one pharmacophore only connected to a linker), displays a CPP profile similar to that of morphine. These data endorse the theory that MDAN bivalent ligands promote antinociception with decreased addiction compared with morphine.\textsuperscript{22, 41} In addition, it has been shown that morphine CPP can be terminated after a long abstinence period but re-instated by repeated injection, which kindles drug-seeking behaviour. This behaviour is not present with MDAN ligands.\textsuperscript{41} Therefore, MDAN ligands may be used not only as antinociceptive agents with reduced addictive properties but also as therapeutic alternatives for already addicted patients.\textsuperscript{12} Tables 2 and 3 include ligands with affinity at the MOP and DOP receptors.

\textbf{KDAN series of ligands}

Portoghese and colleagues proposed a similar nomenclature to the MDAN bivalent ligands, this time for KOP and DOP receptor selectivity, naming the series as KDAN (K for \(\kappa\)-receptor, D for \(\delta\)-receptor).\textsuperscript{13} Multiple ligands with different numbers of atoms in their linker (noted again as
numbers in the series) have been studied, producing the KDN series of ligands [both pharmacophores as (N) antagonists] and KDAN series (a KOP agonist and a DOP antagonist pharmacophore). Tables 2 and 3 include ligands with affinity at the DOP and KOP receptors. KDN-21, a compound with a spacer length of 21 atoms, has been shown to antagonize the \( \kappa_1 \)-receptor agonist bremazocine, while weakly antagonizing the \( \kappa_2 \)-receptor agonist U50488 and potently antagonizing the \( \delta_1 \)-receptor agonist DPDPE. The data confirm the suggestion that the \( \kappa_1 \)-\( \delta_2 \) or the \( \kappa_2 \)-\( \delta_1 \) putative receptors represent the \( \kappa \)-\( \delta \) receptor heterodimer which, in the presence of the KDN and KDAN ligands, are bridged. HEK cell-lines expressing either KOP and DOP receptors or cell-lines co-expressing both KOP and DOP receptors were used to study two ligands: KDN-20 and KDN-21. These displayed a 120- and 200-fold higher affinity, respectively, for the co-expressed receptor when compared with single-expressing systems. The authors of these reports interpreted the data based on the existence of DOP-KOP heteromers in the co-expressed cell lines. Bivalent ligands with a \( \kappa_1 \)-receptor agonist ICI-199 441 and a DOP receptor antagonist naltrindole as pharmacophores have also been synthesized (KDAN series) and evaluated after intrathecal administration in tail flick assays and in binding studies. KDAN-18 showed a maximum of 65-fold higher affinity for a co-expressing DOP-KOP HEK cell line when compared with mixed membranes of single-expressing cell lines. The monovalent ligands ICI-199 441 and naltrindole possessed much lower affinity for all receptors, when either co-expressed or mixed. The optimum spacer length of 18 atoms for the KDAN ligands (KDAN-18) is believed to favour interdimeric bridging of two active sites (one receptor from each of two neighbouring receptor-dimers) rather than intradimeric bridging (two receptors in a receptor-dimer). However, stereochemical and binding data show that such complicated bridging is less possible and more energy-demanding (Fig. 3, Part 6).

Factors affecting bivalent opioid properties

In addition to the pharmacophore chemistry, two other factors that affect optimum interaction of bivalent ligands and receptor binding sites are the spacer type/length and the pharmacophore type/number.

Spacer type and length
As noted earlier spacer type and length between the two pharmacophores is a factor that affects the interaction of a bivalent ligand with its receptor targets. It has been suggested that optimum binding occurs when spacer length corresponds to the distance between the active sites.

Fig 3 Schematic representation of possible binding combinations (1–6) to opioid receptor dimers with respective ligands. Two different opioid receptors (blue and orange) constitute a heteromeric receptor dimer. The pharmacophores A and B of the bivalent ligand A–B (see also Fig. 2) are selective for their respective receptors (blue for A, orange for B) and are linked together through an intermediate spacer. When successful binding occurs, an appropriate—but not necessarily identical—response is evoked (represented by the ‘thunderbolt’ sign under the receptors) through activation of G protein. The schema assumes that the G protein associated with each receptor can trigger a response when activated by pharmacophore binding. (1) Bivalent ligand A–B binds to the receptor dimer blue–orange, using both of its pharmacophores for a perfect match. (2) One pharmacophore (A) of the bivalent ligand A–B binds to the respective receptor monomer (blue). (3) Replacement of pharmacophore B with an inactive (+)-enantiomer (+B) causes only the binding of pharmacophore A, leaving pharmacophore +B unbound because of its stereochemical divergence. (4) Binding of pharmacophore A, combined with binding of an endogenous opioid ligand C, could possibly leave pharmacophore B unbound. Individual pharmacophore affinities and concentration gradients play a crucial role for the competition of B and C over the red-receptor active site. (5) Two endogenous opioids (D and E) bind to an opioid receptor dimer. The evoked response should theoretically be the same as that from combination-1. (6) Stereochemical orientation bridging of two receptors from neighbouring receptor-dimers is less likely to be successful because of the spatial disorientation of pharmacophores relatively to their active sites. The bivalent ligand A–B cannot take the optimum orientation in order to bridge neighbouring receptors, no matter what rotation its approach takes.
of the respective vicinal or dimeric receptors, although this supposition will need rigorous experimental validation. Molecular modelling of opioid receptor dimers shows that the distance between the monomers with an interface in transmembrane domains (TM) 5-6-7 is around 27 Å, while those with a TM 4-5 interface are 32 Å. Physiochemical spacer characteristics (hydrophobicity, molecular size, flexibility, and vulnerability to enzymatic cleavage) also affect the efficacy of bivalent ligands.

**Pharmacophore type and number**

Specific strategies are being used to define the role and function of the presence of a second pharmacophore in a bivalent ligand. Three compounds are usually tested: (i) a single pharmacophore, (ii) a single pharmacophore attached to a spacer, and (iii) two pharmacophores linked with a spacer. Studies have shown that for most pharmacophores, type (ii) ligands usually show lower affinity for the receptor than (iii) or even (i). This observation is attributed to steric hindrance of the active site by the spacer chain.

The choice of using either non-peptidic or peptidic opioid pharmacophores also contributes to bioactivity and physical characteristics. Non-peptidic ligands have increased resistance to enzymatic degradation and thus possess an advantage for accessing the CNS after peripheral administration. Opioid receptors are highly selective for the opioid pharmacophores (noted as a morphine displays 1000-fold lower affinity than (−)morphine). The utilization of enantiomeric forms (isoforms) of the pharmacophores in bivalent ligands provides an additional tool for studying receptor binding and activation. The (−) pharmacophores bind to the receptor but their stereochemistry is probably responsible for the loss in affinity and reduction in potency. Bivalent ligands containing both (−) and (+) pharmacophores (notated as a −/+ ligands) show a significant decrease in activity, compared with the homologous −/− ligands. As only one of the two pharmacophores of the −/+ ligand has affinity for the receptor, the potency of the ligand might be expected to half that of the −/− ligand. This can be true if the bivalent ligand bridges two neighbouring, but not associated receptors, as associated receptors interact in a different manner. Nevertheless, what we see in practice is not a halved potency in the −/+ ligands. For example, the activity of the opioid −/− bivalent ligand is increased from five-fold (in the case of two oxymorphone pharmacophores) to 30-fold (in the case of norBNI ligands) compared with their respective −/+ analogues. This disproportional increase in activity of −/− ligands compared with −/+ ligands could imply the bridging of two active sites that are found in a receptor complex (dimer). The binding of one pharmacophore to one receptor causes a conformational change that possibly affects the binding of the second receptor in a positive allosteric manner; hence, the disproportional increase in activity of the bivalent ligand. Of interest, similar molecules that have different stereochemical orientation (owing to one -C-addition) may show the opposite pharmacological activity profile for a given receptor (i.e. Table 2, compounds 4 and 5, showing both a MOP receptor agonist activity but opposite DOP receptor activity). The use of ligands that contain enantiomeric moieties (thus different stereochemistry) can reveal the role of the stereochemical orientation of its moiety to the compound’s activity. In addition, hydrophobic or aromatic groups embedded in the structure of non-selective DOP/MOP receptor ligands enhance the interaction with their respective receptors. For example, in the non-selective ligand that incorporates one Dmt-Tic pharmacophore linked to -Gly-NH-CH2-, when benzyimidazole (Bid) addition is compared to phenyl (Ph) addition there is a marked loss of MOP affinity with no change in DOP affinity (Table 2, compounds 4 and 7).

**Dimerization and bivalent opioid ligands**

The role of receptor dimerization

Pharmacological data from independent research groups in the past have suggested the existence of different opioid receptor ‘subtypes’ (putative opioid receptors δ1, δ2, κ1, κ2, etc.), based primarily on the pharmacological profiles taken by the combination of different agonists and antagonists. Whilst molecular biology has identified the existence of only three genes for the opioid receptors, multiple splice variants of these genes have been found. Whether these splice variants or different post-translational modifications of the original gene products are the explanation for the observed pharmacological diversity, is unclear. The carboxyl-terminal splicing of the rat MOP receptor has been shown, for example, to modulate receptor internalization and resensitization, rather than its pharmacological profile. The traditional view of opioid receptors sees a single receptor (MOP, DOP or KOP) working alone. However, the mixed pharmacological profile seen for some of the proposed opioid receptor ‘subtypes’, has led to studies that focused on opioid receptor association, co-localization, and oligomerization, as possible mechanisms of explaining the distinct pharmacological profiles found. Further studies using immunofluorescence and immunoprecipitation assays have confirmed that opioid receptors not only dimerize in various combinations but mostly exist as receptor dimers and not monomers, in a variety of different tissues. Other studies have confirmed that dimerization modulates receptor function and that different receptor dimers possess distinct pharmacological profiles. Based on studies in the spinal cord of mice Portoghese and Lunzer characterized the putative δ1 and κ2 opioid receptor as a DOP-KOP heteromeric receptor. In addition, other studies
suggest that the δ2 and κ1 phenotypes might represent neighbouring associated δ and κ receptors. Two different receptor dimerization mechanisms have been proposed: 

**constitutive dimerization** [where dimers are constructed in the endoplasmic reticulum (ER) after RNA translation], and 

**ligand-induced dimerization** (forming on the cell membrane), where dimers are formed on the cell membrane in response to the presence of specific ligands. Opioid receptor dimers have been shown to be formed mainly through constitutive dimerization. Although it has been shown that opioid receptor dimers are coupled allosterically without compromising their binding sites, the binding of a ligand to one active site can cause a conformational change of the receptor that could affect the second binding site. This effect may be positive or negative (positive or negative cooperativity). Portoghese proposes the existence of two recognition sites in an opioid receptor dimer, which modulates receptor activation and antagonism.

### Bivalent ligands and receptor dimers

As noted above, heterobivalent ligands have been shown to possess a higher affinity for their respective receptors when using cell membranes from cells that co-express the two receptors, rather than from a mixture of membranes from cells that express the individual receptors. In addition, various reports mention an increase in the activity and selectivity of bivalent ligands when compared with data from individual binding of their monomeric pharmacophores. These data are open to interpretation and we discuss this further with respect to receptor dimerization. We would stress that much of this general discussion requires experimental evaluation. Bivalent ligand thermodynamics are believed to favour binding of the ligand to receptor dimers rather than receptor monomers, suggested by the disproportional increase in affinity and potency of bivalent over monovalent ligands. It has been postulated that bivalent ligands can bridge the space between receptor dimers and therefore may activate both receptors simultaneously. This simultaneous binding indicates an important role of linker type/length connecting the two pharmacophores, discussed above. Molecular modelling and binding studies with bivalent ligands have suggested that the optimum length between the active sites of receptors within a dimer is around 25 Å for most opioid receptor dimers. However, a few studies have shown different optimum linker lengths for the same bivalent ligands after testing in different cell models. These data may indicate an effect of cell background on receptor dimerization. Nevertheless, dual activation of two receptor active sites by one bivalent molecule is considered a thermodynamically favourable interaction over monovalent binding.

Portoghese and colleagues suggest that there may be a more complex relationship between receptors and bivalent ligands. Homodimerization of different receptors (DOP–DOP, KOP–KOP, and MOP–MOP) favours their neighbouring association and thus bivalent agents may bridge two receptors from different receptor–dimer complexes (Fig. 3). Although there are no studies confirming favourable association of receptor dimers, high expression levels may promote such clustering. Higher order oligomers have also been suggested to affect receptor function, although it has been shown that tetramers are formed as a result of oligomerization of two different dimers, rather than clustering of four individual receptors.

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**Fig 4** Schematic representation of the potential clinical advantages of using bivalent ligands, with the various combinations of pharmacophores (antagonists and agonists). In addition, there are some reports showing reduced tolerance for ligands with mixed MOP/DOP agonist activity.
**Bifunctional opioids and anaesthesia/analgesia**

It is clear from the description above that currently there is a wide range of bivalent ligands available. While some can be useful as experimental tools, most are not suitable as models for further clinical development and therefore further studies are needed. Bivalent ligands, as a class of compounds, have the advantage of single drug administration and this has implications for reduced drug–drug interactions and more practically for patient compliance. In addition, there is good evidence that some bivalents have improved pharmacological profile. The potential in vivo profiles of the various combinations of pharmaco- phores in bivalent ligands are illustrated in Figure 4.

Of the many ligands described in this review, we are particularly interested in bivalent ligands with MOP-agonist activity and DOP-antagonist activity. Such molecules show not only an increased antinociception and reduced tolerance when compared with morphine, but also decreased physical dependence, reduced respiratory depression, and decreased gastrointestinal inhibition. The development of opioid bivalent ligands can also be useful in areas of clinical pharmacology other than analgesia. For example, one of the current therapeutic strategies already used in cocaine abuse is the co-administration of KOP receptor antagonists with MOP receptor agonists. An opioid bivalent ligand that incorporates the activities of the two individual agents could provide a pharmacodynamic/kinetic advantage and therefore reduced side-effects. Nevertheless, the bulk of this work is grounded in anaesthetic pharmacology–medicinal chemistry–small animal behavioural studies. The real test and challenge will be taking suitable molecules into our most important species, humans.

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**Appendix**

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<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>EC50</td>
<td>half-maximal effective concentration: is the concentration of agonist that produces 50% of its maximal effect</td>
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<tr>
<td>Emax</td>
<td>the maximum effect (biological response) that a ligand can produce</td>
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<tr>
<td>Enantiomers</td>
<td>or optical isomers, are compounds that have the same molecular formula but a different spatial arrangement (mirror-images)</td>
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<tr>
<td>IC50</td>
<td>half-maximal inhibitory concentration: is the concentration of an antagonist needed to inhibit a biological response to its 50%</td>
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<tr>
<td>Ligand</td>
<td>a molecule that binds to a biomolecular target (e.g. receptor) and therefore has a biological activity</td>
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<tr>
<td>Ligand affinity</td>
<td>the ability of a drug to bind to a receptor, often expressed by the Ki</td>
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Simultaneous targeting of multiple opioid receptors

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