In vitro and *in vivo* characterisation of the bifunctional MOP/DOP ligand UFP-505.

N. Dietis[1], H. Niwa[1], R. Tose[1], J McDonald[1], V. Ruggieri[2], M. Filaferro[3], G. Vitale[4], L. Micheli[5], C. Ghelardini[5], S Salvadori[6], G. Calo[6], R. Guerrini[7], D.J. Rowbotham[8] and D.G. Lambert[1].

ONLINE SUPPLEMENT

Materials and Additional Methodology

Sources of chemicals

The opioid peptide UFP-505 was synthesised at the Department of Pharmaceutical Sciences at the University of Ferrara as described previously. [A, B] Naloxone (non-selective opioid antagonist) and [D-Pen^2,D-Pen^5]-enkephalin (DOP agonist; DPDPE) were purchased from Sigma. Tritiated diprenorphine (non-selective opioid receptor antagonist; ^{3}H-DPN; 50.9 Ci mmol^{-1}) was purchased from Perkin Elmer. Endomorphin-1 (EM1) was synthesised in house (Department of Pharmaceutical Sciences, University of Ferrara). GTPγ^{35}S (1250 Ci mmol^{-1}) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Morphine and Fentanyl were purchased from Sigma-Aldrich Co. (St. Louis, USA) and S.A.L.A.R.S. (Como, Italy). All tissue culture media and supplements were obtained from Invitrogen (GIBCO, Paisley, U.K.). The ketamine hydrochloride/xylazine hydrochloride solution was purchased by Sigma, USA. All other reagents used were purchased with the highest quality available.

Membrane preparation

Cells and tissues were homogenised and crude membrane pellets obtained following centrifugation at 13500 g for 3 min at 4 °C. These were then resuspended in a homogenising buffer containing 50 mM Tris-HCL pH to 7.4 with KOH (for saturation and displacement binding) or 50 mM Tris and 0.2 mM EGTA pH 7.4 with NaOH (for GTPγ^{35}S binding in hMOP, hDOP), additionally supplemented with 5 mM $MgSO_4$ pH7.4 with KOH (for GTPγ^{35}S on hNOP) as described previously.[C] Suspensions were centrifuged at 20374 g for 10 min at 4 °C. This was repeated three times in total. Pellets were finally resuspended in an appropriate volume of buffer and protein concentration was determined by the method of Lowry.[D]

Opioid receptor mRNA expression

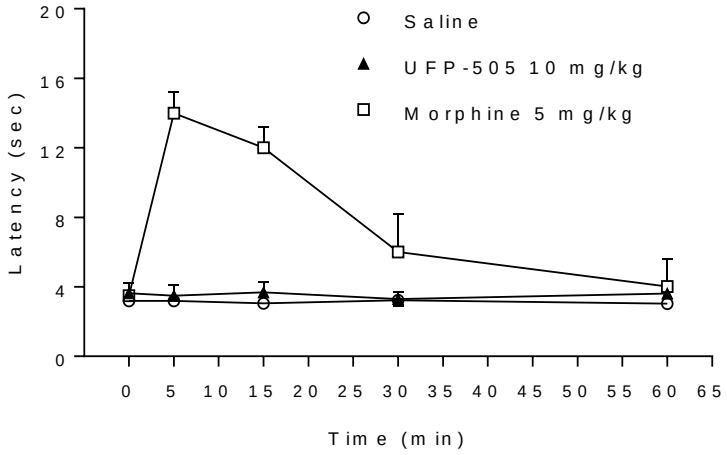
RNA was extracted from animal tissue or cultured cells using a Cells-to-CT™ gene expression kit (Applied Biosystems). mRNA encoding the human MOP and DOP receptors was assessed by quantitative real-time PCR (qRT-PCR) using validated TaqMan primers and compared with a housekeeper (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) as previously described [E] using a StepOne instrument (Applied Biosystems). q-PCR data were expressed as the difference in cycle threshold ($\Delta C_t = Ct^{\text{Gene of interest}} - C_t^{\text{GAPDH}}$: high value, low expression).

Dietis et al, Table 1S
Opioid receptor mRNA expression changes in neural tissue from treated rats

Tissue	Treatment	OPRM1	OPRK1	OPRD1	OPRL1
Spinal cord	*Morphine*	8.08 ± 0.13 ↑	10.89 ± 0.07	9.14 ± 0.07 ↑	12.34 ± 0.05 ↓
	UFP-505	8.54 ± 0.32 ↑	11.13 ± 0.12	9.55 ± 0.50 ↑	12.27 ± 0.17 ↓
	Saline	9.64 ± 0.15	10.87 ± 0.06	11.01 ± 0.24	9.80 ± 0.17
Frontal Cortex	*Morphine*	9.16 ± 0.04	11.73 ± 0.48 ↑	10.82 ± 0.22	10.22 ± 0.11
	UFP-505	9.73 ± 0.46	11.81 ± 1.13 ↑	11.13 ± 0.04	10.12 ± 0.15
	Saline	10.77 ± 0.44	14.05 ± 0.04	11.10 ± 0.18	11.47± 0.15
Rest of the brain	*Morphine*	10.24 ± 0.11	12.44 ± 0.31	11.93 ± 0.09 ↓	9.38 ± 0.05
	UFP-505	10.23 ± 0.01	12.29 ± 0.01	12.70 ± 0.01 ↓	9.34 ± 0.01
	Saline	9.75 ± 0.18	11.09 ± 0.07	10.36 ± 0.16	9.42 ± 0.13

Table 1S legend: Opioid receptor mRNA levels (ΔC_t values) for all opioid receptor genes (mean ± SEM; n=3-5) using GAPDH as a housekeeper, in neuronal tissue from acutely treated rats (A: 10nmol UFP-505, 10nmol morphine or saline-treated; i.t.). Data were produced by RT-qPCR from tissue lysates. Reduction in the ΔC_t indicates upregulation (↑) of receptor mRNA, whereas increase in the ΔC_t indicates downregulation (↓).

Dietis et al, Supplementary Figure 1.



Tail-withdrawal assay. Morphine was dissolved in saline while UFP-505 was dissolved in 1% DMSO and 100 µl/mouse was administered subcutaneously at 30 min before the test to CD1 male mice (25-30g. Antinociception was evaluated by tail-withdrawal assay in a water temperature of 48°C and cut off time 20 sec. Data are mean+SEM, 4 animals in each group.

Supplement specific references

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