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Relationship between 4,5-epoxymorphinan structure and in vitro modulation of cell proliferation

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Abstract

Morphine belongs to the class of compounds known as 4,5-epoxymorphinans, which can alter immune function directly via receptors expressed by immune cells. However, the opioid characteristics of these receptors are not clear. Therefore, the aim of this study was to investigate the in vitro immunomodulatory effects of 24 structurally related 4,5-epoxymorphinans to allow further characterisation of the receptor that mediates the immunomodulation and to ascertain if there is any structure-effect relationship. The immunomodulation of 4,5-epoxymorphinans using isolated mouse splenocytes stimulated with concanavalin A resulted in five types of responses: an inverted bell shaped curve (oxycodone, inhibitory $EC_{50} = 1.6$ nM), an inhibitory concentration response curve (buprenorphine, inhibitory $EC_{50} = 12.6$ μM), an inverted bell-shaped curve with induction (morphine, induction $EC_{50} = 1.7$ μM), an induction concentration response curve (oxymorphone, induction $EC_{50} = 20$ nM), and the lack of any response (e.g. noroxycodone). Non-stereoselectivity, naloxone-insensitivity, naloxone-sensitivity and non-classical opioid rank order of effect were all observed. A structure-effect relationship was developed and significant evidence for non-classical opioid receptor function on immune cells was concluded.

Keywords: Opioid; Immune system; 4,5-Epoxymorphinans; Cell proliferation; Structure–effect relationship

1. Introduction

Morphine, the prototypical opioid agonist belongs to the 4,5-epoxymorphinan class of compounds (Table 1). Other common either clinically prescribed or abused 4,5-epoxymorphinans include codeine, oxycodone and diacetylmorphine (heroin). Apart from their analgesic properties, 4,5-epoxymorphinans and endogenous opioids such as β-endorphin can directly influence immune cell function. Wybran et al. (1979) demonstrated that morphine was able to inhibit T cell rosetting in a naloxone-reversible manner. Subsequently, numerous research groups have used a plethora of both in vitro and in vivo infection and immunity models to investigate the extent and mechanism underlying opioid-induced alterations in immune function (as reviewed by Eisenstein and Hilburger, 1998; Glasel, 2000; Carr et al., 1996). However, few studies have departed from using morphine as the prototypic 4,5-epoxymorphinan.

Centrally, endogenous opioid peptides, 4,5-epoxymorphinans and other synthetic opioids act on the "classical" μ, κ and δ opioid receptors and their subclasses (Dhawan et al., 1996). When administered in vivo, opioids have centrally and peripherally mediated actions (King et al., 2001). Therefore, it is possible for these compounds to alter immune function indirectly via neuronal innervation of lymphoid organs (Felten et al., 1985) or via endocrine pathways such as opioid-induced activation of the hypothalamus-pituitary-adrenal axis which subsequently results in the release of immunosuppressive glucocorticoids (Bryant et al., 1991). A concerted effort has been made to identify the specific receptors that mediate the direct opioid effects on immune cells using biological activity, ligand and fluorescent antibody binding and mRNA detection by reverse transcription-polymerase chain reaction (Madden et al., 1998; Sharp et al., 1998). Low expression levels of neuronal-like opioid receptors on immune cells has made detection difficult; however,

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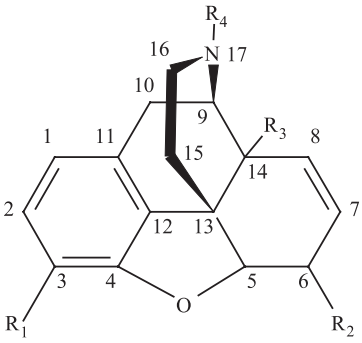
Table 1
Comparative structures of 4,5-epoxymorphinans used in this study



Compound	R_1	R_2	R_3	R_4	Other changes	Degrees of separation
Morphine	OH	OH	H	CH_3		0
6-Hydroxyoxymorphone	OH	OH	OH	CH_3	1	1
Hydromorphone	OH	=O	H	CH_3	1	1
Oripavine	OH	OCH_3	H	CH_3	2	1
Morphine-6-glucuronide	OH	$OC_6H_9O_5$	H	CH_3		1
6-Monoacetylmorphine	OH	$OCOCH_3$	H	CH_3		1
Codeine	OCH_3	OH	H	CH_3		1
Morphine-3-glucuronide	$OC_6H_9O_5$	OH	H	CH_3		1
6β-Naltrexol	OH	OH	OH	C_4H_7	1	2
Oxymorphone	OH	=O	OH	CH_3	1	2
Hydrocodone	OCH_3	=O	H	CH_3	1	2
Thebaine	OCH_3	OCH_3	H	CH_3	2	2
Diacetylmorphine	$OCOCH_3$	$OCOCH_3$	H	CH_3		2
Naloxone	OH	=O	OH	C_3H_5	1	3
Naltrexone	OH	=O	OH	C_4H_7	1	3
Oxycodone	OCH_3	=O	OH	CH_3	1	3
Norhydrocodone	OCH_3	=O	H	H	1	3
Naloxone Methiodide	OH	=O	OH	C_4H_8I	1	4
3-O-Methylnaltrexone	OCH_3	=O	OH	C_4H_7	1	4
Noroxycodone	OCH_3	=O	OH	H	1	4
Buprenorphine	OH	OCH_3	H	C_4H_7	1, 3, 4	
Norbuprenorphine	OH	OCH_3	H	H	1, 3, 4	

1: single bond between C_7 and C_8; 2: double bond between C_6 and C_7 and C_8 and C_{14} and single bond between C_7 and C_8; 3: $C_6H_{13}O$ at position 7; 4: 2 carbon bridge between C_6 and C_{14}.

the current evidence indicates the presence of neuronal like (classical) opioid receptors on some immunocompetent cell types (specifically μ: Sedqi et al., 1995; Chuang et al., 1995; κ: Bidlack et al., 1995; Belkowski et al., 1995; δ: Gaveriaux et al., 1995; Li et al., 1999; and reviewed by Sharp et al., 1998). In addition, non-opioid (non-classical) receptors that do not behave in the same manner as their neuronal counterparts have been identified on immune cells. The differences in their pharmacological properties include non-stereoselectivity (Roy et al., 1991), naloxone insensitivity (Jessop and Taplits, 1991; Roy et al., 1998), decreased affinity for opioid agonists (Roy et al., 1991) and altered optimal ion concentrations for agonist binding (Madden et al., 2001). A receptor which binds opioid alkaloids, but not opioid peptides has also been characterised on immune cells and has been named the μ_3 opioid receptor (Makman et al., 1995). Specific β-endorphin receptors which are naloxone-insensitive and which display other non-classical opioid characteristics are also expressed on immune cells (Hazum et al., 1979; Shahabi et al., 1990, 1992; Woods et al., 1997). Despite the low expression levels and differing pharmacological properties, activation of these receptors is able to alter biological function (Gaveriaux-Ruff et al., 1998).

The direct pharmacodynamic effects of 4,5-epoxymorphinans on immune cells, via both classical and non-classical opioid receptors, depend on the model used and the concentration range of agonist investigated. Morphine caused a naloxone-insensitive concentration-dependent decrease in thymocyte (Roy et al., 1997) and splenocyte proliferation (Jessop and Taplits, 1991), whilst others were unable to alter splenic or peripheral blood lymphocyte proliferation except at high (100 μM) concentrations (Fecho et al., 1996). Conversely, Bidlack and Hemmick (1990) demonstrated a naloxone-insensitive increase in proliferation of lymph node T cells by morphine. Inter-animal strain differences in response to opioids has also been observed (Eisenstein et al., 1995). These conflicting data have lead to confusion regarding the biological and physiological relevance of opioid receptors expressed on immune cells; however, the expression and release of endogenous opioid agonist peptides by activated immune cells during inflammation (Cabot et al., 1997) and stress (Rittner et al., 2001) indicates the potential for a regulatory role. Furthermore, since immune-derived cytokines are integral to the development of opioid tolerance (Raghavendra et al., 2002), and immune status alters the analgesic efficacy of morphine (Kamei et al., 1992) and the severity of opioid withdrawal (Dougherty et al., 1990), suggesting a substantial role for the immune system in opioid pharmacodynamic effects and highlighting the need

to understand the effects of a wider range of 4,5-epoxymorphinans on immune function.

4,5-Epoxymorphinans have varying affinities for different classes of the classical μ, δ and κ opioid receptors. Chen et al. (1991) demonstrated a structure-binding affinity relationship using a large number of structurally related 4,5-epoxymorphinans and displacement of tritiated DAMGO ([D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin) from rat brain homogenate μ opioid receptors. Therefore, if a similar model is applied in vitro, as suggested by Sibinga and Goldstein (1988), and utilised in vivo by Sacerdote et al. (1997), to investigate immunomodulatory effects of 4,5-epoxymorphinans, a better understanding of the receptors which mediate these responses may be gained, and a greater appreciation of how opioid immunomodulation may influence opioid pharmacodynamics. Therefore, the aim of this study was to investigate the in vitro immunomodulatory effects of a large number of structurally related 4,5-epoxymorphinans and to ascertain if any structure-effect relationship is apparent. This also enabled a comparison of the rank order of effect with currently established μ opioid receptor binding affinity and binding efficacy rank order data from previous studies. Selective and non-selective opioid antagonists were also used to determine the opioid receptor subtypes that may be involved. A splenocyte mitogenesis assay was used as the experimental model, however in contrast to many previously applied models, a rapid non-toxic and sensitive proliferation detection method was used (Ahmed et al., 1994), combined with incubation conditions optimised for the detection of altered immune response by in vitro exposure to 4,5-epoxymorphinans.

2. Materials and methods

2.1. Chemicals

RPMI (Roswell Park Memorial Institute) 1640 media with HEPES and L-glutamine modification was purchased from Invitrogen (Mulgrave, Australia). Penicillin-streptomycin solution (10,000 units penicillin, 10 mg streptomycin per ml), concanavalin A, oxycodone hydrochloride, morphine-3-glucuronide, morphine-6-glucuronide, thebaine hydrochloride, hydromorphone hydrochloride, naloxone methiodide, naloxone hydrochloride, naltrexone hydrochloride, 3-*O*-methylnaltrexone, naltrindole hydrochloride, naloxonazine dihydrochloride and nor-binaltorphimine dihydrochloride were purchased from Sigma (St. Louis, MO, USA). Oripavine and the plus inactive isomers of morphine and codeine were obtained from the National Institute of Drug Abuse (Bethesda, MD, USA). 6β-naltrexol was synthesised by the Department of Chemistry at the University of Adelaide (Porter et al., 2000). Hydrocodone base, 6-hydroxyoxymorphone and norhydrocodone base were obtained from Dr. S. Hosztafi (ICN Alkaloida, Tiszavasvári, Hungary). Buprenorphine and norbuprenorphine were obtained from Reckitt Benckiser Healthcare (Hull, UK). Foetal calf serum was obtained from Trace Scientific (Melbourne, Australia). Diacetylmorphine base and 6-monoacetylmorphine were purchased from the National Analytical Reference Laboratory of the Australian Government Analytical Laboratories (Pymble, Australia). Noroxycodone and oxymorphone hydrochloride were obtained from Du Pont Pharmaceuticals (Wilmington, DE, USA). Codeine phosphate was obtained from F.H. Faulding (Adelaide, Australia) and morphine hydrochloride from McFarlane Smith (Edinburgh, UK). AlamarBlue was purchased from Astral Scientific (Gymea, Australia). All other reagents and chemicals were obtained from commercial sources and were of analytical grade quality.

2.2. Preparation of solutions

Dissolving diacetylmorphine base directly into Milli Q water or an equimolar solution of hydrochloric acid caused spontaneous diacetylmorphine degradation of 10–15% (Hutchinson and Somogyi, 2002). To prevent this, diacetylmorphine base was dissolved in pH 5.0 phosphate buffer (monopotassium phosphate 65.9 mM and disodium phosphate 0.8 mM). Once in solution, diacetylmorphine was stored at −80 °C until required. This process was also used for 6-monoacetylmorphine base. Noroxycodone, norhydrocodone, the plus isomers of morphine and codeine, oripavine, norbuprenorphine and hydrocodone were all dissolved in dilute hydrochloric acid such that the molarity of the acid and base were the same, while the remaining compounds were dissolved in Milli Q water. All solutions were store at 4 °C until used.

2.3. Animals

Ethics approval to conduct the studies was obtained from the University of Adelaide Animal Ethics Committee. Male Balb/c mice 6–8 weeks old were purchased from Central Animal Supplies (Waite Campus, University of Adelaide, SA, Australia) and used as donors of splenocytes for use in cell culture. Animals were provided with standard rodent feed and water ad libitum. Animals were housed in a standard 12 h light dark cycle (starting at 7 am) under constant room temperature of 22 ± 2 °C (mean ± range).

2.4. Lymphocyte preparation

The method used is based on that previously described (Hutchinson and Somogyi, 2002). Aseptic techniques were used during the preparation of the lymphocytes. Mice were sacrificed by cervical dislocation followed by prompt removal of the spleen. The spleen was prepared as a single-cell suspension by massaging and washing through a nylon mesh into a 15 ml tube with up to 15 ml of RPMI 1640 (HEPES modification, 0.3 mg·ml^{-1} L-glutamine, 5 ml penicillin-streptomycin solution per l). The cells were centrifuged at 4 °C for 5 min at 1000 rpm, the supernatant was discarded

and the cells resuspended in 1 ml of media followed by the addition of 10 ml of lysis buffer (ice cold 1 ml of 20.56 $g \cdot l^{-1}$ tris base (pH 7.65), 9 ml 0.83% NH_4Cl in H_2O mix just prior to addition to cells). The suspension was placed on ice for 4 min, centrifuged (5 min at 1000 rpm) and the supernatant discarded. The suspensions of cells were pooled (6–10 spleens depending on the size of the assay) and were resuspended in 10 ml of media followed by centrifugation (5 min at 1000 rpm), removal of supernatant and resuspended in 5 ml of enriched RPMI 1640 (RPMI 1640 enriched with 10% foetal calf serum). The number of viable lymphocytes in the suspension was counted using trypan blue and a haemocytometer. Cells were then diluted in enriched media to 1×10^6 cells$\cdot ml^{-1}$ and 100 μl of this suspension was added to each well of the 96 multiwell plates (Nunc, Roskilde, Denmark) with a final volume of 200 μl, comprising 50 μl of concanavalin A (10 $\mu g \cdot ml^{-1}$) and/or 50 μl of opioid ($n = 12$ wells; 0 μM control, 0.4 nM, 4 nM, 0.04 μM, 0.4 μM, 4 μM, 40 μM and 400 μM). Unstimulated mitogen negative control wells were also prepared, using 50 μl of media instead of concanavalin A. The plates were incubated at 37 °C, 5% CO_2 in a humidified incubator (Thermoline, NSW, Australia) for 24 h following which 25 μl of a diluted AlamarBlue solution (5 μl AlamarBlue, 20 μl cell medium) was added to each well. The plates were incubated for a further 4 h following which 175 μl of media was transferred from the clear 96 multiwell plates to white 96 multiwell plates (BMG Labtechnologies, Offenburg, Germany) for fluorescence quantification (Ex 545, Em 590) on a BMG Polarstar microplate reader (BMG Labtechnologies). Each 4,5-epoxymorphinan was assessed on six separate occasions using six separate splenocyte preparations.

2.5. Assay optimisation

Total incubation time, foetal calf serum, concanavalin A, cell and AlamarBlue concentrations, and AlamarBlue incubation time were optimised in order to quantitatively detect alterations in concanavalin A-induced proliferation following exposure to various 4,5-epoxymorphinans. AlamarBlue obtained from a 48 h incubation with cells was prepared at concentrations from 0.01% to 5% (volume/volume) with cells 1×10^5 cell/well ($n = 12$) in supplemented RPMI 1640 and fluorescence was quantified to determine the optimal concentration to minimise quenching of the fluorescence signal. Foetal calf serum, cell numbers and concanavalin A concentration were optimised by preparing cells as described above with 2.5%, 5% or 10% final concentration foetal calf serum; concanavalin A at 0.1, 1, 2.5, 3.75 and 5 $\mu g \cdot ml^{-1}$; and, cell numbers of 1×10^4, 1×10^5, 1×10^6 and 1×10^7 cell per well and were incubated for 24 or 48 h ($n = 3$ for each combination). The plates were then assayed for proliferation as before. A foetal calf serum concentration and cell number that did not substantially increase the background noise of the mitogenesis assay was chosen, whilst a sub-maximal concanavalin A concentration was selected.

Incubation time was optimised by preparing plates as before with final concanavalin A concentrations of 1 and 2.5 $\mu g.ml^{-1}$. The proliferation of the cells was quantified at times 4, 8, 12, 16, 20, 28, 36, 44 and 52 h after initial exposure to concanavalin A (including a 4 h incubation with AlamarBlue) and a time prior to the maximal quantified proliferation was selected.

2.6. Inhibitor studies

Incubation conditions were only slightly modified from those described in Section 2.4. The cells were incubated with 25 μl of antagonist for 15 min prior to the addition of 50 μl concanavalin A and 25 μl agonist (concentrations doubled due to altered volume added). Naloxone was used at four concentrations 0.0001, 0.01, 1 and 100 μM. Specific opioid receptor subtype antagonists naltrindole (δ), naloxonazine (μ) and nor-binaltorphimine (κ) were initially incubated at concentrations from 0.0001 to 100 μM to determine a concentration at which no response from the antagonist alone was observed, as at least naltrindole has been shown to alter cell proliferation (Gaveriaux-Ruff et al., 2001). The concentrations used were 1 μM for naltrindole and naloxonazine, and 0.1 μM for nor-binaltorphimine. All antagonists were used in combination with (-)-morphine (10 and 0.1 μM), (+)-morphine (100 and 0.1 μM), oxycodone (0.01 μM), morphine-6-glucuronide (0.01 μM) and buprenorphine (10 μM).

2.7. Data analysis

The data comprised baseline proliferation (unstimulated proliferation) that was subtracted from all other proliferation data and then expressed as the percent of proliferation of the mitogen control minus one hundred percent. Equations relating modulation of proliferation to drug concentrations were fitted to the data using Prism 4.0 (GraphPad, CA, USA). Two types of equations were used for these calculations:

A basic Hill equation $y = \left(\frac{\text{Bottom}+(\text{Top}-\text{Bottom})}{1+10^{(\log EC_{50}-x)\times \text{Slope}}}\right)$, where Bottom is the minimum and Top is the maximum response (proliferation) and LogEC_{50} the concentration required to achieve 50% of the response, and Slope is the slope of the relationship. Secondly, the sum of two Hill equations were fitted to the data as an equation that fitted both the upward and downward parts of a bell-shaped curve

$$y = \left(\frac{\text{Bottom}_{DT} + (\text{Top}_{DT} - \text{Bottom}_{DT})}{1 + 10^{(\log EC_{50_{DT}} - x)\times \text{Slope}_{DT}}}\right) + \left(\frac{\text{Bottom}_{UT} + (\text{Top}_{UT} - \text{Bottom}_{UT})}{1 + 10^{(\log EC_{50_{UT}} - x)\times \text{Slope}_{UT}}}\right)$$

Parameters were obtained for the downward (DT) and upward (UT) parts of the concentration response relationship. Where possible the Top_{DT}, Bottom_{UT}, Bottom (on a positive slope) and Top (on a negative slope) were set at zero

to simulate a physiological situation where if no drug was present there would be no effect. However, in some cases the data did not allow this, because the responses obtained at low ligand concentrations did not tend to zero resulting in an inappropriate fit; instead these points were constrained as close to zero as the model would allow (upper or lower limits were set in Prism 4.0). Using the best-fit parameters generated by Prism, the maximum proliferative responses and the concentration at which these responses occurred were calculated using Excel 2000 and the Solver analysis pack. Statistical significance was assessed using a one-way analysis of variance with Bonferroni post hoc test comparing the mitogen control with all other data points. All data are presented as mean ± S.E.M. Significance was set at $P < 0.05$.

3. Results

Optimisation of the experimental conditions resulted in using a 24 h exposure incubation time, 5% foetal calf serum, 2.5 $\mu g \cdot ml^{-1}$ concanavalin A, 1×10^5 cells·well^{-1}, 2.5% AlamarBlue and a 4 h incubation with alamarBlue. These conditions gave reduced background proliferative noise in the experimental system and allowed quantification of the response prior to the maximal response.

In vitro exposure of mitogen-stimulated splenocytes to 4,5-epoxymorphinans caused varied responses, however these were divided into five major groups.

1. *Inverted bell-shaped curve*: Inhibition of the proliferative response and subsequent return to baseline response was found for oxycodone (Fig. 1A), morphine-6-glucuronide, diacetylmorphine, 6-monoacetylmorphine, (−)-codeine and (+)-codeine (Table 2). This response was characterised by minimal alteration in response from control at low concentrations (0.0001 μM), significant inhibition (range −22% to −51%, $P < 0.026$) at mid-range concentrations (0.01–1 μM), followed by a return to control proliferation at high concentrations (10–100 μM). The sum of two Hill equations adequately fitted to these data (Fig. 1A). The nadir of proliferation and the concentration at which this occurred varied. The nadir for oxycodone was the greatest (−51%) whilst the lowest concentration at which a nadir occurred was 0.003 μM for (−)-codeine (−27%).
2. *Inhibitory concentration response curve*: Significant inhibition ($P < 0.02$) at high concentrations (>1 μM) was found for oripavine, buprenorphine (Fig. 1B), norbuprenorphine, thebaine and 3-*O*-methylnaltrexone (Table 2). A Hill equation with a negative slope was modelled to these data. The responses were characterised by minimal response over the low and mid-range concentrations (0.0001–1 μM), followed by significant inhibition at high concentrations (100 μM). The concentration at which the nadir occurred was 100 μM for all compounds, however the nadir ranged from −28% to −219%. The inhibitory response had not plateaued at the highest concentration tested indicating even greater inhibition may be possible with higher concentrations.
3. *Inverted bell-shaped curve with induction*: The effect of (−)-morphine (Fig. 1C), 6-hydroxyoxymorphone and (+)-morphine at the low concentrations was similar to that of oxycodone and the first group of compounds. The lowest concentrations did not affect the proliferative response but significant inhibition ($P < 0.022$) at the mid-range concentrations (0.01–1 μM) (Table 2) was found. In contrast with oxycodone and the first group of 4,5-

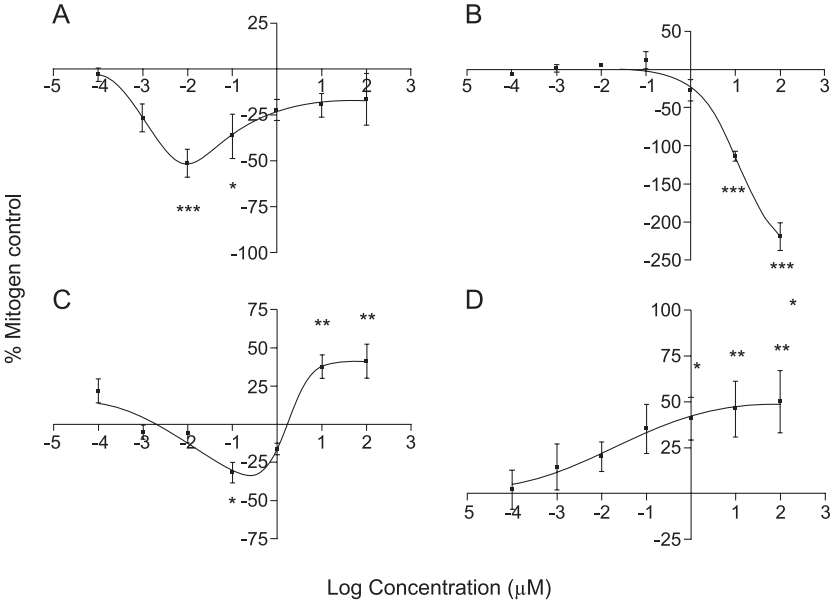


Fig. 1. Effect of opioids on proliferation of splenocytes following 24 h incubation with oxycodone (A), buprenorphine (B), morphine (C) and oxymorphone (D). Error bars are S.E.M. of six independent experiments and statistical significance is indicated by $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Table 2
Data for all 4,5-epoxymorphinan compounds analysed

	Compound	Downward Response			Upward Response		
		EC_{50}	Max effect	Conc	EC_{50}	Max effect	Conc
1	Oxycodone	1.6 nM	− 51%	10 nM	0.03 μM		
	Morphine-6-glucuronide	0.1 nM	− 31%	20 nM	>100 μM		
	Diacetylmorphine	0.1 nM	− 29%	2.0 μM	3.2 μM		
	(−)-Codeine	0.8 nM	− 27%	50 nM	0.1 μM		
	6-Monoacetylmorphine	0.03 nM	− 23%	76 μM	100 μM		
	(+)-Codeine	1.3 nM	− 22%	2.5 nM	1.1 μM		
2	Buprenorphine	12.6 μM	− 219%	100 μM			
	Norbuprenorphine	>100 μM	− 79%	100 μM			
	Thebaine	>100 μM	− 55%	100 μM			
	Oripavine	>100 mM	− 36%	100 μM			
	3-*O*-Methylnaltrexone	20 μM	− 28%	100 μM			
3	(−)-Morphine	25 nM	− 33%	234 nM	1.7 μM	41%	40 μM
	6-Hydroxyoxymorphone	316 nM	− 21%	50 nM	3.5 μM	24%	100 μM
	(+)-Morphine	1 nM	− 11%	40 nM	>100 μM	11%	100 μM
4	Oxymorphone				20 nM	49%	100 μM
	6β-naltrexol				2 μM	33%	100 μM
	Hydromorphone				>100 μM	27%	100 μM
	Morphine-3-glucuronide				25 nM	17%	100 μM
	Naltrexone				40 nM	16%	100 μM
5	Hydrocodone	No response					
	Naloxone	No response					
	Norhydrocodone	No response					
	Noroxycodone	No response					
	Naloxone Methiodide	No response					

Maximum effect responses and EC_{50} concentrations reported were obtained from the modelled Hill equations.

epoxymorphinans, at the high concentrations there was a significant induction of proliferation ($P < 0.026$). The nadir of the responses was greatest for (−)-morphine (−32%), as was the zenith (41%) (Fig. 1C). The lowest concentration at which the nadir and zenith occurred was 0.04 μM for (+)-morphine (−11.2%) and 1.7 μM (−)-morphine (41%), respectively.

4. *Induction concentration response curve*: Significant induction ($P < 0.023$) of the proliferative response was observed when cells were incubated with oxymorphone (Fig. 1D), morphine-3-glucuronide, naltrexone, 6β-naltrexol and hydromorphone (Table 2). A Hill equation with a positive slope was modelled to these data. Oxymorphone had the greatest maximum response (48.9%), at 100 μM.
5. Hydrocodone, naloxone, norhydrocodone, noroxycodone and naloxone methiodide had no significant effect on the proliferative response.

3.1. Comparison of rank orders

1. *Inhibition (downward):* The rank order of maximal inhibitory effect was buprenorphine > norbuprenorphine > thebaine > oxycodone > oripavine > (−)-morphine > morphine-6-glucuronide >diacetylmorphine > 3-*O*-methylnaltrexone > (−)-codeine > 6-monoacetylmorphine > (+)-codeine > 6-hydroxyoxymorphone > (+)-morphine (Table 2). However, the rank order of the EC_{50} values for this response was 6-monoacetylmorphine < diacetylmorphine = morphine-6-glucuronide < (−)-codeine < (+)-morphine < (+)-codeine < oxycodone < (−)-morphine < 6-hydroxyoxymorphone < buprenorphine < 3-*O*-methylnaltrexone < norbuprenorphine < thebaine < oripavine (Table 2).
2. *Induction (upward)*: The rank order for the maximal stimulatory effect was oxymorphone > (−)-morphine > 6β-naltrexol > hydromorphone > 6-hydroxyoxymorphone > morphine-3-glucuronide > naltrexone > (+)-morphine (Table 2). The maximal responses achieved in group 1 were not included as this groups' maximal response was not above baseline. The rank order of the EC_{50} values of the stimulant response including the group 1 data was oxymorphone < morphine-3-glucuronide < oxycodone < naltrexone < (−)-codeine < (+)-codeine < (−)-morphine < 6β-naltrexol < diacetylmorphine < 6-hydroxyoxymorphone < 6-monoacetylmorphine < hydromorphone < morphine-6-glucuronide < (+)-morphine (Table 2).

3.2. Other response characteristics

The responses were not stereoselective since both codeine and to a lesser degree morphine stereoisomers affected

Table 3
Effect of naloxone on immunomodulation by different 4,5-epoxymorphinans

Naloxone	Control	0.0001 μM	0.01 μM	1 μM	100 μM
(+)-Morphine 0.1 μM	-17.3 ± 2^{b}	-6.9 ± 3	1.5 ± 3^{B}	4.5 ± 3^{C}	-12.2 ± 7
(−)-Morphine 0.1 μM	-32.8 ± 6^{c}	16.3 ± 13^{C}	19.2 ± 7^{C}	19.6 ± 2^{C}	-30.2 ± 8
Oxycodone 0.01 μM	-36.2 ± 8^{a}	30.2 ± 13^{A}	31.5 ± 24^{A}	3.7 ± 12	-70.3 ± 14
M6G 0.01 μM	-32.7 ± 12^{a}	22.7 ± 6^{B}	18.8 ± 13^{A}	16.4 ± 15^{A}	-37.2 ± 12
(+)-Morphine 100 μM	51.7 ± 5^{c}	42.8 ± 14	26.7 ± 8	16.4 ± 6^{A}	9.2 ± 13^{B}
(−)-Morphine 10 μM	35.7 ± 8^{c}	32.8 ± 7	39.0 ± 8	43.2 ± 7	29.7 ± 9
Buprenorphine 10 μM	-137.5 ± 7^{e}	-44.7 ± 11^{C}	-56.0 ± 12^{C}	-47.3 ± 11^{C}	-82.2 ± 7^{B}

Data are expressed as the percent of the mitogen control minus one hundred percent ± the S.E.M. from six independent experiments. Statistical differences in the control column are from comparison with the mitogen control and statistical significance is signified as $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$. All other statistical analysis are compared to the control column using a one-way analysis of variance (ANOVA) with Bonferroni post hoc. Statistical significance are signified as $^{A}P<0.05$, $^{B}P<0.01$ and $^{C}P<0.001$. M6G is morphine-6-glucuronide.

proliferation to a similar degree. The response to 0.1 μM morphine and 100 μM buprenorphine were not due to significant reductions in the viability of the cultured cells even after 48 h exposure as assessed by trypan blue exclusion and lactate dehydrogenase leakage (data not shown).

3.3. Antagonists

Naloxone significantly dose dependently antagonised the inhibition caused by (+)- and (−)-morphine (0.1 μM, $P<0.01$), oxycodone (0.01 μM, $P<0.05$) and morphine-6-glucuronide (0.01 μM, $P<0.05$), however it potentiated inhibition at 100 μM (Table 3). Interestingly, the concentrations of naloxone that antagonised the inhibitory responses of (−)-morphine, oxycodone and morphine-6-glucuronide caused proliferation above basal activity (Table 3). Naloxone was only able to partially reverse buprenorphine inhibition, with greater antagonistic effect from 0.0001 to 1 μM naloxone. Conversely, naloxone had no effect on the induction of proliferation caused by (-)-morphine (10 μM). However, naloxone at 1 and 10 μM antagonised (+)-morphine (100 μM) induction of proliferation.

Naloxonazine antagonised the inhibition caused by (+)- and (−)-morphine (0.1 μM, $P<0.01$), oxycodone (0.01 μM, $P<0.001$) and morphine-6-glucuronide (0.01 μM, $P<0.001$) and partially antagonised the inhibition of buprenorphine (10 μM, $P<0.05$), whilst nor-binaltorphimine and naltrindole had no significant effects (Table 4). None of the selective antagonists were able to inhibit the induction of proliferation caused by (−)-morphine (10 μM) or (+)-morphine (100 μM).

4. Discussion

The aim of this study was to characterise the in vitro immunomodulatory effects of a large number of 4,5-epoxymorphinans. The use of AlamarBlue in this investigation allowed a short exposure time (24 h), rapid detection of proliferation (4 h) and the removal of toxic influences from the experimental model. Although previous studies have produced conflicting results with the data presented here (Fecho et al., 1996; Jessop and Taplits, 1991), we have tested a wider range of compounds using the same experimental model. Hence, we are in a position to allow more meaningful comparisons to be made between the effects of different 4,5-epoxymorphinans, thereby allowing elucidation of a structure-effect relationship.

In contrast to other biological systems for evaluating the effects of structurally similar 4,5-epoxymorphinans, five different types of responses were obtained: an inverted bell-shaped curve (e.g. oxycodone), a concentration-dependent inhibitory response curve (e.g. buprenorphine), an inverted bell-shaped curve with induction (e.g. morphine), a concentration-dependent induction response curve (e.g. oxymorphone), and lack of response (e.g. hydrocodone).

Table 4
Effect of the selective opioid receptor subtype antagonists on the immunomodulation by different 4,5-epoxymorphinans

	Control	Naltrindole (δ) 1 μM	Naloxonazine (μ) 1μM	Nor-binaltorphimine (κ) 0.1 μM
(−)-Morphine 0.1 μM	-27.9 ± 5^{c}	-25.7 ± 2	4.0 ± 8^{C}	-36.5 ± 6
(+)-Morphine 0.1 μM	-18.8 ± 3^{a}	-18.2 ± 4	8.3 ± 5^{B}	-19.3 ± 8
Oxycodone 0.01 μM	-30.5 ± 4^{c}	-25.0 ± 5	1.8 ± 4^{C}	-28.5 ± 5
M6G 0.01 μM	-21.8 ± 5^{a}	-16.3 ± 3	8.5 ± 5^{C}	-31.3 ± 6
Buprenorphine 10 μM	-120.0 ± 7^{c}	-109.0 ± 9	-87.3 ± 10^{A}	-131.5 ± 11
(−)-Morphine 10 μM	22.4 ± 4^{b}	5.7 ± 5	7.3 ± 6	11.3 ± 8
(+)-Morphine 100 μM	20.7 ± 3^{a}	8.8 ± 5	13.0 ± 3	15.3 ± 6

Data are expressed as the percent of the mitogen control minus one hundred percent ± the S.E.M. from six independent experiments. Statistical differences in the control column are from comparison with the mitogen control and statistical significance is signified as $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$. All other statistical analysis compare are compared to the control column using a one-way ANOVA with Bonferroni post hoc. Statistical significance are signified as $^{A}P<0.05$, $^{B}P<0.01$, $^{C}P<0.001$.

The diverse characteristics of these responses brings into question the classical opioid nature of the receptor(s) through which these responses are being mediated. If the 4,5-epoxymorphinans were acting via a single classical opioid receptor, the directions of the response would be similar and potency easily assigned. Furthermore, the rank order of the maximal effect and EC_{50} values would be similar to currently available neuronal opioid binding data. However, this was not the case. Table 5 compares the rank order of μ opioid receptor binding affinities for the 24 4,5-epoxymorphinans used in this study and previously reported by others with the rank order of immunomodulatory effects found in this study. It is clearly apparent that the comparative rank orders are dissimilar, implying therefore that the immunomodulatory effects observed here are not mediated by the neuronal μ opioid receptor.

Other classical opioid characteristics such as stereoselectivity and antagonist sensitivity were also inconsistent among the 4,5-epoxymorphinans used. For example, the opioid-active (−) and opioid-inactive (+) stereoisomers of codeine and morphine caused similar immunomodulation, thereby displaying non-stereoselectivity. Furthermore, (−)-morphine caused both naloxone- and naloxonazine-sensitive inhibition, and naloxone- and naloxonazine-insensitive induction of the proliferative response, which has been reported previously for naloxone (Roy et al., 1998), whilst nor-binaltorphimine and naltrindole were without antagonistic effects. These antagonist data would normally suggest the presence of classical μ opioid receptor(s) causing the inhibitory response and non-classical opioid receptor(s) mediating the induction of proliferation. However, the rank order data in Table 5 and the lack of stereoselectivity indicate that the opioid inhibition of proliferation was a non-classical opioid response, although elements of opioid antagonist sensitivity remain. Hence, if only one opioid agonist and antagonist were used in the assay, an incorrect conclusion would have been made, since the available data would have supported the involvement of a classical opioid receptor.

The non-classical opioid hypothesis and proliferation response data found in this study conflict with previously published results. Fecho et al. (1996) found no change in morphine-induced splenocyte proliferation except for significant suppression at high concentrations (100 μM), whilst Jessop and Taplits (1991) only found inhibition of concanavalin A induced splenocyte proliferation. Thomas et al. (1995) were unable to demonstrate any clear dose-dependent relationship in the proliferation response following exposure to morphine, morphine-6- and morphine-3-glucuronides and found the inactive metabolite normorphine possessed more activity than the others. In contrast, our data agree with Bidlack and Hemmick (1990) who reported induction of proliferation at 10 μM morphine but did not report the effect at lower concentrations. The reason for these discrepancies is likely to be experimental differences between the studies, including animal strain and other experimental parameters such as exposure and proliferation time. We considered that proliferation should be quantified before the maximum effect is achieved and well before media depletion. Furthermore, in vitro immune responses to opioids are reduced following longer incubation times (Shahabi and Sharp, 1995) possibly caused by receptor down regulation (Sharp et al.,

Table 5
Rank order of available μ opioid receptor binding affinities gathered from Chen et al. (1991), Codd et al. (1995), Lewanowitsch and Irvine (2003), Metzger et al. (2001), Spetea et al. (1998), Selley et al. (2001), Magnan et al. (1982), Law and Loh (1999) and Huang et al. (2001) and rank orders of responses obtained from the current study

	Rank orders				
Compound	Binding affinity	Downward maximal effect	Downward EC_{50}	Upward maximal effect	Upward EC_{50}
Buprenorphine	1	1	10	NR	
Norbuprenorphine	2	2	12	NR	
Naltrexone	3	NR		7	4
Naloxone	4	NR		NR	
Morphine-6-glucuronide	5	7	3		13
Hydromorphone	6	NR		4	12
Oxymorphone	7	NR		1	1
(−)-Morphine	8	6	8	2	7
Oxycodone	9	4	7		3
Diacetylmorphine	10	8	2		9
6-Monoacetylmorphine	11	11	1		11
Hydrocodone	12	NR		NR	
Naloxone Methiodide	13	NR		NR	
Morphine-3-glucuronide	14	NR		6	2
(−)-Codeine	15	10	4		5
Thebaine	16	3	13	NR	
(+)-Morphine	17	14	5	8	14

NR represents that no deviations from baseline response in that direction was observed.

1997), emphasising the need for a shorter incubation period. The use of AlamarBlue allowed short exposure (24 h) and rapid detection of proliferation (4 h) due to assay sensitivity, thereby avoiding some of these experimental limitations.

Due to the apparent non-classical opioid nature of these responses, an hypothesis for a structure–effect relationship was formulated. There are four different functional group modifications at the R1 position of the 4,5-epoxymorphinans tested: hydroxyl, methoxyl, acetyl and glucuronide. Changing from the polar hydroxyl or glucuronide functional groups to the relatively non-polar methoxyl group (morphine-3-glucuronide or morphine to codeine; oxymorphone to oxycodone; naltrexone to 3-*O*-methylnaltrexone; hydromorphone to hydrocodone) caused loss of induction at high concentrations. The change in response from an hydroxyl to glucuronide exchange (morphine to morphine-3-glucuronide) resulted in loss of inhibition, whilst the induction at high concentrations remained, suggesting the more polar the R1 functional group, the more likely induction of proliferation would occur. The substitution of an hydroxyl to methoxyl at R1 (oripavine to thebaine) was more difficult to explain, as it appeared that the methoxyl at position R2 caused significant inhibition at high concentrations. As highlighted previously (Hutchinson and Somogyi, 2002), it is difficult to draw any conclusions from the use of diacetylmorphine in cell culture as it is rapidly degraded to 6-monoacetylmorphine and morphine. However, the diacetylmorphine response is in keeping with the presence of both morphine and 6-monoacetylmorphine in the media.

At the R2 position, there were five different functional groups hydroxyl, methoxyl, glucuronide, acetyl and ketone. Modifications of the functional group from the polar hydroxyl or glucuronide to the non-polar ketone caused loss of response; however in this case the response was inhibition at low concentrations. For example, morphine or morphine-6-glucuronide to hydromorphone; 6-hydroxyoxymorphone to oxymorphone; and codeine to hydrocodone all showed these characteristics. The scenario is not supported by the changes from 6β-naltrexol to naltrexone, since 6β-naltrexol did not inhibit proliferation, possibly due to the presence of the cyclopropylmethyl group at the R4 position. Changes at R2 from a methoxyl to a ketone (oripavine to hydromorphone and hydrocodone to thebaine), methoxyl to hydroxyl (thebaine to codeine or oripavine to morphine) or methoxyl to acetyl (oripavine to 6-monoacetylmorphine) caused loss of significant inhibition at high concentrations. It appears therefore that a methoxyl functional group at R2 causes significant inhibition at high concentrations. Replacement of an acetyl by a glucuronide, hydroxyl or ketone group (6-monoacetylmorphine to morphine-6-glucuronide, morphine or hydromorphone, respectively) produced no change in function. In the case of morphine to morphine-6-glucuronide, the change of a hydroxyl to the more polar glucuronide caused more pronounced inhibition suggesting that increasing the polarity of the group at the R2 position results in greater inhibition.

Alterations of the functional group at R3 from an hydroxyl to an hydrogen (oxycodone to hydrocodone, 6-hydroxyoxymorphone to morphine and oxymorphone to hydromorphone) caused a reduction in response. Removal of the methyl group at R4 (hydrocodone to norhydrocodone; and oxycodone to noroxycodone) resulted in loss of response and removal of the cyclopropylmethyl at R4 caused a shift to the left of the response for norbuprenorphine (compared to buprenorphine) and loss of response for noroxycodone (compared to 3-*O*-methylnaltrexone). Substitution of a methyl with a cyclopropylmethyl (oxycodone to 3-*O*-methylnaltrexone and 6-hydroxyoxymorphone to 6β-naltrexol) caused loss of inhibition at low concentrations or reduced induction of proliferation (oxymorphone to naltrexone). Finally, the change from a methyl or cyclopropylmethyl to an alkene (oxymorphone or naltrexone to naloxone) caused loss of effect. The substitutions at R4 indicate that more than a hydrogen atom needs to be present for activity to remain, although there is no clear explanation for the change in responses associated with other functional groups at this position.

Overall, these structure–effect data indicate that a relatively polar R1 functional group is responsible for the induction of proliferation, whilst increasing the polarity of the R2 and R3 functional groups appear to be responsible for inhibition of proliferation. A clear role for the R4 functional group is not apparent, although substitution with a hydrogen substantially reduces activity. This structure–effect hypothesis does not correlate well with previous structure affinity data for opioid agonist binding to μ opioid receptors. Chen et al. (1991) demonstrated that for 4,5-epoxymorphinan binding to rat brain homogenate, the functional group at the R1 position was the most important determinant of binding affinity, whilst the group at the R2 position had little effect on binding affinity (Table 5). This is not the case for the responses observed for these compounds' effect on the stimulated splenocyte culture. Indeed, the opposite occurred, with changes at the R2 position resulting in changes in response at significantly lower concentrations, indicating that this part of the molecule is more important for this pharmacodynamic effect (Table 5).

Several parent drugs and their major metabolites were evaluated in this system, enabling a comparison of parent to metabolite relative activities. For example, morphine-6-glucuronide was a more potent inhibitor of proliferation than morphine and morphine-3-glucuronide but a less potent inducer of proliferation than morphine and morphine-3-glucuronide at higher concentrations, indicating that activation of different immunomodulatory potentials may occur in vivo due to metabolism of the parent drug. Similarly, oxycodone was a more potent inhibitor at low concentrations but less potent inducer at high concentrations than its *O*-demethylated metabolite oxymorphone.

Conversely, codeine was a more potent inhibitor at low concentrations than its *O*-demethylated metabolite morphine. These results highlight the immunological significance of the functional groups at R1 and R2, which are metabolically labile, leading to potential changes in immuno-pharmacodynamics across a dosing interval due to the different time courses of concentrations of immunologically active metabolites.

The differences between immune opioid effects and central opioid effects for the 4,5-epoxymorphinans examined may be found in the microenvironments in which immune cells operate. Peripheral immune cells can potentially experience the whole gamete of physiological conditions that exist within the human body due to their ability to move with relative ease from the circulation into peripheral tissues and back into the circulation; therefore, the cell membrane receptors must operate in these varying conditions. The optimal sodium ion concentrations for ligand binding for one of the non-opioid receptors characterised is different to that of neuronal opioid receptors (0.15 mM versus 0.05 mM) and is more closely related to the ionic conditions found peripherally (Madden et al., 2001). It is possible that the varied extracellular conditions that immune cells experience cause the opioid receptors to lose their specificity due to altered tertiary structure or receptor co-localisation. Alternatively, proteins released by immune cells may interact with opioid receptors and alter their affinity, as has been reported for interleukin-1 increasing the specific binding of endogenous opioids to rat brain slices (Wiedermann, 1989) or increased binding affinity of immune opioid receptors after proliferation (Roy et al., 1991). If the latter is the case, then inter-laboratory variability in results could be expected due to the likely differences in availability of effector molecules because of binding to different plastics and blocking of potential binding sites by serum supplemented media (McLure et al., 2000; Obach, 1997). Furthermore, the use of a mixed culture such as splenocytes is likely to produce different effects, since different co-stimulation signals will be available, compared to single cell type or cell line cultures (Nguyen and Miller, 2002). Finally, the presence of splice variants of opioid receptors on immune cells (McCarthy et al., 2001) may also explain the diversity of responses that have been observed, as these types of receptors display altered ligand selectivity and sensitivity (Pasternak, 2001).

In conclusion, the structure–effect relationships formulated here indicate that the R2 functional group at position 6 of the 4,5-epoxymorphinan structure determines inhibition of proliferation, whilst the R1 at position 3 predicts induction of the proliferative response. Despite the inhibition of the response at low concentrations being naloxone and naloxonazine-sensitive, the classical neuronal opioid nature of the response is not supported by the non-stereospecificity and lack of normal rank order of effect to μ opioid receptor binding. The induction of response at high concentrations was not a classical opioid response due to naloxone-insensitivity. These conflicting rank order, antagonist and stereoselectivity data emphasise the need to include multiple opioid agonists to determine the opioid nature of a response, especially when dealing with opioid modulation of these immune responses. This model could be used to evaluate the immunomodulatory effects of non-4,5-epoxymorphinan opioids (e.g. fentanyl, methadone and peptide opioids). Furthermore, the use of these structurally diverse compounds may further clarify the nature of the receptors mediating opioid immunomodulation. As our understanding of the direct effects of opioids on the various cells in the immune system increases and how these responses affect in vivo pharmacodynamics of opioids, an appreciation of the balance between central effects of opioids such as analgesia and dependence and immunomodulation may allow better selection of medication in some clinical settings.

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