ELSEVIER

European Journal of Pharmacology 316 (1996) 369–372

ejp

Short communication

Tramadol, M1 metabolite and enantiomer affinities for cloned human opioid receptors expressed in transfected HN9.10 neuroblastoma cells

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Received 8 August 1996; accepted 27 September 1996

Abstract

Tramadol hydrochloride is a centrally acting synthetic analgesic in widespread clinical use. Despite different degrees of opioid-like characteristics in preclinical tests, it is characterized by lack of full naloxone reversibility or naloxone-precipitated withdrawal in humans. To investigate this apparent discrepancy, the present study measured the affinity of tramadol (and its enantiomers) and an active *O*-desmethyl metabolite (M1) (and its enantiomers) to cloned human opioid receptors of the μ, δ and κ type stably expressed in HN9.10 neuroblastoma cells. At μ sites, the K_i values for tramadol, its (+) and (−) enantiomers, M1, and its (+) and (−) enantiomers were 17000, 15700, 28800, 3190, 153 and 9680 nM, respectively, compared to 7.1 nM for morphine. These results are consistent with the suggestion of a non-opioid contribution to the clinical profile of tramadol.

Keywords: Tramadol; Analgesia; Cloned human receptor; Opioid receptor type

1. Introduction

Tramadol hydrochloride ((1*RS*,2*RS*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol HCl) is the fourth most-prescribed centrally acting analgesic in the world. Yet, to date, its mechanism of action is not fully understood. With apparent opioid-like activity in some preclinical models and antinociceptive and analgesic potency between that of codeine and morphine (Lee et al., 1993; Raffa et al., 1992), tramadol was thought, prior to about 1990, to produce its effects exclusively through an opioid mechanism. More recent studies, however, have suggested a contribution from a non-opioid mechanism (see Raffa et al., 1995 for review). The co-existence of opioid and non-opioid mechanisms might be explained by examining the pharmacologies of the enantiomers of tramadol (Raffa et al., 1993) and its *O*-desmethyl metabolite (M1). The purpose of the present study was to measure the affinity of tramadol, M1 metabolite, and their enantiomers for the recently available cloned human opioid receptors.

2. Materials and methods

Racemic tramadol hydrochloride (molecular weight (MW) = 344.3), its enantiomers, racemic *O*-desmethyl metabolite (M1) hydrochloride (MW = 285.8), and its enantiomers were obtained from Grünenthal (Stolberg, Germany) or were synthesized in-house. (−)U50,488 (*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate) and nor-BNI (nor-binaltorphimine hydrochloride) were gifts from Institut de Recherche Jouveinal (Paris, France). Morphine and DAMGO ([D-Ala^2,*N*Me-Phe^4,Gly-ol^5]enkephalin) were obtained from the NIDA (National Institute on Drug Abuse) drug supply program. CI-977 ((5*R*)-(5,7,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8yl]-4-benzofuran-acetamide monohydrochloride, Enadoline) was a gift from Dr. John Hunter (Roche Bioscience, Palo Alto, CA, USA). DPDPE ([D-Pen^2,D-Pen^5]enkephalin and [D-Ala^2,Glu^4]deltorphin were gifts from Dr. Victor Hruby (University of Arizona, Tucson, AZ, USA) and [^{3}H]CTOP (D-Phe,Cys,Tyr,D-Trp,Om,Thr,Pen,Thr-NH_2) (45.2 Ci mmol^{-1}), [^{3}H]DAMGO (48.9 Ci mmol^{-1}), [^{3}H]U-69,593 ((5α,7α,8β)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-benzeneacetamide) (47.7 Ci mmol^{-1}), [^{3}H]pCl-DPDPE (([Tyrosyl-3,5-^{3}H)[D-Pen^2,D-Pen^5]en-

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PII S0014-2999(96)00770-4

kephalin) (48.6 Ci $mmol^{-1}$) and [^{3}H]diprenorphine (39.0 Ci $mmol^{-1}$) were obtained from New England Nuclear (Wilmington, DE, USA). All other compounds were obtained from commercial sources.

The mouse hippocampal neuroblastoma cell line HN9.10 (Lee et al., 1990) was transfected via calcium phosphate precipitation with cDNAs that encode either human μ-, δ- or κ-opioid receptors subcloned into pcDNA3 that was kindly provided by Dr. Lei Yu (Indiana University School of Medicine, Indianapolis, IN, USA) (μ), Dr. Brigitt Kieffer (Ecole Supérieure de Biotechnologie, Illkirch, France) (δ) and Dr. Eric Mansson (Ohmeda Pharmaceuticals, Murray Hills, NJ, USA) (κ). Transfectants were selected with 1 mg/ml G418 (Gibco BRL, Gaithersburg, MD, USA). Clonal cell lines that exhibited a stable expression of the opioid receptors based on radioligand binding analysis (see below) were used for further analysis. Cells were maintained in 5% fetal calf serum, 5% newborn calf serum, 90% DMEM, 1 mM L-glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin. Membranes were prepared from cell cultures by harvesting cells in ice-cold 50 mM Tris buffer (pH 7.4). The cells were homogenized and immediately centrifuged at 48 000 × g at 4°C for 30 min. The pellet was resuspended by homogenization in ice-cold buffer and the protein concentration was determined by the Lowry method (Lowry et al., 1951). All assays were carried out in duplicate in 50 mM Tris, 5 mM $MgCl_2$, 0.5% bovine serum albumin and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4). All incubations were carried out at 25°C for 3 h in a total volume of 1 ml. Saturation analyses were carried out using 12 concentrations each of: [^{3}H]DAMGO (0.06–10.2 nM) for the human μ-opioid receptor, [^{3}H]pCl-DPDPE (0.01–4.25 nM) for the human δ-opioid receptor, [^{3}H]U69,593 (0.01–3.63 nM) for the human κ-opioid receptor and [^{3}H]diprenorphine (39.0 Ci $mmol^{-1}$; NEN) for all three receptor types. Non-specific binding was defined by the binding of the radioligand in the presence of 10 μM naloxone. For competition assays, at least 10 concentrations of each ligand were used. The incubations were terminated by rapid filtration and the radioactivity in the samples was determined by liquid scintillation counting. The data were analyzed by non-linear regression analysis using GraphPad Inplot. The K_i values were calculated from the IC_{50} values, based on the Cheng and Prusoff equation (Cheng and Prusoff, 1973), from at least two independent experiments. The statistical significance of a one-site or two-site model for a particular competition analysis was determined by an F test (95% confidence level).

3. Results

Stable expression of the three cloned human opioid receptors was achieved in the hippocampal neuroblastoma cell line HN9.10. This cell line has been used previously to generate transfected, stable cell lines that express high levels of the murine κ-opioid receptor (Lai et al., 1994). Initial screening of cells transfected with each of the three human opioid receptor cDNAs identified three clones that expressed high levels of specific binding of the non-selective opioid antagonist [^{3}H]diprenorphine (K_d values ranged from 260 pM to 590 pM). These three cell lines also showed high affinity, saturable binding of the selective agonists [^{3}H]DAMGO (μ), [^{3}H]pCl-DPDPE (δ) and [^{3}H]U69,593 (κ), respectively (Table 1A). Further analysis

Table 1
Cloned human μ- (HMOR), δ- (HDOR) and κ- (HKOR) opioid receptors expressed in neuroblastoma cells (HN9.10)

(A) Saturation analysis of subtype-selective agonists

Receptor	Ligand [a]	K_d (nM) [b]	B_{max} (pmol/mg) [c]
HMOR	[^{3}H]DAMGO	2.99 (1.72–4.63)	0.03 ± 0.01 (n = 3)
HDOR	[^{3}H]pCl-DPDPE	0.47 (0.35–0.65)	3.04 ± 0.03 (n = 2)
HKOR	[^{3}H]U69,593	0.27 (0.12–0.45)	4.42 ± 0.97 (n = 4)

[a] Ligand concentration ranges: [^{3}H]DAMGO (0.06–10.2 nM); [^{3}H]pCl-DPDPE (0.01–4.25 nM; and [^{3}H]U69,593 (0.01–3.63 nM).
[b] The range of K_d values is given in parentheses. [c] B_{max} values are expressed as mean ± S.E.M.

(B) K_i values of opioid ligands

Competitor	HMOR [a]		HDOR [b]		HKOR [c]	
	(K_i, nM)	n_h [d]	(K_i, nM)	n_h	(K_i, nM)	n_h
DAMGO	0.76	0.91	429	0.75	184	0.83
CTOP	0.98 [e]	1.36	n.d. [f]	–	n.d.	–
Morphine	7.1	0.96	150	0.86	14.7	0.93
DPDPE	1860	0.82	1.39	0.87	14 200	0.94
[D-Ala^2,Glu^4]Deltorphin	3390	0.77	2.46	0.87	28 600	1.15
CI977	272	1.07	900	0.72	0.11	1.09
Nor-BNI	34.5	1.22	63.2	0.84	0.13	1.44
(−)U50,488	n.d.	–	11 100	0.66	1.08	1.10

[a] vs. [^{3}H]DAMGO (3.2 ± 0.8 nM). [b] vs. [^{3}H]pCl-DPDPE (694 ± 25 pM). [c] vs. [^{3}H]U69,593 (326 ± 5 pM). [d] Hill coefficient. [e] K_d value determined from saturation analysis. All data were best-fit (F test) by one-site model. [f] n.d. = not determined.

Table 2
K_i values of tramadol, its *o*-desmethyl metabolite (M1) and each enantiomer for cloned human μ- (HMOR), δ- (HDOR) and κ- (HKOR) opioid receptors expressed in HN9.10 neuroblastoma cells

Competitor	HMOR (K_i, nM) [a]	HDOR (K_i, nM) [b]	HKOR (K_i, nM) [c]
Tramadol	17000	n.c. [d]	49700
(+) Enantiomer	15700	n.c.	71500
(−) Enantiomer	28800	n.c.	125000
O-Desmethyl metabolite (M1)	3190	6660	1910
(+) Enantiomer	153	2780	910
(−) Enantiomer	9680	118000	50800

[a,b,c] Concentrations of radiolabeled ligands the same as those in Table 1. All data were best-fit (*F* test) by a one-site model. [d] n.c. = no inhibition up to 1 mM.

using selective ligands (Table 1B) confirmed that the characteristics of the three opioid receptors expressed in these cell lines, based on radioligand binding analysis shown here, were in good agreement with that obtained previously (Mannson et al., 1994; Raynor et al., 1995; Simonin et al., 1994, 1995) and were consistent with that of the classified μ-, δ- and κ-opioid receptors. These cell lines were termed HMOR (μ-opioid receptor), HDOR (δ-opioid receptor) and HKOR (κ-opioid receptor).

Competition analysis using tramadol, its enantiomers, M1 and its enantiomers against the binding of [^{3}H]DAMGO, [^{3}H]pCl-DPDPE and [^{3}H]U69,593 showed low affinity of tramadol for any of the three cloned human receptors (Table 2). The enantiomers of tramadol also had low affinity for these receptors, with the (+) enantiomer having a slightly greater affinity than the (−) enantiomer (Table 2). M1 and its enantiomers had a greater affinity for the receptors than did their corresponding parent compound (Table 2). As was the case for tramadol, the (+) enantiomer of M1 had a greater affinity for opioid receptors than did the (−) enantiomer (Table 2).

4. Discussion

Displacements of isotopically labeled agonists by tramadol, its *O*-desmethyl metabolite, and their enantiomers were monophasic. Out of 48 randomly selected clones that had been transfected with the cDNA for the μ-opioid receptor, the highest expression level was substantially lower than that obtained with clones that expressed the δ- or κ-opioid receptors, despite similar experimental procedures. The reason for this is unclear at present, but the finding is not surprising. Because agonist binding is influenced by the interaction of the receptors with G-proteins, it is possible that the observed low percentage of receptors that exhibited high affinity for the agonists was the result of unavailability of requisite G-proteins in the HN9.10 cells. Alternatively, the low number of high affinity binding sites might result from the distribution of the receptors in the cells. Nevertheless, labeling of the receptor sites with selective agonists, as done in the present study, measured tramadol affinity for only those sites that exhibit high affinity for the respective subtype-selective agonist.

Racemic tramadol had highest affinity for μ-opioid receptors (17000 nM), with greater than 4-fold weaker affinity at κ sites and no measurable inhibition of δ binding. Since the K_i values for morphine were 7.1, 150 and 14.7 nM at μ, δ and κ sites, respectively, the highest affinity of racemic tramadol for any opioid receptor was less than 1/1000th that of morphine. The affinity of the racemate was between that of its enantiomers. The (+) enantiomer displayed a greater affinity than did the (−) enantiomer at the μ site, in agreement with results using rat brain homogenates (Raffa et al., 1993). The nature, or extent of the contribution of the *O*-desmethyl metabolite (M1) to tramadol-induced analgesia is not fully known. In the present study, racemic M1 had only μM affinity for any of the receptor types. Although the (+) enantiomer of M1 had the highest affinity (153 nM at the μ site) of any of the tramadol or metabolite compounds tested (more than an order of magnitude less than morphine), M1 does not appear to play a dominant role in tramadol-induced analgesia in humans (Collart et al., 1994; Lee et al., 1993) and data from rodent and human studies suggest that M1 has difficulty penetrating the blood-brain barrier (unpublished data). Together, these findings are consistent with the clinical experience that: (i) tramadol-induced analgesia in human volunteers is not reversed by naloxone, (ii) tramadol-induced analgesia in human volunteers is substantially attenuated by non-opioid antagonists, (iii) naloxone does not precipitate significant withdrawal signs in patients on long-term tramadol treatment, and (iv) the epidemiological history of tramadol suggests lack of substantial abuse (see Raffa et al., 1995 for review).

In summary, tramadol and its enantiomers bound with only weak affinity to cloned human μ-opioid receptors (K_i values in the μM range) expressed in neuroblastoma cell line HN9.10, and with even less affinity for human δ- or κ-opioid receptors. The M1 metabolite and its enantiomers bound with higher affinity than the parent compounds at μ-opioid receptors (albeit still with much lower affinity than morphine) and, similar to tramadol, with less affinity for δ- or κ-opioid receptors. M1, therefore, might contribute to (or be a major source of) the opioid component of tramadol-induced antinociception or analgesia. Overall, these results are consistent with a duality of mechanism of

action, including a contribution from some non-opioid component, to the analgesic action of tramadol in humans that might help explain its reported favorable clinical attributes (Lee et al., 1993).

Acknowledgements

The authors thank Ellen E. Codd (The R.W. Johnson Pharmaceutical Research Institute, Spring House, PA, USA) for critical review and helpful suggestions on the manuscript.

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