Development and characterisation of novel fentanyl-delta opioid receptor antagonist based bivalent ligands

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Editor’s key points

- There is considerable need for opioids with reduced tolerance.
- A series of bifunctional opioid receptor ligands was tested for receptor binding and function.
- Addition of a linker did not impair receptor binding, but reduced µ opioid receptor functional activity, indicating the need for alternate approaches to reducing tolerance.

Background. Opioid tolerance is a limiting factor in chronic pain. Delta opioid peptide (DOP) (δ) receptor antagonism has been shown to reduce tolerance. Here, the common clinical mu opioid peptide (MOP) (µ) receptor agonist fentanyl has been linked to the DOP antagonist Dmt-Tic (′2,′6-dimethyl-L-tyrosyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) to create new bivalent compounds.

Methods. Binding affinities of bivalents (#9, #10, #11, #12 and #13) were measured in Chinese hamster ovary (CHO) cells expressing recombinant human MOP, DOP, Kappa opioid peptide (KOP) (κ) and nociceptin/orphanin FQ opioid peptide (NOP) receptors. Functional studies, measuring GTPγ35S or β-arrestin recruitment, were performed in membranes or whole cells respectively expressing MOP and DOP.

Results. The new bivalents bound to MOP (pkι : #9:7.31; #10:7.58; #11:7.91; #12:7.94; #13:8.03) and DOP (pkι : #9:8.03; #10:8.16; #11:8.17; #12:9.67; #13:9.71). In GTPγ35S functional assays, compounds #9 (pEC50:6.74; intrinsic activity:0.05) #10(7.13:0.34) and #11(7.52:0.27) showed weak partial agonist activity at MOP. Compounds #12 and #13, with longer linkers, showed no functional activity at MOP. In antagonist assays at MOP, compounds #9 (pKb:6.87), #10(7.55) #11(7.81) #12(6.91) and #13(7.05) all reversed the effects of fentanyl. At DOP, all compounds showed antagonist affinity (#9:6.85; #10:8.06; #11:8.11; #12:9.42; #13:9.00), reversing the effects of DPDPPE ((D-Pen2,5)enkephalin). In β-arrestin assays, compared with fentanyl (with response at maximum concentration (RMC):13.62), all compounds showed reduced ability to activate β-arrestin (#9 RMC:1.58; #10:2.72; #11:2.40; #12:1.29; #13:1.58). Compared with fentanyl, the intrinsic activity was: #9:0.12; #10:0.20; #11:0.18; #12:0.09 and #13:0.12.

Conclusions. The addition of a linker between fentanyl and Dmt-Tic did not alter the ability to bind to MOP and DOP, however a substantial loss in MOP functional activity was apparent. This highlights the difficulty in multifunctional opioid development.

Keywords: delta receptors; fentanyl; mu receptors; opioid; pharmacology - analgesics opioid

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Opioid use in the pain clinic is hampered by development of tolerance, providing a dilemma in the treatment of chronic pain. The underlying causes of chronic pain are complex, the origins of which include a wide range of diseases and/or injuries, making effective treatment difficult.1 For instance, the treatment of chronic pain in cancer has been shown to be ineffective in up to 50% of patients in the last year of their lives.2 3 Most clinically available opioids act solely at the mu opioid peptide (MOP) (µ) receptor, a member of the Gαo-protein coupled opioid receptor family of receptors, which also includes the delta opioid peptide (DOP) (δ), kappa opioid peptide (KOP) (κ) and nociceptin/orphanin FQ opioid peptide (NOP) receptors. Substantial evidence has implicated the DOP receptor3 4–6 in the development of tolerance, hastening the development of multifunctional opioids.

The mechanisms underlying the development of tolerance are varied, involving a multitude of cellular functions, receptor properties and signalling processes, such as receptor internalisation and β-arrestin recruitment.7 A large body of evidence, including various molecular studies8 9 10 and work in
mouse models, has implicated other members of the opioid receptor family, specifically the DOP receptor, as potential mediators and/or instigators in the development of tolerance. The involvement of two, or more, opioid receptors in the progression of tolerance has provided a target for the development of drugs with multiple pharmacophores, i.e. bivalent or bifunctional ligands. Bivalent and bifunctional ligands could present more predictable pharmacokinetic and pharmacodynamic traits than two separately acting ligands. Synergy between two conjoined pharmacophores can also lead to improved potency, as well as a potential reduction in side-effects.

Previous attempts at developing bivalent ligands have focused on adaptations or derivatives of semi-synthetic or peptide structures. We studied chemical adaptations of fentanyl (N-(1-(2-phenylethyl)-4-piperidinyl)-N-phenylpropionamide), a clinically available synthetic opioid, to accept linker molecules and a second pharmacophore (Dmt-Tic (2′,6′-dimethyl-L-tyrosyl-1,2,3,4-tetrahydrisoquinoline-3-carboxylic acid)). Previous studies have reported the synthesis and chemical adaptation of these carboxy-fentanyl molecules through conjugation with DOP agonists (enkephalins) and neurokinin-1 (NK-1) antagonists. Fentanyl is a potent MOP agonist (10 fold greater than morphine) used in the treatment of pain in a clinical setting. Moreover, fentanyl is hydrophobic allowing greater access to the central nervous system than, for instance, morphine. The potent analgesic activity and high bioavailability of fentanyl make it an ideal model on which to base a mixed opioid. The second pharmacophore chosen for the development of these new bivalent compounds is the DOP antagonist Dmt-Tic. Dmt-Tic has a long history of use in the development of multi-pharmacophoric compounds, and it has been demonstrated that C-terminal chemical modifications of Dmt-Tic are tolerated, thus making it a good candidate for the development of a [MOP agonist]-[DOP antagonist] complex.

We characterised the binding and functional activity of five newly synthesized fentanyl (MOP)/Dmt-Tic (DOP) bivalent compounds. As shown in previous studies, linker length can affect the affinity and efficacy of either pharmacophore; with this in mind we have examined spacer length between the MOP and DOP pharmacophores. Finally we explored whether these novel bivalent molecules produce changes in signalling in G-protein activation and β-arrestin recruitment assays.

### Methods

#### Materials

The reference molecules, Dmt-Tic and Nocieptin/Orphanin FQ (N/OFQ) were synthesised in house (Department of Chemical and Pharmaceutical Sciences, University of Ferrara). Tritiated UFP-101 ([3H]-UFP-101) was synthesized as described. Tritiated diprenorphine ([3H]-DPN) was purchased from Perkin Elmer (UK). Morphine and naloxone was purchased from Sigma-Aldrich Co. (Dorset, UK). Fentanyl, norbinaltorphimine (KOP antagonist, norBNI) and [D-Pen²,D-Pen⁵]-enkephalin (DOP agonist, DPDPE) were purchased from Tocris (Abingdon, UK). Tissue culture media and supplements were obtained from Invitrogen (Paisley, UK). Nomenclature and structure are shown in Table 1. Synthesis of the novel bivalent ligands is described in detail in the Supplementary material.

#### Cell culture

Chinese hamster ovary (CHO) cells expressing recombinant human opioid receptors were grown in either Hams F12 (for CHO₉,MOP, CHO₉,DOP and CHO₉,KOP cells) or DMEM/Hams F12 1:1 (for CHO₉,NOP cells). The media contained 10% fetal bovine serum, 100 IU ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 2.5 μg ml⁻¹ fungizone. Stock cultures were maintained through the addition of 200 μg ml⁻¹ G418 (for CHO₉,MOP, CHO₉,DOP and CHO₉,KOP cells). CHO₉,NOP cells were maintained with G418 (200 μg ml⁻¹) and hygromycin B (200 μg ml⁻¹). Cell cultures were maintained at 37°C in 5% CO₂/humidified air. Cells were used for experimentation when confluent.

#### Membrane preparation

Cells were harvested, homogenised, and membrane fragments were resuspended in either a wash buffer consisting...
of 50 mM Tris-HCl pH to 7.4 with KOH, for CHO₂,MOP, CHO₂,DOP and CHO₂,KOP; or additional 5 mM MgSO₄ for CHO₂,NOP cells for saturation and displacement binding assays, or a homogenisation buffer (50 mM Tris and 0.2 mM EGTA pH 7.4 with NaOH) in GTPγ[S] functional assays. Membrane suspensions were centrifuged at 20374 g at 4 °C for 10 min, and this process was repeated thrice. The resulting pellet was resuspended in an appropriate volume of the desired buffer and protein concentration measured using the Lowry assay.²⁶

**Ligand selectivity and binding affinity-displacement binding assays**

Membrane protein (20–40 μg) was incubated in 0.5 ml of 50 mM Tris, 0.5% bovine serum albumin (BSA) and ~0.8 mM [³⁵S]-DPN (for CHO₂,MOP, CHO₂,DOP and CHO₂,KOP) or ~0.8 mM [³⁵S]-UFP-101 (for CHO₂,NOP cells), as well as varying concentrations (1 pm–10 μM) of control ligands and test compounds. Nonspecific binding was determined in the presence of 10 μM naloxone for CHO₂,MOP/DOP/KOP or 1 μM of N/OFO for CHO₂,NOP cells. Samples were incubated for 1 h at room temperature, following which reactions were terminated by vacuum filtration onto PEI-soaked Whatman GF/B filters, using a Brandel harvester (Semat, Germany).

**Ligand functional activity-GTPγ[S] assays**

Membrane protein (20–40 μg) was incubated in 0.5 ml volume of 50 mM Tris, 0.2 mM EGTA, 1 mM MgCl₂, 100 mM NaCl, 0.1% BSA, 0.15 mM bacitracin; pH 7.4, GDP (33 μM for classical; 100 μM for NOP), and ~150 mM GTPγ[S]. A range of concentrations of both control (fentanyl, DPDPDE) and the test compounds (1 pm – 10 μM) was added prior to incubation. Nonspecific binding was determined in the presence of unlabeled GTPγS (10 μM).��⁻¹ Samples were incubated for 1 h at 30 °C with gentle agitation. Reactions were terminated by vacuum filtration through dry Whatman GF/B filters.

**Beta-Arrestin assay**

Assays were prepared as described in the DiscoveRx (UK) PathHunter® assay protocol. Cells were incubated for 24 h (human OPRM1, MOP) or 48 h (human OPRD1, DOP) as suggested in 96 well plates provided. Following the desired incubation period, desired concentrations of compounds were added to the wells, and incubated at 37 °C for 90 min. For morphine and fentanyl, at MOP, and DPDPDE, at DOP, a range of concentrations was used (1 nM – 10 μM). At MOP, only maximum concentrations (10 μM) compounds #9–#13 were used. In antagonist studies at DOP, 10 nM of Dmt-Tic was incubated in a range of concentrations of DPDPDE (1 nM–10 μM). High concentrations of DPDPDE (1 μM) were incubated with 100 nM (~EC₅₀) of compounds #9–#13 to determine antagonist activity. The final development reagent was added and plates were incubated at room temperature for 1 h. Plates were read using a Dynex MLX luminometer, Dynex Technologies, UK, set at 1 sec/well to measure relative light units (RLU).

**Data analysis**

Data are expressed as mean(SEM (n)) experiments. Results were analysed and graphs were fitted using GraphPad PRISM V6.02 (San Diego, USA). In displacement binding studies, the concentration of competitor which produced 50% displacement (IC₅₀) was corrected for competing mass of radiolabel according to Cheng and Prusoff²⁶ using Kᵣ values for [³⁵S]-DPN of 125 pM (MOP); 323 pM (DOP); 134 pm (KOP) and for [³⁵S]-UFP-101 107 pM (NOP).²⁹ ³⁰ GTPγ[S] binding data are expressed as a stimulation factor (agonist stimulated specific binding/basal specific binding).²⁷ Antagonist affinities were calculated using the Gaddum equation.β⁻¹ β-arrestin recruitment is expressed as an activation factor (activated (RLU)/basal RLU). Data were analysed using t-tests and/or ANOVA, with Bonferroni corrections as appropriate with P<0.05 considered significant.

**Results**

Displacement binding assays-Fentanyl analogues RRC2, RRC3 and RRO

RRC2; RRC3; RRO failed to displace [³⁵S]-DPN in CHO cell membranes expressing MOP, DOP or KOP receptors. These compounds also failed to displace [³⁵S]-UFP-101 in CHO cell membranes expressing NOP receptors (Table 2).

Displacement binding assays-Final compounds #9, #10 and #11

At the MOP receptor, #9, #10, #11 displaced binding of [³⁵S]-DPN in a concentration dependent and saturable manner. Binding affinities (pKᵢ) were: #9(7.31), #10(7.58) and #11(7.91) (Table 2). Binding affinities of test compounds #10 and #11 were not statistically different to that of the reference ligand fentanyl (8.13). Compound #9 was significantly different, displaying a lower binding affinity for MOP. At the DOP receptor, all ligands had nanomolar affinity: #9(8.03), #10(8.16) and #11(8.17) (Table 2). Of the test compounds, only #9 was significantly different compared to Dmt-Tic-OH (8.95). Compounds #9, #10, #11 displaced [³⁵S]-DPN binding to CHO₂,KOP membranes [NorBNI (10.16); #9(7.29); #10(7.02); #11(7.13)] [³⁵S]-UFP-101 was also displaced by the test compounds in CHO₂,NOP membranes [N/OFO (10.69) #9(7.28); #10(6.96); #11(7.48)] (Table 2).
Since compounds #9, #10 and #11 showed reduced efficacy at CHO_MOP, these were screened in an antagonist assay (Fig. 1B; Table 2). A fixed concentration (1 μM) of the bivalent compounds was added to varying concentrations of fentanyl in CHO_MOP cells; there was a rightward shift in the fentanyl concentration response curve. Both #10 and #11 displayed E_{max} values similar to fentanyl (Table 2). E_{max} in the presence of #9 was significantly different to fentanyl. The pK_b values were: #9 (6.87), #10 (7.55) and #11 (7.81).

In CHO_DOP membranes DPDPE (D-Pen²,D-Pen⁵ Enkephalin) stimulated the binding of GTPγS (pEC_{50} 7.70; E_{max} 2.76). Compounds #9, #10 and #11 did not stimulate binding of GTPγS (Fig. 2A). When compounds #9, #10 and #11 were co-incubated with the DOP agonist DPDPE, all compounds caused a rightward shift in the concentration-response curves (Fig. 2B). Compounds #10 and #11, produced pK_b values of 8.06 and 8.1, respectively. Compound #9 had a pK_b of 6.85 (Table 3).

Functional assays were performed in cell membranes expressing the KOP and NOP receptor; bivalents #9–#11 showed no agonist or antagonist activity at these receptors (Fig. 3A and B).

Displacement binding assays-Final compounds #12 and #13

Compounds #12 and #13 displaced [³H]-DPN at the MOP, DOP and KOP receptors in a concentration dependent and saturable manner, but failed to displace [³H]-UFP-101 (Table 2). At the MOP receptor, #12 (pK_b: 7.94) and #13 (8.03) were
not significantly different from fentanyl (8.13). At the DOP receptor, #12(9.67) and #13(9.71) both showed increased affinity for the DOP receptor, when compared with both #11(8.37) and the parent compound Dmt-Tic-OH (8.95). Differences in binding affinity between the two Gly-linker extended molecules were evident at the KOP receptor; #12 (8.14) displaying nanomolar affinity. While there was no significant difference in the binding affinities of #11(7.13) and #13(7.35), #12 showed statistical differences from both of these compounds. The Gly extended compounds showed either weak (#13; 6.63), or no affinity (#12; inactive) for the NOP receptor (Table 2).

<table>
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<tr>
<th>Compound</th>
<th>Agonist Activity (pEC50)</th>
<th>Relative Intrinsic Activity</th>
<th>Antagonist Activity pKb</th>
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<tr>
<td>CHOhMOP</td>
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<td>Fentanyl</td>
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<td>0.27</td>
<td>7.81 (0.18)</td>
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<tr>
<td>#12</td>
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<td>Inactive</td>
<td>6.91 (0.08)</td>
</tr>
<tr>
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<td>7.05 (0.11)</td>
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<td>N/A</td>
</tr>
<tr>
<td>CHOhDOP</td>
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<td></td>
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<tr>
<td>Dmt-Tic</td>
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<td>Inactive</td>
<td>8.77 (0.11)</td>
</tr>
<tr>
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<td>Inactive</td>
<td>6.85 (0.19)</td>
</tr>
<tr>
<td>#10</td>
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<td>Inactive</td>
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<tr>
<td>Analysis of Variance</td>
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<td>N/A</td>
<td>significant</td>
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</table>

**Table 3**  
Agonist and antagonist activity of the [fentanyl]–[Dmt-Tic] bivalent pharmacophores at CHOhMOP and CHOhDOP. Antagonist affinity was determined against Fentanyl (MOP) and DPDPE [D-Pen², D-Pen⁵ Enkephalin] (DOP). Relative intrinsic activity was determined by removal of basal activity and as a ratio of full agonist Emax. All experiments are represented as the mean (SEM) with n ≥ 3. If a significant difference was detected (ANOVA), post-hoc testing using Bonferroni multiple comparisons was employed *P<0.05; **P<0.005; ****P<0.0001 compared with fentanyl for MOP and Dmt-Tic for DOP.

Fig 2 (a) DPDPE stimulated GTPγ[S] binding. Compounds (#9, #10, and #11) showed no activity in CHOhDOP cell membranes. (b) DPDPE stimulated binding in the absence and presence of 100 nM of bivalent compounds (#9, #10, and #11). All data are mean (SEM) of 5 experiments. Reference ligand, DPDPE; [D-Pen², D-Pen⁵ Enkephalin].

GTPγ[S] functional assays—Final compounds #12 and #13

The Gly-extended compounds #12 and #13 failed to stimulate the binding of GTPγ[S] with no efficacy at the MOP.
receptor (Fig. 4A; Table 3). At 300 nM, compounds #12 and #13 behaved as weak antagonists with pKb values of 6.91 and 7.05, respectively (Fig. 4B).

Compounds #12 and #13 showed no activity at the DOP receptor (Fig. 5A, Table 3). However, when co-incubated with the DOP agonist DPDPE, both #12 (pKb: 9.42) and #13 (9.00) produced a rightward shift in the agonist concentration response curve (Fig. 5B, Table 3).

Since #12 and #13 displayed binding affinity for KOP, GTP\._{\text{GS}}[35S] functional assays were performed. Both compounds were inactive (Fig. 6A). In antagonist experiments, 300 nM and 1 μM for #12 and #13, respectively, produced a weak but measurable rightward shift in the concentration response curve when co-incubated with dynorphin-A. Compound #12 and #13 produced pKb values of 6.96 and 6.45, respectively (Fig. 6B). In view of the low affinity at NOP for #12 and #13 functional assays were not performed.

### Beta-Arrestin assays

β-arrestin recruitment in MOP cells was determined for fentanyl and morphine in CHO\._{\text{MOP}} cell lines (Fig. 7A). Maximally effective concentrations of bivalent compounds were used to compare against fentanyl and morphine (Fig. 7A). Fentanyl (Emax: 13.62(0.45)) produced a greater β-arrestin recruitment than morphine (Emax: 10.80(0.38)). Fentanyl (pEC50: 7.45 (0.07)) also has increased potency compared with morphine (6.88(0.12)). The potency of fentanyl for β-arrestin recruitment was similar to its potency of GTP\._{\text{GS}} activation (Table 2). When compared with the maximum response of fentanyl, the
bivalent ligands showed poor ability to recruit β-arrestins. Response from the maximum concentration tested in rank order was: #10 (2.72) < #11 (2.40) < #9 (1.58) < #13 (1.58) < #12 (1.29). The ability of these compounds to recruit β-arrestins was significantly different when compared with fentanyl. When compared with fentanyl, the intrinsic activity of these compounds was: #9: 0.12; #10: 0.20; #11: 0.18; #12: 0.09 and #13: 0.12.

In DOP cells, ability of DPDPE to recruit and Dmt-Tic (the parent pharmacophore) to inhibit β-arrestin recruitment were measured (Fig. 8A). DPDPE produced a concentration dependent and saturable increase in β-arrestin recruitment with pEC50 and Emax of 7.56(0.09) and 14.58(0.61) respectively. Dmt-Tic acts as a potent antagonist of DPDPE induced β-arrestin recruitment, with a pKb of 9.20(0.11). All of the [fentanyl]-[Dmt-Tic] bivalent pharmacophores reduced the ability of DPDPE to recruit β-arrestins. When compared with DPDPE at 1 μM (13.46(0.29)), the presence of #9 (7.43), #10 (9.51) and #11 (9.56) significantly reduced the ability of DPDPE to recruit β-arrestins, indicating antagonist activity. The presence of either 10 nM #12 (6.44) or 10 nM #13 (5.56) led to >50% reduction in the ability of DPDPE to recruit β-arrestin, suggesting potent antagonist activity at the DOP receptor (Fig. 8A).

**Discussion**

Linking of the Dmt-Tic pharmacophore to fentanyl produced compounds that displayed binding affinities at MOP comparable to that of the parent compound fentanyl, with the...
exception of #9. Compound #9 showed a decrease in binding affinity for MOP. Final compounds #9–13 displayed affinity for the DOP receptor consistent with the presence of a Dmt-Tic pharmacophore. Compounds #10 and #11 displayed similar binding affinity to that of Dmt-Tic, whilst compound #9 again showed a decrease in binding affinity. Compounds with glycine-extended linkers (#12 and #13) showed a relative increase in binding at DOP. In functional studies, all compounds showed either weak partial agonist activity or no functional activity at MOP relative to fentanyl, with compounds #9, #10 and #11 acting as weak partial agonists, while the extended linker compounds (#12 and #13) showed no functional activity at this receptor. In antagonist assays at MOP, compounds #10 and #11 antagonised fentanyl, producing pK₆ values similar to both their pKᵢ and pEC₅₀ values, inherent qualities of a partial agonist. Compounds #12 and #13 displayed low antagonist affinity at MOP, but displayed increased antagonist affinity, when compared with Dmt-Tic, at DOP. The antagonist affinity displayed at DOP by #12 and #13 matched their pKᵢ values for DOP. The weak intrinsic activity, regarding GTPγ35S binding, was mirrored in their ability to recruit β-arrestins. Compounds #10 and #11 produced the highest recruitment; however this was approximately 5-fold lower than that of the parent compound fentanyl.

Fig 7 (a) Fentanyl and morphine concentration response curves for β-arrestin recruitment. (a) shows the recruitment of β-arrestin in the presence of 10 μM of fentanyl, morphine and the various bivalent ligands in CHO/DOP cells. The activation factor is a ratio relative to basal β-arrestin activity. Data were significant (ANOVA) and *P < 0.0001 compared with fentanyl post hoc Bonferroni test. Data are shown as mean (SEM) for n ≥ 5.

Fig 8 (a) Depicts a concentration response curve in the absence, and presence, of 10 nM Dmt-Tic. (a) Shows the inhibition caused by selected concentrations of the various bivalent ligands in CHO/DOP cells. Data were significant (ANOVA) and *P < 0.0005; **P < 0.0001 compared with DPDPE post hoc Bonferroni test. Data are shown as mean (SEM) for n ≥ 5.
Prior to the development of the [fentanyl]-[Dmt-Tic] ligands, the carboxy fentanyl series had been conjugated with both DOP agonists (enkephalins) and NK-1 antagonists. In the case of [fentanyl]-[NK-1 antagonist], a series of compounds where tested using radioligand binding and ex vivo tissue functional studies. In agreement with the present study, functionalised fentanyl derivatives showed a loss of functional ability at MOP. Following conjugation with an NK1 antagonist, most compounds showed a reduction in binding affinity for MOP and functional activity in guinea pig ileum contraction assays. Further studies, using the RRC3 fentanyl derivative demonstrated significant analgesia in warm water tail flick tests, following spinal administration, after conjugation with enkephalins. This compound produced antiallodynic and thermal antihypersensitive effects in nerve injured animals.

The involvement of DOP receptors in MOP receptor functional activity, trafficking and endocytosis has been demonstrated in a large number of studies. Initially, experiments involving antagonism of the DOP receptor during morphine treatment demonstrated an attenuation of tolerance. The role of DOP in tolerance was further demonstrated in studies involving either DOP knockout or ppENK knockout mice, whereby removal of either the receptor or endogenous agonist led to a reduction in morphine tolerance. Furthermore, DOP receptor expression and trafficking is also up-regulated during long-term morphine treatment. Co-expression of both MOP and DOP in neurons and cells in the pain pathway has led to the hypothesis of a heterodimer, with unique signalling properties that influence opioid tolerance. The involvement of the DOP receptor in this heterodimer is believed to include differential phosphorylation and recruitment of β-arrestins, such that selectivity for the ubiquitination pathway is favoured after endocytosis. This makes it an ideal target for a drug with dual selectivity. Due to the more predictable pharmacokinetics and pharmacodynamics of a single drug, previous work has either focused on novel bifunctional drugs (UFP-505) or bivalent pharmacophores (MDAN21). In the development of drugs with dual agonist targets, it is important that the individual affinities produced by the drug for different receptors are similar. This would remove any favourability for a particular receptor, thereby increasing the possibility of the drug acting at both target sites. In the case of both our compounds and UFP-505, their affinities for DOP are higher than their affinities for MOP. However, since both have agonist activity at DOP, this could be beneficial as a higher affinity for DOP ensures that DOP is fully blocked when MOP is activated.

Due to the differences in functional activity seen with compounds #9, #10 and #11 compared with fentanyl, it was initially hypothesised that the distance between the pharmacophores affected their ability to interact with MOP. The importance of spacer molecules (size, distance, charge) has been previously demonstrated in a number of models, none more so effectively than in the development of the MDAN series of compounds. In order to validate this, we extended the linker molecules, using the amino acid glycine, of the most efficacious compound, #11. Either a single glycine molecule (#12) or two glycine molecules (#13) were added to the linker structure. The increase in spacer length between the MOP and DOP pharmacophores completely prevented MOP receptor activation. In contrast, DOP antagonist activity was retained. These results inferred that the changes of efficacy determined here, most likely lay in a combination of adaptation of the chemical structure of the fentanyl conjugates and linker length. It should be emphasized that simple functionalization of fentanyl with an acidic moiety as in RRC2, RRC3 and RRO produced loss of MOP binding.

β-arrestin recruitment has been implicated in the internalisation and recycling or ubiquitination of opioid receptors. In this study, bivalent compounds showed poor efficacy in general and substantially reduced ability to recruit β-arrestins to the MOP receptor, collectively suggesting a limited ability to promote receptor internalisation. Interestingly, the bivalent ligand with the shortest linker length (#9) displayed weaker antagonist activity at DOP. The main limitation of our study is the lack of antagonist activity at DOP, this could be beneficial as a higher affinity for DOP ensures that DOP is fully blocked when MOP is activated.

Limitations

While the compounds demonstrated the desired antagonist affinity at DOP, they demonstrated poor, or no, efficacy at MOP in our high expression system. Coupled with a modest antagonist affinity at the MOP receptor, these compounds would be predicted to have absent or ultra-low analgesic action. In the clinical setting the goal would be to design a high efficacy MOP agonist (retain the activity of fentanyl) coupled with DOP antagonism. The main limitation of our study is the lack of in vivo testing. However, we would argue that as the MOP agonist activity is lost there would be little point in undertaking antinociceptive testing in vivo.

In conclusion, the development of clinically available opioids with reduced tolerance profile is of high importance; MOP agonist - DOP antagonist ligands are possible candidates. In this study, the potent clinical MOP opioid fentanyl was linked to the DOP antagonist, Dmt-Tic to produce a new series of bivalents. This series of compounds was unable to retain the potent functional activity of fentanyl at MOP whilst retaining potent antagonist activity at DOP.
Supplementary material
Supplementary Material is available at British Journal of Anaesthesia online.

Authors’ contributions

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Mark F. Bird is a British Journal of Anaesthesia/Royal College of Anaesthetists funded PhD student.

Declaration of interest
D.G.L. and D.J.R. are board members and directors of British Journal of Anaesthesia. D.G.L. is also Administration Director and Company Secretary for British Journal of Anaesthesia.

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References
3 Colvin LA, Lambert DG. Pain medicine: advances in basic sciences and clinical practice. Br J Anaesth 2008; 101: 1–4
4 Abdelhamid EE, Sultana M, Portoghese PS, Takemori AE. Selective blockade of delta opioid receptors prevents the development of morphine tolerance and dependence in mice. J Pharmacol Exp Ther 1991; 258: 299–303
6 Schiller PW. Bi- or multifunctional opioid peptide drugs. Life Sci 2010; 86: 598–603
28 Cheng Y, Prusoff WH. Relationship between the inhibition constant (K) and the concentration of inhibitor which causes 50 per cent inhibition (50) of an enzymatic reaction. Biochem Pharmacol 1973; 22: 3099–108

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33 Ong E, Cahill C. Molecular Perspectives for mu/delta Opioid Receptor Heteromers as Distinct, Functional Receptors. Cells 2014; 3: 152–79
34 Liggett SB. Phosphorylation Barcoding as a Mechanism of Directing GPCR Signaling. Sci Signal 2011; 4: pe36–

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