ORIGINAL ARTICLE

Clemens Gillen · Michael Haurand
Dieter Johannes Kobelt · Stephan Wnendt

Affinity, potency and efficacy of tramadol and its metabolites at the cloned human μ-opioid receptor

Abstract The present study was conducted to characterise the centrally active analgesic drug tramadol hydrochloride [(1RS,2RS)-2-[(dimethyl-amino)-methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride] and its metabolites M1, M2, M3, M4 and M5 at the cloned human μ-opioid receptor. Membranes from stably transfected Chinese hamster ovary (CHO) cells were used to determine the four parameters of the ligand-receptor interaction: the affinity of (±)-tramadol and its metabolites was determined by competitive inhibition of [^{3}H]naloxone binding under high and low salt conditions. The agonist-induced stimulation of [^{35}S]GTPγS binding permits the measurement of potency (EC$_{50}$), efficacy (E_{max} = maximal stimulation) and relative intrinsic efficacy (effect as a function of receptor occupation). The metabolite (+)-M1 showed the highest affinity (K_i=3.4 nM) to the human μ-opioid receptor, followed by (±)-M5 (K_i=100 nM), (-)-M1 (K_i=240 nM) and (±)-tramadol (K_i=2.4 μM). The [^{35}S]GTPγS binding assay revealed an agonistic activity for the metabolites (+)-M1, (-)-M1 and (±)-M5 with the following rank order of intrinsic efficacy: (+)-M1>(±)-M5>(-)-M1. The metabolites (±)-M2, (±)-M3 and (±)-M4 displayed only weak affinity (K_i>10 μM) and had no stimulatory effect on GTPγS binding. These data indicate that the metabolite (+)-M1 is responsible for the μ-opioid-derived analgesic effect.

Key words Affinity · Analgesics · Efficacy · GTPγS · Human μ-opioid receptor · Tramadol

Introduction

Tramadol hydrochloride is a centrally acting analgesic drug of widespread clinical use that acts as a μ-opioid-re-

ceptor agonist and inhibits the reuptake of noradrenaline and serotonin (Raffa et al. 1992, 1993). It is a racemic mixture of the (+)-enantiomer (1R,2R)-2-[(dimethyl-amino)-methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride and the (-)-enantiomer (1S,2S)-2-[(dimethyl-amino)-methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride. Biotransformation of tramadol in man and animals takes place via N- and O-demethylation (phase I reactions) and conjugation of O-demethylated compounds (phase II reactions). The phase I metabolites are mono-O-demethyl-tramadol (M1), mono-N-demethyl-tramadol (M2), di-N-demethyl-tramadol (M3), tri-N,O-demethyl-tramadol (M4) and di-N,O-demethyl-tramadol (M5). M1-conjugates and M5-conjugates formed by glucuronidation and sulphation are the main phase II metabolites (Lintz et al. 1981). In-vitro receptor binding studies with membrane preparations from rat brain showed only a moderate affinity of tramadol to the μ-opioid receptor site, whereas its O-demethyl metabolite [(±)-M1] was about 300-fold more potent (Frink et al. 1996). Opioid agonists differ in their analgesic effects not only because of different affinities to the μ-opioid receptor, but also because of their different ability to activate the μ-opioid receptor. Therefore, efficacy studies are necessary for a complete understanding of opioid action. Opioid receptors transduce their signal via pertussis toxin-sensitive heterotrimeric G-proteins (Fedynyshin and Lee 1989; Selley and Bidlack 1992; Chakrabarti et al. 1995) that can act on several effectors through inhibition of adenylate cyclase (Frey and Kebabian 1984; Yu and Sadee 1988; Childers 1991), activation of inwardly rectifying K$^+$ currents (Aghajanian and Wang 1986; North et al. 1987) or inhibition of voltage-dependent Ca^{2+} channels (Moises et al. 1994; Rhim and Miller 1994). Receptor-mediated G-protein activation can be measured by the ability of agonists to stimulate [^{35}S]GTPγS binding in isolated membranes (Hilf et al. 1989). This assay relies on the agonist-induced GDP/GTP exchange occurring at the G$_α$-proteins within the receptor/G-protein complex. The [^{35}S]GTP binding is used to determine the potency (EC$_{50}$) and relative efficacy (maximal stimulation) of a drug. A parallel measurement of

C. Gillen (✉) · M. Haurand · D. J. Kobelt · S. Wnendt
Department of Molecular Pharmacology, Grünenthal GmbH,
Zieglerstrasse 6, D-52078 Aachen, Germany
e-mail: Clemens.Gillen@grunenthal.de,
Fax: +49-241-5692430

binding affinity under [^{35}S]GTPγS binding conditions allows the calculation of the relative intrinsic efficacy (effect as a function of receptor occupation). Here we report on the determination of the four parameters of ligand-receptor interactions for tramadol and its metabolites: potency, efficacy, relative intrinsic efficacy and affinity.

Materials and methods

Drugs

Racemic tramadol hydrochloride [(1RS,2RS)-2-[(dimethyl-amino)-methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride], the racemic tramadol metabolites M1 (mono-O-demethyl-tramadol), M2 (mono-N-demethyl-tramadol), M3 (di-N-demethyl-tramadol), M4 (tri-N,O-demethyl-tramadol) and M5 (di-N,O-demethyl-tramadol) and the (+)- and (-)-enantiomers of the metabolite M1 were synthesised in-house. Morphine sulphate, levo-methadone [R(-)-methadone hydrochloride], fentanyl citrate and DAMGO ([D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin) were obtained from Research Biochemicals International (Natick, Mass., USA).

Receptor affinity studies

Competition analysis under low salt conditions. Membranes from CHO-K1 cells transfected with human μ-opioid receptor (Receptor Biology, Beltsville, Md., USA) were thawed rapidly, diluted 90-fold with 50 mM Tris/HCl (pH 7.4) and resuspended by homogenisation. All incubations were carried out in triplicate at 20°C for 1 h in a total volume of 250 μl, consisting of 200 μl membrane suspension with 10 μg protein, 25 μl of 50 mM Tris/HCl (pH 7.4) containing 20 nM [^{3}H]naloxone (58 Ci/mmol; Du Pont NEN), and 25 μl of 50 mM Tris/HCl (pH 7.4). Non-specific binding was defined in the presence of 10 μM naloxone. For competition assays, ligands were dissolved in 50 mM Tris/HCl (pH 7.4) and tested at six concentrations of each ligand. Incubations were started by addition of membrane suspension and were terminated by rapid filtration with a Brandel 96 sample harvester using GF/B-UniFilter-plates (Packard) pre-soaked with 50 μl of 50 mM Tris/HCl (pH 7.4) per well, followed by washing three times with 250 μl of 50 mM Tris/HCl (pH 7.4). After the filter plates were dried at 50°C for 1 h, 35 μl per well of Microscint 40 (Packard) was added and the radioactivity in the samples was determined with a Wallac 1450 MicroBeta scintillation counter (Wallac). Data were analysed by non-linear regression analysis (Figure P version 6.0c; Biosoft, Cambridge, UK). K_i-values were calculated from IC$_{50}$-values, based on the Cheng and Prusoff equation (Cheng and Prusoff 1973).

Saturation analysis. Membranes from CHO-K1 cells transfected with human μ-opioid receptor were thawed rapidly, diluted 120-fold with 50 mM Tris/HCl, 0.02% (w/v) bovine serum albumin (pH 7.4), and resuspended by homogenisation. All incubations were carried out in duplicate at 25°C for 2 h in a total volume of 500 μl containing 450 μl membrane suspension with 16.3 μg protein, 25 μl of 50 mM Tris/HCl (pH 7.4) with [^{3}H]naloxone, and 25 μl of 50 mM Tris/HCl (pH 7.4). Saturation analysis was carried out with ten concentrations of [^{3}H]naloxone in the range of 0.125–10 nM. Non-specific binding was defined in the presence of 10 μM naloxone. Incubations were started by addition of membrane suspensions and were terminated by rapid filtration with a Brandel harvester type M-24R using Whatman GF/B filter mats pre-soaked in 50 mM Tris/HCl (pH 7.4), followed by washing three times with 1.5 ml of 50 mM Tris/HCl (pH 7.4). The filter discs were incubated with scintillation fluid (Ready Protein, Beckman) for 12 h at room temperature and the radioactivity in the samples was determined by liquid scintillation counting. K_D-values

were determined by use of the program Ligand (Biosoft, Cambridge, UK).

Receptor efficacy studies

Competition analysis under high salt conditions. Five micrograms of membranes were incubated with the appropriate concentrations of agonist drugs (10^{-10}–10^{-4} M) in the presence of 4 nM [^{3}H]naloxone under the same conditions of assay buffer (except that non-radioactive GTPγS was used instead of [^{35}S]GTPγS), incubation time and post-incubation procedure that were employed in the [^{35}S]GTPγS binding experiments described below.

[^{35}S]GTPγS binding. Membranes from CHO-K1 cells transfected with human μ-opioid receptor were thawed rapidly, diluted in 20 mM HEPES, pH 7.4, and resuspended by homogenisation. For each assay equivalents to 5 μg membrane protein were incubated in 20 mM HEPES, pH 7.4, 10 mM MgCl$_2$, 100 mM NaCl, 1 mM EDTA, 10 μM GDP, 0.4 nM [^{35}S]GTPγS (Amersham), 1 mg WGA-PVT beads (Amersham), and the agonist in a final volume of 200 μl. After 1 h of incubation at room temperature the assay plate was centrifuged for 10 min at 3200 rpm. The bound activity was determined with a Wallac1450 MicroBeta scintillation counter (Wallac).

Data analysis. Data were analysed by non-linear regression analysis using Figure P (version 6.0c; Biosoft, Cambridge, UK). K_i-values were calculated from IC$_{50}$-values based on the equation of Cheng and Prusoff (1973). Calculations of intrinsic efficacy were based upon the equation suggested by Ehlert (1985), where efficacy $\varepsilon = E_{max-A}/E_{max} \times (K_i/EC_{50}+1) \times 0.5$ and E_{max} is the maximum stimulation of [^{35}S]GTPγS binding by [D-Ala^2, N-Me-Phe4, Gly-ol^5]-enkephalin (DAMGO), E_{max-A} is the maximum stimulation in the presence of the agonist A, EC$_{50}$ is the concentration of the agonist A resulting in 50% [^{35}S]GTPγS stimulation and K_i is the concentration of agonist A resulting in 50% occupancy of the receptor. Each assay was performed in triplicate in two separate experiments ($n=3$, $N=2$).

Results

Affinity studies

The binding of [^{3}H]naloxone to the human μ-opioid receptor was saturable and of high affinity. Scatchard analysis was performed by best fit and single-site analysis and demonstrated that [^{3}H]naloxone bound to the human receptor with a K_D of 0.5±0.02 nM (mean ± SD). Competition studies against the binding of [^{3}H]naloxone to the cloned human μ-opioid receptor with tramadol and its phase I metabolites M1, M2, M3, M4 and M5 revealed that the metabolite (+)-M1 has the highest affinity to this receptor. The K_i-value for (+)-M1 is 3.4±0.2 nM (mean ± SD). (±)-Tramadol itself is about 700-fold less potent than (+)-M1. The low affinity of (±)-tramadol to the human μ-opioid receptor is demonstrated by the K_i-value of 2.4±1.1 μM (mean ± SD). Another metabolite with a greater affinity than (±)-tramadol to the human μ-receptor is (±)-M5. For this compound a K_i-value of 100±40 nM (mean ± SD) was determined. Thus, (±)-M5 is about 30-fold less potent than (+)-M1. In comparison with (±)-tramadol, the affinity of (±)-M5 to the human receptor is about 24-fold greater than the affinity of (±)-tramadol. The (-)-enantiomer of M1 also showed a submicromolar

Table 1 Affinity of tramadol and its metabolites at the human μ-opioid receptor. The K_i-value was determined from [³H]naloxone competitive inhibition experiments using the Cheng-Prusoff equation (Cheng and Prusoff 1973). *n.d.* not determined)

Ligand	K_i low salt (nM)	K_i high salt (nM)
Morphine	0.62±0.13	415±55
(±)-Tramadol	2400±1100	>10,000
(±)-M1	5.4±1.4	n.d.
(+)-M1	3.4±0.2	3200±790
(-)-M1	240±40	>10,000
(±)-M2	12,000±3000	>10,000
(±)-M3	>10,000	n.d.
(±)-M4	>10,000	n.d.
(±)-M5	100±40	5700±1140

affinity to the human μ-opioid receptor with a K_i-value of 240±40 nM. The metabolites (±)-M2, (±)-M3 and (±)-M4 have lower affinities than (±)-tramadol with K_i-values greater than 10 μM.

Morphine was studied as a reference compound and showed a K_i-value of 0.62±0.13 nM (mean ± SD). The affinity of morphine determined in this study is in good agreement with that obtained previously for the human μ-receptor (Raynor et al. 1995). Thus comparing the affinities of morphine with (+)-M1, morphine is 5.5-fold more potent (Table 1).

Efficacy studies

To optimise measurement of specific GTPγS binding, preliminary experiments were conducted using variable concentrations of SPA beads and GDP in the assay, as well as variable incubation times and temperatures for reaction
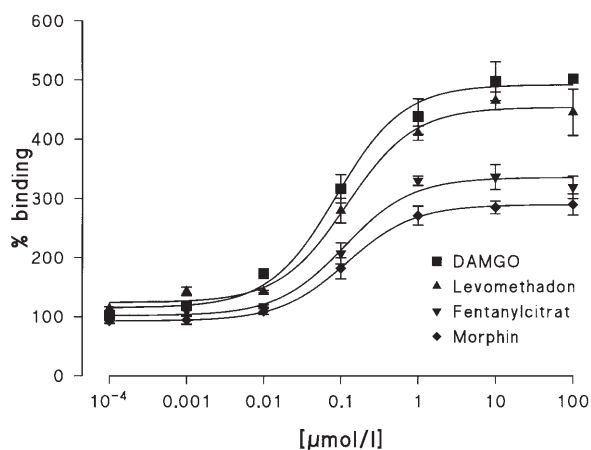

Fig. 1 DAMGO-, fentanyl-, levo-methadone- or morphine-promoted stimulation of [³⁵S]GTPγ binding to membranes of CHO cells stably expressing the human μ-opioid receptor. The membranes were incubated with 0.4 nM [³⁵S]GTPγS, 10 μM GDP and various concentrations of the agonists (each *n*=3; see Materials and methods). The data shown are mean values ±SD expressed as percent stimulation of [³⁵S]GTPγS binding with basal [³⁵S]GTPγS bound set as 100%. The EC₅₀- and E_{max}-values from curve fitting of these data are shown in the upper part of Table 2

(data not shown). With the optimised protocol a dose-response curve was established for the reference compounds DAMGO, fentanyl, levo-methadone and morphine. All compounds stimulated GTPγS binding but with different relative efficacy (E_{max}; Fig. 1).

DAMGO showed the highest E_{max} (set to 100%) followed by levo-methadone (E_{max}=88%), fentanyl (E_{max}=62%) and morphine (E_{max}=0.52). All compounds exhibit similar potencies in the range of 0.1 μM. For the determination of intrinsic efficacy, the binding of the compounds has to be measured under the same assay conditions that were used in the GTPγS binding assay. High-affinity binding was reduced by the addition of sodium, and guanosine diphosphate in the GTPγS binding assay reduces the affinity of the compounds towards the μ-opioid receptor, yielding K_i-values for the reference compounds in the range of 0.32–0.42 μM. With these K_i-values the relative intrinsic efficacy was determined according to the formula of Ehlert (1985) and retained the rank order: DAMGO > levo-methadone > fentanyl > morphine (Table 2).

[³⁵S]GTPγS binding studies with (±)-tramadol and its metabolites exhibited great differences in GTPγS binding: (+)-M1, (-)-M1 and (±)-M5 stimulated GTPγS binding (Fig. 2), whereas (±)-tramadol and the metabolites (±)-M2, (±)-M3 and (±)-M4 did not do so (data not shown). Since the maximal GTPγS binding achieved with (+)-M1, (-)-M1 and (±)-M5 was below that obtained with DAMGO, the tramadol metabolites behave as partial agonists compared to DAMGO. There was a clear correlation between potency and efficacy: the most potent compound (+)-M1 (EC₅₀=0.86±0.21 μM) showed the highest efficacy (E_{max}=52±2%) followed by (±)-M5 (EC₅₀=7.7±1.9 μM,

Table 2 Efficacy and relative intrinsic efficacy of reference compounds, and tramadol and its metabolites at the cloned human μ-opioid receptor. Efficacy was calculated by dividing the efficacy of each agonist by the efficacy value for the most efficacious agonist (DAMGO), and the EC₅₀ value was measured in the [³⁵S]GTPγS stimulation experiments. The K_i-value was determined from [³H]naloxone competitive inhibition experiments using the Cheng-Prusoff equation (Cheng and Prusoff 1973). Relative intrinsic efficacy was calculated according to the equation of Ehlert (1985). For details see Materials and methods

| Agonist	Efficacy (E%E_{max} ±SD)	EC₅₀ (nM)	K_i high salt (nM)	Relative intrinsic efficacy
Reference compounds				
DAMGO	100±4	87±19	320±120	2.33
Levo-methadone	88±3	118±25	355±155	1.76
Fentanyl	62±3	110±30	355±126	1.25
Morphine	52±0.3	118±23	415±55	1.17
Tramadol and its metabolites				
DAMGO	100±6	98±22	346±124	2.26
(±)-Tramadol	<5	–	>10,000	–
(+)-M1	52±2	860±210	3200±790	1.23
(-)-M1	>40	>50	>10,000	–
(±)-M2	<5	–	>10,000	–
(±)-M5	44±2	7700±1900	5700±1140	0.38
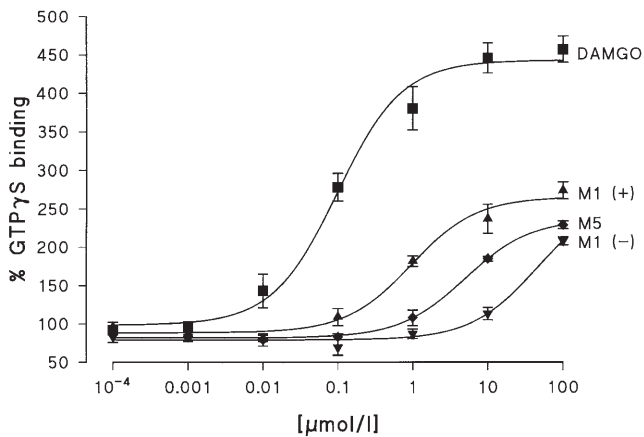
Fig. 2 (+)-M1-, (-)-M1-, (±)-M5- or DAMGO-promoted stimulation of [^{35}S]GTPγS binding to membranes of CHO cells stably expressing the human μ-opioid receptor. The membranes were incubated with 0.4 nM [^{35}S]GTPγS, 10 μM GDP and various concentrations of each agonist. Data shown are mean values expressed as percent stimulation of [^{35}S]GTPγS binding with basal [^{35}S]GTPγS bound set as 100%. They are representative data from one experiment out of a set of two experiments (n=3). The EC_{50}- and E_{max}-values from curve fitting of these data are shown in the lower part of Table 2

E_{max}=44±2%) and (-)-M1 (EC_{50}>50 μM, E_{max}>40%). In the binding studies under the same conditions the metabolite (+)-M1 showed the highest affinity with a K_i=3.2±0.79 μM followed by (±)-M5 with a K_i=5.7±1.1 μM. For (-)-M1 a decrease in [^{3}H]naloxone binding was only seen for the highest concentrations tested (10 μM and 100 μM), so its K_i-value could not be estimated. The relative intrinsic efficacies of (+)-M1 and (±)-M5 were 1.23 and 0.38, respectively.

Discussion

Affinity studies

Competition studies with the isotopically labelled antagonist naloxone and tramadol and its phase I metabolites revealed only a weak affinity of (±)-tramadol to the human μ-opioid receptor in the μM range (K_i=2.4±1.1 μM). The (+)-M1 metabolite and to a lesser extent also the (±)-M5 and (-)-M1 metabolites show a higher affinity to this receptor with K_i-values in the nanomolar range (3.4 nM for (+)-M1, 100 nM for (±)-M5 and 240 nM for (-)-M1). The metabolites (±)-M2, (±)-M3 and (±)-M4 displayed little or no affinity to the human μ-opioid receptor. Thus it can be concluded that only (±)-M1 and (±)-M5 can contribute to the μ-opioid action of the drug. It has to be noted that the affinity of the racemic metabolite M1 determined in this study with a K_i of 5.4 nM does not correspond to the K_i-value of 3.19 μM given in another study also using a cloned human μ-opioid receptor (Lai et al. 1996). Given the fact that the M1 racemate is composed of the equimolar amounts of the (+)- and the (-)-enantiomer, com-

bined with the fact that the (-)-enantiomer displays only poor affinity to the μ-opioid receptor, the K_i-value of the (+)-enantiomer should be approximately half of the K_i-value of the racemate (for a review see Crossley 1995). This holds true for our study with a K_i-value of 3.4 nM for (+)-M1 vs. 5.4 nM for (±)-M1. However, Lai et al. (1996) reported a K_i-value of 153 nM for the (+)-enantiomer of M1, which is 20-fold lower than the K_i-value of the racemate reported by them. Although the reason for this discrepancy between the present results and those of Lai et al. (1996) remains unclear, it should be noted that affinity studies with membranes from rat brain yielded K_i-values for (±)-M1 and (+)-M1 in the low nanomolar range with K_i=17 nM for (+)-M1, 1.8 μM for (-)-M1 and 28 nM for (±)-M1 (Frink et al. 1996), which are in good agreement with binding data reported here and confirm the arithmetic relationship between racemate and active enantiomer.

Efficacy studies

The measurement of efficacy of the reference compounds in the GTPγS assay yielded a rank order (DAMGO > levomethadone > fentanyl > morphine) which is in good agreement with the literature. Payza et al. (1996) analysed different agonists on the cloned human μ-receptor and reported the following rank order: DAMGO (E_{max}=100%) > fentanyl (E_{max}=59%) > morphine (E_{max}=53%). The same rank order (DAMGO > fentanyl > morphine) was found by Traynor and Nahorski (1995) on the human SH-SY5Y cell line which expresses the human μ-opioid receptor. The measurement of the affinity of μ-receptor ligands under the conditions used for the GTPγS assay yielded K_i-values that were much higher than the K_i-values reported for the human μ-opioid receptor measured under low salt conditions (Payza et al. 1996). However, it has been shown for many G-protein-coupled receptors that an increase in sodium chloride and guanosine nucleotide concentrations leads to decreased affinity of agonists for receptor binding sites (Childers and Snyder 1980; Emmerson et al. 1996; Kenakin 1997), probably by reducing the agonist-independent coupling of receptor and G-protein (Wieland and Jacobs 1994). From the potency and relative efficacy obtained in the GTPγS assay together with the affinity measured under the same conditions the relative intrinsic efficacy could be calculated (Ehlert 1985). The calculation of relative intrinsic efficacy indicates highest efficacy for DAMGO, followed by levomethadone, fentanyl and morphine. Thus, morphine behaves like a partial agonist at the μ-opioid receptor, which has also been reported by other groups (Selley and Bidlack 1992; Traynor and Nahorski 1995; Emmerson et al. 1996). This partial agonism of morphine can also be demonstrated in antinociceptive tests after an increase in the intensity of the stimulus (Walker et al. 1993) or a decrease in receptor density by β-funaltrexamine (Adams et al. 1990).

The analysis of tramadol and its metabolites in GTPγS binding revealed agonistic activity for (+)-M1, (-)-M1 and

(±)-M5, but with different relative intrinsic efficacies. The fact that (±)-tramadol showed no increase in basal GTPγS binding can be explained by a decrease in μ-receptor affinity under GTPγS assay conditions in combination with the low affinity of (±)-tramadol for the human μ-opioid receptor (K_i=2.4 μM). The metabolite (+)-M1 showed a relatively high intrinsic efficacy (ε=1.23) that is between the relative intrinsic efficacies of morphine and fentanyl. (±)-M5 displayed a much lower relative intrinsic efficacy (ε=0.38). The high relative intrinsic efficacy of (+)-M1 combined with the fact that (+)-M1 showed the highest affinity of all metabolites tested indicates that also in vivo this metabolite may account for the μ-opioid component of the analgesic effect. The contribution of the different tramadol metabolites vs. tramadol itself seems to be different between rodents and humans. The analgesic effect of tramadol in the mouse or rat tail-flick test can be antagonised by the specific opioid receptor antagonist naloxone, confirming a central site of action mediated by opioid receptors (Carlsson and Jurna 1987; Raffa et al. 1992; Mattia et al. 1993). The strong analgesic effect after i.v. application of the (+)-M1 enantiomer in tail flick confirms this enantiomer as the major opioid component of tramadol (Kögel et al. 1999). In humans, naloxone only partially inhibited the analgesic effect of 100 mg tramadol p.o. in a double-blind, placebo-controlled study in 12 healthy volunteers (Collart et al. 1993a), which suggests a greater importance of the non-opioid mechanisms in humans. In order to reveal the role of M1 in humans, the analgesic effect of tramadol was evaluated in extensive vs. poor metabolisers of sparteine, since the cytochrome P450 2D6 (sparteine oxygenase) is involved in the *O*-demethylation of tramadol, yielding M1 (Collart et al. 1993b; Poulsen et al. 1996). Whereas Collart et al. (1993b) reported decreased M1 levels in poor metabolisers with no effect on tramadol analgesia, Poulsen et al. (1996) could show that the decreased M1 levels in poor metabolisers coincide with a decreased analgesic effect of tramadol given orally (2 mg/kg).

Due to the increased polarity of M5 with respect to M1, M5 would be expected to penetrate the blood-brain barrier less well than M1. Together with the fact that it displays a K_i-value of 100 nM at the human μ-opioid receptor and has a threefold lower intrinsic efficacy than (+)-M1, it might be suggested that interaction of (±)-M5 with the central μ-opioid receptors is of no importance. However, to better evaluate the contribution of tramadol and its metabolites M1 and M5 with respect to the activation of the μ-opioid receptor in vivo, the brain levels of these compounds need to be measured, for instance from cerebrospinal fluid analysis or in PET studies.

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